BIOTECHNOLOGY SUMMIT

3rd Biotechnology Summit

ITSON

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INSTITUTO TECNOLÓGICO DE SONORA
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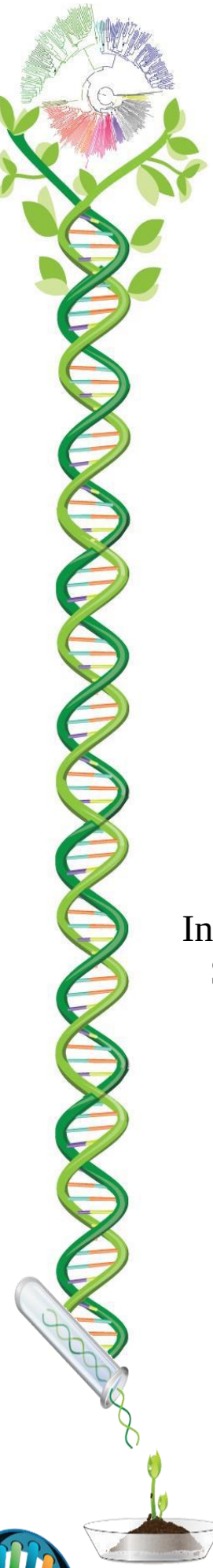

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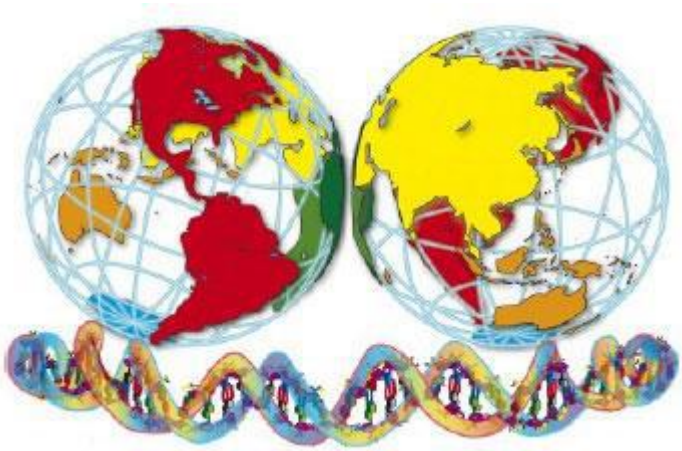

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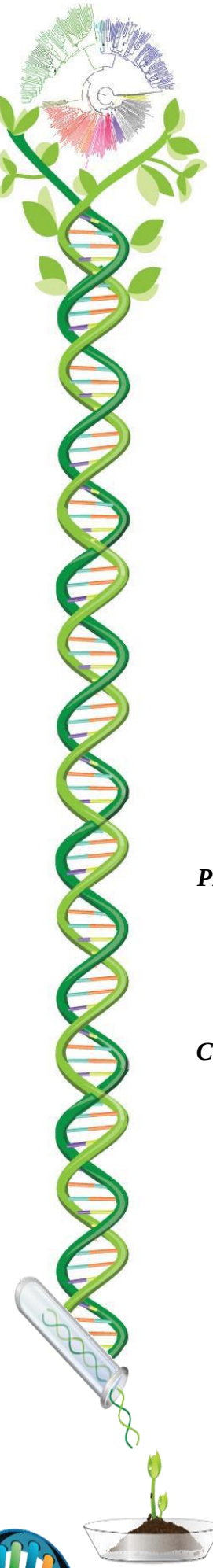

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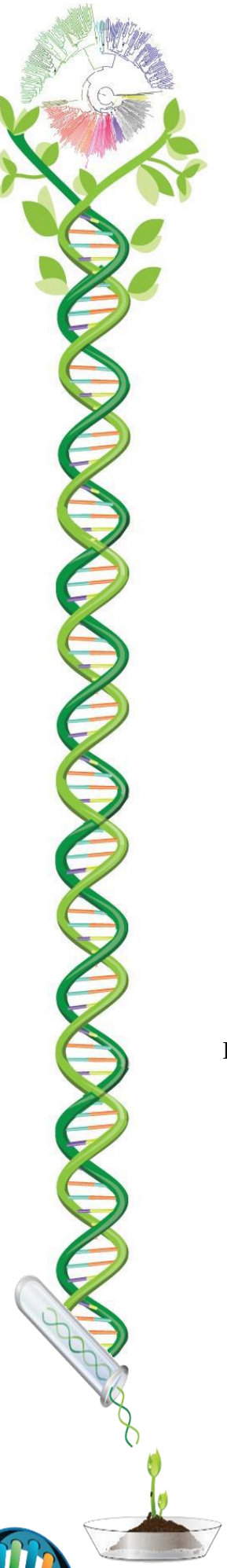

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Welcome to the 3rd. Biotechnology Summit, 2016

The 3rd. Biotechnology Summit (2016) was organized by the Instituto Tecnológico de Sonora (ITSON) and the International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society. This event was held at ITSON (Ciudad Obregon, State of Sonora, Mexico) on October 24 – 28, 2016.

The aim of this Summit was the exchange of cutting-edge knowledge among researchers, students and biotechnology professionals, being an excellent opportunity to establish strong collaboration for the global biotechnology development under critical points of view focused on the solution of International biotechnological concerns.

The scientific areas discussed during this Summit were:

1. ***BLUE Biotechnology:*** *Aquaculture, Coasts and sea, Fish health and nutrition, Aquatic animal reproduction, Cloning and genetic modifications, Aquaculture and fisheries pest and Disease control.*
2. ***BROWN Biotechnology:*** *Space and geomicrobiology, Arid Zone and Desert Biotechnology.*
3. ***DARK Biotechnology****: Human and animal and pest control, Bioterrorism, Biowarfare, Biocrimes and Anticrop warfare.*
4. ***GREEN Biotechnology:*** *Production, Processing and storage of agricultural and Livestock production, Biofertilizers and agrobiochemicals, Agri-Agrocultural pest and Disease control, Ecology and rational wild life management, preservation of biodiversity. Plants, Pets and Farm-animal disease, health, nutrition, reproduction, and cloning and genetic modification. Plant micropropagation and plant tissue culture. Sustainable Design, Renewable energy generation: Resource-saving and energy-efficient, Bioremediation & Environmental Biotechnology, bio-fuel production and sustainable biotechnology development. Biotechnologies for competitive production. New materials and new energy sources.*
5. ***GREY Biotechnology:*** *Focus on the Industrial biotechnologies: Classical Fermentation & Bioprocess/Bioengineering. Engineering and technology re-equipment for bioproduction, output of science-intensive bioproducts. Downstream processing. Control and Simulation of bioprocesses.*
6. ***PURPLE Biotechnology:*** *Strategy for the intellectual property protection, Patents, Publications, Inventions.*
7. ***RED Biotechnology:*** *Human Health & disease, Medical, Diagnostics and Tissue engineering.*
8. ***WHITE Biotechnology:*** *Gene-based Industrial biotechnologies.*
9. ***YELLOW Biotechnology:*** *Food, Nutrition Science and Nutraceuticals.*
10. ***PLATINUM Biotechnology:*** *Synthetic Biology.*
11. ***SILVER Biotechnology:*** *Biobusiness, BioEntrepreneurship & Marketing, Development Economics, Biobusiness and Marketing.*

12. ***IRIS Biotechnology:*** *Multidisciplinary Area on Biochemistry, Molecular Biology & Biotechnology and Applications based on omic's.*
13. ***TRANSPARENT Biotechnology:*** *Bioethics, Biotechnology and Society: tools for asses the support to the scientific sector, including its biotechnological potential and human resources.*
14. ***GOLD Biotechnology:*** *Bioinformatics, Nanobiotechnology, Microelectronic and Microelectromechanical systems (MEMS), Micro Systems Technology (MST), Nano Electro Mechanical Systems (NEMS) and micromachines.*
15. ***INDIGO Biotechnology:*** *Education & Early Childhood Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, BioTechnology & Society as Information and telecommunication technologies TIC´S: for integrating science, education and manufacturing.*

The successful implementation of the Biotechnology Summit 2016 was possible due to the support of prestigious International and National Biotechnology Institutes and Companies, *i.e.* Applikon Biotechnology Inc., Bio-Rad Laboratories Inc., Canitec S.A. de C.V., CINVESTAV, Centro Interamericano de Certificación de Habilidades Jurídicas S.C., CRUNO-Chapingo, Colección de Microorganismos Edáficos y Endófitos Nativos (COLMENA), ICGEB, CABCA-ITSON, Logitlab S.A. de C.V., MasAgro-Biodiversidad, Open Agriculture Journal, Probiotek S.A. de C.V., Sociedad Mexicana de Biotecnología y Bioingeniería A.C., Tetrarium S.A. de C.V., UNAM, Universidad del Papaloapan, Waters S.A. de C.V., Dismant S.A. de C.V., Merck S.A de C.V., Patronato para la Investigación y Experimentación Agrícola del Estado de Sonora A.C. , and H. Ayuntamiento de Cajeme.

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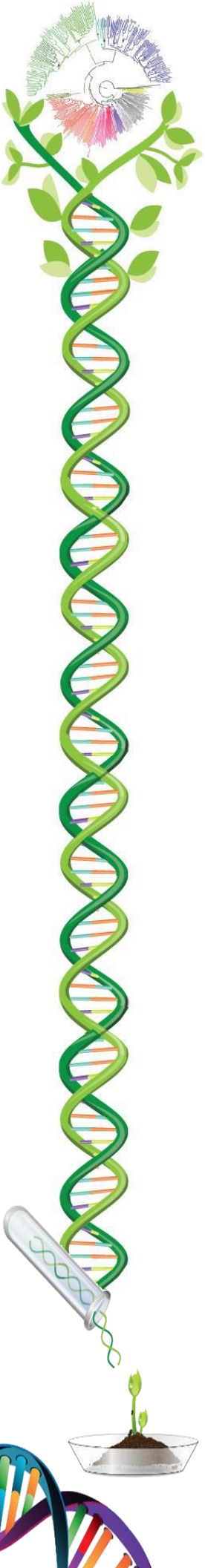

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3rd BIOTECHNOLOGY SUMMIT

October 24-28, 2016

INSTITUTO TECNOLÓGICO DE SONORA

Ciudad Obregón, Sonora, México

Area I
RED

Human Health & Diseases, Biomedicine, and Human Tissue Engineering.

Production and characterization of several mouse monoclonal antibodies for the rapid diagnosis and vaccine development of Hepatitis C virus

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Abstract:
Hepatitis C virus (HCV) is a global health problem particularly in Egypt and it causes chronic C hepatitis, which can develop to liver cirrhosis and hepatocellular carcinoma. Monoclonal antibodies have several applications in immunodiagnosis and immunotherapy for several infectious diseases. Herein, we try to use it for rapid immunodiagnosis for HCV infection based on detection of HCV envelope antigen in the infected patient's sera. Also we try to study the neutralizing activity of these monoclonal antibodies in different vitro cell culture systems. We established by hybridoma technology by immunization of C57BL/6 Mice by plasmid expressed HCV envelope, 8 hybrid cell lines produced several mouse monoclonal antibodies (mAb) by hybridoma technology. ELISA and Dot ELISA were used to evaluate the sensitivity and specific of the one (7G9 mAb) of these antibodies. Neutralization assay were used to evaluate the antibody neutralizing activity. We established 8 IgM producing hybrid cell lines where antibodies from four of them showed highly neutralizing activity using different neutralization assay. One of these antibodies, 7G9, showed high specificity (88%), sensitivity (87%), and efficiency (87%). Conclusion: the generated mAbs can be used for rapid immune diagnosis for HCV infection and HCV vaccine development.

Keywords: Hepatitis C virus● Monoclonal antibodies● Diagnosis● Vaccine development● Envelope.

Introduction:

HCV is the health problem in Egypt and infected more than 170 million individuals worldwide. The diagnosis of HCV is based on detection of HCV antibodies as a first screening step. But detection of anti-HCV antibodies cannot differentiate between past and recent infection. On the other hand, PCR for detection of HCV RNA is expensive and needs more sophisticate equipment in addition to good training. So we need simple and rapid assay for diagnosis and based on detection of viral antigens as an accurate assay and without the necessity of using sophisticated equipment. The detection of HCV circulating antigens will base on specific monoclonal antibodies generated by hybridoma technique. On the other hand until

now there is no available vaccine for HCV infection (Fauvelle *et al.,* 2016). Here we will present our data about generation of several mouse monoclonal antibodies targeting HCV envelope region and study its neutralizing activity for preventing the viral entry to the target hepatoma cell line in *in vitro* system. The epitope for the neutralizing mouse monoclonal antibodies can be used as a candidate HCV vaccine.

Materials and methods

We used hybridoma techniques establishment of several hybrid cell line producing mouse monoclonal antibodies. We established, by hybridoma technology by immunization of C57BL/6 Mice by plasmid expressed HCV envelope, 8 hybrid cell lines that produced several mouse monoclonal antibodies (mAb). ELISA and Dot ELISA were used to evaluate the sensitivity and specific of the one (7G9 mAb) of these antibodies. Neutralization assay were used to evaluation the neutralizing activity of the generated monoclonal antibodies. We established 8 hybrid cell lines. All antibodies are IgM. ELISA was used to screening of the generated antibodies. ELISA was carried out for detection of HCV E1/E2 antigen. Peptide ELISA was used to study the antibody mapping. Neutralizing activity of the generated mouse monoclonal antibodies was evaluated by different neutralization assays (HCV pp, HCV cc and using HCV Infected Sera) as follows:

1-Neutralization assay using HCV pp system

We used the same procedures as reported in our previous study (Tabll *et al*., 2013). HCV pseudotype particles were used to study the early stages of viral life cycle. This virus like particles was used for testing the neutralizing activity of the generated human antibodies. In brief, HCV pp infects Huh 7 cells and infectivity is evaluated by quantification of the amount of luciferase expressed in Huh-7 cells. To measure luciferase activity, Huh 7 cells were washed twice with PBS and lysed with 100 µl cell culture lysis reagent (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Luciferase activity was measured with the luciferase assay system (Promega, Madison, WI, USA) using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany).

2-Testing the Neutralizing activity of the generated mAb by HCV cc system

To check whether the mAb neutralize the J6-JFH replication, culture supernatants containing mAbs pre-incubated with HCV cc prior to transfection of Huh7.5 cells. In all experiments RNA was extracted from cell lysate followed by HCV RNA detection by nested RT-PCR as the procedures reported by Wakita *et al.,* (2005)

3-Natural Infection of Huh7 Cell Line using HCV Infected Sera

The cell line (Huh-7) was cultured into 6-well plates and then infected with HCV genotype 4 positive sera, which were confirmed to be infected with HCV by nested RT-PCR and amplification of HCV positive strand. Infection was done according to protocols described by Song *et al.,* (2001) and Seipp *et al.,* (1997) Infected cell lines were grown in culture, and were tested for the presence of HCV RNA by RT-PCR at time intervals starting from 48 h post infection and reaching to one-month post-infection.

Blood Samples: A total of 138 serum samples of HCV infected patients confirmed positive by PCR and 25 negative healthy samples were kindly obtained from National Research Centre, Giza, Egypt. All the HCV-RNA tested sera used in this study were collected from Egyptian patients.

Results and discussion

ELISA was performed in order to select the spleen of mouse, which gave the higher antibody response for cell fusion (Figure 1). After that we generated 8 hybrid cell lines that produced 8 mouse monoclonal antibodies (Figure 2)

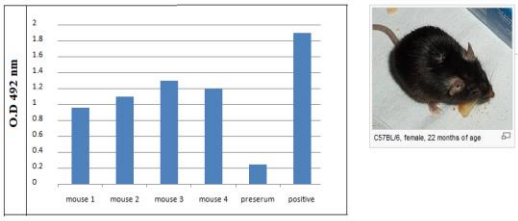

Figure 1. Antibody response in mice vaccinated by plasmid expressed HCV E1/E2 and measured by ELISA

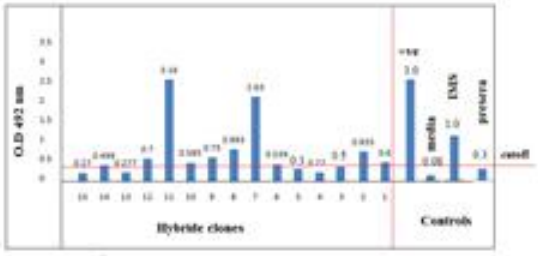

Figure 2. Screening of the generated mAb by ELISA

All generated mAb are IgM and kappa light chain (Figure 3).

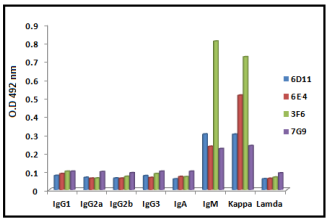

Figure 3. Antibody isotyping of the generated mAbs

We used peptide ELISA to test the antibody mapping of the generated monoclonal antibodies. We found that mAb 7G9 was reacted mainly with region HCV E1 (315-323) and also with region HCV E2 412-419 and HCV E2 517-531 (Figure 4).

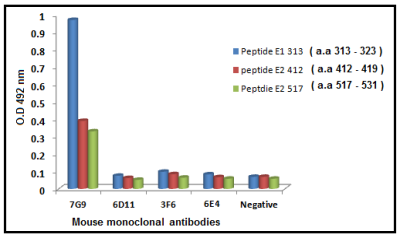

Figure 4. Peptide mapping for the generated mAbs

The Neutralizing activity of the generated mAbs showed that there are 4 antibodies were neutralizing and 4 mAbs were Non-neutralizing as evaluated by HCV Pp and HCV cc neutralization systems (Figures 5-6)

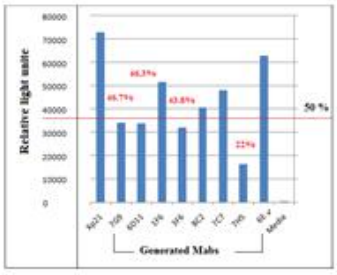

Figure 5. Neutralization activities of the generated mAbs by HCV pp system.

However, each mAb was tested in triplicate tissue culture wells

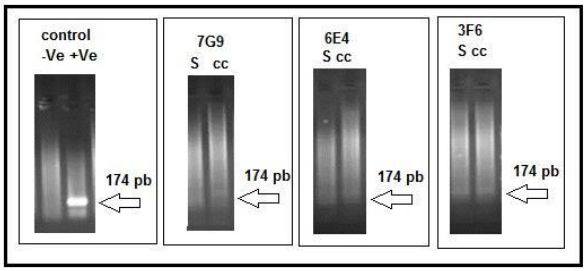

Figure 6. Neutralization activities of the generated mAbs by HCV cc system

The neutralization means ability of the antibody to prevent viral particle to binding and entry to the target cell and / or prevent intracellular replication of the virus in the target cell. So we expect that the neutralizing antibodies are very important to understanding the epitope of the virus which can be used as a candidate protective vaccine for HCV infection. Also we tested the diagnostic value of the one of the generated mAb “7G9” ELISA based on 7G9 mAb showed sensitivity of 80 %, specificity 96 % and efficacy 82%. Virus isolation, viral antigen and viral nucleid acid detections are direct methods of the detection of the virus Thus, detection of antigens

is important for detecting the acute phase leading to early treatments (Muller-Breitkreutz *et al.,* 1999, Attallah *et al.,* 2003, Tabll *et al.,* 2008). All the HCV-RNA molecular tests used are too expensive. In the present study, a new in-house ELISA was presented based on the use of a novel specific mouse monoclonal antibody (7G9) for detection HCV-E1/E2 antigen in serum of HCV infected patients in comparison with HCV-RNA detection using PCR. So we can concluded that generated mouse monoclonal antibodies can be used for rapid immune diagnosis for HCV infection and also for study of HCV vaccine development.

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Evaluation of casp-3 transgene efficacy as a potential biopharmaceutical for gene therapy

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Abstract: Cancer is a term used to define a specific set of diseases where cells have unlimited potential for replication, resistance to apoptosis, immune evasion and metabolic reprogramming. The study of resistance to cell death has allowed to establish new therapeutic targets for the treatment of different types of cancer. The purpose of this work was to evaluate the efficacy of casp-3 transgene into the breast cancer cell line MCF-7 as part of the strategy to test such a molecule as a biopharmaceutical. Transgene expression was determined by PCR and Western blot, the efficacy was determined by MTS assay. Characterization of cell death was performed by next assays: AO/IP staining, DNA fragmentation and flow cytometry. The obtained results indicated that casp3 gene transfection induces programmed cell death in the breast cancer cell line MCF-7 becoming a potential biopharmaceutical for breast cancer that could be used as a transgene in adenoviral gene therapy.

Keywords: MCF-7 ● cell death ● casp-3 ● adenoviral vector.

Introduction: High mortality rate due to breast cancer remains as a major problem in the worldwide health. The development of new therapeutic strategies such as gene therapy, suggests an important alternative against conventional therapies (chemotherapy, radiotherapy and / or surgery), which continuously generate adverse reactions that affects the quality of patient life (Cheang *et al*. 2008). Gene therapy (GT) is a novel alternative to combat diseases where current therapies are ineffective (Chun *et al*. 2014). In general, such a therapy involves the introduction of genetic material into a target cell in order to produce a therapeutic effect (Kootstra and Verma 2003). Currently, GT comprises multifactorial disorders such as cancer, cardiovascular diseases, neurodegenerative disorders and infectious diseases among others (Naldini 2015). One GT “neck of bottle“ is the introduction of genetic material into the target cell. Different systems have been developed including viral vectors. It has been demonstrated that adenoviral vectors are useful because their efficacy in the delivery of therapeutic transgene into the cell nucleus (Wold 2015).

In another hand, MCF-7 cell line was obtained of a pleural effusion from a patient with metastatic breast cancer. This cell line has been used for the study of the estrogen receptor (ER) alpha, due to the fact that it is an excellent model to express substantial levels of ER, mimicking the majority of invasive human breast cancers that express such a receptor (Ballestar *et al*. 2006). Another important characteristic is its deficiency in the expression of caspase 3, which acts as an effector caspase playing a crucial role in apoptosis (Kagawa *et al*. 2001). As known, the regulation of apoptosis mediated by the expression of caspases is critical in maintaining the cell homeostasis. Loss of expression or deregulation of aberrant caspases promotes cell survival and, in some cases, cancer development (Ballestar *et al*.

2006). So, the study of caspases roles to reset the apoptotic mechanism focuses on deficient tumors caspases. For example, the cell line MCF-7, does not express caspase 10 (involved in the extrinsic pathway) or executing caspase 3 (Jänicke 2009). So, the aim of this work is to demonstrate that the reset of Caspase-3 gene expression induces apoptotic cell death in MCF7 cells which are used as a model of breast cancer considering such a molecule as a potential biopharmaceutical for gene therapy.

Materials and methods:

Culture and treatment of human breast cancer MCF-7: MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% fetal bovine serum (FBS) at 37ºC and 5% CO_2.

Plasmid characteristics: Expression vectors for human active caspase-3 (pcDNA3-Casp3-myc, 6429 pb) and human mutant caspase-3 (pcDNA3-Casp3-C163A-myc, 6396 pb) were obtained from Addgene (numbers 11813 and 11814) (Stennicke 1997) and pcDNA3+ plasmid used as a negative control was kindly donated by PhD. Oscar Peralta-Zaragoza (INSP-SS).

Transfection of MCF-7 cells with pcDNA3-Casp3-myc, pcDNA3-Casp3 C163A-myc and pcDNA3+: MCF-7 cells were inoculated into 6-well plates and transfected by Lipofectamine 2000 reagent (Invitrogen) used 6 µg of each DNA plasmid and their expression were monitored at 72 h. Samples were collected for further testing.

Trypan blue staining: Cells were counted using the trypan blue staining. Harvested cells were collected and a 1:4 dilution was done with the trypan blue staining. Then, sample was set on the hemocytometer and counted using an optical microscope 20X.

MTS assay: The MTS (CellTiter 96® AQueous - Promega) assay was used to detect the effect of plasmids pcDNA3-Casp3-myc, pcDNA3-Casp3-C163A-myc and pcDNA3+ over the growth of MCF-7 cells to determine cell viability and % of growth inhibition. Cells were plated into 96-well plates and allowed to grow for 72 post-transfection.

Acridine orange (AO)/propidium iodide (PI) double staining cell morphological assessment: Approximately 2×10^5 cells/mL were inoculated into 6-well plates and allowed to attach. Then, 72 h post-inoculation cells were exposed to 5 µL of AO (50 µg/mL) and PI (50 µg/mL) and placed on a glass slide for observation under a fluorescence microscope (Nikkon Eclipse E400). Positive control of apoptosis was induced by 3 mM H_2O_2 for 4 h (positive control of death).

Agarose gel electrophoresis: DNA samples were obtained by the alkaline lysis method, phenol/chloroform extraction and later precipitated using isopropanol. Then DNA was resuspended into TE buffer and stored at -20ºC. 1% agarose gels were prepared following standard protocols. Later, the gel was revealed in a transilluminator (Bio-Rad, ChemiDocXRS).

Western blot: To identify Casp3 protein, MCF-7 cell cultures were transfected with the plasmids, and a SDS-PAGE of cell lysates was performed. Subsequently, proteins were

transferred to a nitrocellulose membrane following standard protocols. Membrane was blocked using 2% of powder milk. Then, membrane was incubated with anti-casp3 for 4 h. Finally, it was incubated with a secondary antibody coupled to peroxidase by 2 h and it was revealed in a transilluminator.

APC Annexin-V staining: IP-Annexin-V apoptosis detection kit (BD Biosciences) was used according to the manufacturer's instructions. Cells were harvested and washed twice with cold PBS and then re-suspended in 1X binding buffer. Cells were stained with APC Annexin-V staining for 15 min at room temperature in the dark. Cells were analyzed without washing on a flow cytometer within 1 h.

Results and discussion:

It was observed that percentage of viability of MCF7 cell line, determined by trypan blue exclusion and by MTS after different transfections, drops dramatically in cultures transfected with pcDNA3-Casp3-myc (active caspase) at 72 h. Transfected cells with pcDNA3-Casp3 C163A-myc (mutated caspase) plasmid showed activity even when there is a mutation in the active site of such a caspase. Obtained viabilities were compared to pcDNA3+ transfected cultures where no effect was observed (Figure 1).

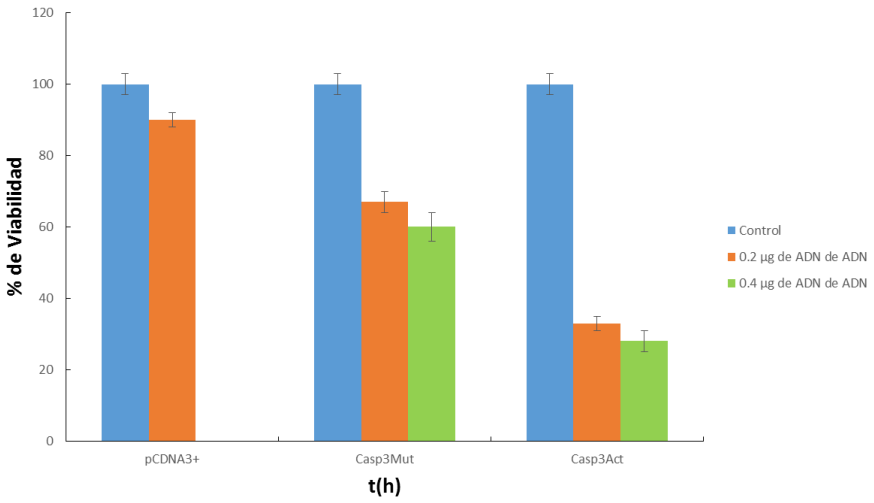

Figure 1. Percentage of viability determination by MTS technique in MCF-7 cells.

Determination of casp-3 gene in transfected MCF-7 cells with pcDNA3-myc-Casp3, pcDNA3-myc-C163A Casp3 and pcDNA3 + at 72 h plasmids was done by PCR. The result is shown in Figure 2. As observed, transfected cells with Casp-3 gene (8 and 9 lines) show the amplified gene in contrast to control cells where there is not DNA amplification (6 line).

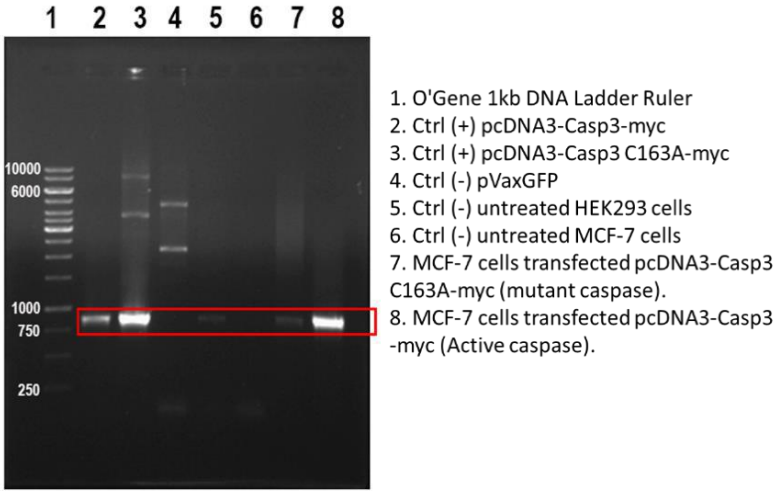

Figure 2. Agarose gel amplified PCR for casp3 (864 bp) gene.

Nuclei morphology of MCF-7 transfected cells with pcDNA3-myc-Casp3, pcDNA3-myc-C163A Casp3 and pcDNA3+ were also observed by epifluorescence microscopy. As shown in Figure 3, cultures transfected with active caspase have characteristic apoptotic features, contrary to the cultures transfected with the mutated caspase nor in control cultures where the pattern is not apoptotic. Such a morphology has been reported in protocols where cell death in MCF7 cells was induced by another compounds (Mandal *et al.* 2013).

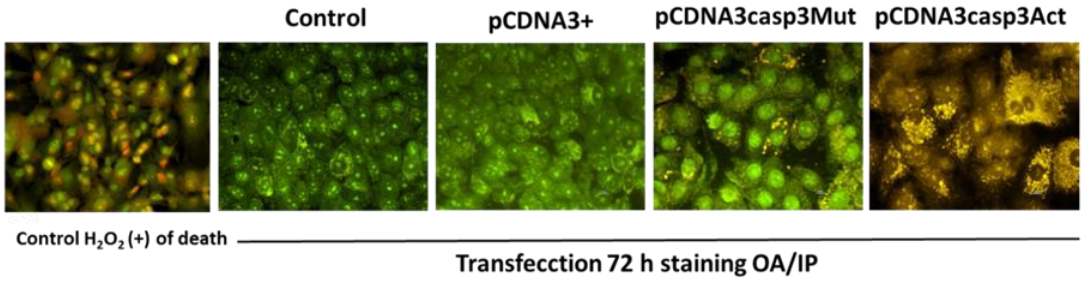

Figure 3. Epifluorescence microscopy staining with OA / IP at 72 h post-transfection.

Apoptosis was also determined by Annexin-V assay by flow cytometry. Transfected cells with active caspase plasmid shown an apoptotic pattern in early stages but a secondary necrotic pattern was present at 72 h. Transfected cells with the mutated caspase plasmid showed an apoptotic pattern due to the delay in the apoptosis development. Control cells did not show any type of cell death. Finally, DNA fragmentation was determined into an agarose gels pattern. MCF7 cells do not develop a specific fragmentation pattern due to the fact that casp-3 is not present (Ofir *et al.* 2002). Obtained results shown that in transfected

cells with active-caspase 3 gene and mutated-caspase 3, typical fragmentation patterns of 180 pb and multiples were observed in contrast to the control culture (Data not shown).

Conclusions

It was observed that the reset of Casp-3 gene expression in MCF7 cells allows to induce programmed cell death in an efficient way, causing a significant decrease in cell viability. Apoptotic cell death pattern was corroborated by nuclei morphology (fragmentation), Anexin-V staining and DNA fragmentation pattern. The activities of active and mutated caspases 3 were also corroborated observing that the last one has lower activity. No significant effect was observed in cultures transfected with pcDNA3+ so the effect of pcDNA3-myc-Casp3-Casp3 pcDNA3 (active caspase) vector is attributed to the transgene casp-3, nor to the backbone plasmid.

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Detection of antibodies against *Borrelia burgdorferi* in dogs from southern of Sonora

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Abstract

Lyme disease, a debilitating chronic infection in humans and dogs caused by the spirochete *Borrelia burgdorferi* is the most common of diseases transmitted by vectors in USA. Since Sonora is borderline state with USA, studies focused to determine the presence of the disease in this region are necessary. Because is complicated perform a sampling serological systematic in human populations, it was opted to use the pets as sentinels based in that dogs share same environment with their owners as well as the same environment. The aim of this study was determine the presence of antibodies against *Borrelia burgdorferi*, by the indirect immunofluorescence method, in dogs from three municipalities in southern Sonora. Overall, 21/90 (23.33%) serum samples were positive to the test, of which a 12/30 (40.0%) were from Alamos and 9/30 (10%) from Navojoa with 0/30 (0%) from Guaymas. These results are first report of serological evidence of Lyme disease in Sonora State and allow recognize geographic expansion of a new endemic area where both dogs and humans are at risk of infection.

Keywords: ● Lyme disease ● Borrelia burgdorferi ● dog ● serology.

Introduction

Currently, in veterinary medicine as human medicine, boost in the incidence of positive cases to tick-borne diseases around the world is reported. Lyme disease, a debilitating chronic infection in humans and dogs caused by the spirochete *Borrelia burgdorferi*, is the most common of diseases transmitted by vectors in USA (Little *et al.* 2012; Brian *et al.* 2014). *Borrelia burgdorferi* is transmitted by ticks of the genus Ixodes, mainly of the species *Ixodes ricinus* in United Kingdom (Smith *et al.* 2012; Coipan *et al.* 2016), while in North America, the tick black-footed *Ixodes scapularis (deer tick)* is the vector of the disease, which have a life cycle of three hosts, with larvae and nymphs feed on small rodents and passerine birds, while adults feeding on large mammals (white-tailed deer, humans) (Nelder *et al.* 2016). The nymph stage is responsible for the majority of the cases reported in humans (Brian *et al.* 2014). Other authors mentioned also that the majority of cases are transmitted by ticks soft (species of Ornithodoros and Argas), but there are exceptions (Lin *et al.* 2005).

In humans, clinical signs include features red circular rashes, erythema migrans, which spreads from the site of the tick bite, followed by a flu-like condition. If untreated, the disease progresses to neurological problems and arthritis (Smith *et al.* 2012; Coipan *et al.* 2016). Lyme post-disease syndromes and chronic forms of the disease can last for many years. The disease is zoonotic and circulates within populations of reservoirs of wild animals, human infection is relatively casual. In Europe, 32 species of vertebrate reservoirs has been identified (Smith *et al.* 2012). In dogs, usually clinical signs have not yet described, but they

could include fever, arthritis, anorexia, lymphadenopathy and glomerulonephritis (Wagner *et al.* 2015). Ticks are relatively more abundant in forests, heaths or moors, although they can also be found in city parks where the hosts are abundant (Smith *et al.* 2012).

The fact to have found seropositive animals *Borrelia burgdorferi*, suggests the presence of the disease in the human population. According to others studies (Smith *et al.* 2012), is difficult and complicated perform serological sampling in populations human; then, based in that dogs live in the same environment with their owners and use to visit the same areas outdoors, pets as sentinels, for the detection of antibodies against the disease of Lyme is an option (Smith *et al.* 2012). In this way, it is possible to infer the possible prevalence of disease in the human population.

This study was performed using an indirect immunofluorescence assay (IFA) because this method has been used previously in several studies, and because it is the standard serology test for Lyme disease, which provides useful results (Hao et al. 2013). The aim of this study was determine the presence of antibodies against *Borrelia burgdorferi* in dog residents on South of Sonora expecting infer about the presence of the disease in the human population.

Material y methods

This study was developed including three towns located at south of Sonora State, considered that these towns presented the environment conditions to develop of Lyme disease in humans and dogs (Álamos, Navojoa and Guaymas). A total of 90 dogs without distinction of race, sex or age, 30 for each town were included.

Blood samples were collected from the cephalic vein, and deposited in tubes without anticoagulant. The samples were preserved fresh in a cooler with frozen gels, maintaining a temperature of 4°C. Once sampling was completed, blood samples were moved to the laboratory of pathological anatomy of the Department of Agronomic and Veterinary Sciences of the Technological Institute of Sonora, Ciudad Obregon, Sonora; there, they were centrifuged at 1500 rpm for 10 minutes for the extraction of the serum. Immediately, serum extracted was settled in transparent plastic vials, identifying them with an appropriate number. Subsequently serum samples were frozen at -20°C until tested.

Tests were conducted using a commercial kit of immunofluorescence indirect (IFA) for *Borrelia burgdorferi* (VMRD Inc). The procedure was performed as recommended in the kit instructions. In brief, tempered serum samples were diluted at ratio of 1:64. Thereafter, once plates were tempered, 10 µl of diluted serum were placed in wells leaving free the wells 1 and 7, where the positive and negative controls were placed, respectively. The next step was to incubate the plate at 37°C for 40 minutes inside a humidity chamber. Thereafter, plates were drained and dry face dorsal plate with absorbent strip containing the kit, then settled 10 µl of reagent anti-IgG or IgM into each of the wells later turned to incubate the plate in the same way for 37°C for 40 minutes. Plates were washed with a buffer with a pH of 9.0 and maintained there for 10 minutes. Finally, plates were dried with 10 µl of mounting fluid in each well, was placed over a coverslip and proceeded to observe fluorescence microscope with 20 X, 40 X and 100 X objectives.

Results and discussion

Overall, a total of 21/90 (23.33%) samples were positive to the test. Results by municipality, showed total of 12/30 (40%) positive dogs in Alamos, 9/30 (30%) positive dogs in Navojoa, while 0/30 positive dogs were found in Guaymas.

Table 1. Distribution of percentage of positive dogs by town.

Town	Positive/total	Percent of positive
Alamos	12/30	40%
Navojoa	9/30	30%
Guaymas	0/30	0%

Based in previous studies (Bowman *et al.* 2009) that confirmed that canine exposure to *Borrelia burgdorferi* mimics the geographic distribution of cases in humans, these results of 23.3 % of positive dogs could be an estimate of the presence of this disease in human population in south of Sonora.

Current climate change models predict increased precipitation extremes that could provide conditions to increase of *Ixodes scapularis* populations. Bergerr *et al.* (2014) provided information on effect of environmental moisture on tick survival. These environmental conditions will increase the risk of Lyme disease for human and animal populations.

Conclusions

The results obtained indicate the presence of canine seropositive to antibodies against *Borrelia burgdorferi* in southern Sonora. This is the first report demonstrating the presence of Lyme disease in Sonora State.

Although this is not an epidemiological study because it was only oriented to find at least one positive dog, results suggest that this disease is present and should be considered as differential of diseases that may be confused due to clinical signs and usually are not considered as possible because the absence of reports.

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Effect of gamma irradiation on stored red blood cells

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Abstract
Blood transfusion is one of the most important therapies in various diseases, being vital to maintain the quality of the blood (Sparrow 2010). In patients with compromised immune system the use of gamma irradiated blood is required to prevent graft versus host disease associated with transfusion (TA-GVHD) (Andreu and Fressy 1995). However, in the blood gamma irradiated oxidative damage in red blood cells occurs, including abnormalities in cell membranes causing hemolysis. In this work we study the effect of gamma irradiated in red blood cells. Different doses of gamma radiation were used the most recommended dose is 25 Gy (Treleaven, Gennery et al. 2011) and two higher dose 50 and 100 Gy. We calculated osmotic pressure, Dose lethal 50 (DL_{50}) and force with data obtained of fragility osmotic and size. The obtained results show changes in structural integrity of the erythrocyte cell membrane components as a result of oxidative damage due to gamma radiation.

Key Works: Red Blood cells ● Gamma irradiated ● Osmotic fragility ● Dose lethal 50

Introduction

Red blood cells (RBCs) better known as erythrocytes are biconcave discs of approximately 7.5μm diameter and 2.5 μm thick, its shape is important for its function. These cells have a high deformability, allowing them to pass through the capillaries. Erythrocytes are responsible for oxygen transport; the hemoglobin is the heteroprotein responsible of this process biochemical (Hb), the main component of red blood cells. Furthermore, the RBC is relatively impermeable maintaining osmotic equilibrium with a physiological environment when not subjected to stressors. When subjected to a hypotonic medium, the cell is filled with fluid which will result in lysis. Glycation studies show that pathological or subjected to stressors cells decrease their ability to deformation of the membrane compared to healthy red blood cells (Soares, Folmer et al. 2014). In the same sense, recent studies indicate that gamma radiation produces free radicals, which can alter the state of lipids and proteins. Since it can produce lipid peroxidation, disulfide bond formation, etc., detrimental to membrane proteins could therefore reduce deformation ability of the membrane and increase its rigidity. Resulting loss of membrane elasticity and an increase in osmotic fragility (Das, Chakraborty et al. 2013). Also as a product of the interaction of the RBC with hemi ionizing radiation which is an oxidation product of hemoglobin occurs, ionizing radiation also leads to the destruction of the membrane (Kozlova, Chernysh et al. 2014). Oxydation reports have shown that hemi echinocytes form at low concentrations and at high concentrations sferoechinocytes (Chiu, Huang et al. 1997), which ultimately leads to hemolysis. Recent

studies have reported increased hemolysis in red blood cells stored for more than 30 days. However, this effect is accelerated by irradiation due to the susceptibility induction membrane as observed by osmotic fragility.

Materials and Methods.

Blood irradiation and storage

RBCs are the principal component of whole blood obtained from the median cubital vein of healthy adult volunteers. The blood was collected and stored using standard plastic tubes (BD Vacutainer *1* K2EDTA anticoagulant). The whole blood was gamma irradiated using a ^{60}Co source for a 25, 50 and 100 Gy doses at 0.7 Gy/s rate (Nordion, Gamma cell 220 Excell). Each irradiated blood was stored separately at 4°C and analyzed after 0, 5 and 13 days.

In vitro RBC osmotic fragility was also evaluated using the method described by Faulkner and King with modifications and using different amounts of sodium chloride (2, 3, 4, and 8 g/L) in PBS, pH 7.4. Osmotic fragility and shape of erythrocytes don't show because are in arbitration process. However, it´s considered as the lethal dose 50 (DL_{50}) when it was 50% erythrocyte lysates, this result was obtained of osmotic fragility. In the same way, the pressure osmotic was calculated using the equation of Van't Hoff, which it requires ideal gas constant, temperature and a difference of concentration, these were calculated at DL_{50}. Finally, with the formula that involves pressure, force and area, osmotic pressure was calculated at LD_{50}. The pressure data were obtained by osmotic pressure and the area (size) was obtained by Atomic Force Microscopy (AFM) was determined.

Result and discussion

In a study of our group osmotic fragility and size of erythrocytes gamma irradiated and storage are analyzed, this information is in arbitration process, parameters such as the osmotic pressure, DL 50 and the force resisting these cells were determined in this work. Some studies shown that the deformability of RBC decreases progressively during storage and increase the erythrocyte hemolysis (Hess, Sparrow et al. 2009, Moroz, Chernysh et al. 2015). This biochemical event is probably accelerated by irradiation (Reverberi, Govoni et al. 2006). In the same way the ionizing radiation affects the membrane susceptibility and provokes the osmotic fragility increase. In this work we were used different concentrations of NaCl to evaluate the effect of radiation in osmotic fragility and consequently with the osmotic pressure and force.

We observed that storage and irradiated RBC are more susceptibility a differences of concentration more near of isotonic, while red blood cells unirradiated and not stored resist more hypotonic concentrations. With the data of fragility osmotic and size of gamma irradiated and storage RBC which are being subjected erythrocytes and indirectly calculate the force applied at that point. Similarly, if we treat osmotic fragility and susceptibility of erythrocytes to hemolysis when increasingly exposed to hypotonic saline. In these solutions water goes into the erythrocyte which swells until the capacity is exceeded and cell membrane bursts. To perform the calculations will be established as the LD_{50} (dose in which they have hemolysate 50% of the cells) concentration of 2.5 g/L although this measurement was not performed was taken as an approximation due to 3g/L NaCl was one 6.32% hemolysis while a 2 g/L corresponds the 95.58% hemolysis. The results in Table 2 are

approximate; we need to perform a curve with different concentrations of NaCl between 3-7 g/L to have a more accurate data.

Table 1. LD_{50} for gamma irradiated red blood cells and stored.

	DL_{50}		
	Day 0 NaCl (g/L)	**Day 5** NaCl (g/L)	**Day 13** NaCl (g/L)
RBC	2.5	2.6	2.9
30Gy- RBC	2.6	2.8	6
50 Gy- RBC	2.7	2.9	6.3
100 Gy- RBC	3.2	3.6	6.5

With approximate results in Table 1 we can interpret that as RBCs are exposed to higher doses of gamma radiation are more susceptible to changes in concentrations, so the membrane becomes more fragile, also the same phenomenon is observed in stored RBC. Moreover, it is interesting to determine the osmotic pressures (∏) which will relate to LD_{50} of each of the measurements. We will use the equation of Van't Hoff, which is used for solutions with different concentrations which in our case.

Osmotic pressure formula: $\prod = R \cdot T \cdot \Delta C$

We use the following data: T = 298.15 K R 8314 Pa * L / mol K

Table 2. Concentration difference between different concentrations of LD_{50}.

	Concentration		
	Day 0 ΔC moles	**Day 5** ΔC moles	**Day 13** ΔC moles
RBC	0.09409	0.09238	0.087254
30Gy- RBC	0.09238	0.088960	0.034217
50 Gy- RBC	0.09067	0.087254	0.029084
100 Gy- RBC	0.08212	0.075278	0.025662

Now with the data in Table 2 we can determine the osmotic pressure which are exposed RBCs. Table 3 it can be seen as increasing the radiation erythrocytes resist less osmotic pressure in relation to control (erythrocytes unirradiated). This analysis provides us with valuable information since as mentioned at the beginning RBCs have the great strain capacity which an abnormal in that capacity variation, could result in the difficulty of passing through capillaries as you normally do and cause its hemolysis (Kim, Kim et al. 2012). To go deeper into it will determine the force that resists an erythrocyte to the LD_{50}, this calculation was done with our experimental data.

Table 3. Osmotic pressure gamma irradiated cells in the LD_{50}.

	Osmotic pressure DL_{50}		
	Day 0	Day 5	Day 13
	Π (atm)	Π (atm)	Π (atm)
RBC	2.3023	2.2605	2.1353
30Gy- RBC	2.2605	2.1768	0.8372
50 Gy- RBC	2.2187	2.1353	0.7116
100 Gy- RBC	2.0094	1.8420	0.6279

Table 4. Strength in red blood cells in the LD_{50}.

	Force		
	Day 0	Day 5	Day 13
	(μN)	(μN)	(μN)
RBC	11.0081	10.2220	9.1021
30Gy- RBC	12.1295	9.8435	4.3447
50 Gy- RBC	12.7278	11.6048	2.6642
100 Gy- RBC	11.1770	7.9872	2.4806

Table 4 shows the force to which the RBC are subjected when they are in their lethal dose 50, can be seen as the ability of erythrocyte to resist this force decreases as the days go storage, however this value was not achieved establish a relationship between different doses of radiation not seen a parameter to follow. In the same sense, it is considered necessary to evaluate other parameters such as deformability and viscosity to make more precise information on the effect of radiation on biomechanical properties (Kim, Kim et al. 2012), a finding which could be affected in the cells red gamma irradiated.

Conclusion

We used the pressure osmotic and resisting forces in RBC as parameters to evaluate the damage to blood cells gamma irradiated. Stressing agents such as the radiation exposure and the storage of blood tissue enhanced the damage to the membrane integrity of RBC. We observed at DL_{50} that osmotic pressure in irradiated and storage RBC decreased along the time in contrast to no stored and non-irradiated RBCs. On the other hand, we observed DL_{50} shows that cells are more sensitive to osmotic effect.

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Association of MTHFR gene polymorphisms with the Methotrexate toxicities in Bangladeshi Acute Lymphoblastic Leukemia patients

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Abstract

The objective of this present study was to investigate the correlation between methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms with methotrexate (MTX) induced toxicities and plasma homocysteine level in Acute Lymphoblastic Leukemia (ALL) in the patients of Bangladesh. Reduced MTHFR activity was generated by several polymorphisms thus manifested in the impaired remethylation of homocysteine to methionine and causes abnormal MTX metabolism especially in tissues with high turnover. Therefore, the risk of elevated plasma homocysteine as well as MTX induced toxicities become higher with MTHFR polymorphisms. During our pharmacogenetic study conducted we recruited 160 ALL patients receiving MTX containing chemotherapeutic protocol, and they were genotyped for MTHFR C677T and A1298C polymorphisms with polymerase chain reaction- restriction fragment length polymorphism. We also measured plasma homocysteine level of 51 patients by AxSYM Homocysteine Assay method. We found 68.1% CC, 26.3% CT and 5.6% TT genotype for MTHFR C677T polymorphism and 39.3% AA, 46.9% AC and 13.8% CC genotype for MTHFR A1298C polymorphism in ALL patients. Our study prognosticated that MTHFR C677T and MTHFR A1298C polymorphism ($P < 0.05$) are significantly associated with MTX induced mucositis and diarrhea as well. Although the risk of elevated plasma homocysteine level was 5 to 6 folds higher for both polymorphisms, but may be due to small sample size we didn't find it statistically significant.

Keyword: MTHFR ● Homocysteine ● Methotrexate ● Toxicities● Acute lymphoblastic Leukemia.

Introduction

Clinical studies indicate that when polymorphisms arise in the genes coding for enzymes involved in folate metabolism affect the sensitivity of patients to folate-based chemotherapeutic drugs such as methotrexate. This may be a direct effect or indirect due to shifts intracellular folate distribution (de Jonge *et al.* 2005).

Catalytic conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate is mediated by Methylenetetrahydrofolate reductase (MTHFR). The enzyme is also known to involve in folate metabolism by creating a co-substrate for homocysteine remethylation. MTHFR reaction is considered as the only way for the synthesis of 5-methyltetrahydrofolate which is utilized by methionine synthase to convert homocysteine to methionine (Kasap *et al.* 2006).

Humans with inherited defects in 5,10-MTHFR gene exhibit features of accelerated aging and a marked propensity for several age-related diseases due to the elevated level of

homocysteine through enhancing the accumulation of DNA damage by inducing a methyl donor deficiency state and impairing DNA repair. Such DNA damage can lead to cancer in mitotic cell, while promotes cell death in postmitotic cells such as neurons (Mattsom *et al.* 2002). There are two frequently observed MTHFR polymorphisms detected in human (Skibola *et al.* 1999), where C677TMTHFR variant results from C to T substitution at nucleotide 677 which converts alanine to a valine (rs1801133, A222V). The functional consequences of this polymorphism are implicated as a risk factor for neural tube defects, pathogenesis of cardiovascular diseases, neurovascular diseases, neurodegenerative disorders, infertility in men, ischemic and hemorrhagic stroke, osteonecrosis, breast cancer, diabetic nephropathy, and others. A second polymorphism, an A to C change at nucleotide position A1298C (rs1801131) converts glutamate to alanine, though is not as well characterized, leads to diminished enzyme activity (Kasap *et al.* 2006). he risk of acute lymphoblastic leukemia (ALL) has been also related to Inheritance of mutant alleles of MTHFR. On that case, folate deficiency leads to double-strand DNA breaks thus that is presumably the onset of the leukemogenic process via increasing the risk of chromosomal aberrations. It is supposed that genetic polymorphisms and environment interaction might play a role in the susceptibility to ALL (Zanrosso *et al.* 2006). Methotrexate (MTX) is an important chemotherapeutic drug in the treatment of ALL and other malignancies. Thus, common polymorphisms in genes coding for enzymes involved in folate metabolism affect the sensitivity of patients to folate-based chemotherapeutic drug such as MTX (Jone *et al.* 2005). By considering all of these aspects, this study was carried out in the Bangladeshi ALL patients in order to investigate the association of rs1801133 and rs1801131 polymorphisms with methotrexate induced toxicities and also the relationship of these polymorphisms with hyperhomocysteinemia.

Method and materials

Patient selection. Total one hundred and sixty (n=160) clinically diagnosed acute lymphoblastic leukemia cases of Bangladesh were recruited from different private and public hospitals of Bangladesh. These patients were experiencing MTX-containing treatment protocol UK ALL regimen A and B, BFM (modified) 1995 and HDMTX. This study was performed from April 2014 to July 2015 and it was completed in accordance with Declaration of Helsinki and its further amendments. Written consents for participating in this study were obtained from all patients before entering the study and they were free to withdraw from the study at any time without any obligation.

Treatment protocol for ALL. Three types of multi-agent treatment protocols were used in the selected group of patients including modified Berlin-Frankfurt-Münster (BFM) (64.4%), Medical Research Council Acute Lymphoblastic Leukaemia Trial XII (UKALL XII) Regimen A and B (31.8%) and High dose Methotrexate (HDMTX) (3.8%). Most of the patients (96%) were on long-term low dose MTX maintenance therapy whereas the rest (4%) of them received high dose MTX. The chemotherapy-induced toxicities were graded according to the Common Terminology Criteria for Adverse Events (CTCAE v4.0)(NCI, 2009).

Plasma homocysteine concentration. Venous blood (2 ml) was collected from each patient in a sterile serum tube containing clot activator. The serum layer from the blood was separated after centrifugation within 30 minutes of collection and the concentration of

homocysteine was determined by AxSYM Homocysteine Assay for only 51 patients excluding the other 109 patients.

DNA isolation and Genotyping. From each selected patients 3ml of venous blood was collected in a Na_2-EDTA tube and was stored at -80^0 C until isolation. Genomic DNA was extracted from blood samples of 161 colorectal cancer patients by using methods as described by Islam *et al.* (2014). Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method was employed for genotyping of MTHFR rs1801133 and rs1801131 (Dulucq *et al.* 2008; Weisberg *et al.* 1998).

Statistical analysis. Associations of the genotypes with the toxicities of chemotherapy was estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from multivariate logistic regression with adjustment for age, sex, BMI, area of residence and the patients with higher toxicities (grade III and grade IV) were compared with that of the lower toxicities (≤grade II).

Result

Patient characteristics and overall toxicities. Among the ALL patients, about 37% were under the age of 15 years and more than 70% of the patients were under 25 years old. 68% of the patients carried wild type (CC), 26% carried heterozygous (CT) whereas 6% carried mutant homozygous (TT) genotypes of rs1801133 polymorphism. On the other hand, the genotype distribution was 39.37%, 46.87% and 13.75% for wild type (AA), heterozygous (AC) and mutant homozygous (CC) respectively in case of rs1801131 polymorphism. The most common toxicities were Gastrointestinal toxicities more specifically diarrhea (47%) followed by mucositis (38%) whereas, in case of hematological toxicities, 24.38% of the patients had anemia and only 7.50% and 9.38 % patients got leukopenia and thrombocytopenia respectively. We also identified 3 patients (1.88%) with peripheral neuropathy and no patient was identified with grade 4 toxicity in case of thrombocytopenia and neurological toxicities.

Effect of rs1801133 polymorphisms on MTX-induced Toxicities. An elevated risk of mucositis was found in case of the patients carrying CT, TT and CT+TT genotypes [OR= 2.97, 95 % CI= 1.36-6.46, p=0.006; OR= 8.48, 95 % CI= 1.41-50.90, p= 0.019 and OR= 3.41, 95 % CI= 1.64 to 7.10 p= 0.001, respectively]. An increased risk of diarrhea was found in the carriers of TT and CT+TT genotypes [OR= 6.03, 95 % CI= 1.11 - 32.77, p=0.037; OR= 2.40, 95 % CI= 1.18-4.89, p=0.016]. No association of s1801133 was found with other toxicities like nausea, vomiting, constipation, anemia, leukopenia, thrombocytopenia and peripheral neuropathy. An elevated level of homocysteine was found in the variant genotype but, statistically no significant association was found.

Effect of rs1801131 polymorphism on MTX induced Toxicities. An increased risk of mucositis was found in the carriers of AC, CC and AC+CC genotypes of rs1801131 polymorphism [OR= 2.21, 95 % CI= 1.04-4.69, p=0.040; OR= 4.39, 95 % CI= 1.41-13.69, p=0.011 and OR= 2.59, 95 % CI= 1.27 - 5.26, p=0.009, respectively]. A statistically significant relationship was also obtained between CC genotype of rs1801131 polymorphism and diarrhea [OR= 6.03, 95 % CI= 1.11-32.77, p=0.036]. The AC and AC+CC genotypes of rs1801131 were also associated with anemia [OR= 2.47, 95 % CI= 1.03-5.94, p=0.043; OR= 2.63, 95 % CI= 1.13–6.10, p=0.025]. The rs1801131 polymorphism was not associated with other toxicities

like nausea, vomiting, constipation, leukopenia, thrombocytopenia, and peripheral neuropathy in the studied population. No statistically significant association was found with the level of homocysteine.

Discussion

The role of folate in cancer is due to defective cell division caused by the shortage of thymidine for DNA synthesis and a shortage of methyl groups for DNA methylation. These pathways have been correlated with chromosome instability as change in the folate supply influences nucleic acid synthesis, DNA repair, and methylation. Thus Chromosomal damage, formation of fragile sites, and micronuclei are the major consequences of folate deficiency and sometimes found an association with tumorigenesis (Kim *et al.* 2006). The goal of this study was to detect whether there is any association of the MTX-induced toxicities in the ALL patients with the MTHFR rs1801133 and rs1801131 polymorphisms.

It was found that rs1801133 and rs1801131 polymorphisms reduce the activity and thermolability of MTHFR, changed intracellular folate distribution, accelerated cellular growth rate and increased thymidylate synthase activity (Sohn *et al.* 2004). MTX inhibits the conversion of folate to their active form tetrahydrofolate by blocking dihydrofolatereductase, while other folate enzymes like MTHFR are also inhibited by MTX metabolites (Sohn *et al.* 2004). Therefore, there is the possibility of an association between toxicities of MTX and MTHFR gene polymorphism. Several studies have been carried out to detect the association of rs1801133 and rs1801131 polymorphisms with MTX sensitivity (Caliz *et al.* 2012; Tukova *et al.* 2010). No such studies have been performed on Bangladeshi ALL patients and therefore we investigated the relationship of rs1801133 and rs1801131 polymorphisms with MTX induced toxicities and plasma homocysteine level in ALL patients. In our study, we found a significant association of rs1801133 and rs1801131 polymorphisms with mucositis and diarrhea. A meta-analysis reported the risk of MTX induced oral mucositis, increased with rs1801133 polymorphism; however, the effect of rs1801131 was controversial (Yang *et al.* 2012). Eissa *et al.* (2013) found a significant association of MTX induced gastrointestinal toxicities, neutropenia and hepatic toxicities with rs1801133 and Tantawy *et al.* (2010) found association of rs1801133 with increased risk of mucositis in pediatric ALL treated with high dose MTX. Tantawy *et al.* (2010) also found a higher risk of diarrhea in association with rs1801133 genotype in pediatric ALL patients. Ongaro *et al.* (2009) and Liu *et al.* (2011) demonstrated increased risk of gastrointestinal toxicities including mucositis and other hematologic toxicities with rs1801131 polymorphism in adult European ALL and pediatric Chinese ALL patients respectively. We found an elevated risk of anemia with rs1801133 polymorphism whereas we did not find any association of rs1801131 polymorphism with anemia. Lopez-Lopez *et al.* also found no association of rs1801131 polymorphism with anemia. Eissa *et al.* (2013) found an increased risk of hematologic and other toxicities in Egyptian adult ALL patients with low dose MTX intake and Spanish pediatric ALL patients with high dose MTX intake respectively which is in agreement with previous studies (Ongaro *et al.*, 2009, Liu *et al.*, 2011)

Conclusion

Our results indicate that rs1801133 and rs1801131 polymorphisms were associated with elevated risk of MTX-induced gastrointestinal toxicities like mucositis and diarrhea. Our results also indicate that rs1801131 was associated with elevated risk of hematological toxicities like anemia.

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Effect of *Annona muricata* on the phenotype of a mutant strain of *Caenorhabditis elegans*

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Abstract

Annona muricata extracts, and its synthesized active components, have been found to have anti-cancer and anti-tumor effects, through experiments in vivo and in vitro. This study evaluated the effects *Annona muricata* on the phenotype the mutant strain *Caenorhabditis elegans*. The results showed that active components of *Annona muricata* decreased locomotion, from 17.5 (control strain) to 13 undulations in 30 seconds under 5 mg/ml. The results presented in this study suggest that *Annona muricata* can produce neurotoxic effects at concentrations equal to or greater than 5 mg/ml. In addition, it was demonstrated that *Annona muricata* could not produce positive effects on the mutant strain of *Caenorhabditis elegans* phenotype. This strain is a knockdown model dic-1 gene whose absence or low regulation of in its human counterpart is reflected in lung and prostate cancer.

Keywords: • *Caenorhabtidis elegans*• Strain mutant •*Annona muricata* •Locomotion.

Introduction

Annona muricata (*A. muricata*), commonly known as Soursop-Graviola, have been recognized and used in ethnomedicine worldwide (Moghadamtousi et al, 2015). This has led to increasing attention on the use and impact of *A. muricata* in the population, including Latin American countries, where the fruit and leaves extract are marketed. Scientific literature has reported several phytoconstituents such as Acetogenins, whose biological activity is focused on the inhibition of mitochondrial complex I (NADH: ubiquinone oxidoreductase) (Zafra-Polo et al, 1995). Also, these Acetogenins have cytotoxic properties against different tumor cell lines such as lung, prostate, liver, and colon (Yang et al, 2015).
Over the past decades, various mutant strains of *C. elegans* have been found to be ideal for the study of specific diseases, including cancer (W.H.Organization, 2015; Lans et al, 2015). This worm model allows the analysis of signaling pathways, study of genes and proteins involved in embryogenesis, and its complete development. Moreover, this model could facilitate the understanding of the mechanisms of drug and other substance action (Sánchez-Blanco et al, 2014). In this study the *C. elegans* mutant strain was used. This study evaluated the extract in vivo effects on the *C. elegans* mutant strain. The phenotypic aspects such as locomotion were analyzed.

Materials and Methods

Maintenance and cultivation of *C. elegans* mutant strain. The mutant strain of *C. elegans* was obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota

(USA). Phenotypic characterization (size, locomotion and viability) of the mutant strain was performed using a Leica DM IL inverted microscope, Olympus light microscope CX31, and an Olympus SZ51 stereoscope. The nematode was maintained on NGM Nematode growth medium (Porta-de-la-Riva et al 2012), the Escherichia coli strain OP: 50 used as a food source (donated by Calixto A, Chile Mayor University), and kept at 20°C, according to Brenner standardization (Brenner, 1974). The synchronization protocol was standardized according to the characteristics of the mutant strain, adjusting the concentrations of NaOH 1M/Cl2 5%/H2O solution (5: 3.8: 1.2) (Porta-de-la-Riva et al 2012).

Extraction of EEAML.

A. muricata were collected in La Mesa, Cundinamarca (Colombia), wrapped in Kraff paper (226.43 g dry weight) under shade and dried at room temperature for two months (Castro et al, 2008). Subsequently, the *A. muricata* were ground and placed in ethanol 95% for 3 weeks. A stock solution (141 mg/ml) was obtained and used in the preparation of NGM media for different tests. The Minimum Inhibitory Concentration (MIC) identification was performed. by disk diffusion testing on Mueller Hinton Agar (MHA), under the Clinical and Laboratory Standards Institute (CLSI) protocols (Castro et al,2008).

Synchronized nematodes were placed in NGM media with EEAML concentrations (1 and 5 mg\ml), then controls were placed in the media without EEAML, and other with 0.2% DMSO. The bioassays of locomotion assay were performed in triplicate, Initially locomotion was determined by the number of undulations made by the head of the adult stage nematode in 30 seconds. Undulation photography taken as a reference of the mutant strain locomotion (*Undulation head) and locomotion comparison between mutant and N2 wild strain (Zhuang et al, 2014). The tests were performed in triplicate and analysis was performed using Graph Pad Prim sortware version 6 (One-way ANOVA). Significant differences were determined as $P < 0.05$. Photographs taken by the Olympus microscope light CX31 40X and Leica DM IL inverted microscope 40X.Statistical analysis as mean +/- standard deviation of the triplicate tests.

Results and discussion

Phenotypic comparison between the mutant strain and the wild strain (N2). Larval stages of C. elegans strain NB327 were initially characterized (Fig. 1A). The eggs the mutant was large, round and symmetrical, whereas the wild strain N2 eggs had an elongated shape and were smallers (Fig. 1B). All larval stages were recognized in the mutant strain (Figure 1C).

Figure 1. Phenotypic characterization of the differences between the mutant strain and the wild strain N2.

A. muricata belongs to the Annonaceae family and has been a traditional plant remedy in America and Africa. Phytochemicals trials have shown that Acetogenins are the main constituent and active component for the developing of anti-cancer and anti-tumor effects, which have been demonstrated through experimentation in vivo and in vitro. However, neurotoxicity has been reported as a side effect (Moghadamtousi et al, 2015). In this study, *C. elegans* strain was exposed to two *A. muricata* doses. Initially, the phenotypic strain characterization was performed by checking abnormal eggs morphology with similar results having been reported (Sánchez-Blanco et al,2014)

The *A muricata* affects the mutant strain locomotion, but not its length. The mutant strain NB327 was exposed to 1 mg/ml and 5 mg/ml concentrations for 6 days, where subsequently no significant alteration of the nematode body length was found, compared to the controls. However, when the mutant strain was exposed to 5 mg/ml concentration, it showed a significant decrease in average locomotion, resulting in 13 undulations in 30 seconds, in contrast to the control strain's 17.5 undulations in 30 seconds, meanwhile, the nematode exposed to 1 mg/ml concentration did not show any locomotion changes (Fig 2). These data suggest that active compounds of *A muricata* can produce neurotoxic effects in concentrations greater than or equal to 5 mg/ml.

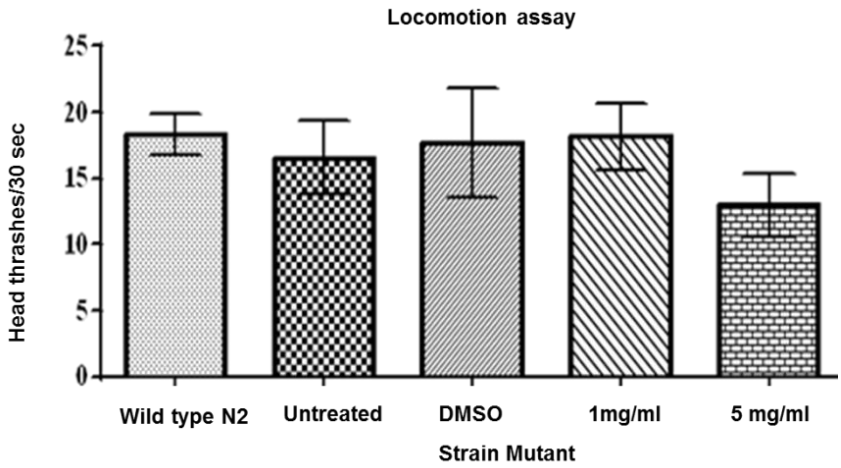

Figure 2. Locomotion of mutant and N2 wild strain.

The results of NB327 strain locomotion, showed undulations from 15 to 20 in 30 seconds, which were similar to those described (Calahorro,2011) . In this study, the NB327 strain locomotion behaviour was increased with the addition of 1 mg/ml concentration, compared to the control. However, the addition of 5 mg/ml concentration presented a reduction of 22% compared to the presented control, and these data are similar to the those in the study described by Moron Francisco Rodriguez J et al (2010). This study argued that a possible neurotoxic effect of the *A. muricata* extract, with the appearance of Parkinson atypical forms, could be associated with the consumption of fruit (15 mg/fruit), and plant leaves (140 mg/cup of tea). The Acetogenins mechanism is based on the inhibition of mitochondrial complex I (NADH: ubiquinone oxidoreductase), which is necessary in ATP synthesis, increasing

oxidative stress, cytotoxic effect, and cell-proliferation in neoplastic cell lines. (Schlie-Guzmán et al,2009).

Conclusions

Furthermore, this study found that the *A. muricata* extract and isolated Acetogenins (especially Annonacina) produced a neurotoxic effect *in vivo* or *in vitro*. It was also determined that Acetogenins, as an environmental neurotoxin, could be responsible for atypical sporadic Parkinson's and dementia in tropical zones (French West Indies) (J. Le Ven et al, 2014). This agrees with various other research studies carried out since 2002.

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LACE1 interacts with YME1L protease and mediates degradation of nuclear-encoded cytochrome c oxidase subunits

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Abstract

Several classes of proteins possessing chaperone and/or proteolytic activities are involved in maintenance of mitochondrial protein homeostasis, which is crucial for cellular function and integrity. Here, we focused on characterization of human LACE1 (lactation elevated 1) function in the maintenance of mitochondrial protein homeostasis. LACE1 is the human homologue of yeast mitochondrial Afg1 (ATPase family gene 1) ATPase with unknown cellular role. Yeast Afg1 is involved in proteolytic degradation of mitochondrially encoded complex IV subunits, and, on the basis of its similarity to CDC48 (p97/VCP), it was suggested to facilitate extraction of polytopic membrane proteins. Using RNAi knockdown, immunoblot analysis, fluorescence microscopy and expression analyses on a HEK293 cell model we demonstrate that LACE1 mediates degradation of nuclear-encoded complex IV subunits COX4 (cytochrome c oxidase 4), COX5A and COX6A, and is required for normal enzyme activity of complexes III and IV of the mitochondrial respiratory chain. Using affinity purification of LACE1-FLAG expressed in a LACE1-knockdown background, we show that the protein interacts physically with COX4 and COX5A subunits of complex IV and with mitochondrial inner-membrane protease YME1L. Thus our study establishes LACE1 as a novel mitochondrial factor with a crucial role in mitochondrial protein homeostasis maintenance.

Keywords: Mitochondria•LACE1 •Proteolysis•YME1L •Cytochrome C Oxidase

Introduction

Mitochondria are essential cellular organelles containing their own DNA (mtDNA) of non-nuclear origin. They take part in a variety of fundamental cellular processes, including heme biosynthesis, programmed cell death (apoptosis), iron-sulphur complex assembly, calcium signalization, and most importantly, they are at the center of cellular energetics (Scheffler, 2008). Afg1 (ATPase family gene 1) is a yeast mitochondrial ATPase, a member of SEC18-NSF, PAS1, CDC48-VCP, TBP family of ATPases (Khalimonchuk et al., 2007). It is a well evolutionary-conserved protein with a robust mammalian homologue LACE1 (lactation elevated 1). The protein is composed of five domains and contains an ATP/GTP binding P-loop (Walker A motif) (Khalimonchuk et al., 2007). Afg1 homologues structurally resemble mitochondrial FtsH/AAA family proteases but do not contain the conserved zinc protease domain (Anand et al., 2013). Yeast Afg1 deletion strain had reduced respiratory growth rate and diminished activities of respiratory chain complexes III and IV. The protein was found to facilitate degradation of mitochondrially-encoded complex IV subunits COX1, COX2 and COX3 (Khalimonchuk et al., 2007). RT-PCR of mouse tissues showed marked tissue-dependent LACE1 expression with the highest mRNA levels in heart, kidney and lactating vs. inactive breast tissue.

Materials and Methods

Cell culture and transfection - Human embryonic kidney cells (HEK293, CRL-1573) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in high-glucose DMEM (PAA Laboratories, Pasching, Austria) supplemented with 10% (vol/vol) fetal bovine serum Gold (PAA Laboratories) at 37°C in a 5% (vol/vol) CO2 atmosphere.
shRNA, ORF constructs and mutagenesis - A negative control (scrambled) pGIPZ shRNAmir construct and five different pGIPZ shRNAmir constructs targeting the human LACE1 transcript (NM_145315) were obtained from Open Biosystems (GE Dharmacon). To generate stable LACE1 KD cells, subconfluent HEK293 cells (107) were transfected by electroporation using Nucleofector™ (Lonza, Walkersville, MD) with cell specific kit according to manufacturer's instructions, and stably expressing cells were selected using puromycin at a concentration of 1.5 µg/ml over a period of 3 wk. Western blot analysis was used to evaluate the efficiency of LACE1 knockdown at the protein level in each of the stable cell lines (monoclonal anti-FLAG M2 antibody, Sigma). Co-immunoprecipitation and affinity purification - For anti-FLAG co-immunoprecipitation the LACE1 KD cells were transiently transfected with the LACE1-FLAG construct, with the CLPP-FLAG construct or with the empty parental pCMV6 vector. Equivalent transfection efficiency was monitored by Western blotting with the anti–FLAG M2 monoclonal antibody (Sigma-Aldrich, USA). Antibodies - The monoclonal antibody against human LACE1 was obtained from Abcam, UK. The M2 monoclonal antibody to FLAG was from Sigma Aldrich, Germany. Antibody to mtHSP70 was from Lonza, Switzerland. Antibodies to ATPase F1-α, SDHA, Core 2, NDUFB6, COX1, COX2, COX4, COX5A, COX6A, citrate synthase, Spg7 and LON were obtained from Abcam, UK. The antibody to OPA1 was purchased from BD Biosciences (Oxford, UK). Antibodies to OXA1L and YME1L were from previous studies (Stiburek et al., 2012; Stiburek et al., 2007). Mitochondrial isolation and subfractionation - Mitochondrial fractions were isolated from HEK293 cells using cell disruption by hypo-osmotic swelling coupled to Dounce homogenization, removal of nuclear contamination by low-speed centrifugation (2,000 × g) and fractionation in discontinuous sucrose density gradient (1-1.5 M) by ultracentrifugation (85,000 × g) essentially as described herein [29]. Enzyme activity assays - The enzyme activities of respiratory chain complexes I-IV were measured spectrophotometrically in isolated mitochondria essentially as previously described (Stiburek et al. 2007).

Results and Discussion

1. Loss of LACE1 leads to increased accumulation of nuclear encoded complex IV subunits and to reduced enzyme activities of complexes III and IV

As yeast LACE1 homologue Afg1 was found to facilitate turnover of mitochondrially encoded cytochrome oxidase (complex IV) subunits, we therefore assessed levels of complex IV subunits in whole cell lysates of LACE1 knockdown cells using western blot analysis. We found unaffected levels of mitochondrially encoded complex IV subunits, but markedly increased levels of three (COX4, COX5A, COX6A) of the nuclear-encoded components of complex IV (Fig. 1A). In contrast, steady-state levels of other respiratory chain subunits were found unaltered in LACE1 KD cells (Fig. 1A). To assess the effects of loss of LACE1 on respiratory chain function we measured activities of respiratory complexes I-IV using spectrophotometry on isolated mitochondria. We found significantly reduced activity of complex III (68.9% of controls) and complex IV (67.5% of controls) in mitochondria of LACE1

KD cells (Fig. 1B). The activity of complex I was not significantly affected in these cells, but the activity of complex II was markedly elevated (138.5% of controls) (Fig. 1B). Yeast Afg1 was shown to facilitate degradation of mitochondrially-encoded complex IV subunits COX1, COX2 and COX3, and yeast Afg1 deletion strain showed respiratory-growth impairment and diminished activities of respiratory complexes III and IV (Khalimonchuk et al., 2007). The fact that nuclear-encoded complex IV subunits COX4, COX5A and COX6A were markedly elevated in LACE1 knockdown cells, but the mitochondrially-encoded subunits were completely unaffected suggests changes in substrate specificity of LACE1 throughout evolution. Similar, but more profound shift in substrate specificity between yeast and mammalian orthologue concerning complex IV subunits was found for the inner membrane translocase OXA1L (Stiburek et al., 2007).

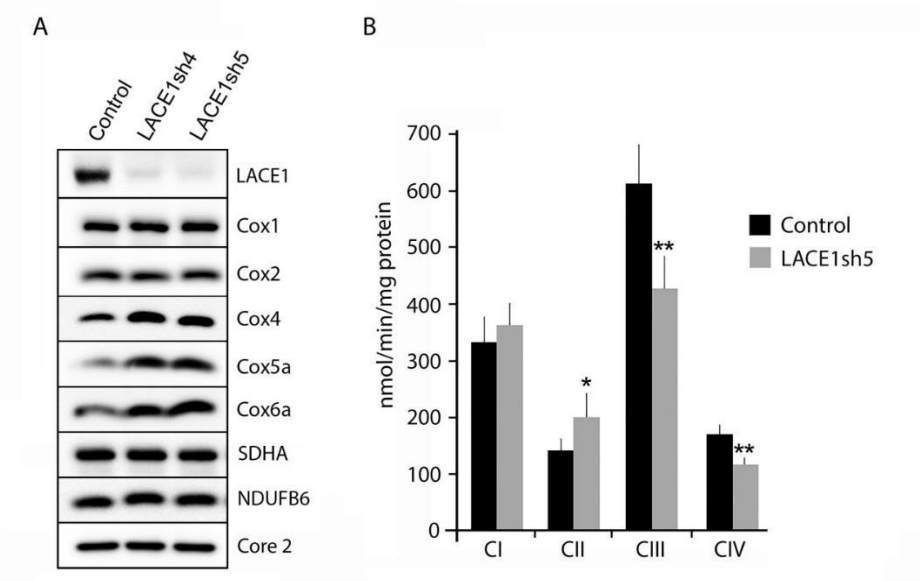

Fig. 1. Loss of LACE1 leads to increased accumulation of COX4, COX5A and COX6A, and to diminished enzyme activity of complexes III and IV

2. Walker A and Walker B motif LACE1 mutants fail to support clearance of complex IV subunits

To address functional significance of both Walker A and Walker B motifs of LACE1 and to confirm specificity of the observed RNAi phenotypes we expressed wt LACE1 as well as K142A and E214Q LACE1 in LACE1 KD background. Whereas, expression of wt LACE1 led to significantly reduced mitochondrial accumulation of COX4, COX5A and COX6A, ectopic expression of both LACE1 variants did not led to suppression of this phenotype (Fig. 2A, B). Western blots of identical samples also showed diminished levels of LON protease and increased levels of YME1L protease in mitochondria of LACE1 KD cells (Fig. 2A, B). In contrast to requirement of wt LACE1 re-expression for clearance of excess COX4, COX5A and COX6A, alterations of LON and YME1L in LACE1 KD cells were effectively suppressed by expression of both LACE1 variants (Fig. 2A, B). The ectopic expression of K142A LACE1 mutant shows the essential character of the Walker A motif for LACE1 involvement in degradation of excess nuclear-encoded complex IV subunits. Indeed, the essential lysine of Walker A motif is known to support binding of nucleotides (Leonhard et al., 1999).

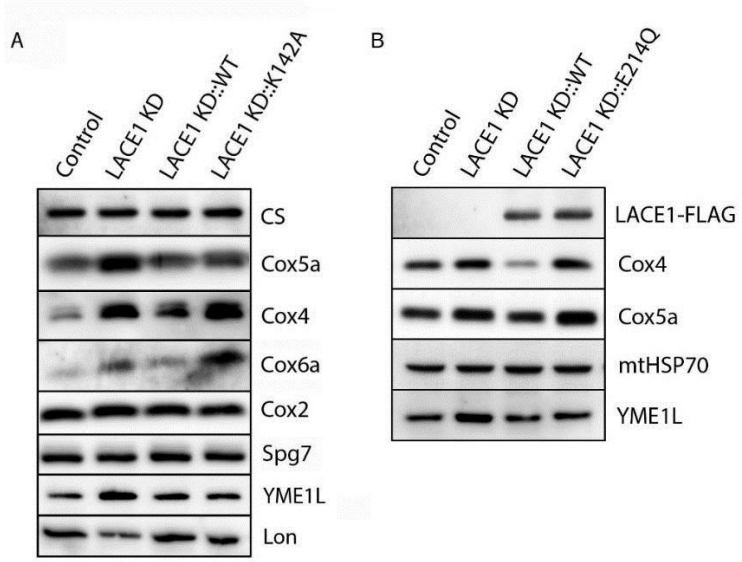

Fig. 2. Ectopic expression of wild-type LACE1 but not of K142A or E214Q variants rescues the increased accumulation of complex IV subunits. A-whole cell lysates; B-isolated mitochondria

3. COX4, COX5A and YMEL co-purifies with wt LACE1 expressed in LACE1 KD cells

To find out which of the observed effects associated with manipulation of cellular LACE1 levels are based on direct protein-protein interactions we used mitochondria from LACE1 KD cells transfected with wt LACE1-FLAG construct to perform anti-FLAG affinity purification. Subsequent western blot analysis revealed significant amount of COX4 and COX5A subunits to specifically co-purify with LACE1-FLAG, but not CLPP-FLAG protein (Fig. 3). Importantly, additional western blotting screen also identified significant amount of YME1L protease in LACE1-FLAG purification sample (Fig. 3). We were not able to identify any of the mitochondrially-encoded complex IV subunits or additional nuclear-encoded complex IV components in LACE1-FLAG affinity preparations using western blot analysis.

LACE1 is shown here to physically interact with COX4 and COX5A subunits of complex IV and with the inner-membrane protease YME1L. In addition, loss of LACE1 is associated with marked upregulation of YME1L. Indeed, COX4 subunit is one of the previously identified proteolytic substrates of YME1L protease (Stiburek et al., 2012). This fact provides further functional link between LACE1 and YME1L (Stiburek et al., 2012). On the other hand, the matrix-localized LON protease, which was reported to degrade COX4 and COX5A, exhibited marked reduction in LACE1 knockdown cells (Hansen et al., 2008). In contrast to increased YME1L that could stem from simple compensatory response to LACE1 deficiency, the observed downregulation of LON is more difficult to explain.

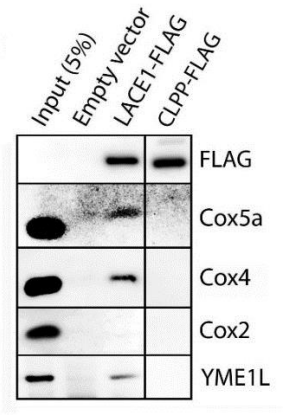

Fig. 3. LACE1 co-immunoprecipitates with COX4, COX5A and YME1L

Conclusions

We have identified mammalian LACE1 as a mitochondrial factor required for clearance of excess nuclear-encoded subunits of complex IV. We further show here that loss of LACE1 leads to diminished activity of respiratory complexes III and IV. Using ectopic expression of K142A and E214Q mutant variants, LACE1-mediated degradation of nuclear-encoded complex IV subunits is shown to depend on functional Walker A and Walker B motifs. In addition, affinity purification of ectopically expressed wt LACE1-FLAG provides physical link between LACE1 and the mitochondrial i-AAA protease YME1L. Collectively, our results establish a crucial role for LACE1 in the maintenance of mitochondrial protein homeostasis and identify possible novel accessory protein of the inner membrane i-AAA complex.

Acknowledgments

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Fast and reliable DNA extraction protocol for identification of species in meat products sold on the commercial market using PCR

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Resumen

In this work a protocol for DNA extraction was established from meat of different animals, (beef, pork, horse), using TE lysis buffer with different concentrations of phenol and chloroform as a base reagent in different times and temperatures. It analyzed commercials samples meat purchased in local region. The methodology was optimized with 12 samples (10 for each of the samples) with 30 repetitions per sample. Purity results obtained in the three species of 1.7 and a concentration at 260nm for 100 µl/ml of DNA once optimized, the protocol was tested in 465 different samples meat of different animal species and their presentation was fresh and processed. The results showed purity 1.35±0.076 and a concentration 70±0.31 µl/ml, and a time of 1.5 hours. These results were tested with PCR as reported by several authors. The extract was tested in different reactions of PCR with the specific primers for horses result 39 positive samples. The proposed methodology provides DNA concentration and purity suitable for amplification PCR efficient thereof, besides quick and easy to perform.

Keywords: DNA extraction Standardization meat products.

Introduction

Animal cells have a greater amount of integral proteins in their lipid layer, therefore implement reagents necessary to generate a cell lysis, turn as the implementation of some nucleases; It is important to control the volume of reagents, not to degrade the genetic material. Currently the use of molecular DNA analysis techniques for identification and detection of diseases of species has become conventional, because of its ease of operation, high accuracy and efficiency. In Mexico, meat products have wide acceptance there are several companies that make up the meat industry in charge of processing these foods (Hernández-Chávez *et al.,* 2011). Extracting genetic material is a key part in different molecular techniques to address the after mentioned problematic. By their protein structure meat tissues exhibit much greater resistance to some buffers and/or reagents for cell lysis, turn as much lipid that impede DNA purity, this leads to long periods to develop a good extraction (Carlos F et al., 2001). Some commercial kits expose an easy and simple

extraction though some are too expensive. Other standard protocols proposed extraction with a large amount of time and low purity (Klein *et al*., 1998). To extract nucleic acids from biological material must cause cell lysis; inactivate cellular nucleases and separating nucleic acids from cell debris. The ideal lysis procedure usually consists of a balance of techniques and must be strong enough to break the complex starting material (a meat tissue), but gentle enough to preserve the nucleic acid (Hargin et al., 1996). The wavelength of 260 nm is the peak absorbance for both DNA and RNA for, however the purity analysis. It is done with 230/260 and 260/280 relations to assess DNA purity with respect to contaminating proteins as phenol and the resulting DNA extraction (Maniatis *et al*, 1992). Therefore, the aim of this study was to standardize a protocol for extracting DNA samples in fresh meat and processed using homogenization buffer and organic solvents.

Metodology

The meat samples of the first part of the work (optimization) were obtained from commercial establishments in the region. 465 samples were provided as a service to a private institution in the center of the country. All samples were processed at the Laboratory of Food Quality and Authenticity of Animal Origin from Department of Agricultural and Veterinary Sciences (ITSON). The samples were handled and ice detail transported in safe conditions preventing cross-contamination. The cell lysis buffer used was Tris-HCl, EDTA, SDS and NaCl, with different concentrations (Table 1). Phenol was used to help break the cell wall, chloroform and isoamyl alcohol to precipitate the DNA, and proteinase K to provide improved purity. For DNA extraction of the meat samples, a sample between 90-110 mg of lean meat was weighed. It was ground in 250 µl of lysis buffer. Once all samples were crushed to eppendorf tubes with 750 µl of buffer, adding the missing volume. It was vortexed for 2min at 3000 rpm to homogenize the sample. It was incubated at 50-60 ° C for 30min.

Table 1. Concentration and reagents lysis buffer.

Reagent	Concentration
Tris-HCl pH 8.0	**10mM**
EDTA	**1mM**
SDS	**0.1%**
NaCl	**10mM**

600 µl subsequently added solution of phenol-chloroform-isoamyl alcohol proportions 25:24:1. It was vortexed for 10 min at 3000 rpm. Step followed centrifuged at 14,000 rpm for 10 min at 4 ° C. The supernatant was transferred to a new vial. It was added 10 µl of proteinase K. Then the sample at 20-50 ° C was incubated for 15 to 30 minutes depending on the species. Processed meat lysis temperature was 60 ° C. 600 µl of solution phenol-chloroform-isoamyl alcohol (24: 1 25) it is again added. It was vortexed for 10 min at 3000 rpm. It was centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new vial.

500 µl of isopropanol was added, mixing with gentle agitation. It was centrifuged at 14,000 rpm for 10 min at 4 °C. The DNA pellet was recovered by pouring the liquid and investing vials on absorbent paper. After eliminating waste alcohol, DNA was rehydrated with 50mL of Milli-Q water or TE buffer (Tris10 mM, 1 mM EDTA, pH 7.5). Then were stored at -20 ° C for further analysis. Quantification of DNA extract obtained was performed using a 1800 model spectrophotometer SHIMADZU with a cell-quartz, 5 µl DNA sample was taken which was diluted with 495 µl of pure water milli-Q, with a dilution factor of 1:100 (Clark *et al*., 2001) at a wavelength of 280 nm and A260 nm. Subsequently, the extract was analyzed by conventional electrophoresis to verify the extraction yield and size approximately the purified nucleic acids (Rivas et al., 1997).

Results and discussions

DNA purity was measured to determine the effectiveness of protocol and amount extracted was quantified using a spectrophotometer. A total of 25 samples were analyzed statistically to determine the effectiveness of the protocol, which was obtained for the sample of a total of 25 samples were analyzed to statistically determine the effectiveness of the protocol. The purity was 1.75±0.035 for horse, beef 1.79±0.031, pork 1.77±0.035 and processed meats was 1.23±0.026 (30 for each of the samples). The purity in 3 tissues suitable values 1.75 to 1.79, being 1.8 values the perfect purity value (Somma, 2004). For 465 samples results of 1.35±0.076 purity and a concentration of 70 ± 0.31. µl / ml they were obtained, in a time of 1.5 hours. As concluded that the effectiveness of
the protocol is excellent for molecular studies. Only for processed meats was very low purity (average 1.2), although the minimum amplification is 1.0 (Bourke *et al.,* 1999). Thus giving a robust protocol for meat in poor condition. Once optimized conditions. The gel electrophoresis analysis with the following results proceeded; for pork 4 tests were made at different incubation times with lysis buffer (15 min, 30 min, 45 min, and 60 min), this due to the high amount of fat in the cell structure in pork, giving a better result at 45 min (Figure 1). Once selected the best time, extracted 30 times to check the effectiveness of the protocol statistically.

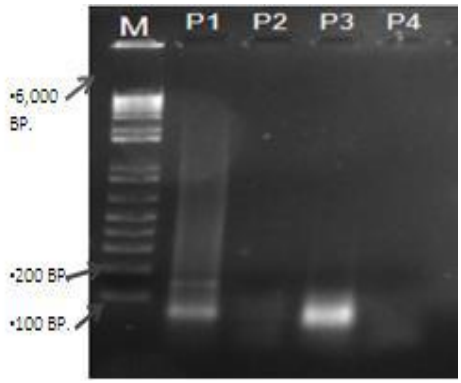

Figure 1. Test different incubation times in cell lysis for pick was; Molecular market 1kb Plus (M); (15 min (P1), 30 min (P2) 45 min (P3), 60 min (P4)

For horse meat and beef, 3 tests were implemented in time incubated with proteinase K, because in these tissues meats contain less fat, likewise, the cells that make these muscle tissues contain more structural protein which is necessary to promote protease activity, favoring the time of 30 min and 15 min horse in beef (Figure 2 and 3), once selected the best time, it extracted 30 times to check the effectiveness of the protocol.

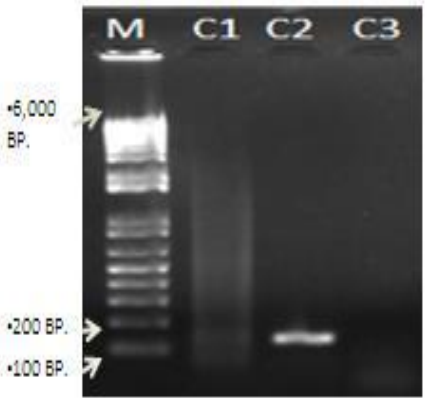

Figure 2. Extractions with different incubation temperatures with proteinase K, horse: Molecular market 1kb Plus (M); 15 min (C1), 30 min (C2), 45 min (C3).

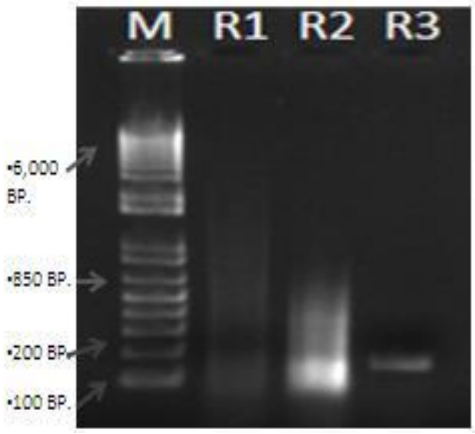

Figure 3. Extractions with different incubation temperatures with proteinase K, beef: Molecular market 1kb Plus (M); 5 min (R1), 10 min (R2), 15 min (R3).

In processed meats due to cell damage caused by high cooking temperatures and other treatments in the flesh that alter the pH of the sample, it was decided not to marinate the meat, as it is degraded in the mortar In addition decreased the temperatures of incubation (Figure 3), once selected the best time, extracted 10 times to check the effectiveness of the protocol statistically. PCR was developed to assess the effectiveness of the protocol in processed meats performing a statistical quantity of 24 tests, using Eqqus1 and Eqqus2 primers, which amplify a conserved sequence in the mtDNA region cytochrome B (Figure 4). Although the amount of DNA is very low just a single molecule to be used in molecular studies.

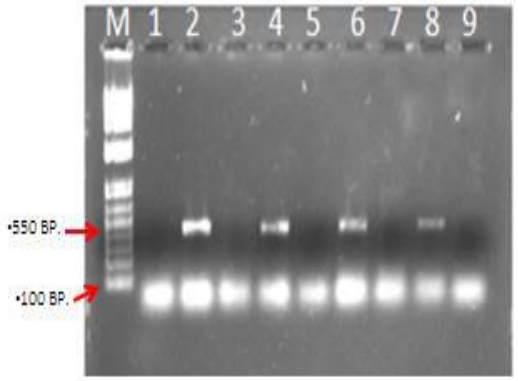

Figure 4. PCR meat products. Molecular market 1kb Plus (M); Negative control with beef (1), the positive control on horseback (2), and lane 3 to 9 different processed samples.

Conclusions

The results show that this DNA extraction protocol using phenol and chloroform, is fast and easy to perform, obtaining better results for fresh samples. Not so for processed samples they had very low values of purity and concentration. Fat of some species (such as pigs), limit the right extracting DNA from the samples. However, the amounts obtained in this work can be used in molecular studies (PCR, sequencing, and genetic mapping, among others).

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Molecular marker for detecting slaughter pain in electro-stunned sheep

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Abstract

Advocacy against animal cruelty and promotion of humane slaughter in meat industry has been in circulation for many years. The use of appropriate stunning method is introduced to meat industry as one of the statutory requirements of achieving humane slaughter. Despite the introduction and adoption of various stunning methods by the meat industry, the correlation between electrical stunning and slaughter pain still remains unaddressed. In this study, ovine ubiquitin C-terminal hydroxylase (ovUCHL1 mRNA) was used as a novel biomarker for quantification of pain expression resulting from electrical stunning of merino sheep. Higher impedance exhibited by ewes showed that it reacted differently to electrical insults during slaughter. Real time quantification of ovUCHL1 mRNA therefore suggested its potential use as a biomarker for detecting slaughter pain in electro-stunned sheep.

Key words: Biomarker· Merino· stunning.

Introduction

Advances in biotechnology is behind the discovery of molecular markers and its applications in many fields of science. Biomarkers are detectable from biological samples as indicators of normal physiologic activities, pathogens or response to therapeutic interventions under *in vitro* or *in vivo* conditions. A panel of candidate markers from blood or body tissues have been detected for pain quantification using high-throughput omics-technologies. Ubiquitin C-terminal hydroxylase (UCH-L1), substance P (SP), protein S-100, neuron-specific enolase and glial fibrillary acidic protein are typical for understanding neuronal damage or traumatic pains. However, UCH-L1 is one of the most abundant and highly expressed brain-specific protein, which is present and localised in the neurones and neuroendocrine cells in vertebrates. Brain specificity of this biomarker suggests its concentration in the brain more than other tissues (Day & Thompson, 2010; George, et al., 2011). Under conventional, kosher or halal slaughter procedures, meat species still experience pain which invariably affects the humanness of animal slaughter and quality of meat. Although the mechanism of actions and signalling pathways of many biomarkers have been identified yet, there is still scanty evidence on pain expression by meat species during slaughter. Understanding the severity of slaughter pain and its correlation with animal stunning will help in improving stunning efficiency and processes involving conventional, religious or traditional slaughter methods. For the first time, real time quantification of ovine Ubiquitin C-terminal hydroxylase (ovUCHL1 mRNA) was used to determine pain expression by sheep following slaughter at a high throughput abattoir.

Materials and Methods

The Research Ethics Committee of the University of Fort Hare (UFH/UREC, 7-REC-270710-028) granted approval for the execution of this study. Blood samples (5-10ml) were collected from the jugular veins of merino castrates (n=30) and ewes (n=30) during slaughter and stored in ultra-low freezer (-80°C) prior to ovUCHL1 mRNA assay. Total mRNA was rapidly extracted from the blood samples using Zymo whole-blood RNA MiniPrep™ kit (Zymo Research Corporation, Irvine, CA 92614, U.S.A.). The kit was chosen for having the ability to extract high quality RNA (A260/A280 >1.8, A260/A230 >1.8) that are suitable for all downstream RNA-based manipulations. Master Mix was used with real-time thermal cyclers [LightCycler® 480 SYBR Green I Master LightCycler® 480 instrument] using two-steps Real Time polymerase chain reaction (qPCR) for quantitative determination of ovUCHL1 mRNA. Partial mRNA sequences from GenBank accession (*Ovis aries* UCH L1-S27), a protein mRNA and partial cds (ACCESSION AY566307, VERSION: AY566307.1) were used to BLAST the long Expressed Sequence Tags (EST) to represent nearly the full-length of mRNA sequences.

Results and Discussion

In Table 1, the GC-content of 58.33% for forward primer and 54.17% for reverse primer were within the normal range of 45-60%. This GC range depicts a high annealing strength affirming that the GC-content for forward primer and reverse primer were within the normal GC range of 45-60%. While the derivative reporter (-Rn) value was higher (86.94°C), that of primer-dimer had characteristically lower Tm value of 65°C and thus implying that at a higher inflection point (Tm), 50% of the primer was annealed.

Table 1 Primer used for Ovine Ubiquitin C-terminal hydroxylase (ovUCHL1 mRNA) quantification

Primer characteristics	Template strand	Length (bp)	Start	Stop	[a]Tm	[b]GC (%)
Forward primer	Plus	24	19	42	59.69	58.33
Reverse primer	Minus	24	231	208	59.71	54.17
Product length	217					
Two primers complementarily	Max complementarity in continuous: 3 bp, free energy= 1.50 Kcal/mol 5'-TCCGGGTCTCATCTGTCTCCTCCT-3' ||| 3'-ACCGTTCGTTGACCTTCTACCTGC-5'					
Two primers complementarily	Max complementarity in discontinuous: 8 bp 5'-TCCGGGTCTCATCTGTCTCCTCCT-3' || || ||| | 3'-ACCGTTCGTTGACCTTCTACCTGC-5'					

[a]Tm: Melting temperature; [b]GC: Guanine-Cytosine

Using SYBR Green Master mix in the temperature-dependent dissociation between DNA-strands showed a typical primer-dimer formation (Figure 1). The dissociation curve in Figure 1A implies that 50% of dsDNA fragment from sheep that exhibited slaughter pain was attained at 82.13^{o}C melting point (Tm). Negative melting curve showing absence of ovUCHL1 mRNA expression in slaughtered sheep has Tm value of 76.1^{o}C which is typical of dissociation reaction with a baseline fluorescence signal (Figure 1B). A high degree of interactive stability between primer-target gene and a rise in absorbance intensity thus produced ideal single stranded UCHL1 mRNA amplicon. It is obvious that the fluorescence signal produced was characteristic of a curve consisting of baseline region, exponential growth and linear phases. Also, positive amplification occurred at 11.25ΔR after successive thermal cycling. The presence of single curve at the peak of amplification showed the absence of contaminating products such as DNA or primer-dimer that could have appeared as additional peaks, different from the desired amplicon.

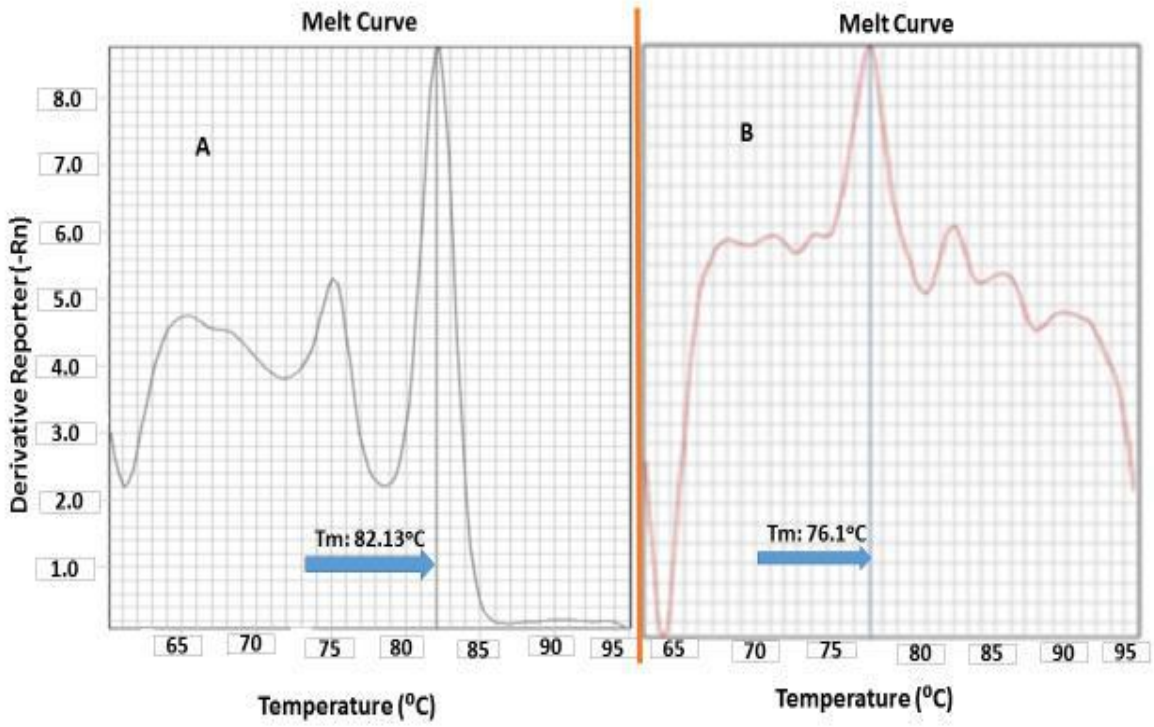

Figure 1 Melt curves showing presence and absence of UCHL1 mRNA expressions by stunned Merino sheep [Note: A=Presence of pain expression & B= Absence of pain expression]

From these results, it can be deduced that Tm value depends on the length, sequence order and GC content of the dsDNA fragment. Thus, supporting the fundamental principle that the fluorescence intensity proportionally increases with the amplicon (dsDNA) concentration in real-time polymerase chain reaction. The melt peak-resolution and amount of UCHL1 mRNA product from the animals followed a pattern that was expected to

validate the expression of this biomarker from any biological sample (Wilhelm & Pingoud; 2003). Generation of a single product especially, ovUCHL1 mRNA during a melt run suggested an association between 'time-temperature binding pattern' of the SYBR green 1 and the growth of the peak. This result is similar to previous findings by Aniko & Delano (2006). Also, the use of intercalating dyes such as SYBR Green1 for melting curve analysis, was in tandem with a report on amplicon detection and differentiation by Papa *et al.* (2010).

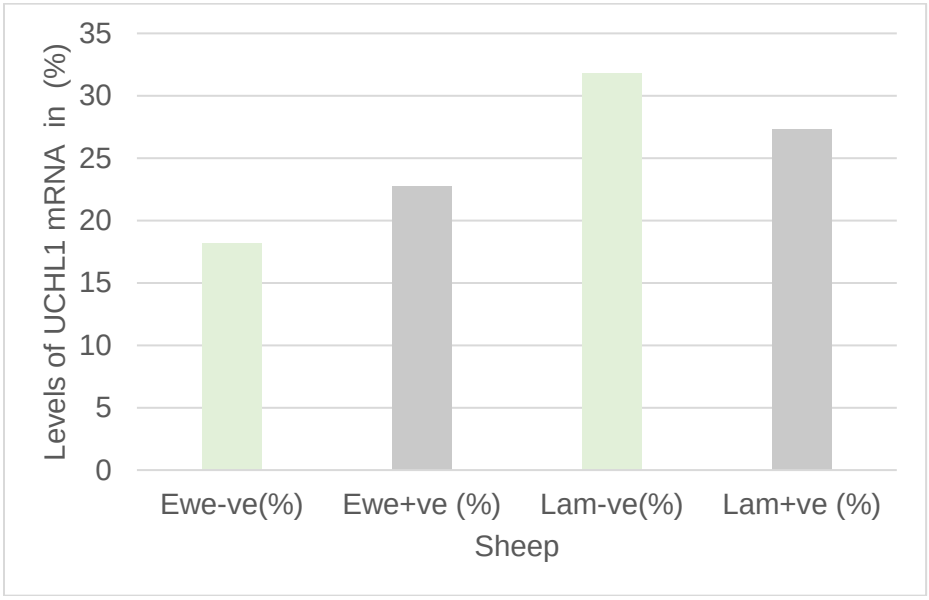

Figure 2 Comparative expressions of UCHL1 mRNA by merino sheep after stunning at the abattoir [Note: Lam-ve (%) and Ewe-ve (%) = Lambs and Ewes that did not express ovUCHL1 mRNA; Lam+ve (%) and Ewe+ve (%) =Lambs and ewes that expressed ovUCHL1 mRNA]

The post-stunning expression of ovUCHL1 mRNA was not the same among all the stunned sheep (Figure 2). While the expression of this marker was relatively higher from lambs (27.27%), the value obtained from ewes (22.72%) was lower. This result can be linked with variations in electro-dermal activities of the stunned animals. Observation during exsanguination suggested that some factors influenced animal's sensitivity to pain and initiation of ovUCHL1 mRNA expression. The amplitude of stunning current was an average of 137.50amps and most of the sheep were held within 10-20 seconds during stunning before exsanguination. Some sheep were stunned more than once due to mis-stunning, emotional excitability, poor head-stunner conductivity or improper contact between the animal and head-stunner. Current passing through the brain during stunning causes substantial depolarisation of the neurons and consequently triggers neuronal excitability across the brain cells (Farouk, 2013). Animal age at slaughter, body weight, intra-muscular fatness deposition, degree of dehydration and amount of wool on the animal can affect the impedance or amount of voltage-current required to cause grand mal seizure.

Conclusion

It is evident from this study that ovine Ubiquitin C-terminal hydroxylase is a potential biomarker for detecting slaughter pain in electro-stunned sheep. Real time quantification of this marker revealed that ewes and lambs react in a different way to electric insults during slaughter. Future studies should focus on correlation of slaughter pain with meat quality and even compare different slaughter or stunning methods for the same animal species. Doing this will assist in characterising pain sensitivity of merino genotypes and develop stunning efficiency models that can give top meat quality traits.

Acknowledgement Authors are grateful to Govan Mbeki Research and Development Centre, Red Meat Research and Development [RMRD-SA] and National Research Foundation [NRF] of South Africa for support on this study.

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Evidence of green fluorescent protein and growth hormone expression in red abalone (*Haliotis rufescens*) larvae

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Abstract

The red abalone *Haliotis rufescens* is highly appreciated mollusk in the national and international markets. Due to its natural over-exploitation and low growth rate, aquaculture research has made special efforts in improving its production process. One of the main objectives of abalone producers is to increase their growth in a shorter period of time in order to elevate its production due to the fact that it takes four to five years to reach commercial size. This investigation explores the use of green fluorescent protein (GFP) fused with growth hormone (GH) using pAcGFP1-N vector and CMV promoter. *H. rufescens* sperm was electropored using three voltages (0.5, 0.75 and 1.0 v). The highest expression of GFP-GH (40%) was obtained at 0.75v. No significant differences ($p < 0.05$) were found in the fertilization rate experiments. This rapid methodology will provide the basis for the modification and/or over expression of different key genes in the growth or regulation process of red abalone using a potent CMV promoter.

Keywords: • mollusk • GFP • transfection • CMV promoter • electroporation.

Introduction

The abalone adductor muscle or foot is highly appreciated as a delicatessen worldwide. However, their wild population has been overexploited ~~it~~, so, rearing methods for abalone have been developed and now there are more countries interested in its cultivation. One of the main drawbacks facing the abalone cultivation process its slow growth rate; farms must wait until the abalone reaches commercial sizes (between 7-8 cm of shell length and 80 g of weight) and this can take up to 4 to 5 years. In the last decades, genetic modifications have been used to try to resolve their growth problem creating hybrids, polyploidy or transgenic abalones (Elliot, 2000). Transgenic technics have improved over the years especially for vertebrates, however, they have not been standardized to marine invertebrate cells. The sperm-mediated gene transfer (SMGT) using an electroporation device has been of great utility and success as a gene vehicle, compared with eggs and larvae electroporation (Lavitrano et al., 2013). Sperm electroporation has been used successfully in *Haliotis diversicolor* (Tsai et al., 1997; Tsai, 2000) and *Haliotis iris* (Sin et al., 1995). However, it has never been used in *Haliotis rufescens*. Electroporation, as a mass gene transfer, is a very simple technique that allows the introduction of exogenous

DNA into cells; however, it is a method that should be optimized for each cell type. That is why, this paper successfully presents the standardization of sperm electroporation with an expression vector that contains a fish Cobia-GH, GFP as a marker and CMV promotor using the MicroPulser-Biorad electroporator.

Materials and methods

Abalone gametes were obtained from commercial farms located at Eréndira, Baja California, México. Plasmid pACGFP1-N ready vector (CLONTECH ®) was used. The synthetic sequence of cDNA of Cobia-GH (GenScript, USA Inc.; NCBI, GenBank ID: GQ861507.1) was attached to the vector via ligase independent cloning (Jeong et al., 2012). Three positive clones were sequenced to confirm the correct ORF's. Sperm (5×10^6 cel mL^{-1}) was electropored in filtered sea water with voltages of 0.5, 0.75 and 1.0 Kv, two pulses of 0.5 millisecond, into 0.4 cm cuvettes, in a total volume of 1 mL 500 ng of circular form of plasmid DNA was added, except to control, experiments were done by triplicate. Fertilization was done in a 1:100 oocyte-sperm ratio. After that, they were incubated with constant aeration at room temperature (18-20 °C). Samples were collected after 2 h of being fertilized to assess the fertilization rate and 48 h after to evaluate in vivo expression. GFP expression was observed and register in a confocal microscope after 48 h to be fertilized (Olympus IX81). PBS prewashed pool of larvae was used to purify DNA for PCR. Western blot was done with 5 µL of defrosted larvae pellet from each treatment and control samples. Fish Cobia-GH protein was detected by Western blot using polyclonal anti-GH, followed by donkey anti-rabbit IgG peroxidase conjugate and TMB stabilized substrate for HRP. Data were analyzed by Kruskal-Wallis test and a multiple comparison Z' test. A $p < 0.05$ was chosen as a significance level (STATISTICA 7.1 software, StatSoft, Inc. 1984-2005).

Results and discussion

Average fertilization rates were of 75 to 80% for the three treatments and control. Eggs fertilized with electropored sperm demonstrated no significant difference as compared to the control ($p < 0.05$). Transgene expression analysis measured by fluorescence in larvae was positive in an average of 9.7% for 0.5 Kv, 40% in 0.75 Kv, while in 1.0 Kv was 27.7% (Figure 1, a-f). Fluorescence was observed evenly in the cells of the larvae but not on the control group (Figure 1, g-h). Fluorescence between treatments showed significant differences.

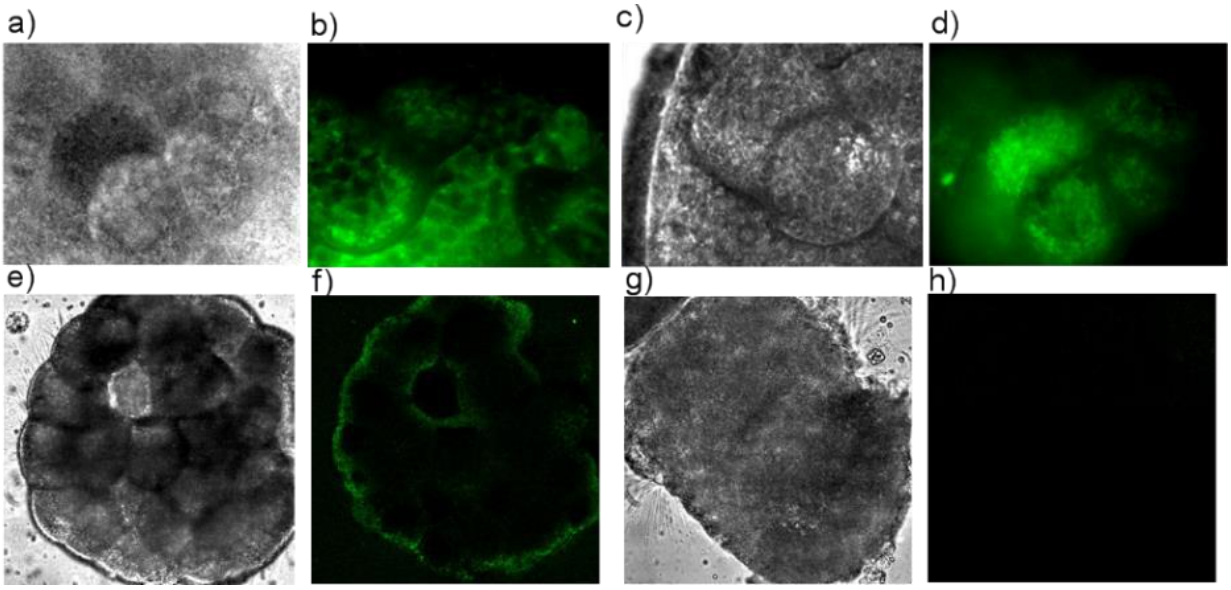

Figure 1. GFP-GH expression; a)- b), c)-d), e)-f), positive larvae, g)-h) negative control, without and with fluorescence excitation.

Fish Cobia-GH protein was observed by Western blot (WB) obtaining an intense band near to 35.8 KDa in each of the three treatments (lines 1-3); negative results were obtained in the control sample (line 4, Figure 2).

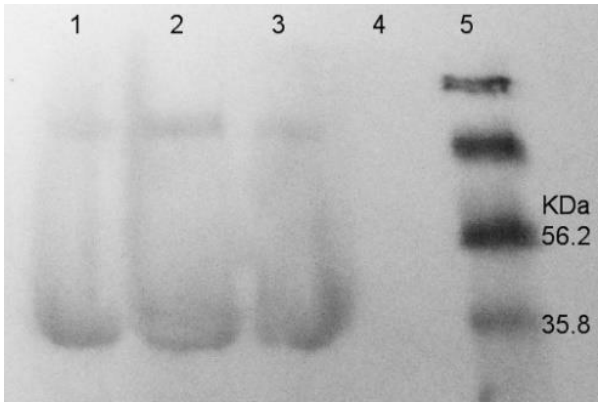

Figure 2. WB showing positive band at 35.8 KDa; 1) 0.5 v, 2) 0.75 v, 3) 1.0 v, 4) negative control, 5) protein marker.

The transgenesis evaluated by PCR from each treatment resulted in a positive band of 642 bp and 711 bp for GFP (data not shown). The transgenesis rate obtained in this study is quite similar to those reported in others aquatic animals using sperm as a vehicle (amphioxus 15% (Yu and Peijun, 2005), fishes 23-38% (Li et al., 1996; Sin et al, 2000), abalone 28-65% (Tsai et al., 1997; Tsai, 2000). Previous studies have shown that sperm electroporation made in fishes and marine invertebrates (Sin et al., 1995; Yu and Peijun,

2005; Kuznetsov et al., 2001) was done using voltages between 0.2 to 4 Kv, except for abalone sperm, where the voltage was increased to10 Kv (Tsai et al., 1997; Tsai, 2000). Since we observed that abalone sperm lost mobility as the voltage was increased, the decision was made to use voltages up to 1 Kv. On the other hand, most of the SMGT and electroporation studies used PCR, Southern blot and enzyme activity as a transgenesis detection technique. A few invertebrate studies exist that report GFP as a live expression marker, such is the case of amphioxus (Yu and Peijun, 2005) where GFP was expressed in 15% of the larvae when the sperm was electroporated with 0.5 Kv, in contrast, our results show a success rate of 40% using 0.75 Kv. In conclusion, we can state that the use of electroporation-SMGT and GFP as a marker in the expression of genes in the red abalone *H. rufescens* is a useful technique to determine if the expressed protein is uniform or in mosaicism into the cell embryos. This rapid methodology will provide the basis for the modification and/or overexpression of different key genes in growth regulation of red abalone.

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Antibacterial properties from fungi isolated from marine sediments

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Abstract

Marine fungi biodiversity as new precursor of biotechnology products are poorly studied compared to terrestrial counterpart who has been used as a rich source of bioactive compounds. Since their importance, we presented here the first fungi report from sea sediments of twenty-six stations at Southern California gulf, Mexico. Seventeen selected halotolerant fungi were isolated and identified by sequencing the ITS 1 and 2 genes. Identified fungi belonged to the phylum Ascomycota and Basidiomycota (16 and 1 strains, respectively). *Cladosporium* was represented by 29%, *Aspergillus* by 24%, *Talaromyces* by 12% and other species by 35%. Two fungi showed antibacterial activity against *E. coli* and *S. aureus*.

Keywords: • marine fungi • antimicrobial •Ascomycetes • ITS.

Introduction

Marine microbial biodiversity has been underestimated, since there are few organisms that could be cultured. Metagenomics studies are now discovering new species however; marine fungi are less known than marine bacteria. Sea sediments represent an extreme environment to microorganisms since they have reduced solar irradiation, low temperatures and high atmospheric pressure. Those conditions could be responsible for genetic and metabolic adaptations to produce interesting bioproducts. Marine fungi studies have been registered different metabolites properties such as: i) anticancer, ii) antibacterial, iii) antiviral, iv) antifungal and v) antimalarial. Those molecules with biological activity where isolated from the fungi genus: *Curvularia*, *Trichoderma*, *Cladosporium*, *Holorosellinia, Ascochyta, Stachybotrys, Phoma, Alternaria, Stillbella, Aspergillus, Emericella, Penicillium, Microsphaeropsis, Zopfiella* and *Fusarium* (Hasan *et al*., 2015). Since there are only a few marine fungi reported from Mexican sediments. The objective of this study was isolate and identified the fungi biodiversity of Southern California gulf sediments. Also, they were analyzed to antibacterial activity.

Materials and methods

Sediment samples were taken from 26 stations at Loreto Bay. The stations were choose based on previously study from Becerril-Espinosa *et al*. (2013), where they found new actinobacteria species with biological activity. Sediments were taken from 43 to 311 m deep using a modified Kahlsico grab sampler (model # 214WA110). Each sample was transferred to a sterile bag (Nasco whirl-pack) and stored at 4 °C. Before use, sediment samples were dried in a laminar flow hood for 24 h. Sample sediments were diluted 1:10 in

2% saline solution and 0.5% glucose. Dilutions were incubated for 1 h at RT. Then, they were inoculated in YEPD medium (1% Yeast extract, 2% peptone, 2% dextrose, 2%, agar) and ampicillin. Samples were also inoculated in YEPD with 50% of sea water. Plates were grown for two months and examined every 48 h. Mycelia were transferred to new YEPD with 100% of sea water. Only the fungi that grow under the last condition were selected. They were observed under stereoscopic microscope (Carl Zeiss® Semi DV4) and optical microscope (Motic® B1). Fungi strains were label from C1 to C42. Experiments were done by duplicate. Isolated strains were selected by their salt tolerance and divided in 4 categories: No halotolerant - unable to grow in medium with sea water; lightly halotolerant - able to grow in medium with 50% or less of sea water; halotolerant - able to grow in medium with 0 to 100% sea water and halophyte - do not grow in medium without sea water. Broth culture mycelium was collected after seven days to purified DNA using Michiels *et al*. (2003) protocol. PCR reactions were performed in 25 µL volume; 1X Go-Taq green master mix (PROMEGA®), 10 µM of each primer (ITS 1F 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS 2R 5′-GCTGCGTTCTTCATCGATGC-3′), 20 ng of DNA, 10 µM of BSA, final concentration. Amplicons were from 200 to 235 bp. Amplification condition were 1 cycle of 3’ at 95 °C, 30 cycles of; 1’ at 95 °C, 1’ at 59 °C, 1’ at 72 °C followed by a final extension of 10’ at 72 °C. PCR amplicons were verified by 2% agarose electrophoresis and visualized by UV light. They were purified and sequenced by Allele biotech at San Diego CA, USA. ITS sequences were subjected to BLASTn search to confirm specific amplification and similarity with other fungi. Sequences were aligned using MUSCLE from MEGA 7.0 (Kumar *et al*., 2015), phylogenetic tree were done based on 1000 repetitions of bootstrap and Jukes and Cantor model (1969) using the maximum likelihood method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (6 categories (+G, parameter = 6.4873)). All sequences were deposited in Genbank (NCBI), their accession number is next to the strain label in figure 1.
Antimicrobic sensitivity testing were done using collected broth after 8 days fungi culture. Sterile 0.6 mm Whatman™ paper disc was moist with collected broth. Discs were deposited on Muller Hilton agar plates, previously spread with pathogenic bacteria (*E. coli* (ATCC ®11775™), *Staphylococcus aureus* subsp *aureus* (ATCC®25923™) and *Pseudomonas aeruginosa* (ATCC®27853™). Those were incubated by 48 hours at 37°C. Once the incubation time was elapsed, the inhibition diameter was measured and registered. Each assay was performed by duplicated. Results and discussion A total of 38 filamentous fungi were isolated from 26 stations. We had a good amount of cultural fungi recovery from sea sediments compared to other authors. Singh *et al*. (2010) isolate 28 fungi from 496 sediments, Damare *et al*. (2006) obtained 181 fungi from 672 sediments and Singh *et al*. (2010) 28 fungi from 20 sediments; all samples were from the central Indian basin. Singh *et al*. (2010) suggested that differences on recovery must rely on oceanographic conditions, nevertheless we also observed dependency to the culture media, antibiotic, darkness and temperature. Salt tolerant strains frequency was: 26% No halotolerant; 29% lightly halotolerant; 11%, halotolerant and 34% halophytes. Taking, the classification purposed by Kohlmeyer & Kohlmeyer (1972), the no halotolerant and lightly tolerant fungi were consider as no marine; the halotolerant as facultative marine and the halophytes as marine. We were able to identify 14 strains by sequencing the ITS1-ITS2 region and we couldn’t three strains since we didn’t get any amplification, even when we tested others fungi universal primers (ITS4, NS1, NS2, ITS5, LR5, LROR, nuSSUF, nuSSUR) those were only identified by mycelia morphology as *Cladosporium* (C7, C14

and C37). In figure 1 we present the phylogenetic tree of the isolated fungi. Most of them belonged to the phylum Ascomycota (16 strains), they were clustered into six genera (*Aspergillus, Cladosporium, Chaetomium, Epiccocum, Talaromyces and Phialosimplex*). Two of them were identify only to the order level (Pleosporales and Microascales) and only one to Basidiomycota (*Pheniophora).* The highest frequency of the phylum Ascomycota in marine media has been previously reported (Singh *et al*., 2010; Raghukumar and Damare, 2008; Damare *et al*., 2006) as it was in this study were we found *Cladosporium* and *Aspergillus* (29% and 24%, respectively). All fungi founded in this study have been previously reported in marine samples, however Phialosimplex is the first report in México. These species was reported by first time in 2014 in Germany salt mines. It is interesting that it best growth conditions was at 15% NaCl media as Greiner *et al*. (2014) reported, these osmotic characteristics can be exploited later to be found new genes that can provide saline tolerance to plants. Reports from Mexican marine fungi started in 1968 by Kohlmeyer, who isolated fungi from mangroves, González *et al*. (1998, 2000, 2001) from coastal beaches. It's important to emphasis that all Mexican fungi previously reported were identified only by morphological characteristics. Valderrama *et al*. (2016) identify by molecular techniques non culturable fungi from different waters sources, including marine, however they only found in common to this study, Aspergillus and Pleosporales. Others studies in México reported *Aspergillus, Cladosporium* and *Scopulariopsis (González et al., 1998; González et al., 2000), Phoma* (Kohlmeyer, 1968) in marine samples. We report by first time the presence of *Talaromyces, Phialosimplex, Epiccocum, Chaetomium and Pheniophora* in Mexican marine sediments. Antibacterial activity was shown by two strains: C38 KX219724 identified as *Epiccocum* inhibit S*. aureus* (inhibition diameter 155 mm) and *Peniophora* (C11 KX219725) against *E. coli* (inhibition diameter 145 mm) (Figure 2). Baute *et al*., (1978) isolated the antibiotic epicorazine A and B from *Epiccocum nigrum* strains, moreover Xing *et al*. (2010) and Henriquez *et al*., (2014) reported that this species produces an antibiotic that showed a superior activity than ampicillin sodium when it was tested against *S. aureus*. We must to say that those species were isolated from marine algae and sponges and not from sediments as us. The bioactive compound, Masmaric acid has been isolated from *Peniophora species*, specifically *P. latea* (Kupka *et al*., 1982) and *P. affinis* (Gerber *et al*., 1980), they produces Peniophorin A and B, and those compounds showed a broad antimicrobial activity against gram positive and negative bacteria. Another antibiotic molecules are Drosophilin A and Drosophilin A methyl Ether, both of them purified from *P. fastuosa* (Teunissen *et al*., 1997).

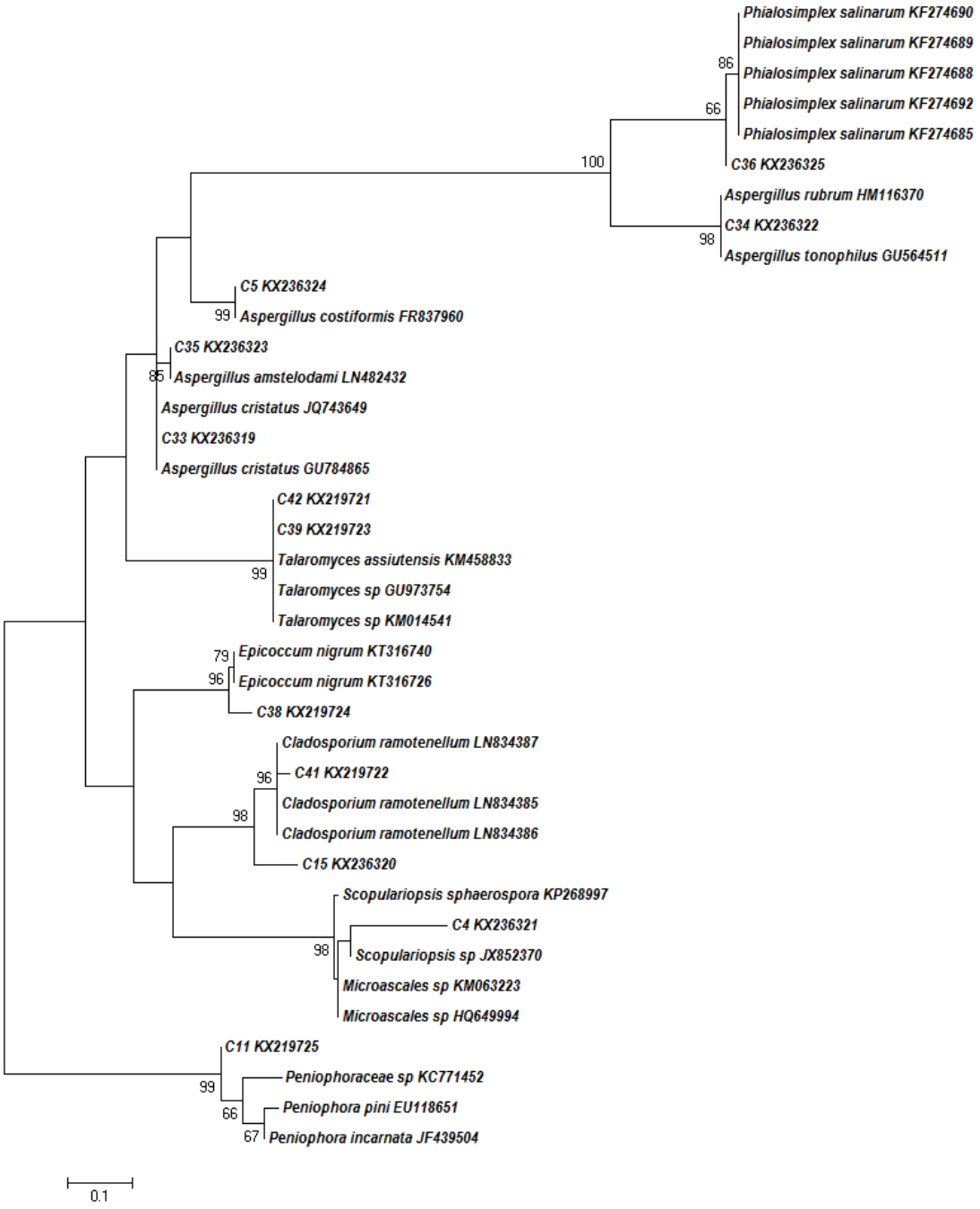

Figure 1. Molecular Phylogenetic tree based on maximum likelihood method

Biodiversity of marine fungi in sea sediments represent a versatile reservoir of bioactive metabolites such as antibiotics, anti-inflammatory, anti-cancer, anti-viral among others, that could be potentially used in biotechnology that is why this study focus in the isolation and identification of such interesting microorganism. We reinforced the importance to continue with this kind of studies in Mexican ecosystems.

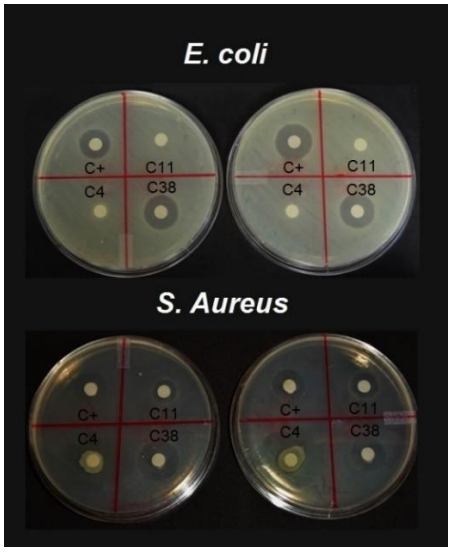

Figure 2. Antimicrobic sensitivity testing showing fungi strains with positive inhibition (C38 and C11); negative C4; C+ positive control (*Penicillium chrysogenum*)

Acknowledgements

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Procedure for the identification of sexual differentiation genes in *Hemichromis guttatus* females

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Abstract

The identification of sexual DNA markers has served many purposes, from basic knowledge on sexual development and differentiation, to the assessment of sex reversion of hormonal exposed fish. In the present study several candidate genes were analyzed to be validated as sexual markers for the cichlid *Hemichromis guttatus* a well-known invasive species whose genome or any genetic information is not yet known and that has important ecological impacts in a natural protected area characterized by several endemisms. A way to eradicate invasive species is the Trojan Y-Chromosome hormonal strategy in which males with two Y chromosomes are breed with females of the invasive population to lead to the decline or disappearance of the invasive population. The identification of candidate marker genes in the cichlid genome was confirmed by PCR essays using genomic DNA, and the expression of these same genes using mRNA extractions. Genomic material was extracted from gonadal tissue from *H. guttatus* specimens, regardless of their sex, size or age. Four candidate genes have been selected for sexual identification of phenotypically differentiated females, and thus primers for the orthologues genes: *Foxl2a*, *Figla*, *Wnt4a* and *Ctnnb1b* have been obtained. All genes have been successfully amplified using both nucleic acids.

Keywords: *Foxl2a* • *Figla* • *Ctnnb1b* • *sexual identification* • *Cichlidae*.

Introduction

Hemichromis guttatus, commonly known as jewel cichlid, is an ornamental fish native to Africa that has been introduced to different regions of North America, including the Natural Protected Area Cuatrociénegas of Coahuila, Mexico, and Austria. At the present it represents an evident threat to the local biodiversity, as a result of its high reproduction rate, its aggressive behavior and its ability to outcompete endemic naïve native species that constitute almost the totality of its diet. Consequently, native populations have drastically declined Until now, efforts to continuously fish the invasive populations have been unsuccessful (Área de Protección de Flora y Fauna Cuatrociénegas, 2008), mostly as a result of their high reproduction rate and larvae survival. Considering this context, the development of strategies leading more effective and long-lasting results is paramount. One of these solutions relays in the Trojan Y-Chromosome strategy which consists in exposing invasive males to female sexual hormones so as to obtain males with two Y chromosomes (YY), which after breeding with females of the invasive population will result in a male-dominant population that will eventually lead to the decline or disappearance of

the invasive population. A crucial step in this strategy is the identification of genetic sexual markers for the early and precise identification of reverted males as well as the offspring that carry the YY chromosomes. Identification of sexual markers poses a particular challenge as they are sex specific and they vary among species. In the present study genes involved specifically in female gonadal development in the cichlid *Hemichromis guttatus* were analyzed in order to identify sexual markers for this species.

Material and Methods

Wild invasive jewel cichlid was caught and transported to the Ecophysiology laboratory, FCB, UANL. A total of 10 adult males and 10 females were selected for gonadal tissue extraction. Fish size ranged from 3 to 5cm. For genomic DNA (gDNA) extraction, 100mg of muscle were dissected and placed in 15ml Falcon tubes containing of 70% ethanol to cover the whole tissue sample. Thereafter a traditional phenol:chloroform extraction was performed (Chen, et al., 2007). Because of the high content of lipids in muscle samples several extractions were needed. Gonad samples ranging from 20 to 50mg were placed in 1.5mL Eppendorf tubes containing RNAlater solution (enough to cover the whole tissue), for the subsequent extraction of total RNA using either the trizol method (Sambrook and Russell, 2006) or columns of the RNeasy® Kit by Qiagen. For the preparation of cDNA from the RNA extraction the Accuscript (Qiagen) protocol was used employing reagents from the Omniscript Kit (Qiagen). Finally, DNA, total RNA and cDNA were stored at -20 °C until further use. The resulting genomic material was used in PCR reactions that were prepared inside a Telstar Bio II Advance biosecurity cabinet, and then were run in either an Axygen Maxygen Thermocycler from Axygen Biosciences or a T100 Thermal Cycler (BIORAD) for the amplification essays. The standard PCR protocol consisted of 3 min at 95 °C, followed by 35 cycles under the following conditions:Denaturation: 95 °C for 30 seconds; Annealing: optimum temperature for each pair of primers for 30 seconds; Extension: 72 °C for 30 seconds. Finally, after the 35 cycles a final extension of 72 °C for 1 minute was performed. Results were visualized using a Gel Doc EZ Imager (BIO-RAD). Oligonucleotides used for the PCR essays of each candidate gene were taken from Böhne *et al*., (2013). And their synthesis was requested to T4OLIGO-Novik. Once the genes were amplified purification protocols directly from PCR reactions followed, using a Qiaquick Kit from Qiagen, while for products treated from agarose gels a QiaEX II Kit was used. Purified products were used for the cloning essays using different protocols: preparation of calcium competent cells (DH5-α), ligation, transformation and preparation of LB medium with ampicillin IPTG and X-Gal, from Cloning plus kit (Qiagen). And from this clones a Sanger automatic sequencing protocol is nowadays being performed for obtaining the genomic sequences of some candidate genes.

Results and Discussion

PCR protocols were carried out for the amplification of orthologue genes, *Ctnnb1b*, *Figla*, *Wnt4a* and *Foxl2a*, in gDNA and cDNA, aimed at verifying the presence of the markers in females of H. guttatus. For the standard PCR protocols, performed with gDNA and cDNA, the temperature gradient selected for the annealing step was based on the melting temperature for each primer pair, obtained by the formula (A+T) 2 + (C+G) 4 = Tm – 5, and by oligo analyzer from IDT.

All fragments could be amplified in both templates and ranged within the molecular weight estimated by *in silico* essays with the orthologue gene sequences of all genes, in this way the best temperature to amplify only one product for every gene was selected. PCR protocols started with the gDNA template to find out if the genes were present in *H. guttatus* genome, and once the amplification in gDNA was achieved cDNA was used for exploring gene functionality or gene expression purposes. Fig. 1 shows the amplification product of *Wnt4a* as an example of a positive PCR assay.

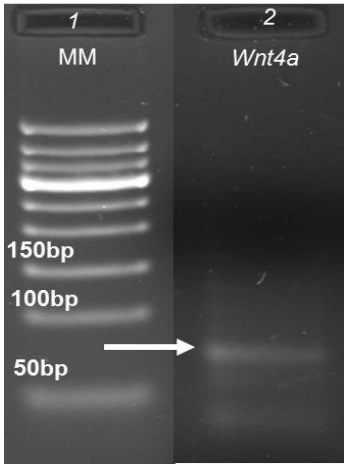

Figure 1. 2% Agarose gel stained with ethidium bromide showing standard PCR protocol results from reactions for the *Wnt4a* gene. Lane 1 (MM) corresponds to a 50bp molecular marker. Lane 2 shows the *Wnt4a* amplified product with an estimated size of 75bp, using cDNA as template.

Once the amplification of the products succeeded, purification from PCR reactions followed, as above mentioned, and the results are shown below (Fig. 2).

Purified PCR products

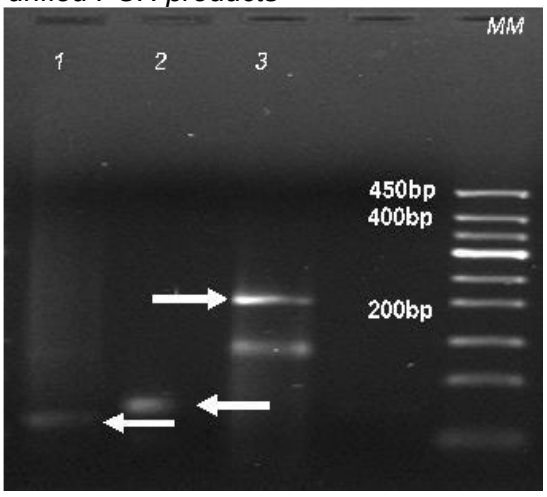

Figure 2. 2% Agarose gel stained with ethidium bromide showing purification results from PCR reactions of *Foxl2a*, *Figla* and *Ctnnb1b* genes. Lane 1 shows the *Foxl2a* amplified product with an estimated size of 59bp. Lane 2 shows the *Flgla* amplified product with an estimated size of 70bp. Lane 3 shows the *Ctnnb1b* primers with an estimated size of 200bp. An extra fragment near 150pb is observed as a result of a possible degradation of the gene since PCR assays usually demonstrate only one amplified product. Lane 4 (MM) corresponds to a 50bp molecular marker.

Once the products were purified cloning assays were initiated for sequencing. To date, only the results from the *Ctnnb1b* gene are available, and as stated in the methods section a cloning protocol where *E. coli* DH5α were transformed with the purified gene ligated to

pDrive plasmid was used. Several cell cultures were performed, and those colonies thought to be successfully transformed according to the cloning kit instructions were selected, until the transformation was fully achieved. Once this was accomplished a standard plasmid purification was done. Two PCR protocols were performed using the purified plasmid as template, one with *Ctnnb1b* primer pairs and the other with plasmid primers. PCR assays were done to verify if the plasmid contained the fragment of interest. Using *Ctnnb1b* primers it coul be observed that the gene was correctly ligated in the plasmid agreeing with the molecular weight of the product previously estimated in other PCR assays, and with the plasmid primers. Taken into account the size of the gene plus the size of the cloning site of the plasmid, it could be confirmed that the fragment was ligated to the plasmid. Both PCR results helped to identify the gene of interest as a function of its estimated molecular weight and to assess that it was ligated to the plasmid by comparing and adding the molecular weight of both plasmid cloning sites and the gene fragment.

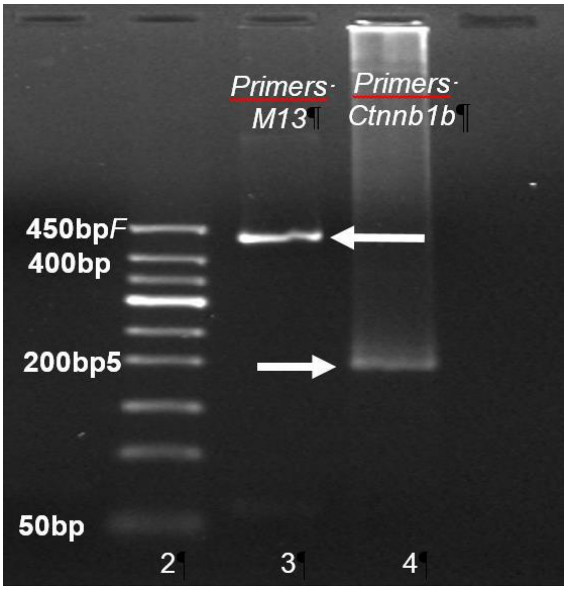

Figure 3. Agarose gel at a 0.8%concentration, stained with ethidium bromide. Lane 2 shows a molecular marker of 50bp. Lane 3 represents the PCR product obtained with the primers from pDrive plasmid showing a weight close to 438bp corresponding to the estimated weight resulting from adding the 238bp cloning site of the plasmid to the 200bp of the fragment to which it was ligated to, Lane 4 shows a PCR product obtained using *Ctnnb1b* primers where the product of the gene can be seen at 200bp.

In this way the results of PCR protocols using both gDNA and cDNA as templates were useful to confirm that the orthologue genes selected, *Ctnnb1b*, *Figla* and *Foxl2a*, were in fact present in the genome and expressed in *Hemichromis guttatus*. And even though the specific sequence of every gene is still unknown it seems very likely that the primer pairs may not vary. A possible critical observation to the procedure could be the amplification of only one PCR product for every analyzed gene considering that their expression will be measured using qPCR. This imply that if the primers anneal with more than one region on the template the quantifications would not be directly correlated with the product of interest. In the case of *Ctnnb1b,* there is a need to design specific primers once we the gene sequence is obtained.

Conclusions

All orthologue genes are functionally present, meaning that these are actually expressed in *H. guttatus*. The cloning and sequencing analysis of these genes in *H. guttatus* are pending in order to provide new genomic information for the eradication of this invasive species.

Acknowledgements We would like to thank all technical help we have had, especially from Elías Lozano, Sergio Luna and Carlos Barriga, who participated in the capture, maintenance and reproduction of the specimens used in the study. We would want to especially acknowledge CONABIO for the financial support given to the FCB, UANL, project LI003.

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Identification of potential sexual development markers on *Hemichromis sp.* females

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Abstract

The use of DNA markers to identify products involved in sexual differentiation is still under research in some organisms as the *Teleostei* fishes, which are important due to their use for consumption, aquarism, genetic and phylogenetic studies. Even with all these researches about sexual development related genes reported to date, there's still much yet to understand about these mechanisms in important genus as *Hemichromis* given their status not only as a well-known aquarium fish, but as one invasive species that is causing trouble in Florida and México, jeopardizing endemic species. This is why identifying females by genotype and phenotypic expression of certain genes is important to develop a suitable control method and a better understanding for the sexual development on this genus. The presence, fragment amplification and sequencing of *Dax1a* as a specific chromosomal marker will be evaluated along with *CYP19A1a* as a potential female marker and 18s rRNA for its possible use as a Housekeeping gene.

Keywords

Cichlidae • *CYP19A1a* • *Dax1a* • *18s rRNA* • Sexual differentiation.

Introduction

The genus *Hemichromis* refers to sweet water fish species that belong to the *Cichlidae* family. Some of these fish are considered economically relevant, mostly for aquarism, this is why the cichlids *Hemichromis sp.*, also known as African jewel fishes and described for the first time by Peters in 1858, have been vastly used for this purpose. Even though this species is not native in Mexico, it has been detected in the Churince pool, and this is the reason why it's suspected was introduced intentionally or accidentally in early 90's in this region. Due this issue, the CONANP (National Commission of Natural Protected Areas) carries out population monitoring because the species is already considered as invasive, due to its impressive adaptation to the Churince pool environment, endangering the endemic cichlid of this region: *Herichthys minckleyi*. For all this is that the search of control strategies and possible eradication of *Hemichromis* are a priority for the conservation and equilibrium of this ecological system (Área de Protección de Flora y Fauna Cuatrociénegas, 2008). Even though the species presents sexual dimorphism it's hard to phenotypically differentiate between genders, unless they are already in reproductive stage, when males present a brownish color and a bigger size than females, which presents a bold red color. One way to achieve their differentiation gender differentiation is by histologic tests of their gonads, which are clearly differentiated after sexual maturation (Área de Protección de Flora y Fauna Cuatrociénegas, 2008). This difficult identification of

sex is what makes it hard for a population control program to be established, because many of the strategies as the Troyan chromosome and vectors carrying lethal genes require an strict sex monitoring so they assure the environment is not further damaged (Wise de Valdez *et al*. 2011; Cotton & Wedekind 2007). Due this urgent need to establish a control program to stop the growth of the invasive species in the Churince pool at Cuatro ciénegas, it's a priority to identify specific chromosomal genes, that are involved in sexual differentiation and a housekeeping gene to successfully stablish a program for their control.

Materials and Methods

2 Male and 2 female cyclids, all collected from the Churrince pool in Cuatrociénegas, Coahuila on August 8th, 2015.

DNA isolation from Hemichromis sp. Muscle. The extraction was made by the Phenol-Chloroform method reported by Chen *et al.* (2007). The tissue was dissected and kept in EtOH 70% if needed (Doorenweerd & Beentjes, 2012). After the extraction, the final product was kept at -20°C until its quantification and use.

RNA isolation from Hemichromis sp. The isolation was made by the Trizol protocol and the RNAeasy Quiagen kit. The RNA obtained was kept at -20°C until its quantification and use.

Oligonucleotide design and synthesis. Sequences for the *18s rRNA* were taken from the publications of Geng *et al*., (2013) and Burmeister, Kailasanath and Fernald (2007). Primers for the *CYP19A1a* (Genbank ID: 100708217) and Dax1a (Genbank ID: 100534476) gene, were designed with the help of the software SnapGene viewer and NCBI's software: PrimerBlast. The oligonucleotide synthesis was made with the T4OLIGO-Novik department in Guanajuato, México.

PCR protocol. The PCR assays were made to stablish the optimum temperatures for each primer, modifying the conditions according to the results obtained. The primer sequences used were: *CYP19A1a:* Fw: 5´- CAT GAA CGA GAG AGG CAT CA -´3, Rv: 5´- AGA TGT CCA CCA CAG TGC AG -´3. *18s rRNA*: Fw: 5'- GGACACGGAAAGGATTGACAG -3', Rv: 5'- GTTCGTTATCGGAATTAACCAGAC-3'. *Dax1a:* Fw: 5'-TTTGCAGGAGGTCCGACA-3', Rv: 5'-TCCACAACAGAGACGCC G -3'. The DNA concentration used was 50 ng/µl, 1.33µl dNTPs 4 mM, 3 µl $MgCl_2$ 10 mM, 1 µL Primers 5 mM, 1 µL PCR Buffer 10x and TaqPolymerase and a variable amount of MQ-H_2O so PCR assays were made of minimum 10 µl for better results with the CFX96 thermal cycler from Biorrad. The program used consisted on an initial DNA denaturation at 95°C for 3 minutes, followed by a second denaturation at the same temperature for 30 seconds; the annealing was made either in a gradient for the standardization of the primers used, or at the optimum temperature decided for them for 30 seconds, then the extension was made at 72 °C for 30 seconds. All this was repeated for 35 cycles and then followed by a final extension of 2 minutes at 72 °C. All samples were migrated in an agarose 2% gel stained with ethidium bromide.

Retrotransciption, purification of PCR products, cloning and sequencing. cDNA synthesis was needed for the assays and it was achieved by the use of the Accuscript Invitrogen's kit and Omniscript from Quiagen. This cDNA was used as template for the *CYP19a1A* and *18s rRNA* primers to be standardized. The purification of the products obtained for the three candidate genes was carried out using both QUIAQUICK and QUIAEXII Quiagen's kits. Products where cloned with the Quiagen Cloning plus kit and using the pDrive cloning vector using the Ecoli DH5-α strain for the process.

The white colonies were selected and tested with the M13 primers to corroborate the presence of the fragment of interest before being sent for automatic Sanger's sequencing. After this, the sequences were analyzed and curated with *SerialCloner* and *SnapGene viewer*. All sequences were compared with the NCBI and EMBL databases, using related species to corroborate their identity and the primers were adjusted according to their differences with the obtained sequence for their proper use for future q-PCR assays.

Results and discussion

Three candidate genes were selected for this project: *DAX1a, CYP19A1a* and *18s rRNA*. *Dax1a* was selected given its status as a sexual master gene located in the X chromosome in mammals and its shared and conserved functions in fish from the *Teleostei* group (Shirak *et al*., 2006), which made it a potential chromosomal marker for this work to differentiate between males and females. On a similar note, *CYP19A1a*, the gene responsible for the aromatase production was selected, its involvement in the estrogen production and the development of the ovarian ducts in both fish and mammals; also, its expression is higher in the gonads of females from cichlid fishes that are phylogenetically near to *Hemichromis*, and it's relatively low on the few tissues that also present it, which makes it a female specific expression marker (Heule *et al.*, 2014). For this study, a control gene is required to properly evaluate the expression of *CYP19A1a*, this is why *18s rRNA* was selected, it's known as a housekeeping gene that is stable between tissues in cichlids like *Oreochromis niloticus* and *Astatotilapia burtoni* (Geng *et al.*, 2013) in normal conditions, thus is needed to evaluate how it reacts under this experimental conditions. Firstly, it was needed to determine whether the selected genes were or not present in the genome of *Hemichromis sp.*, given the fact the designed primers were either designed with the use of orthologues genes available in the databases or taken from papers that focused on related species due the lack of available *Hemichromis sp.* sequences. The tree genes were present in the genome (Figure 1), and the size they presented in the PCR amplification was the expected for the designed primers for both *DAX1a and CYP19A1a* and the *18s rRNA* primers obtained from Geng *et al.*, (2013). This made possible to continue with the cloning assays for both *Dax1a* and *18s rRNA* to corroborate their identity (Figure 2) and adjust the primers for the PCR and q-PCR assays for highly specific primers for the proper differentiation between sexes; *CYP19A1a* is already cloned but is yet to be sequenced. Only *Dax1a* and *18s rRNA* are were sequenced to verify the identity of the sequence, CYP19A1a is yet to be sequenced.

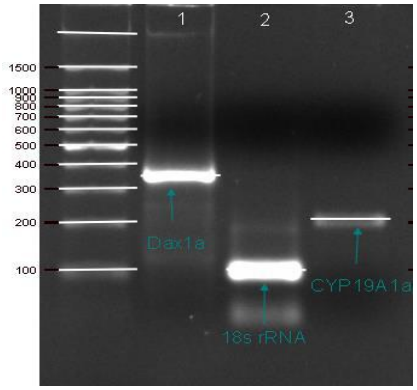

Figure 1: Agarose gel 2% stained with ethidium bromide showing the amplified genes. In lane 1 Dax1a is shown with an approximate weight of 354 pb, in lane 2 18s rRNA is shown with a weight of 111 pb and lane 3 shows CYP19A1a with an approximate weight of 215 pb.

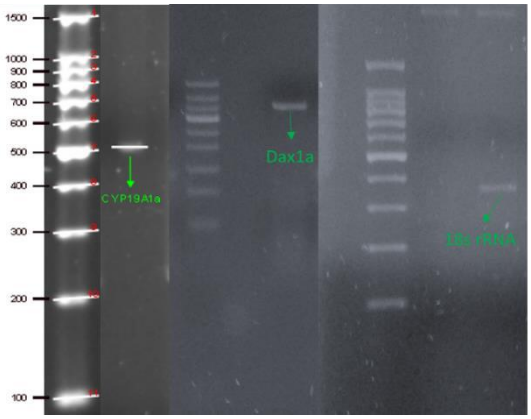

Figure 2: Agarose gel 2% stained with Ethidium Bromide showing the Candidate genes cloned in pDrive plasmid. The cloning site of pDrive has a molecular weight about 231pb, in lane 2 *CYP19A1a* is shown with a weight of 488 pb, lane 3 shows the cloned *18s rRNA* with a weight about 350pb and in lane 4 *Dax1a* is shown with a weight of 585 pb.

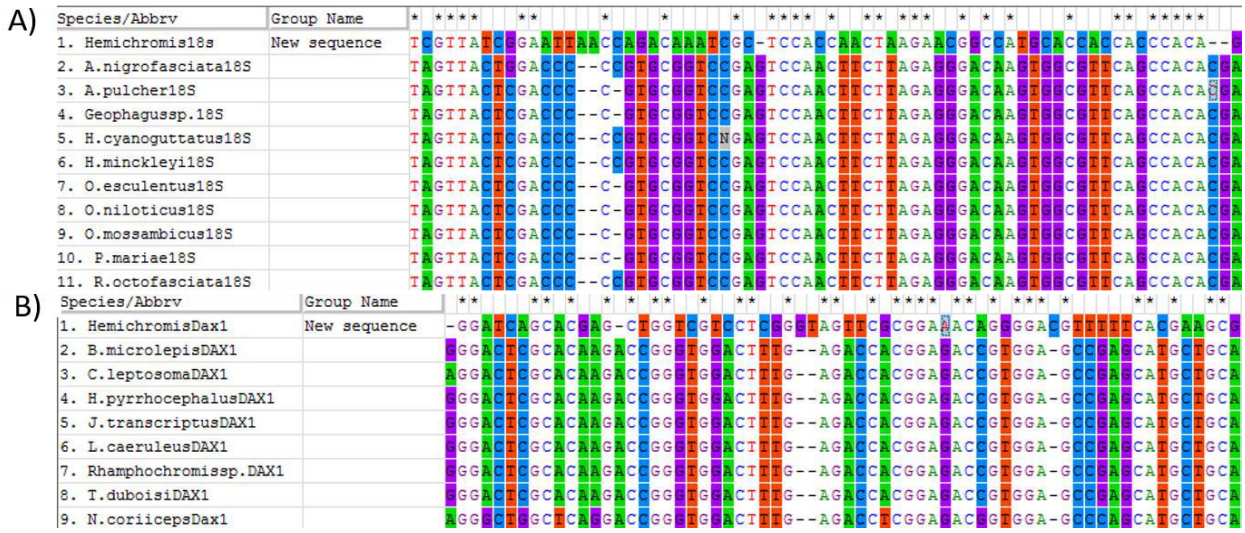

Figure 3: Sequence alignments generated in MEGA6 using a built-in CLUSTALW of the sequences obtained in the NCBI with the use of blastn and the sequence obtained from the sequenced vector, A showing the ones obtained for *18s rRNA* and B the ones for *Dax1a.*

After this, a Sanger sequencing was made and the resulting sequences for both *18s rRNA* and *Dax1a* were curated with the use of both SerialCloner and Snapgene viewer software. The identity of both genes was corroborated, finding the *Dax1a* specific binding both genes were indeed the genes we were looking for. There were slight differences between the *Dax1a* primers and the obtained sequence, thus a redesign is needed to assure the specificity; in *18s rRNA*'s case, the primers were found to be specific and no changes are needed on their sequence. Figure 3 shows that, in both cases part of the sequence has conserved sites, that added to the values of similarity between sequences given by the program blastn, which are no lower than 99% for *18s rRNA* and 97% for *Dax1a* showing that both are, indeed fragments of the genes of interest for this study.

Conclusions

The presence of *18s rRNA*, *Dax1a* was and *CYP19A1a* was confirmed with the use of PCR; only 18s rRNA and Dax1a were sequenced for the corroboration of their identity which was proved. At day 95, the expression of the *CYP19A1a* will be evaluated and it is expected to be significantly different between male and female cichlids of this genus, also the presence of *Dax1a* might be higher in females than in males. The suitability of *18s rRNA* as housekeeping gene for this study is still being determined.

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Feminization of the invasive jewel cichlid (*Hemichromis guttatus*) by dietary administration of 17β-estradiol

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Abstract
The Cuatrociénegas invasive jewel cichlid *Hemichromis guttatus* was exposed to 17β-estradiol-enriched Artemia nauplii at a concentration of 200 mg/L. A total of 75 larvae were exposed to the treatment during 60 days starting at 15 day post hatching (DPH) resulting in 100% feminization, although survival was low (21%) compared to the control (60%).

Keywords Estradiol • feminization • invasive • cichlid • selco • nauplii • Cuatrociénegas

Introduction

The jewel cichlid (Hemichromis guttatus) is an exotic invasive species that has been introduced since 1996 in the valley of Cuatrociénegas, Coahuila, Northeastern Mexico (Contreras & Ludlow, 2003). The lack of predators, pathogens and parasites allowed jeweled cichlid populations to become dominant, consequently, the jewel cichlid has outcompeted crucial endemic cichlid species (Connor & Rothermel, 2013). The present study is part of a control strategy to eradicate jewel cichlid populations by introducing YY males, Trojan Y Chromosome fishes; Fig. 1 (Carrillo, 2009) which involves an initial sex reversion step (Gutierrez & Teem, 2006).

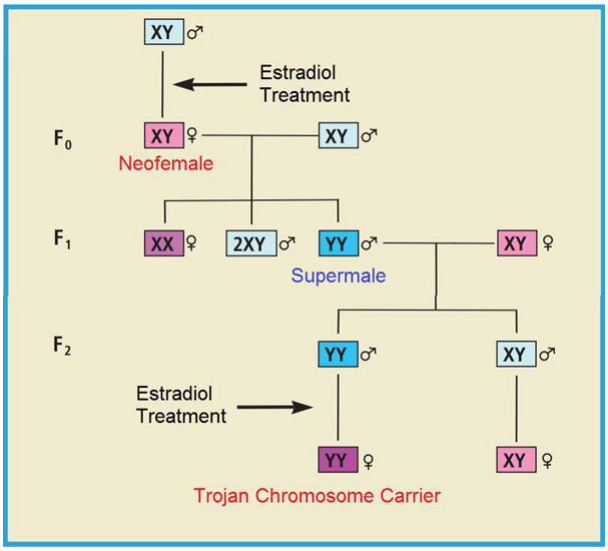

Figure 1. Diagram illustrating the production of YY super-males and Trojan chromosomes carriers after being treated with estrogens and retrogression.

Hormone administration to induce sexual reversion of fish is usually employed in aquaculture in order to generate monosexual populations aimed at obtaining higher yields (Gutierrez & Teem, 2006). Sex reversal of males by estrogens is commonly used, such as

the employment of 17-β-estradiol, estrone, 17-α-ethinylestradiol, diethylstilbestrol or anti-androgens. Estradiol was chosen in order to induce feminization; since it is the mayor estrogen in vertebrates related to the onset and maintenance of the female reproductive system (Welshons et al., 2003) and because it has been successfully used for sex reversion in the case of cichlids (George & Pandian, 1996; Marín Ramírez et al., 2015; Yousefian, Laghab, Irani, & Makhdoomi, 2012). After inducing sex reversal in males, the neofemales (F_{xy}) were crossed with normal males (M_{xy}), in order to produce super males (M_{yy}). When these super males breed with normal females (F_{xx}) they will generate only males, because they possess a disomy of the sexual Y chromosome (Beardmore, Mair, & Lewis, 2001). Also if they are released in the place where the invasion is ongoing super males will negatively affect the gender proportion of the invasive populations since their F1 will consist only of males. This will prevent females from being present in the populations and consequently from mating. With the time and after several releases of super males the invasive population will turn monosexual after some generations have passed and eventually the population will become extinct (Cotton & Wedekind, 2009; Gutierrez & Teem, 2006). The objective was to induced the feminization of males of *Hemichromis guttatus* to females.

Material and Methods

Larvae were obtained from the reproduction of jewel cichlid adults collected in Cuatrociénegas, Coahuila which were captive breed at FCB-UANL Ecophysiology Lab. Larvae were and transferred to 1-L aquaria at the beginning of the experimental treatments. Each treatment was carried out in triplicate, consisting of 25 individuals set into an aquarium, which were exposed during 60 days starting from day 15 post-hatching (DPH) (Vidal-López, Álvarez-González, Contreras-Sánchez, & Hernández-Vidal, 2009). Artemia nauplii enrichment was carried out according to the method reported by (Vidal-López et al., 2009) consisting in the use of estradiol dissolved in SELCO at a concentration of 200 mg/L. Larvae were administered continuously through a feeder; 3,000 nauplii per replicate every 3 h, (Fig. 2).

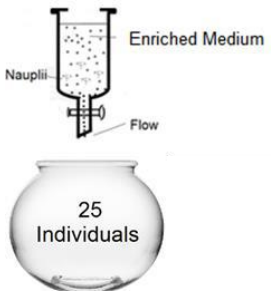

Figure 2. Nauplii feeder.

Artemia cysts were hydrated for 30 minutes, there after these were decapsulated in a 10% sodium hypochlorite solution for 30 seconds. The reaction was stopped using sodium thiosulfate. Finally, the cysts were hatched in a saline solution (30 ppm) aereated from the bottom. A stock solution of 500 mg of 17-β-estradiol in 75 ml of Selco was prepared to reach a final concentration of 6.66 mg/ml and keep at 4 °C. After hatching, nauplii were enriched for two hours in a solution made of Selco and water with a concentration of E2 of 200 mg/l (Vidal-López et al., 2009). The control group was fed non-enriched nauplii. After

the treatment was over the larvae accepted commercial diet regime so the diet was change to commercial feed until dissection. In order to determine the effectiveness of the treatments, each individual gonad was determined by gonadal squash with aceto-carmine (Wassermann & Afonso, 2002), and sex ratio were contrasted with Fisher´s exact test with a statistical significance of 0.05, in R (version 3.3.2).

Results

Survival. The mean survival for the treatment of estradiol was 21% and 60% for the control. *Size.* The estradiol treatment was highly different in weight and total length than the control treatment with means of 421.46 mg ± 114.24mg in weight for the control and 352.3 mg ± 67.21 mg for estradiol (P>0.001), moreover the mean of the total length for estradiol was 26.31 mm ± 1.64 mm and 28.36 mm ± 3mm for control (P>0.001). The normality and homoscedasticity of the groups was checked by the test of Shapiro - Wilk and Bartlett respectively. *Condition Factor.* The condition factor it´s use to compare the relationship between the length and weight and it is related to the general wellbeing and health of the fish (Reynold, 1968). The condition factor of the control treatment females was 1.80±0.18, 1.74± 0.15, 1.91±0.15, for males of the control treatment and females of the estradiol treatment respectively. *Sex ratio.* Sex ratio from the control group was not statistically different from the expected 1:1 sex ratio (P=0.6732). However, the estradiol group was different (P=0.0024) when compared with the theoretical proportion, and also highly different from the control group (P=0.00005). Microscopic examination of the gonads in all groups revealed that females and males presented normal gonadal development and no intersex fish were identified. The fry were sex by the method of squash (Wassermann & Afonso, 2002) from the 81 dph to 84 dph. Fig.3

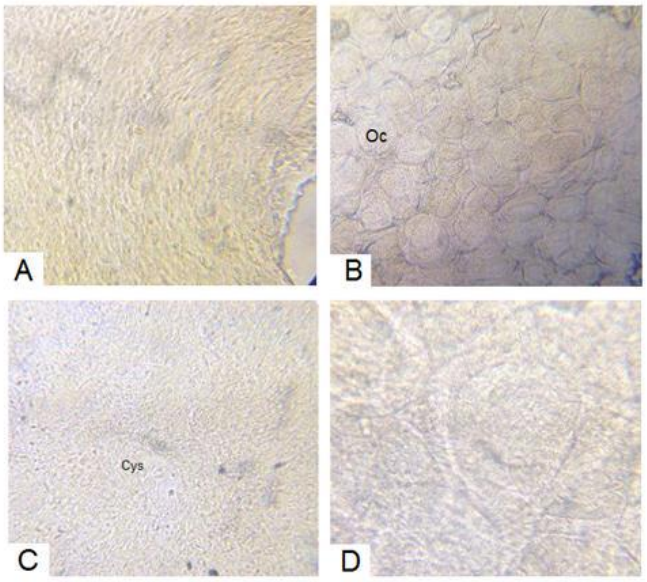

Figure 3. Phenotypic sexes of the jewel cichlid fry using the aceto-carmine technique. Male and female gonads at 100x (A and B), 200x (C and D). Cys, Cyst; Oc, Oocytes.

The results were analyzed with Fisher´s exact test using the software R against the theoretical sex proportion 50/50; the analyses were made by comparing each replica; after that the individuals of each treatment were added up to form groups and then the proportions of both treated and untreated groups were compared (Table 1).

Table 1. Comparison of the sex ratio against the theoretical proportion using Fisher´s exact test showing p value for significance.

Treatment	Male	Female	P Value
Control	25	20	0.6732
Estradiol	0	16	0.0024

Discussion

The negative effect of estradiol on survival has previously been reported (George & Pandian, 1996), however different factors could have influenced the survival rate such as the sensitivity of the species, time of exposition and concentration, among the most important ones. In addition to this the estradiol has been shown to negatively influence fish growth (George & Pandian, 1996; Karsli, Aral, & Yes, 2016), but only during the time of exposure to the steroid because a normal growth was reported months after retrieval of the treatment (Babiak et al., 2012; Schill, Heindel, Campbell, Meyer, & Mamer, 2016; Wang et al., 2008)**.** This negative effect on growth is attributed to the decline in the quantity of somatostatin receptors, since estradiol affects negatively on the pituitary (SRIF-14) thus affecting the secretion of GH in goldfish (Cardenas et al., 2003). Highly significant differences ($P<0.001$) were observed when the condition factor of males and females of the control treatment was compared with that of the females of the estradiol treatment. In contrast no significant differences were found when comparing the condition factor of males and females of the control treatment. Similar results were found in tilapia. (Khallaf et al., 2003). The condition factor may vary according to the seasons (El-Sayed et al,.2007) and decreases significantly after the reproductive season, these changes are attributed to the release of the gonadal content (Khallaf, 1986). This indicates a correlation between condition factor and gonadal development, similar results were found by Le Cren (1951) when comparing the condition factor with the size of the gonads on *Perca fluviatilis.* Therefore, these differences could be a consequence of the development of the gonads caused by the estradiol treatment.

Conclusions

The E2 treatment resulted in 100 % feminization and is highly different from the control group which proves this method of feminization effective. However, we found using this concentration and time of exposure the survival is low.

Acknowledgements The authors thank CONABIO for the financial support.

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Marine bacteria population and diversity on reverse osmosis pretreated water

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Abstract
Specific marine bacteria survive water desalination pretreatment processes and reach reverse osmosis membranes. Once there, bacteria attach to the membrane surface, proliferate, form colonies, excrete polysaccharides, and in a last stage, form a biofilm. Biofilm formation represents 30% of the fouling found on reverse osmosis membranes, producing clogging. In order to analyze membranes' colonizing bacteria, seawater proceeding from Cortes Sea was fed to a Reverse Osmosis (RO) desalination pilot plant. As the RO installation operated, water samples were taken throughout the process, with the aim of isolating marine bacteria, surviving each stage of pretreatment. Only bacteria surviving last stage, ultraviolet radiation, are those which immediately reach the RO membrane. The six resultant samples provided bacterial isolates, form a new marine microbial collection, for which microbial population will be count, and diversity will be determined. Water pretreatment is intended to progressively decrease water salinity before it reaches the membrane, present work explores any impact of this same pretreatment, over bacteria population. Combined, the first three pretreatment stages manage to detain the largest amount of bacteria (up to 66.66% of cultivable bacteria extracted from raw sea water samples). However, once this water reaches UV radiation, bacteria population duplicates. Bacteria diversity from shows no relation to the sequence of these stages throughout desalination process.

Keywords: *reverse osmosis* ● *pretreatment* ● *marine bacteria.*

Introduction

Specific marine bacteria is proven to survive water pretreatment processes aimed at reverse osmosis desalination equipment, finding its way to the central membranes. Once there, bacteria will attach to the membrane surface, proliferate, form colonies, excrete polysaccharides, and in a last stage, form a biofilm (Pavarina *et al.* 2011). Biofilm formation represents 30% of the biofouling found on reverse osmosis membranes (Del Vigo 2012). Major negative impacts of biofilm formation are the clogging of membranes, resulting in an increase of pressure applied needed to perform reverse osmosis, a decrease on the resultant permeate flux (Nguyen *et al.* 2012), and a general reduction of the membrane's shelf life. In order to analyze membranes' colonizing bacteria, later responsible for biofouling, 280 L of seawater collected at Cortes Sea were introduced into a RO desalination pilot plant, as a feed water fluent. As the RO installation operated, water samples were taken throughout the process (Figure 1), with the aim of isolating marine bacteria, surviving each stage of pretreatment, such as sand, and activated carbon filters.

From these, only bacteria surviving last stage, ultraviolet radiation (UV) are those which reach the RO membrane.

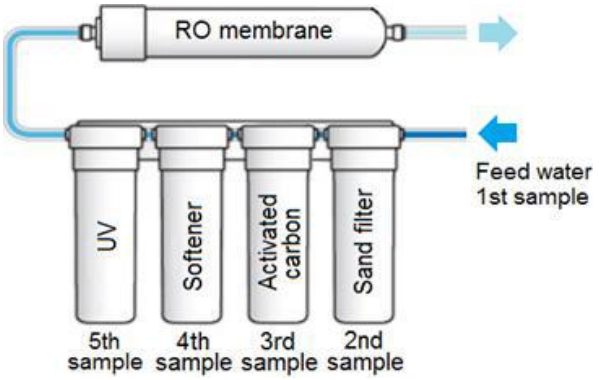

Figure 1. Water sampling positions along reverse osmosis pilot plant.

The five resultant samples provided bacterial isolates, form a new marine microbial collection. Water pretreatment is intended to progressively decrease water salinity before it reaches the membrane, present work will determine any impact of this same pretreatment, on bacteria population. Marine bacteria are generally classified as culturable or nonculturable bacteria, only the first category will be subject of the present study, given that only culturable bacteria are able to colonize the RO membrane surface.

Materials and methods

Microbial isolation. Serial dilutions were performed to enumerate bacterial populations and diversity, thus, samples were taken at depth of 4 m, through a manual water pump, and were stored at 4 °C. Microbial growth was completed within 24 h after the water samples were collected. In order to obtain the major population of marine bacteria from those extractions, a modified marine growth medium was designed, matching the salinity conditions found on this marine ecosystem (Vu *et al.* 2009). Microbial growth. Previously reported growth medium (Romero *et al.* 2015) was used to grow both marine bacteria and fungi, extracted from the collected water samples. 0.25 mL of each sample was applied and spread directly on Petri dishes containing the culture medium used, and incubated at 33°C for 12 h. Independent medium growth will be prepared for each sample site, due to possible salinity adjustments on water samples, after withdrawing each stage. Microbial Population. The quantitative determination of marine bacteria populations was carried out using plate count method (Reynolds *et al.* 2011). On the subject of direct cultures from sea water samples, colony forming units (CFU) located inside the same cm^2 cannot always be counted, therefore serial dilutions (Reynolds *et al.* 2016) may be commonly performed, until CFU's are able to enumerate. Diversity. Each CFU presenting distinctively macroscopic characteristics was isolated into different Petri dishes. Several bacterial characteristics were analyzed, such as: color, size, edges' roughness, texture, and turbidity. This screening is reported on a relative abundance chart for marine bacteria (Vijayan *et al.* 2012).

Results and discussion

Once seawater ran through the reverse osmosis plant, independent water samples were taken along the pretreatment process. Each sample offered the following information:

Table 1. Salinity decrement along water pretreatment

	Raw water	Sand filter	Activated carbon	Softener	Ultraviolet radiation
Total dissolved solids (mg/L)	36, 600	36, 520	36, 100	35, 512	35, 510
Salinity percentage	100%	99.78%	98.63%	97.02%	97.02%
Decrement	0%	0.22%	1.37%	2.98%	0%

Preliminary observations. Even when reverse osmosis pretreatment is not intended to decrease salinity levels found on feed water (Table 1), it achieves to reduce up to 3% of the total dissolved solids throughout desalination process as appreciated on table 1. For this, separately medium growth preparations were required for each sample site, in the aim of not restraining any bacterial growth, due to salinity stress on marine bacteria.

Table 2. Marine bacteria population, along water pretreatment.

Sampling site	UFC/mL	Percentage
1) Raw water (RW)	3.8 E+04	100%*
Pretreatment		**Decrement**
2) Sand filter (F)	3.3 E+04	13.32%
3) Sand filter, activated carbon (FC)	3.0 E+04	20.03%
4) Sand filter, activated carbon, and softener (FCS)	2.5 E+04	33.33%
5) Sand filter, activated carbon, softener, and ultraviolet radiation (UV)	1.3 E+04	66.66%

*100% of cultivable bacteria on raw water sample.

Combined, the first three pretreatment stages manage to detain the largest amount of bacteria (up to 66.66% on bacterial population decrement, in relation to raw water sample calculated population) (Table 1). Once this water reaches the last stage, corresponding to ultraviolet radiation, bacteria population increases near to the point of duplication (Table 2).

Table 3. Marine bacteria diversity, along RO pretreatment stages.

Sample site	RAW	F	FC	FCS	UV
No. of bacterial isolations	16	10	8	6	10

Naturally, greater diversity was found on the sample collected directly from raw water (16 different strains, as seen on table 3), while bacterial diversity on every other stage tend to decrease along the pretreatment process. Sidewise, these strains displayed the following repetitions:

Table 4. Chart of marine bacteria relative abundance.

Distinctive Strains	Repetitions per stage					
	R	F	FC	FCS	UV	
I	9		3		2	Greater growth
II	8		1			
III	7				1	
IV	3					
V	4				3	
VI	2					
VII	1					Standard growth
VIII	1					
IX	4	1				
X	1	3				
XI	1	3				
XII	9	1		6		
XIII		3		5		
XIV	5					
XV			4			
XVI	1	3	1	5	2	
XVII	3	4	1	2	3	
XVIII					3	
XIX		1				
XX		3			2	
XXI			6		1	
XII					1	
XIII			3			
XIV			4			
XV					6	
XVI	1					
XVII			1	3		Low
XVIII		2		3		
	60	24	24	24	24	156

Within a total of 156 grown bacteria cultures, only 28 of them displayed distinctively characteristics. Not all of these strains are present since raw water, pointing to possible pollution. Bacteria diversity on water samples show no trend in relation to the sequence of the four stages throughout the desalination process. Two of the strains are proven to be present on each pretreatment stage (lines XVI and XVII shown on table 4), while nine of

them are considered site-specific, referring to those bacteria that are only found on a single stage, with no repetitions on any others. Separately, ultraviolet radiation is well known to induce mutation on bacteria cells, and might increase strains' diversity (Zion *et al.* 2006). Since not the entire bacteria collected will reach the RO membranes, only those culturable specimen proceeding from the last pretreatment stage will colonize the membrane's surface. Ten of these strains observed different macroscopic characteristics from each other (Figure 2).

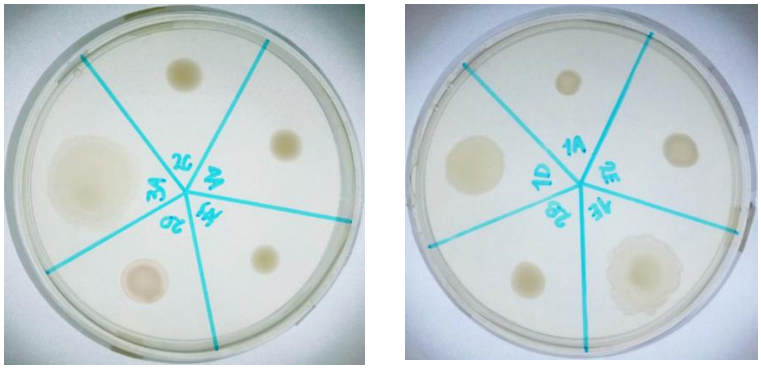

Figure 2. Bacterial strains isolated from last stage of sea water pretreatment.

Conclusions

Reverse osmosis pretreatment progressively decreases available nutrients for marine bacteria, before it reaches the membrane, having a direct impact on bacteria population diminishment. Bacterial diversity and population numbers demonstrate to have a trend on the first three pretreatment stages, while the last stage shows no relation. Increasing diversity on colony forming units (UFC) after UV stage, implies that bacteria surviving pretreatment, mutated into two or more different strains. The possible presence of bacterial contamination, or even biofilm formation on the system's water pipes is not discarded as another responsible for this increment on bacterial diversity, however, previous pretreatment stages did not show this behavior. Bacterial strains surviving last stage of water pretreatment (UV) will reach to the RO membrane, and ignite biofouling. Future work will focus on these specific bacteria, in order to achieve biofilm control.

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Endemic Plants from Mauritius Islands as Potential Phytomedicines

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Abstract
The endemic floristic wealth of Mauritius represents a reservoir of new biologically active ingredients. *Sideroxylon* species and *Diospyros* species have been traditionally used for the treatment of minor ailments. The present study describes the phytomedicinal profile of these species through antioxidant and antibacterial assays. *Diospyros chrysophyllos* exhibited the highest amount of phenolics (221 mg gallic acid equivalent /g and *Diospyros boutoniana* exhibited strong reducing power (946.22 mmol Fe2+/g extract). Promising antibacterial activity was noted with *Sideroxylon puberulum* and *D. boutoniana* (minimum inhibitory concentration of 39.06 and 78.125mg/ml) respectively. These results validate the phytochemical and bioactive richness of *Disopyros* species endemic to Mauritius and reveal their potential for pharmacological exploitation. The genetic diversity of selected *Sideroxylon* species is also described to endorse their uniqueness as Mauritian endemic bioresources. We also noted that the Mauritian endemic *Sideroxylon* species are genetically related to Argan oil tree.

Keywords *Diospyros. Sideroxylon*. Phenols. FRAP. Antibacterial.

Introduction

The genus Diospyros is one of the most important sources of bioactive compounds and exclusively 1, 4-naphthoquinones and flavonoids (Nematollahi *et al*. 2012; Mallavadhani *et al*. 1998). 1, 4-naphthoquinones are reported for their anticancer, antiinflammatory and antibacterial activity whereas flavonoids display important antioxidant activity (Kauroo *et al*. 2015). The genus Sideroxylon is characterised by the presence of isoquercitrin, quercetin, (+)-catechin and luteolin (Gurib-Fakim *et al*. 1992). Presently, research is geared towards the anti-oxidant activity of plant extracts due to diseases caused by oxidative stress (Saradhi *et al*. 2014). Multiple lines of evidence have implicated oxidative stress and free radical damage to the pathogenesis and possible etiology of Alzheimer disease, heart diseases, and diabetes. Non communicable diseases such as diabetes (50.3%), heart diseases (38.5%) and cancer (19.3%) are imposing large economic burden on the Island of Mauritius. Mauritius in the Mascarene Islands is a biodiversity hot spot which represents a reservoir of new biologically active ingredients (Baider et *al*. 2010). So far, most have not yet been scientifically validated for their bioactivities and are not yet exploited. The present research study validates the use of selected endemic plants in the traditional medicine of Mauritius and reveals their potential for drug development.

Materials and Methods

Plant material and extraction procedure.

The leaves from *Diospyros chrysophyllos* (MAU 0016596), *Diospyros boutoniana* (MAU 0016538) and *Sideroxylon puberulum* (MAU 0016536) were collected from Natural Parks

in Mauritius. Voucher specimens were deposited at the Mauritius Herbarium and 150 g were extracted using cold methanol and the filtrate was concentrated in a rotary evaporator under reduced pressure and low temperature.

Determination of total Phenolic content.
Total phenolic content of organic extracts was determined as per Singleton and Rossi (1965).

Determination of total flavonoid content.
Total flavonoid content was determined as per Kaur and Mondal (2014).

Determination of proanthocyanidins. Total proanthocyanidins content of organic extracts was carried out as per Price *et al*. 1978.

Ferric Reducing antioxidant power (FRAP).
The reducing ability was determined as per Benzie and Strain (1996).

Anti-bacterial assay.
The serial dilution technique was used (Eloff, 1998).

DNA Extraction.
DNAs were extracted as per Doyle and Doyle (1990).

PCR Amplification of segment of the chloroplast genome.
PCR amplification was performed in 25 µl reactions with the following components: 1.5 total genomic DNA, 2.5 µl reaction buffer, 2.5 µl DNTP`s, primer (matK 1R and 3F) and (trnL E and F), 0.5 µl, Taq polymerase 0.2 µl and 16.8 µl millipore water.

Regions were amplified using the following cycling conditions.
30 cycles of 95 °C 5 min,94 °C 1 min followed by a annealing of 55 °C for 1 min,72°C for 2 min and elongation at 72°C at 10 min.

Statistical analysis.
All experiments were conducted in triplicate. A subset of the PCR products was sequenced and aligned using Bioedit software.

Results and discussion

Total Phenolic and Total Flavonoid and Total Proanthocyanidins content.
Diospyros chrysophyllos and *D. boutoniana* had highest levels of phenolics and flavonoids (69.2 mg quercetin/g extract). The proanthocyanidin content varied from 29.9 to 32.4 mg cathecin /g extract. Similar results have been obtained in *D. blancoi* from India (Khan *et al*. 2016).
Ferric reducing ability.
Diospyros species showed the highest activity in the order *D.boutoniana* > *D.chrysophyllos* > *S.puberulum.* Epidemiological studies strongly support the contribution of polyphenols to

the prevention of many diseases such as cancers and neurodegenerative diseases (Scalbert *et al*. 2015).

Antibacterial activity.

Plant extracts with an MIC value less than 100 µg/ml are usually considered as promising candidates for further pharmacological studies (Eloff, 2004). We noted promising activity with *S. puberulum* and *D. boutoniana* (MIC value 39.06 to 78.125 respectively). Flavonoid (myricetin) isolated from *D. virginiana* from Egypt showed strong antibacterial activity (Rashed *et al*. 2014).

Amplification of noncoding cpDNA.

Primers showed good amplification (a single strong band) in all the *Sideroxylon* ssp screened (Figure 1). The PCR annealing temperature was optimised from 50 ^{0}C to 55 ^{0}C to improve the amplification of those regions. The amplification products were sequenced and BLAST was used to search the GenBank for matching sequences. Sequences of *Sideroxylon* and *Minusops* had maximum similarity with *Argania* and *Manilkara* sequences from GenBank (Figure 2).

Table 1. Antimicrobial activity of tested plants (MICµg/ml).

Methanolic extact	**Gram Negative Bacteria**		
	Klebsiella pneumoniae (KP) ATCC 13883	*Pseudomonas aeruginosa* (PA) ATCC 27853	*Escherichia coli* (EC) ATCC 25922
EDB	78.125	39.06	39.06
EDC	78.125	156.25	39.06
SSP	39.06	156.25	78.125
Chloramphenicol µg/ml)	15.63	125	62.5
	Gram Positive Bacteria		
	Staphylococcus aureus (SA) ATCC 29213	*Enterococcus faecalis* (EF) ATCC 29212	*Bacillus cereus* (BC) ATCC 11778
EDB	39.06	39.06	78.125
EDC	39.06	78.125	156.25
SSP	39.06	39.06	78.125
Chloramphenicol µg/ml)	15.62	3.91	31.25

EDB: *D. boutoniana;* EDC*: D. chrysophyllos;* SSP: *Sideroxylon puberulum*

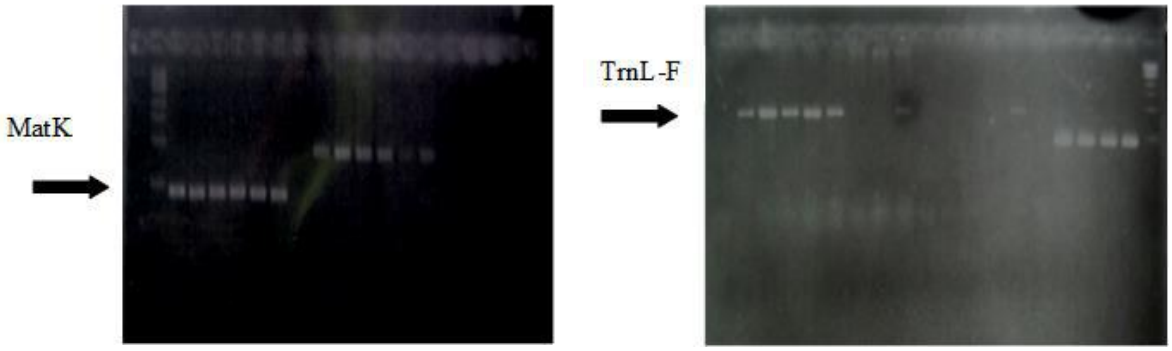

Figure 1: Gel photos showing the amplification success of the cpDNA (MatK and Trn L-F) regions tested in *Sideroxylon* species.

Sideroxylon species are genetically more distant from the genus *Manilkara* spp and *Mimusops* spp than they are from *Argania spinosa* endemic to Morocco. Traditionally, Argan oil from the tree is used for many purposes such as in cosmetic as medicine and edible oil (Babili *et al*. 2010).

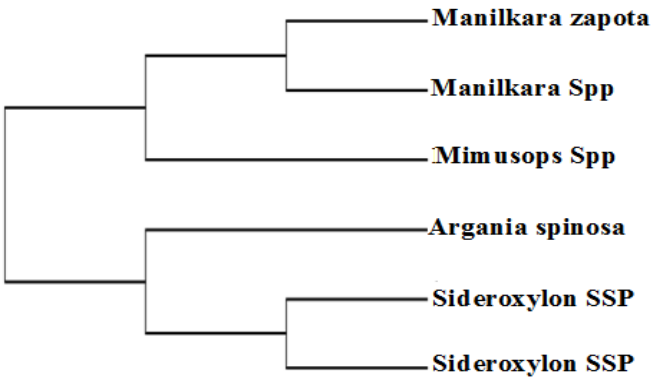

Figure 2: Dendogram illustrating genetic similarity among selected species from the Sapotaceae family marker.

Conclusions

Mauritian endemic plants, particularly the species *Diospyros* methanolic extracts, contain significant levels of phenolic compounds and exhibit considerable ferric reducing capacity. *Sideroxylon puberulum* and *Diospyros boutoniana* exhibited promising antibacterial activity and they can be considered as potential sources of antimicrobials worth of pharmacological exploitation. Moreover, the Mauritian endemic *Sideroxylon* species are genetically related to Argan oil tree.

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Molecular identification of *Vibrio parahaemolyticus* strains responsible of Acute Hepatopancreatic Necrosis Disease

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Abstract
Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) is an emerging disease diagnosed in penaeid shrimp. Affects the digestive system of shrimp, mainly in the Hepatopancreas. Its acute progressive degeneration ultimately leads to the shrimp die. Recently has been diagnosed the cause as a virulent strain of *Vibrio parahaemolyticus* (called Vp_{AHPND}). Vp_{AHPND} has a plasmid (pVA1) which expresses a binary toxin called PirABvp. In this work we will carry out the identification of strains VpA_{HPND} through amplification of specific sequences by PCR.

Keywords: AHPND • *Vibrio parahaemolyticus* • toxin PirAvp •toxin PirBvp.

Introduction

Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS), is an emerging disease diagnosed in penaeid shrimp (particularly *Penaeus monodon* and *Litopenaeus vannamei*). Has been officially reported in Asian Pacific countries including China (2009), Vietnam (2010), Malaysia (2011), Thailand (2012) and recently in Mexico (2013) (Chamberlain 2013). AHPND affects the digestive system of shrimp, mainly the Hepatopancreas. This acute progressive degeneration ultimately leads to the affected shrimp die for hepatopancreas dysfunction. Initial studies showed that the inflammatory response usually triggered by a pathogen; it is absent in the early stage of the disease. So this strongly suggests an etiology for toxins (Han *et al*. 2015). Recently at the Arizona University, Lightner *et al.* (2013) diagnosed the cause as a virulent strain of *Vibrio parahaemolyticus* (called VP_{AHPND}). VP_{AHPND} has a plasmid of 70 kbp (called pVA1), which expresses a binary toxin PirABvp. This toxin consists of two subunits PirAvp and PirBvp (Lee *et al.* 2015; Han *et al.* 2015). *V. parahaemolyticus* strains recently were isolated in Baja California Sur. In order to identify strains producing AHPND we conducted identification based on the detection of specific sequences of genes encoding PirAvp (AP3) and PirBvp toxins. This genes, are only found in strains causing AHPND. Oligonucleotides that were used are shown in Table 1.

Materials and methods

V. parahaemolyticus strains

Different strains of *V. parahaemolyticus* causing AHPND isolated in Baja California Sur and Sinaloa, as well as reference strains without the plasmid pVA1 were used for this study.

Strains isolation

In 2015-2016 we made a sampling from shrimps *Litopenaeus vannamei* in differents farms from B.C.S. Also it was conducted microbiological analysis from hepatopancreas from each organism collected. The first step was the disruption of tissue in 10 ml seawater sterile. A volume from 100 ul from this suspension it was spread in TCBS agar (Agar Thiosulfate Citrate Bilis Sucrose), which is a medium selective from differentiation for isolation of *Vibrio*. Serological and biochemical tests for the identification of the strains were conducted.

Plasmid isolation

Plasmids were extracted using CTAB protocol from a cell culture with approximately 12 hours of incubation at 30°C and 200 rpm. DNA integrity was analyzed on 1% agarose gel.

Vp_{AHPND} Strains identification

The used method for identification of VP_{AHPND} strains, were through the PCR. The identification of each strain was based on the detection of genes encoding PirAvp (AP3) and PirBvp toxins, which are only found in Vp_{AHPND}.

Table 1. Oligonucleotides used for detection of AHPND strains.

Name	Sequence (5′-3′)
AP3-F	ATGAGTAACAATATAAAACATGAAAC
AP3-R	TTAGTGGTAATAGATTGTACAG
PirB-F	ATGACTAACGAATACGTTGTAAC
PirB-R	CTACTTTTCTGTACCAAATTCAT

Results

V. parahaemolyticus strains that are being used in this study were:

C(-) as reference negative control; positive control C(+), PA1a, PA1b, PA2, PA3, PA4, PA6, PA7, PA8, PA9, PA10, PA11, isolates from La Paz, B.C.S. PB2, PB4, PB5, PB6, PB7, PB8, PB9, PB10, PB11, PB14, PB15, PB16, PB18- Isolates of a second outbreak in La Paz, BCS. M8, M22, M30, M37 were isolated from Sinaloa.

Plasmid isolation

Plasmid isolation was carried out using the methodology described above. In Figure 1 and 2, 1% agarose gels were performed for the visualization of plasmids from each strain isolated from AHPND outbreaks. In these figures it is achieved visualize differences between purified plasmids from each strains, this because of the different plasmids could have each bacterium. Figure 1A: Lane 1.-Reference Strain, 2.-S8, 3.- C(+), 4.- S22, 5.- S30, 6.- S37, 7.- PA1a, 8.-PA1b, 9.- PA2, 10.- PA3,11.- PA4, 12.- PA6,13.- PA7, 14.- PA8, 15.- PA9, 16.- PA10, 17.- PA11. Figure 1B: Lane 1.-Reference Strain, 2.- PB2, 3.- PB4, 4.- PB5, 5.- PB6, 6.- PB7, 7.- PB8, 8.- PB9, 9.- PB10, 10.- PB11,11.- PB14, 12.- PB15,13.- PB16, 14.- PB18.

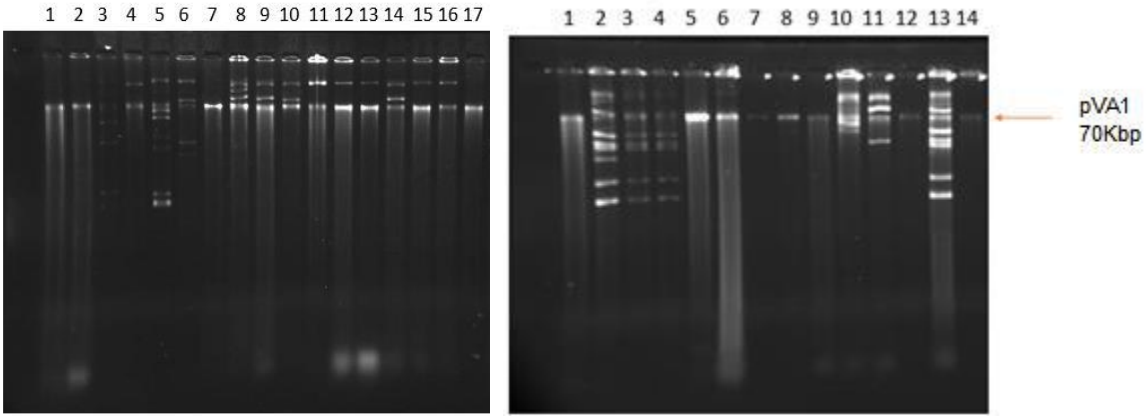

Figure1. Plasmids from *V. parahaemolyticus.*

Identification of VP_{AHPND}

Identification was performing with specific sequences encoding PirAvp (AP3) and PirBvp. The analysis shows that 27 strains have sequences encoding for PirAvp and PirBvp toxin. Figure 2, amplification of the gene encoding PirAvp (AP3), which has a length of 336 bp. In figure 2A, nine strains were positive: Lane 1. Molecular weight marker, 2.- Strain S8, 3.- C(+) ,4.- S22, 5.- S30, 6.-S37, 7.- PA1a, 8.- PA1b, 9.- PA2, 10.- PA3, 11.- PA4, 12.- PA6.
In figure 2B, Sixteen strains were positive: Lane 1.- Molecular weight marker, lane 2.- C (-), lane 3.- C (+), lane 4.- S22, lane 5.- S30, lane 6.- S37, lane 7.- PA1a, lane 8.- PA1b, lane 9.- PA2, lane 10.- PA3, lane 11.- PA4, lane 12.- PA6.

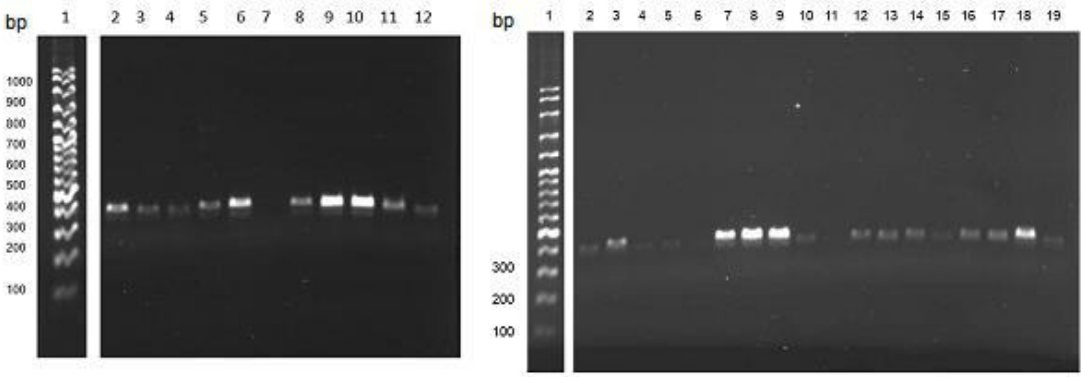

Figure 2. Amplification of the specific sequence of PirAvp (AP3).

Finally, was conducted amplification of fragment PirBvp. Figure 3, amplification of the gene encoding PirBvp, which has a length of 1317 bp. Figure 3: Lane 1. Molecular weight marker, 2.- Strain C (-), 3.- S8 ,4.- C (+), 5.- S22, 6.-S30, 7.- S37, 8.- PA1a, 9.- PA1b, 10.- PA2, 11.- PA3, 12.- PA4, 13.- PA6, 14.- PA7, 15.- PA8, 16.- PA9, 17.- PA10, 18.- PA11,

19.- PB2, 20.- PB5, 21.- Molecular weight marker, 22.- PB6, 23.- PB7, 24.- PB8, 25.- PB9, 26.- PB10, 27.- PB11, 28.- PB14, 29.- PB15, 30.- PB16, 31.- PB18, 31.- CVP2.

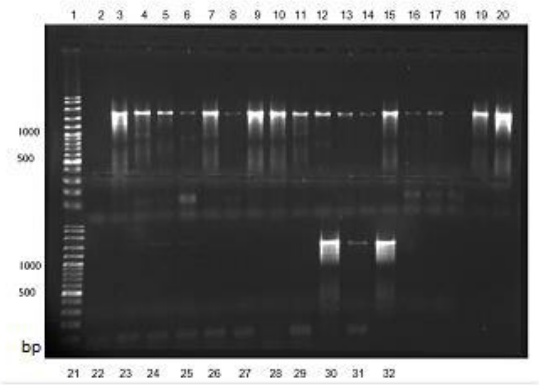

Figure 3. Amplification of the specific sequence of PirBvp.

Sequencing of PirAvp and PirBvp gene

Figure 4 show the alignment of *V. parahaemolyticus* strain S37 PirAvp with the sequence encoding PirA (AP3) from in GeneBank. Note that the sequences are homologous. On the other hand, we also carry out the sequencing and alignment of PirBvp. We found that have 100% of identity respect to PirB gene deposited in GeneBank (data no shown).

```
Cepa_S37_           --ATGAGTAACAATATTATGCAATAAACCCATTATTCTCACGATTGGACTGTCGAACCAA
SECUENCIA_AP3       --ATGAGTAACAATATAAAACATGAAACTGACTATTCTCACGATTGGACTGTCGAACCAA
                      ************************************************************

Cepa_S37_           ACGGAGGCGTCACAGAAGTAGACAGCAAACATACACCTATCATCCCGGAAGTCGGTCGTA
SECUENCIA_AP3       ACGGAGGCGTCACAGAAGTAGACAGCAAACATACACCTATCATCCCGGAAGTCGGTCGTA
                    ************************************************************

Cepa_S37_           GTGTAGACATTGAGAATACGGGACGTGGGGAGCTTACCATTCAATACCAATGGGGTGCGC
SECUENCIA_AP3       GTGTAGACATTGAGAATACGGGACGTGGGGAGCTTACCATTCAATACCAATGGGGTGCGC
                    ************************************************************

Cepa_S37_           CATTTATGGCTGGCGGCTGGAAAGTGGCTAAATCACATGTGGTACAACGTGATGAAACTT
SECUENCIA_AP3       CATTTATGGCTGGCGGCTGGAAAGTGGCTAAATCACATGTGGTACAACGTGATGAAACTT
                    ************************************************************

Cepa_S37_           ACCATTTACAACGCCCTGATAATGCATTCTATCATCAGCGTATTGTTGTAATTAACAATG
SECUENCIA_AP3       ACCATTTACAACGCCCTGATAATGCATTCTATCATCAGCGTATTGTTGTAATTAACAATG
                    ************************************************************

Cepa_S37_           GCGCTAGTCGTGGTTTCTGTACAATCTATTACCAC
SECUENCIA_AP3       GCGCTAGTCGTGGTTTCTGTACAATCTATTACCAC
                    ***********************************
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Figure 4. Alignment of fragments from S37 strain and PirAvp (AP3) in the GeneBank.

Discussion

Most *Vibrio parahaemolyticus* strains that were isolated were positive for the presence of the PirAvp, PirBvp genes. Similar results obtained by Lee *et al*. (2015) in their analysis of strains AHPND, this analysis shows that strains possessing the genes encoding for a band is observed PirAvp up to 336 bp and PirBvp band up to 1317 bp. However, some of the

strains positive in PirAvp while for PirBvp were negative. This occurs because some strains have only some of the toxin genes or part of them (Tinwongger *et al.* 2016). The absence of the genes may be justified because the plasmid pVA1 presents transposase recognition sites, these sites are located at the ends where the genes are located which allows the horizontal transfer of these genes. These results are similar to those obtained by Lee *et al*. (2015) in a comparison of plasmids of positive strains AHPND and a strain isolated from an outbreak of AHPND, which had the plasmid pVA1 but not genes encoding binary toxin PirABvp. Thus to corroborate both negative and positive will be confirmed by qPCR. This method quantitatively will let us know if it is likely that the number of copies is insufficient to achieve visualize the product in a gel or just have one of the genes. Expectations in this work is the purification of toxins to carry out studies that allow us to learn more about the mechanism of action.

Conclusions

In the plasmid purification, we observe distinct concentration and pattern distribution; these differences can be due to the number of plasmids and isoforms in each strain.

With the sequencing of the PCR products PirAvp was able to identify if the sequences were homologous or if they had some kind of mutation which could explain the difference in the degree of virulence between strains. Based on this data it is defined that the products of such strains (PirAvp) do not show any difference in sequence.

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Costal changes due to subglacial volcanic eruptions on Deception Island (West-Antarctica)

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Abstract

We have examined radiocarbon ages, carbon and nitrogen isotopic compositions, and particle-size distributions in subsoil horizons from coastal areas of Whalers Bay and between Wensleydale Beacon and Cross Hill, Deception Island, Antarctica. The findings indicate that the Deception Island's landforms were preserved until 5.5 kyr cal. BP (BP means Before Present = 1950). From this time, the volcanic eruptions began to occur, producing landscape changes in its coastal environments. Additionally, the coastal changes are consistent with the presence of old ^{14}C ages of subsoil organic matter and so the occurrence of intense cryogenic disturbance processes (alluvial sedimentation), such as those caused by jokulhlaup.

Keywords: volcanic eruptions • alluvial sedimentation • cryoturbation process • jokulhlaup.

Introduction

Deception Island can be considered as an outdoor laboratory for volcano geophysics, debris-flow processes and related phenomena due to the large number of multidisciplinary studies to understand the origin and morphology of the South Shetland Islands archipelago. Deception is an active Quaternary volcanic island located in the Bransfield Strait, between the South Shetland Islands and the Antarctic Peninsula. It has traditionally been considered as a collapsed caldera formed by subsidence into a magma chamber of a group of overlapping volcanoes along arcuate and radial faults (Smellie, 2001). During the Holocene, the island's geomorphic evolution was controlled by different volcanic eruptions, by modifying both the inner and the outer shoreline and the appearance of new volcanic edifices like cinder cones and crater-lakes from maar and phreatomagmatic-type eruptions (Smellie, 2001; Liu *et al*., 2015). Unfortunately, there is a few published descriptions of well-recorded geomorphic events and our knowledge of its reconstruction of palaeo-volcanic surfaces is still lacking because it is usually related to recent eruptions (Torrecillas *et al.*, 2012). The objective of this work is therefore to determine the geomorphic variation undergone by Deception Island, based on coastal changes associated with the eruptions occurred throughout its existence. Therefore, this study addresses the temporal modification of sedimentary deposits and their organic accumulation rates, assessing particle size, organic content, carbon and nitrogen isotopic analysis and radiocarbon dating in 50-cm soil profiles from coastal soils in ice-free subpolar areas of Whalers Bay

(62°58'42" S, 60°33'30"W) and between Wensleydale Beacon and Cross Hill (62° 56' 41" S, 60° 41' 27" W).

Materials and methods

The sampling sites are Holocene beaches, located within a distance of 200 m from the shoreline and shows almost purely barren volcanic sandy soils (Figure 1). Soil profiles were collected from 20×20 cm pits excavated down to a depth of 50 cm. Individual sediment samples were extracted at depth intervals of 5 cm. Permafrost was not present in these soil profiles. Samples (200-300 g) from each layer of the soil profiles were dried overnight in an oven at 60 °C. Dried samples were then homogenized after the removal of large rock fragments and biological remains through a 2 mm mesh sieve. Subsample sets were created to determine the soil compositions and perform radio- and-stable isotope analyses. In order to reduce old carbon contamination in radio- and-stable isotope analyses from bedrock-derived lignite and potentially large inputs of penguin guano, soil samples were also sieved at 0.5 mm. In turn, the interference of inorganic C in both analyses was addressed by removing carbonates after acid addition (1.0 M HCl), with subsequent removal by washing with ultrapure water and drying. Soil physical and mineralogical properties were analyzed at UFF. Soil moisture was determined by the gravimetric method. Matrix grain size was measured using a laser particle size analyzer. Radiocarbon dating was determined by Accelerator Mass Spectrometry (AMS-^{14}C). Age calibration was performed with OxCal software using the Southern Hemisphere atmospheric curve SHCal13 (Hogg *et al.*, 2013). Therefore, radiocarbon ages are expressed in units calibrated dates before present = 1950 or cal. BP. Carbon and nitrogen isotopic analyses were performed at UC Davis Stable Isotope Facility, USA. Details of the sample preparation and analysis can be seen in Muniz *et al.* (2016).

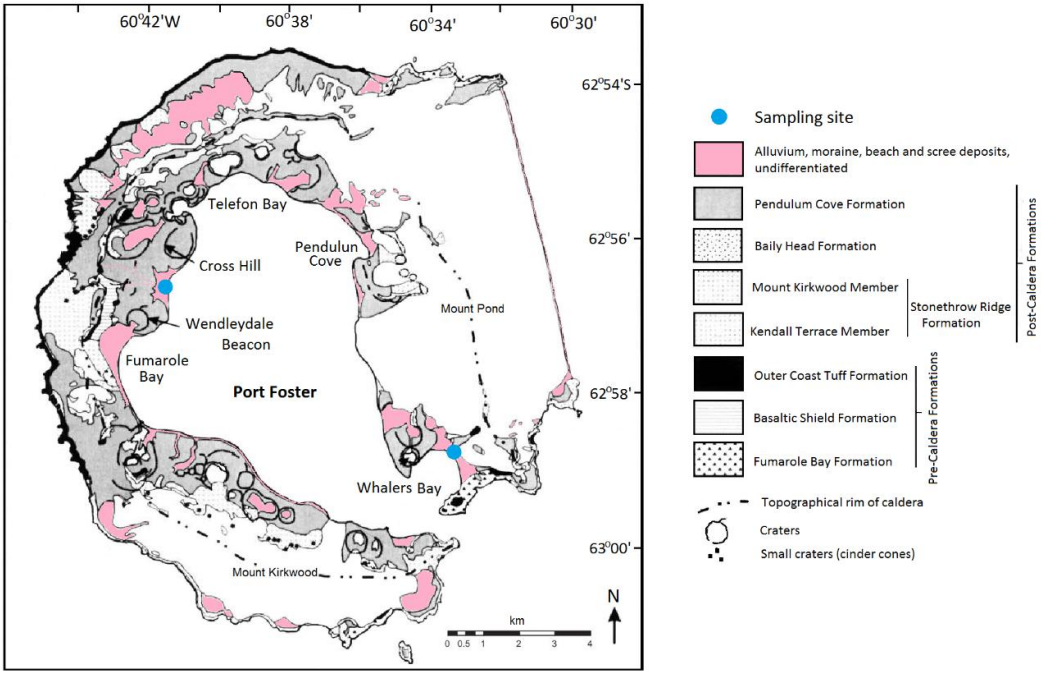

Figure 1. Geological map and sampling sites of Deception Island (Smellie, 2001).

Results and discussion

The layers of Whalers Bay's soil profile have high gravel contents (30-90%) and a sandy-skelectic texture. Clay contents in the < 2mm fraction are very low (1.3-2.9 %, i.e. 30-50 g kg^{-1}) and its textural group can be classified as muddy sand. The soils have very little horizon differentiation, so that the soil profile discloses a homogeneous section. The layers have grayish colors with a few gravels of yellowish hue and very low chroma, indicating a low degree of oxidation. Soil horizons from costal area between Wensleydale Beacon and Cross Hill show similar behavior. However, there is an intermediary subsoil layer enriched in mud (clay contents ranging from 7.3-11.3%). Muddy-sand soil has a low water-holding capacity, poor ability to store plant nutrients, rapid decomposition of organic matter, and high pollutant leaching potential (Haynes, 1998). Then, the coastal area of Whalers Bay and between Wensleydale Beacon and Cross Hill have currently a high susceptibility to wind and water erosion. This behavior is confirmed by Figures 2a and 2b that shows the presence of low values of organic matter and nitrogen contents from Wallers Bay's soil profile. Similar behavior is observed from soil profiles of costal area between Wensleydale Beacon and Cross Hill. This result can also be interpreted as evidence of increased overland flow from the surroundings, probably caused by increased precipitation or erosion effects.

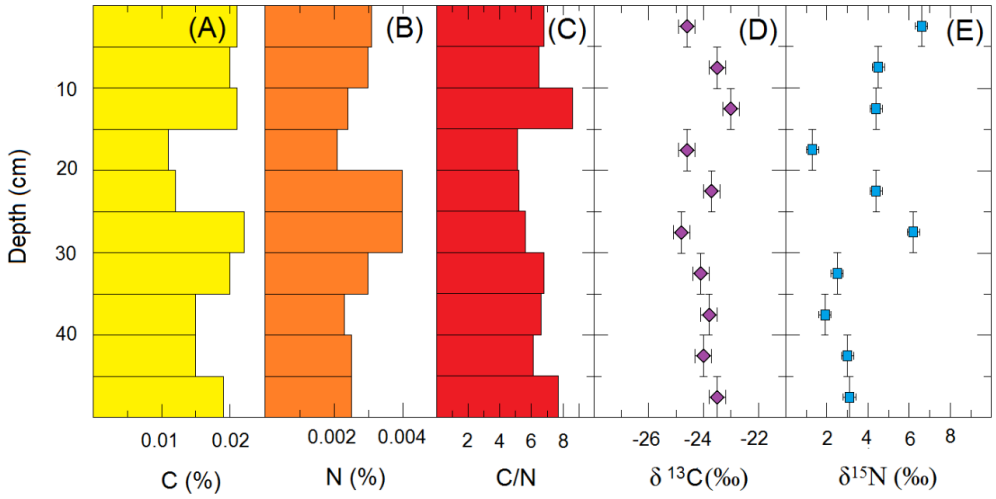

Figure 2. Distributions of C, N, C/N, $\delta^{13}C$, and $\delta^{15}N$ from Wallers Bay's soil profile.

Based on previous studies (Schmidt *et al.*, 2011) about the nature of the Soil Organic Matter (SOM) in subsoils, the ^{14}C age usually increases with soil depth, and carbon present in the subsoil horizons is characterized by a low ^{14}C activity. Old ^{14}C age of organic matter (OM) in subsoils may be observed as a result of continuous microbial recycling of labile material. Low-density C and microbial phospholipid fatty acids get older and decomposition rates slow with depth. For SOM in subsoils, it must additionally be considered that the ^{14}C activity may be influenced by the contribution of substrate inherent geogenic carbon, which is usually carbon dead (= older than 50,000 years), not showing ^{14}C activity. Generally, C in deep soil horizons is characterized by high mean residence times of up to several thousand years. With few exceptions, the SOM content and its carbon-to-nitrogen (C/N) ratio are decreasing with soil depth, while the stable C and N

isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of SOM are increasing, indicating that OM in deep soil horizons is highly processed. An increase in C/N with depth in a few soils may be explained by the presence of charred material, such as volcanic islands. From Figures 2a and 3a, one can note that the behavior expected for the vertical distribution of C and ^{14}C, respectively, is observed below 27.5 ± 2.5 cm depth in the Whalers Bay's soil profile. There is a good linear relationship between the depth and the ^{14}C age for organic matter. Additionally, organic carbon content is decreasing with soil depth, while C/N (Fig. 2c), $\delta^{13}C$ (Fig. 2d), and $\delta^{15}N$ (Fig. 2e) of SOM are increasing. Therefore, the accumulation of organic matter is stable from 5,486 ± 78 yr cal. BP to 10,868 ± 157 yr cal. BP and the beach of Whalers Bay was not altered (or perturbed) by volcanic eruptions during this period. These findings suggest therefore that the volcanic eruptions on Deception Island began from the last 5.5 kyr cal. BP and are consistent with previous publications in which reveal that the island's geomorphic evolution was controlled by different volcanic eruptions from 5,000 yr cal BP to 1970, by modifying both the inner and the outer shoreline (Smellie, 2001; Lee *et al.*, 2007; Torrecillas *et al.*, 2012; Liu *et al.*, 2015). Coastal changes occurred in Whalers Bay can also be understood from Figure 3a. It shows that the oldest ^{14}C ages of subsoil OM (between 13.5 and 11 kyr. cal. BP) are observed in upper soil horizons (above 25 cm depth). Taking into account the complex history of Deception Island during the Holocene, this result suggests that upper part of the section may have originated from elsewhere and resedimented on Whalers Bay. Old ^{14}C forms the bulk of the permafrost carbon pool that accumulated over thousands of years. Permafrost thaw (or glacier melting) stimulates the release of old ^{14}C that will contribute to the occurrence of old ^{14}C ages in SOM (Schmidt *et al.*, 2011). Therefore, the upper soil horizons could be related to cryoturbation processes in these maritime periglacial environments with lots of freeze-thaw cycles. Sedimentological processes, such as alluvial sedimentation, can also be from lahars. Lahar is a moving mixture of volcanic debris and water. It may be hot (few hundred °C) or cold. A flood caused by a subglacial volcanic eruption can release a lahar, also called glacier run or jökulhlaup (Jakob and Hungr, 2005). Jökulhlaups are also sources of old C (low ^{14}C activities).

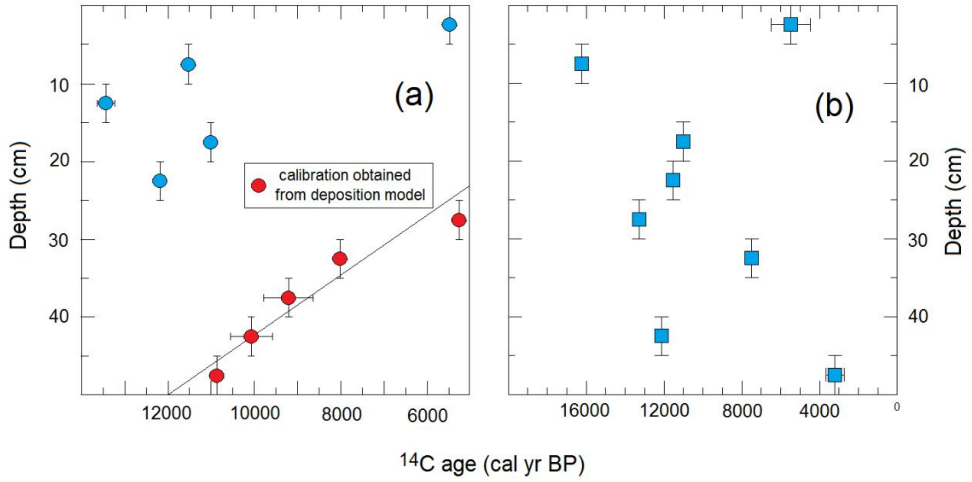

Figure 3. *^{14}C age for organic matter from soil profiles of coastal areas of **a)** Wallers Bay and, **b)** between Wensleydale Beacon and Cross Hill.*

Comparing the behavior for the organic carbon content, C/N, $\delta^{13}C$, and $\delta^{15}N$ (Figures 2a, 2c, 2d, and 2e, respectively) of the upper soil horizons (above 25 cm depth) with deep soil horizons (below 25 cm deep) from the Whalers Bay profile, we observed that they did not exhibit similar shaped. Upper soil horizons show fluctuations, suggesting that above the 25 cm depth, the physical and chemical properties of the soil and the biogeochemical reactions in the soil are not stable and, consequently, this soil layer may have been subjected to mixing processes over time. Taking into account the soil characteristics observed for the Whalers Bay and that the subsoil discloses very little horizon differentiation, it is not possible to state whether the material reworked/resedimented in the upper part of the section (above 25 cm deep) is relative to one single deposition event or if the depositional pattern has not been changed over time. However, it is possible to state that costal area on Deception Island has been changed since the last 5.5 kyr cal. BP. On the other hand, taking into account the soil characteristics observed for the soil horizons from the coastal area between Wensleydale Beacon and Cross Hill and their respective ^{14}C ages values of bulk soil organic matter (Figure 3b), we observed that subsoil showed horizon differentiations and there is not a noticeable relationship between the burial depth and the ^{14}C age for organic matter. Moreover, old ^{14}C ages are observed throughout the soil profile. Both information suggests, therefore, that this coastal area may have been subjected to over one reworked/resedimentation event during the Holocene and it is consistent with the occurrence of permafrost thaw/glacier melting phenomena due to volcanic eruptions.

Conclusions

Using radio- and stable-isotope methods, such as carbon and nitrogen isotopic compositions and radiocarbon dating in soil profiles from the Deception Island, we seek to understand the coastal changes occurred on Deception Island during the Holocene. From analysis of the persistence of soil organic matter as an ecosystem property, it is possible to predict how its costal area responded to subglacial volcanic eruptions. Deep soil horizons from Whalers Bay indicated that the Deception Island's landscape was preserved until 5.5 kyr cal. BP. From this time the volcanic eruptions began to occur. This result is in agreement with previous tephra measurements from solitary beds in lake sedimentary sequences. Even though it was not possible to state whether the upper soil horizons (above 25 cm deep) from Whalers Bay is relative to one single deposition event or if the depositional pattern has not been changed over time, our results suggest that the coastal area on Deception Island has been changed since the last 5.5 kyr cal. BP. However, they indicate that coastal area between Wensleydale Beacon and Cross Hill was changed during the Holocene by more than one reworked/resedimentation event. This is important information that can help the understanding of landscape changes on Deception Island's. Moreover, this kind of study can also assist in understanding of conditions for cryogenic soil processes that are predicted to dramatically change in response to the ongoing climate warming.

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Genetic diversity of snail *Lobatus gigas* (GASTROPODA: STROMBIDAE) in Yucatan, Mexico

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Abstract

The genetic population structure of the pink snail *Lobatus gigas* in the Yucatan Peninsula, Mexico, was determined by isozymes expression in polyacrylamide gels. Muscle samples of 50 organisms, captured at four sites of the Yucatan Peninsula, were used to characterize the genotypic expression as revealed by the expression of 55 loci in 30 enzymatic systems. The TFPGA program was used to analyze genic frequency data. The following parameters were determined: descriptive statistics, F statistic, genetic distances, Hardy-Weinberg equilibrium, UPGMA and the number of migrants as indicator of gene flow. Heterozygosity values ranged with a mean value of 0.0366; F_{IS} values ranged with a mean value of –0.0492; and F_{ST} values ranged with a mean value of 0.1039, suggesting heterozygote deficiency. The number of migrants were 2.156 per generation, which suggests a certain degree of variability among populations and corroborates the low values obtained for Nei's genetic distance, of 0.0053 for the node showing the separation of the population from Arrecife de Alacranes from the other populations. We conclude that the *Lobatus gigas* populations studied here do not present genetic fragility for their subsistence.

Keywords: Population genetic● Gene flow● Isozymes● *Lobatus gigas.*

Introduction

The characterization of the population genetic structure is a criterion of gran utility for the preservation of the important commercial and ecologic species, considering this is the way to show the heterogeneity and homogeneity of the population on big geographic location (Utter, 1991; Bates y Innes, 1995; Casu *et al*. 2012). The ecology of the larvaes influence highly the structuration of the population and exist a lot of speculation about the evolutionary advantages that the larvas develop, the panmixis is characteristic in the marine species of extend disperse, and this is owing to the movement of the larvs for the currents and events related to them. This way in species where the larvas take the same cavity than the adults, the dispersion can be very limited and the ability of move in big live areas for the adults can be lost (Rodriguez and Tello 2011). Queen conch *Lobatus gigas* L. is a big marine gastropod mollusk of significant economic importance for the marine area in the Caribbean. Nervertheless, as a result of intense effort of fishing and the destruction of the habitat of this mollusk. *L. gigas* fisheries in the most of the Caribbean areas has been seriously reduces and in some cases has been reached the disappear of the resource (Stoner y Ray, 1993). This led to *L. gigas* was considerate a commercially endangered species of global level in 1983 (Stoner 1994) and added in 1992 to the II

appendix of The Convention on International Trade in Endangered Species (CITES) (Stoner *et al*., 1996), promoting with this measure that the fishery of this mollusk has been closed seasonably in multiannual periods in areas of Venezuela, México, Belice, Cuba, Colombia and the United States. (Stoner *et a*l. 1996). This problem of Over exploitation in this organism increase by the lack of information about the specie in some areas of the Caribbean, mainly that information about the abundance in juveniles and adults, the distribution and abundance of larvae and the population genetic structure. *Lobatus gigas* in Mexico, is actually managed with a ban that goes from April to November and apparently match with his reproductive cycle. Another regulatory measurement is the minimum size of extraction, that decrease from 22 cm to 20 cm of the siphonal length because of the practices exercised from the fishermen dedicated to its capture. It is clear that his new size also affects to the reproduction and the recruitment. Finally, a catch quota of 2.5 tons per month was fixed, which not being respected by the fishermen, that also complement their assigned quotas with juvenile's snails. Actually have been ordered the total closure of the resource.

Methodology

The obtaining of the organisms was performed in four places in the coasts of the Yucatan Península, in Punta Allen, Banco Chinchorro, Arrecife de Alacranes and Isla Mujeres. Mollusk samples were macerated using the extraction buffer TEB, adjusting to a pH of 7, polyacrylamide gels were prepared at 7.7%, for being used with the native system (Brewer 1970). The phenotypic presence was determinate following the Shaw and Prasad (1970) and Brewer (1970) procedures. Conceited loci and alleles were designated according to the naming system proposed for Shaklee and Keenan (1986). Multiple loci of a particular enzyme were designated numerically (1, 2, 3, etc.) considering faster at lower anodal mobility. Alleles of a particular locus were appointed by their anodic relative mobility and naming the most frequent allele as 100 and the other above and below thereof with the respective values. loci and alleles were designated according to that proposed by Shaklee and Keenan (1986) nomenclature system. A locus was considerate polymorphic if the allele most common has a minor probability than 95%. (Towsend and Shing 1984) and the level of hererozygosity was determinated in regarding to the Hardy-Weinberg equilibrium. TFPGA program edition 1.3 (Tools for population genetic analyses), Miller 2000), was used for the analysis of genetic data population allozyme. Two alternatives were used to test the balance of the Hardy-Weinberg equilibrium, the testing calls goodness of fit Chi - Square and exact tests Haldane (Miller 2000). To assess the genetic differentiation among population we apply the statistical F developed by Wright (Sokal & Rohlf 1995, Weir 1990), the extent of variation between individuals within populations, f = F_{IS} or as fixing and measurement variation among populations, Q = F_{ST} or coefficient coancestry. The similarity measures used by this program are given for the distance from Nei options, (1972) and unbiased Nei (1978), the Wright distance (1978), the Rogers modified (1972) and the Reynolds distance and coancestriy. Bootstrapping analysis was used for to have a graphic or dendrograms representations of the genetics distance results of genetic distances and the inference about the possible relationships between the sites analysed (Sokal and Rohlf 1995).

Results and discussion

The values obtained with the Chi Square and Haldane allow us to appreciate that the loci ST2, G6PDH, LAP 2 and MDH 2 had significance for the first test while ST2, LAP 2 and MDH 2 were significantly different with the second test established that of the 8 loci that showed variation in muscle tissue in the four populations analyzed. Fis values and the fixation index Fst, in Table 1, ranging from 0.0835 to 0.3600 OCTDH until for 2 FUM with an average value of -0.0492, for the first parameter and the second value have an average 0.1039 with a range of 0.0082 to 2 to 0.1967 LAP for MDH 2. The values of genetic distance of all populations are compared paired manner established by the model of distance unbiased Nei (1978) were those who had the lowest values in all populations and away established with the model Rogers (1972) and modified by Wright (1978) were the largest. In the first case the Banco Chinchorro relationship - Isla Mujeres had the lowest value of 0.0011 and Arrecife Alacranes relationship - Punta Allen was the highest value, in the second case the Banco Chinchorro relationship - Isla Mujeres was the lowest value 0.0378 and Arrecife Alacranes relationship - Punta Allen with 0.0834 was the largest. In Fig. 1, the dendrogram obtained by the Cluster analysis using the model of the original distance of Nei (1972) in which it is observed that the maximum distance of 0.0053 was introduced by the node that relates to populations Reef presents Alacranes with others and the lowest value is present node Banco Chinchorro-Isla Mujeres 0.0015.

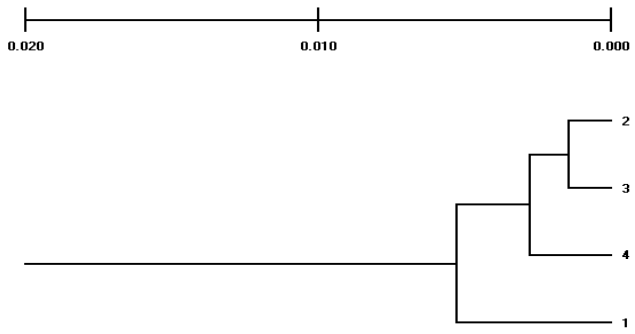

Figure 1. Dendogram pointing the relationship locations of *Lobatus gigas* in the Yucatán Península. 1.- Arrecife Alacranes 2.- Banco Chincorro 3.- Isla Mujeres 4.- Punta Allen.

Widespread occurrence of deficiency of heterozygosity on the expectations of the Hardy-Weinberg equilibrium has been fully reported in studies of isozymes in marine mollusks and other invertebrates (Maltagliati et al, 2002a, Hmida *et al*., 2012), as was presented in *Lobatus gigas* with a value of global average heterozygosity of 0.0366, which although low, is within the values determined for species of marine invertebrates in general since the heterozygote deficiency is a common feature found in these animals (Mamuri *et al*. 1998; Boisselier *et a*l.1999).The average value of Fst of 0.1039 or value of standardized variance in allele frequency was not significantly different from zero and therefore evidence of a certain degree of structure, meaning that 10.39% of the total genetic variation results from the differences between populations and the remaining 89.61% is the reflection of the variation within populations and relative cohesiveness and

conspecificity (Camilli *et al.,* 2001; Casu *et al*., 2002). The positive and significant values for Fis indicate a deviation from the Hardy-Weinberg equilibrium due to a deficit of heterozygotes may be due this to a fecundation between relatives. It has been reported to present a marine mollusk multiple paternities which may have a genetic effect on populations, however the selective pressures against heterozygotes, the presence of null alleles and sampling errors cannot be totally excluded. Analysis for the genetic differentiation of the population of *L. gigas* were carried out using estimators Nei's, Roger's and Reynold's (Wright 1978). UPGMA Cluster analysis reflect the degree of structure of the population and do not express a clear geographic pattern in the distribution of genetic variation in this species. Distance values and identity obtained for *Lobatus gigas* indicate that are typical values for species or populations that are well mixed (Maltagliati *et al*. 2003, Rodriguez and Tello 2011). Although the low observed heterozygosity may hinder discrimination stocks *L. gigas* using parameters such as identity and genetic distance are useful when the values of heterozygosity found in species under investigation are low and have a sufficient number of loci with which the problem of having a small sample size (Bierley *et al*. 1996) is remedied. Despite the relatively low values obtained in genetic distance, determined by the above estimates, there are signs of some sub-structuring of the population as demonstrated with the determined values of Fst and support the idea of seeing the population from the perspective of being a simple genetic unit. From a taxonomic perspective it is generally assumed that identity values above 0.9 or away below 0.1 indicate conspecificity and identity values below 0.8 and distance above 0.22 correspond to an interspecific differentiation with a dark zone between these values (Maltagliati *et al*. 2003).

Conclusions

Based on this study we can conclude that the four sites where *Lobatus gigas* is located at the Yucatan Península, doesn´t exists genetically significant differences to have a low value heterozygisity, a level of characteristic polymorphism of the invertebrate species, flow moderate genes, a geographic structuration not clearly defined, the typical distance value of the invertebrates and a specific connectivity between the population, as lead to establish a same panmitic unity and consequently suggest that the same population of *L. gigas* throughout the influence area can managed in the same manner and with the same criteria for use and conservation.

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BIOTECHNOLOGY SUMMIT

Antioxidant activity of *Pycnoporus* sp. grown in solid-state fermentation using polyurethane foam as support

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Abstract

Several studies have been carried out in order to isolate and identify active substances of fungi including basidiomycetes and the goal is to evaluate their potential therapeutic use, within these fungi are of the genus *Pycnoporus*. These fungi have shown antimicrobial, antiviral and antitumor properties, in addition, recent studies have reported that these fungi contain substances with antioxidant properties, which represent a natural source of antioxidant derivatives that are helpful in reducing and preventing cell damage (aging). The aim of this study was to evaluate the antioxidant activity of the native strain of *Pycnoporus* sp. (HEMIM-63) which is from state of Morelos, Mexico. The strain was grown on polyurethane foam impregnated with potato-dextrose medium and the growth parameters and antioxidant activity (using DPPH and ABTS) were determined. The maximum biomass produced was 6.5 g/L with a μ 0.015 h^{-1}. *Pycnoporus* sp. showed antioxidant activity, the highest values were 64.34 and 52.28% for ABTS and DPPH, respectively. This fungus is a source of natural antioxidants and its content could be increased by improving growing conditions.

Keyword: Antioxidant activity ● free radical scavenging ● solid-state fermentation ● polyurethane foam ● *Pycnoporus.*

Introduction

There is increasing evidence of damage caused by free radicals, since they have an important role in the development of different diseases, due to oxidative stress. Oxidative stress is caused by all these chemical species, with or without charge, that in their atomic structure have an unpaired electron in the outer orbital, creating great chemical instability contributing or feeding back the development of degenerative diseases (Halliwell and Gutteridge 2015). A major problem is generated when our body has to endure an excess of free radicals for prolonged periods, those produced mainly by external pollutants (air pollution, cigarette smoke, UV radiation, etc.) (Finkel and Holbrook 2000). So, external consumption of antioxidant compounds to counteract the harmful effects of these products is recommended. Currently fungi are of great interest as potential protective agents against oxidative damage because they produce a variety of bioactive compounds including phenolic compounds, carotenoids, polypeptides and terpenes, which are being investigated as relevant biological materials for the pharmaceutical industry (Di Piero *et al*. 2010). The basidiomycetes are attracting great attention because of the secondary metabolites that they

produce; the genus *Pycnoporus* is within this group (Lomascolo *et al*. 2003). There are several research papers on this genus, but focused mainly in their ability to degrade lignin (Ryvarden 1991), but they have also been studied by their antimicrobial, antiviral and antitumor properties (Harborne and Williams 1992). Recent studies report that *Pycnoporus* sp. is a natural producer of compounds with antioxidant capacity, so it can be a potential source of bioantioxidantes that would delay or would prevent oxidation as a result of the production of free radicals and cellular damage (Di Piero *et al*. 2010). In this study the antioxidant activity of extracts of *Pycnoporus* sp. grown in solid-state fermentation was quantified by mean of the DPPH and ABTS antioxidant activity determination.

Materials and Methods

Microorganism: The strain of *Pycnoporus* sp. (HEMIM-63) was used. The strain acquired from the Center for Biological Research at the Universidad Autónoma del Estado de Morelos (UAEM), Cuernavaca, Morelos, México, was kept on potato-dextrose agar under refrigeration (4°C) until its use. *Culture Conditions:* The solid-state fermentation (SSF) was carried out in Erlenmeyer flasks of 250 mL containing 0.5 g of polyurethane foam of low density (PUF; 17 kg/m^3) cubes (0.5×0.5×0.5 cm) as an inert support (Zhu *et al*. 1996; Díaz-Godínez *et al*. 2001). This inert support was impregnated with 15 mL of sterile culture medium of potato-dextrose. Three mycelia plug (4 mm diameter), taken from the periphery of colonies of *Pycnoporus* sp., were grown for 7 days at 25ºC in Petri dishes containing potato-dextrose agar (DIFCO^{TM}). These cultures were used as inoculum for each flask. The polyurethane cubes were washed twice with hot distilled water, oven-dried at 60°C for 24 h, and then autoclaved at 120°C for 15 min, before the culture. All inoculated flasks were incubated at 25°C during 27 days and the samples were collected every 24 h after the first 72 h of culture. All cultures were made by triplicate. *Extract preparation and biomass evaluation:* The crude extract (CE) was obtained by filtration of each culture using filter paper (Whatman No. 4). The pH of each CE was measured by a pH-meter and the biomass (X) was determined as the difference of dry weight (g/L) (Díaz-Godínez *et al.* 2001). The assay of biomass $X = X(t)$ was done using the Velhurst-Pearl logistic equation,

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X \qquad (1)$$

where μ is the maximal specific growth rate and X_{max} is the maximal (or equilibrium) biomass level achieved when dX/dt = 0 for X > 0. The solution of equation 1 is as follows,

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}} \qquad (2)$$

where $C = (X_{max} - X_0)/X_0$, and $X = X_0$ is the initial biomass value.

Estimation of kinetic parameters for the previous equations was performed using the non-linear least square-fitting program “Solver” (Excel, Microsoft) (Téllez-Téllez *et al*. 2008).
Determination of protein and sugars: Protein was measured in each CE by the Bradford method (Bradford 1976), using bovine serum albumin (SIGMA) as standard and reported as mg of protein per gram of X (mg/gX). The residual sugar content in the CE was determined using a refractometer. Each extract was measured by triplicate in separated experiments.

Antioxidant activity using 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical method: This assay was determined according to Moraes-De-Souza *et al*. (2008) with some modifications. The reaction mixture consisted of 0.5 mL of CE, 3 mL of methanol, and 0.3 mL of 0.5 mM DPPH radical solution in methanol. After incubation for 45 min, absorbance was spectrophotometrically determined at 517 nm. The antioxidant activity was calculated by using the following equation:
% inhibition = [(Acontrol – Asample)/ Acontrol] x 100
Acontrol = Absorbance of negative control, to=0, Asample = Absorbance of sample after 45 min.

Antioxidant activity of 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) radical: ABTS radical scavenging activity was determined according to Re *et al*. (1999) with some modifications. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS•+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS•+ solution was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm. The reaction mixture consisted of 0.07 mL of CE and 3 mL of the ABTS radical. The absorbance was determined at 734 nm after a 6 min incubation. The antioxidant activity was calculated by using the following equation.
% inhibition = [(Acontrol – Asample)/ Acontrol] x 100,
Acontrol = Absorbance of negative control at the moment of solution preparation, Asample = Absorbance of sample after 6 min.

Results and Discussion

Growth of Pycnoporus sp. in SSF

Figure 1 shows the biomass production over a period of time, the correlation coefficient between experimental data and mathematical model was R^2=0.83. The highest value of biomass was 6.5 g/L with a μ 0.015 h^1. The pH was maintained around 4.3 (Figure 2). Figure 3 shows that the consumption of the carbon source was reduced over culture time; at the end of culture only 5 g/L was observed. Regarding the content of soluble protein (Figure 4), it showed highest concentration at 336 h (0.14 mg/mL) and the lowest amount of protein was 0.07 mg/mL at 72 h. This fungus produced more biomass than *Pleurotus ostreatus*, when it grew in a culture medium with glucose, yeast extract and mineral salts (4.5 g/L and 0.033 μh^{-1}) (Téllez-Téllez *et al*. 2008), suggesting that *Pycnoporus* sp. intended the carbon and nitrogen sources more efficiently to the production of biomass than *Pleurotus ostreatus*.

Antioxidant activity

Figure 5 shows the antioxidant activity using ABTS technique; it can be observed the lowest percentage of inhibition with 22.7% at 408 h, and the maximum value was observed at 72 h with 64.3%. However, the lowest percentage of DPPH inhibition was at 168 h with 16.9% and the maximum was of 52.28% at 120 h (Figure 6).

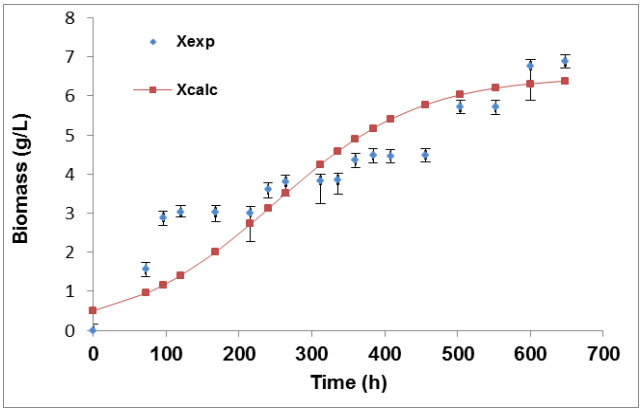

Figure 1. Profile of Biomass growth of *Pycnoporus* sp. (HEMIM-63) in SSF.

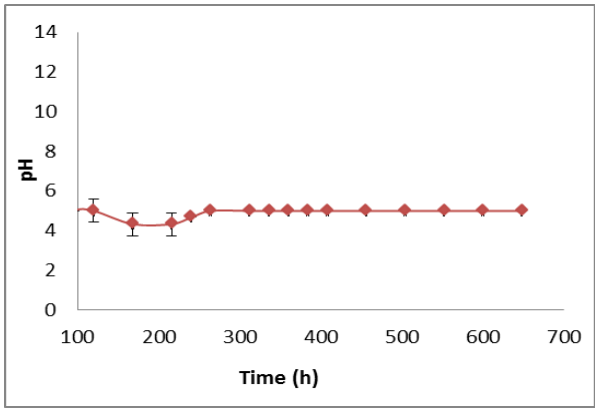

Figure 2. pH profile of *Pycnoporus* sp. culture (HEMIM-63) in SSF.

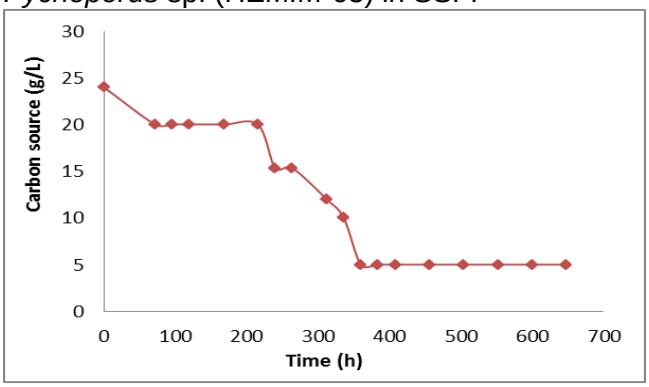

Figure 3. Consumption of carbon source of *Pycnoporus* sp. (HEMIM-63) in SSF.

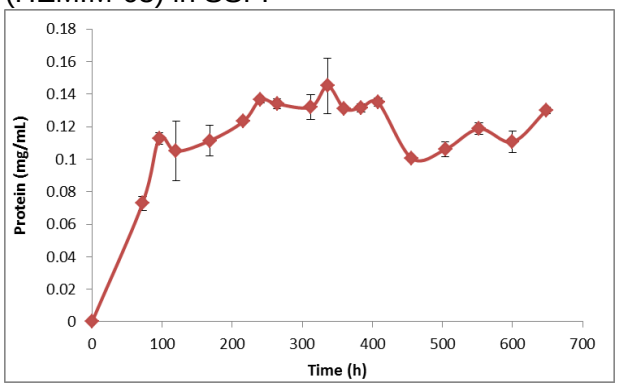

Figure 4. Soluble protein content in SSF of *Pycnoporus* sp. (HEMIM-63).

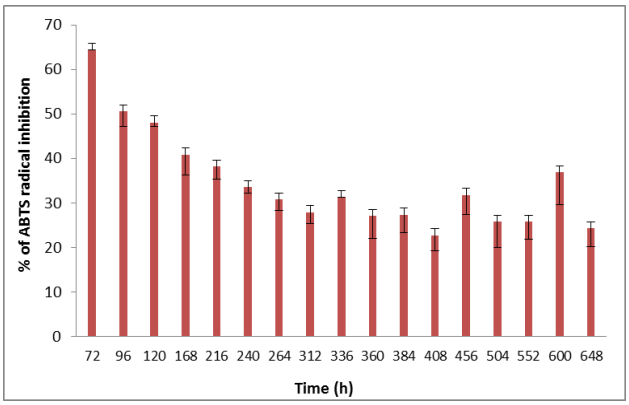

Figure 5. Percentage of ABTS radical inhibition.

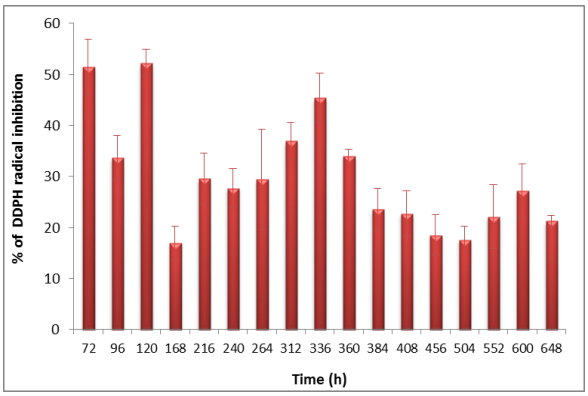

Figure 6. Percentage of DPPH radical inhibition.

Border *et al*. (2011) reported the antioxidant activity (DPPH method) of *Pycnoporus sanguineus* grown in liquid culture using dextrose-potato broth. The extracts obtained from the mycelium at different times of culture (5, 10, 15, 20, 25 and 30 days) were analyzed, all extracts showed antioxidant activity, however, the extract of mycelium of day 30 showed the highest antioxidant activity with 80% inhibition of DPPH radical. There are few reports where the antioxidant activity is measured in extracts of the culture medium. Díaz-Godinez *et al*. (2016) reported the antioxidant activity of extracts of *Pleurotus pulmonarius* and *Pycnoporus cinnabarinus* grown in AirLift reactor. The first fungus exhibited about 50% inhibition of the radical ABTS at 240 h and with DPPH was 32% at 72 h. *Pycnoporus cinnabarinus* reported approximately 55% for ABTS and DPPH at 168 h. In this study we probed an antioxidant activity higher at the same time (168 h). Base on this results, we consider Genus *Pycnoporus* as a promising candidate for antioxidants production but studies are needed in order to optimize culture conditions.

Conclusion

Pycnoporus sp. was efficiently cultured in a solid-state fermentation using polyurethane foam as support. The HEMIM-63 strain showed promising antioxidant activity (DPPH and ABTS methods) in solid-state fermentation on potato-dextrose medium. This antioxidant activity could be increased by optimizing the growing conditions.

Acknowledgements Thanks to PRODEP for sponsoring this research through de project UAEMOR-PTC-336.

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Activity of hydrolases and laccases of *Pleurotus djamor* var. *roseus* grown in submerged fermentation

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Abstract

Some edible fungi produce lignocellulolytic enzymes which degrade lignin and cellulose. However, in terms of biodiversity, it has been estimated that there are 1.5 million different species of fungi, but only about 5% of the total have been described, so it is necessary to know the capacity of production of metabolites of industrial importance from fungi, as is the case of enzymes. So in this work, the activity of hydrolases and laccases from *Pleurotus djamor* var. *roseus*, a native strain of Morelos state, were determined. The fungus was grown in submerged fermentation with a culture medium without addition of inducers. The strain produced 8 g/L biomass and μ 0.015 h^{-1}, the excreted protein was 0.06 mg/mL. The hydrolase activity values were: xylanase 1.49 IU/L, cellulases 122 IU/L and pectinases 140.6 IU/L; laccases at pH 4.5 and 3.5 showed 1330 U/L and 633 U/L, respectively. The native strain of *Pleurotus djamor* var. *roseus* is a good candidate as a source for obtaining lignocellulolytic enzymes.

Keyword: Constitutive enzymes ● submerged fermentation ● lignocellulolytic enzymes ● *Pleurotus djamor* var. *roseus*

Introduction

In the planet large quantities of lignocellulosic materials are discarded, these compounds contain of 40-50% cellulose, 25-40% hemicellulose, 25-35% lignin and other monomers in minor proportion (Pointing 1999). Such materials cause pollution problems, however, can be used to generate products of biotechnological importance. Some fungi have an enzymatic system to degrade lignin that allow its mineralization to CO_2 and water. The lignocellulolytic enzymes are used in the conversion of lignocellulose complex fermentable sugars for the production of fuel alcohol (Sánchez and Cardona 2008). In paper recycling, a mixture of cellulases, pectinases, xylanases and lipases are used; in extracting juices, flavors, oils and pigments, cellulase, pectinases and xylanases are added; also are used in the preparation of non-caloric additives (Joshi and Pandey 1999). The ligninases are used to increase the digestibility of lignocellulosic materials for ruminant feeding (Weinberg 2000; Koutinas *et al.* 2004). So it is important to follow in the search for producing organisms of lignocellulolytic enzymes, so they can take advantage of waste. The aim of this study was to determine the activity of hydrolases and laccases of *Pleurotus djamor* var. *roseus* grown in submerged fermentation without the presence of inducers.

Materials and Methods

Organism and culture conditions. The strain of *Pleurotus djamor* var. *roseus* (HEMIM-103) was used. The strain acquired from the Center for Biological Research at the Autonomous University of Morelos State (UAEM), Cuernavaca, Morelos, México. Submerged fermentation was carried out in Erlenmeyer flasks of 125 mL containing 50 mL of potato dextrose broth. Three mycelial plugs (4 mm diameter) taken from the periphery of colonies of fungus grown for 7 days at 25ºC in Petri dishes containing potato dextrose agar were used as inoculum for each flask. The culture was incubated at 25ºC for 27 days on a rotary shaker at 120 rpm.

Enzymatic extract preparation and biomass evaluation. The enzymatic extract (EE) was obtained by filtration of the cultures using filter paper (Whatman No. 4). The pH of each EE was measured by potentiometry and the biomass (X) was determined as difference of dry weight (g/L) (Díaz-Godínez *et al.* 2001).

The assay of biomass $X = X(t)$ was done using the Velhurst-Pearl logistic equation,

$$\frac{dX}{dt} = \mu\left[1 - \frac{X}{X_{\max}}\right]X \qquad (1)$$

where μ is the maximal specific growth rate and X_{max} is the maximal (or equilibrium) biomass level achieved when $dX/dt = 0$ for $X > 0$. The solution of equation 1 is as follows,

$$X = \frac{X_{\max}}{1 + Ce^{-\mu t}} \qquad (2),$$

where $C = (X_{max} - X_0)/X_0$, and $X = X_0$ is the initial biomass value.

Estimation of kinetic parameters in the above equations was performed using the non-linear least square-fitting program “Solver” (Excel, Microsoft) (Téllez-Téllez *et al*. 2008).

Determination of protein and sugars. Protein was measured in each EE by the Bradford method (Bradford 1976), using bovine serum albumin (SIGMA) as standard and reported as mg of protein per gram of X (mg/gX). The residual sugar content in the EE was performed using a refractometer. Each extract was measured by triplicate in separated experiments.

Enzymatic assays. Three hydrolase activities in EE were assayed by quantifying reducing sugars using the DNS (3,5-dinitrosalicylic acid) method (Miller 1959). The assay mixture contained 475 µL of substrate (0.5% polygalacturonic acid or 1%, 0.5% birch xylan in 0.1 M acetate buffer at pH 5.3, and 1.0% carboxymethylcellulose in 0.1 M acetate buffer at pH 5.0, for pectinase, xylanase, and cellulase activities, respectively) and 50 µL of EE. The reaction temperatures were 45 and 50 °C, respectively. One international unit (IU) of hydrolase activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per minute under assay conditions. Laccase activity was determined in each EE by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol (DMP) as substrate. The assay mixture contained 950 µL substrate (2 mM DMP in phosphate buffer pH 6.5, acetates buffer pH 5.5, 4.5 y 3.5) and 50 µL EE, which was incubated at 40 °C (Téllez-Téllez *et al*. 2005). One enzymatic unit (U) of laccase activity was defined as the amount of enzyme that gives an increase of 1 unit of absorbance per min in the reaction mixture. The activity was expressed in U/L of EE.

Results and discussion

Pleurotus djamor var. *roseus* (Figure 1) showed 8 g/L of biomass and a μ of 0.015 h^{-1}, the pH of the initial medium was 5.5, during growth of the fungus was increased reaching 6.8 at 648 h (Figure 2), It was observed at the end of culture 8 g/L residual glucose (Figure 2). Regarding the soluble protein concentration was 0.06 mg/mL at 504 h (Figure 3).

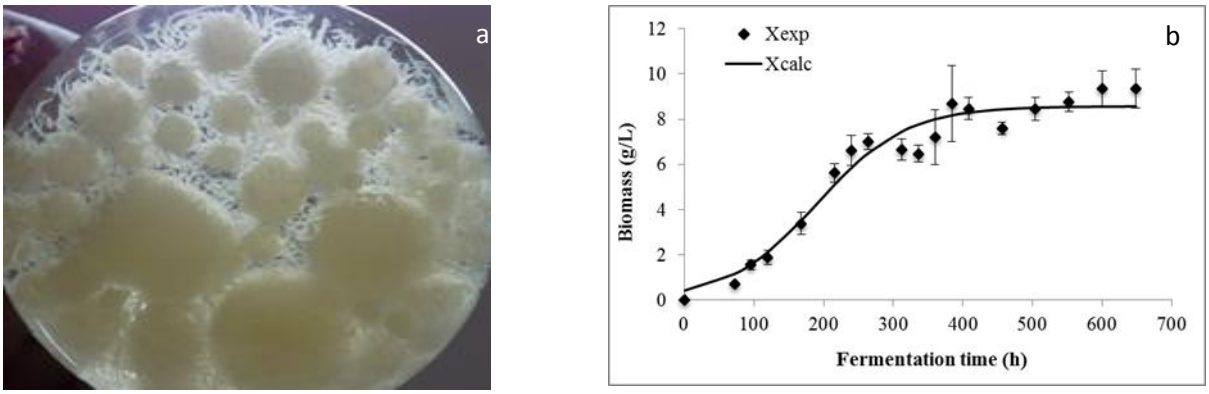

Figure 1. Growth of *Pleurotus djamor* var. *roseus* in submerged fermentation. a) mycelium, b) growth curve.

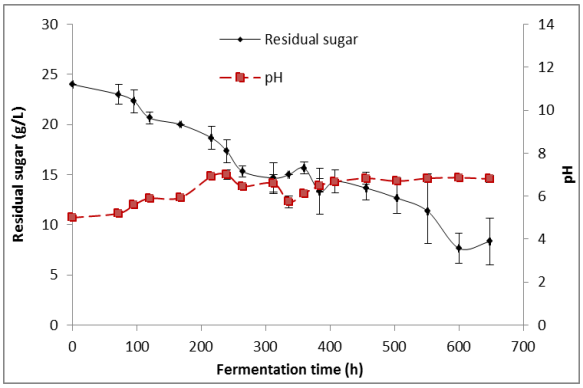

Figure 2. Residual sugar and pH in the culture of *Pleurotus djamor* var. *roseus* grown in submerged fermentation.

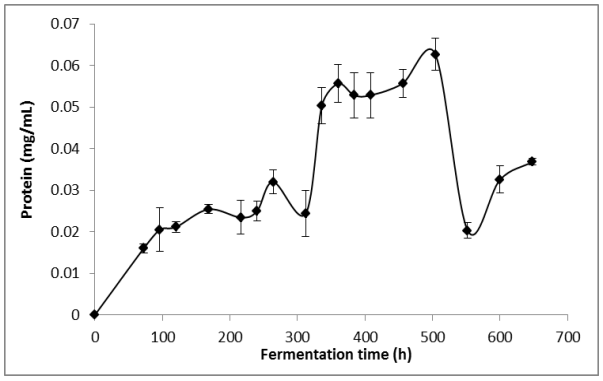

Figure 3. Soluble protein content in the culture of *Pleurotus djamor* var. *roseus* grown in submerged fermentation.

Téllez-Téllez *et al.* (2008) grew *Pleurotus ostreatus* in a liquid culture medium with glucose, yeast extract and mineral salts, showing 5.5 g/L of biomass and μ of 0.022 h^{-1}, the total consumption of the carbon source was at 430 h. This study presented higher biomass and lower μ values. Díaz-Godínez *et al*. (2016) grew *Pleurotus pulmonarius* in a reactor AirLift with the same liquid culture medium used in this study; they reported the enzymatic activity of hydrolases and laccases, the activity of cellulases was the highest (900 U/L), followed by pectinase (600 U/L), xylanase (550 U/L) and laccase (550 U/L) in the first 200 h of growth. *Pleurotus djamor* var. *roseus* reported lower values of enzymatic activity than those reported in the previous work. The xylanase activity was 1.49 IU/L (264 h), cellulases was 122 IU/L (72 h) and pectinases was 40.6 IU/L (456 h); the three enzymes were detected at the beginning or end of the growth phase (Figure 4), which could suggest that they are constitutive

enzymes and important for nutrition. Laccase activity was evaluated at four different pHs (Figure 5). It had highest activity at 120 h of culture, the values were at pH 4.5 (1330 U/L), followed by pH 3.5 (633 U/L), pH 6.5 (518 U/L) and pH 5.5 (505 U/L) in all pH 's. In general, the maximum laccase activity was at the beginning of the exponential phase and gradually declined.

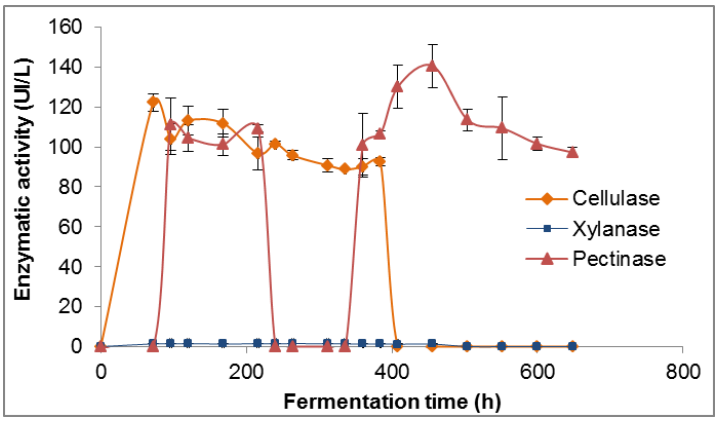

Figure 4. Hidrolytic activity produced by *Pleurotus djamor* var. *roseus* in submerged fermentation.

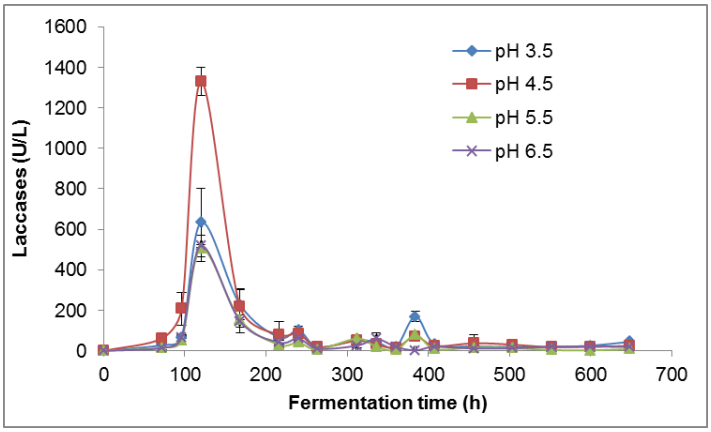

Figure 5. Laccases activity produced by *Pleurotus djamor* var. *roseus* in submerged fermentation

A commercial strain of *Pleurotus ostreatus* was grown on rice straw and sawdust in plastic bags. Endoglucanases, carboxymethylcellulases, and pectinases activities produced on sawdust (6.0, 13.5, and 22 U/g, respectively) were higher than those observed in rice straw (4.0, 5.0, and 14.0, respectively). Xylanase activity produced in the sawdust showed higher activity in the mycelium (21 U/g) than in the fruiting stage (11 U/g). The exoglucanase activity was about the same in both residues (Sherief *et al*. 2010). Cellulolytic activities were compared in two white-rot fungi, *Bjerkandera adusta* and *Pycnoporus sanguineus*, that were cultivated on wheat straw agar. *Bjerkandera adusta* showed 1.6 times higher carboxymethylcellulase activity than *Pycnoporus sanguineus* (Quiroz-Castañeda *et al*. 2009). In our study, the activity of hydrolase enzymes was lower, but the activity of laccases was higher without the presence of inducers. These results suggest that enzyme activity values depend of the strain and the culture conditions.

Conclusion

Pleurotus djamor var*. roseus*, a native strain of Morelos state, presented hydrolases and laccases activities without the presence of inducers, which could increase the activity by optimizing the culture conditions and by induction.

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Chemical and enzymatic pretreatment evaluation on the steviosides extraction from *Stevia rebaudiana*

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Abstract

Stevia rebaudiana accumulates steviol glicosides, a group of diterpen glycosides known for being non-caloric sweeteners with potential health benefits. Their widespread use requires an effective extraction method. In this work, the use of chemical and/or enzymatic pretreatments on steviosides extraction was evaluated. Chemical and enzymatic pretreatments were selected according to *S. rebaudiana* leaves composition evaluated on based of lignocelluloses content. Chemical NaOH pretreatment was observed to give the highest stevioside and rebaudioside A yield in comparison to methanol and simple water extraction. The extracted stevioside and Rebaudioside A, were identified and quantified by HPLC using corresponding standards. Enzymatic extraction with commercial CTec3 displayed a higher stevioside and rebaudioside A extraction in comparison to controls, suggesting that an enzyme-based process could be an option for steviosides extraction.

Keywords: *steviosides, extraction, biomass pretreatment*

Introduction

Steviol glycosides (SG's) are diterpene glycosides used as natural low-calorie sweeteners. They are obtained mainly from the leaves of *Stevia rebaudiana* Bertoni, a native plant from Paraguay. SG's are 300 times sweeter than sucrose and have been used in medicine and food industries (Rao *et al*. 2012). Stevia leaves accumulate in their vacuoles eight different SG's which differ from each other in the type and number of glycosilation (Geuns, 2003; Brandle & Telmer, 2007). Stevioside (Stev) and Rebaudioside A (RebA) are the most abundant glycosides found in *S. rebaudiana* leaves therefore; are the commercial SG's generally used. Procedures for SG's extraction involve aqueous or alcohol solvent, followed by precipitation or crystallization, supercritical fluid methods, pressurized liquid extraction, among others (Puri *et al*. 2011). Recently, enzyme assisted extraction have been reported in the bioactive compounds extraction, including SG's extraction (Puri *et al*. 2012). SG's are synthesized in the cytosol and storaged into the vacuole cell (Brandle & Telmer, 2007), therefore, enzymatic assisted extraction may be promising since cell wall degrading enzymes aid to break down or modify the plant cell wall, rendering intracellular compounds more accessible for extraction. Puri *et al*., (2012) optimized a process for extracting SG's using hemicellulases, reporting 3-times more production than not optimized procedure. The modification and disruption of cell wall have been quite studied in terms of bio fuels

production (Kumar *et al.* 2009). It is known that a combination of chemical and enzymatic procedures may increase the yield production of sugars release. The most studied biomass pretreatment includes ammonia fiber explosion, steam explosion, chemical treatment and biological treatment, being a combination of the last two quite promising (Rosgaar *et al*. 2007). Nevertheless, the choice of the pretreatment used depends on the biomass composition and the stability of the product. In the case of pretreatment application to biofuels obtainment, the goal of the process is to break down the lignin and disrupt the hemicelluloses complex so the enzymes can access and hydrolyze cellulose. However, no studies have been documented about the use of pretreatments in the extraction of bio active compounds such as SG's. In this work we studied the effect of chemical and enzymatic pretreatment on the methanol based SG's extraction. For this purpose, we evaluated the structure of *Stevia rebaudiana* leaves and applied pretreatments used for other biomass with similar structures.

Materials and methods

Sample. Stevia rebaudiana leaves were purchased from a local provider in Orizaba (Veracruz, México). Leaves were dried and grinded into particles of size 1-2mm. Chemical reagents were acquired by Sigma or Baker ®. Enzyme preparation CTec3 was bought from Novozymes ®.

Characterization of Stevia rebaudiana residues leaves. S. rebaudiana leaves, were characterized after methanol extraction, for identification and quantification of structural sugars and lignin content. For this purpose, the National Renewable Energy Laboratory (NREL) methodologies for biomass characterization, were applied (Sluiter *et al*., 2008). Briefly, biomass was prepared by drying sample at 60°C then after, ashes and extractives were determined according to NREL protocol. Structural Sugar content was analyzed by acid hydrolysis followed by HPLC quantification. For HPLC quantification a Carbohydrate Analysis Aminex HP-column was used. Lignin determination was performed by UV-absorption spectroscopy after acid hydrolysis (Chen, 2014).

Chemical pretreatments. For all the experiments 2g of biomas (*S. rebaudiana* leaves) were used. Acid pretreatments were set at H_2SO_4 1.5% (w/v) into covered glass bottles. Then, samples were autoclaved at 121°C, 1h. After pretreatment, pH was adjusted with ($CaCO_3$) (Dien, *et al.* 2006). Alkali pretreatment with NaOH was settled at two concentrations: 5 g/100 g biomass and 20 g/100 g biomass. Biomass was submerged into solution and incubated at 25°C, 24h, in constant agitation 120 rpm. After pretreatment, pH was adjusted with (HCl) 1 M (Sills & Gossett, 2011). Pretreatment with $(NH_4)_2SO_4$ was settled at 0.07% final concentration and incubated at 30°C, 24h and at 100°C, 5 min (EP 2 179 085 B1, 2014).

Enzymatic pretreatment. 1g of biomass chemically pretreated was incubated with 21.5 µL CTec3 enzyme at 50°C, 72h. Control pretreatment was used without enzyme incubation

Stevioside extraction. 1g of biomass, pretreated or not, were incubated with methanol 1g/10ml at room temperature. Then after, samples were filtrated and the filtrated was dried at room temperatura for 4-6 days. (Bondarev, 2001).

Stevioside and Rebaudioside A quantification by HPLC. Quantification was performed in a HPLC Perkin Elmer. A column SPHERI 5 RP-18 5 µm 250x4.6 mm was used under isocratic conditions at 30°C. PDA detector was used for the identification of steviosides. Mobile phase was acetonitrile:water in a 70:30 proportion. Identification and quantification was performed by calibration of method used with Stev and Reb A standards (Sigma).

Results and discussion

Characterization of *Stevia rebaudiana* residues after methanol extraction is summarized in Table 1. The presence of glucose and galactose suggests the hemicellulosic composition of a typical non woody plant but, similar to grass and cereals content (Puls *et al. 1997)*. *S. rebaudiana* have been classified as a perennial herb, a branched bushy shrub in the sunflower family (Lemus-Mundaca *et al.* 2012). Therefore, these results are in accordance to this type of family structural composition which is similar to Graminae and bark (Chen, 2014). Due to the relative high content of lignin (45%) this biomass may be classified as a recalcitrant biomass (Zhao *et al.* 2012). The content of ash varies with the varieties of materials, it depends on the kind of plant, organs, age and so on. Ash represents elements in the growth environment of the plant. Generally, ash content is less than 1% in wood and higher in Graminae (Chen, 2014). The ash content in these results is in accordance to the similarity of the plant to the latter group. The stevioside and rebaudioside A quantification after chemical or chemical/enzymatic pretreatment are shown in Table 2. Chemical pretreatments promoted a higher steviosides and rebaudioside extraction, in comparison with the enzymatic extraction. However, the quantity obtained by simple enzyme hydrolysis suggests a potential use for lignocellulolytic enzymes in this process. Previous works have reported the increase of extraction by using hemicellulolytic enzymes (Puri *et al.* 2012) however; no experiments had been done with the use of lignocelluloses activity. Further works may incorporate the study of synergistic enzymatic action on extraction.

Even though Stev extraction was similar to controls, NaOH pretreatment at the higher concentration used displayed the higher RebA extraction. NaOH is known for removing amorphous substances such a lignin and hemicelluloses (Kumar *et al.* 2009). Besides, it plays an important role in removing lignin and making cellulose more expose which might increase the porosity of the cell wall making aiding the extraction of Reb A. Kong *et al*., (1992), reported that alkalis remove acetyl groups from hemicelluloses thereby reducing the steric hindrance and enhancing accessibility, which may contribute to the extraction of Reb A in this study.

Table 1. Content of *S. rebaudiana* leaves residues.

Component	**Percentage (w/w)**
Total solids	93.14(±12.5)
Humidity	6.98(±1.2)
Monosacharides	
Glucose	0.776(±0.123)
Galactose	0.134(±0.045)
Fructose	0.785(±0.10)
Total lignin	44.91(±10.39)
Soluble	19.8(±12.5)
Non soluble	25.08(±8.94)
Ash	1.7(±0.02)

Table 2. Stevioside and rebaudioside A quantification after pretreatments.

Pretreatment	Stevioside (µg/mL)	Reb A (µg/mL)	Stev Yield	Reb A Yield
Methanol extraction	17,108.82	2,278.12	17.11	2.28
H2SO4				
control	11,319.65(±1579)	544.45(±183)	11.32	
sample	544.32 (±30)	ND		
			0.54	
NaOH				
control	40,006.11(±381)	4,529.67(±219)	40.01	4.53
Sample 5%			38.82	
	38,824.43(±1087)	3,406.48(±200)		3.41
Sample 20%	26, 326.36 (±271)	10,043.55 (±247)	26.33	10.04
$[NH_4]_2SO_4$				
Control	7,616.70(±1687)	831.54(±167)	7.62	0.83
Sample 100°C	10, 899(±615)	ND	10.90	
Sample 30°C	6,818.94(±999)	764.35(±130)	6.82	0.76
Enzymatic Hydrolysis	6,154.46(±150)	4,629.95 (±580)	6.1	4.6
Control				
	5, 457.60(±980)	3,790 (±*605*)	5	3.8
+ NaOH 5%			0.17	0.24
	170.881(±180)	241.28(±33)		
+NaOH 20%	135.65 (±7)	484.57(±120)	0.14	0.48

+Enzymatic hydrolysis after described pretreatment
ND= No detected

Conclusions

These results support the feasibility of chemical and enzyme assisted extraction for steviosides and rebaudioside A. A chemical pretreatment with NaOH gave a better yield than that obtained by conventional solvent extraction methodology for Reb A, which is economically important. The highest recovery of stevioside was achieved with NaOH pretreatment at 5% in 24h at 25°C, which was similar to its control. Further experiments for optimization of the enzymatic hydrolysis are being performed.

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Bacillus subtilis FSQ1 as a promising biological control agent against *Sclerotinia sclerotiorum* in common bean (*Phaseolus vulgaris* L.)

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Abstract

The common bean (*Phaseolus vulgaris* L.) is an important legume used for human consumption; however, several factors diminish its yield, such as: the white mold disease caused by the fungal pathogen *Sclerotinia sclerotiorum.* The aim of this work was to isolate, identify and characterize promising biological control agents against this pathogen. The soil and plant tissue sampled for the isolation of *S. sclerotiorum* and biological control agents was collected in a commercial bean crop, located at Gabriel Leyva Solano, Sinaloa, Mexico, in 2014. *S. sclerotiorum* was successfully from sclerotia presented in the plant tissue collected, the macro, microscopic and molecular identification show that the isolated obtained belongs to *S. sclerotiorum*. In addition, the strain FSQ1 (23 bacterial strains were isolated in total) presented a high antagonistic activity against *S. sclerotiorum*, observing a fungal growth inhibition > 30% using confrontation assays *in vitro*. The strain FSQ1 was identified as *Bacillus subtilis*, by the amplification of the 16S rRNA gene. Furthermore, greenhouse assays were conducted for quantify the impact the co-inoculation of the pathogen and the biological control agent on common bean seedlings, the results show that the strain FSQ1 reduces totally the presence of the white mold disease caused by *S. sclerotiorum*. These results show the potential biological control of white mold disease caused by *S. sclerotiorum* in the field, through the inoculation of *B. subtilis* FSQ1.

Keywords: *Sclerotinia sclerotiorum* ● biocontrol ● rhizosphere

Introduction

The common bean (*Phaseolus vulgaris* L.) is the most important legume for human consumption, it comprises ~ 50% of total legumes consumed by the world population (Broughton *et al.* 2003). In addition to its high protein content, the common bean has interesting nutritional properties, such as: high content of fiber, carbohydrates, vitamins, and minerals (Chen, 2015). Mexico has a production of $23x10^6$ tons per year, contributing with 6% of the global production (FIRA, 2015). Unfortunately, several factors such as nutrients deficiencies, acidic soils, and diseases particularly white mold, have been important constraints to the common bean production around the world (Schwartz and Singh, 2013). White mold disease, caused by the fungus *Sclerotinia sclerotiorum*, is an endemic disease affecting cold humid highlands in Mexico, as well as countries having similar climatic conditions. This disease is characterized by the formation of white-cottony mycelium on plants, causing small, circular and dark lesions, which diminish up to 50% of the crop

production when climatic conditions are optimal for the fungal development (Schwartz and Singh, 2013; Lopez, 2010). In advanced fungal development stages, *S. sclerotiorum* produces sclerotia, which are structures used as a main conservation and dispersion mechanism, generated by the aggregation of hyphae and continuous layers of melanised pseudoparenchyma cells (Henson *et al.* 1999). To date, different strategies have been used to control the white mold affecting the common bean production around the world. Thus cultural practices play a key role in the integrated management, however, these do not completely suppress this disease. Therefore, the application of synthetic fungicides (Benomyl, Carbendazim and/or Iprodione) is the traditional solution for controlling *Sclerotinia sclerotiorum* (Chitrampalam, 2010). However, the application of these fungicides is not a sustainable alternative way, because their prolonged use generates pathogen resistance, as well as several negative environmental effects such as soil contamination (Porter and Phipps, 1985). Thus, a promising alternative way for diminishing the negative impact of this pathogen as well as its traditional control practices is the use of biological control agents. Those comprise the use of microorganisms or their metabolites for controlling o diminishing the negative impact caused by pathogens affecting agronomic crops (Rincón *et al*. 2014).The aim of this work was the isolation and characterization of a promising soil bacterial strain, *Bacillus subtilis* FSQ1, as a biological control agent against *S. sclerotiorum*, the causal agent of the white mold disease in common bean crop, which were isolated from a commercial field located in the state of Sinaloa, Mexico.

Materials and methods

Sampling site. The sampling site was locate at Gabriel Leyva Solano community, located in the municipality of Guasave, Sinaloa state, Mexico (25º 37' 51″ N, 108º 39' 28″ W), during the 2013-2014 common bean productive cycle. Ten plants were randomly collected, which showed symptoms of the white mold disease. In addition, 250 grams of rhizosphere soil were collected. These samples were transported to the laboratory at 4 ^{0} C in sterile plastic bags (SAGARPA, 2016).

Isolation of microorganism. Pathogen isolation: Collected plants presenting the white mold disease were surface sterilized using 1% NaClO for 1 min, and cut into 1 cm^2 pieces, which were placed on Petri dishes containing Potato Dextrose Agar (PDA), and incubated for 48 h at 28 ± 2 °C. Each mycelial colony was isolated using the same culture medium. Fungal colonies with the characteristics of *Sclerotinia* were confirmed microscopically according to Willetts and Wong (1980). Rhizosphere bacteria isolation: 10 grams of rhizosphere soil were placed in 90 mL of sterile distilled water and homogenized at 250 rpm for 1 h, and then 0.1 mL of serial dilutions up to 1x10^{-4} were spread on Petri dishes containing Nutrient Agar (AN) supplemented with 80 ppm of terbinafine (Suarez and Rangel, 2013). The inoculated Petri dishes were incubated for 48 h at 28 °C. All experiments were performed in triplicate.

Confrontation assays. Rhizosphere bacteria were confronted against the pathogen. Thus 5x10^3 Colony-Forming Unit of both each bacterial strains and the pathogen were placed 6 cm apart on Petri dishes containing PDA, and incubated for seven days at 28 °C. The assay was performed using three independent replicates and one negative control without the inoculation of potential biological control agents. The percent inhibition was determined according to Schmid *et al.* (2009).

Molecular identification. Genomic DNA from microorganisms presenting biological control as well as the pathogen was extracted according to Reader and Broda (1985). The amplification of the 16S rRNA and the internal transcribed spacer 1 (ITS1) gene were performed using a

Polymerase Chain Reaction (PCR), respectively. The 16S rRNA gene was amplified using the primers FD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and RD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'). The PCR conditions used had an initial denaturation step at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 40 s at 57 °C and 2 min at 72 °C, and a final elongation step of 5 min at 72 °C (Weisburg, 1991). For the amplification of the ITS1 gene the primers used were ITS1F: (5´ -TCCGTAGGTGAACCTGCGG- 3´) and ITS4R: (5´-TCCTCCGCTTATTGATATGC- 3´). The PCR conditions had an initial denaturation step at 95 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and a final elongation step of 10 min at 72 °C (White *et al*. 1990). The PCR products were visualized by 2% of agarose gel electrophoresis, purified and sequenced using a Sanger platform (Sanger ABI 3730 XL, Applied Biosystem). All Sequences were compared to those deposited in the NCBI Genbank. Sequences generated in this study were aligned with those retrieved from Genbank using CLUSTAL W, and analyzed by the neighbor-joining method, using MEGA 6. Stability of clades was assessed with 1000 bootstrap replications.

Greenhouse assay. Three treatments were applied to common bean seeds, which were planted in sterile soil obtained from a common bean farm in active production, located in the state of Sinaloa, Mexico. The treatments evaluated were: A) Control, seeds without microbial inoculation, B) Seeds inoculated only with 24 mL containing $1.2x10^5$ CFU/mL, of the pathogen, and C) Seeds co-inoculated with the pathogen and the biological control agent (24 mL and 38 mL containing $1.2x10^5$ CFU/mL and $2.3x10^8$ CFU/mL, respectively). Then, the plant growth and the white mold disease were measured every 15 days during 2 months, at 28 ± 5 °C. This assay was performed using 14 independent replicates per treatment.

Statistics analysis. Data were analyzed by ANOVA analysis of variance, using Fisher LSD test (P= 0.5), using the Statgraphics Plus 5.1 software.

Results and discussion

The molecular identification – amplifying the ITS1 gene by PCR- of the pathogen FRM18 isolated in this study, showed a 100% of similarity to *Sclerotinia sclerotiorum* (Figure 3). It confirms this specie as the causal agent of white mold disease observed in the field. Twenty-three bacterial stains were isolated from the soil collected in this study, however, only one of them (FSQ1) presented a biological control against *S. sclerotiorum* isolated previously, showing a fungal growth inhibition of 35%, after 7 days of incubation (Figure 1). The behavior of the biocontrol by the strain FSQ1 against the pathogen, suggests that several mechanisms could be involved in this activity such as the competition for nutrients and/or the production of diffusible antifungal metabolites (Duncan *et al.* 2003).

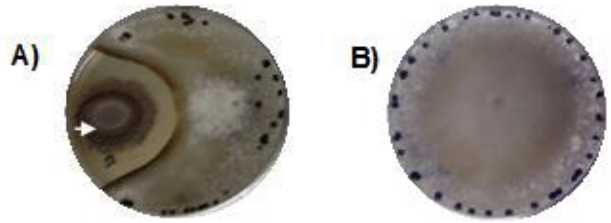


Figure 1. Confrontation assay, **A)** bacterial strain FSQ1 (arrowhead) against *S. sclerotiorum*, **B)** *S. sclerotiorum* (Negative control).

The molecular analysis of the strain FSQ1, based on the amplification of the 16S rRNA gene, reveals that this strain belongs to *Bacillus subtilis* with a similarity of 99.5% (Figure 3). *Bacillus subtilis* has been reported as a producing strain of biological control metabolites of broad spectrum (Araiza and Sanchez, 2012).The interaction between microorganisms studied and common bean plants under greenhouse conditions, shows that the pathogen inoculation to plants (treatment B) presented significant reduction of plant growth (23%) and presence of white mold symptoms compared to un-inoculated plants (treatment A), however plants co-inoculated with *S. sclerotiorum* and *B. subtilis* FSQ1 (treatment C) did not reduce the growth of the plant, getting 11.3 ± 0.9 cm vs. 14.7 ± 1.7 cm and 14.6 ± 1.2 cm, respectively , in addition, the disease was not observed (Figure 2). Thus, the results show that *S. sclerotiorum* reduces the development of common bean plants by the presence of white mold disease; in addition, *B. subtilis* FSQ1 inhibits the impact of this pathogen by its biocontrol, probably due to hydrolytic enzymes, antibiotics, polypeptides, among others (Tejera *et al*. 2011).

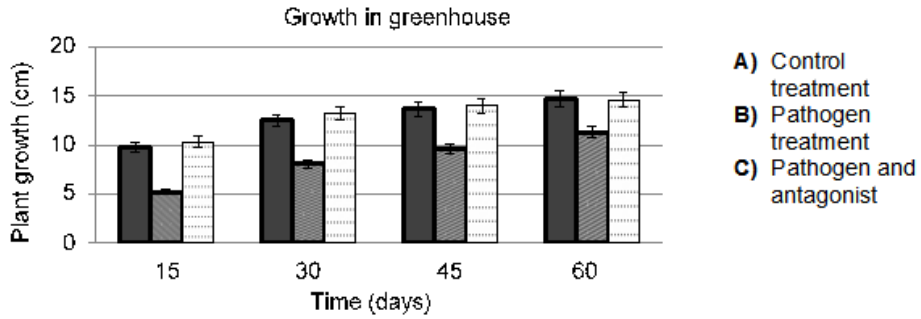
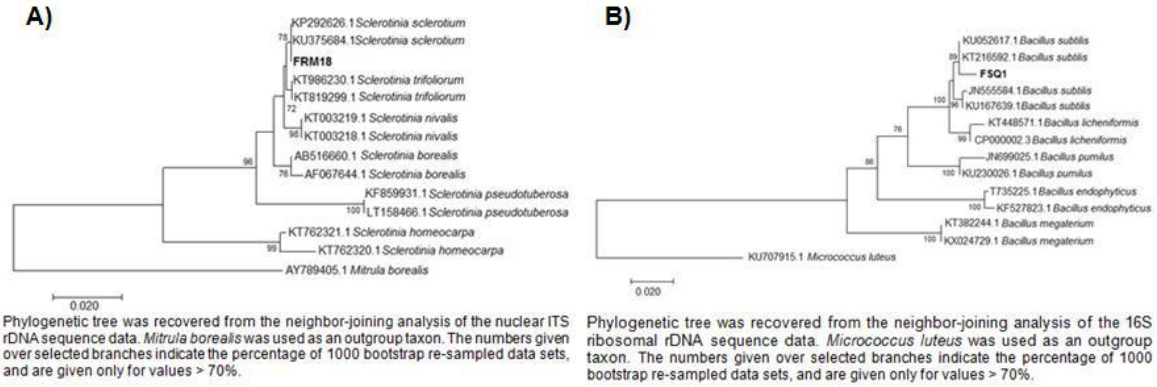


Figure 2. The interaction between microorganisms studied and common bean plants under the greenhouse conditions.



Phylogenetic tree was recovered from the neighbor-joining analysis of the nuclear ITS rDNA sequence data. *Mitrula borealis* was used as an outgroup taxon. The numbers given over selected branches indicate the percentage of 1000 bootstrap re-sampled data sets, and are given only for values > 70%.

Phylogenetic tree was recovered from the neighbor-joining analysis of the 16S ribosomal rDNA sequence data. *Micrococcus luteus* was used as an outgroup taxon. The numbers given over selected branches indicate the percentage of 1000 bootstrap re-sampled data sets, and are given only for values > 70%.

Figure 3. Phylogenetics relationship of **A)** *S. sclerotiorum* FRM18 and **B)** *B. subtilis* FSQ1.

Conclusions

The native soil microbial communities associated to common bean crop are a potential source of biotechnological alternative for controlling the white mold disease in the field. *B. subtilis* FSQ1, a rhizosphere bacterium, is a promising biological control agent against the causal agent of this disease, presenting an inhibition of *S. sclerotiorum* growth of 35% *in vitro*, and 100% of this disease in plants grown under greenhouse conditions. Thus, the strain

FSQ1 is a potential biological control agent against this disease, however several assays need to be developed for understanding its ecological role.

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Identifying bacterial biocontrol agents against *Bipolaris sorokiniana*, the causal pathogen of spot blotch on wheat in the Yaqui Valley, Sonora

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Abstract

Bipolaris sorokiniana is a devastating wheat pathogen. Recently, it was indentified in the Yaqui Valley causing spot blotch. In order to identify potential biological control agents 195 bacterial strains were isolated from bulk and rhizosphere soil used for wheat production in the Yaqui Valley. Fourteen strains showed inhibition against *B. sorokiniana*, ranging from 37 to 65 %. The isolates TSO2 and TSO1 showed the highest inhibition with 65 and 60 %, respectively. Although the antagonistic strains were able to produce common compounds involved in the biological control against fungal pathogens such as lytic enzymes and siderophores, additional studies are needed to identify a potential mechanism of action.

Keywords: ● Biological control, ● fungal plant diseases, ● metabolic characterization.

Introduction

The Yaqui Valley, located at the southern end of Sonora State, is the most important agricultural region for wheat (*Triticum durum* L) in Mexico, with a contribution of ~40% ($1.2x10^6$ ton y^{-1}) of the national production (Córtes-Jímenez *et al.,* 2011). Historically, karnal bunt and leaf rust caused by *Tilletia indica* and *Puccinia triticina*, respectively, have been the major biotic constraints to wheat (Córtes-Jímenez *et al.,* 2011). However, in 2015 *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) was identified causing spot blotch in farmer's fields with spring wheat (Villa-Rodriguez *et al*., 2016). This pathogen infects several plant compartments including steam, leaves, and seeds, being a devastating disease diminishing up to 85 % of the wheat production in warmer areas (Mehta, 2014). The use of common fungicides such as Captan, Mancozeb, Pentachloronitrobenzene (PCNB) and Tricyclazole have shown to be effective to control this pathogen, however several hazardous effects have been related to this agricultural practice, *i.e.* enviromental problems and genotoxicity to humans and animals (Georgian, 1983; Snyder, 1992). The global pathogen distribution and the potential climate change suggest that *B. sorokiniana* become a serious biotic constraint to wheat production in the Yaqui Valley (Bebber and Gurr, 2015). Thus, the development of eco-fiendly alternatives for mitigating future outbreaks is determinant to the global wheat prodduction. The use of biological control agents is a promising alternative way for the plant protection under field conditions in a broad range of crops, including maiz (*Zea mays* L.), grapevine (*Vitis vinifera* L.), and tomatoe (*Solanum lycopersicum* L.) (Ji *et al*., 2006; Magnin-Rober *et al*., 2007; Lizárraga-Sánchez *et al*., 2015). These microorganisms naturally colonize

different plant compartments limiting the pathogen establishment, through the excretion of growth inhibitory metabolites such as antibiotics, lytic enzymes, toxins, and lipopeptides, competition for trace nutrients by siderophores, and inducing plant systemic resistance (Pérez-Montaño et al., 2014; Pandya & Saraf, 2014).Thus, the aim of this study was to identify antagonistic soil bacterial strains against *B. sorokiniana,* isolated from commercial wheat fields located in the Yaqui Valley, Sonora, evaluating their potential metabolic mechanisms of biocontrol.

Materials and methods

Sampling sites: Soil samples were collected from 36 commercial wheat fields randomly distributed in the Yaqui Valley.One kilogram from bulk (0-30 cm depth) and rhizosphere soil were collected using the zig-zag method according to SAGARPA and Servicio Nacional de Sanidad (2015).

Isolation and preservation of bacterial strain: The bacterial isolation was carried out using the serial dilution plating method. For this, 10 g of each individual sample (bulk or rhizosphere soil) were homogenized using 90 mL of sterile distilled water, and 180 rpm for 1 hour in a rotary shaker. Serial dilutions up to 10^{-4} were spread on Petri dishes containing nutrient agar as a culture medium (supplemented with 80 mg/L terbinafine), and incubated during 2 days at 28°C. Bacterial colonies having different morphological traits were purified using the same culture medium. The isolates obtained were cryo-preserved (70% of glycerol at -80°C) in the "Colección de Microorganismos edáficos y Endófitos Nativos - COLMENA" (www.itson.mx/colmena).

Inoculum preparation: The bacterial isolates were grown in nutrient broth during 24 h at 180 rpm and 28 °C. Then, these were harvested by centrifugation at 3500 rpm during 10 min. The pellets were washed three times with sterile distilled water, and adjusted to optic density of OD_{630}=0.8. These bacterial suspensions were used for the biological control assay and their metabolic characterization.

In-vitro biological control assay: This assay was performed using an agar plug of growing *B. sorokiniana* TPQ1 (0.5 cm of diameter), which was obtained from COLMENA (Villa-Rodríguez *et al.,* 2016). It was placed on the center of Petri dishes containing PDA, and incubated for 24 h at 25 °C. Then, 10 µL of bacterial suspension was inoculated onto each of three equidistant points at 2.5 cm from the pathogen (Li *et al.,* 2014). After 8 days of incubation at 25°C, the percentage of inhibition was calculated using the equation:

$$I\,\% = \frac{Fw_1 - Fw_2}{Fw_1} X\ 100$$

Where *I %*= percentage of inhibition (%); Fw_1 = fungi growth without bacterial inoculation (cm); Fw_2 = fungi growth with bacterial inoculation (cm). The assay was performed by triplicate.

Metabolic characterization of identified biological control agents: Qualitative assays were carried out to evaluate the capabilities of the selected isolates to produce indoles, siderophores, and hydrolytic enzymes (chitinases, glucanases and proteases). The Indole production was tested treating the bacterial supernatants with Salkowsky´s reagent as indicated by Glickmann and Dessaux (1995). Siderophore production was detected by inoculation in Chrome Azurol Sagar medium (Schwyn and Neilands, 1987). Chitinase, glucanase and protease production was determined using solid media according to Chernin *et al.* (2002), Teather and Wood (1982), and Wang *et al*. (2015), respectively.

Statistical analysis: Data were analyzed by one-way analyses of variance (ANOVA) test and Tukey–Kramer method (P = 0.05) using Statgraphics software.

Results and Discussion

One hundred and ninety-five bacterial strains were isolated from bulk (52%) and rhizosphere (48%) soil of commercial fields used for wheat production in the Yaqui Valley. The slight decrease observed in the rhizosphere bacterial diversity comparing with the bulk soil, could be explained by the selective influence of the root exudates, *i.e.*inorganic acids and phenolic compounds (Weisskopf *et al.,* 2005).The confrontation assays showed that 14 bacterial isolates exhibited antagonistic activity against *B. sorokiniana* TPQ1, through diffusible metabolites (identified by biocontrol halo formation), which 43 % correspond to gram-negative bacteria and 57 % to gram-positive. These isolates showed different inhibition values ranging from 37% to 65 % (Table 1). The greatest growth inhibition was observed by the isolate TSO2, followed by TSO1, which reached 65 and 60 %, respectively. These results support the antifungal characteristic of several bacterial genera, mainly *Pseudomonas, Bacillus, Serratia, and Burkholderia*, which have been reported as biological control agents against a wide range of important plant pathogen such as *Fusarium spp, Rhizoctonia solani, Colletotrichum sp and Verticillium dahlia* (Pandya &Saraf, 2014).

Table 1. Results of biological control assay and metabolic characterization of the tested isolates.

	Isolate	Gram	% Inhibition	Indole	Siderophore	Chitinase	Glucanase	Protease
Bulk Soil	TSO1	+	60.7 ± 2.5^{e,f}	-	-	-	+	+
	TSO2	+	65.9 ± 3.3^{f}	-	-	-	+	+
	TSO13	+	57 ± 3.3^{c,d,e,f}	-	-	-	+	+
	TSO22	+	59.2 ± 3.3^{d,e,f}	+	-	-	+	+
	TSQ24	+	54.8 ± 1.4^{b,c,d,e}	-	-	-	+	+
	TSM45	-	54.0 ± 2.5^{b,c,d,e}	-	-	-	+	+
	TSO56	-	37 ± 1.2^{a}	-	-	-	+	+
Rhizosphere	TRM2	-	52.5 ± 2.5^{b,c,d,e}	-	-	-	-	+
	TRM3	+	54.8 ± 3.3^{b,c,d,e}	-	-	-	-	-
	TRQ3	-	49.6 ± 3.3^{b,c,d}	-	+	-	-	+
	TRQ6	-	45.9 ± 1.2^{a,b}	-	+	-	-	-
	TRQ9	-	46.6 ± 1.2^{a,b}	-	+	-	-	+
	TRO9	-	59.2 ± 3.3^{d,e,f}	+	+	-	-	+
	TRO10	-	48.1 ± 6.4b^{b,c}	-	+	-	-	+

*Mean values ± SD are shown. *Superscript letters indicate statistically significant differences between isolates when the Tukey HSD test was applied (p < 0.05). *(+/-) symbols indicates positive/negative results in metabolic screening and gram stain.

With the aim of exploring potential metabolites involved in the observed antifungal activity by the bacterial isolates, the production of several metabolites was evaluated. Thus, glucanase activity was observed in all isolates obtained from bulk soil (Table 1). The production of proteases was a generalized feature for almost evaluated strains, except for TRM3 and TRQ6. Contrastingly, strains showing chitinase production were not detected in this study. It is well known that cell-wall degrading enzymes such as glucanases, chitinases and proteases are involved in the antagonistic activity of bacterial biocontrol agents against fungal pathogens (Pérez-Montaño *et al.*, 2014), suggesting their potential role as a biocontrol metabolite produced by the evaluated strains. The siderophore production was observed in rhizosphere isolates TRQ3, TRQ6, TRQ9, TRO9 and TRO10, detected by orange halo formation in the CAS medium. The siderophores are metabolites with high iron affinity that can inhibit the growth of microorganisms by the depletion of this element in the environment (Pandya &Saraf, 2014).In terms of indoles production, only the isolates TSO22 and TRO9 were able to produce this phytohormone, however, the low concentration produced by these strains (data no shown) suggests their scarce participation in the pathogen growth inhibition, since high concentrations (400 ppm) has been reported as antifungal compound against fungal pathogens (Leelavathy, 1969). The strain TRM3 was not able to produce lytic enzymes, siderophores or indole; however, it exhibited antifungal activity against *Bipolaris sorokiniana* (54.81 ± 3.39). The inability showed by this strain to produce the evaluated metabolites suggests the potential role of others compounds in the inhibition of *B. sorokiniana*. Several studies have shown that bacterial biocontrol agents produce a wide number and diversity of antibiotics, which, in turn, are responsible of the suppression of a great range of fungal pathogens (Raaijmakers *et al.,* 2002). More recently Cawoy *et al.* (2015) revealed the critical role that plays the non-ribosomal cyclic lipopeptides (LPs), specially iturins and fengicyn families in the broad spectrum of antifungal activity displayed by *Bacillus spp*. Thus, the production of these metabolites must be considered for future experiment with the aim to elucidate the potential mechanism of action by the evaluated strains against *B. sorokiniana.*

Conclusions

Potential biological control agents against *Bipolaris sorokiniana* were isolated from commercial wheat fields located in the Yaqui Valley. The isolates TSO1 and TSO2 showed promising results as biological control agent, inhibiting > 60 % the growth of *B. sorokiniana in vitro*. The strains evaluated in this work were able to produce metabolites involved in biocontrol of fungal pathogens; however future assays needs to be developed in order to identify the main compound directly involved in the biocontrol activity observed.

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Biocontrol of damping-off diseases in *Carica papaya* (Linnaeus) seedlings under greenhouse conditions using *Trichoderma* spp.

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Abstract

Carica papaya (Linnaeus) is the fifth most important fruit in Mexico in terms of production volume. The principal diseases affecting *C. papaya* seedlings are caused by several naturally occurring phytopathogenic soil fungi, referred to as damping-off diseases. Although the use of chemical fungicides can reduce this disease, more environmentally friendly strategies are needed. This study aimed to evaluate the effectiveness of native strains of *Trichoderma* spp. isolated from the *C. papaya* rhizosphere to control the incidence of damping-off in *C. papaya* seedlings. At the end of evaluation, Trico-SP6 (2.9%) was able to reduce damping-off at the same level as the chemical fungicides [*Copper* (*II*) *Sulfate Pentahydrate* (CSPH)=1.7% and Captan=1.4%]. Furthermore, application of Trico-SP6 resulted both in a higher height (21.6 cm) and stem diameter (0.46 cm) in seedlings in comparison with CSPH (height=19.6 cm and stem diameter=0.39 cm) and Captan (height=19.2 cm and stem diameter=0.39). The inoculation of Trico-SP6 directly in the plant growth substrate can be a good strategy for the biological control of damping-off diseases and to increase plant growth in *C. papaya* seedlings.

Keywords: biological control • chemical fungicides • plant disease • plant growth

Introduction

The main problem in *Carica papaya* seedlings under greenhouse conditions is the presence of phytopathogenic fungi, in particular of the genera *Fusarium, Pythium, Rhizoctonia*, and *Phytophthora*. These fungi affect seedling growth and invade and block the vascular system of plants, causing the yellowing of leaves and necrotic streaks at the base of stems; these symptoms commonly produce damping-off, resulting in the death of seedlings and producing significant losses in nurseries (Huey-Ling *et al*. 2012). To avoid this problem, farmers need to apply chemical fungicides, although these are sometimes inefficient. In addition, these products have high costs and contribute to environmental pollution. The replacement of fungicides with biological control agents such as phytopathogenic fungi is an alternative management strategy for producing food that is safe for consumption and for reducing environmental pollution (Cubillos-Hinojosa *et al*. 2011). In this sense, the *Trichoderma* genus presents good qualities for use in the biological control of plant diseases caused by phytopathogenic fungi due to its capability to colonize roots and the rhizosphere, improve of the health of plants, and stimulate root growth (González-Cardenas *et al.* 2005). Therefore, the main objective of this work was to evaluate the effectiveness of native strains of

Trichoderma spp. isolated from the *C. papaya* rhizosphere in controlling of the incidence of damping-off in *C. papaya* seedlings.

Materials and methods

Maintenance, reactivation, and mass production of Trichoderma spp. Two strains of *Trichoderma* spp. were isolated from a *C. papaya* (cv. Maradol) rhizosphere in a semi-conventional orchard located in Tecoman (Colima, Mexico). Fungal strains were deposited in the mycological collection of the Faculty of Biological and Agricultural Sciences of the University of Colima. Strains were isolated and maintained (25 °C and 75% relative humidity) on potato dextrose agar. Conidia mass production was carried out on rice grains in polyurethane plastic bags according to the method described by Lezama-Gutiérrez *et al*. (2006). Conidia concentration was measured using 1 g of dry powder (conidia) in 10 mL of water plus Tween 80® (0.05% V/V). The conidia produced by each strain were counted in a Neubauer chamber and were adjusted according to **Table 1**. *Evaluated treatments***.** Five treatments and one control were evaluated (**Table 1**). The application of the treatments was performed with a watering hose (10 L) directed to the *C. papaya* seedling stems. Then, stems were rinsed with irrigation water to direct the products to the root area. Applications were performed early morning (7:00 am) at 7, 14, 21, and 28 days (d) after seedling emergence.

Table 1. Evaluated treatment for damping-off control in *Carica papaya* seedlings.

NUMBER	TREATMENTS	DOSE	CONCENTRATION
1	Trico-SP6 (*Trichoderma* sp.)	50 g/200 L	$1x10^{13}$ conidia/mL
2	Trico-SP12 (*Trichoderma* sp.)|	50 g/200 L	$1x10^{13}$ conidia /mL
3	Co-application (Trico-SP6 and Trico-SP12)	50 g/200 L	$1x10^{13}$ conidia/mL
4	Captan	500 g/200 L	1250 ppm
5	Copper (II) sulfate pentahydrate	400 mL/200 L	500 ppm
6	Control (water)	-	-

Trichoderma strains and the co-application were applied in water plus Tween 80® (0.05% V/V).

*Response variables, experimental design, and data analysis***.** Damping-off incidence (expressed in % of plants), damping-off severity, seedling height, and stem diameter were evaluated. Incidence was calculated using the formula: % incidence=(number of total seedlings/number of diseased seedlings)×100. Severity was evaluated by the diagrammatic scale proposed by St-Arnaud *et al*. (1997) and the formula: $S=\Sigma(nxb)/(NxB)\times100$, where n=number of plants in the same class, b=range, N=number of total plants measured, and B=total number of ranges. Seedling height and stem diameter were measured every 10 d after seed germination using a caliper. The experimental design was completely randomized and had six treatments and four replicates; each replicate consisted in 200 seedlings (one tray formed an experimental unit). The response variables were analyzed through an analysis of variance and a comparison of the multiple range test using the least significant difference (LSD) test ($P>0.05$).

Results and discussion

Damping-off incidence. Four evaluations were made after seedling emergence at 10, 20, 30, and 40 d. However, damping-off was only detected at 30 and 40 d. Incidence at 30 d showed a significant difference (*F*=12.04, *P*=0.00001) between the treatments. The chemical fungicides resulted in the lowest incidence (CSPH=1.7%; Captan=1.4%) in comparison to *Trichoderma* applications (Trico-SP6=2.9%, Trico-SP12=3.3%, and co-application=2.9%). The control had the highest damping-off incidence at 4.8% (**Figure 1A**). In the evaluation at 40 d, significant differences in the incidence of damping-off were also found between the treatments (*F*=17.20; *P*=0.00001). The chemical fungicides resulted in the lowest incidence (Captan=1.4%, CSPH=1.7%); however, these incidence values were statistically equal to the incidence of damping-off in Trico-SP6 (2.9%) treatment. This indicates that the *Trichoderma* strain was able to reduce the natural incidence of damping-off at the same level as the chemical fungicides. Meanwhile, the control resulted in the highest incidence in 4.8% of seedlings, statistically equal to that of Trico-SP12 and the co-application in 4.6 and 3.6% of seedlings, respectively (**Figure 1B**). It is well known that *Trichoderma* species successfully control plant diseases due to their direct and antagonistic effects on pathogen activity and/or by inducing the resistance response of hosts (Kobori *et al*. 2015). According to Ezziyyani *et al*. (2004), *Trichoderma* has three mechanisms by which it can displace phytopathogenic fungi, including: a) direct competition for space and nutrients, b) production of secondary metabolites of volatile or nonvolatile nature, and c) direct parasitism of certain species of *Trichoderma* on phytopathogenic fungi. In recent studies, Kobori *et al*. (2015) applied *Trichoderma harzianum* to control *Rhizoctonia solani* in *Cucumis melo* seedlings and found a damping-off incidence of 24.1%, lower than that of non-treated seedlings (66.7%). In *C. papaya* seedlings, González-Cárdenas *et al*. (2005) suggested that the application of *Trichoderma* at a concentration of $1x10^6$ conidia/mL can reduce the natural incidence of damping-off. In this study $1x10^{13}$ conidia/mL produced in solid fermentation was added; this amount of conidia was able to reduce the incidence of damping-off.

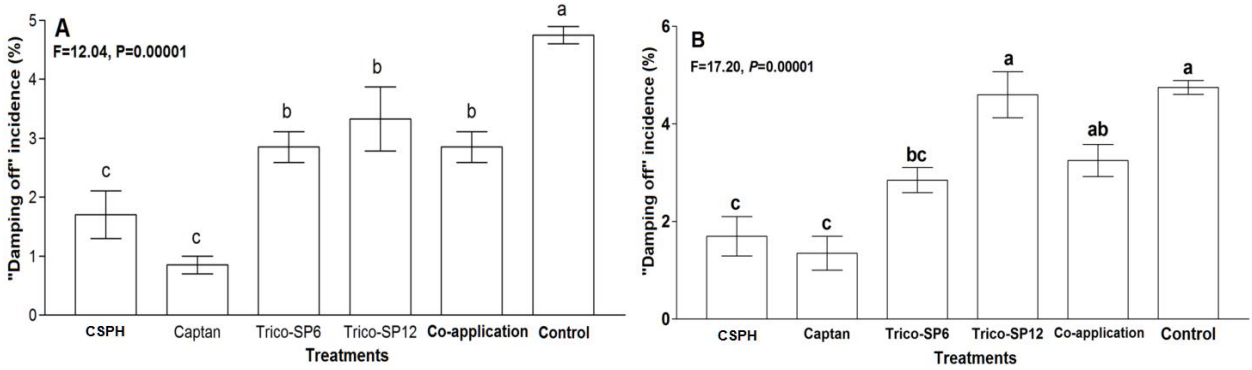

Figure 1. Damping-off incidence in *Carica papaya* seedlings after applications of chemical and biological fungicides at 30 (A) and 40 (B) d. Values were transformed to √X+1 (mean ± standard error, n=80).

Damping-off severity. At 30 d of evaluation, damping-off severity was statistically equal between the applications of *Trichoderma* spp. (Trico-SP6=4.5% and Trico-SP12=6.0%) and chemical fungicides (Captan=0.75% CSPH=1.75%); meanwhile, the control demonstrated the highest severity (17.0%, **Figure 2A**). However, at the end of evaluation (40 d), Captan,

CSPH, Trico-SP6, and the co-application (Trico-SP6 and Trico-SP12) statistically resulted in the lowest severity of 1.3%, 2.5%, 8.8%, and 9.3%, respectively, while the control resulted in the highest severity at 22.5% (**Figure 2B)**. Disease severity is the percentage of relevant host tissues or organs demonstrating symptoms or that are covered by lesions or damages due to disease. The application of *T. harzianum* in *Solanum lycopersicum* seedlings reportedly reduces damping-off severity by 100% when added at a concentration of $1x10^7$ conidia/mL (Majorie *et al*. 2016). In other study, Otadoh *et al*. (2011) reported that the application of *Trichoderma* ($1x10^6$ conidia/mL) isolates on *Phaseolus vulgaris* roots can reduce the number of root lesions and the severity of damping-off caused by *Fusarium oxysporum* f. sp. *phaseoli*. The conidia concentrations used by Majorie *et al*. (2016) and Otadoh *et al*. (2011) are lower compared to those used in this study; however, the production method of these studies used rice grains. In this sense, we can explore the development of a formulated product using Trico-SP6 to achieve lower incidence and severity of damping-off.

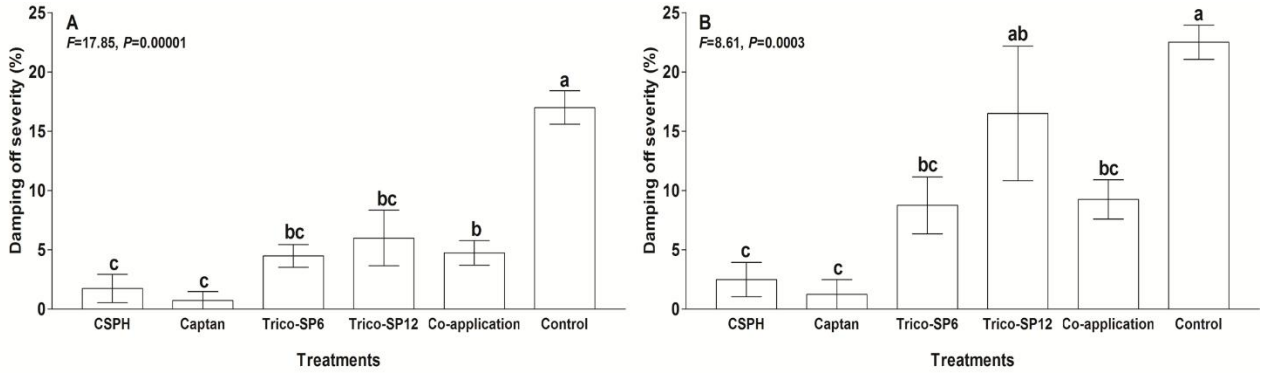

Figure 2. Severity of damping off (%) in seedlings of *Carica papaya* at 30 (A) and 40 (B) d of evaluation. Severity was calculated according to St-Arnaud *et al*. (1997).

Seedling height and stem diameter. At 10 and 20 d significant differences ($P>0.05$) were not found in the height of seedlings between the evaluated treatments (**Table 2**). However, at 30 d the height of Trico-SP6 (14.7 cm) and Trico-SP12 (14.6 cm) significantly increased ($F=10.5$, $P=0.0001$) in comparison to the chemical fungicides (CSPH=13.4 cm, Captan=13.1 cm) and the control (13.2 cm). At the end of evaluation (40 d), the application of Trico-SP6 (21.6 cm) and Trico-SP12 (21.3 cm) significantly increased ($F=9.91$, $P=0.0001$) the height of *C. papaya* seedlings in comparison to Captan (19.2 cm) and CSPH (19.6 cm). However, the co-application of the two *Trichoderma* strains (20.8 cm) increased the height of *C. papaya* seedlings at the same level as Trico-SP6 (21.6 cm), Trico-SP12 (21.3 cm), and CSPH (19.6 cm, **Table 2**). Regarding the stem diameter, at 10 d of evaluation no significant differences were found. However, at 20 d the biological treatments (Trico-SP6=0.22 and Trico-SP12=0.21 mm) allowed for a greater stem diameter in comparison to the other treatments (**Table 2**). At 30 (0.33 mm) and 40 d (0.46 mm) of evaluation, only Trico-SP6 led to a greater stem diameter in *C. papaya* seedlings when was compared to the rest of treatments (**Table 2**). This result suggests that Trico-SP6 can promote the plant growth of *C. papaya* seedlings. In a previous study, Donoso *et al*. (2008) reported that the application of *Trichoderma* spp. in crops resulted in more vigorous plants of higher fresh and dry weight and better root system development. More recently, when *Trichoderma* sp. was applied as a growth promoter, Camargo and Ávila (2014) reported a favorable effect in the growth of pea plants as well as

greater leaf area and root length. Future studies with Trico-SP6 are needed to evaluate its potential for controlling damping-off in soil applications under field and greenhouse conditions. In addition, the viability of conidia in formulated products is important to evaluate before their eventual use.

Table 2. Height (cm) and stem diameter (cm) of *Carica papaya* seedlings under different applications of chemical and biological of fungicides.

Treatments	Evaluation period (d)							
	10		20		30		40	
	SH	SD	SH	SD	SH	SD	SH	SD
CSPH	4.5	0.10	6.7	0.19 c	13.4 c	0.28 d	19.6 bc	0.39 c
Captan	4.2	0.11	6.6	0.19 c	13.1 c	0.28 cd	19.2 c	0.39 c
***Trico* SP6**	4.8	0.11	7.5	**0.22 a**	**14.7 a**	**0.33 a**	**21.6 a**	**0.46 a**
***Trico* SP12**	4.8	0.11	7.2	**0.21 a**	14.6 ab	0.31 b	**21.3 a**	0.45 b
Co-application	4.3	0.11	7.0	0.20 b	13.7 bc	0.30 c	20.8 ab	0.43 b
Control	4.2	0.11	6.8	0.19 c	13.2 c	0.29 cd	19.6 bc	0.40 bc
VC (%)	< 11.7	< 2.9	< 10.1	< 2.1	< 6.5	< 9.0	< 5.10	< 6.8

Means between lines with different literal are significantly different in each other (LSD test *P*=0.05, N=80). SH=seedling height, SD=Stem diameter and VC=variation coefficient.

Conclusions

The application of Trico SP6 was able to reduce the natural incidence and severity of damping-off in *C. papaya* seedlings to the same extent as Captan and copper (II) sulfate pentahydrate. In addition, the *Trichoderma* sp. strain Trico SP6 was able to promote the growth of *C. papaya* seedlings, increasing their height and stem diameter. Therefore, the *Trichoderma* sp. strain Trico-SP6 is a good candidate to develop formulated products for application in papaya plantations.

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Solubilization of inorganic phosphates by soil borne micromycetes isolated from the rhizosphere of *Coffea arabica* plants

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Abstract
Soil phosphorus (P) can be found in insoluble forms such as $Ca_3(PO_4)_2$ and $FePO_4$, which are unavailable for plants. To release the inorganic P of the soil requires the action of P-solubilizing microorganisms. The objective of this study was to evaluate the solubilization of $Ca_3(PO_4)_2$ and $FePO_4$ by soil borne micromycetes isolated from the rhizosphere of *Coffea arabica* plants. In Pikovskaya-agar culture medium with $Ca_3(PO_4)_2$ and $FePO_4$ was evaluated the P-solubilization through the solubilization index (SI) in sixteen isolates. In liquid culture using the same culture medium was evaluated five isolated for $Ca_3(PO_4)_2$ and $FePO_4$ solubilization. For $Ca_3(PO_4)_2$ the isolate SC12 achieved the highest (P=0.00001) SI (1.8) in comparison to other isolates. However, in the qualitative test for $FePO_4$, halo solubilization was not found and was not possible to calculate the SI. In liquid cultures, the best isolation for $Ca_3(PO_4)_2$ solubilization was SC25 with 98.5 mg/L of released P, at the end of evaluation (144 h). However, in $FePO_4$ solubilization, only the isolate SC20 was able to solubilize a considerable amount of $FePO_4$ (17.3 mg/L) after 6 days of culture. Results suggest that the $Ca_3(PO_4)_2$ is more easy to be solubilized by the studied isolates in comparison to $FePO_4$.

Keywords. Biofertilizers, *Penicillium* sp., phosphorus, plant growth.

Introduction

Phosphorus (P) is one of the most important nutrients for growth and development of plants; this nutrient is absorbed in the rhizosphere as dihydrogen phosphate ($H_2PO_4^-$) and trihydrogen phosphate ions ($H_3PO_4^-$). However, near to 95-99% of soil phosphorus is unavailable for plants, because it is in insoluble forms such as $Ca_3(PO_4)_2$, $FePO_4$ or $AlPO_4$ (Khan *et al*. 2014). In order to release the soil P is necessary the biochemistry activity of soil microorganism (Zúñiga-Silva *et al*. 2016). Native soil microorganism such as bacteria, soil borne micromycetes, arbuscular mycorrhiza and microalgae has been reported as P-solubilizing microorganism (Khan *et al*. 2007). One of the most conserved agroecosystems in Mexico is the coffee plantations (*Coffea arabica* L.), which can be found under different production systems such as rustic, under rain forest, simple policulture (i.e. coffee-banana) and multiple policulture (i.e. coffee, banana, macadamia, Heredia-Abarca and Arias-Mota 2008). In Mexico, the production of *C. arabica* is principally in soils of volcanic origin, where P-sources are unavailable for coffee plants (Hernando-Posada *et al*. 2012). The bioprospecting in native soil borne micromycetes to search fungal isolates with high capacity to solubilize inorganic P such as $Ca_3(PO_4)_2$ and $FePO_4$ is a preponderant activity in the develop of biofertilizers; especially, in Colima State, Mexico, where the challenges of the

coffee plantations is the low productivity due to the lack nutrients such as P (Díaz-Cárdenas 2005). Therefore, this study aimed to isolate native soil borne micromycetes from the rhizosphere of *C. arabica* plants and to evaluate their ability to solubilize $Ca_3(PO_4)_2$ and $FePO_4$ in agar-plates and liquid cultures.

Materials and Methods

Soil sampling and fungal isolation. Soil samples for soil borne micromycetes isolation were collected in an organic coffee plantation in "Rancho Nextia", situated in "Cofradía de Suchitlán" in Comala, Colima, Mexico. Plants were chosen randomly in order to collect a representative sample of the soil rhizosphere at 25 cm in deep. Samples were maintained at 5 °C until its processing. Soil borne micromycetes was isolated through soil washing particle technique described by Bills *et al*. (2004). Fungal isolations were grouped according to their phenotypic characteristics (Samson and Gams 1984). Only the five studied isolates of the experiment in liquid cultures were identified at genera level through microcultures

*P-solubilization in agar plates***.** Pikovskaya-agar culture medium was used to evaluated the ability of sixteen isolates to solubilize $Ca_3(PO_4)_2$ and $FePO_4$ as a qualitatively test. Pikovskaya-agar culture medium and spore inoculations were made as described by Zúñiga-Silva *et al*. (2016). Each 24 h during eight days was measured the diameters of the colony and halo solubilization using a millimeter square. Both variables were used to calculate the Solubilization Index (SI) for $Ca_3(PO_4)_2$ and $FePO_4$ according to Zúñiga-Silva *et al*. (2016).

*P-solubilization in liquid culture medium***.** Pikovskaya culture medium without agar was used to quantify the $Ca_3(PO_4)_2$ and $FePO_4$ solubilization by five isolated of soil borne micromycetes, which was selected in the qualitative assays. Liquid cultures were maintained at 25 °C, 120 rpm and 75% of relative humidity during six days for $Ca_3(PO_4)_2$ and nine days for $FePO_4$. P-soluble (mg/L) in fungal extracts was measured using the phosphomolybdenum blue method (Watanabe and Olsen 1965).

Data analysis. Both in agar plate and liquid cultures each isolate were established with four replicates. A one-way analysis of variance and a Low Significant Difference test (LSD, P=0.05) were used to found the best isolate for $Ca_3(PO_4)_2$ and $FePO_4$ solubilization.

Results and discussion

P-solubilization in agar plates. For $Ca_3(PO_4)_2$ was able to calculate the SI, which was the relation between the diameter of halo solubilization and the colony diameter. The halo solubilization of $Ca_3(PO_4)_2$ was clearly and reliable (**Figure 1**). Data analysis revealed that there were significant differences (F=35.08, P=0.00001) in the SI between the studied isolates. The isolate SC12 achieved the highest SI with 1.8 (**Figure 2**); in the other isolates the SI ranging from 1.2 to 1.5. However, in Pikovskaya-agar with $FePO_4$ was not able to calculate the SI; because, solubilization halo for $FePO_4$ were not found in the sixteen studied isolates. Solubilization index is a qualitative parameter to find P-solubilizing fungi; this parameter is easy to calculate and is economically feasible, if we need to test a great number of isolates. However, it is an arbitrary parameter, because it is not precise by its qualitative nature (Zuñiga-Silva *et al*. 2016). In *C. arabica* rhizosphere there is few reports regarding to the P-solubilizing fungi; Hernando-Posada *et al*. (2012; 2013) reported 76 isolates with ability to solubilize $Ca_3(PO_4)_2$ and $FePO_4$ using the relative efficiency of P-solubilization, which is an qualitative parameter such as the SI. Some genera of soil microfungi which have abilities to solubilize P-sources are *Chaetomium*, *Cylindrocarpon*, *Fusarium*, *Penicillium* and *Paecilomyces* (Hernando-Posada *et al*. 2012; 2013). In other study, Vera *et al*. (2002) studied

the solubilization of inorganic P-source ($Ca_3(PO_4)_2$ and $FePO_4$) by soil microfungi isolated from *Eugenia stipitata* rhizosphere and found lees species with the ability to solubilize $FePO_4$ that $Ca_3(PO_4)_2$; the authors reported that *Aspergillus oryzae*, *Paecilomyces* strain 3, *Gongronella* and *Fusarium rodolens* were able to solubilize $FePO_4$. This solubilization was only based in a qualitative test (relative efficiency of solubilization) and not in a quantitative test.

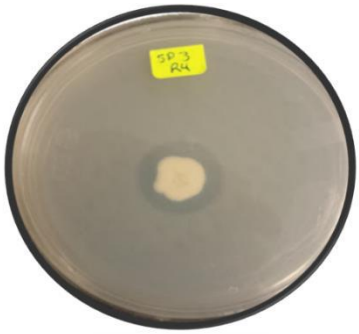

Figure 1. Solubilization halo for $Ca_3(PO_4)_2$ in Pikovskaya-agar.

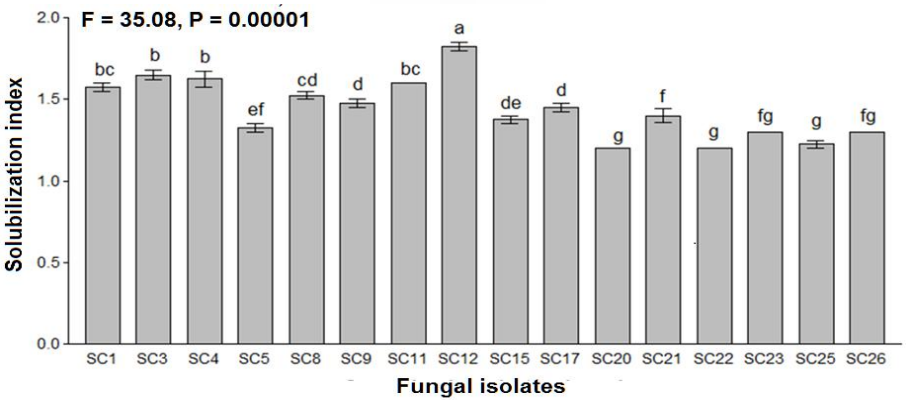

Figure 2. Solubilization index of $Ca_3(PO_4)_2$ by soil borne micromycetes isolated from the rhizosphere of *Coffea arabica* plants.

P-solubilization in liquid culture medium. Five isolates were tested in liquid cultures (isolates: SC3, SC4, SC12, SC20 and SC25). At 48 h of culture the strain SC20 was able to solubilize the higher amount (F=10.8, P=0.0001) of $Ca_3(PO_4)_2$ with 18 mg/L, when was compared to the other strains (**Figure 3**). At 96 h, both SC20 (87 mg/L) and SC25 (89 mg/L) were able to solubilize the $Ca_3(PO_4)_2$ in higher amount (F=88.3, P=0.00001) that the rest of the studied strains (**Figure 3**). At the end of evaluation (144 h), the SC25 isolate was able to solubilize at 100% (98.5 mg/L, F=37.66, P=0.00001) the $Ca_3(PO_4)_2$ (**Figure 3**). Regarding to the $FePO_4$, only in SC25 and SC20 was able to solubilize a few amount of P contained in $FePO_4$. At 72 h of culture, only the strain SC20 achieved P-solubilization with 1.6 mg/L; however, the control showed 6.5 mg/L of P. At 144 h of culture, only the strains SC20 and SC25 showed P-solubilization with 17.3 and 7.8 mg/L, respectively; however, the control again achieved the most $FePO_4$ solubilization (21.6 mg/L). At the end of evaluation, SC20 reduced its P-solubilization at 0.8 mg/L (**Table 1**). This variation in $FePO_4$ solubilization probably was due to: A) the amount of Fe^{2+} contained in $FePO_4$ is causing toxicity to the fungal isolates, B) the amount of Fe^{2+} is interfering in the spectrophotometric lecture, or C) the P-solubilized was used to the fungi for to develop its biomass against the stress caused by the Fe^{2+} contained

in the $FePO_4$. In previous studies has been reported that metal ions such as Fe^{2+} and Fe^{3+} are considered as fungal stressors (Baldrian, 2003). According to the results, the $Ca_3(PO_4)_2$ was easy to solubilize by studied isolates in comparison to $FePO_4$. Regarding to the soil borne micromycetes isolated from the rhizosphere of coffee plants, Hernando-Posada *et al*. (2013) reported the ability of native isolates from Veracruz to solubilize both $Ca_3(PO_4)_2$ and $FePO_4$; some reported genera were *Chaetomium*, *Cylindrocarpon*, *Aspergillus*, *Penicillium* and *Humicola*. In liquid cultures with $Ca_3(PO_4)_2$ Hernándo-Posada *et al*. (2013) found low P-solubilization with *Penicillium janthinelum* (7.0 mg/L), *Paecilomyces marquandii* (1.9 mg/L) and *Cylindrocarpon didymun* (9.69 mg/L). In this sense, the isolates of this study achieved the highest P-solubilization of $Ca_3(PO_4)_2$.

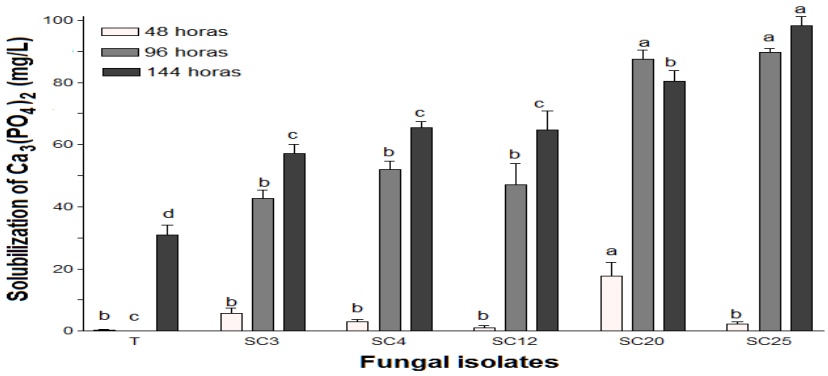

Figure 3. Solubilization of $Ca_3(PO_4)_2$ in liquid cultures by five soil borne micromycetes isolated from the rhizosphere of *Coffea arabica* Plants. Means with different literal in bars are significant different to each other according to LSD test (P=0.05).

Table 1. Solubilization of $FePO_4$ (mg/L) by soil borne micromycetes isolated from the rhizosphere of *Coffea arabica* Plants.

	Evaluation period		
Isolates	**72**	**144 h**	**216 h**
SC3	ND	ND	ND
SC4	ND	ND	ND
SC12	ND	ND	ND
SC20	1.6 b	17.3 a	0.8 b
SC25	ND	7.8 b	ND
Control	6.5 a	21.6 a	7.7 a

Means with different literal in lines are significant different to each other according to LSD test (P=0.05). ND=no detected.

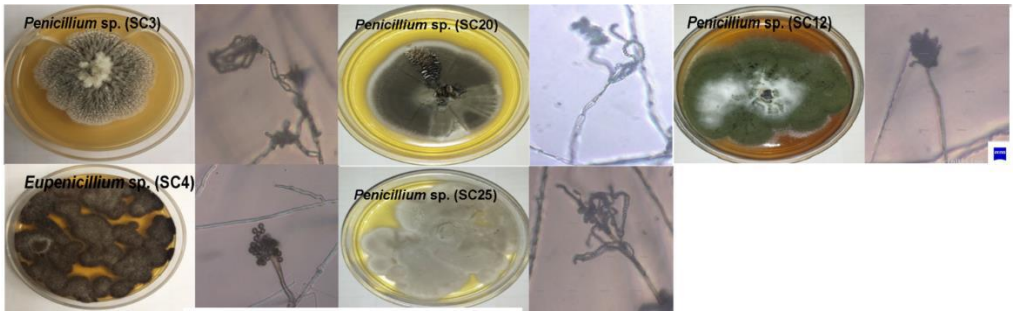

Figure 4. Identification at level of genus of the studied isolates in liquid cultures.

Fungal identification. **Figure 4** shows the aspect of the fungal colony and microcultures of the studied strains. The strains SC3, SC12, SC25, and SC20 correspond to the genus *Penicillium* and the strain SC4 corresponds to *Eupenicillium*. Both genera have been reported has P-solubilizing fungi (Vyas *et al*. 2007).

Conclusions

Native soil borne micromycetes isolated from the rhizosphere of *Coffea arabica* plants are more efficient to solubilize the $Ca_3(PO_4)_2$ than $FePO_4$, both in semi-solid and liquid cultures. The isolate SC12 (*Penicillium* sp.) showed the highest solubilization index for $Ca_3(PO_4)_2$ in semi-solid cultures; however, it is not the best P-solubilizing fungi, because in liquid cultures it was not achieved the highest $Ca_3(PO_4)_2$ solubilization. In contrast, the strains SC20 and SC25 were the best solubilizers of $Ca_3(PO_4)_2$. By other hand, $FePO_4$ was not solubilized efficiently by the studied isolates both in semi-solid and liquid cultures.

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Biofilms formed in different carbonaceous materials used as anodes for microbial fuel cells (MFC)

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Abstract

The Microbial Fuel Cells (MFC), are biolectrochemical devices, where the microorganisms use the substrate chemical energy in order to produce electrical energy. There are electroactive microorganisms which have the capacity to use de cell metabolism for this energy conversion and generate electron transfer to the anode. It is well known that modifying the anode with low cost materials and easy access might help to make more attractive these devices for energy generation and also for waste water remediation. In this research, the Ordered Mesoporous Carbon (OMC) was functionalized with methanol as chemical agent, it was electrochemically evaluated and compared with other commercial carbonoceous materials as Vulcan XC-72 (V-XC-72) and Graphite flakes (GF), which were also functionalized with methanol.The voltammograms show that OMC functionalized and with *Bacillus subtilis* biofilm increased the current density of this material, compared with V-XC-72 and GF.

Keywords: biodegradable substrate • Microbial fuel cell • biotechnology for energy generation • renewable energy •waste water treatment.

Introduction

The microbial fuel cells (MFC) are bioelectrochemical devices with the capacity to contribute to face the energetic crisis and the scarcity of good water quality, in order to increase the renewable energy generation and shape a sustainable development. These devices use microorganisms capable to produce electric energy from the chemical energy present in a substrate (Lovley 2011). According to Revelo *et al.* (2013) the architecture, the microbial function and the type of substrates are the most important issues that affect the performance of MFC. The MFC can remediate the contaminated water with xenobiotic compounds and heavy metals, due that to their microorganisms can transfer electrons to the electrodes (anode) and reduce different pollutant compounds (Revelo *et al.* 2013). However, it has to be remembered that the MFC have many operational and functional advantages over another technologies used actually for energy generation from biodegradable, reduced compounds (Rabaey and Verstraete 2005). The use of low-cost materials with electrochemical properties is helpful for redox reactions in a bioelectrochemical system, which have the improvements required for MFCs, while the porous carbon materials are interesting due to its large superficial area and physicochemical properties. The conventional synthesis produces porous materials with less control of pore size distribution, besides of the mesostructures. However, the synthesis of some materials with a large superficial area and an ordered mesostructure

was reached recently (Liang *et al*. 2008). In previous studies, the MFC performance was enhanced when functional groups containing oxygen were latched to the carbon surface, which might help to the bacteria to have a better direct physical contact with the material (Chen *et al*. 2015). In this research, OMC was synthesized and modified with methanol as a chemical agent and its catalytic activity was compared with V-XC-72 and GF, both of them of commercial use, while the biofilm formed by *Bacillus subtilis* was also evaluated electrochemically in a half-cell, in order to determinate the activity of carbonaceous electrocatalysts.

Materials and methods

The OMC was synthesized with the self-ensemble employing the pyrolysis technique. A mixture of resorcinol/formaldehyde was used as carbon source, while the copolymer Pluronic F127 was used as a template to promote the hexagonal structure. The resin carbonization was done at 900 ^{o}C. After that, the material was ground in a planetary mill, where dust was the result. The OMC was treated to obtain functional groups over the material surface to promote the adsorption of species that can participate in the electron transfer, improving the kinetic of the anodic reaction. Commercial V-XC-72 and GF were employed in two ways, functionalized and unfunctionalized. Their electrochemical behavior was compared with OMC, using Cyclic Voltammetry (CVs). The electrodes were fabricated with 1 cm^2 carbon cloth, used as support matrix and covered with commercial resin, on one side, with the objective that electrolyte (KOH pH 9.6), the pharmaceutical wastewater, and the bacteria are in direct contact with the electrode area where the carbon catalyst was previously deposited in order to obtain a controlled electrochemical characterization. The electrode was weighed when its resin was dried. After that, the brush technique was used to paint with the catalytic ink over the electrode. Then, it was treated ultrasonically for 30 min and deposited over the carbon cloth. After that, the electrodes were weighed again to verify that the catalyst was adsorbed over the carbon cloth. In order to stimulate the biofilm formation over carbon electrodes, six flasks were prepared with 90 mL of sterile nutrient broth, inoculated with *Bacillus subtilis* and left in incubation for 6 days at 28 ^{o}C. Each electrode was put in each flask in an anaerobic environment. After 6 days of incubation, the electrochemical tests were done for the electrochemical characterization. Then Scanning Electron Microscope (SEM) characterization was realized to observe biofilm formation over the electrode. The catalyst evaluation was done in an anodic half cell, where 80 mL of pharmaceutical wastewater (PWW) were used as a substrate. In this chamber, the electrodes containing the carbonaceous catalysts were put and the catalytic activity was evaluated. The catalysts were characterized using a Voltalab Potentiostat, while the catalytic activity was evaluated using Cyclic Voltammetry (CV). The materials were evaluated utilizing KOH and PWW (pH 9.6), resulting CVs that were utilized as comparatives of carbonaceous materials.

Results and Discussions

Figure 1 shows the SEM images of catalysts functionalized and unfunctionalized with CH_3OH. V-XC-72 unfunctionalized and functionalized (Figs. 1a and 1b) present a semispherical morphology. The unfunctionalized and functionalized GF show a laminated morphology (Figs. 1c and 1d), which is typical of this kind of material. OMC unfunctionalized and functionalized (Figs. 1e and 1f) show irregular particles, as cubic morphology. The methanol functionalization did not show morphological changes between treated and untreated materials.

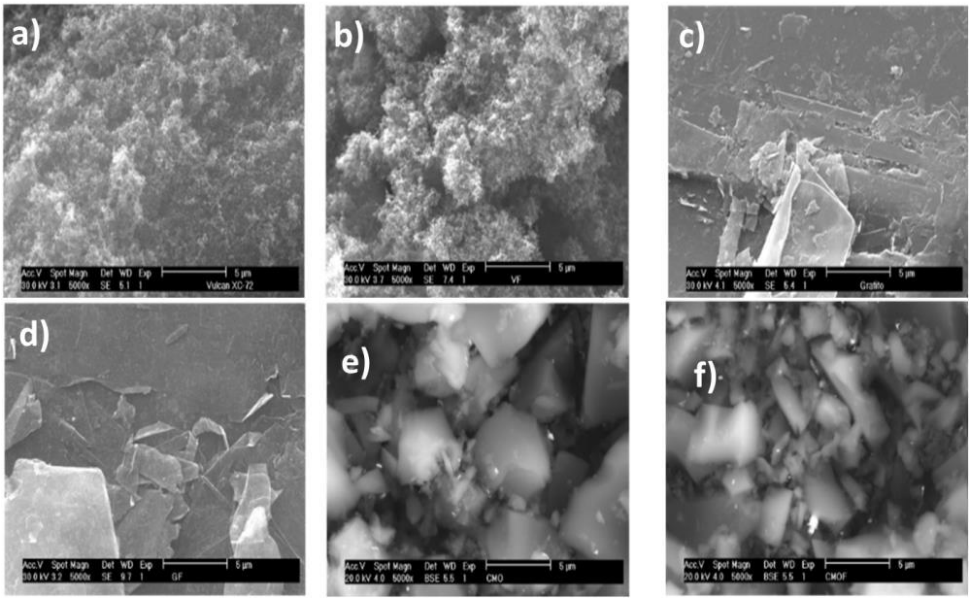

Figura 1 SEM images: a) V-XC-72, b) functionalized V-XC-72, c) GF, d) functionalized GF, e) OMC, and f) funcionalized OMC.

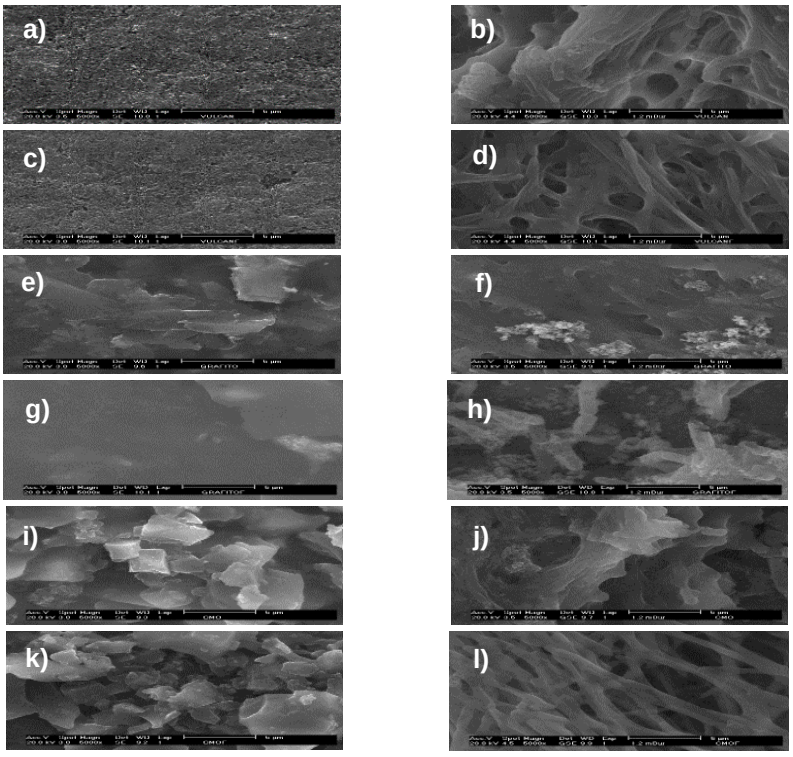

Figure 2. SEM images of electrodes. V-XC-72 unfunctionalized, without (a) and with (b) biofilm; V-XC-72 functionalized, without (c) and with (d) biofilm; GF unfunctionalized, without (e) and with (f) biofilm; GF funcionalized, without (g) and with (h) biofilm; OMC unfunctionalized, without (i) and with (j) biofilm; OMC functionalized, without (k) and with (l) biofilm.

The Figure 2 shows SEM images of fabricated electrodes with the different carbonaceous materiales. In the left column a), c), e), g), i), and k) are the untreated carbonaceous materials before the formation of biofilm over the electrodes. In the right column b), d), f), h), j), and i) show the biofilm by *Bacillus subtilis* over the electrodes, after 6 days of incubation. The SEM images show that the materials had biocompatibility with bacteria, allowing the bacterial adhesion over the electrodes. It is important to mention that the functionalization of the materials with methanol i.e. add –OH groups over the materials, enhanced the bacterial adhesion on the electrodes and improved the processes involved in the bacterial biofilm growth cycle.

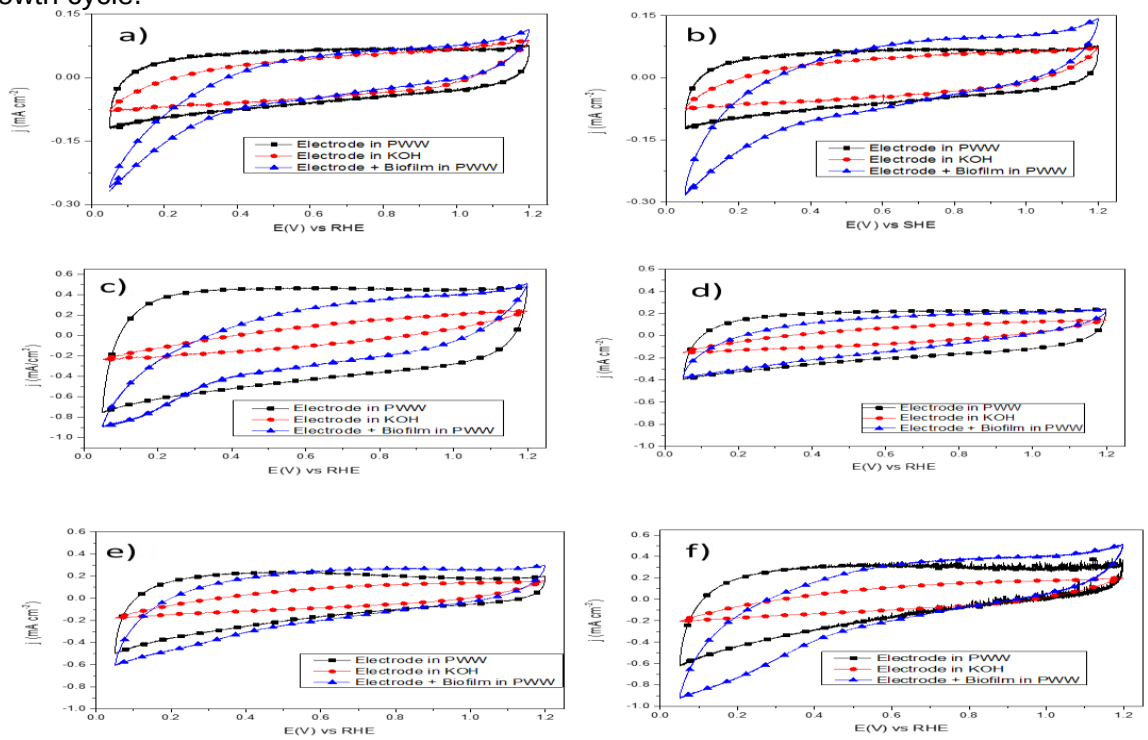

Figure 3. VC´s of electrodes of a) GF, b) functionalized GF, c) V-XC-72, d) functionalized V-XC-72, e) OMC, f) functionalized OMC in PWW, in KOH pH 9.6, and a third electrode with biofilm from *B.subtilis* in PWW.

The Figure 3 shows the CVs of the electrodes contaning carbnonaceous materials without and with treatment as well as the comparative in different substrates as PWW and KOH pH 9.6, and a third electrode containing the carbonaceous material with the biofilm formed by the bacteria *B.subtilis* over de electrode. In all cases, the materials had a capacitive behavior when they were tested in PWW, this also means that they didn´t show any faradaic currents. (a) Graphite and (b) Functionalized Graphite in two different solutions such as PWW and KOH pH 9.6. In contrast, the electrochemical behavior in presence of *B. subtilis* indicates a change in the mechanism in which the current densities are generated (j_f) due to redox reactions. This suggest the biofilm had a positive effect enhancing the electron transfer from the sustrate to the anode. Also it is important to mention that Graphite functionalized enhance the attachment of the bacteria over the electrode and also the electron transfer. CVs of (c)

Vulcan XC-72 and (d) Functionalized Vulcan XC-72 in PWW and KOH pH 9.6. In the electrode with biofilm from *B. subtilis* in PWW, the behavior is different because the current density in Vulcan XC-72 showed increasing results in comparison with Functionalized Vulcan XC-72, opposite case than Graphite. The CV´s of (e) OMC and (f) functionalized OMC in the PWW and KOH pH 9.6 solutions, without and with biofilm. The electrochemical behavior of the electrode with the biofilm in PWW changed to generates current densities due to redox reactions. This suggest that a biofilm was formed over the electrode. In the case of functionalized OMC, it increased CV in comparison with the OMC untreated. Comparing all the electrodes, the one that has the enchanced electrochemical activity was the functionalized OMC.

Conclusions

The biofilms formed on the electrodes of functionalized Graphite and functionalized OMC increased the current density due to redox reactions in the substrates and the electron transfer to the anode. Also the functionalization of the materials enhanced the CVs of OMC and Graphite, opposite case than Vulcan XC-72. Additionally, the materials that were utilized had biocompatibility with the bacteria and permitted the formation of the biofilm over the electrodes.

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TiO_2, ZnO, and Fe_2O_3 nanoparticles effect on *Rhizobium leguminosarum* - *Pisum sativum* L. symbiosis

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Abstract

The effect of nanoparticles (NPs) of TiO_2, ZnO, and Fe_2O_3 at increasing doses on the rhizobia-legume symbiosis (RLS) was evaluated in pea plants (*Pisum sativum* L.) and their symbiotic partnership *Rhizobium leguminosarum*. TiO_2-NPs did not affect the morphological features of pea plants. However, ZnO-NPs at 6 gL^{-1} significantly decreased the development of plants as witnessed by the plant fresh weight and by the root and shoot length. In addition, Fe_2O_3-NPs at 3 gL^{-1} significantly decreased the plant height, while Fe_2O_3-NPs (6 g L^{-1}) significantly decreased the fresh root weight. Infection by *R. leguminosarum* also was affected by NPs, so that the development of nodules decreased in all treatments, suggesting that NPs induced morphological alteration of pea plants, and inhibition or retardation of the symbiosis. Therefore, NPs pollution might be potentially dangerous for the development of crops, the symbiosis, and/or for the biological nitrogen fixation.

Keywords: • pollution • biological nitrogen fixation • environment • nanomaterial • nanotoxicology.

Introduction

Nanoparticles (NPs) are particles with at least one dimension between 1 and 100 nm, whose production has been increased in recent years because their use in a wide range of nanoproducts and applications worldwide, including agriculture, coatings and paints, cosmetics, electronics, medicine, etc. (Keller *et al*. 2013). However, despite the advantages which involves the use of NPs, there is now concern that handling NPs between their production, use and disposal is leading to their inevitable release into the environment (Xu *et al*. 2015), but the environmental impacts are not fully understood (Keller *et al.* 2013), so that biological systems might be affected by the presence of them. One of the biological systems that play a role of paramount importance for plants, particularly for agricultural crops is the rhizobia-legume symbiosis (RLS), which is a mutually beneficial interaction between two species for biological nitrogen fixation (BNF; Musarrat *et al*. 2010), *i.e.* plant obtains nutrients and microorganisms exploit the root exudates of host plants. However, it has been reported that NPs have positive or negative effects on higher plants, which are based mostly on evidences such as percentage of seed germination and root elongation, where the effects

assessed the morphology of cultivated plants which were exposed to different concentrations of NPs (Parveen and Rao, 2015), but on the effect of NPs in the symbiosis *Rhizobium leguminosarum-Pisum sativum* L. it has been few publications so far.Therefore, the release of NPs due to anthropogenic activities, has become a serious threat to the environment, so, this research determines the toxicological effects of TiO_2, ZnO, or Fe_2O_3 NPs, at concentrations of 0, 3 or 6 gL^{-1}, on the growth of *P. sativum* L. and on the rhizobia-legume symbiosis between pea plants and their symbiotic partnership *R. leguminosarum.*

Materials and methods

Commercial pea seeds were used, which were purchased from '*Los Molinos*', Tepoztlan Mor. *Rhizobium leguminosarum* strain was obtained from the '*Colección Nacional de Cepas Microbianas y Cultivos Celulares, Cinvestav-Zacatenco*' and maintained in TY culture medium until its use. Nanoparticles of TiO_2, ZnO and Fe_2O_3, which were used to carry out this research were acquired from 'ID-nano; Investigación y Desarrollo de Nanomateriales, S.A. de C.V., San Luis Potosí, S.L.P., México'. Eighty-four red plastic nursery pots (10.1 × 9.3 × 7.1 cm; upper Ø × height × bottom Ø, respectively) were filled with 60 g of vermiculite and saturated with tap water. Three days later, three pea seeds were sown at 1.5 cm depth at each pot and watered with NPs suspensions of TiO_2, ZnO or Fe_2O_3, at concentrations of 0, 3 or 6 gL^{-1}. Ten days after sowing (DAS) thinning was performed to leave a seedling in each pot, which was inoculated with 10 mL of the bacterial suspension of *R. leguminosarum* at the bottom of stem of each seedling and maintained in greenhouse for 35 DAS. Subsequent irrigations were performed with NPs suspensions previously indicated at 5 and 15 DAS, at concentrations of 0, 3 or 6 gL^{-1}. Furthermore, the plants were irrigated using the nutrient solution proposed by Fernández-Luqueño *et al.* (2008) in order to attend the crop water needs. Destructive samplings were conducted at 20 and 35 DAS, in which six pots of each treatment for each sample were randomly selected. Subsequently, the plant was extracted from its respective container and removed from the substrate (vermiculite) carefully, avoiding the damage to the root system. The characterization of the root, stem and nodules of each plant, and statistical analysis were performed according to Fernández-Luqueño *et al.* (2008).

Results and discussion

The ZnO-NPs (6 gL^{-1}) and Fe_2O_3-NPs (3 gL^{-1}) significantly decreased root length at 20 DAS, compared with control treatment (CCT) (Table 1). Similarly, plants exposed to ZnO-NPs (3 gL^{-1}) decreased significantly root length at 35 DAS, CCT. Additionally, plants amended with ZnO-NPs (6 gL^{-1}) significantly decreased the shoot length at 20 DAS, CCT. Similarly, the presence of both concentrations of ZnO-NPs and Fe_2O_3-NPs (6 gL^{-1}) decreased the shoot length at 35 DAS, CCT. Meanwhile, the presence of ZnO-NPs and Fe_2O_3-NPs (6 gL^{-1}) significantly decreased the fresh weight of the root at 20 DAS, CCT; likewise, plants exposed to ZnO-NPs (6 gL^{-1}) significantly decreased their shoot fresh weight at 35 DAS, CCT. Furthermore, the TiO_2-NPs and ZnO-NPs at 3 and 6 gL^{-1} significantly decreased the root colonization by *R. leguminosarum* at 20 and 35 DAS, however the presence of Fe_2O_3-NPs (3

gL^{-1}) increased the formation of symbiotic nodules, indicating that Fe_2O_3-NPs favored the symbiotic interaction between the roots of pea and *R. leguminosarum* at 35 DAS, CCT (Table 1). The results obtained are consistent with those reported by Fan *et al*., (2014), who found that the presence of TiO_2-NPs at concentrations up to 1000 mgL^{-1} did not show significant effects on the root length of pea plants. Additionally, Fan *et al*. (2014) also found that the shoot length and the average surface area of the leaves were not affected by the presence of TiO_2-NPs, which also coincide with the results obtained in this investigation. Fernández-Luqueño *et al.* (2014) also reported that some crops (*Petroselinum sativum* Hoffman, *Antirrhinum majus* L., and *Origanum vulgare* L.) were affected by at least one of five types of NPs evaluated (Fe_3O_4, Fe_2O_3, FeOOH• x H_2O, TiO_2, and ZnO), when the crops were growth into a plant growing chamber. Meanwhile, Huang *et al.*, (2014) reported that the exposition of pea plants to ZnO-NPs concentrations up to 1000 mgL^{-1}, decreased the root length and trigger some significant changes in the morphology thereof, *i.e.* ZnO-NPs decreased the number of roots of the first and second order. Additionally, these authors reported that ZnO-NPs decreased significantly the shoot length and decreased the average surface area of the pea leaves. Similarly, Mattiello *et al*., (2015) found that a concentration of 2000 mgL^{-1} of CeO_2-NPs and TiO_2-NPs did not affect significantly the germination and root elongation of barley plants. However, they reported that the CeO_2-NPs induced genotoxic effect since reduced the mitotic index of the treated plants, demonstrating the negative effect that the CeO_2-NPs have in the cell cycle. Our results are according with those reported by Fan *et al*., (2014) and Huang *et al*., (2014). They observed that TiO_2- or ZnO-NPs on pea plants, which were inoculated with *R. leguminosarum*, had a toxic effect on the symbiosis of pea-*R. leguminosarum*, since the process of root infection was delayed by the presence of both NPs *i.e* TiO_2- or ZnO-NPs trigger a slow development of root nodules, and therefore a delay in the BNF process. However, the presence of Fe_2O_3-NPs increased the RLS in this study, which coincides with that reported by Burke *et al.*, (2015), who observed a significant increase in the colonization of soybean roots by rhizobia, when they were exposed to Fe_3O_4-NPs, suggesting that some Fe_3O_4-NPs are able to set Fe free and increase the root colonization because the Fe is an essential micronutrient for plants and nitrogen-fixing bacteria *i.e.* Fe_3O_4-NPs might trigger some biochemical signals in order to increase the colonization and growth of nodules.

These finds are an important factor to take into account with regard to the applicability of NP for long-term use in crops but, the selection of the proper NP at their proper concentration is important for realizing higher benefits for a target agrosustainable. Additionally, there is the need of generating more data on chronic effects from long terms and concentration exposure of nanoparticles in plants, which is important for a better understanding of the potential hazard or risk of this nanoparticles, while more studies are also necessaries in order to identify the highest potential of NP in the rural sector and in the agro-food industry worldwide.

Table 1. Morphological characteristics and nodulation of *Pisum sativum* L. inoculated with *Rhizobium leguminosarum,* and irrigated with suspensions of TiO_2, ZnO, or Fe_2O_3 nanoparticles, at concentrations of 0, 3 or 6 gL^{-1}.

Characteristics	DAS[ϕ]	Treatments [μ]							MSD[Ω]
		Ti3	Ti6	Zn3	Zn6	Fe3	Fe6	Control	
Root length (cm)	20	26.5 a	27.4 a	25.5 ab	19.5 c	21.1 bc	25.1 ab	27.9 a	7.8
	35	22.9 b	28.7 ab	34.3 a	27.4 ab	24.7 b	28.0 ab	25.8 b	11.1
Shoot length (cm)	20	19.5 ab	18.3 ab	19.9 ab	12.4 b	21.9 ab	16.4 ab	22.7 a	14.5
	35	22.3 ab	19.2 ab	12.9 b	12.6 b	19.0 ab	16.4 b	26.5 a	14.2
Root fresh weight (g)	20	3.0 ab	3.7 a	3.2 ab	2.2 c	3.4 a	2.4 bc	3.6 a	1.3
	35	6.0 a	4.6 ab	5.1 ab	4.4 b	5.0 ab	5.2 ab	5.7 ab	1.9
Shoot fresh weight (g)	20	3.5 a	3.8 a	3.6 a	3.3 a	4.6 a	3.5 a	4.4 a	2.0
	35	5.9 ab	5.7 ab	5.7 ab	4.6 b	6.0 ab	6.0 ab	7.4 a	2.8
Root dry weight (g)	20	0.26 abc	0.28 ab	0.28 abc	0.22 c	0.32 a	0.24 bc	0.23 bc	0.07
	35	0.36 a	0.32 ab	0.31 ab	0.29 b	0.30 b	0.32 ab	0.29 b	0.07
Shoot dry weight (g)	20	0.50 a	0.61 a	0.49 a	0.48 a	0.76 a	0.56 a	0.64 a	0.4
	35	1.03 a	0.90 ab	0.80 ab	0.65 b	0.89 ab	0.84 ab	1.03 a	0.4
Nodules number (units)	20	3.00 c	0.66 d	1.16 d	0.33 d	3.83 bc	5.16 ab	5.83 a	2.8
	35	7.17 b	3.67 c	2.00 c	2.17 c	10.33 a	9.3 ab	6.83 b	4.1
Nodule diameter (mm)	20	1.14 ab	0.49 b	1.21 a	0.68 ab	1.12 a	1.20 a	0.75 ab	0.9
	35	2.00 a	1.37 b	0.97 cd	0.88 d	0.96 cd	1.21 bc	1.03 cd	0.4

[ϕ] Day after sowing; At 20 DAS n=6, at 35 DAS n=6.
[μ] Ti3=TiO_2 a 3gL^{-1}, Ti6=TiO_2 6 gL^{-1}, Zn3=ZnO a 3gL^{-1}, Zn6=ZnO a 6gL^{-1}, Fe3=Fe_2O_3 a 3gL^{-1}, Fe6= Fe_2O_3 a 6gL^{-1}
[Ω] Minimum significant difference,
[†] Values with the same letter, *i.e*. within the rows, are not significantly different between the treatments (Tukey α=0.05).

Conclusions

It was found that pea crop exposed to ZnO and Fe_2O_3 nanoparticles changed its morphology as witnessed by the root and shoot length as well as the fresh weight of root and shoot. Additionally, it was observed that the presence of TiO_2 and ZnO nanoparticles in the rhizosphere delayed the process of infection by *R. leguminosarum*, which caused the late formation of nodules on the roots, therefore the biological nitrogen fixation was also delayed; however, it was observed that the Fe_2O_3 nanoparticles accelerated and increased the production of nodules by *R. leguminosarum.* The environmental exposure of nanoparticles cannot be overlooked because they decrease the plant growth and affect the processes involved in the rhizobia-legume symbiosis, *i.e.* pea plants are vulnerable to the nanoparticles used in this research. These findings might be especially important because the pea crop has social, economic, and environmental value throughout Mexico, but also has a low efficiency in the process of biological nitrogen fixation. Although the causes of our findings remain unclear, it is suggested that the nanoparticles interfere with some physiological and morphological processes, which are determined by the type of nanoparticles, exposure times, and the nanoparticles concentration.

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BIOTECHNOLOGY SUMMIT

Simultaneous removal of carbamazepine and ibuprofen by electrooxidation using Ti/PbO_2 anode

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Abstract

Emerging contaminants such as pharmaceuticals, personal care products, surfactants, industrial additives, plasticizers, pesticides and several chemical compounds; affect the health of humans and animal species even when they are present in low concentrations. The objective of this work was to assess the effect of current intensity (CI), electrolysis time (ET), electrolyte concentration (EL), and electrolyte type (ELT) on the removal of Carbamazepine (CBZ) and Ibuprofen (IBP) from water. Electrooxidation process was investigated using Ti/PbO_2(anode) and Ti(cathode) cylindrical electrodes in the presence of sodium sulfate ($NaSO_4$) and ammonium sulfate ($(NH_4)_2SO_4$) as electrolyte. The initial concentration in all treatments was 5 mg/L for each pollutant. A cylindrical reactor with concentric electrodes was used. The electrodes were distributed from center to periphery (cathode-anode-cathode). A Box-Behnken design was carried out to evaluate CBZ and IBP removal with a total of twenty different experimental conditions. Pollutants removal was affected for two of four variables studied (CI and ET), showing significant difference. Optimum results were obtained with CBZ reaching a removal of 92.92%, in the case of IBP only 65.85% of removal was found. These results were obteined with 1.26 Amperes of CI, 80 minutes of ET, 3 g/L of EL and $(NH_4)_2SO_4$ as ELT. It was probed that the ET and CI are variables responsible for the pollutants removal. The effect of the ELT is only significant in the removal of ibuprofen. The EL showed no significant difference between treatments.

Key words: ●electrooxidation ●pollutant ●treatment ●Carbamazepine ●Ibuprofen.

Introduction

Today, it is common to speak about emerging contaminants such as pharmaceuticals, personal care products, surfactants, industrial additives, plasticizers, pesticides and several chemical compounds. Although these compounds are usually found in concentrations of mg/L or ng/L (Lapworth *et al.* 2012). They cause significant biological effects such as endocrine system disruption, hormonal blocking functions (García *et al.* 2011). Carbamazepine is an important pharmaceutical drug due to its use in clinics in the treatment of epilepsy and neuropathic pain, its high recalcitrance, and its ecotoxicological potential (García *et al.* 2014). Ibuprofen is a non-steroidal anti-inflammatory drug that has been widely used to treat pain and inflammation in rheumatic disease and other musculoskeletal disorders (Rezaeifar *et al.* 2016). Some wastewaters have extremely high chemical oxygen demand (COD), on average about 2000 mg/L, displaying a strong odor and dark color. Therefore, proper treatment of these effluents is essential before being discharged into water bodies (Zodi *et al.* 2010). The

main methods for removing contaminants from wastewaters are physical, chemical and biological processes that occur in conventional processing methods. However, these methods are not efficient to remove micropollutants. Electrochemical technologies have reached a promising stage of development and they also can be used effectively to remove these compounds (Farhadi *et al.* 2012). Furthermore, Zaroual *et al*. (2008) reported that in recent years, the electrocoagulation has been successfully used to treat a variety of industrial wastewaters. Aleboyeh *et al.* (2007) mentioned that electrocoagulation process has been applied to treat a variety of dye effluents. Electrooxidation is a process that degrades pollutants without forming other waste contaminants such as blood clots or flocs. Electrooxidation requires a supporting electrolyte to enhance electrical conductivity. Recent work has shown that sodium sulfate is the best supporting electrolyte for the electrochemical process considering economy, efficiency, and environmental aspects (García *et al.* 2011).
Box-Behnken have been previously using for determining the optimum operating conditions of COD (Ahmadi and Ghanbari, 2016). This design includes uniformly distributed points in the space of encoded variables. One advantage is the ability to explore the entire experimental region and the usefulness of response interpolation. Besides the matrix allows the description of a region around an optimal response. Because of the importance of emerging contaminants removal from aquatic systems, the target for this research was to assess the effect of current intensity, electrolysis time, electrolyte concentration, and electrolyte type on the removal of Carbamazepine and Ibuprofen from water.

Materials and methods

Synthetic solution. CBZ and IBP analytical grade were used, to prepare a standard calibration curve. A stock solution (10 mg/L of each pollutant) was prepared using distilled water. Pharmaceuticals were solubilized by stirring for 24 hours at room temperature. This solution was kept at 4°C. Subsequently, 500 mL of stock solution were diluted with distilled water until reach a volume of litter. Then it was kept under stirring for 10 minutes. The resulting mixture with 5 mg/L of each pollutant was used to carry out all the experiments.
Experimental unit. A cylindrical electrooxidation reactor was manufactured using acrylic material with 10 cm of radius, 20 cm of height, and 1500 mL of total volume. The trading volume was 1100 mL. Two circular titanium mesh electrodes were used as cathode and a circular titanium mesh electrode lead dioxide coated was used as anode. All electrodes were placed in concentric arrangement with interposed the center to the periphery (cathode-anode-cathode) to maximize conductivity efficiency. The spacing between electrodes was 1 cm.
Studied variables. The effect of four variables, CI, ET, EL and ELT over carbamazepine (CBZR) and ibuprofen removal (IBFR), and Energy consumption (EC). A calibration curve of known concentration (0.1 to 5 mg/L) versus relative absorbance was used to calculate the concentration of the pharmaceuticals and to estimate removal efficiency. A total of 34 different experiments or treatments resulted using Box-Behnken design. Samples of each experiment were obtained at the beginning and at the end of each treatment. Carbamazepine and Ibuprofen concentration in the solution was determined by UV spectrophotometry at 285 and 210 nm respectively. Removal efficiency was calculated according to the following equation:

$$\%R = \left[\frac{C_0 - C_f}{C_0}\right] x100 \qquad (1)$$

%R : Percentage of pollutant removed.
C_0 : Initial concentration of pollutant.
C_f : Final concentration of pollutant.

Results and discussion

Results obtained are listed in Table 1, which shows the variables effect on pollutants removal. The last column represents power consumption according to experimental conditions of each treatment.

Table 1. Removal of pollutants and energy consumption for treatment.

Treatment	CI (A)	ET (min)	EL (g/L)	ELT	CBZR (%)	IBPR (%)	EC (KW-h/m^3)
1	1.00	40.00	2.00	$NaSO_4$	61.47	14.33	2.73
2	2.00	40.00	2.00	$NaSO_4$	80.60	27.25	6.00
3	1.00	80.00	2.00	$NaSO_4$	90.84	60.38	5.73
4	2.00	80.00	2.00	$NaSO_4$	96.64	53.64	13.33
5	1.00	60.00	1.00	$NaSO_4$	77.65	22.10	4.30
6	2.00	60.00	1.00	$NaSO_4$	90.54	42.10	11.20
7	1.00	60.00	3.00	$NaSO_4$	76.25	31.42	4.60
8	2.00	60.00	3.00	$NaSO_4$	94.27	21.76	9.20
9	1.50	40.00	1.00	$NaSO_4$	76.23	18.03	5.00
10	1.50	80.00	1.00	$NaSO_4$	87.98	79.26	10.20
11	1.50	40.00	3.00	$NaSO_4$	76.28	32.32	4.30
12	1.50	80.00	3.00	$NaSO_4$	95.44	62.58	8.80
13	1.50	60.00	2.00	$NaSO_4$	95.25	57.45	6.90
14	1.50	60.00	2.00	$NaSO_4$	87.21	47.87	6.90
15	1.50	60.00	2.00	$NaSO_4$	79.45	56.39	6.60
16	1.50	60.00	2.00	$NaSO_4$	88.04	49.66	6.45
17	1.50	60.00	2.00	$NaSO_4$	88.71	23.11	6.90
18	1.00	40.00	2.00	$(NH_4)_2SO_4$	53.99	32.38	2.66
19	2.00	40.00	2.00	$(NH_4)_2SO_4$	85.36	56.87	6.26
20	1.00	80.00	2.00	$(NH_4)_2SO_4$	83.17	58.67	5.33
21	2.00	80.00	2.00	$(NH_4)_2SO_4$	94.37	63.23	12.53
22	1.00	60.00	1.00	$(NH_4)_2SO_4$	73.49	42.88	4.30
23	2.00	60.00	1.00	$(NH_4)_2SO_4$	90.27	57.63	11.40
24	1.00	60.00	3.00	$(NH_4)_2SO_4$	87.93	48.88	3.90
25	2.00	60.00	3.00	$(NH_4)_2SO_4$	90.65	63.41	8.40
26	1.50	40.00	1.00	$(NH_4)_2SO_4$	77.27	44.67	5.10
27	1.50	80.00	1.00	$(NH_4)_2SO_4$	91.86	65.14	9.80
28	1.50	40.00	3.00	$(NH_4)_2SO_4$	79.36	48.90	4.20
29	1.50	80.00	3.00	$(NH_4)_2SO_4$	91.03	68.73	8.40
30	1.50	60.00	2.00	$(NH_4)_2SO_4$	84.15	52.79	6.45
31	1.50	60.00	2.00	$(NH_4)_2SO_4$	86.30	58.33	6.75
32	1.50	60.00	2.00	$(NH_4)_2SO_4$	87.97	59.59	6.60
33	1.50	60.00	2.00	$(NH_4)_2SO_4$	86.80	60.80	6.90
34	1.50	60.00	2.00	$(NH_4)_2SO_4$	89.02	62.19	6.90

Results were analysed with Design Expert 7 software. Optimal values for the studied variables, maximizing pollutants removal and minimizing energy consumption, were obtained, which are presented in Table 2.

Table 2. Optimum values by statistical analysis.

Current intensity (A)	Electrolysis time (min)	Electrolyte concentration (g/L)	Electrolyte type	Carbamazepine removal (%)	Ibuprofen removal (%)	Energy consumption (KW-h/m^3)
1.26	80.00	3.00	$(NH_4)_2SO_4$	92.92	65.85	6.74

Also the effect of ET and CI on the CBZ and IBP removals can be observed using the response surface plot (Figure 1 and figure 2 respectively). In addition, significant effect of the variables is demonstrated for the ANOVA analysis ($F < 0.05$).

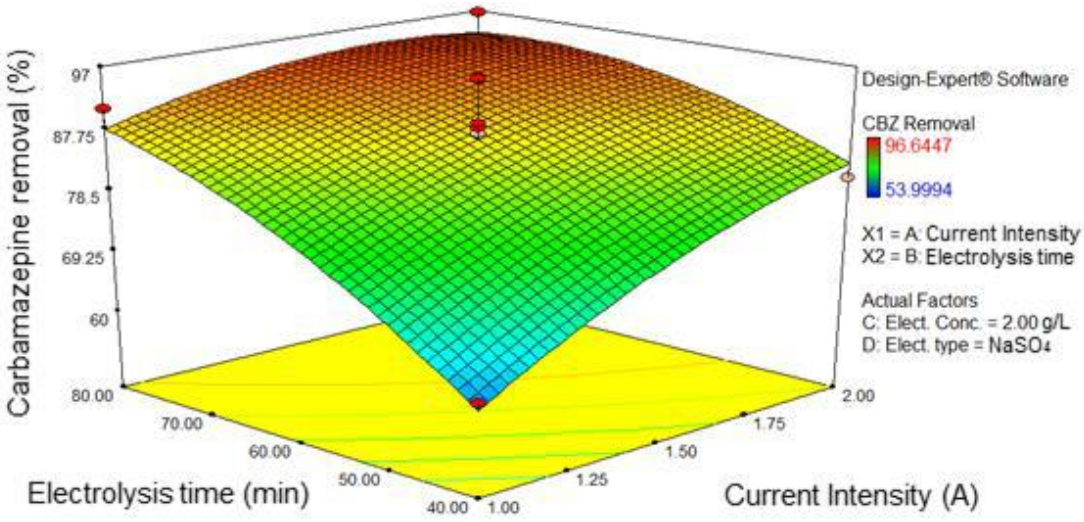

Figure 1. Effect of the current intensity and time on the Carbamazepine removal.

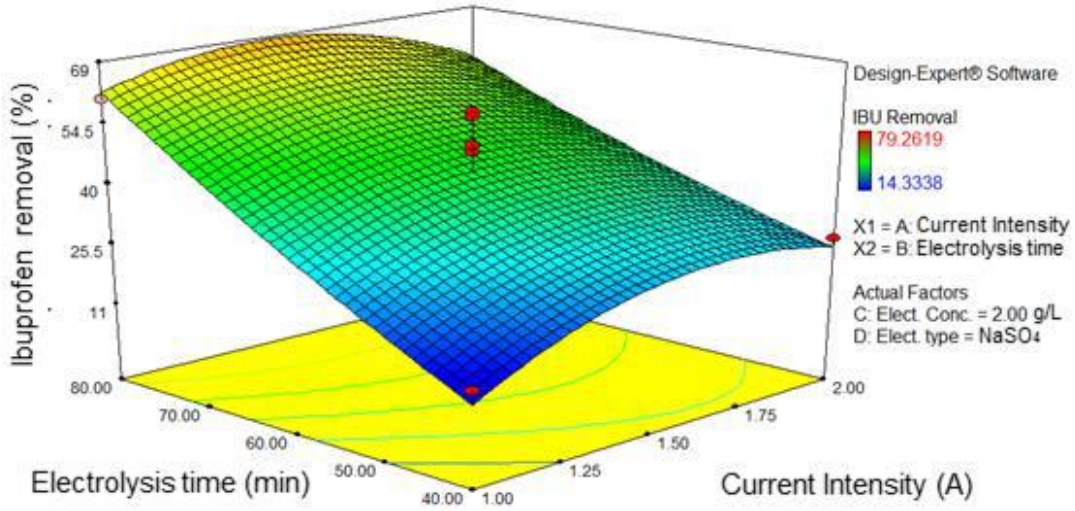

Figure 2. Effect of the current intensity and time on the Ibuprofen removal.

Conclusion

CBZ and IBP removal is affected mainly by two variables, CI and ET, showing significant difference. Optimum remotion for CBZ was 92.92%, while for IBP only 65.85% of removal was found. These results were achieved with 1.26 Amperes of CI, 80 minutes of ET, 3 g/L of EL and $(NH_4)_2SO_4$ as ELT. It was probed that the ET and CI are the main variables responsible for the pollutants removal. The effect of the ELT is only significant in the case of f ibuprofen removal. The EL showed no significant difference between treatments. This process seems to be a promising technology that could be used as tertiary treatment to remove emerging contaminants from the water.

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Activity of lipases, chitinases and proteases of the entomopathogenic fungus, *Metarhizium anisopliae* developed in different culture media

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Abstract

Sugar cane (*Saccharum officinarum*) represents one of the most important crops in our country, loss caused by screwworm of stalk are estimated in 50%. *Metarhizium anisopliae* is an entomopathogenic fungus with potential use against insect pests of sugar cane. Several studies have been carried out in order to evaluate the enzymatic activities of the entomopathogen fungi and relate it with their virulence, because they represent a viable alternative to control the loss caused in the production of sugar cane. The aim of this research was to grow *Metarhizium anisopliae* in different culture media and evaluate its enzymatic activity. Culture media were potato dextrose broth, sucrose-yeast extract broth, extract of banana-grape-potato and of banana. The results show that the potato-dextrose broth produced the highest activity of lipases and proteases and chitinase activity was similar in all culture media. It was observed that the last enzyme is constitutive and proteases and lipases are inducible. These results suggest that the chitinases act first in the processes of micelial invasion on the insects and the activities of proteases and lipases are expressed at different level depending their induction by insect components, however it is necessary to make experiments of virulence *in situ* to establish correlations.

Keyword: Antioxidant assay ● free radical scavenging ● solid-state fermentation ● polyurethane foam ● *Pycnoporus.*

Introduction

Sugar cane (*Saccharum officinarum*) represents one of the most important agricultural crops on an industrial level in Mexico (Hernández-Velázquez *et al*. 2012). Losses are estimated of up to 50% by damage caused by screwworm of stalk, the major pest of this crop. The main species of these insects belong to the order Lepidoptera. Currently, biological control including the use of entomopathogenic fungi is a viable alternative (Cortez-Madrigal *et al*. 2014). Delgado *et al*. (2001) evaluated the activity of proteases and chitinases as well as the percentage of pathogenicity of various strains of *Bauveria bassiana* and *Metharizium anisopliae* against *Hypothenemus hampei*, they observed that there was some degree of relationship between the highest values of pathogenicity and protease activity and of chitinase. *Metarhizium anisopliae* is an entomopathogenic fungus with potential use against insect pests of sugar cane where their extracellular enzymes including lipases, proteases, chitinases and laccases are related to pathogenicity of the strain. In this study the activity of

lipases, proteases and chitinases of *Metarhizium anisopliae* (EH156) grown in liquid culture using different culture media were evaluated.

Materials and Methods

Organism and culture conditions: *Metarhizium anisopliae* strain EH156, was used. The strain was obtained from the Center for Biological Research of the Universidad Autónoma del Estado de Morelos (UAEM), Cuernavaca, Morelos, Mexico. It was grown in submerged fermentation using 250 mL Erlenmeyer flasks with 50 mL of dextrose-potato broth (BDP) or sucrose-yeast extract broth (SY) or infusion of banana (10%) (B), or of banana-grape-papa (BPG) also 10%. The inoculum consisted of $1x10^7$ conidia per flask. They were incubated at 26 °C with orbital shaking at 150 rpm for 72 h.

Enzymatic assays: the activity of lipases, proteases and chitinases in the fermentation broth was evaluated. For lipases, the reaction mixture was: 10 µL of substrate (p-nitrophenylbutyrate) (p-NPB), 890 µL of phosphate buffer 0.1M pH 7.2 and 100 µL of simple, the reaction mixture was incubated at 30 °C for 5 min, then absorbance was read at 400 nm (Margesin 2005). One international unit of lipase activity (IU) was defined as the amount of enzyme required to hydrolyze 1 µmole of p-NPB per minute under the conditions described above (Guisán 1998). Protease activity was determined by quantitation of aromatic amino acids released in the reaction mixture (450 µL of 1% casein in phosphate buffer 0.1 M at pH 6.0 and 50 µL of sample); was incubated at 35 °C for 15 min, the reaction was stopped with 750 µL of trichloroacetic acid (Baker) 5%, then centrifuged at 15,000 rpm for 5 min and the absorbance at 280 nm of the supernatant was read. One unit of protease activity was defined as the amount of enzyme required to release aromatic amino acids to cause a change of 1.0 absorbance unit per min of incubation (kunitz 1947). Chitinase activity was determined by quantifying the concentration of N-acetylglucosamine (NAG) released in the reaction mixture (900 µL of 1% chitin in acetate buffer 0.1M pH 5.0 and 100 µL of sample). It was incubated at 37 °C for 4.5 h, the reaction was stopped by addition of 870 µL of the p-dimethylaminobenzaldehyde (DMAB) (10:01 v/v 0.67M DMAB in glacial acetic acid and 10 M HCl), incubated again for 20 min, the absorbance was read at 585 nm (Coudron *et al*. 1984). One IU of chitinase activity was considered as the amount of enzyme which liberates 1 µmole of NAG per min. Variance analysis was carried out to determine significant differences between means of three replicates.

Results and Discussion

Metarhizium anisopliae (EH156) showed differences in the activity of lipases (Figure 1), the broth of BDP showed the highest value (about 64 IU/L), followed by SY with half the activity approximately. The broth BPG reported one third of the activity value observed for the broth BDP and minimal activity was observed in the medium B (4.6 IU/L). Coca *et al.* (2001) evaluated the activity of lipases produced by different bacteria, yeasts and fungi in the presence of olive oil. The lowest values were shown by bacteria such as *Escherichia coli* (25 IU/L), followed by yeast as *Saccharomyces cerevisiae* (50 IU/L) and the highest values were in fungi such as *Aspergillus niger* and *Aspergillus fumigatus* with values above 200 IU/L.

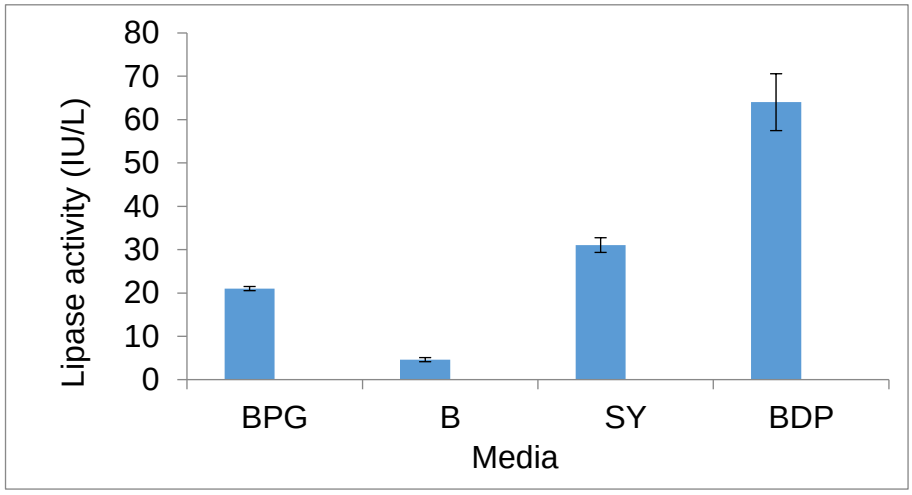

Figure 1. Activity of lipases of *Metarhizium anisopliae* (EH156) grown in liquid fermentation.

Protease activity (Figure 2) was very high in all cases but also showed differences depending on the culture medium and a similar pattern to that observed with the lipase activity was observed. The highest value of protease activity was in the medium BDP (973 U/L), followed by SY (663 U/L) and finally the media BPG and B (368 and 307 U/L, respectively). Chitinase activity was similar in all cases with values of 1.0-1.7 IU/L (Figure 3), no significant difference between the values was found ($p>0.05$). Recently, to understand the relationship between pathogenicity and enzymatic activity of fungal isolates associated with *Diaphorina citri* Kuwayama, three isolates of *Beauveria bassiana* (Bals.) Vuill. and six of *Hirsutella citriformis* Speare from southeastern and Central Mexico were characterized enzymatically. A positive relationship (r = 0.84 to 0.98) was found only between intracellular proteases versus pathogenicity (Cortez-Madrigal *et al*. 2014).

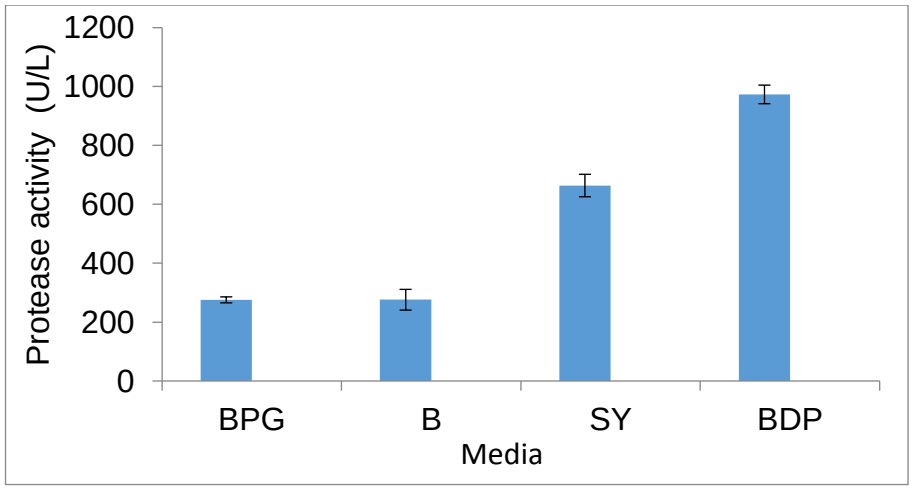

Figure 2. Activity of proteases of *Metarhizium anisopliae* (EH156) grown in liquid fermentation.

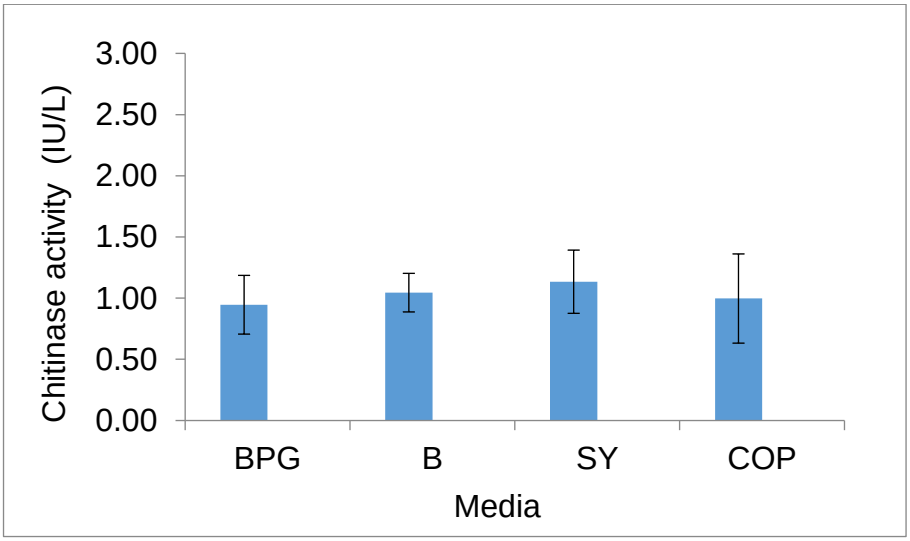

Figure 3. Activity of chitinases of *Metarhizium anisopliae* (EH156) grown in liquid fermentation.

Conclusions: The lipase activity of *Metarhizium anisopliae* (EH156) was low, the protease activity was high, however, it was found to be depend on the substrate on which the fungus grows, suggesting that there was induction for these enzymes, while chitinases were presented as constitutive enzymes. This could set different virulence levels depending on the substrate on which fungi will grow. Enzymatic activities evaluated suggest that the virulence of the fungus on insects is given mainly by the ability to produce proteases, and the attack in the first instance may be based on the activity of chitinases, however it is necessary to make experiments of virulence *in situ* to establish correlations.

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TiO_2, ZnO, and Fe_2O_3 nanoparticles effect on *Bradyrhizobium japonicum-Glycine max* [L.] Merr. symbiosis

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Abstract

In this research, the effect of nanoparticles (NPs) of TiO_2, ZnO and Fe_2O_3, at concentrations of 3 and 6 gL^{-1} on rhizobia-legume symbiosis (RLS), using soybean plants and their symbiotic partnership *Bradyrhizobium japonicum* was determined. Exposure of soybean plants to TiO_2-NPs and Fe_2O_3-NPs did not show significant changes in the shoot length and in the root elongation, in contrast, the presence of ZnO-NPs (3 gL^{-1}) increased significantly the length of the shoot and root length. The RLS, determined by the number of nodules, was not significantly different when plants were treated with increasing doses of NPs at 20 days after sowing (DAS), however, in case of the ZnO-NPs a significant decreased in the size of nodules was observed, while TiO_2-NPs (6 gL^{-1}) and Fe_2O_3-NPs (3 gL^{-1}) increased it significantly at 35 DAS, compared with the control treatment. The results showed that the effect of NPs is dependent on the type and concentration of NPs and exposure time, which means that the NPs released into the environment might affect the crop development and some important biological processes such as the biological nitrogen fixation.

Keywords: • pollution • biological nitrogen fixation • environment • nanomaterial • nanotoxicology.

Introduction

Nanoparticles (NPs) consist of atomic and molecular assemblies having nanoscale dimensions (*i.e.* 1-100 nm), whose production has been increasing steadily in recent years; today nanoproducts are used in various specific applications worldwide. However, despite the advantages that involve the use of NPs in various applications, there is now concern that their environmental impact is not fully understood (Keller *et al*., 2013). In addition, NPs handling between production, use and disposal is leading to its inevitable release into the environment (Xu *et al.,* 2015). Although NPs are used worldwide by their innovative and promising properties, the fate and impacts of the NP have not yet been fully studied. There is a serious concern that NPs might migrate to the environment and therefore into humans. In addition, many products that employ NP are not labeled to alert consumers about the potential risk, eliminating the right to choose or avoid using these products. The lack of both government surveillance and regulation in this new technology is further compounded by the lack of data

and appropriate safety tests, reinforcing the potential negative effects that might occur on health and the environment (León-Silva *et al*., 2016). Rhizobia-legume symbiosis (RLS) is an interaction between two species for biological nitrogen fixation (BNF), this partnership is mutually beneficial (Musarrat *et al*., 2010), *i.e.* plant obtains nutrients and microorganisms exploit the root exudates of host silver. Based on the above, the release of manufactured NPs has become a serious threat to the environment, so studies have been reported which demonstrate the negative effects of NPs on the plant shoot system, mainly in crops of high economic, social and, environmental value. It is of utmost importance to assessing the toxicity of NP on the seed germination and changes in the morphology of plants exposed to NPs (*Parveen and Rao*, 2015). However, at present a small number of related effects of NPs on the RLS studies, therefore, this research determines the toxicological effects of NPs TiO_2, ZnO and Fe_2O_3, at concentrations of 0, 3 and 6 gL^{-1} on the symbiosis *Glycine max* [L.] Merr-*Bradyrhizobium japonicum,* and the morphological changes of plants exposed to NPs.

Materials and methods

Soybean (*Glycine max* [L.] Merr.) seeds of 'Huasteca 400' variety were acquired from the experimental field '*Las Huastecas, INIFAP Tamaulipas*'. The bacterial strain *Bradyrhizobium japonicum* was obtained from the '*Colección Nacional de Cepas Microbianas y Cultivos Celulares, Cinvestav-Zacatenco*' and maintained in TY culture medium until its use. Nanoparticles of TiO_2, ZnO and Fe_2O_3 were acquired from? 'ID–nano; Investigación y Desarrollo de Nanomateriales, S.A. de C.V., San Luis Potosí, S.L.P., México'. Eighty-four red plastic nursery pots (10.1 × 9.3 × 7.1 cm; upper Ø × height × bottom Ø, respectively) were filled with 60 g of vermiculite and saturated with tap water. Three days later, three soybean seeds were sown at 1.5 cm depth at each pot and watered with NPs suspensions of TiO_2, ZnO or Fe_2O_3, at concentrations of 0, 3, or 6 gL^{-1}. Ten days after sowing (DAS), thinning was performed to leave a seedling in each pot, which was inoculated with 10 mL of the bacterial suspension of *B. japonicum* at the bottom of stem of each seedling and maintained in greenhouse for 35 DAS. Subsequent irrigations were performed with NPs suspensions previously indicated at 5 and 15 DAS, at concentrations of 0, 3, or 6 gL^{-1}. Furthermore, the plants were irrigated using the nutrient solution proposed by Fernández-Luqueño *et al.* (2008) in order to attend the crop water needs. Destructive samplings were conducted at 20 and 35 DAS, in which six pots of each treatment for each sample were randomly selected. Subsequently, the plant was extracted from its respective container and removed from the substrate (vermiculite) carefully, avoiding the damage to the root system. The characterization of the root, stem and nodules of each plant, and statistical analysis were performed according to Fernández-Luqueño *et al.* (2008).

Results and discussion

None of the evaluated NPs significantly change the shoot length, the number of nodules or the diameter of the nodules at 20 DAS, compared to the control treatment (CCT; Table 1); similar behavior was observed in the length and root fresh weight at 35 DAS. However, TiO_2-

NPs, ZnO-NPs and Fe_2O_3-NPs significantly increased the shoot fresh weight at 35 DAS and the shoot dry weight at 20 and 35 DAS, CCT. The suspension of ZnO-NPs (3 gL^{-1}) significantly increased the root length at 20 DDS and the shoot length at 35 DAS, CCT. The TiO_2-NPs and ZnO-NPs at both concentrations, and Fe_2O_3-NPs (6 gL^{-1}) significantly increased the root fresh weight at 20 DAS, CCT. The NPs of ZnO and TiO_2 and Fe_2O_3 (3 gL^{-1}) significantly decreased the fresh weight of the aerial part at 20 DAS, CCT

The TiO_2-NPs and ZnO-NPs, at both concentrations, and Fe_2O_3 (6 gL^{-1}) significantly decreased root dry weight and the number of root nodules at 35 DAS, CCT. ZnO-NPs (3 gL^{-1}) and Fe_2O_3-NPs (6 gL^{-1}) significantly increased root dry weight at 20 DAS, CCT. Additionally, the ZnO-NPs significantly decreased the diameter of nodules at 35 DAS, while TiO_2-NP (6 gL^{-1}) and Fe_2O_3-NPs (3 gL^{-1}) significantly increased it at 35 DAS, CCT. Similar studies report that exposure of pea plants toTiO_2-NPs show no significant effects on plant morphology (Fan *et al.,* 2014), which coincides with that obtained in this study at 20 DAS. Fernández-Luqueño et al. (2014) also reported that some crops (*Petroselinum sativum* Hoffman, *Antirrhinum majus* L., and *Origanum vulgare* L.) were affected by at least one of five types of NPs evaluated (Fe_3O_4, Fe_2O_3, $FeOOH{\bullet}xH_2O$, TiO_2, and ZnO), when the crops were grown in a plant growing chamber.On the other hand, ZnO-NPs (3 gL^{-1}) increased the root length in soybean plants, in contrast with those reported by Huang *et al.,* (2014), who indicate that exposure of pea plants to ZnO-NPs (1 gL^{-1}), showed significant decrease in root length and significant changes in the morphology of plants.

Burke *et al.,* (2015) reported that Fe_3O_4 nanoparticles can affect the root system as wells as leaf phosphorous content from soybean plants (*Glycine max* (L.) Merr.), but Quoc *et al.* (2014) found that iron NPs increased up 16% the yield of soybean in comparison with the control sample. In addition, Martinez-Fernandez *et al.* (2016) found reduction of the root functionality from sunflower plants (*Helianthus annuus* L.) by iron oxide nanoparticles. Several studies on the application of nanoparticles in a relatively broad range of species have attempted to understand the effect on plant growth. For instance, Lin and Xing (2007) reported that ZnO nanoparticles can inhibited seed germination of ryegrass. With respect to the results obtained on the RLS, in the first stage of the experiment (20 DAS), no significant changes in the development and number of nodules were observed, however, at 35 DAS, it was observed that the presence of NPs significantly reduced the number of nodules, which coincides with that reported by Fan *et al.,* (2014) and Huang *et al.,* (2014) in their research, where both authors observed that exposure of pea plants inoculated with *Rhizobium leguminosarum* had a toxic effect on the symbiosis of pea-*R. leguminosarum*, having the effect of delaying the process of BNF.

Table 1**.** Morphological characteristics and nodulation of *Glycine max [L.] Merr* inoculated with *Bradyrhizobium japonicum,* and irrigated with suspensions of TiO_2, ZnO and Fe_2O_3 nanoparticles, at concentrations of 0, 3 or 6 gL^{-1}.

Characteristics	DAS[ϕ]	Treatments [μ]							MSDΩ
		Ti3	Ti6	Zn3	Zn6	Fe3	Fe6	Control	
Root length (cm)	20	25.0 b[†]	26.1 a	28.2 b	25.8 a	25.1 b	24.2 b	21.4 b	10.66
	35	35.9 a	31.3 a	36.5 a	31.3 a	35.3 a	35.8 a	35.4 a	11.99
Shoot length (cm)	20	9.1 b	8.7 b	8.1 b	8.3 b	7.6 b	8.9 b	8.2 b	1.92
	35	11.9 a	12.0 a	9.8 a	10.7 a	11.7 a	12.1 a	11.1 a	2.55
Root fresh weight (g)	20	1.9 b	1.9 b	2.2 b	2.3 b	0.6 b	2.0 b	0.8 b	0.80
	35	3.9 a	4.1 a	3.9 a	3.5 a	4.2 a	3.6 a	3.8 a	1.33
Shoot fresh weight (g)	20	1.1 b	1.2 b	1.1 b	1.1 b	0.8 b	1.2 b	1.3 b	0.31
	35	2.5 a	3.0 a	2.3 a	2.1 a	2.9 a	2.4 a	3.8 a	0.80
Root dry weight (g)	20	0.12 b	0.11 b	0.13 b	0.12 b	0.09 b	0.14 b	0.10 b	0.04
	35	0.26 a	0.29 a	0.28 a	0.26 a	0.34 a	0.30 a	0.37 a	0.08
Shoot dry weight (g)	20	0.17 b	0.17 b	0.18 b	0.17 b	0.14 b	0.19 b	0.23 b	0.05
	35	0.49 a	0.48 a	0.39 a	0.40 a	0.55 a	0.48 a	0.77 a	0.14
Nodules number (units)	20	0.0 b	0.0 b	0.0 b	0.0 a	0.0 b	0.0 b	0.0 b	0.0
	35	0.5 a	2.5 a	1.17 a	0.16 a	5.83 a	2.66 a	7.3 a	7.10
Nodule diameter (mm)	20	0.0 b	0.0 b	0.0 b	0.0 a	0.0 b	0.0 b	0.0 b	0.0
	35	1.22 a	0.47 a	0.14 a	0.50 a	2.59 a	1.56 a	1.59 a	1.52

[ϕ] Day after sowing; At 20 DAS n=6, at 35 DAS n=6.

[μ] Ti3=TiO_2 3gL^{-1}, Ti6=TiO_2 6 gL^{-1}, Zn3=ZnO 3gL^{-1}, Zn6=ZnO 6gL^{-1}, Fe3=Fe_2O_3 3gL^{-1}, Fe6= Fe_2O_3 6gL^{-1}

[Ω] Minimum significant difference,

[†] Values with the same letter, *i.e.* within the rows, are not significantly different between the treatments (Tukey α=0.05).

Conclusions

The significant effects of NPs on the morphology of soybean plants depended on the response variable, i.e. root and shoot length, fresh and dry weights of root and shoot, as well as the exposure time that the plants were exposed to NPs, while different morphological behaviors were observed with respect to the above. According to the morphological variables of soybean plants, it was noted that the main effects occurred at 35 DAS. Therefore, it is confirmed that NPs have different behaviors once these come into contact with biological systems, so it is suggested to carry out additional studies in order to evaluate the potential effects of NPs in crops of high economic and social value, and furthermore explore the RLS processes in the presence of NPs to have a clear idea of the behavior of NPs in this biological systems of great importance such as the BNF.

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Identification of native antagonistic fungi against *Bipolaris sorokiniana,* causal agent of Spot blotch in Wheat, isolated in the Yaqui Valley, Sonora

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Abstract

Wheat is one of the most important crop worldwide. Unfortunately, several factors reduce its yield, such as high temperatures, pests and diseases. Spot blotch, caused by *Bipolaris sorokiniana*, is an important plant disease in humid field environments, with relative humidity above 90%. The aim of the present work was the identification of potential native fungi with antagonistic traits against *B. sorokiniana*. Fifty-nine fungal strains were isolated from wheat in the Yaqui Valley (this region contributes about 50% of the wheat national production). These strains were evaluated against *B. sorokiniana,* by dual culture technique (9). Two *Trichoderma harzianum* strains (TSM39 and TSO39) were the best antagonist showing a *B. sorokiniana* growth inhibition values > 80%, by mycoparasitism. These BCA strains represent a promising sustainable alternative for controlling this disease in the Yaqui Valley.

Keywords ● Biocontrol ● *B. sorokiniana* ● Spot blotch

Introduction

Wheat is one of the most important crops around the world, due to its protein concentration (Mehta *et al.* 2014). Mexico has a wheat production of $3.78x10^6$ tons per year (Wheat Atlas. 2016), where the State of Sonora contributes about 50% of this production, being the Yaqui Valley the major producer in this State (Cortés *et al*. 2011; SAGARPA. 2016). Unfortunately, several external biotic and abiotic factors negatively affect its global production, such as high temperatures, salinity, pests and diseases (Anderson *et al.* 2004). Thus, plant pathogens have a significant negative impact on the production of this cereal, due to wide distribution (Mehta *et al.* 2014). For example, the development and severity of fungi diseases are related to climatic conditions presented in the field, *i.e.* the temperature and the atmospheric humidity (Manamgoda *et al*. 2011). Spot blotch caused by *Bipolaris sorokiniana*, diminish the wheat production in humid environments, mainly in countries as India, Brazil, and China (Fuentes *et al*. 2009). Villa *et al*. (2016) reported the first symptoms of Spot blotch in wheat plant growing in the Yaqui Valley, Sonora during the 2014-2015 crop season, which showed a yield reduction of 1.9 ton-ha^{-1}, being associated to a significant temperature (2.1°C) and relative humidity (3.2%) increase (Felix and Fuentes. 2015). These conditions promoted the development of *B. sorokiniana*, which has been reported as a semi-biotrophic fungus with worldwide distribution affecting small grain cereals (especially wheat and barley), grasses and others (Chowdhury *et al*. 2013).

Actually, the agricultural management for plant diseases has been focused on the chemical control (tebuconazol and epoxiconazol), having a good inhibition of plant pathogens. However, the application of these fungicides is highly associated with numerous negative environmental effects: a) toxicity, b) pesticide residually, c) resistance of the pathogen, and d) soil and water contamination (Chowdhury *et al*. 2008; Usta. 2013). Thereby, it necessary to develop eco-friendly alternatives for controlling these plant diseases, diminishing the negative impact caused by the use of traditional practices. The use of microorganisms as biological control agent (BCA) is a promising alternative due its effectiveness against plant pathogens. However, this strategy is still unexplored on wheat production in the Yaqui Valley. The aim of this work was to identify promising native fungi with antagonistic trails against *B. sorokiniana*, causal agent of Spot blotch of wheat in the Yaqui Valley.

Materials and methods

Study site. The study site was located at the Yaqui Valley, Sonora, Mexico (108° 53' y 110° 37' west longitude and 26° 53' y 28° 37' north latitude), during 2013-2014 season having spring wheat cv. CIRNO C2008, where spot blotch symptoms were observed.

Microorganisms used in this study. Pathogen strains: Four pure cultures of *B. sorokiniana* (Villa *et al.* 2016) were obtained from the "Colección de Microorganismos Edáficos y Endófitos Nativos" COLMENA (www.itson.edu.mx/colmena). These cultures were transferred into Potato Dextrose Agar (PDA), and were incubated at 28 ± 2ºC for 10 days.

Microorganisms isolation: 10 grams of soil were placed in 90 mL of sterile distilled water and homogenized at 200 rpm for 1 h, then serial dilutions (1:10) method until 10^{-4} was used (Cordova-Bautista *et al.* 2009). Later, 0.1 mL of these dilutions was inoculated on Petri dishes containing PDA, supplemented with 80 ppm of naxilidic acid. The inoculated Petri dishes were incubated for 48 h at 28°C ± 2 °C.

Confrontation assays in vitro. Fifty-nine fungi strains soil were evaluated against *B. sorokiniana* by dual culture technique on Petri dishes containing PDA as culture medium (de los Santos *et al.* 2013). $1x10^3$ spores of each strain of *B. sorokiniana* were inoculated separately at the center of the Petri dishes, having a distance of 3 cm from the potential fungal BCA ($1x10^3$ spores). All Petri dishes inoculated were incubated at 28 ± 2ºC for 6 days. The percent of growth inhibition of *B. sorokiniana* by the BCA was measurement according to Sibounnavong *et al.* (2009).

Molecular identification. DNA from each isolates showing biocontrol against all strains of *B. sorokiniana* was extracted according to Reader and Broda (1985). The 5.8s rRNA gene was amplified using the primers ITS1F: (5´ -TCCGTAGGTGAACCTGCGG- 3´) and ITS4R: (5´-TCCTCCGCTTATTGATATGC- 3´). The Polymerase Chain Reaction (PCR) conditions had an initial denaturation step at 95 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and a final elongation step of 10 min at 72 °C. PCR products were verified by 2% agarose gel electrophoresis, purified and sequenced by Sanger platform. Sequences were compared and deposited in the NCBI Genbank. ITS1-ITS4 rRNA gene sequences of isolates were aligned with the software Clustal W and analyzed by the Neighbour-joining method, using MEGA. ITS1-ITS4 rRNA of *Saccharomyces cerevisiae* (AB180469.1) was used as outgroup. Stability of clades was assessed with 1000 bootstrap replications.

Statistics analysis. Data were analyzed by ANOVA analysis of variance, using Fisher LSD test (P= 0.5), using the Statgraphics Plus 5.1 software.

Results and discussion

Fifty-nine strains of fungal strains associated to wheat were isolated from soil samples collected in the Yaqui Valley. However, only nine of those (15%), showed antagonistic activity against all four strains of *B. sorokiniana* studied in this work. The percentage of growth inhibition ranges were between 60% - 98% (Table 1), being the strain TSM39 the best BCA, which suppressed >80% the growth of *B. sorokiniana in vitro*. In addition, the strain TSO39 was the second best antagonist with inhibition values between 69.2-96.8%, showing mycoparasitism (Figure 1). On the other hand, the lowest growth inhibition was observed by strains TSO53 and TSO52 showing values ranging between 55.8-82.8% (Table 1). The behavior of the antagonistic strains suggests the production of diffusible metabolites as biocontrol molecules, such as lytic enzymes, toxic metabolites, and antibiotics (Figure 1) (Duffy *et al.* 2003).

Table 1. Inhibition percent of *B. sorokiniana* by dual confrontation on PDA after 6 days

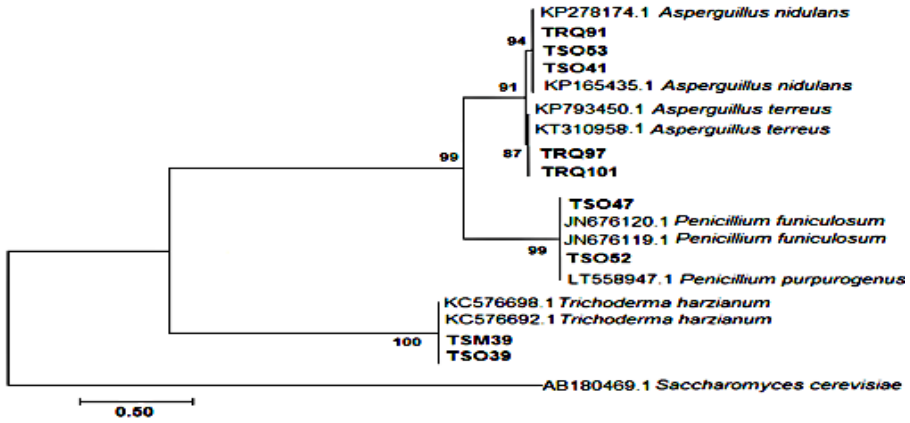
	% inhibition of *B. sorokiniana*								
	TSM39	**TSO39**	**TSO53**	**TSO41**	**TRQ91**	**TRQ97**	**TRQ101**	**TSO47**	**TSO52**
TPQ1	80.8 b	69.2 c	55.8 c	65.4 b	61.5 c	67.3 c	67.3 c	53.9 c	55.8 b
TPQ6	96.9 a	96.9 a	79.7 a	76.6 a	93.8 a	93.7 a	90.6 a	79.7 a	82.8 a

Mean values with same letters (column) are not significantly different as ANOVA test (P=0.05).

TSM39 vs TPQ1 TRQ101 vs TPQ6 Control TPQ1 Control TPQ6

Figure 1. Confrontation assays against *B. sorokiniana*, and control strains of *B. sorokiniana*.

The molecular characterization of promising BCA against *B.* sorokiniana were carried out by ampliflying the ITS1 gene using PCR. The strains TSM39 and TSO39 showed a 100% of similarity to *Trichoderma harzianum,* the strains TRQ91, TSO53 and TSO41 had similarity to *Aspergillus nidulans,* the strains TRQ47 and TRQ101 to *Aspergillus terreus,* and the strains TSO47 and TSO52 to *Penicillium funiculosum* and *Penicillium purpurogenus*, respectively (Figure 2). Several strains of *T. harzianum* have been evaluated against plant pathogens such as *B. sorokiniana*, and *Fusarium graminearum,* with inhibition ranges of 45.45% and 40%, respectively (Hasan, 2013). The result founded was in accordance with the tendency of *T. harzianum* to being mycoparasitic to pathogens, and its mechanisms are the production of lytic enzymes, degrading cell walls of pathogens, thus, they are recognized for not being selective to plant pathogens (Infante *et al.* 2009).



Phylogenetic tree was recovered from the neighbor-joining analysis of the nuclear ITS rRNA sequence data. Saccharomyces cerevisiae was used as an outgroup taxon, The numbers given over selected branches indicated the percentage of 1000 bootstraps resampled data sets, and given only for values >87%.

Figure 2. Phylogenetic relationships of antagonistic fungi isolates.

In contrast to *Trichoderma* spp, the biological control of *Aspergillus* spp and *Penicillium* spp has been evaluated against *Sclerotinia sclerotiorum* and *Fusarium oxysporum* by several disease-suppressive mechanisms such as antibiosis, metabolites, competition, and mycoparasitism. In case of *Aspergillus* spp showed a particular selectivity through some plant pathogens. However, some *Aspergillus* spp have an impact on agricultural practices to produce several mycotoxins (aflatoxins, sterigmatocystin, and ochratoxins) characterized for causing carcinogenic effects (Quiroz *et al*. 2008; Yang *et al*. 2008; Gautam and Bhadauria. 2012; Barocio *et al.* 2013; Hu *et al.* 2013; Radhakrishnan *et al.* 2014).

Conclusions

The species *T. harzianum, A. nidulans, A. terreous, P. purpurogenus*, and *P. funiculosum* present strongly inhibition of radial growth of *B. sorokiniana,* with inhibition values between 53.85- 96.88% by different suppressive biological activity as mycoparasitism and production of metabolites and antibiotics. *B. sorokiniana*, causal agent of Spot blotch has become a severity disease mostly associated in warm regions with high temperature and relative humidity, as the Yaqui Valley. Thus, new sustainable alternatives for plan disease management that includes native fungi and their ecological role impact should be explored in further investigations as promising biological control agents in the Yaqui Valley.

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Evaluation of consortia microalgae-bacteria for the treatment of municipal wastewater effluents

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Abstract

In this investigation it was studied the nutrients removal of NH_3-N, NO_3-N and PO_4-P from municipal wastewater using artificial consortia of *Chlorella vulgaris*, *Bacillus cereus* and *Pseudomonas putida*. A wastewater model was prepared simulating the physicochemical characteristics determinate at the wastewater plant in Chapala Jalisco, México. In the experiments we added 900 mL of wastewater model and 100 mL of the corresponding inoculum of the consortium. The nutrients removal with the consortium of *Chlorella vulgaris-Bacillus cereus-Pseudomonas putida* were 23.8% of NO_3-N, 2.5% of NH_3-N and 9.6% PO_4-P. With the consortium of *Chlorella vulgaris-Bacillus cereus* the results were 40% of NO_3-N, 22.9% of NH_3-N and 9.4% of PO_4-P. The removals with the consortium of *Chlorella vulgaris-Pseudomonas putida* were 8.7% of NO_3-N, 10.2% of NH_3-N and 8.8% of PO_4-P. The highest biomass production was found with the consortium of *Chlorella vulgaris-Pseudomonas putida* (227 mg L^{-1}) followed by the consortium of *Chlorella vulgaris-Bacillus cereus-Pseudomonas putida* (205 mg L^{-1}) and *Chlorella vulgaris-Bacillus cereus* with (66.3 mg L^{-1}). The present study highlighted the utility of these microorganisms for wastewater treatment and the promise of sewage as a growth for biomass production.

Keywords ●Wastewater treatment, ● Nutrient removal, ● Consortia, ●Biomass production.

Introduction

Environmental issues are becoming profound problems because of the possible risk of pollution of ecosystems due to the increasing population, urbanization and industrialization. One of the most important matters, includes the excessive production of wastewater. The liberation of this wastewater without the appropriate treatment into freshwater resource is giving serious environmental problems. For these and others problems the scientific communities are studying how to improve the sustainability of our planet. Therefore, the mixing of untreated wastewater in aquatic bodies is one of the major issues that are changing the stability of ecosystems (Renuka *et al*. 2013). Further, the presence of excess of nutrients, as Nitrogen and Phosphorus in untreated wastewater, causes the growth of aquatic plants, the lack of oxygen causing the death of aquatic animals, leading to a total degradation of the water bodies (Khan and Ansari, 2005). These practices also cause negative effects, such as; an increment of consumption of dissolved oxygen, and the contamination by toxic chemicals. The increases of eutrophication by the large amounts of Phosphorus and Nitrogen, result in the degradation of aquatic ecosystems for the deterioration of water quality and its potential

danger to human health. The above is due to the fact that the nitrate in the gastrointestinal tract can be reduced to ions of nitrite (Wang *et al*. 2010). In Mexico, during the year of 2010, it was generated 3,380, 326 m^3 s^{-1} of wastewaters; from this amount only 208 m^3 s^{-1} were collected. México has 2,186 wastewater treatment plants, but it was only giving a treatment to 93.6m^3 s^{-1} of wastewaters produced. However, in the last 7 years the volume of treated water has been tripled to 61.7% (INEGI. 2010). In the natural aquatic environment, microalgae always coexist with bacteria. The interactions between microalgae and bacteria are many (Cole, 1982). Combined systems of algae-bacteria can take up Nitrogen and Phosphate for growth (He *et al*. 2009), improving the water quality (Gonzalez *et al*. 1997). The bacteria can break down organic matter and assimilate phosphorous using the O_2 produced by photosynthesis of algae (Munoz and Guieysse, 2006). The Algae release a variety of organic substances, composing of proteins, lipids and nucleic acids; all these molecules serve as substrates for the bacterial growth (Abed *et al*. 2007). Previous reports mentioned that the interaction of the consortium *Chlorella vulgaris*/*Bacillus cereus*/*Pseudomonas putida* increases the growth of the microalgae (Qu *et al*. 2014). This study was conducted to evaluate the potential of the artificial consortia of *Chlorella vulgaris*, *Pseudomonas putida*, and *Bacillus cereus* in nutrients removal from municipal wastewater, to try to find an alternative method to the improvement of water and its quality. Thus, the aim of this work is to study the capacity of these microorganisms to remove NO_3-N, NH_3-N and PO_4-P in municipal wastewater.

Material and Methods

Wastewater collection and analyses. The wastewater was collected from a treatment plant located at Chapala, Jalisco, Mexico. Physicochemical parameters such as alkalinity, carbonate, hardness, dissolved oxygen (DO), biochemical oxygen demand (BOD), concentration of NO_3-N, NH_3-N and PO_4-P, were determined using the Standard Methods for Water and Wastewater (APHA, 1998). Once the characterization of wastewater (Table 1), was carried out a model of wastewater with the same characteristics was formulated.

Table 1. Physicochemical characteristics of municipal wastewater.

Municipal wastewater parameters		
	Standard Methods	
Determinations	**APHA 1998**	**Concentration**
pH	4500-H^+ B	7.7
Hardness	2340 C	293 mg $CaCO_3$ L^{-1}
Alkalinity	2320 B	293.32 mg $CaCO_3L^{-1}$
$CaCO_3$	2330 B	0.0069 mg HCO_3 L^{-1}
DBO	5210 B	3.2 mg L^{-1}
Total chlorine	4500-Cl-G	140.98 mg L^{-1}
NO_3-N	4500-NO_3- B	5.54 mg L^{-1}
NH_3-N	4500-NH_3- C	20.72 mg L^{-1}
PO_4-P	4500-P-C	2.66 mg L^{-1}

Cell cultures. The organisms used were *Chlorella vulgaris* ATCC (UTEX 29), *Pseudomonas putida* (ATCC 700007) and *Bacillus cereus* (ATCC 9592), the microorganism were obtained from The Global Bioresource Center, USA. For *Chlorella vulgaris,* it was inoculated in a Liquid Bold's Basal Medium (BBM) and for the bacteria they were inoculated in tryptone soy broth. Both cell cultures were carried out at 25 °C during 21 days.

Experimental setup. The inocula were adjusted to 0.5 Optical Density (OD) at 600 nm for *Chlorella vulgaris, Pseudomonas putida* and *Bacillus cereus*. However, the preparation of the inoculum for the consortium was prepared with a ratio of 3:1 of optical density (Zhi and Yen, 2014) The culture of *Chlorella vulgaris* has an optical density of 0.5, *Pseudomonas putida* and *Bacillus cereus* have an optical density of 0.16 respectively. The experiments were carried out in 1L bottles, containing 900 mL of model wastewater and 100 mL of the corresponding consortium. The bottles were maintained at 25 °C with an agitation of 100 rpm and photoperiods of 12:12 light/dark. The systems were analyzed every 24 hours. Determination of the concentration of NO_3-N, NH_3-N and PO_4-P in the model wastewater were carried out, using the methods specified in Table 1 (APHA, 1998). The experiments were conducted by triplicate.

Biomass determination. The determination of biomass, was carried out with 6 ml of sample which was centrifuged at 14,000 rpm for 15 minutes and the pellets obtained were dried at 60 °C until a constant weight.

Results and discussion

Nutrients removal. Removal of nutrients from the model wastewater was carried out in three consortium systems, composed for *Chlorella vulgaris, Pseudomonas putida and Bacillus cereus*. In the Figure 1 shows the comparison of the different nutrients removal by the three consortium systems. The results of the accumulative removal of NO_3-N for the consortium in the model wastewater are show in the Figure 1a. The consortium with the microorganism *Chlorella vulgaris/Pseudomonas putida/Bacillus cereus* was able to remove the 40%(1.1026 to 0.6174 mg), the consortium of *Chlorella vulgaris/Pseudomonas putida* was able to remove the 8.7% (1.1026 to 1.007 mg) and the consortium of *Chlorella vulgaris/Bacillus cereus* was able to remove the 8.9% (1.1026 to 1.007 mg). *Chlorella vulgaris* was the dominant microorganism in the consortium and both bacteria gave the robustness to the systems (Ruiz *et al*. 2011). However, the consortium with *Pseudomonas putida* and *Bacillus cereus* were able to remove a small percent of the NO_3-N, this happens when there was not enough organic matter available in the medium and the metabolism of the bacteria decreases, since the medium was not supplemented with glucose, it causes that the bacteria systems make an adjustment for the maintaining energy (Shumiao *et al*. 2009). The results of the accumulative removal of NH_3-N are showed in the Figure 1b. The removal of NH_3-N in the model wastewater. The consortium *Chlorella vulgaris/Pseudomonas putida/Bacillus cereus* was able to remove the 2.5% (40.85 to 39.82 mg), *Chlorella vulgaris/Pseudomonas putida* remove the 10.2% (40.85 to 36.70 mg) and the consortium of *Chlorella vulgaris/Bacillus cereus* was able to remove the 22.9% (40.85 to 31.10 mg). Zhijie *et al*. (2013) found that the artificial consortium of *Chlorella* and *Bacillus* has an efficiency of 78% of NH_4 in the wastewater removal, which contained glucose like carbon source. However, in our study, we remove a small percent of the nutrient, because our wastewater model was not supplemented with glucose or glycerol. The consortium of *Chlorella* and *Pseudomonas* had already been investigated previously by Ghulam *et al*. (2015), they found in their study that the consortium of these two microorganism are apt for the removal of NH_3-N and are capable to remove a

bigger amount when the wastewater have a high content of organic matter. In our experiments this not happen owing that our wastewater it does not have organic matter. The results of the accumulative removal of PO_4-P are showed in the Figure 1c. The removal of PO_4-P by the consortium of *Chlorella vulgaris/Pseudomonas putida/Bacillus cereus* was able to remove the 9.6% (0.002359 a 0.0021336 mg), *Chlorella vulgaris/Pseudomonas putida,* remove the 8.7% (0.002359 a 0.002153 mg) and the consortium of *Chlorella vulgaris/Bacillus cereus* was able to remove the 9.4% (0.002359 a 0.002133 mg). In previous studies (Hernandez *et al*. 2009) have been found that the elimination of 92% of total phosphorus in wastewater, this in 168 hours of processing. We obtained a removal of the 9.36% of PO_4-P in the wastewater model (in which their nutrients were not increased). The elimination of the PO_4-P could be for the absorption and photosynthesis processes carried out by the microalgae in the system and which causes the removal of phosphate in the wastewater, whereas the removal of phosphate by the bacteria is due to the excess of ingestion of the nutrient (Hernandez *et al*. 2009). Ghulam *et al*. (2015) studied the artificial consortium of *Chlorella* and *Pseudomonas* in the removal of total Phosphorus, nevertheless they did not specify the percentage of removal that could be reached with this.

Biomass production. Biomass was monitored as dry weight (Figure 1d), for the consortium of *Chlorella vulgaris*/*Pseudomonas putida/Bacillus cereus*, *Chlorella vulgaris/Pseudomonas putida* and *Chlorella vulgaris/Bacillus cereus*. The consortia were able to produce a maximum of concentration of biomass. The first one was able to produce 205 mg L^{-1}, the second consortium produce 227 mg/L and the last one was able to produce 66.3 mg L^{-1}. These results can show that the entire consortium used in this study, are capable to growth in the model wastewater and they are efficient in the removal NO_3-N, NH_3-N and PO_4-P, without any external source of nutrients.

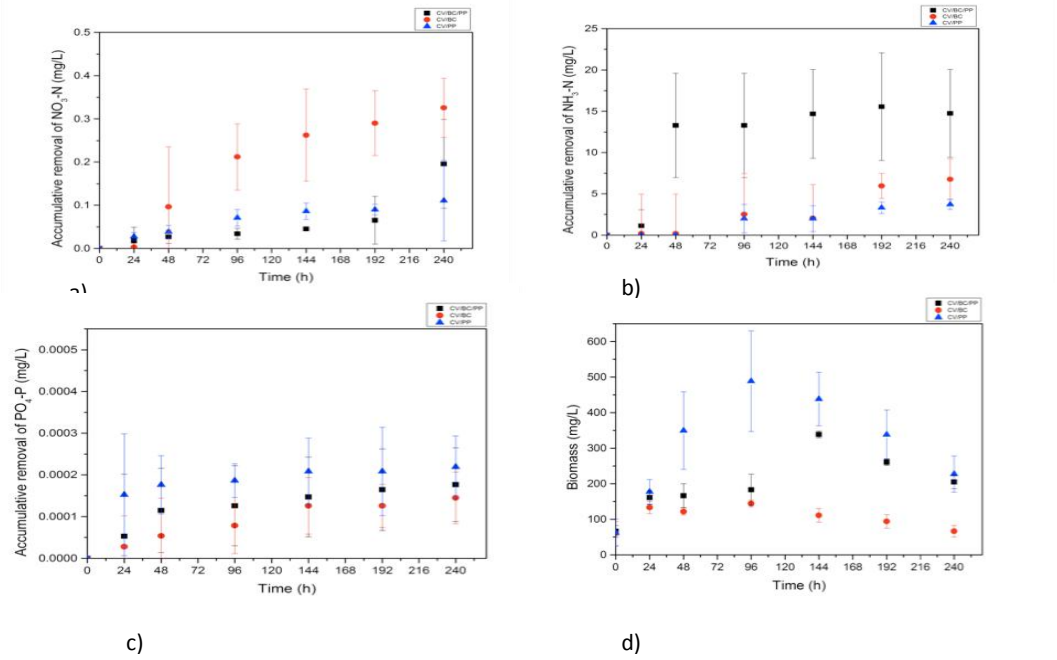

Figure 1. Accumulative removal of nutrients in model wastewater with artificial consortium. a) accumulative removal of NO3-N, b) accumulative removal of NO3-N, c) accumulative removal of PO4-P, d) biomass production.

Conclusions

Based on the analysis of removal of NH_3-N, NO_3-N and PO_4-P in municipal wastewater treated model, it was found those microalgae-bacteria consortia are suitable for the removal of these nutrients and simultaneously the production of biomass. For the consortia studied *Chlorella vulgaris/ Bacillus cereus / Pseudomonas putida*, *Chlorella vulgaris/ Bacillus cereus* and *Chlorella vulgaris/ Pseudomonas putida*, the second consortium is the greater removal for NH_3-N (23.85%) and NO_3-N (40%), however in the removal of PO_4-P the consortium with a higher percentage was *C. vulgaris / B. cereus/ Pseudomonas putida*. For biomass production, the consortium of *Chlorella vulgaris*/*Pseudomonas putida* was the one that produced the highest amount of this (227 mg L^{-1}). With the results presented, we can recommend the use of the consortium *Chlorella vulgaris / Bacillus cereus*, when the concentration of nitrogen increased and the Phosphate concentration are lower in this wastewater. However, if in these waters would find a high concentration Phosphate, then we recommend to use the consortium of Chlorella vulgaris / Bacillus cereus / Pseudomonas putida. It is very important to note that these treatments need not add glucose or other external.

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Study of the action mode of *Wickerhamomyces anomalus* against *Colletotrichum gloeosporioides*

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Abstract

Colletotrichum gloeosporioides is the causal agent of anthracnose in fruits and vegetables representing a global problem. However, the use of biocontrol agents has proved effective against these diseases. In this work the antifungal activity of *Wickerhamomyces anomalus* isolated from contaminated avocados against *Colletotrichum gloeosporioides* was evaluated. The yeast *W. anomalus* could reduce the growth of *C. gloeosporioides* in plate and by volatile compounds. The conidia germination and mycelia growth occurs from a 1:1 ratio of the pathogen/antagonist. By SEM the damage caused by *W. anomalus* to the mycelium structure was evidenced. The antagonist cells showed adhesion to the mycelium by a polysaccharide biofilm. The presence of mycelium stimulates the hydrolytic enzyme production with maximal activity of chitinases at 24 h and 60 hours for glucanases. These results show that *W. anomalus* use together different mechanisms to express its antifungal action against *C. gloeosporioides*. This study reports for the first time, the results with a phytopathogen isolated from avocado fruits, which represents an opportunity to establish biocontrol into this agricultural product.

Keywords: ●*Colletotrichum gloeosporioides*, ●*Wickerhamomyces anomalus*, ●Biocontrol, ●Avocado

Introduction

Phytopathogen microorganisms that cause decay in fruit and vegetables with commercial value represent a global problem, since the percentage loss of these products its about 20% to 35%. (Abano *et al.* 2013). The disease known as anthracnose, caused by *Colletotrichum* gender affects fruits, vegetables, legumes and ornamental plants (Ashwiniy and Srividya, 2013). These diseases are usually controlled with the use of chemical fungicides (Nelson, 2008, Garbanzo, 2011). Direct application to food is considered dangerous to human health and a solution to this has encouraged the search for safer and friendly alternatives to the environment, to stop or reduce post-harvest decay (Kwasiborski *et al.* 2014; Ashwini and Srividya 2013). An effective alternative is the use of biocontrol agents, which involves the participation of antagonistic microorganisms applied directly to the fruits, which compete directly with the phytopathogenic microorganisms to prevent establishment and growth (Haissam, 2011). A yeast with a history of biocontrol in various fruits is *Wickerhamomyces anomalus* (*Pichia anomala*) due to its antagonistic effect against fungal pathogens (Haissam, 2011). This study reports for the first time, the results with a phytopathogen isolated from avocado fruits, which represents an opportunity to establish biocontrol into this agricultural product.

Materials and methods

Biological material: An isolate of *C. gloeosporioides* from infected avocados collected in the field was used. The yeast *W. anomalus* was obtained in the laboratory of Phytopathology of CEPROBI-IPN and was provided for this study by Dr. Ana Niurka Hernandez Lauzardo. *W. anomalus* strain is registered in the database of the NCBI as KP238319.

Antagonistic capacity: Antagonism was evidenced in Petri dishes with YMA medium (Yeast-Malt Agar) or PDA (Potato Dextrose Agar) with both microorganisms in different ends of the dish.

Growth inhibition of C. gloeosporioides by volatile compounds generated by W. anomalus: A double chamber composed my two Petri dishes was made according to the methodology by Vero *et al*. (2012) containing *C. gloeosporioides* on top and *W. anomalus* at the bottom of the chamber.

Inhibition of conidial germination of C. gloeosporioides by W. anomalus: Flasks were inoculated with PDB medium containing different number of cells of *W. anomalus*. Inoculating 1OD unit ($5x10^5$ cells) and making serial dilutions (1:10). Conidia of *C. gloeosporioides* was inoculated into relationships 1/1, 10/1, 100/1 and vice versa. Cultures were maintained under stirring for 72 h.

Scanning electron microscopy of the W. anomalus interaction with the phytopathogen C. gloeosporioides: mixed cultures were performed with a 1:1 ratio pathogen / antagonist. Aliquots were taken at 0, 24, 48 and 72 h. The samples were processed with a standard procedure to observe in scanning microscopy.

Evaluation of the production of hydrolytic enzymes, chitinase and glucanase from W. anomalus: The evaluation was made according to the methodology of Tayel *et al.* (2013). The activities of the β-1,3-glucanase and exo-chitinase were measured by the DNS method using laminarin as substrate for the activities of the exo-β-1,3-glucanase, and N-acetyl glucosamine for exo-chitinases. Incubating *W. anomalus* (1 UDO) as a control, *W. anomalus* with conidia of *C. gloeosporioides* and *W. anomalus* with mycelium from *C. gloeosporioides* grown for 72 h previously. Determinations were performed in the supernatant. For chitinase activity, 1 U of activity was defined as the amount of enzyme required to liberate 1µmol of N-acetyl-β-D-glucosamine per minute per mL from 1% solution of colloidal chitin. And a unit of β-1,3-glucanase was defined as the amount of enzyme which liberates 1 µg of glucose equivalent per minute per mL of enzyme solution.

Determination of biofilm formation by W. anomalus: It was performed according to the methodology of Vero *et al*. (2012). Using a 96-well plate containing 200 µL PDB medium, inoculating *W. anomalus* for 48 h. Subsequently the wells were emptied; they rinsed with distilled water and were dried. The biofilm was stained with a crystal violet solution 1%, wells were rinsed and dried. The stained biofilm was diluted with ethanol and the absorbance at 600 nm was measured in an ELISA reader. Biofilm formation was considered positive if the absorbance of the wells was greater than the mean of the negative controls plus three standard deviations.

Results and discussion

Antagonism tests showed a significant reduction (65%) in average on PDA medium and showed no significant reduction in YMA medium. In our case, both media containing glucose as carbon source and nutrients difference given by potato dextrose and malt extract showed involvement in antagonistic activity. Jijakli and Lepoivre (1998) described the biocontrol exercised by *W. anomalus* is subject to the type of nutrients. The inhibition by volatile

compounds decreased 10% growth over the control, however, the identity of the volatiles that inhibited growth of the fungus was not yet determined. Volatile inhibition has been demonstrated previously in different antagonists (Vero *et al*. 2012, Druvefors *et al.* 2005). Coda *et al.* (2011) identified in *W. anomalus* the ethyl acetate and ethanol as volatile compounds that cause inhibition. *W. anomalus* didn't allow the conidia germination of *C. gloeosporioides* in any of the tested relationships. While in the control this germination and mycelial growth can be seen. *W. anomalus* presents antagonism against filamentous fungi such as *Penicillium expansum*, *Botrytis cinerea*, *Aspergillus flavus*, all fruit or vegetable pathogens, inhibiting the germination of conidia and mycelial growth (Jijakli and Lepoivre, 1997). Colonization of the hyphae of C*. gloeosporioides* by *W. anomalus*, causing structural damage and adhering directly to the hyphae was observed by SEM. This suggests that *W. anomalus* is using several mechanisms to cause damage to the wall of *C. gloeosporioides*, such as the micoparasitism, degradation by extracellular enzymes and adhesion processes. Tayel *et al*. (2013) reported damage on hyphae, micoparasitism and finally complete lysis of *Aspergillus flavus* by *W. anomalus*. This effect may vary depending on the plant pathogen against which the yeast is facing. Chitinase activity (Figure 1) reached maximum activity only in the presence of mycelia with 21.4 U / mg at 24 h and 48 h minimum to near the 0.4 U / mg values. The presence of conidia of *C. gloeosporioides* did not stimulate the production of this enzyme, and the values obtained were similar to that provided little activity in the absence of antagonist phytopathogen. This suggests that chitinase is induced by the presence of chitin from the mycelium which its amount is greater than in conidia.

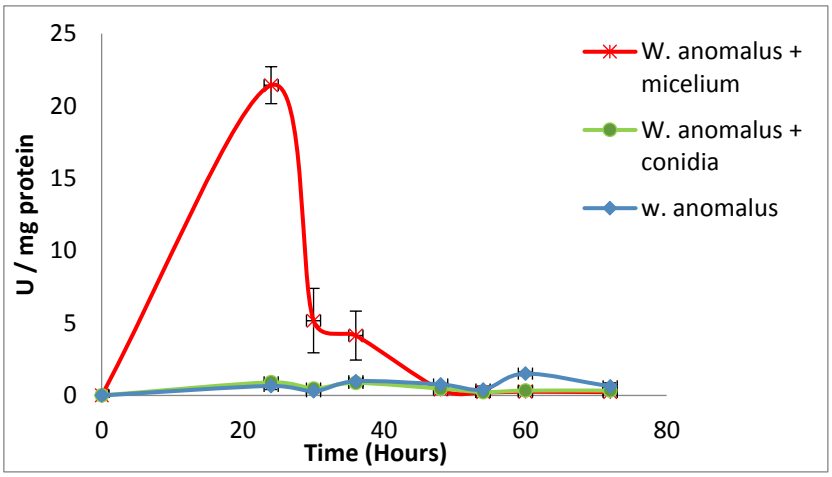

Figure 1. Specific activity of *W. anomalus* chitinases in presence of mycelium and conidia of *C. gloeosporioides*

Glucanase activity (Figure 2) presented 2 peaks of activity in the presence of mycelium , one at 30h with 6.5 U / mg and another at 60h with more than 10 U / mg. The presence of conidia produces the lower activity (6.4U / mg). Unlike chitinase, the antagonist produces glucanase in absence of the fungal stimulus. Similar results were found by Haissam and Lepoivre (1998), which found that the activity of glucanase from *W. anomalus* in the presence of cell wall fragments of *Botrytis cinerea* was increased in the same way to decay upto 72 hours. It has also been reported that the activities of *Pichia guilliermondii* are also higher when the

media are supplemented with the cell walls of phytopathogenic fungi (Chanchaichaovivat *et al*. 2008).

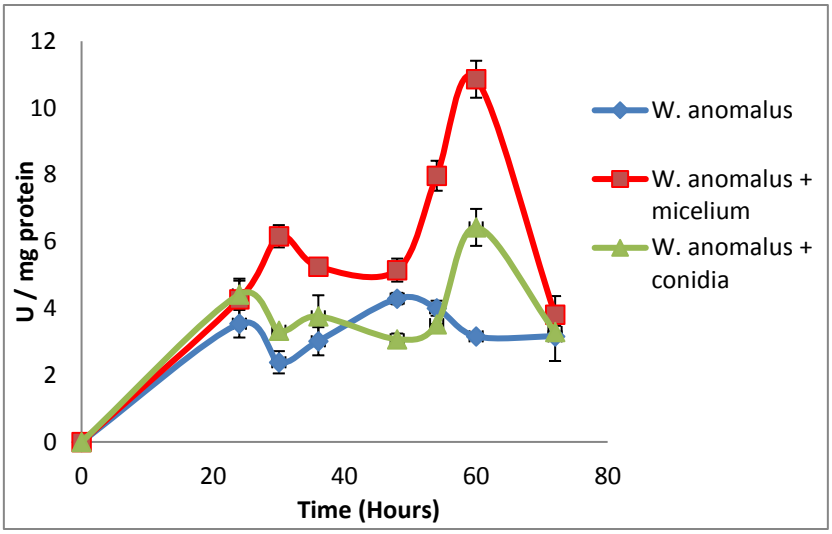

Figure 2. Specific activity of *W. anomalus* glucanases in presence of mycelium and conidia of *C. gloeosporioides*

The average value of biofilm formation was OD 0.3025 ± 0.0988 while the controls showed a value of OD 0.0979 ± 0.0292. Biofilm formation is considered an important attribute for antagonists, as it helps them to successfully colonize and protect both the wound and the intact fruit (Droby *et al.* 2009). These biofilms are also a mechanism that gives the yeast's ability to withstand stress and improve endurance (Vero *et al.* 2012). Biological control agents and their mechanism of action have aroused great interest in the last twenty years, to prevent post-harvest diseases of fruits and vegetables caused by pathogenic fungi. In this study we focus on the mechanism of action presented by the antagonist yeast *W. anomalus* against *C. gloeosporioides* isolated from avocado, causes anthracnose. These fungus infections affect a lot of post-harvest crops, generating significant economic losses. Microorganisms as *Streptomyces sp* have shown antifungal activity for *C. gloeosporioides*, *Penicillium italicus* and *Fusarium oxysporum* among others (Chen *et al.* 2015). *Trichoderma* species, also exhibit antagonism toward some phytopathogen (Kumar *et al*. 2011). However, antagonistic yeasts are of the most promising because they do not produce other harmful molecules that could affect the stored products.

Conclusions

Regarding the results obtained it can be concluded that *W. anomalus* is an efficient antagonist to inhibit growth of *C. gloeosporioides* isolated from avocados. The presence of the pathogen stimulates the production of hydrolytic enzymes and biofilm that allow the development of the biocontrol effect. Glucanases and chitinases enzymes were needed to inhibit the germination of conidia and their production is an important factor for causing structurally damage to the hyphae of the phytopathogen. The biofilm allowed *W. anomalus* invading and parasitizing the mycelium of *C. gloeosporioides*. Indirectly, it can use its ability to produce volatile compounds and inhibit mycelial growth. The mechanisms used by *W.*

anomalus work together and complement each other to achieve biocontrol activity of *C. gloeosporioides*. Finally, this study reports for the first time, the results with a phytopathogen isolated from avocado, which represents an opportunity to establish biocontrol into this agricultural product.

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Organogenesis in vitro of *Jatropha curcas* variety AL JC01 obtained from a plantation in Sucilá, Yucatán

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Abstract
Jatropha curcas is a second-generation energy crop that produces up to 40% oil in its seeds and by a transesterification process it can to obtain quality biodiesel. Despite these desirable features, there is no a variety generate high seed production therefore makes it unprofitable; one of the tools that help keep pure genetic lines would be the in vitro culture of elite varieties, However, the low reproducibility of micropropagation protocols do a difficult task for this specie; until now it has obtained shoot induction variety ALJC01 using MS medium (Murashige and Skoog, 1962) supplemented with adenine sulfate, 6- (γ, γ-Dimethylallylamino) purine (2iP), indoleacetic acid (AIA), myo-inositol and thiamine; the shoot elongation was achieved on MS medium supplemented with benzylaminopurine (BAP), indoleacetic acid (IAA) and adenine sulfate.

Keywords: *Jatropha curcas*●Organogenesis●Micropropagation●*in vitro.*

Introduction

Jatropha curcas is a perennial oleaginous plant belonging to the family Euphorbiaceae, its importance in recent years is because the seeds have about 30 to 40% oil (Soares *et al*. 2016) and by transesterification can be obtained biodiesel high quality (Pan *et al*. 2016). This species has an impressive genetic diversity (Rincón *et al*. 2016; Pecina *et al*. 2011), which may partly explain the different responses of an explant of an accession morphogenesis *in vitro* culture (Rajore and Batra, 2005); Furthermore, studies *in vitro* culture, include supplementation of organic additives such as polyvinylpyrrolidone (PVP) and citric acid to the culture medium for indirect morphogenesis by establishing immature embryos (Varshney and Johnson, 2010) or sulfate adenine for direct organogenesis from nodes (Datta *et al*. 2007), somatic embryogenesis on leaf explants (Baran-Jha *et al*. 2007), including the addition of coconut water for cell culture suspension from leaf and hypocotyl (Soomro and Memon, 2007).due to this specie (registered in Mexico) has a productivity of 2.5 ton / ha, length approximately of 1.5 m and is tolerant to pests and diseases, the aim of this study was to develop a protocol for *in vitro* morphogenesis of *Jatropha curcas* variety ALJC01 for future research and agricultural applications.

Materials and Methods

Establishment. the seeds of *J. curcas* variety ALJC01 were harvested of a plantation in Sucilá, Yucatan, Mexico; seeds were surface sterilized with benzalkonium chloride (0.5%) and chloride by stirring with commercial detergent (1 mg / 100 mL) for 15 min, allowed to dry on paper towels for 30 min. Later the seed coat was removed and the embryos were disinfected in laminar flow cabinet; by immersion in a solution of Extran (5%) for 5 min, then

transferred to 70% ethanol for 1 min, followed by stirring in sodium hypochlorite (30%) for 15 min, and finally three rinses with sterile distilled water. Embryos disinfected were planted in Magenta with 40 mL of MS medium at 100% of its ionic strength which were incubated in the dark for 15 days, then transferred to photoperiod to 16/8 (light / dark) with 23 ± 2 ° C and 60% RH approximately 15 days.

Shoot induction: From seedlings *in vitro*, the cotyledon leaves were extracted and cut explants approximately 1cm^2, which, were placed in a MS medium in different percentages of its ionic strength (75, 100, 125 and 150 %); all treatments were added with adenine sulfate, 6-(γ, γ-Dimethylallylamino) purine (2iP), indoleacetic acid (IAA), myo-inositol and thiamine; each treatment had 20 replications, being one explant the experimental unit per bottle with 25mL of culture medium; the control was MS medium basal without plant growth regulators (PGRs) or organic additives. The experiment was performed threefold and were evaluated 40 days after the sowing, evaluated the percentage of explants lives and number of induced shoots.

Shoots elongation: Elongation of shoots was evaluated on MS medium with BAP, AIA and supplemented with different concentrations of adenine hemisulfate (271, 543, 814 and 1357 µM), the experiment were performed with 10 repetitions and the experimental unit was a fragment of calli with shoots induced (approximately 1 cm^2) placed in a flask with 25 mL of culture medium. After 30 days, the response rate and size of shoots was evaluated, the experiment was put in a growing room with 16/8 (light / dark) of photoperiod, 23 ± 2 °C and 60% of RH.

Results and discussion

Establishment of embryos was 100% pathogen-free; embryos germinated about 20 days of culture *in vitro* and their transfer to photoperiod improve the development and growth. For induction of shoots, in Figure 1 can be seen that the response rate was between 60 and 100% for treatments with 75 and 100% of the ionic strength of MS; in contrast, treatments at 125 and 150% of the ionic strength of MS, showed low response except treatment to 125% of the ionic strength of MS in the reply 3 which had a rate of approximately 60% response to induction.

The ANOVA indicated differences statistically significant, showed in Table 1 with the Tukey test where values with the same letters correspond to the treatments statistically equal.

Table 1. Average of shoots induced in leaf explants of cotyledon of *J. curcas* during 40 days of culture.

Treatment	Replica1	Replica 2	Replica 3
BG1	31.3±16.76^a	20.30±25.92^a	14.8±11.89^a
BG2	3.9±6.380^b	19.50±20.31^a	15.8±11.11^a
BG3	0.0±0.000^b	1.80±5.020^{ab}	14.9±11.91^a
BG4	2.1±6.640^b	0.00±0.000^b	0.0±00.00^b
T	0.0±0.000^b	0.00±0.000^b	0.0±00.00^b

Same literal is statistically equal according Tukey ($p < 0.05$).

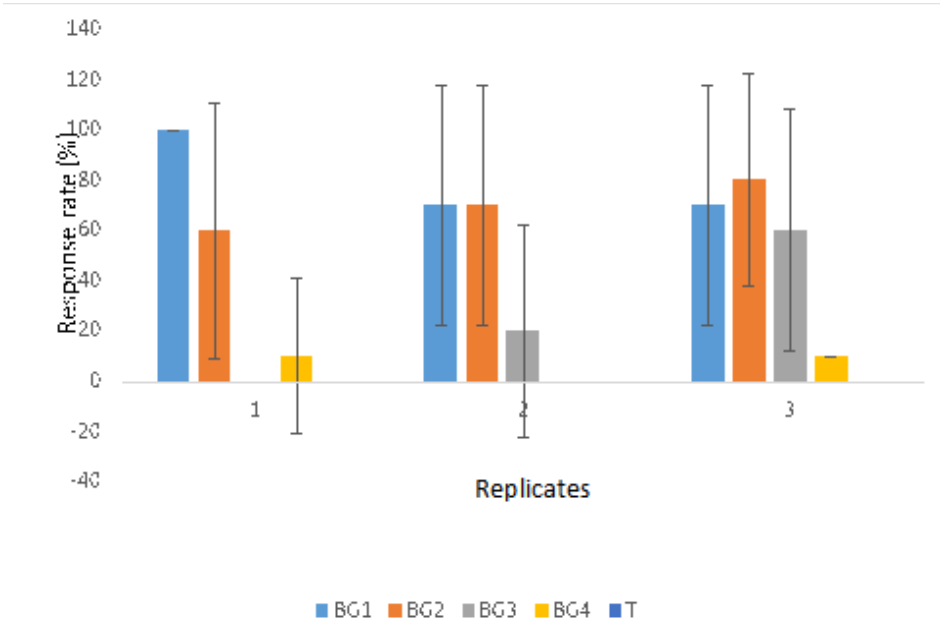

Figure 1. Response rate in explants of *Jatropha curcas* to induction medium of shoots.

Shoot induction is generated by various factors such as concentration of the medium, the type of explant and the application of plant PGRs, as to the salt concentration of the basal medium, several authors mention that the ideal medium is MS in 100% of its ionic strength like the investigation realized by Soares *et al*. (2016) when used a MS medium complete with the addition of BAP, IBA and GA_3 to acquire organogenic competence; similary, Kumar *et al.* (2010), obtained organogenesis direct from cotiledonar leaves with half full MS adding TDZ, kinetin (Kin), BAP and ANA, this shows the full MS medium is suitable for the development of shoots similar to the results obtained in this work, on the other side, the addition of auxine IBA and cytokinin BAP are required for *in vitro* morphogenesis in *Jatropha curcas* (Kumar *et al*. 2010); in our results, the use combined of auxin IAA and cytokinin 2ip were the PGRs that generated response, perhaps the application of the organic additive adenine sulfate improve the response of the explants.

For elongation, likewise the use of the organic additive adenine sulfate in combination of BAP more AIA (Figure 2), helped elongation (approximately 2 cm in length) of shoots induced at 40 days of culture.

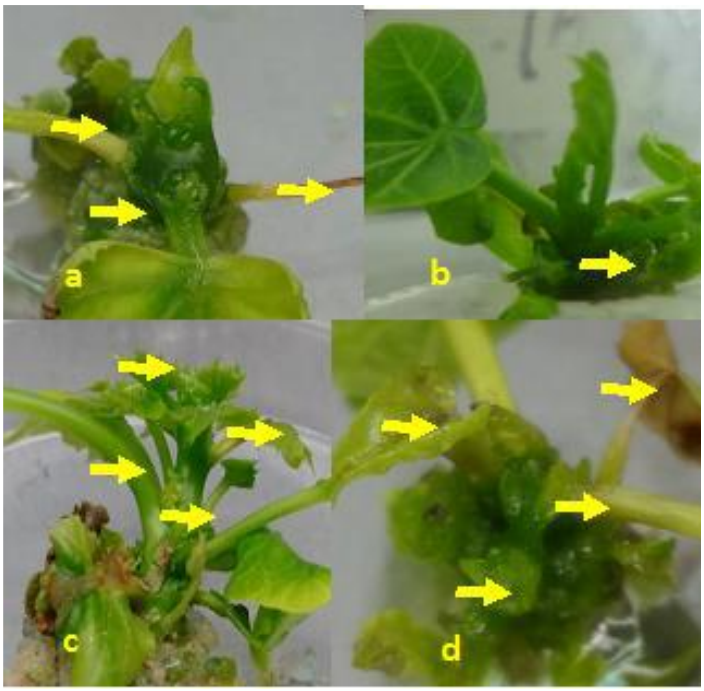

Figure 2. Shoots elongation with different concentrations of adenine sulfate during 40 days of culture; a) 271µM, b) 543µM, c) 814µM and d) 1357µM. Arrows in a) and d) indicate excessive growth of callus, necrosis and death in explant; in b) a growth excessive of callus and c) organogenesis of leaves and stem normal.

In the present work was observed that sulfate adenine is an organic additive that has an effect on the induction and elongation of shoots in micropropagation of *Jatropha curcas*, this effect, possibly due to the interaction of adenine sulfate with cytokinins that helps the growth and development of shoots (Nwankwo and Krikorian, 1983) Accordig to Murashige (1974) adenine sulfate can stimulate cell growth and enhance shoot formation, due that the adenine sulfate, acts like a natural precursor on synthesis of cytokinin (Khan *et al*., 2014), also, it provides an additional source of nitrogen to the cells, which can be absorbed more quickly as inorganic nitrogen (Husain, *et al*., 2006). this is corroborated by the work realized by Shrivastava and Banerjee (2008), when obtained the induction and development of shoots of *J. curcas* combining adenine sulfate BAP, 3-indole butyric acid (IBA) and other additives such as glutamine, L-arginine and citric acid.

Conclusions

A protocol for induction and elongation of shoots of *Jatropha curcas* variety ALJC01 was obtained on cotyledonary leaf explants; for induction is recommendable to use a concentration of MS medium in 75 or 100% of its ionic strength adding adenine sulfate, AIA, 2ip, myo-inositol and thiamine; for elongation it is advisable to add 814 µM of adenine sulfate to shoot elongation medium. It concluded that the MS medium in 100% of its ionic strength and the use of adenine sulfate as organic additive in the culture medium, helps to the induction and growth of shoots.

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Symbiotic interaction study between *Chlorella vulgaris and Pseudomonas aeruginosa* through the use of metabolic extreme pathways

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Abstract

Microalgae and bacteria can have a mutual benefit relationship named symbiosis, in order to survive in fluctuating environments lacking of a carbon source or nutrients. The symbiotic relationship between microalgae and bacteria has been widely studied in open and under controlled enclosed systems to discover fluxes among them that could explain a biomass increase in both microorganisms under a photoautotrophic condition. However, the relation between both microorganisms especially in term of metabolic behavior have not been studied thoroughly *in silico* way. In this project it has developed the genomic scale reconstruction (*in silico*) of the main co-culture metabolisms of the microalga *Chlorella vulgaris* and the bacteria *Pseudomonas aeruginosa.* By applying the extreme pathways metabolic engineering tool, we figured out that microalga provides an endogenous organic carbon source (maltose) come from the starch resulted from the active metabolism of Calvin-Benson cycle and also oxygen resulted from the photosynthesis; and the bacteria instead returns inorganic CO_2, product of its respiration. Finally, this work can serves as a template to know the metabolic capability of this co-culture for biotechnological approaches, for example predict production or removal of important metabolites before a previous experimental investigation step saving time and costs.

Keywords: Symbiotic ● co-culture ● *Chlorella vulgaris* ● *Pseudomonas aeruginosa* ● extreme pathways.

Introduction

Research projects related with the microalgae-bacteria interaction, agree with an increase in biomass growth of both microorganisms due to a co-culture between them under photoautotrophic conditions, rather than their separate metabolic activity in pure culture (Watanabe *et al*., 2005; Choix *et al*., 2012; Guo and Tong, 2013). In a natural aquatic process a microalgae always coexists with a bacteria, due to simultaneous interactions between them, in which bacteria benefit from microalgae exudates like oxygen and an endogenous carbon source, and microalgae growth is promoted by bacterial products such as inorganic carbon dioxide and others growth factors (Qu *et al*., 2014). The symbiotic relationship between microalgae and bacteria has been widely studied in open and under controlled enclosed systems to discover fluxes among them that explain a biomass increase in both microorganisms. Nevertheless, those studies even when they have obtained many

interesting results, they lack surely discussions due to the absence of experimental data or mostly information about how cells realize the adaptation of metabolic operation in response to changing nutrient availability or environmental factors, namely, the interaction mechanism (Heinemann and Sauer, 2010). Moreover, a co-culture has not been yet studied by means of metabolic engineering tools.

Currently, most of the molecular information from one specific microorganism is available from biological databases which collect it from high-throughput technologies describing the network components (genes, proteins, metabolites). From above, the availability of annotated genome sequences has allowed to reconstruct genome-scale biochemical reaction networks for microorganisms which then can be analyzed (Price *et al*., 2004) to determinate the biological range capabilities and their characteristics under governing constrains.

One specific *in silico* technique of metabolic engineering is called Extreme Pathways, which are vectors used to characterize the phenotypic potential of a defined metabolic network, the study of all possible metabolic active pathways in a stationary state under certain conditions (Bell and Palsson, 2005). Study the behavior of one microorganism is considerate a hard issue, and more difficult is to trace the conduct of a group of microorganisms like it happens in symbiosis, because of when there is a co-culture the performance of every single microorganism changes by shifting a feasible metabolic pathway to adapt to such conditions. Consequently, an ExPa can mark out which are the resulted active metabolisms due to varying conditions or in this case the constraints that could be imposed by the researcher. Therefore, all the combinations of possible reactions inside the metabolic network, the paths with better yields and the active enzymes are obtained by means of an Extreme Pathway Analysis *in silico* models.

In the present project it has been made the reconstruction of a metabolic model of a stoichiometric model that is able to simulate the central metabolism of *Chlorella vulgaris* and *Pseudomonas aeruginosa* to estimate the phenotypic potential and the growth of these microorganisms under certain conditions. Therefore, by means of the analysis of their metabolism it can be simulated, first, its growth under different culture conditions. Through this, we can get to know enzymes and metabolites that are keys of the built network for symbiosis interaction. Secondly, information about the energetic consumption from photons and the minimum requirement of inorganic carbon source under autotrophic conditions can be obtained, allowing the comparison between yields regarding other nutrients or microorganisms, instead of making experimental studies to know how carbon and minerals minimal requirements affects biomass yields or lipids content (Lohman et al., 2014; Li *et al*., 2015). Finally, this work can serves as a template to know the metabolic capability of this co-culture for biotechnological approaches, for example predict production or removal of important metabolites before a previous experimental investigation saving time and costs.

Materials and Methods

Metabolic network reconstruction. The first step was the obtaining of the stoichiometric reactions of the different metabolisms for *C. vulgaris* and *P. aeruginosa*. The electronic data bases used as supporting media for the construction of the model were BRENDA, NCBI, MetaCyc , KEGG and *Pseudomonas* database (Kanehisa, 2016) also, the information found in the referenced literature (Allen, 2002; Oberhardt et al., 2008). The metabolism included for microalgae were: photosynthesis, Calvin-Benson cycle, starch metabolism, glycolysis/gluconeogenesis, TCA cycle, fatty acids synthesis, triglycerides synthesis,

oxidative phosphorylation, pentose phosphate pathway, protein synthesis (18 amino acids), nucleic acids synthesis, carbohydrate synthesis, glycerophospholipids, chlorophyll synthesis (Chla and Chlb), transport reactions, maintenance and biomass formation. For *P. aeruginosa*: starch metabolism, glycolysis, TCA cycle, glyoxylate cycle, pentose phosphate pathway, oxidative phosphorylation, synthesis of acetic acid, amino acid synthesis, nucleic acid synthesis, peptidoglycan synthesis, synthesis of fatty acids and polyhydroxyalkanoates, biomass formation, transport and exchange reactions. After that all the stoichiometric reactions were ordered in a matrix system, denominated as stoichiometric matrix S, where every row m from S represents one metabolite and every column n is a reaction (Figure 1b). The last columns of the S matrix are the columns that determine the exchange fluxes of the system (20 exchange fluxes), meaning, the inlet and outlet metabolites of this metabolic network. Extreme Pathway Analysis. The calculated ExPas in the research work herein were obtained by means of an algorithm developed within a research group, based on the principles of analysis of ExPas and Metabolic Pathway Analysis by using MATLAB platform (The Mathworks, Inc., USA). From every ExPa obtained, substrate yield and energy source were calculated with respect to the biomass to find the best theoretical yields and the active reactions in those vectors

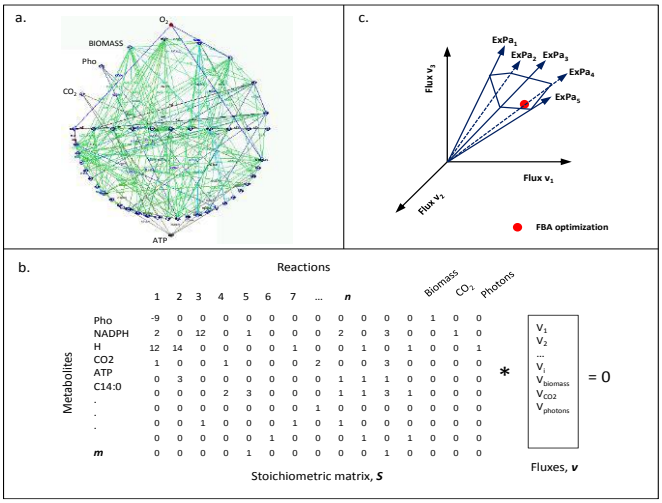

Figure 1. Schematization of the methodology used.

Results and discussion

The size of the matrix S was 296 x 306 (metabolites x reactions). From the 293 columns, the last 20 represented the exchange fluxes, where were included entries such as photons, glucose, phosphate, ammonia nitrogen, sulfate, magnesium, potassium, iron, calcium, zinc, copper and manganese; as well as outputs like biomass from each microorganism, polyhydroxyalkanoates production, carbon dioxide and oxygen.

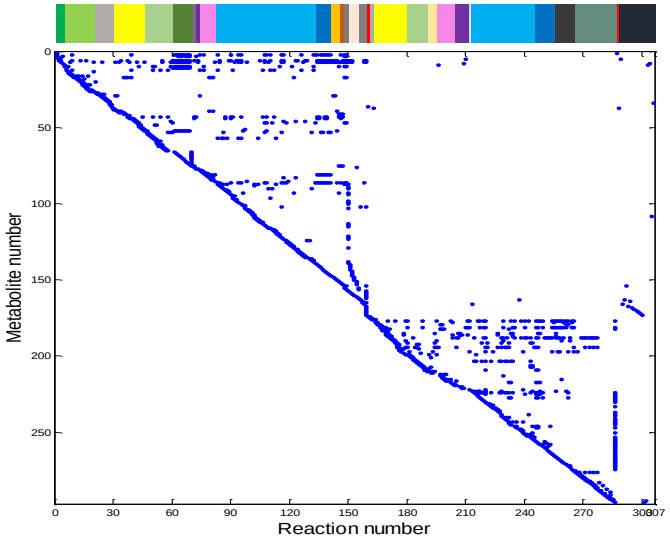

Figure 2. Connectivity in the metabolic network.

The resulted matrix S can be graphically represented (Figure 2) to notice which metabolites have greater participation in the network, or also which reactions include a greater number of those metabolites. Furthermore, in this representation of S it can be observed that the different metabolites are shown in a color scale, where the columns having the same one take part of a particular metabolism (see Figure 3 for color nomenclature). Therefore, from the microalga side ATP_Cv cofactor was the metabolite that shows greater connectivity, due to its participation in all biological reactions that require energy, followed by Pi_Cv. NADPH_Cv has greater connectivity than NADH_Cv, due to the synthesis of fatty acids, where large amounts of reducing power are consumed, as well as with ACCOA_Cv. In protein and nucleic acid synthesis ammonia is mainly consumed, followed by other intermediary metabolites such as OA_Cv, R5P_Cv and PG3_Cv. Finally, CO_2 and O_2 can intervene both as substrate as well as product, depending on the enzyme that is working or the type of growth that is being simulated. In the other hand, the reactions with more participation were protein synthesis reactions and the biomass formation in 147 and 273 for microalga and bacteria, respectively. Metabolic Pathway Analysis threw 2844 ExPas contained in a matrix (Pt) where the coefficients were the fluxes that fulfill the stoichiometry of the metabolic network, and also, which satisfy the requirements per definition of an ExPa. In figure 3, it can be observed which metabolism is more active in the majority of ExPAs. Thereby, Calvin-Benson and Photosynthesis were the most active metabolism. Unlike, polyhydroxyalkanoates (PHAs) synthesis was the metabolism with less participation due to the bacteria cannot produce PHAs because it rather uses the energy for maintenance and growing when there is no external carbon source.

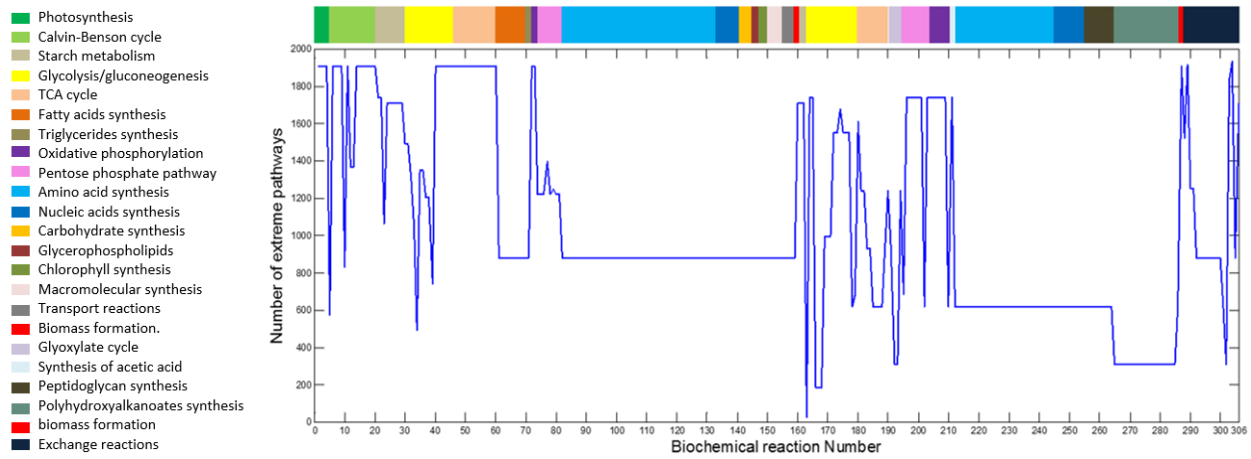

Figure 3. Biochemical reaction participation in extreme pathways.

From the whole set of ExPas obtained, 332 ExPas were selected based on which had zero in the external glucose (GLUext) flux exchange and an active entry of photons which represents a photoautotrophic condition, so that the carbon source for microalga would be only the carbon dioxide (CO2ext) that is producing the bacteria, and the organic carbon source for bacterial growth come from microalga metabolism. According to the concept of symbiosis there is a biomass growth of both microorganisms so from the last quantity, we rest the ones which had zero in the biomass flux exchange (BIO_Cv and BIO_P), and we obtained 96 ExPas. So that Expas represents the solutions set for a symbiotic condition. Also, the yields for desired metabolite with respect to biomass can be analyzed, in this case the yields for CO_2 fixed and O_2 generated in photosynthesis seem to be close to 39.8 mmol CO_2 g-1 DW and 59 mmol O_2 g-1 DW.

Conclusions

This project provides a fundamental approach to enhance our understanding of biological systems where microalgae and bacteria coexist like it happens for wastewater nutrient removal or the production of certain metabolite. The study of carbon flux can elucidate a symbiotic interaction between both microorganisms through the obtaining of theoretical yields or the analysis of active metabolism which explains what happens in a metabolic level. Further, the metabolic model will be validated in an experimental step using a laboratory scale reactor to link key experimental data with the exchange fluxes (*in silico* step) under a photoautotrophic scheme.

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Life cycle assessment for renewable energy generation systems

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Abstract

Life Cycle Assessment (LCA) is used to evaluate environmental impacts generated thorough the life cycle of a product (cradle to grave), which is from primary resources to consumption and the final disposal, in order to identify critical points in the process. The goal of this study was to evaluate two systems of bioenergy production using LCA, bioethanol production and bioelectricity production from non-food waste. The initial step determine input and output flows of each process for system integration. With this information the study objectives, scope of studio system, function and functional unit, which will be used for referred inventory analysis.

Keywords: Life Cycle Assessment • Biorefinery • Bioethanol • Bioelectricity.

Introduction

The global increasing on energy security and environmental degradation has allowed new research on sustainable and renewables alternatives, in order to reduce the use of fossil fuels (Adesanya et al., 2014). A promising alternative among renewable energy options had been the generation of energy from biomass.Biofuels and bioproducts produced from renewable resources could help to reduce the amount of fuels and also CO_2 production (Naik et al., 2010). For this reason, bio fuels and bioproducts are considered sustainable and renewable sources of energy. In this regard, another alternative is bioelectrical systems (BES), prominent among these microbial electrolysis cell (MEC) and the microbial fuel cell (MFC), these BES offer an excellent alternative for wastewater treatment and also the production of electrical current (Kim et al., 2014). However, it is necessary to consider environmental negative facts related to biological processes which represent challenges for the future (Mata et al., 2010). This approach includes supply capacity and use of resources that could claim up waste generation. In this sense, the integration of processes to optimize resources and minimize waste generation (Zhu et al., 2015) is possible using biological, chemical or thermal processes in a biorefinery, which have an important role in climate change according to the report "The future of industrial biorefineries" of World Economic Forum (WEF). Even so, it is considered beneficial to know in detail those effects that production processes cause to the environment, which may assess environmental impacts that these new forms of energy production can produce to the environment. For this purpose, LCA is a tool that allow identify, characterize and quantify environmental impacts associated to each stage of the life cycle of a product (ISO 14040: 2006). LCA provides information on the total environmental performance of a process, which can be used as a decision-making, this one of the main benefits to take advantage (Adesanya et al., 2014). LCA also helps to

identify energy and emission "bottlenecks", i.e. the life cycle stages of a process that are critical to the overall environmental burden and thus require further improvement (ISO 14044: 2006). Nowadays several studies on LCA had reported the application for bioenergy production systems, which discussed the environmental benefits of biodiesel and bioethanol production from renewable raw materials. Some authors had reported the use of LCA for lignocellulosic feedstock in the production of bioethanol, their reports indicate significant results taking into account different factors (type biomass, conversion technologies and product use) that affect the results of the analysis (Naik et al., 2010, Pehnt, 2006, Singh et al., 2010). The objective of this work, was to determine the environmental performance analysis of second generation bioethanol fuel production and combustion, from agro-industrial waste using fermentation for conversion of cellulose to bioethanol coupling the use of a waste in Microbial Fuel Cell (MFC) for the production of bioelectricity.

Materials and methods

The LCA methodology used in this study followed the framework of ISO 1040:2006 and ISO 14044:2006 standards.

LCA studies includes four phases. The relationship between the phases is illustrated in Figure 1. Direct applications of the results of LCA studies, i.e. the applications intended in the goal and scope definition of the LCA study are depicted in Figure 1.

The principal stages in an LCA study are listed like:

- The goal and scope definition
- Inventory analysis
- Impact assessment
- Interpretation

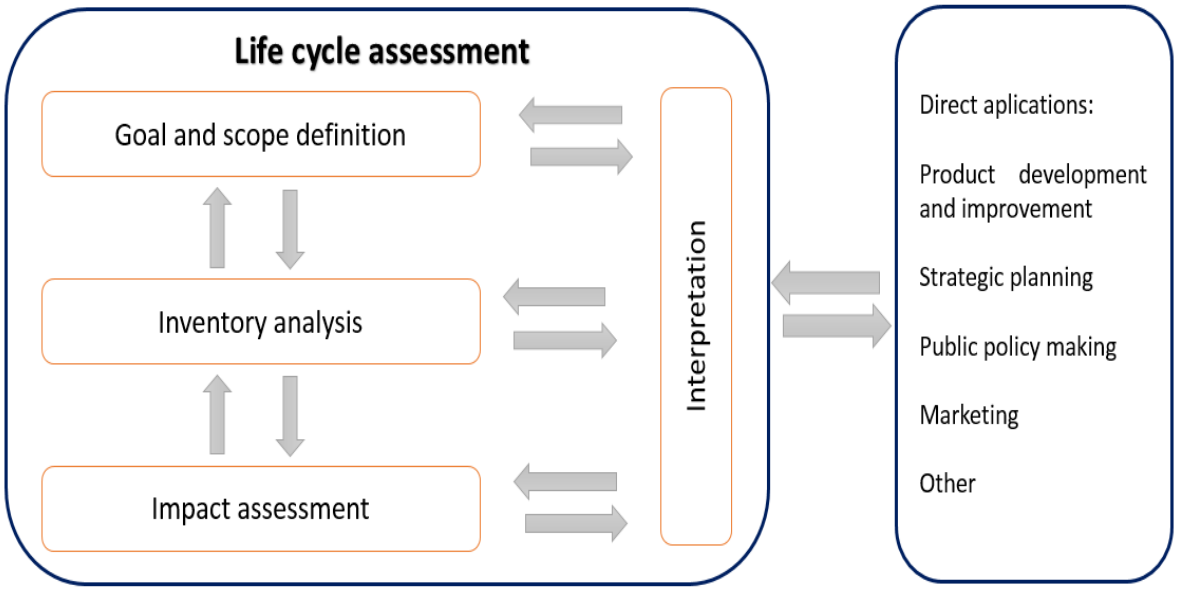

Figure 1. Principal stages of a life cycle assessment.

The scope, including the system boundary and level of detail, of a LCA depends on the subject and the intended use of the study. The depth and the breadth of LCA can differ considerably depending on the goal of a particular study. The life cycle inventory (LCI phase) is an inventory of input/output data with regard to the system being studied. It involves collection of the data necessary to meet the goals of the defined study. The purpose of life cycle impact assessment phase (LCIA) is to provide additional information to help assess a product system´s LCI results also well understand their environmental significance. Life cycle interpretation is the phase of the LCA procedure, in which the results of a LCI or a LCIA, or both, are summarized and discusses as a basis for conclusions, recommendations and decision-making in accordance with the goal and scope definition.

Results and discussion

Biodiesel, bioethanol and bioelectricity production were analyzed. Information about process like resources, amounts and products were incorporated into a preliminary database. Also elementary flows into and out of each unit process and product flows entering and leaving the system were obtained. Integration of process in a system is present in Figure 1. Initial process is about biodiesel production (from vegetable oils). A first effluent represented by glycerol is discarded. This effluent is incorporated into bioethanol production. This process use agro-industrial waste and fermentation with *Saccharomyces cerevisiae*. Weekly biodiesel production is 200 L, with a yield of 92 to 94% and a volume of 40 L. For bioethanol process, it was obtained 8 L of ethanol (99%) from 200 L of wort (yield process 4%). In fractional distillation step of bioethanol production was obtained a stream with glycerol and water. This effluent is being tested in a microbial fuel cell for production of bioelectricity.

Table 1 shows the goal, scope function and functional unit considered for LCA analysis, as the primary steps of the study.

Table 1. Determination of the objective and function of the LCA.

Goal	Function	Unit Function
To assess environmental impacts of the bioethanol generation from agro-industrial waste.	Cover 10% of the fuel for C.U.-Amazcala (32.5 km) route.	1.25 L of bioethanol

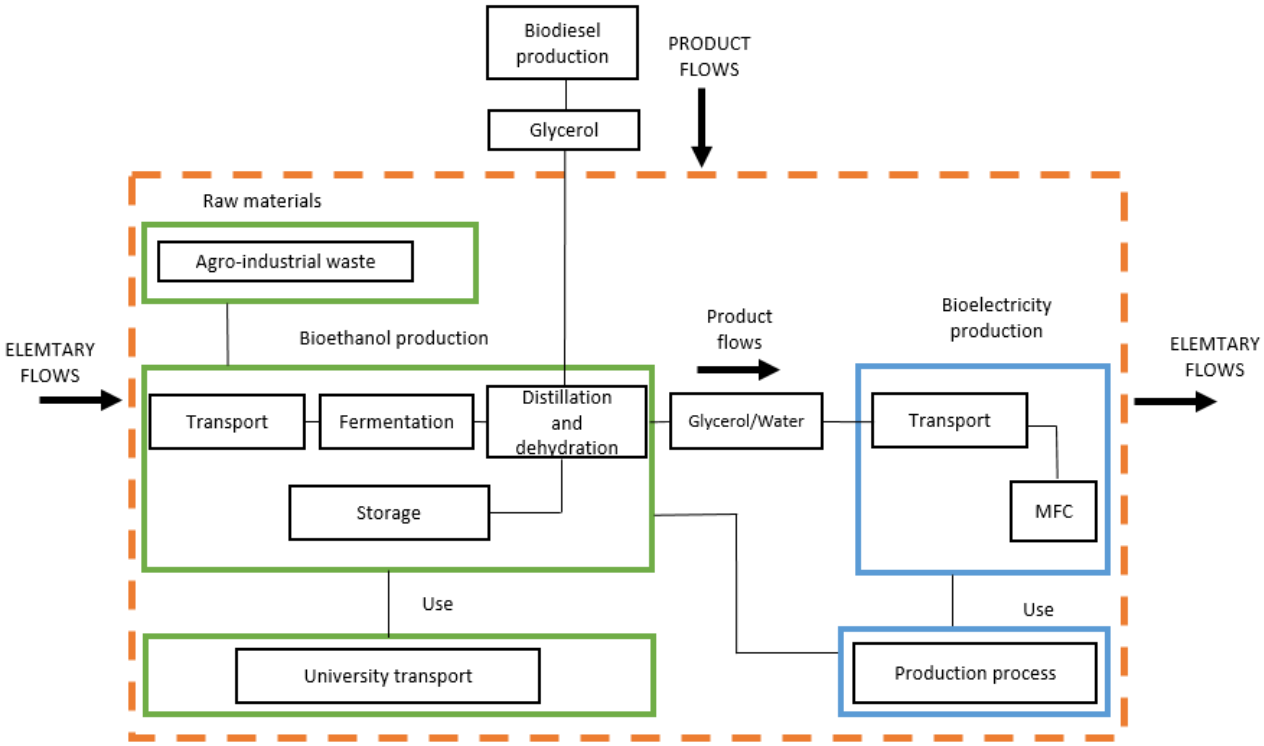

Figure 2. Flow chart for integrated processes studied by LCA.

Conclusions

The first phase of LCA was determined, this includes objective and scope, system boundaries, function and functional unit. Defining system boundaries is critical to appropriately conducting an LCA study. Although the system boundaries of biomass ethanol can vary from study to study depending on the inclusion or exclusion of some specific processes. Our analysis aim was to compile an inventory of inputs and outputs of bioenergy production cycle in order to evaluate and compare different origin of biomass initial. LCA will be use to estimate the energy and climate change impacts.

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Enzyme activity of phosphatase, urease and dehydrogenase in soil under the application of herbicides

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Abstract
The activity of three enzymes in soil was analyzed under the effect of two agrochemicals 2,4-D and glyphosate for four different soils. The enzymes were phosphatase, dehydrogenase and urease. To carry out this work, samples of four different points at a depth of 0 to 10 cm were collected in an area of 4 x 4 m from the community of Marin, Nuevo Leon, Mexico. Physicochemical tests were performed to characterize the soil, such as: texture, bulk density, particle density, organic matter, cation exchange capacity, pH, electrical conductivity, anions, cations, heavy metals and density of microorganisms.
As a result, more of 80% enzyme inhibition assay was obtained, so it is concluded that the addition of these herbicides at a dose 10 times higher than the normal field application rates caused changes to soil enzymatic activity.

Keyworks*:* • Enzymes • Herbicides • 2, 4 D • Glyphosate.

Introduction

Enzymes are a special type of proteins that combine with a specific substrate and act to catalyze a biochemical reaction, without experiencing changes in their structure; generally in the soil, enzymes are essential for the transformation of energy and nutrient cycling. Due to its protein nature they can be affected by environmental factors such as temperature and pH (Alexander 1980, Coyne 2000, Paul and Clark 2007). Plants, animals and microorganisms produce soil enzymes. May be present in dead cells and cellular debris that are absorbed and incorporated into clays humic substances (Baležentienė y Klimas 2009). In recent years, great advances in the field of chemistry there have been, with the synthesis of many new chemicals, among which include plastics, pharmaceuticals, petroleum products, fertilizers, herbicides and pesticides. The production and use of these substances have improved the conditions of human life, which has led to population growth; however, the development of synthetic chemical industry, has major implications for the environment, therefore the large amounts of organic and inorganic which, on several occasions, its effects on the environment you will break down substances. One of the most widely used agricultural chemicals worldwide is glyphosate [N- (phosphonomethyl) glycine] is a, post-emergent, systemic and broad spectrum non-selective herbicide, which binds rapidly and strongly to matter organic when in contact with the soil and aquatic sediments suspended particles. The herbicide 2,4-D is one of the oldest synthetic pesticides. It was released in the forties and sadly became famous because it was part of the chemical defoliant "Agent

Orange" in the Vietnam War. 2,4-D is even today widely used around the world. The objective of this study was to evaluate the response of enzyme activity in soil under three agrosystems herbicide application.

Materials and methods

A study in which enzyme activity in soil was assessed using the method was performed by Zabaloy et al. (2008), which were collected ten points, statistically random sample, in an area of 4 x 4 m with an area of 0 to 10 cm in 4 different sites in the town of Marin, Nuevo Leon, Mexico, for 6 months at different times. This sites are:

Site A: Agricultural field where are set annual crops of sorghum and maize, this site was used wastewater from the municipal seat of Marin, N.L

Site B: temporary meadow where the dominant species is buffel grass (Cenchrus ciliaris L.).

Site C: Mainly agricultural land planted to oats (Avena sativa) is irrigated with water from storm water runoff captured in two bodies of surface water.

Site D: Rangeland with sparse native vegetation where cattle less dough.

The experiment was conducted in two phases, first the different physicochemical properties to characterize the soil, the parameters determined were texture, bulk density, particle density, organic matter, cation exchange capacity, pH, electrical conductivity, anions, cations and heavy metals (table 1), all methods were based in Dawson et al. (2007). The second part of the studies was enzymatic analysis in soils that are based on different methods proposed by Dawson et al. (2007), and Margesin (1997) is used to quantify chemical derivatives of enzyme activity, dehydrogenase, alkaline phosphatase and urease, respectively. Ten samples were taken by parcel and collected from 0 to 10 cm of deep.

The cores were placed in plastic bags and transported to the laboratory in ice bucket to keep low temperature during transportation. For the enzymatic quantitation two pollutants were added at a concentration of 125 mg L^{-1} for glyphosate and 4 mg L^{-1} to 2,4-D, this corresponds to 10 times more the recommended dose (represented as H) each herbicide for hectare. Microcosms were incubated at 28 ° C for 21 days and analyzed at 0, 7, 14 and 21 days (Dawson et al. 2007).

Determination of Phosphatase. In the method for determination of phosphatase enzyme was performed first weighing 1g sample of each sample or soil and were introduced into 25 ml Erlenmeyer flasks, after that was added 0.25mL of toluene, 4 mL of pH 11 buffer and 1 mL of P-nitrophenylphosphate, stirred and incubated at 37 °C for 1 hour, back extracted from the incubator and is added 1mL 0.5M $CaCl_2$ and 0.5M NaOH 4 mL was stirred and gravity filtered with Whatman # 1 paper and finally was analyzed spectrophotometrically at a wavelength of 405 nm.

Determination dehydrogenase. The enzyme dehydrogenase is based on two periods first, 6 g sample of each soil was weighed and deposited into 4 vials of 50 mL, two are added 0.5% glucose (the weight of soil), and other two vials without glucose and more soilless vial which was used as a target, all the vials was added 1 mL of 3% TTC 2.5 mL deionized water and including white, then the soil is mixed and reagents with a glass rod, capped and incubated for 1 week. When passing week of incubation are added to all vials 10 mL of methanol (MeOH) and filter through Whatman # 42. After the filtrate was collect in 250 mL Erlenmeyer flask. All vials were washed with two or more than 10 mL of methanol and reading was

performed uv-vis spectrophotometer at 485 nm and through a calibration curve the concentrations were determined.

Determination urease. Five grams of soil were place into each of three 50-mL incubation flasks, and treat two of them with 2.5 mL of substrate solution and 20 mL of borate buffer; pipette only 20 mL of borate solution into the third flask (control). The flasks were stoppered and incubated for 2 h at 37°C. After incubation, the control samples were treated with 2.5 mL of substrate solution and all samples with 30 mL of potassium chloride (2 M)–hydrochloric acid (0.01 M) solution. The samples were shaked for 30 min on a rotatory shaker. Then, the soil suspensions were filtered using folded filters.

Table 1. Physical and chemical properties of the soils studied.

	Soils			
Parameter	**A**	**B**	**C**	**D**
Organic material (mL)	3.42	3.39	2.77	3.16
Texture	Clay	Clay	Clay	Clay
CO_3 (me L^{-1})	0.2	0.25	0.1	0.2
HCO_3 (me L^{-1})	3	2.65	2.8	2.75
Cl (me L^{-1})	228.44	320.41	99.14	230.50
Relative density (g cm^{-3})	2	1.53	1.81	2.5
pH	moderately alkaline	moderately alkaline	moderately alkaline	moderately alkaline
Cation exchange capacity (me L^{-1})	25.5	20.5	23.65	29.5
Mg(mg L^{-1})	4.8975	6.8425	7.54	2.875
K(mg L^{-1})	24.4875	34.2125	37.7	14.375
Pb(mg L^{-1})	0.063	0.086	0.0965	0.0975
Na(mg L^{-1})	31.9745	60.8065	31.718	28.334
Ca(mg L^{-1})	44.895	44.7475	49.5125	57.6475

Results and discussion

The results obtained with the two-herbicide treatments, indicated that there was a lowering effect on the three enzymes activities studied. Practically the final concentration of the enzyme phosphatase to be comparative with the initial concentration shown as decreases, despite being different soils, their behavior is consistent in the inhibition of enzymes with both herbicides, and has effect with samples having concentrations 10 times higher than the concentration with the standard established, only for glyphosate it was slightly lower effect. Table 2 show the behavior of the enzyme for both pollutants, the concentrations are represented as L (low concentration) and H (High concentration, 10 times more the recommend).

Table 2. Effect of 2, 4 D and Glyphosate herbicides on Phosphatase enzyme. Concentrations were interpreted as: 1) 2, 4 D L, 2) 2, 4 D H, 3) Glifosate L, 4) Glifosate H.

	µg p-nitrophenol g^{-1} soil															
Site	**A**				**B**				**C**				**D**			
days	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
0	133.21	85.52	98.02	75.79	29.54	84.43	25.34	19.97	28.08	17.65	45.29	68.20	30.18	41.40	30.18	41.40
7	122.82	73.56	113.70	121.60	115.33	108.67	67.74	104.54	88.08	16.12	79.01	67.88	25.40	22.13	25.40	22.13
14	57.60	61.75	194.55	119.28	94.60	191.08	63.74	73.30	136.03	73.26	41.20	29.09	24.81	21.18	26.30	33.12
21	54.33	32.24	214.70	184.96	229.56	173.96	61.98	58.25	35.93	21.86	35.93	21.86	32.12	2.31	77.05	44.13

Table 3. Effect of 2, 4 D and Glyphosate herbicides on urease enzyme. The concentrations were interpreted as: 1) 2, 4 D L, 2) 2, 4 D H, 3) Glifosate L, 4) Glifosate H.

	µg N g^{-1} soil															
Site	**A**				**B**				**C**				**D**			
days	**1**	**2**	**3**	**4**	1	2	3	4	**1**	**2**	**3**	**4**	1	2	3	4
0	16.51	13.85	8.48	9.56	11.99	4.05	8.94	8.47	3.00	2.11	3.585	2.89	4.78	6.41	12.03	11.61
7	13.29	12.84	10.60	9.56	12.85	9.75	7.54	5.52	4.35	3.86	5.32	1.65	7.05	1.49	12.84	8.99
14	8.43	11.82	9.34	8.94	11.44	7.18	6.55	5.16	3.54	3.13	4.35	1.62	6.00	1.42	12.30	8.27
21	8.03	2.87	8.21	8.43	9.61	5.64	6.11	4.38	2.06	1.18	3.72	2.37	3.95	0.30	10.59	9.72

Table 4. Effect of 2, 4 D and Glyphosate herbicides on dehydrogenase enzyme. The concentrations were interpreted as: 1) 2.4D L, 2) 2.4D H, 3) Glifosate L, 4) Glifosate H.

	µg TPF g^{-1} soil															
Site	***A***				***B***				***C***				***D***			
days	**1**	**2**	**3**	**4**	1	2	3	4	**1**	**2**	**3**	**4**	1	2	3	4
0	120.11	117.84	181.39	179.48	118.18	74.43	37.71	36.62	23.94	21.84	19.38	18.20	28.45	74.70	19.29	16.68
7	100.64	93.77	169.19	118.40	98.97	53.67	35.96	30.57	19.27	18.60	17.32	14.72	23.13	25.44	16.43	12.78
14	88.43	79.83	154.27	149.74	92.28	75.78	35.67	16.73	20.83	17.14	16.85	15.28	17.12	25.37	72.92	53.97
21	64.26	59.93	120.00	114.42	64.28	56.08	24.2	13.05	13.50	10.70	13.47	8.83	7.75	3.62	47.94	41.72

Urease enzyme shows us clearly that was affected by the herbicide being inhibited by both pollutants, but compared with dehydrogenase and phosphatase enzymes concentrations steadily declined, on the other hand to add the contaminates high concentration values decrease even more, and for urease enzyme was not, its concentration of the initial to the final was not very significant, although equally were added on both low methane concentrations as high. The table 3 shows the behavior of the urease enzyme concentration over time.

The effect that indicate values thrown dehydrogenase enzyme with the addition of herbicides over time, were way too high a concentration higher to 100 mg/L, more than the aforementioned enzymes, the effect could cause this behavior could be by reactive because for this enzyme could receive conditions appropriate for this phenomenon is exercised, in the table 4 behavior shows that exercises this enzyme.

Conclusions

During this study, the importance of the effect of enzymes depending on the soil type was observed, so the different physic each plant in order to determine what type of soil is also the same as the pH of each was made a of them, cation exchange capacity, bulk density, relative density and humidity, among others, all were considered to be able to present the results shown.

Considering the physicochemical properties of each soil, enzymatic analysis, which threw us desired data, because that for phosphatase, β-glucosidase and urease enzymes initial concentrations were between 15 and 8 mg L^{-1} and after the effects of the was held herbicides could obtain values to 0 mgL^{-1} and were dehydrogenase initial concentrations above 100 mg L^{-1} high triple aforementioned enzymes and obtaining an inhibition of more than 80% for all enzymes operated.

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Genetic stability of *Vitis vinifera* L. after cryopreservation

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Abstract

Grapevine is an economically important fruit crop worldwide. In México, Sonora State leads in the production and exportation to international markets. Cryopreservation is a good alternative for the long-term conservation of plant genetic resources, although it very often can induce injury that can produce changes in genome. Evaluate the genetic stability of buds cryopreserved using Inter-Simple Sequence Repeats (ISSR) markers. Axillary buds cv. 'Flame seedless' were cryopreserved by vitrification and stored in liquid nitrogen (LN) for: one hour, one week and one month. From ten ISSR primers evaluated, only two primers produced distinct and reproducible bands. These primers produced fragments ranging from about 300 to 2000 bp. Different ISSR fragments were detected between control and cryopreserved buds, revealing polymorphism. These results suggest that cryopreservation and vitrification-cryopreservation affect genetic stability in grapevine in all the times of storage.

Keywords: ISSR • PVS2 • table grape • vitrification.

Introduction

Grapevine (*Vitis vinifera* L.) is considered one of the most economically important crops in the world (Wang *et al.* 2004). Species conservation requires efficient and cost-effective *ex situ* methods, which can be complemented with *in situ* preservation programs (Ganino *et al.* 2012). Cryopreservation is a very efficient alternative for the long-term storage of germplasm and is based on the reduction of metabolic functions at -196°C, the temperature of liquid nitrogen (LN). Application of cryopreservation combined with *in vitro* methodologies has offered new opportunities for long-term conservation of vegetative propagated crops (Benson *et al.* 2011). Protocol for cryopreservation in *Vitis vinifera* L. was first reported for Dussert *et al.* (1991) using embryogenic cell suspension. Later, different cryopreservation techniques, such as encapsulation-dehydration and vitrification (Wang *et al.* 2004; Benelli *et al.* 2013; Markovic *et al.* 2013; Vasanth and Vivier 2011), have been tested in grapevine tissues, but only recovery, viability and regrowth have been evaluated. Cryopreservation of axillary buds in grapevine, it has not been developed. Only using an embryogenic cell suspension (Wang *et al.* 2004; González-Benito *et al*. 2009; Ben-Amar *et al*. 2013) and shoot-tips tissue (Markovic *et al.* 2013; Matsumoto and Sakai 2003) with regeneration or viability frequencies after LN treatment ranged from 37 to 78%. Cryopreservation process involves many manipulations of the tissues, culture initiation, proliferation, acclimation, dehydration, cryoprotection, LN exposure, vitrification, rewarming, recovery, and

regeneration; the study of genomic alterations becomes essential (Berjak *et al.* 2011). For that, it is desirable to assess the genetic integrity of the plants after surviving cryogenic storage to determine if they are true-to-type after cryopreservation. The most commonly used marker systems for genetic stability study are Random Amplified Polymorphic DNA (RAPD) (Srivastava *et al*. 2004), Amplified Fragment Length Polymorphism (AFLP) (Wang and Yu, 2001), and Inter-Simple Sequence Repeat (ISSR) (Vijayan *et al.* 2006). In grapevine, Zhai *et al.* (2003) cryopreserved shoot-tips from 'Cabernet franc', 'Chardonnay', 'Fengh 51' and 'LN33' cultivars using encapsulation-dehydration method. Using RAPD marker, no differences were found between the DNA patterns obtained with plantlets regenerated from control and cryopreserved plantlets. Only one report is about genetic stability in grapevine, for that reason is needed to test the effect of grapevine plants after the protocol. The present research was carried out to evaluate the genetic stability of grapevine axillary buds cv. 'Flame seedless' subjected to vitrification-cryopreservation using inter simple sequence repeat (ISSR) markers.

Materials and methods

Axillary buds of grapevine (*Vitis vinifera* L.) cv. 'Flame seedless' were used. Sample collection of grapevine rootstocks containing 5 to 7 axillary buds from the "Casas Grandes" vineyard located 40 km from Highway 36 North to the coast of Hermosillo, Sonora, México (29°02'41.0"N, 111°43'59.3"W). The axillary buds were dissected with a sterile razor blade and disinfected in commercial chlorine solution at 25% (1.3% NaOCl) with 0.1% Tween 20 for 5 min and then rinsed three times with sterile distilled water.

Cryopreservation procedure. For cryopreservation, the disinfected buds (five replicates per treatment with five buds each, n=25) were transferred to sterile 2-mL polypropylene cryovials and immersed directly into LN. The sampling was carried out after storage for an hour, a week, and a month in LN. After each freezing period, cryovials containing frozen buds were thawed rapidly in a water-bath at 38°C for 3 min.

Vitrification-cryopreservation procedure. For vitrification-cryopreservation, the buds were treated with the plant vitrification solution N° 2 (PVS2) contained 30% (w/v) glycerol, 15% (w/v) ethylene-glycol, and 15% (w/v) dimethyl-sulfoxide in MS medium with 0.4 M sucrose at pH 5.8 (Sakai *et al.* 1990). The buds were disinfected as described above and transferred into 2-mL cryovials (five replicates per treatment with five buds each, n=25) containing 1 mL of PVS2 solution. The control treatment did not include the PVS2 solution. The samples were incubated at 25±2°C with agitation for 180 min. This was the best incubation time according to a previous viability assay using grapevine buds (data not shown). The cryovials were directly immersed in LN, and stored for an hour, a week, and a month. After each treatment, was thawed in a water-bath at 38°C for 3 min.

Genetic stability assessment. DNA was extracted from 0.5 g tissue using the CTAB method according to Japelaghi *et al.* (2011). For ISSR analysis, a set of 10 primers (reported by Seyedimoradi *et al.* (2012)) was carried out in DNA from axillary buds derived from cryopreserved and vitrified-cryopreserved experiments. PCR amplification was carried out in a total volume of 50 µL containing 5 µL of 10X PCR buffer, 1 µL of 10mM PCR nucleotide mix, 3 µL of 25mM $MgCl_2$ solution, 80 pmol primer, 0.25 µL GoTaq® DNA polymerase and 25 ng DNA (Hassan *et al.* 2011). All amplifications were carried out in a Swift™ MaxPro (ESCO, Singapur) as follows: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50ºC and 2 min at 72°C, and 8 min at 72°C for final extensión. Amplified products were

electrophoresed in 1.2% agarose gels for 1.5 h at 50 V in 1X TAE buffer and visualised by ethidium bromide staining.

Data analysis. Scoring of ISSR data was perfomed using a 1.2% agarose gel electrophoresis profile. Amplified bands obtained with all the molecular markers were scored visually for the presence and absence of bands for all the treatments. Polymorphic information contented values were calculated for each ISSR primers for treatment.

Results and discussion

In this study, ISSR markers were employed to test the genetic variability of cryopreserved grapevine axillary buds (*Vitis vinifera* L.) cv. 'Flame seedless'. ISSR markers have been tested to analyze grapevine diversity because of their properties of genetic co-dominance, high reproducibility, high abundance, high overall mutation rate and high polymorphism (Hyppolyte *et al.* 2012). We analyzed eight treatments of cryopreservation of grapevine buds. In order to assess their genetic variability of cryopreserved buds ISSR patterns were compared with control buds without treatment. From ten primers tested, eight (80%) generated amplicons. Of these eight primers, only two (20%) produced reproducible and good quality bands that were used in the present study to analyze the genetic stability. Of the two primers, one (Figure 1A: ISSR1 5'AGAGAGAGAGAGAGAGT3') detected one to seven bands and the second (Figure 1B: ISSR6 5'GAGAGAGAGAGAGAGAA3') from five to six bands. The size of the amplified products ranged from 300 to 2000 bp (Figure 1). The Figure 1, line C: control buds without treatment, 1: Buds after 180 min in PVS2, 2: 1 hour in LN without PVS2, 3: PVS2 and 1 h in LN, 4: 1 week, 5: 1 week in LN, 6: 1 month in LN, 7: PVS3 and 1 month in LN, 8: PCR control and M: 1 Kb plus ladder marker.

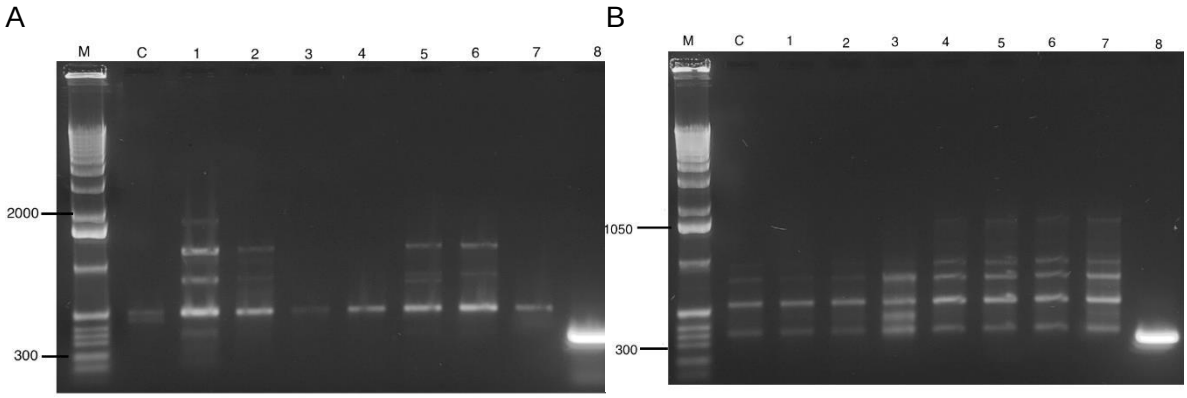

Figure 1. ISSR-PCR profiles of grapevine buds (*Vitis vinifera* L.) cv. 'Flame seedless'.

At the present, increasing evidences have demonstrated that materials maintained genetic stability during the period of cryopreservation (Zhai *et al*. 2003; Baránek *et al*. 2010; Condello *et al.* 2009); one reason may be that in most cases only a very small fraction of the genome (0.001-1%) is analyzed (Benson *et al.* 2013). In our analysis we found significant changes occurred when buds were used in vitrification with PVS2 and in cryopreservation in all the different times in LN (Figure 1). Probably for the series of stresses (dehydration, osmotic pressure, low temperature) throughout the cryopreservation process

may result in genetic variation (Engelmann 2004; Hazubska-Przbyl *et al.* 2010). Many other factors are capable of inducing genetic variation in cryopreserved materials, including the type of explants selected, the freezing procedure employed and regrowth pattern achieved (Aronen *et al*. 1999). In our study, axillary buds were the explants selected for freezing because they are programmed to directly develop into shoots, and we can avoid changes for effect of tissue culture. However, the changes found in bands profiles in the grapevine buds are very evident in the treatments of vitrification and cryopreservation. Like our results, few cases of genetic variability were observed at RAPD or ISSR loci (Urbanová *et al*. 2006; Martín *et al*. 2005; Ai *et al.* 2012), but it has been attributed to the toxic effect of PVS2 and/or the regeneration phase (Akdemir *et al.* 2013) and probably occurred in non-coding regions that did not affect phenotypic characteristics (Kaity *et al.* 2008). Results from the present study signify the importance of carrying out genetic stability studies; preferably, a combination of techniques should be used to determine the stability status of cryopreserved material.

Conclusion

The results from the present study using two primers shows that the process of cryopreservation and vitrification cause genetic variability in grapevine axillary buds (*Vitis vinifera* L.) cv. 'Flame seedless' in all storage times in LN when assessed by ISSR markers.

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Determination of laccases activity of *Ganoderma lucidum* grown in liquid culture

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Abstract

The white-rot fungus *Ganoderma lucidum*, is able to degrade lignin and other compounds in the growing substrates. In this study the ability of a strain of this species for producing the laccase enzyme in two culture media (one with inductor) at different pHs were evaluated. In both culture media, the biomass production was very similar (3.5 and 4.0 g/L) and there was little consumption of sugars, however, there was great difference in laccase activity, in the culture medium with the presence of inducer, the activity increased by at least 10 times in all pHs, the maximum activity was 8573 U/L (312 h) in the presence of inducer at pH 4.0, while in the medium without inducer was observed at approximately 13 times less (653 U/L) to 120 h. This results suggest that *Ganoderma lucidum* is a good laccase producer, and the activity values could be increased by changes in the inducer concentration or modifying other parameters of the culture medium. This medicinal mushroom is great alternative for the production of laccases which has great biotechnological applications.

Keywords: Copper (●) *Ganoderma lucidum* (●) Laccases (●) pH

Introduction

Fungi are a diverse group and with largest number of species in terrestrial environments after insects, due to its wide distribution and association with various organic and inorganic substrates, they are in all ecosystems (Betancur *et al.* 2007). Fungi are considered natural recyclers because they use an enzyme complex that can degrade various substrates and turn them into resources for their survival (Lindequist *et al.* 2005). White-rot fungi are characterized by their ability to degrade lignin. Since this is a heteropolymer made up of units of different structure (generally phenolic compounds), is required special enzymes that are able to oxidize these links without high specificity. Among those enzymes are the laccases. Each enzyme has specific characteristics, including the optimal pH (it has been reported for fungal laccases in acidic ranges). For example, the optimum pH for the oxidation of ABTS is generally less than 4.0, the phenolic compounds as the DMP, guaiacol and syringaldazine have values between 4.0 and 7.0. The optimum pH of the enzymes of different fungi for catechol and hydroquinone are 3.6-4.0 and 3.5-6.2, respectively (Azila *et al.* 2008). The stability of fungal laccases is generally at acidic pH (Simmons & Singleton 1996), although there are exceptions (Kulikova *et al.* 2011). The aim of this study was to quantify at different pHs the laccase activity of *Ganoderma lucidum* grown in liquid culture in both medium with and without laccase inducer.

Materials and methods

Organism

The strain of *Ganoderma lucidum* (CP-145) was used, which was donated by Dr. Edmundo Arturo Pérez Godínez from the Autonomous University Chapingo. For the inoculum, the strain was grown on potato-dextrose agar (PDA) for 8 days at 25° C.

Liquid fermentation

Erlenmeyer flasks of 125 mL with 50 mL of culture medium (potato-dextrose broth, DP) or optimized medium (IND) for laccase production, which contains glucose (10 g/L), yeast extract (5 g/L) and mineral salts including $CuSO_4$-$5H_2O$ (0.4 g/L) where copper acts as inducer (Téllez-Téllez *et al.* 2008) were used. Each flask was inoculated with 3 mycelial pellets of 4 mm taken from the peripherial of a colony grown as above was described. The cultures were incubated in orbital shaking of 120 rpm at 28ºC for 17 days.

Enzymatic extract and biomass quantification

The enzymatic extract (EE) was obtained by filtration of broth using filter paper (Whatman No. 4), and the retained biomass was determined as difference of dry weight (g/L) (Díaz-Godínez *et al.* 2001).

The assay of biomass X = X(t) was done using the Velhurst-Pearl logistic equation,

$$dX/dt = \mu(1-X/Xmax)X \qquad [1]$$

Where μ is the maximal specific growth rate and Xmax is the maximal (or equilibrium) biomass level achieved when dX/dt = 0 for X> 0. The solution of equation 1 is as follows,

$$X = Xmax/1+Ce-\mu t \qquad [2]$$

Where C = (Xmax - X0)/X0, and X = X0 is the initial biomass value.

Estimation of kinetic parameters in the above equations was performed using the non-linear least square-fitting program “Solver” (Excel, Microsoft) (Díaz-Godínez et al. 2001; Viniegra-González et al. 2003; Téllez-Téllez *et al.* 2008).

Laccase activity

Determined by changes in the absorbance at 468 nm, using 2,6-dimethoxyphenol as substrate (DMP). The assay mixture contained 950 µL of substrate (2 mM DMP in 0.1 M acetate buffer at pH´s 4.5 and 4.0 or glycine-HCl buffer at pH´s 3.5 and 3.0) and 50 µL EE, which was incubated at 40 ºC for 1 min (Téllez-Téllez *et al*. 2008). One enzymatic unit (U) of laccase is defined as the amount of enzyme, which gives an increase of 1 unit of absorbance per min in the reaction mixture.

Results and discussions

Biomass production in both culture media are shown in Figure 1a and 1b, for DP medium was 3.5 g/L and in the IND medium was 4 g/L at 408 h of culture, there were no important changes of pH in both media (Figure 1c). It is possible, that the biomass was higher in IND medium than in the DP medium, since the first contains a nitrogen source of high biological value and its carbon chains could be metabolizing for biomass production.

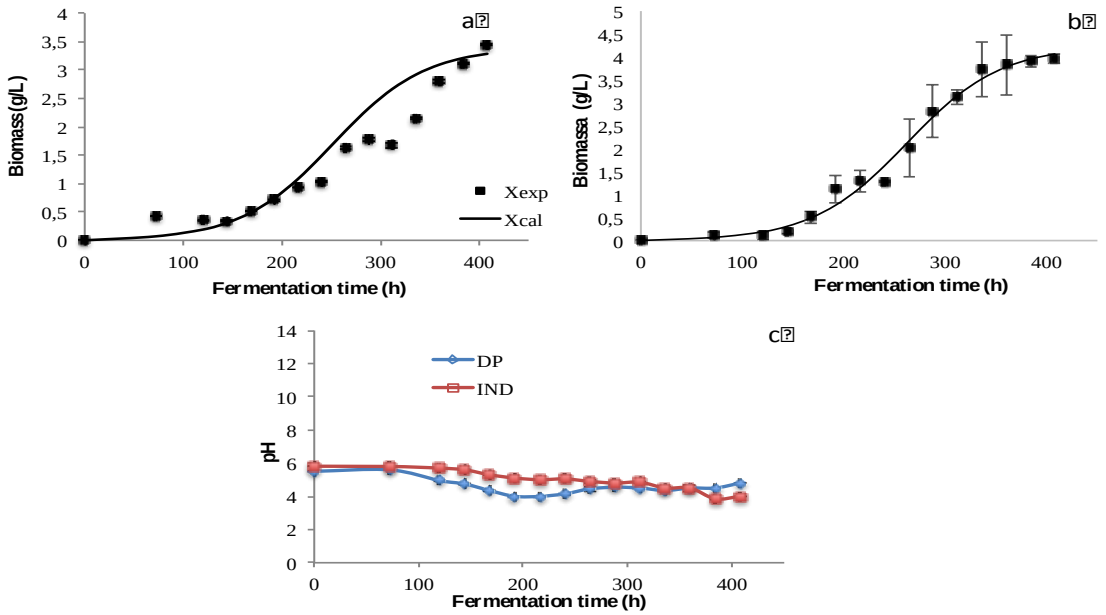

Figure 1. Biomass evolution of *Ganoderma lucidum* grown in DP (a) and IND (b) media and pH of the cultures (c).

Figure 2 shows the activity of laccases obtained in both culture media. The maximum activity in the DP medium was 679 U/L at pH 3.5 at 192 h and 653 U/L at pH 4.0 at 120 h. In general, in the IND medium, the activity was about 10 times higher in all pHs with respect to that observed in the medium DP; the highest value was 8573 U/L at pH 4.0 to 312 h (Figure 2b).

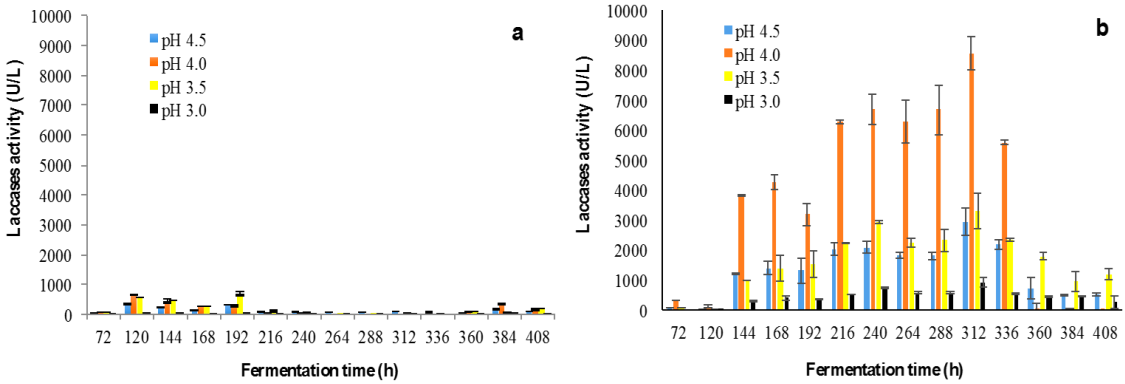

Figure 2. Laccase enzymatic activity of *Ganoderma lucidum* grown in DP (a) and IND (b) media.

Rothschild *et al.* (1999) reported that high concentrations of both copper and lignocellulosic waste can affect the mycelial growth. In this study, the biomass production (maximum of 4 g/L) could be affected in the medium IND by the presence of copper and in the DP medium by the increase of broth density perhaps by the production of polysaccharides. Wang & Ng (2006) reported that the pH regulates the production of laccases in *Ganoderma lucidum*

being higher production at acidic pH (3-5), at pH of 5 and 6 there is decreased activity and pH of 6 and 7 little activity is detected, but at pH of 8 and 9 activity is imperceptible, consistent with the results of this work, since most laccase activity was at pH 4.0 and 3.5. Songulashvili *et al*. (2011) reported a high production of laccase (149 600 UL^{-1}) in medium supplemented with starch and 3 mM Cu^{2+}, which was also observed in this work as the laccase activity increased in the presence of copper.

Conclusion

The laccase activity was higher in the medium added with inducer and it was observed that the pH for activity was around 4.0-4.5, in that sense the culture conditions could be optimize mainly for the carbon source, inducer concentration and pH of the culture medium to increase the activity values.

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The overexpression of an *Amaranthus hypochondriacus* NF-YC gene modifies growth and confers water deficit stress resistance in Arabidopsis

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Abstract

Nuclear factor-Y (NF-Y), is a plant heterotrimeric transcription factor constituted by NF-YA, NF-YB and NFYC subunits. The function of many NF-Y subunits, mostly of the A and B type, has been studied in plants, but knowledge regarding the C subunit remains unknown. Here, a water stress-induced NF-YC gene from *Amaranthus hypochondriacus* (*AhNF-YC*) was further characterized by its overexpression in transgenic *Arabidospis thaliana* plants. A role in development was inferred from modified growth rate in root, rosettes and inflorescences recorded in *AhNF-YC* overexpressing Arabidopsis plants, in addition to a delayed onset of flowering. An altered expression of the latter in water stressed and recovered transgenic plants, suggested that their increased water stress resistance was partly ABA-dependent. An untargeted metabolomic analysis also revealed an altered metabolite pattern, both in normal and water stress/recovery conditions. These results suggest that AhNFYC may play an important regulatory role in both development and stress, and represents a candidate gene for the engineering of abiotic stress resistance in commercial crops.

Key words: • grain amaranth • growth and development • NF-YC • water stress.

Introduction

Abiotic stress as drought, salinity and extreme temperatures has a negative impact on growth, development and yield in plants. To minimize cellular damage caused by abiotic stress, plants have evolved complex, well-coordinated adaptive responses that operate at molecular and physiological levels (Lindemose *et al*., 2013). The physiological flexibility of plants required to adapt to adverse environmental conditions has generated an interest on the identification of genes responsible of conferring diverse kinds of abiotic stress tolerance, preferably from plants that can grow in challenging habitats (Rozema and Schat, 2013). Transcription factors (TFs) are master regulatory elements that activate the expression of signaling cascades or whole networks of genes through their binding to distinct cis-regulatory elements. They control intrinsic developmental processes, and some families regulate responses to external stimuli, including stressful environmental conditions. Due to their central regulatory role in various stress responses, TFs have become attractive targets for engineering stress-resistance in plants (Valliyodan and Nguyen, 2006). The nuclear factor Y (NF-Y) TFs are rapidly emerging as important regulators of numerous plant

developmental and stress induced responses. NF-Ys are sequence-specific TFs with histone like subunits, which have the unique characteristic of binding DNA at CCAAT sites as heterotrimeric complexes composed of single subunits from each of three different protein families: NF-YA, NF-YB, and NF-YC (Stephenson *et al.,* 2007). Recent reports have elucidated some of the functions performed by the NF-Y TF family. These include a possible role of NF-YB and NF-YC members in Arabidopsis, tobacco, and wheat in the regulation of light responses and flowering time (Hackenberg *et al*., 2012; Han *et al*., 2013). In addition, it has been observed that the overexpression of the NF-YA10 subunit from wheat and NF-YB subunits from Arabidopsis conferred salinity and drought tolerance in maize and Arabidopsis plants (Nelson *et al*., 2007; Siefers *et al*., 2009) without affecting seed yield. Even though the involvement of both NF-YA and NF-YB in plant drought resistance has been reported, it is still important to determine how the NF-YC subunits contribute to establish, or even enhance, the function(s) of the NF–Y complex(es) in plants. Recently the interest in the study of grain amaranth has increased for their inherent tolerance to high temperatures and drought, traits which are closely associated with their C4 photosynthesis, indeterminate flowering habit and superior water use efficiency derived, in part, by their ability to grow long tap roots and develop an extensive lateral root system (Omamt *et al*., 2006). The recently generated *Amaranthus hypochondriacus* (Ah) de novo transcriptome (Délano-Frier *et al*., 2011) was used as a tool to identify grain amaranth genes induced under one or more stress conditions. One of the genes identified was the *AhNF-YC* gene, which was found to significantly increase its expression in leaves of young *A. hypochondriacus* plants, subjected to several types of stress. In contrast to the known physiological role played by a number of NF-Y subunits in other plant species, the role of this particular grain amaranth NF-YC is unknown. Therefore, the *AhNFYC* gene was overexpressed in Arabidopsis as a strategy to gain a deeper knowledge of its function in planta. The results obtained from the analysis of transgenic Arabidopsis plants that constitutively overexpressed the *AhNF-YC* gene were suggestive of its role both in developmental and abiotic stress resistance processes.

Materials and Methods

A. hypochondriacus var. Revancha was used in this study (Délano-Frier *et al*., 2011) and Arabidopsis thaliana (Columbia ecotype) were employed. For *in vitro* culture, seeds were surface sterilized and were subsequently sown on half-strength MS medium. Plates were sealed and incubated in a growth chamber at 21 ± 1 ◦C under a 16 h light/8 h dark photoperiod. In Arabidopsis, after germination on MS plates, 8-day-old seedlings of transgenic lines were planted in pots containing a soil mixture and placed in a conditioned growth room at temperature of 22 ± 1 ◦C, 70% humidity, and 16 h light/8 h dark photoperiod. For the water stress resistance test, plants were grown in soil under a normal watering regime for 3 weeks. Watering was then withdrawn, and observations were subsequently recorded for 8–10 d. At this stage of the experiments, WT plants exhibited severe dehydration effects. The survival rate was determined 1 d after watering was re-established. *Morphological characterization of transgenic Arabidopsis plants.* For root elongation analysis, seedlings were cultured in MS medium, and the seeds were germinated in a growth chamber vertically for 7 d under a 16 h/8 h light/ dark photoperiod at 21 ± 1 ◦C. Root length was measured at 3, 5 and 7 d post germination. Rosette area, leaf number and inflorescence length was measured in 5- to 6-weeks-old transgenic Arabidopsis plants to determine the

effect of AhNF-YC overexpression on vegetative growth and flowering time. The onset of flowering was also recorded.

Metabolic profile of Arabidopsis plant leaves. Lyophilized leaves of WT and AhNF-YC OE Arabidopsis plants were finely ground and sieved. Subsequently, 25 mg of plant powder was extracted with 0.5 ml of a solution containing HPLC grade methanol 75% v/v and 0.15% v/v formic acid. The mixture was sonicated for 15 min and then centrifuged at 10,000 × g for 5 min. The resulting supernatants were filtered prior to analysis by direct-injection electrospray ionization mass spectrometry (DIESIMS). Continuous spectra were collected in a range of 80–1300 m/z during 1 min, with a scan time of 10 s. The metabR program was used to obtain the metabolite heat map.

Results and discussion

The root length of seedlings of two AhNF-YC OE Arabidopsis lines grown in vitro became significantly longer than WT plants as they developed. The number and length of root hairs in 5-day-old seedlings, although fewer and shorter than WT seedlings, also varied between the different *AhNF-YC* OE lines. On another hand, a larger rosette area, compared to WT plants, was observed in AhNF-YC OE Arabidopsis, in particular the L5 line, had a much delayed flowering time compared to WT plants. However, L5 plants grew significantly larger inflorescences and had higher fresh weights once their life-cycle was completed (Figure 1).

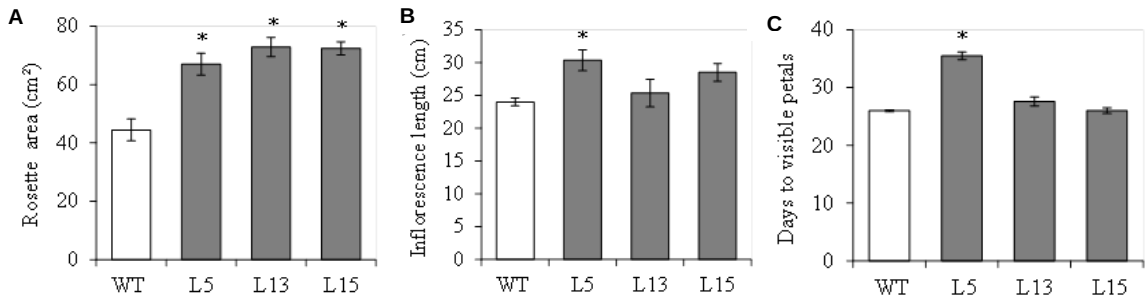

Figure 1. The overexpression of *AhNF-YC* in Arabidopsis modifies its morphology and growth rate. **A)** Rosette area, **B)** inflorescence length, **C)** time elapsed between germination and the appearance of the first petals, after a 4-week growth period, in wild type plants (WT) and in different transgenic plant lines (L5, L13,L15) overexpressing the *AhNF-YC* gene.

The induced expression of the *AhNF-YC* gene under water stress conditions in grain amaranth, suggested that this gene could be a factor involved in plant water stress resistance. In order to test this possibility, survival to these conditions was evaluated in 3-weeks-old WT and *AhNF-YC* OE L5 plants which were subjected to severe water stress by witholding watering for several days. The plants were later allowed to recover by restoring water supply in order to score the survival rate. Under these conditions, *AhNF-YC* OE L5 plants showed a significantly increased water stress survival rate as compared with WT plants (90.7% vs. 64%). It was observed, also, that *AhNF-YC* OE L5 plants showed lesser signs of damage than WT plants after exposure to 8 d of water stress conditions. This behavior was consistent with slower water loss in detached rosette leaves of *AhNF-YC*

transgenic plants, and to their increased turgor and decreased wilting under water stress conditions (Figure 2).

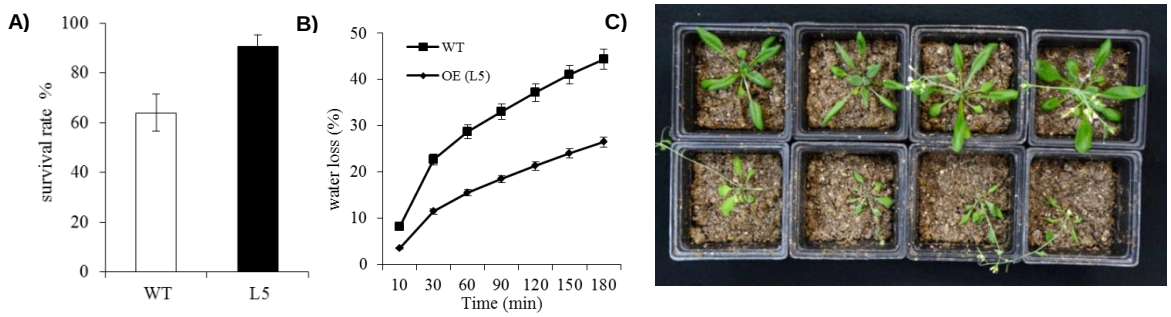

Figure 2. Water stress condition and recovery. **A)** The survival rate determined one day after watering was reestablished. **B)** Water loss rate in leaves of *AhNF-YC* transgenic [OE (L5)] and wild type (WT) plants, and the fresh weight (FW) was measured at the time intervals indicated. Water loss was calculated from the decrease in FW compared with time zero. **C)** The aspect of the transgenic [(OE-(L5)] and WT plants, 8 d after water stress treatment.

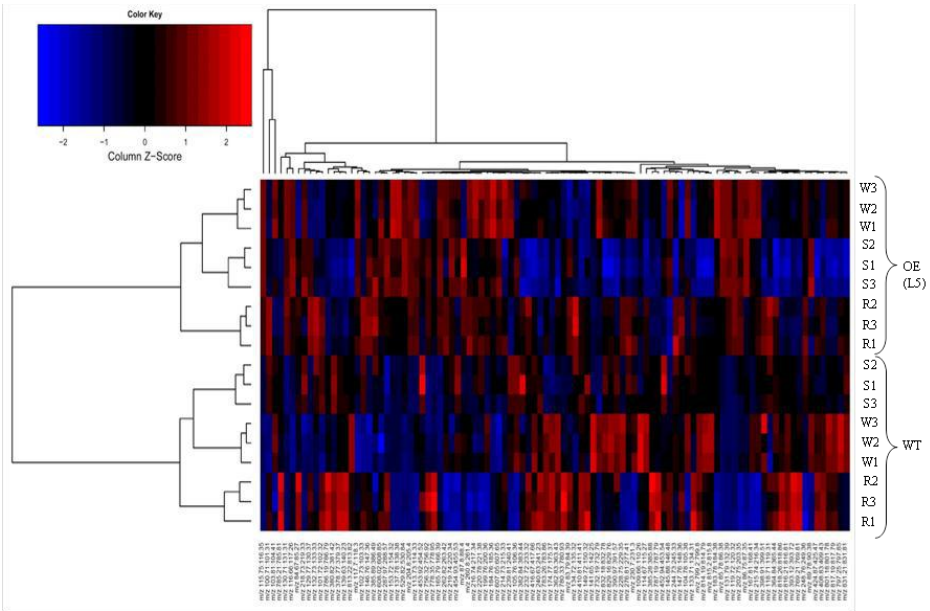

Figure 3. The overexpression of AhNF-YC in Arabidopsis modifies its metabolic pattern in well-watered conditions and in both water stress and recovery conditions.

Untargeted metabolic analysis was used to evaluate the metabolomic variability between WT and *AhNF-YC* OE L5 plants in well-watered conditions, under water stress and following recovery after stress. The metabolite fingerprint included more than 900 different ionizable molecules. From these, the 100 most abundant metabolites were selected to obtain a metabolic heat-map, from which a dendrogram was constructed (Figure 3). The analysis of

the metabolomic data using this approach indicated that both genetic modifications and water stress strongly influenced the metabolome. Thus, the results showed a well-defined grouping of samples according to the genotype, clearly separating WT from AhNF-YC OE L5 plants. Besides, the metabolite profiles produced by water stressed plants, were also grouped apart in a genotype-dependent manner. These clearly differed from those derived from recovered plants, which also clustered according to their genotype.

Conclusions

The visible effects on development and abiotic stress responses caused by the ectopic expression of the AhNF-YC gene in Arabidopsis were indicative that this gene may be an important regulator of these processes in plants. Based on the experimental evidence obtained in this study, it may be concluded that this particular NF-YC subunit may exert these effects in plants, at least in part, by regulating phytohormone biosynthesis, sensitivity and/or signaling, particularly with respect to ABA, by a still unknown mechanism(s). A first step toward its elucidation, now in progress, will be to determine the ability of this AhNF-YC subunit to associate with other NF-YA and NF-YB subunits in grain amaranth and, perhaps also, in Arabidopsis.

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Isolation and characterization of microorganisms with biodegradation activity on the pesticides atrazine, chlorothalonil and chlorpyrifos

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Abstract
Pesticides in soil present a grave pollution problem, as they can remain immobilized for long periods of time. In the present work, soil samples with both a history of pesticide application (atrazine, chlorothalonil, chlorpyrifos) and untreated were collected in order to isolate microorganisms capable of biodegrading such compounds, to be used as bioremediation agents. By using growth tests in solid and liquid media spiked with pesticide concentrations ranging from 100 to 500 mg L^{-1}, several bacteria capable of growth were selected, including from the genders *Acinetobacter calcoaceticus, Pseudomonas* sp*., Enterobacter* sp*., Pantoea ananatis* and *Bacillus thuringiensis.*

Keywords: Bioremediation • Soil • Pesticides

Introduction

Pesticides are organic synthetic compounds used for agricultural pest control. Even using the best agricultural practices, these are often misapplied, and thus released in the environment at high concentrations, causing soil and water contamination, with severe health effects in human, animals and plants; therefore, bioremediation is required (Zablotowicz *et al*. 2006). Bioremediation is an environmental cleaning technique which uses microorganisms to degrade or neutralize chemical compounds such as pesticides (Hindumathy and Gayathri 2013). One of these compounds is atrazine, an herbicide used worldwide either alone or in combination with others herbicides. It is an active environmental pollutant due to its low biodegradability, having high potential to contaminate soils and surface and ground water (Azizullah *et al*. 2014). Chlorpyrifos is a type of organophosphorated pesticide that has been used worldwide since the 60´s for the control of foliar insects, producing dangerous effects on the environment. The contamination has been found up to 24 km away from the point of use. If the pesticide is not degraded or detoxified quickly, it could affect rivers, lakes and many organisms (Kumar 2011). Chlorothalonil is a broad spectrum chlorinated fungicide, which is extremely toxic to fish, and it is considered a probable human carcinogen. The objective of this study was to isolate and characterize native strains of bacteria capable of degrading pesticides from contaminated soils with atrazine, chlorpyrifos and chlorothalonil and establishing bioremediation schemes of contaminated soils.

Materials and methods

Samples of the rhizosphere in agricultural soils where the pesticides [atrazine (6-cloro-N2-etil-N4-isopropil-1,3,5-triazina-2,4-diamina), chlorothalonil (1,3-dicianotetraclorobenceno) y chlorpyrifos (O, O-dietil, O-3,5,6-tricloropiridina-2-il fosforotioato)] are routinely applied were collected, as well from untreated soils. Approximately 1 kg of soil was taken from the rhizosphere, at a depth of 0-10 cm, using polyethylene bags for each sample.

A serial dilution (1:1000) was performed, and this sample was extended over LB media mixed with each pesticide at 200 mg $L^{-1.}$ Colonies that grew in the plates were selected and score in a plate for further purification in LB media. Once purified, they were inoculated again in LB media, but this time the pesticide concentration was increased to 400 and 500 mg L^{-1} (Akbar and Sultan 2016). Additionally, the isolated strains were also incubated in minimum medium added with 200 mg L^{-1} of each pesticide to observe their growth. The isolated microorganisms were kept at -70°C in 20% glycerol.

The structure of the cell wall was analyzed by Gram staining. McConkey agar was used to identify the morphology and typo of cell wall of the isolated strains. The morphology of the colonies and its pigmentation was identified in LB medium, as well as fluorescence (characteristic of *Pseudomonas* sp.) using UV light in minimum media. Finally, to further characterize the bacteria, an antibiogram using the protocol of Kirbi-Bauer (disc diffusion) using oxacillin (1 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg) and cefoxitin (30 µg).

A growth curve was obtained for each strain, by measuring the optical density at 620 nm in LB medium added with 100 mg L^{-1} of pesticide. DNA was extracted of each pure strains, and approximately 500 pb fragment of the 16S rRNA gene was amplified by PCR using the following specific primers: PLB16-F 5´-AGA GTT TGA TCC TGG CTC AG-3´ and MLB16-R 5´-GGC TGC TGG CAC GTA GTT AG-3´. The PCR products were sent to an external sequencing service (Institute of biotechnology, UNAM). The returned sequences were matched by a BLAST query in the gene bank of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Results and discussion

A total of 39 isolated microorganisms were capable of growing in different pesticide concentration. Most soil bacteria were Gram negative (20 grew on the selective media) and five of they were able to ferment lactose. Additionally, four isolated tested positive for fluorescence after exposure to UV light. In the antibiogram, all microorganisms were resistant to clindamycin, and 96% to cefoxitin y oxacillin.

After the BLAST query, we found that some of the isolated microorganisms correspond to the genera *Acinetobacter calcoaceticus, Pseudomonas* sp*., Enterobacter* sp*., Pantoea ananatis* y *Bacillus thuringiensis*, all of these genera are typically used in bioremediation and biological control schemes. Figure 1 shows a phylogenetic tree built using four of our isolated strains as well as other soil and reference bacteria.

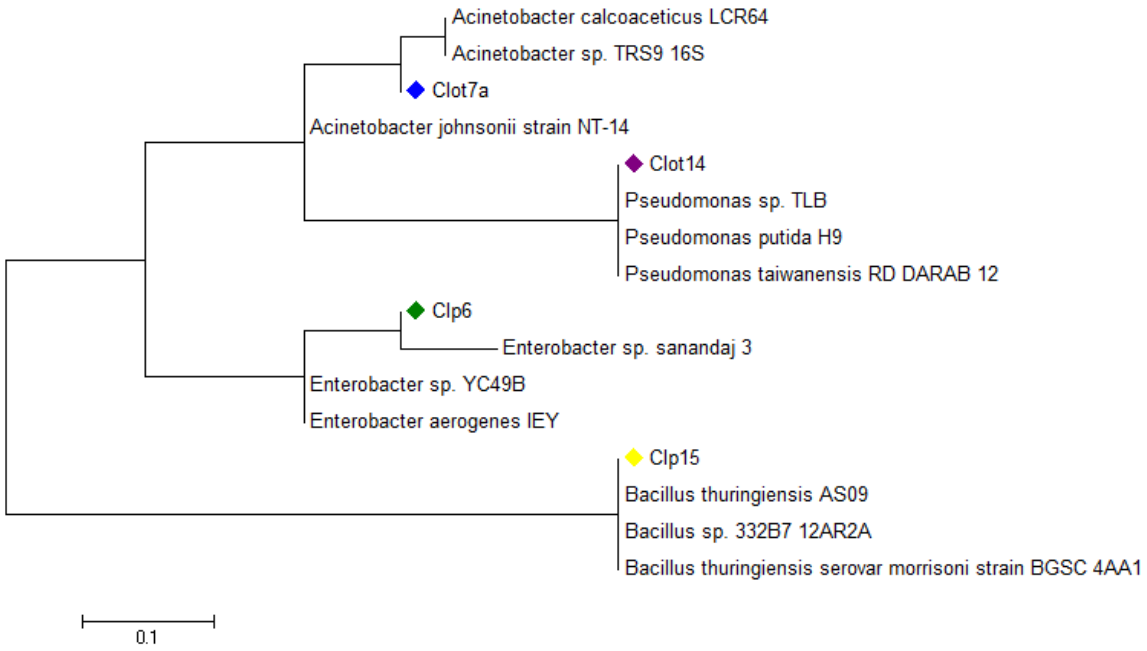

Figure 1. Molecular phylogenetic analysis obtained by the method of maximum likelihood using the Jukes-Cantor model.

An assay in minimal medium added with three different compounds, atrazine, chlorpyrifos and chlorothalonil, was developed with the purpose to identify bacterial strains that could grow in the presence of pesticides. The results showed that many of them could be developed being in contact with more than one type of pesticide (Table 1).

Table 1. Strains of study that have higher growth in minimal medium supplemented with pesticides.

	Minimal	M+Atrazine	M+ Chlorpyrifos	M+ Chlorothalonil
Riz 3	+	+	+	+
Riz 4	+	+	+	+
Riz 6	+	+	+-	+
Riz 10	+	+	+	+
Clp 6	+-	+	+	+
Clp 14	++	++	++	++
Clp 15	+	+	++	+
Clot 7a	+	+	+	+
Clot 9b	+	+	+	+
Clot 14	+	++	+	+

++ abundant growth, + moderate growth, +- poor growth.

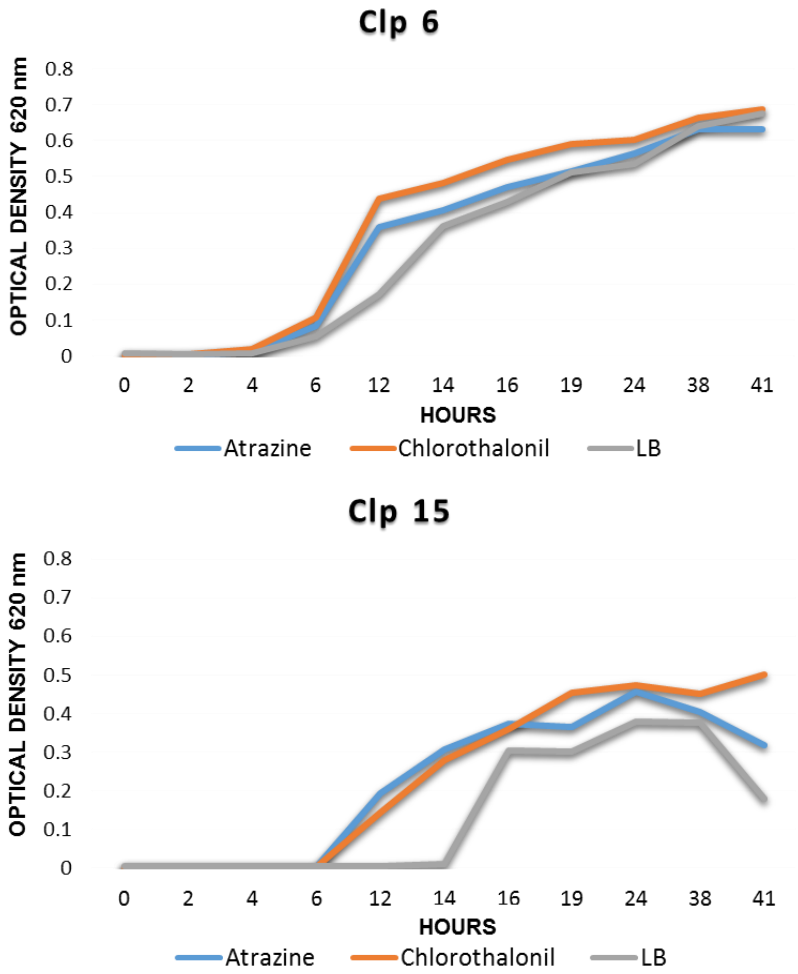

Figure 2. Kinetics of growth of two bacillus Gram negative.

Growth kinetics using LB medium supplemented with 100 mg L^{-1} of pesticide, were developed to identify whether the bacteria can grow and reproduce in these conditions. The results obtained (Figure 2) shows two strains that obtained the highest growth being in contact with pesticides compared to medium without the compound.

Conclusions

The results of isolation, identification and ability to growth in media with pesticide, demonstrates the potential of microorganisms isolated in this work to be used as agents for bioremediation of soils contaminated with pesticides.

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Impact of agricultural practices on soil fungal diversity associated to durum wheat (*Triticum durum*) production in the Yaqui Valley, Sonora

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Abstract
The wheat production concentrates in the state of Sonora, representing 48% of national production, due to Yaqui Valley's contribution, where 170 thousand hectares are sown. Yaqui Valley is characterized by its limited contents of organic matter, alkaline soils, semi-arid weather, low precipitation, and excessive chemical fertilizer additions, driving to degradation and desertification of soils. The objective of the present work was to quantify the impact of the agriculture practice on soil fungal population and diversity in the Yaqui Valley. Sampling recollection was on soil with 3 types of farming practice: chemical, mixed and organic fertilizer. The analyses were focused in organic matter, fungal diversity and population. The microorganisms' isolation was performed with serial dilutions method and organic matter content was measured under NOM-021-SEMARNAT-2000. Sites with organic and mixed farm management vs. inorganic farm management shows a higher percentage of organic matter, higher fungal population and greater diversity, that due to the incorporation of organic fertilizers. A lower fungi population was observed on chemical site is possibly due to low content organic matter.

Keywords ● farming practices ● organic matter ● fungal genders

Introduction

The Yaqui Valley, located in the State of Sonora, México, contributes with 48 % of the national wheat production. This production has been characterized by the use of high levels of synthetic fertilizers, *i.e.* N (263 kg/ha), P_2O_5 (120 kg/ha), and K_2O (60 kg/ha), generating low use efficiency of these nutrients by plants (38% for nitrogen fertilizers), representing high costs for farmers, by leaching and volatilization (Cortes *et al.* 2011). Excessive uses of synthetic fertilizers has led to increase the generation of greenhouse gases, pollution of groundwater, and ecological imbalance in the soil, characterized by low availability of nutrients (C, N, P and K), and modifying the native soil microbiome associated to the crops (Mohammadi, 2011).

At present, organic fertilizers are appealing methods for carrying out a sustainable agriculture, because they improve the soil structure by increasing the porosity, the water retention capacity, and increase nutrient avaibility (Priyadharsin *et al*. 2015). In addition, they contribute with organic matter to the soil, which modify the native and/or inoculated soil microbial community. These microorganisms are responsible of the organic matter degradation, plant growth, nutrient cycling, and degradation of pollutants and pesticides in the soil (Suhr *et al*. 2014; Liu *et al.* 2014). Thus, organic matter with a C/ N lower favors the activation of microorganisms' short term; on the other hand, the C/ N high helps

microorganisms long term (Rodríguez-Romero *et al.* 2011). Thus, the microbial diversity in these organic fertilizers has a relevant impact on its potential function in the soil x crops interaction, where fungi represent 70% of the total microbiome. The fungi community is involved in solubilization of nutrients (mainly phosphorus), tolerance to various stresses (biotic and abiotic), diseases control and improve quality and soil structure. The population of fungi is high when the soil is rich in plant residues due the fungi are saprophytic (Julca-Otiniano *et al*. 2006; Priyadharsin *et al.* 2015), however, the Yaqui Valley is characterized by low content of organic matter, alkaline soils, semi-arid weather, low precipitation, and excessive chemical fertilizer, driving to degradation and desertification of soils, which potentially modify microbial communities, mainly fungi (Cervantes, 2014). In this context, the aim of the present work was to quantify the impact of the different agriculture practices on soil fungal population and diversity in the Yaqui Valley.

Materials and methods

Sampling site. This study was carried out selecting three wheat commercial fields located in the Yaqui Valley, Sonora, having different agricultural practices in terms of fertilizer sources: a) traditional chemical fertilizer, b) organic matter incorporation plus chemical fertilizer (mixed), and c) only incorporation of organic matter (Table 1).

Table 1. Sources of fertilizer applied to the soil in each study site in the Yaqui Valley.

Chemical	Mixed	Organic
Ammonia gas	Urea	Chicken manure
Phosphorus	Bat guano	Earthworm humus
	Phosphorus	Fish concentrate

A total of 25 soil samples were collected (30 cm depth) according to SAGARPA & National Sanity Service (2015). The samples were transported at 4°C for their microbial and organic matter analysis. The organic matter content was measured under NOM-021-SEMARNAT-2000, it was carried out with the method of Walkley and Balck.

Fungal isolation. This assay was performed through dependent culture techniques. It was conducted using the serial dilutions (1:10) method until 10^{-4}. Later, 0.1 mL of these dilutions was inoculated on Petri dishes containing Potato Dextrose Agar as culture media, supplemented with 80 ppm of nalidixic acid. Inoculated Petri dishes were incubated for 2 days, at 28°C, and Colony Forming Unites (CFU) were counted, in order to estimate and the fungal population for each site (Cordova-Bautista *et al.* 2009).

Fungal molecular identification. Fungal DNA was extracted with a phenol-chloroform method reported by Raeder *et al*. (1985). Molecular identification of fungal isolates obtained was performed amplifying the 5.8S rRNA gene by primers ITS1F (5´ - TCCGTAGGTGAACCTGCGG-3´) and ITS4R (5'- TCCTCCGCTTATTGATATGC-3'). 50 µL PCR mixture containing 100 ng DNA template, 0.2 µmol of each primer, and 4 U MyTaq DNA polymerase (Bioline). The PCR condition had an initial denaturation step at 94°C(3 min), 35 cycles of denaturation at 94°C(30s), followed by annealing at 55°C(30 s), and extension at 72°C(1 min), and a final step at 72°C(10 min). The PCR products were verified by electrophoresis on gel agarose/TAE (2%), and amplicons were sequenced by the Sanger platform. The sequences obtained were edited using the software FINCH TV, and analysis of the sequences was performed by BLAST.

Statistical analyses. The data obtained were analyzed with the analysis of variance test (ANOVA) one-way and Tukey-Kramer method (P ≤ 0.05) using the Statgraphics Plus 5.1 software.

Results and discussion

The organic matter content among the study sites showed significant differences, being the lowest value (0.45%) observed in the soil under chemical fertilizer. The study sites receiving organic fertilization had high organic matter content, *i.e.* mixed site 1.5% and organic site 1.6%, due to these agricultural practices increase the organic matter content in the soil around 13g/ kg soil (Ramirez *et al.*, 2002). Lopez-Martinez *et al.* (2001) reported that the addition of manure into the soil changed its organic matter contents, showing an initial content from 0.6 - 0.9%, and increasing from 1.1 to 1.5%. The fungi community is a main component in this type of organic fertilizers, which are involved in the organic matter decomposition releasing nutrients by its saprophytic traits (Priyadharsin *et al.* 2015). In this study, we found that the fungal population (Figure 1) and diversity (Figure 2) were higher in study sites incorporating organic matter compared to the traditional chemical fertilization. Zhen *et al*. (2015) reported around 50% more fungi population in the soil under organic fertilization (manure and manure + bacterial) than soil under chemical fertilization (Urea). Zhang *et al.* (2015) determined by PLFA (Phospholipid Fatty Acid Analysis), that the highest PLFA was found in the soil under organic fertilization (37-40 nmol/g soil) compared to the soil under chemical fertilization (33 nmol/g).

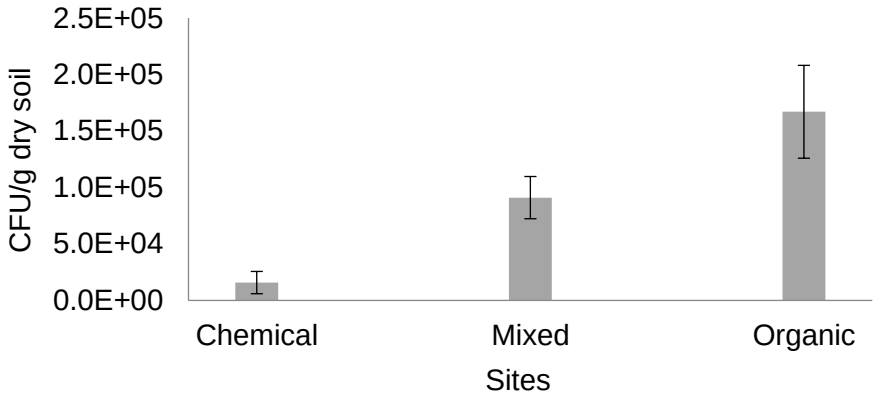

Figure 1. Fungi population quantified in the study sites under different gradient of organic matter incorporation in the Yaqui Valley, Sonora

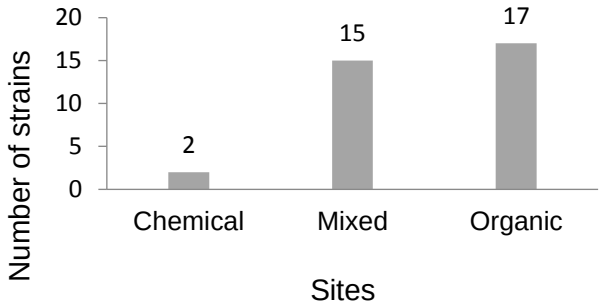

Figure 2. Numbers of strains isolated from the soil under different farming practices in the Yaqui Valley, Sonora

The molecular analyses of the isolates by the amplification of the 5.8S rRNA gene showed 8 fungal genera for the field under organic and mixed fertilization, and 2 different fungal genera for the chemical site (Table 3). The fungi diversity in field under chemical fertilization is potentially affected by the low soil organic matter content (0.45%), due to the biological activity of soil depends directly on the organic matter content (Maganhotto *et al*. 2016). In arid zones (as the Yaqui Valley, Sonora) have been reported the presence of the genera *Aspergillus* and *Penicillium* (Samaniego-Gaxiola *et al*. 2007), which are reported as plant growth promoters, these genera were isolated only in the soil under organic and mixed fertilization. In addition, the study sites under organic fertilization were isolated *Trichoderma* and *Penicillium* characterized as biological control agents (Felix *et al*. 2008). The fungal genera isolated in the study site under organic fertilization (*Aspergillus, Cladosporium, Penicillium and Rhizopus*) have been associated to the chicken manure (Escobar *et al.* 2012). On the other hand, in the mixed site the genera *Aspergillus, Penicillium and Rhizopus* have been associated to bat guano *(*Ogórek *et al.* 2016). Only 2 genera were isolated from the soil under chemical fertilization, probably due to negative impacts of applied inorganic ammonia gas and phosphorus.

Table 3. Fungal genera diversity in the study sites.

Genus	Chemical	Mixed	Organic
Acremonium			x
Aspergillus			x
Chaetomium		x	
Cladosporium			x
Dothideomycetes		x	
Myrothecium		x	x
Mortierella	x		
Penicillium		x	x
Pyrenochaeta		x	
Rhizopus			x
Setophoma	x		
Stachybotrys		x	
Talaromyces			x
Trichoderma		x	x
Volutella		x	

Conclusion

Commercial wheat farms under organic and mixed fertilization management showed a higher organic matter content, fungi diversity and population compared to the study sites under chemical fertilization, probably to the incorporation of external sources of organic matter. However, the ecological impact of the external fungal populations on the native microbiome and the nutrient cycling need to be studied, in order to develop eco-friendly alternatives for the wheat production in the Yaqui, Valley.

Acknowledgements The authors acknowledge support by the Cátedras CONACyT Program through Project 1774 “Alternativas agrobiotecnológicas para incrementar la competitividad del cultivo de trigo en el Valle del Yaqui: desde su ecología microbiana hasta su adaptabilidad al cambio climático”, and the CONACyT Project 253663 “Fortalecimiento de la infraestructura del Laboratorio de Biotecnología del Recurso Microbiano del ITSON para la creación de COLMENA: COLección de Microrganismos Edáficos y Endófitos NAtivos, para contribuir a la seguridad alimentaria regional y nacional”.

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Micropropagation in vitro evaluation of the culture media WPM and MS; and molecular characterization of genotypes of *Rubus*

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Abstract

A comparison of culture media WPM and MS, for Autumn bliss raspberry and blackberry Tupi, in homogeneous temperature and photoperiod conditions added with 30 g L^{-1} sucrose, 8 g L^{-1} of Bacteriological agar, 1.0 mg L^{-1} of PVP and 1.0 mg L^{-1} BA. Efficiency based on the ratio of shoot multiplication was evaluated, obtaining the best results with MS medium, sequencing of a wild organism blackberry collected in Cerro Viejo Jalisco was sequenced and identified as *Rubus glaucus* a bank formed free wild germplasm and commercial species of blackberry and raspberry, molecularly characterized ISSR PCR with oligos, achieving a specific grouping including through a dendrogram.

Keywords • Culture Media • *Rubus fruticosus* • *Rubus ideaus* • Berries • ISSR.

Introduction

Blackberry (*Rubus fruticosus*) are shrubs with woody stems, armed with thorns, flowers 3 cm white or pink, fleshy fruit called "polidrupa". Raspberry (*Rubus ideaus*) are perennial shrubs, reaching 2 meters high, have woody, rounded, erect, reddish, equipped with slender spines stems, flowers 1 cm in diameter, white and gathered in inflorescence up to 10 flowers each , fruit "polidrupa" called raspberry. (González & Céspedes *et al.* 2010). They are of great economic importance, are considered as functional foods for its antioxidant and disease prevention components contain phenolic compounds, anthocyanins, flavonoids, to name a few.

High consumption of these fruits, vegetable cultivation *in vitro* offers a solution to the demand for seedlings of blackberry and raspberry for agronomic crop. So it is necessary to start with the establishment micropropagation protocols in large-scale systems for mass multiplication and allow the rapid introduction in the field of new genotypes elites disease resistant and high productivity, which can be initiated or improved from genebank, molecular characterization to be important to determine the proximity of individuals, achieving a grouping potentiation species and genetic improvement, including. By obtaining a gene bank of wild species and free commercial varieties, the study of the genus *Rubus* be expanded in our country, highlighting the economic importance it represents for the state of Jalisco and Michoacán; and opening doors to new scientific research relevant to the biotechnology and genetic development of these crops.

Both blackberry and raspberry are woody species for plant tissue culture important to the success of micropropagation factor is the culture media and are two most commonly used one is the WPM (Lloyd and McCown *et al.* 1980) is a culture medium of low concentration of salts particularly suitable for woody species, compared to MS medium (Murashige & Skoog *et al.* 1962), contains a greater amount of macroelements and salts which have higher concentrations of nitrogen. Regeneration of adventitious shoots from leaf discs, assessed macronutrient composition of the media WPM and MS variety of raspberry Autumn bliss, obtaining 4.1 buds per explant for the average WPM and 5.0 shoots per explant for MS medium. (Ambrozic *et al.* 1994). Using meristem buds the best result with MS medium containing 2 mg l-1 BA and GA3 0.5 was obtained, obtaining a number of outbreaks of 3.3 (Najaf-Abadi *et al.* 2009). Leaf sections were used as explants for regeneration of plantlets obtained raspberry 7% regeneration, using 2.0 of benzyladenine (BA) and 0.1 of indolebutyric acid (IBA). (McNicol & Graham *et al.* 1990).

Materials and methods

This work was carried out in the laboratory of Molecular Biology Institute of Technology Tlajomulco (ITTJ). Field material that was collected of Cerro Viejo Jalisco was obtained, whereas commercial varieties (free) of raspberry herintage and autumn bliss were also taken; and varieties dasha, brazos and Tupy blackberry.

For plant tissue culture the Autumn bliss variety of raspberry and Tupy variety of blackberry were used, 10 explants of 2 cm disinfection treatment consisting of washing with detergent 1% for 30 seconds was performed were cut, these explants were disinfected with chlorine to 20% with two treatments, T1 for Blackberry with 10 min of exposure and raspberry T2 for 20 min; once completed three rinses of sterile distilled water were performed before placing the explants in media MS and WPM culture for comparison were added with 30 g L^{-1} sucrose, 8 g L^{-1} of Bacteriological agar, 1.0 mg L^{-1} PVP and 1.0 mg L^{-1} BA. This treatment was performed in triplicate and the same conditions of temperature 26.5 °C and 16 hour photoperiod light incubation, evaluating the effectiveness of the culture media in relation to the multiplication coefficient of new outbreaks.

DNA extraction from plant material was performed, as the protocol says Doyle & Doyle *et al.* (1987). PCR was performed using oligos ITS-1 (-TCCGTAGGTGAACCTGCGG-) and ITS-4 (-TCCTCCGCTTATTGATATGC-) for the DNA sample of wild blackberry plant collected in Cerro Viejo Jalisco was purified with the kit SV Gel and PCR Wizard® Clean-up system, was sent to be sequenced and compared to the NCBI, GenBank. The ISSR oligos were tested for polymorphic bands and build a dendrogram with NTSYSpc 2.20 program to achieve in grouping among agencies. To make the dendrogram a DNA sample cranberry biloxi variety and a sample of *Agave tequilana* added.

Table 1. PCR conditions for ISSR.

TEMPERATURE	TIME	CYCLES
94°C	4'	1
94°C	1'	35 cycles
Temperature of primer	1'	
72°C	2'	
72°C	7'	1
4°C	∞	1

Results and discussion

The disinfection treatment for blackberry Tupy variety 15% contamination by fungus and 4% oxidation, the multiplication coefficient of MS medium was 7.18 I get over the WPM being 6.51 respectively and obtained from the averages of the treatments was obtained. Meristem regeneration in Tupy variety 7.02 was obtained under similar conditions with the exception of PVP, by Bobrowski et al. (1996). In the case of disinfection treatment raspberry variety Autumn Bliss 18% fungus contamination by bacteria and 4%, the multiplication coefficient was 8.13 and 6.20 with MS medium with WPM medium was obtained. In raspberry with this variety with MS medium it obtained an average multiplication rate of 6.40, study by Pedroso *et al.* (2008) The results for the two varieties indicate that the MS medium (Murashige & Skoog *et al.* 1962) outstripped the WPM (Lloyd and McCown *et al.* 1980) for the two treatments performed for each of the species blackberry and raspberry, similar results were found by Ambrozic *et al.* (1994).

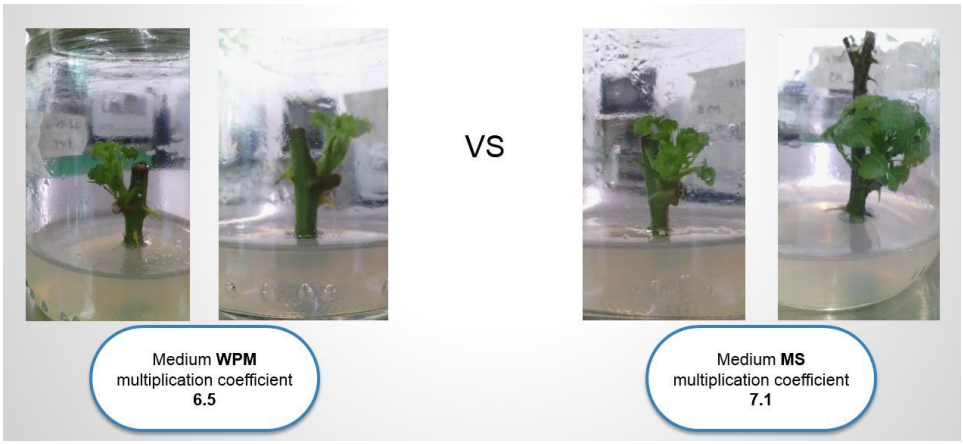


Figure 1. Coefficient multiplication of blackberry Var. Tupy.

The presence of DNA extraction was checked by agarose gel 1%. We performed the with ITS1 and ITS4 oligos, the result of sequencing for wild blackberry cerro viejo Jalisco was obtained and was analyzed and compared in the database NCBI GenBank, resulting in 100% coverage and 92% identity at the species level with *Rubus glaucus*. Raspberry Autumn Bliss for 95% coverage of the total sequence shown and identity to species of *Rubus ideaus* with 100% was obtained.

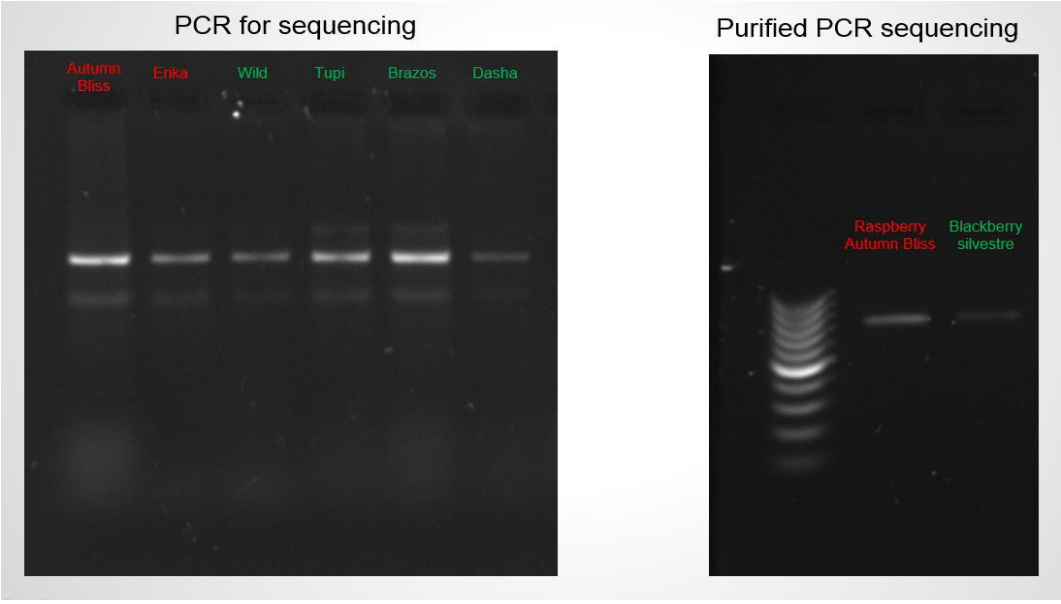
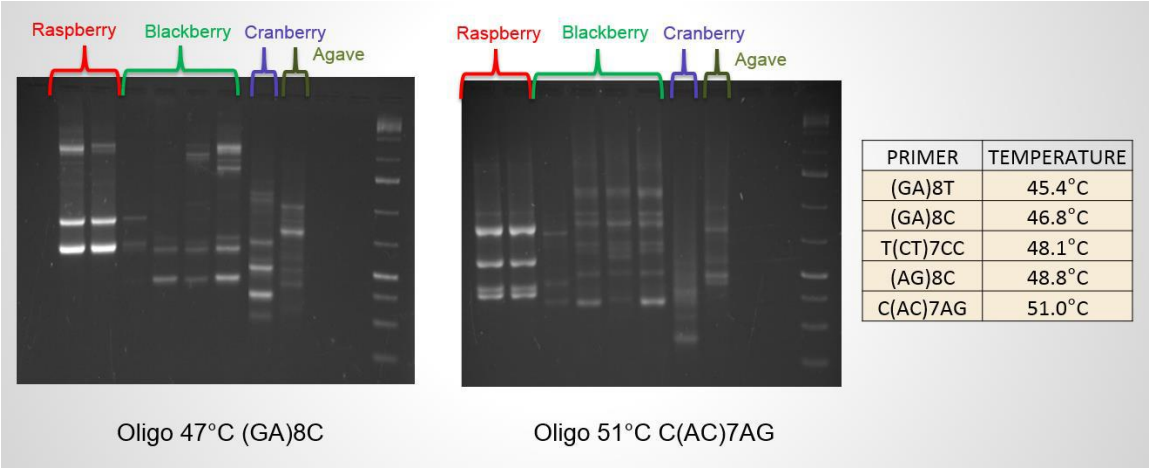


Figure 2. PCR with oligos ITS1 and ITS4 and purification.

The ISSR oligos of which five oligos which gave a greater number of polymorphic bands were chosen were tested (GA) 8T (GA) 8 C, T (CT) 7CC (AG) 8C and C (AC) 7AG. It based on the coefficient of Jaccard similarity observed species grouping raspberry and blackberry was constructed one dendrogram.



PRIMER	TEMPERATURE
(GA)8T	45.4°C
(GA)8C	46.8°C
T(CT)7CC	48.1°C
(AG)8C	48.8°C
C(AC)7AG	51.0°C

Figure 3. Oligos PCR ISSR.

Raspberries represent the first group in the dendrogram, which are linked to the next group are blackberries and within this there is a narrower molecular similarity between varieties Tupi and brazos and turn are linked to the wild blackberry Cerro Viejo Jalisco and Dasha farthest variety; In the third group is the blueberry and end in the fourth *tequilana agave* group. These grouping allow us to discover the similarity or genotypic differences between each of the varieties of raspberry and blackberry, giving the tools for genetic improvement based on this molecular analysis.

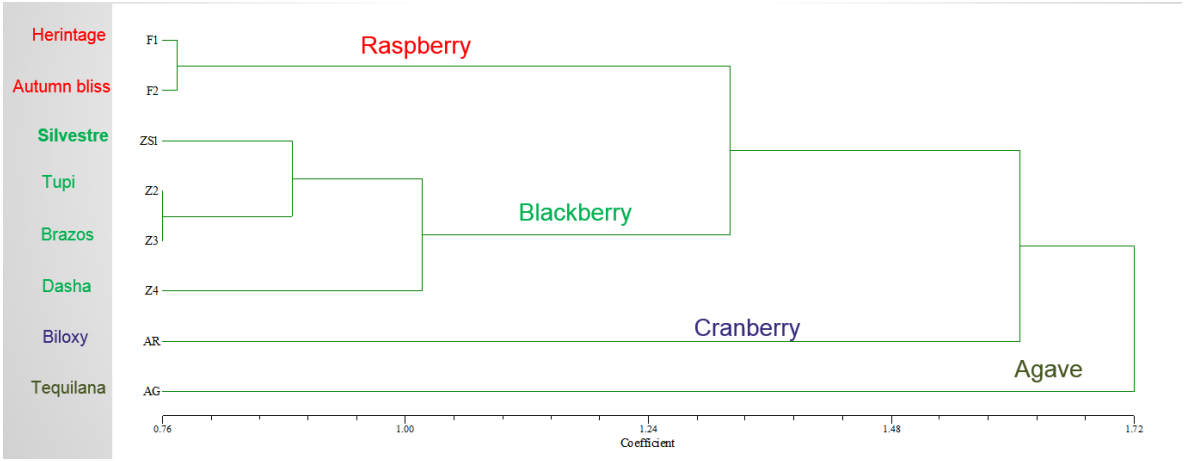


Figure 5. Dendrogram grouping raspberries and blackberries.

Conclusions

It is checked with the conditions established in the treatment of *in vitro* culture of Tupi blackberry and raspberry Autumn bliss, which in culture medium MS have a better efficiency in increasing the multiplication coefficient of outbreaks in these varieties compared to the WPM medium. One old wild plant native to Cerro Viejo Jalisco, same as was sequenced and identified as *Rubus glaucus* molecularly, obtaining a gene bank of wild material that develops in a producing region berries as Jalisco was collected. Based on the genebank a dendrogram that indicates the grouping of each species and its closeness between them took place, wild blackberry worth noting that Cerro Viejo Jalisco has a greater closeness molecularly speaking with blackberries varieties Tupi and brazos; opening doors for genetic improvement.

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Identification of compounds involved in plant growth-promotion in *P. fluorescens* and insights from the genome sequence

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Abstract

Four new rhizospheric isolates were identified as *Pseudomonas fluorescens* UM16, UM240, UM256, and UM270, based on their complete 16S ribosomal gene sequencing. These pseudomonads showed a high degree of antagonism against the phytopathogen *Botrytis cinerea*. In addition, all strains significantly increased *Medicago truncatula* biomass and chlorophyll content. These activities were exerted by the emission of either diffusible organic compounds or VOCs. In biocontrol experiments, the four strains were able to protect *M. truncatula* plants from *B. cinerea* infection. Genetic and biochemical analysis revealed the potential activity and production of phenazines, cyanogens, and ACC deaminase, biofilm, siderophores, proteases, indole-3-acetic acid and sulfur-containing volatiles. Comparative genomic analysis of strain UM270 with other pseudomonads revealed unique features involve in rhizosphere colonization plant-bacteria interaction. In conclusion, the *Pseudomonas* strains analyzed here exert multiple antagonistic and PGP mechanisms, and represent an excellent option to be used as either biocontrol or biopromoting agents in crops.

Keywords *Pseudomonas* ● Fungi ● Biocontrol ● Volatile Organic Compounds ● *Medicago truncatula.*

Introduction

Various studies have reported the capacity of diverse bacterial genera to control fungal diseases; however, the *Pseudomonas* genus appear frequently in the literature as biocontrol agents (Santoyo *et al.* 2012). Diverse *Pseudomonas* spp., which are common and abundant inhabitants of the rhizosphere, have the capacity to inhibit or suppress plant diseases are important actors in suppressive soils (Mendes *et al.* 2011). Similarly, when fluorescent pseudomonads are directly applied to seed or soil inoculation treatments, excellent biocontrol activities have been reported (De La Fuente *et al.* 2006). In the same sense, some pseudomonads also have the capacity to colonize the internal plant tissues, living as endophytes and exerting protective and plant growth-promoting activities (Santoyo *et al*. 2016).

Recently, comparative genomic analysis of *Pseudomonas* strains reveals the presence of unique genetic characteristics among strains, as well as a huge genetic diversity and traits involved in multitrophic interactions (Loper *et al.* 2012). The unique known work of genomic sequencing of any *Pseudomonas* strain in Mexico is the one published by Hernández-Salmerón and colleges (2016). It is expected that that more genomic work related to fluorescent pseudomonads be done in years to come, since there is a high climate and soil diversity in Mexico, requiring the use of rhizobacteria with several capacities to adapt and survive in multiple agro-conditions.

Rhizobacteria exhibit direct and indirect mechanisms to beneficiate plant growth promotion (Santoyo *et al.* 2012; Glick 2014). Direct promotion of plant growth occurs when a bacterium either facilitates the acquisition of essential nutrients or modulates of level of hormones within a plant, while indirect mechanisms comprise antagonism toward phytopatogens. Very few works in literature report strains containing both mechanisms. Here, we present four novel strains containing both, direct and indirect mechanisms of plant-growth promotion. We also describe the comparative genomic analysis of several fluorescent strains, including the UM270 isolate, which contains unique genetic elements involve in rhizosphere colonization.

Materials and Methods

Bacterial isolates with antifungal capacities were surveyed from *Medicago* spp. plant rhizospheres in an agricultural field in Morelia, Mexico. Genomic DNA was isolated from the four *P. fluorescens* strains, and the 16S ribosomal subunit DNA (rDNA) gene was amplified using PCR. The PCR products were purified and sequenced at the LANGEBIO (Accession Numbers: KJ801565–KJ801568). The evaluation of fungal antagonism was performed as previously reported on petri dish bioassays (Hernández-León *et al.* 2015). To evaluate whether the four isolates exhibit direct PGP activities, we employed leaflets of *M. truncatula* (ecotype Jemalong A17) plants and followed a protocol previously reported by Orozco-Mosqueda *et al.* (2013). Biocontrol experiments also involved *M. truncatula* plants and *B. cinerea* phytopathogen as previously (Martínez-Absalón *et al.* 2014).

Strains UM16, UM240, UM256, and UM270 were analyzed for diverse biocontrol and/or plant-growth promotion traits. Siderophore production was determined by the chrome azurol S (CAS) assay, while Skim Milk agar (SM) plates were used to detect protease production. Biofilm formation capacity in bacteria. IAA production was analyzed in filtered-culture supernatants from the four strains by GC–MS (Gas Chromatograph 6850 Series II – Mass Spectrometry detector 5973; Agilent, Foster City, CA, USA) analysis. NA cultures were supplemented with tryptophan and incubated with agitation (250 rpm) at 30 °C for 48 h. The identity of IAA was confirmed by comparison of the retention time in the bacterial extracts with samples of the pure IAA standard (Sigma–Aldrich). IAA amounts produced by strains were estimated using calibration curves for each strain.

VOCs produced by strains UM16, UM240, UM256, and UM270 were analyzed by solid phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS) on PDMS/DVB fibers (Supelco, Inc., Bellafonte, PA, USA). All experiments described in this section were performed in triplicate.

Genomic comparison was made using Mega 5. Original genome annotations were obtained directly from the NCBI nucleotide database. Previous to the analysis, all homologous sequences were identified by using Ublast algorithm, contained in USEARCH, against the non-redundant database, with an e-value 1e-6. SEED database was used to identify orthologs function between the *Pseudomonas* genomes. All unique genes found in UM270 strain were identified and blast-searched for an assigned function.

Results and Discussion

Identification of diffusible and volatile compounds

By sequencing the complete ribosomal 16S ribosomal genes, strains were characterized as *Pseudomonas fluorescens* species (UM16, UM240, UM256 and UM270). *Pseudomonas* strains exhibited differential growth promotion of *Medicago truncatula* plants, either by synthesis of diffusible compounds or by volatile organic compounds (VOCs) emission.

Chromatographic analysis showed that strains produce indolacetic acid (AIA) at different concentrations. Plant-bacteria interacting experiments revealed that strains produce diverse VOCs, including the dimethyl disulfide (DMDS) and dimethylhaxadecylamine, which has been previously assigned in our work group as a plant-growth promoting compound. *Pseudomonas* strains also exhibited antagonism toward the *fungi Fusarium oxysporum, Rhizoctonia solani, Diaporthe phaseolorum, Coletotricum linthemutianum* and plant protection from *Botrytis cinerea* (Figure 1). Respect to the latter, all strains showed strong mycelial inhibition in Petri dish assays, covering the plates with a red color pigment which has been related to the synthesis of 2,4-diacetylphloroglucinol (DAPG). PCR amplification showed the presence of the complete operon *phlACBD*, responsible of DAPG synthesis in all strains. As expected, we also detected genes related to phenazines, HCN, as well as the synthesis of siderophores and protease activity. Interestingly, *B. cinerea* induced *phlD* and *hcn* expression in *Pseudomonas* strains, which suggests bacterial strains are sensing and defending from the pathogen trough synthesis of this antibiotic.

Comparative genomic analysis

A total of six complete genomes of *P. fluorescens* and *Pseudomonas* sp. UW4 were downloaded from GenBank and compared against the recently sequenced strain UM270 (Hernández-Salmerón *et al.* 2016). Similar abundance of genes involved in primary metabolisms, such as amino acids, carbohydrates, proteins and DNA processing were found. Nevertheless, important gene function differences were analyzed between strains. *P. fluorescens* F113 and UM270 genomes displayed major abundances of functions involved in motility and chemotaxis. Presence of virulence, disease and defense are common activities among these strains similarly to metabolism of potassium, phosphorus, sulphur and iron. On the other hand, several functions vary between the members of this group of bacteria, such as metabolism of nitrogen, respiration, cell wall and capsule, stress response, secondary metabolites biosynthesis, regulation and cell signaling; this variation of gene functions could help to understand the great ability of colonization and adaptability.

¨Unique¨ genes in strain UM270

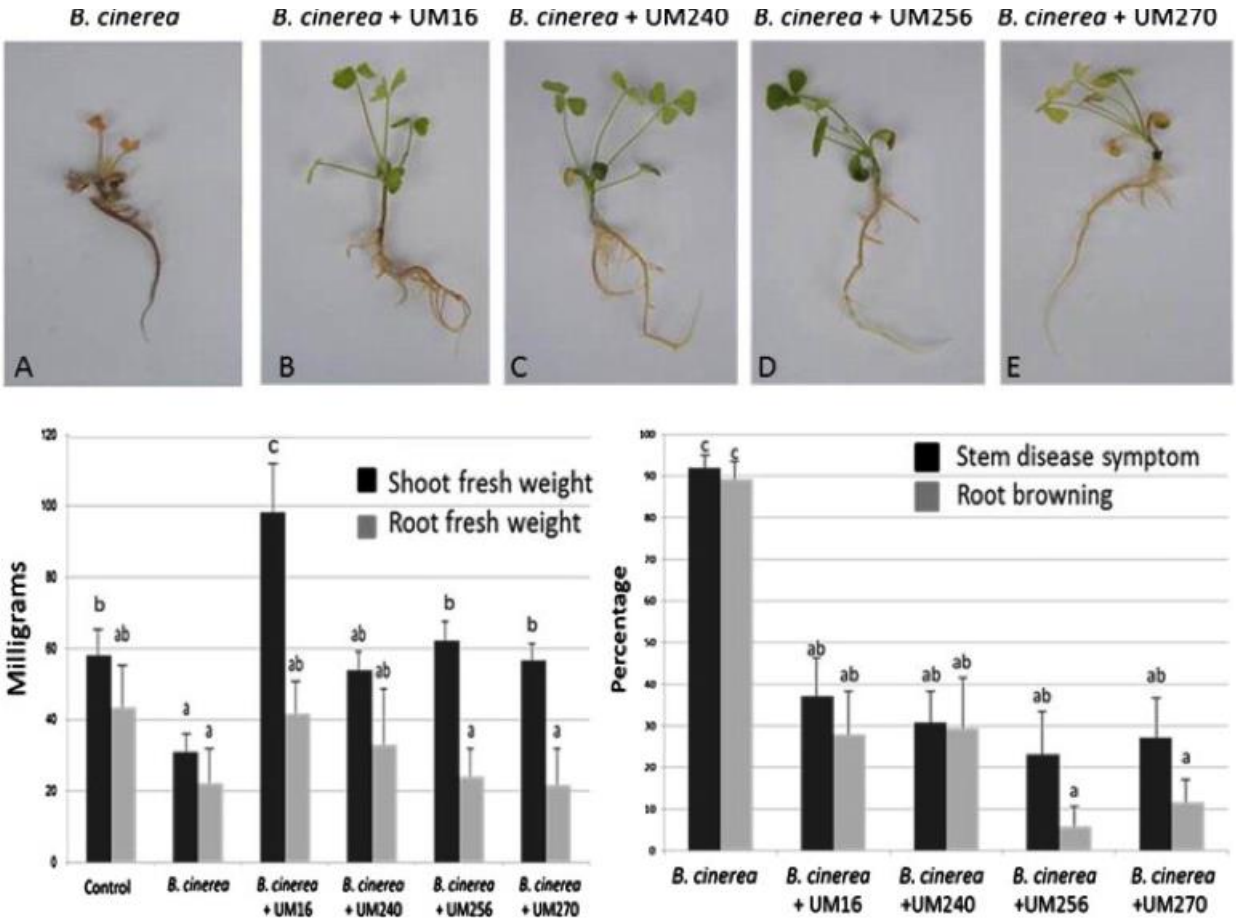

Figure 1. Biocontrol capacity of the *P. fluorescens* strains UM16, UM240, UM256, and UM270 against *B. cinerea* in *M. truncatula* seedlings. Representative plants (n = 12) are shown in different panels (A–E). (A) Control plant inoculated with *B. cinerea* fungus. (B) Plants coinoculated with *B. cinerea* and UM16 (B), UM240 (C), UM256 (D) and UM270 (E). Left graphic shows the shoot (black bars) and root fresh weights (gray bars). Right graph shows stem disease symptoms (black bars) and root browning (gray bars). Letters indicate that means differ significantly by Duncan's multiple range test ($p < 0.05$). Taken from Hernández-León *et al*., 2015.

A total of 599 unique predicted CDSs in UM270 strain were identified. These protein-coding genes are not contained in any of the rest of pseudomonads strains surveyed in this study. From the total, 407 CDS were of unknown function. Therefore, only 192 could be functionally examined. These ¨unique¨ genes coded in the UM270 genome we related to rhizosphere colonization, competence and survival, but full list will be presented in the conference.

Conclusions

Pseudomonas fluorescens UM16, UM240, UM256 and UM270 have a high potential to be used as either biocontrol or biopromoting agents in crops.
Comparative genomic analysis of strain UM270 with other fluorescent strains reveal unique features involve in rhizosphere colonization and plant-bacteria interaction.

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Enrichment of nitrogen fixing free life bacteria from municipal wastewater treatment plants

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Abstract

Biological nitrogen fixation loss of soils additionned with the increasing use of pesticides and chemical fertilizers is an environmental problem that affects the quality and fertility of these ones. Previous studies focused on the implementation of nitrogen fixing bacteria (NFB) in agricultural systems but those techniques were limitated by the ammonium and salts remnants in the soils. For the first time at laboratory scale the enrichment of NFB from municipal waste water treatment plants (MWWTP) was performed. The experimental phase consisted in the enrichment of four MWWTP's sludges. The two sludges presenting the highest amount of fixed nitrogen were exposed to concentrations of salts and ammonium. These experimental conditions were characteristics of salt degraded soils. The presence of NFB, possibly establishing consortia with other microorganisms associated to the nitrogen cycle, was proved. The exposition of the sludges to high concentrations of salts and ammonium was not a NFB population depletion factor. The results suggest a potential use of these sludges as a complementary alternative to regenerate salt degraded soils.

Keywords: Nitrogen Fixing Bacteria● Sequential Batch Reactor● Enrichment● Municipal Waste Water Treatment Plants●

Introduction

78% of the troposphere composition is dinitrogen (N2). There are two ways to fix nitrogen: **1)** Anthropogenic (54%): a) Haber Bosh process (H-B); b) fossil fuel digestion; c) culture media of Nitrogen Fixing Bacteria (NFB) by bioreactors and 2) Natural (46%): a) oxidation of nitrogen by electric storm and b) symbiotic and free living nitrogen fixation (Fowler *et al.* 2013). The nitrogen produced anthropogenically, is used for 80% within chemical fertilizer and for 20% in industry (Yara International, 2014). The Haber-Bosh process and fossil fuel digestion, are the most widely used for the production of NH3, but are associated with negatives environmental impacts. This process emits more of 300 million of metric ton of carbon dioxide (Cherkasov *et al.* 2015; Tanabe and Nishibayashi, 2013) and the use of this product provokes loss of biodiversity in the soils, the eutrophication of superficial and groundwater, the imbalance of the nitrogen cycle (Grageda *et al.* 2012), problems of toxicity in aquatic organisms, soil salinization and reduced productivity (Estupiñan and Quesada, 2010).

Different alternatives strategies of fixation are known, and it has been focused on the isolation of NFB from fertile soils and their enrichment into liquid culture media later implanted in the degraded soil as microbial inoculants (Carvajal-Muñoz and Mera-Benavides, 2010; Grageda *et al.* 2012). These inoculants decrease the use of chemical agro products by 10% (Ahemad and Kibret, 2014; Bhattacharyya and Jha, 2012). Recently the

presence of NFB in WWTP's has been shown and cultivated (Pérez-Peláez *et al.* 2011). These systems have high concentrations of ammonia (>50 mg.L-1 NH_4^+) and salts (>2 dS.m-1) (Becerra-Castro *et al.* 2015). The presence of these microorganisms suggest their resistance and adaptability to extreme conditions and make them suitable candidates for the production of nitrogen compounds ex-situ. For what we know, no example has been found in the related literature of previous intents of diversity bacteria enrichment from WWTP's sludges to produce nitrogen compounds with potential use on degraded soils. We worked on an innovative strategy solution to agricultural problems using the native microbial biodiversity with engineering and molecular tools.

Material and Methods

Selection of Inoculum. Inoculum was taken from four different kind of MWWTP's. Which were Activate Sludge (AS), Biodisc (B), Constructed Wetlands (CW) and Facultative Pond (FP). All the MWWTP are located in Cali, Colombia. 1L of Inoculum was taken and it was sampled in dry season (January 2013).

Bioreactor operation. Phase 1. Enrichment of NFB. All the inoculums were enriched in an aerobic Sequential Bach Reactor (SBR) with a Hydraulic Time Retention (HTR) of 24 h. The solution used by (Rojas-Tapias *et al.* 2013) was used as culture media. This solution was nitrogen free. Operation lasted 110 days. The two inoculums with most nitrogen fixation capacity were carried to the next experimental phase (Phase 2).

Phase 2. Experimental Phase. The enriched inoculums were exposed to high concentrations of salinity (<4.5 $dS.m^{-1}$) and ammonia (<450 $mg.L^{-1}$). The culture media was the same used in the Phase 1, but was added a source of ammonium. The source of ammonium was $(NH_4)_2SO_4$. Concentrations were 0, 50, 150, 300, 450 $mg.L^{-1}$ in the 0, 1, 2, 3 y 4 weeks respectively. . Each bioreactor had its duplicated and Control. Control consist a bioreactor without source ammonia. Operation lasted 35 days.

Bioreactor measurements. Twice per week was quantified the concentration of total nitrogen (NTK), ammonia (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) following the protocol by (APHA *et al.* 2012) in both phases (Phase 1 and 2), also, pH, Dissolved Oxygen (DO) and Electrical Conductivity (EC) measurements were taken. All the measurements were made in the Microbiology and Environmental Biotechnology Laboratory (Microbiota), Universidad del Valle, Cali, Colombia.

Molecular Analysis. 0.25 gr of each inoculum was taken at the beginning and end of each Phase for acid nucleic extraction. The DNA extraction was made with the Power Soil DNA Isolation kit (MO BIO®). The amplification of *nif*H gene was made by nested PCR. The first and second PCR was carried out with the forward PoLF and reverse PolR primers (Poly *et al.* 2001), but, in the second PCR one was carried out with the forward primer PolF containing a GC clamp. The conditions of both reactions was: Preheating 95 °C 5 min, Melting 94 °C 30 seg, Annealing 52 °C 30 seg, Elongation 72 °C 30 seg, Final Elongation 72 °C 10 min. The PCR products (20 µl) were loaded onto 8% (w/v) polyacrylamide–bisacrylamide gel with denaturation gradients from 40% to 70%. Electrophoresis was carried out at 100 V at 60 °C for 16 h. Gels.

Results and Discussion

According with the operational conditions, all the amount of nitrogen found in the bioreactors is due to biological nitrogen fixation. There wasn´t loss appreciable of ammonia (Chevallier and Toribio, 2005; Jones *et al.* 2013). The Figure 1 shows the amount of total nitrogen in

the bioreactors. No significant differences were found between inoculums (pvalue = 0.69). The metabolic functions of nitrogen transformation are conserved and the little differences in the bioreactors suggest an adaptation of consortia at the given conditions. The bioreactor that fixed the less amount for nitrogen was FP (42.18 mg.Ld-1), but the most fixator bioreactor was AS (59.51 mg.Ld-1).

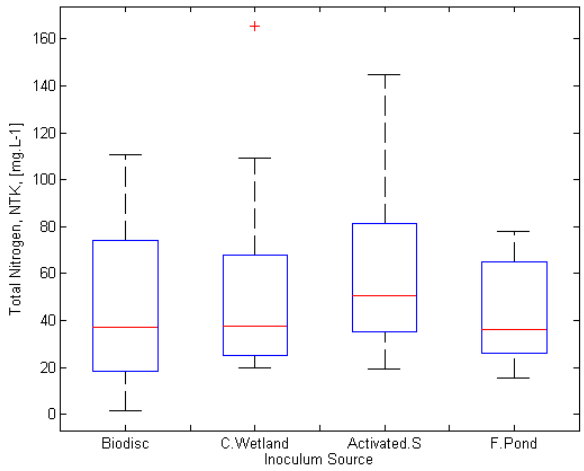


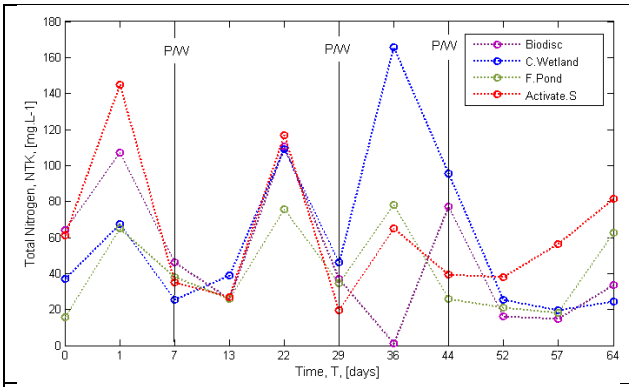
Figure 1. Total nitrogen found in the four bioreactors.

The amount of NTK found in the bioreactor was higher compared with the results of Pratt *et al.* (2007). They could produce 27 mg.Ld^{-1} of NTK. Therefore, the nitrogen available in the medium was produced by NFB and is used immediately for the cellular growth (Baca *et al.* 2000). Table 1 shows the found concentration of different physic-chemical parameters. The bioreactors were under conditions that favored nitrification (Cervantes *et al.* 2000; Pacheco *et al.* 2002). The NFB allowed the presence of other microbial groups involved in the nitrogen cycle as the nitrifying bacteria and their possible metabolic association forming consortia.

Table 1. Concentration of DO, NH_4^+, NO_2^-, NO_3^- (mg.L^{-1}), and ranges of pH (unids).

Parameter	Inoculum			
	B	CW	FP	AS
DO	2.78 ± 1.17	3.38 ± 1.81	2.57 ± 1.07	2.72 ± 1.06
pH	7.60 - 6.50	7.61 – 6.17	7.58 – 6.48	7.73 – 6.03
NH_4^+	8.08 ± 5.17	7.52 ± 5.02	8.66 ± 5.50	7.27 ± 5.15
NO_2^-	1.29 ± 1.07	1.55 ± 1.48	0.97 ± 0.80	1.44 ± 1.23
NO_3^-	23.45 ± 11.65	19.88 ± 12.11	21.54 ± 11.76	21.62 ± 10.23

These associations makes the transfer of materia an energy more efficient (Frioni, 1999). Despite, we couldn't evidence the presence of this microorganism by dependent or independent culture technique, the physic-chemical results and experimental conditions suggest the presence of nitrifying bacteria in the bioreactors. The Figure 6 shows the concentration of total nitrogen in the time of operation.



*P/W: Purges and Wash.
Figure 2. Nitrogen concentration across the time of operation.

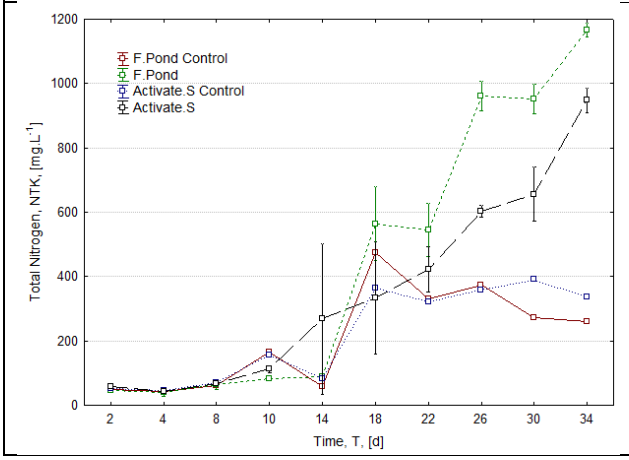
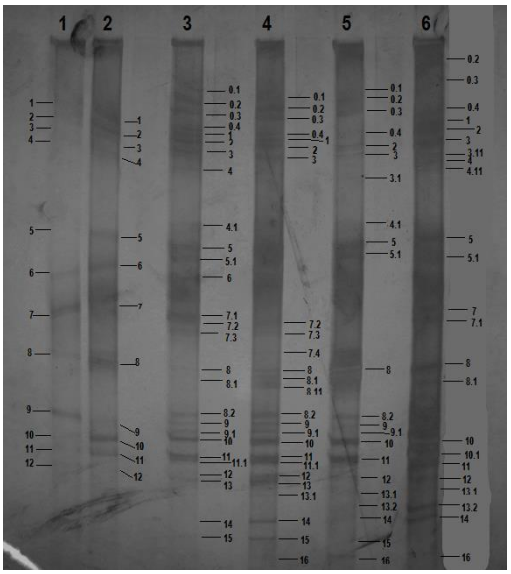


Figure 3. Total Nitrogen found in the FP, AS and their Controls bioreactors.

Approximately, each 10-15 days the bioreactors were purged and washed. These activities of operation generated a negative impact in the microbial consortia, then, some microbial community was lost and with the time of operation the bacteria start to recover itself. Thus, other ways to make purges and wash must be adopted without make damage the microbial population. However the inoculums from FP and AS at the end of Phase 1 were the inoculums which the best adaptation to the operational conditions and they were carried on to the Phase 2.e inoculums FP and AS were exposed at high concentration of salts and ammonia, conditions similar found in a degraded soil by salinity. The Figure 3 shows the amount of nitrogen found in the bioreactors. The increase of ammonia didn't had effect in the response of microbial community the first 22 days, but, after that (300 $mg.L^{-1}$ NH_4^+) started to increase the concentration of ammonia in the supernatant. The ammonia was not consume due unbalance of C/N ratio. The C/N ratio must be approximately 100:3.5 according with Pratt *et al.* (2007) to assure the uptake of nitrogen source. Each bioreactor with its control had similar behavior the first 22 days, showing that the bacteria with nitrogen source will take the nitrogen available and if the bacteria doesn't have any nitrogen source, will fix it from the atmosphere. This suggests that the bacteria are mixotrophic, showing metabolic versatility. Otherwise, the salinity can be measured as Electrical Conductivity (EC). Normally, a degraded soil has a >2 $dS.m^{-1}$ of CE and for example in special cultives such as sugar cane, since 1.7 $dS.m^{-1}$ the productivity is affected (10%). In the bioreactors the EC at beginning of SBR cycle was 1.33 $dS.m^{-1}$ and at the end was 1.70 ± 0.05 $dS.m^{-}$

[1,]Hence, the inoculums enriched and exposed at similar conditions of degrade soil could be adapted successfully. On the other hand, the presence of *nif*H gene was seen in all inoculums. The Figure 4 shows the diversity of NFB by DGGE. The presence of NFB demonstrated that ammonia concentrations weren't a factor that inhibited the microbial population and were conserved across both Phases (1 and 2). The inoculum from AS had more diversity of NFB (27 OUT's) than FP (12 OUT's).



*1) FP Phase 1; 2) FP Phase 2; 3) AS Phase 1; 4) AS Phase 2; 5) FP Control Phase 2; 6) Control AS Phase 2.

Figure 4. DGGE gel visualization.

However, 92.6% of total population of NFB in AS were conserved. The environmental and operational conditions which were exposed the inoculum could affect the NFB population, but, it still conserved the majority of microbial populations. Their success adaptability at the stress condition make it a good alternative of recuperation of degraded soils by salinity and ammonia excess.

Conclusions

The addition of high salinity and ammonia concentration wasn't a factor that negative affect the population of NFB. The methodology employed in the enrichment and adaptation of high concentration and salinity would be a procedure to obtain an alternative agriculture bio-product. This work is the start of all a line of research in the valorization of native biodiversity found in WWTP sludge to produce value-added products, but also knowledge on the little explored microbial biodiversity of sludges.

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Microbial communities associated with the maize crop in the Yaqui Valley, and their potential use as microbial inoculants

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Abstract
The maize is the most important crop in Mexico. In southern Sonora, specifically in the Yaqui Valley, where maize is the second crop after wheat with more hectares planted, this crop is an excellent alternative for spring-summer cycle in this region. However, this valley is severely affected due to the excessive application of agrochemicals in response to the high demands of production. An alternative to counter the negative impacts and in turn increase crop yields, is the use of beneficial microorganisms, mainly bacteria, actinomycetes and fungi native. For these reasons the present work aims to isolate and characterize microorganisms with agronomic characteristics of interest and to establish with them a collection that in the future may serve as a basis for the formulation of microbial inoculants. It is managed to get 203 isolates, which 84% produce siderophores, 33.5% solubilized phosphates and 100% can produce indoles. It is also important to highlight that 8 of those isolates possess the three metabolic characteristics which make them good candidates for future assays focused on the development of a microbial inoculants that contributes to a sustainable agriculture.

Keywords. Indoles•Siderophore•Phosphate solubilization•Beneficial microorganisms.

Introduction

In Mexico, the maize is the most important crops according to its nutritional, economic and social role. This crop represents 50% of food consumed a year, providing 50% of calories required for humans (Massieu-Trigo y Lechuga-Montenegro, 2002). In addition, the national maize production in 2014 reached 24 million tons, representing 4 billion dollars, which were cultivated on 7.4 million hectares (SIAP-SAGARPA 2016), where in northwestern México stands out for its production, especially in the state of Sinaloa. Eventhough it in southern Sonora corn is not the main crop, if an alternative especially for crops in summer (Ortega-Corona *et al*., 2003). The Yaqui Valley, placed in this region, has a 235,000 ha of a cultivable area, being the maize the second main crop, after wheat. Due to the Yaqui Valley is an agricultural area of vital importance for México, it must provide a high demand for production. However this action is affected by the continued population growth and climate change, for this reason it has been necessary to the excessive use of agro-chemicals to increase crop yields and thus satisfy this demand. To diminish the damage caused by the use of large amount of these products, it is necessary to develop strategies aimed to maintain the sustainability of the farming system through rational use of natural resources and the application of appropriate laws for preserving the environment (Shankar Singh J., 2011; All-

Taweil H. I., 2009).The use of microbial inoculants is a potential alternative to be used in the agriculture, focus on to increase the crop production reducing the application of inorganic fertilizers. The microbial inoculants contain plant growth promoting microorganisms with the ability to synthesize compounds that promote plant growth, *i.e.* fixing atmospheric nitrogen, synthesizing several enzymes, solubilizing iron and inorganic phosphorus, producing antibiotics and phytohormones, improving tolerance to drought, salinity, toxic metals stress, (Lucy, 2004). For identification and characterization of plant growth promoting microorganisms is important to explore their trails involved in this behavior, such as indole acetic acid (IAA), a plant hormone promoting root development to increase the availability of nutrients in the soil, such as phosphorus, by producing organic acids capable of solubilizing the insoluble phosphate in soil, and the iron, by producing siderophores sequestering iron to the plant (Compant, 2005; Goldstein, 2007; García F. *et al*, 2010). The objective of this work was to characterize metabolically soil microorganisms associated to the maize in the Yaqui Valley, Sonora, through several biochemical test and classical microbiology techniques, thus identifying promising strains for growth promotion and biological control.

Materials and methods

The sampling was carried out collecting soil at a depth of 30cm at in different points of the Yaqui Valley, growing corn. The selected sites were the block 609 lot 18 (Site 1), block 810 lot 31 (Site 2), block 1501 lot 26 (Site 3), block 2012 lot 35 (Site 4) and block 1115 lot 5 (Site 5). 5 kg of soil in each study site were collected for microbiological analysis. The samples were transported to the laboratory, stored at 4 ° C until their analysis. In order to determine the population of cultivable microorganisms, a count of colony by planting serial dilutions in factors of 1:10 was conducted. Then 0.1 mL sample was taken from the dilution 10^{-3} and inoculated in Petri dishes containing Potato Dextrose Agar, supplemented with 80 μgml^{-1} nalidixic acid for actinomycetes, also 0.1 mL of sample dilution was taken and inoculated into nutrient agar added with 80 µgml-1 terbinafine for the other bacteria, incubated for 72 hours at 28°C, the experiment was conducted in triplicate. All isolates obtained were cryo-preserved and deposited in COLMENA (Colección de Microorganismos Edáficos y Endófitos Nativos, www.itson.mx/colmena). For the determination of metabolic capabilities of each isolate were evaluated in three different tests such as production of indoles, phosphate solubilization and production of siderophores. The analysis of indoles production was carried out according to De los Santos-Villalobos *et al.* (2013) using three independent replicates. For measure the capacity of phosphate solubilization of each isolated were inoculated in duplicate in Pikovskaya (PVK) medium according to Onyia and Anyanwu (2013). The analysis production of siderophores of each isolated were inoculated in duplicated in Crhomeazurol S (CAS) medium according to Alexander and Zuberer, (1991). All tests were replicated three times independently. The obtained data were analyzed by one-way analysis of variance (ANOVA) and Tukey-Kramer method (P=0.05) using the Statgraphics Plus 5.1 software.

Results and discussion

In the sampled soil (Figure 1), it was found that site 4 had the greatest abundance of bacteria, while the site 3 presented fewer quantity of CFU (Colony Forming Unit)/g soil. Similarly, when determining the abundance of actinomycetes, it was observed that the site 4 had present a higher number of CFU additionally shown a significant difference from the

rest of the sites; while in the remaining four sites there were no significant differences in the abundance of these microorganisms.

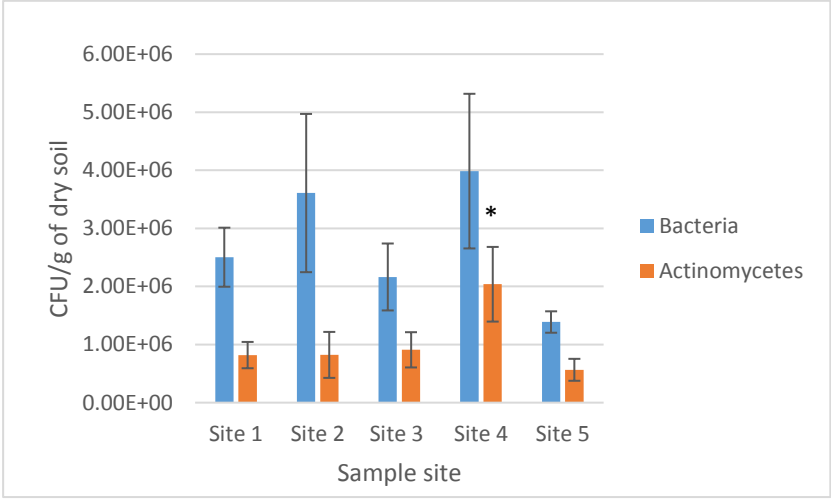

Figure 1. Microbial population of bacteria and actinomycetes in CFU/g of soil. * With their respective significance difference.

Once the microorganisms were isolated, we performed an assessment of PGPB (Plant Growth Promoting Bacteria) mechanisms for all our isolated with the aim of the selection proper PGP (Plant Growth Promoting) strains. We tested three different PGPB mechanisms: (i) indoles production, (ii) siderophore production, and (iii) phosphate solubilization. Several edaphic microorganisms have showed the ability to promote plant growth through several of those mechanisms (Lera *et al*, 2016). The positive results of PGPB mechanisms determination of 203 isolates are shown in Figure 2. Several studies reported that auxins are produced in plants from tryptophan catabolism being this compound the usual precursor of indoles. Different bacteria have been also described with the ability to synthesize indoles in the presence of tryptophan, being this ability common in plant pathogenic microorganism and in beneficial plant growth promoters (Taiz, 2006; Duca *et al*, 2014: Vandeputte O. *et al*, 2005). The strain with the highest production of indoles was isolated from site 2 with a production of 11 ppm labeled with the number 22B45, however, the site 4 turns out to be the site with the highest number of positive strains (Figure 2). The production of siderophore showed that, 84.72% of the edaphic strains were positive (Figure 2), forming a colored halo around the colonies, according to Alexander and Zuberer, (1991). The strain with the highest production of siderophores was isolated from site 3 with a production halo of 300% labeled with the number 31B5. Siderophore production among edaphic microorganism may be common, since edaphics have to compete for Fe supply, and therefore, siderophore production may be highly important for microbial growth due to the siderophores form complexes with other bivalent heavy metal ions increasing the metal availability and the plant metal uptake (Carrillo-Castañeda *et al*, 2002; Rajkumar *et al*, 2010). The determination of the capacity for solubilization of phosphates, 33.49% of the edaphic strains were positive (Figure 2), forming a colored halo of production according to Onyia and Anyanwu (2013). The strain with the highest solubilization of phosphates was isolated from site 4 with a production halo of 130% labeled with the number 41B3. Insoluble phosphates that cannot

be absorbed by plants, can be brought to soluble forms by the action of the microorganisms. The main routes of solubilization is by producing organic acids and the production of acid or alkaline phosphatases (Henao, 2008; Mendoza, 2010).

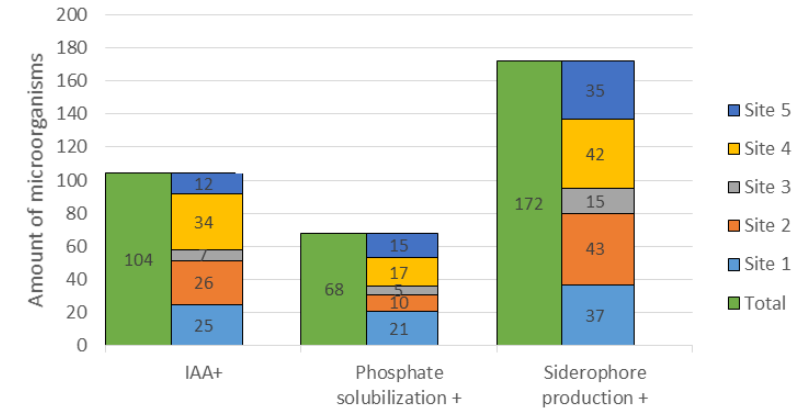

Figure 2. Results of biochemical test per site.

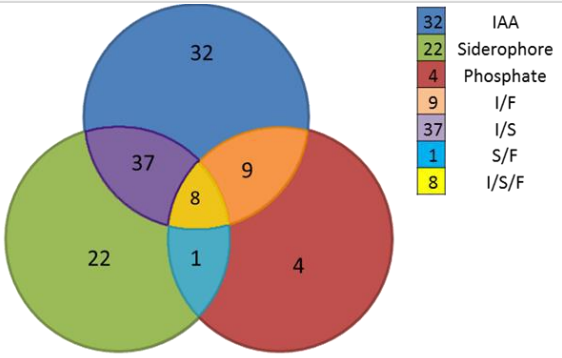

Figure 3. Strains with multiple metabolic capabilities.

The results showed strains with cross biochemical capabilities (Figure 3). Eight strains were capable of producing IAA, induce solubilization of phosphates and the siderophore production in a transversal way. These capabilities indicate that these strains have a great potential in plant growth promoting or to be used in biocontrol.

Conclusion

The presented results indicate that despite the intensive agriculture that has been done in the Yaqui Valley, exist a variety of native microorganisms which can be isolated and characterized, and those that present promissory characteristics can be used as microbial inoculants to strengthen crops interest in a friendly way with the environment.

Acknowledgements The authors acknowledge the INIFAP by support to the project 12315932912 “Aislamiento y caracterización de microorganismos promisorios para fortalecer el cultivo de maíz en el sur de Sonora y norte de Sinaloa”.

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Contamination of organochlorine pesticides in the estuarine zone in the South of Sonora

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Abstract

Levels of thirteen different organochlorine pesticides (OCPs) in surface water were studied: chlordane, gamma chlordane, α-endosulphan, β-endosulphan, lindane, aldrin, dieldrin, Isodrin, α-endosulfan, 4,4' DDD, 4,4' DDT, 4,4' DDE, methoxychlor, mirex; sixteen samples were strategically taken in Lobos, Sonora and examined using gas chromatography with a micro electron capture detector (μ-ECD). Concentrations were obtained within the limits of detection in a range of 0.3708 to 2.426 μgL^{-1} the highest concentrations were found for gamma chlordane (4.1514 μgL^{-1}) and DDT (2.426 μgL^{-1}) and the lowest were found of lindane, dieldrin and 4,4' DDE. These results show the presence of organochlorine pesticides, in the Bay of Lobos, Sonora, which can pose a seriuous threat to humans, endemic species and the ecosystem.

Keywords: Organochlorine pesticides. ● Gas chromatography.

Introduction

Due to its physicochemical characteristics Organochlorine Pesticides may be transported long distances, in the process they're deposited on the soil and in a living organism, according to United Nations Environment Program (UNEP) this phenomenon is causing a negative effect on the environment and human health, because of this the use of Organochlorine pesticides is being highly questioned for controlling crop's pests (Leal, 2014). OCPs are considered by the Environmental Protection Agency (EPA) as pollutants of aquatic ecosystems (Garcia and Rodriguez, 2012). Agricultural activity in the state of Sonora is known for being one of the most developed in the country, the region has a planted area of 419,985 hectares, with a crop's value of 22,794.76 million pesos for the agricultural year 2013-2014 (CONAGUA). The Yaqui Valley is located in the south of the state, it is a region where intensive agriculture is practiced and whose natural water supply and drainage is the Yaqui River which empties into the Gulf of California. The Yaqui River basin has been modified by the use of channels to meet the demands of the Valley, because of this some parts have been removed from the natural riverbed (INECC, 2008) to provide the water demand of crops, the flows drain into one of the largest estuarine areas of the state, known as Bahia de Lobos (Ahrens et al, 2008).

Materials and methods

Study site Lobos' bay is located in the south of Sonora state, at the geographic coordinates 27°21'05.7"N 110°27'15.5"W.

Sample collection. Sampling was carried out by seasons, for the winter samples were collected in the months of November and December 2015, and for the summer season during the months of May and June 2016. They were taken from three strategic points for the winter season and from five points for the summer season. The samples were collected in glass bottles of 500 ml from the most superficial layer of water, they were subsequently stored in an ice box and kept at 4°C then transported to the Environmental Toxicology Laboratory located at CIIBAA building of Sonora Institute of Technology. They were stored in a freezer (GENERAL ELECTRIC 7DTA WH) until analysis.

Extraction procedure. The samples were purified with a modified version of the method described by Espinosa *et al*, (1998). Pesticide extraction was carried out in a Florisil® disposable extraction columns (J.T.BAKER) for each sample and two spiked for internal control and a negative control was used. For the activation of Florisil® column the following solvents were added: i) dichloromethane (5 ml) ii) acetone, iii) hexane. Once the columns are activated, we added 500 µL of the water sample and approximately 40 mL of a mixture of dichloromethane-hexane (30:70) in the syringe for elution. The eluate was collected in a conical glass tube 50 mL to later take it to dryness in a rotary evaporator (N-EVAP-111) and the sample reconstituted with 100 μgL^{-1} of hexane for the analysis at the GC. A gas chromatograph (Agilent Technologies 7890A) with a micro electron capture detector specific for organochlorine pesticides were used, specifications and operating conditions for the quantitative analysis are showed in Table 1.

Table 1. Gas Chromatograph conditions for organochlorine pesticide analysis

Item	Condition
Detector	Micro Electron Capture Detector (µ-ECD)
Column	DB-5 column of 30m length, 0.25mm inner diameter, film of 0.25 µm
Oven Programing	110 °C (2 min hold) to 280 °C
Total run-time	14.33 min

based on table by Lari *et al,* (2014).

Results and discussion

Linearity. The correlation coefficient (R^2) was determined for each analyte (Table 2) using different concentrations of 50, 100, 150, 250 y 1000 μgL^{-1} resulted in a calibration curve, then we observed that the micro electron capture detector (µ-ECD) remained linear obtaining greater correlation coefficients of 0.99 for each analyte according to the guidelines of the USDA.

Quality control and quality assurance. The accuracy of the method was calculated by analyzing water samples, spiked 100 μgL^{-1} of a standard mixture of pesticides with 1000 μgL^{-1} by sextuple. The average recovery (%) obtained was within 70 – 120% defined by US EPA; the limit of detector (LOD) was evaluated injecting low concentrations of the standards, in order to evaluate the sensitivity of the detector to the minimum concentration. The quantification limits (LOQ) were determinated by different injections with minimum concentrations that can be extracted from each pesticide showed in Table 2.The results of analysis of water samples from Lobos bay affirmed the presence of chlordane, Dieldrin, lindane, 4,4' DDE, 4,4' DDT and γ-chlordane (Table 3). The highest concentration detected was for γ-chlordane (4.151 μgL^{-1}) in December 2015, followed by 4,4' DDT (2.426 μgL^{-1}) and its metabolite 4,4' DDE (0.367 μgL^{-1}). The pesticides in the Superficial water samples

from Lobos bay exceeded the US EPA National Recommended Aquatic Life Criteria (Table 4) showing alarming levels of chlordane and DDTs.

Table 2. Retention time, correlation coefficient (R^2), average recovery, limit of detection (LOD), limit of quantitation (LOQ) of OCPs in superficial water (100 µg/L).

	Retention time	Correlation coeficient (R^2)	LOD (µgL^{-1})	LOQ (µgL^{-1})	Average (%) Recovery
Lindane	8.047	0.996	0.11	1.1	76
Aldrin	9.511	0.997	0.1	1	83
Dieldrin	10.823	0.995	0.009	0.9	99
Isodrin	9.897	0.995	0.1	1	89
clordane	10.518	0.995	0.1	1	94
Gamma clordane	10.325	0.995	0.1	1	96
α-endosulfan	10.495	0.998	0.5	5	95
β-endosulfan	11.208	0.999	0.5	5	100
p,p´ DDE	10.789	0.992	0.009	0.9	95
p,p´ DDD	11.261	0.999	0.1	1	11
p,p´ DDT	11.723	1.000	0.1	1	115
Methoxychlor	12.384	1.000	1	10	141
Mirex	13.073	1.000	0.1	1	104

Table 3. Concentrations of pesticides (µgL^{-1}) in superficial water of Lobos bay.

OCPs	n=6		n=10	
	Winter		Summer	
	nov-15	Dec-15	May-16	Jun-16
Lindane	N. D	≤ LOQ	N.D	N. D
Aldrin	N. D	N. D	N. D	N. D
Dieldrin	N. D	N. D	≤ LOQ	N. D
Isodrin	N. D	N. D	N. D	N. D
α-clordane	N. D	N. D	N. D	N. D
γ-chlordane	3.2402	4.1514	N. D	N. D
α-endosulphan	N. D	N. D	N. D	N. D
β-endosulphan	N. D	N. D	N. D	N. D
4,4´ DDE	≤ LOQ	N. D	N. D	N. D
4,4´ DDD	N. D	N. D	N. D	N. D
4,4´ DDT	2.426	N. D	N. D	N. D
Methoxychlor	N. D	N. D	N. D	N. D
Mirex	N. D	N. D	N. D	N. D

These results are consistent with those reported by Villegas *et al*. (1986), where they obtained high Concentrations of lindane, aldrin, heptachlor and DDT in Lobos bay. In a similar study in the coastal water of China reported 4,4' DDE, 4,4' DDT and γ-chlordane in all samples showed concentrations of 4.6, 4, 2.2 pg^{-1} respectively (Lin *et al* 2012). However, compared with other study in the Caribbean and pacific sea concentrations reported by Menzies *et al* (2013) for Chlordane related compounds (range < 0.0011– 0.28512 µgL^{-1}) and DDT related compounds (range <0.0018 - 0.0966 µgL^{-1}) were lower than our study. The concentrations in this study were higher than those presented in the Italian coast by Montuori *et al* (2016) in the Tiber river and estuary with concentrations range of all OCPs detected in range of 0.000009 - 0.00553 µgL^{-1}. All this studies shows that even this compounds have been banned in the Stockholm convention, are continue finding in the environment, one of the possible causes could be their persistence that is from several months to 20 years, according to Ramirez et al. (2001) organochlorine pesticides were used during the Green

Revolution (Diaz and Contreras, 2013) in the principal crops at Yaqui Valley (CONAGUA). Figure 2 shows the distribution of the pesticides by sampling points and the highest concentrations, where the point one (P1) of the months of November 2015 and December 2015 shows the major concentration detected and the most of the pesticides. This could be related to the seedtime autumn-winter in the Yaqui Valley. Although, during the month of May 2016 dieldrin concentration was very low and June 2016 None of the analyzed pesticides were detected.

Table 4. National recommended aquatic life criteria (US EPA) and selected OCPs total concentrations

	US EPA		
	Saltwater CMC* (acute) (µg/L)	**Saltwater CCC** (chronic) (µg/L)**	**Concentrations Lobos Bay (µg/L)**
chlordane	0.09	0.004	7.3916
dieldrin	0.71	0.0019	0.37086
lindane	0.16	—	0.7978
∑DDTs	0.45	0.032	2.793

*CMC criterion máximum concentration
**CCC criterion continuous concentration

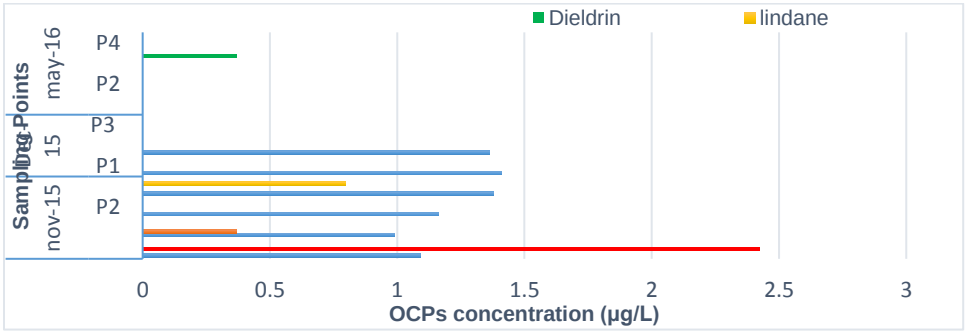

Figure 2. Organochlorine pesticides and concentrations detected in Lobos Bay.

Conclusions

The Organochlorine Pesticides 4,4 DDT, 4,'4 DDE, gamma chlordane, lindane and dieldrin were detected in the water samples extracted from Lobos' Bay in Sonora. The concentrations detected within the limits of quantitation are in a range of 0.3708 to 2426 μgL^{-1} then it can be said that the method and validation parameters (linearity, detection limits) are reliable compared with the parameters set by the EPA. The presence of pesticides in the water samples of the study indicate the persistence and stability of these compounds in the environment. It can conclude that the Lobos bay, Sonora is contaminated with the presence of organochlorine pesticides and this represents a threat to endemic species.

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Genetic diversity of *Bacillus* spp. associated to wheat in the Yaqui Valley, Sonora

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Abstract

A total of 165 bacterial strains were isolated from bulk (5.7 $X10^5$ $CFUg^{-1}$ dry soil) and rhizosphere soil (7.1 $X10^4$ $CFUg^{-1}$ dry soil), used for wheat production in the Yaqui, Valley. The macroscopic and microscopic traits show that 40% of theses strains share characteristics to the *Bacillus* genus. The 16S rRNA gene analysis confirmed that these strains belong to the genus *Bacillus*, specifically grouped into nine species*: B. subtilis, B. amyloliquefaciens, B. pumilus, B. endophyticus, B. megaterium, B. cereus, B. thuringensis, B. licheniformis* and *B. sonorensis*. All species were isolated in both types of samples, except the last three, which were found only in rhizosphere soil. The total cultivable bacterial population in bulk soil was greater than rhizosphere soil; however, the *Bacillus* diversity showed an opposite correlation. This behavior might be attributed to the effect of plant exudates, providing an optimum environment for specific species. Metabolic characterizations need to be developed in terms of identify the plant growth promotion and/or biocontrol traits of this *Bacillus* collection, or those the three rhizosphere *Bacillus* species, focused on their potential use as bioinoculants for enhance wheat production under edaphoclimatic conditions of the Yaqui Valley.

Keywords: Rhizosphere ●bulk soil● wheat ● *Bacillus* spp.

Introduction

Soil is a basic natural resource for food production due to its reservoir of microorganisms, with about 10^9 cells per gram of soil. The functions performed by soil microbiota have considerable direct and indirect effect on growth, health, fecundity, and stress tolerance (Buée *et al*., 2009). Bacteria are the most abundant microorganisms in soil (10^9 CFU g^{-1} soil *vs*. 10^6 CFU g^{-1} soil fungi and 10^4 CFU g^{-1} soil protozoa) (Hoorman and Islam, 2010). The most abundant genera of soil bacteria are: *Arthrobacter, Streptomyces, Pseudomonas, Bacillus, Clostridium, Azomonas, Azospirillum, Azotobacter, Beijerinckia, Rhizobium, Bradyrhizobium, Agrobacterium, Nitrosomonas, and Nitrobacter*, which have been reported as important players in all soil ecosystem services (Abbott, 2015). *Bacillus* is a ubiquitous soil genus due to its capability to form spores, an extreme survival strategy that confers adaptability to extreme conditions (high temperature, high UV irradiation, desiccation, chemical damage and enzymatic destruction (Nicholson *et al.* 2000). Furthermore, several strains of this genus present plant growth promotion (PGP) and/or biological control traits, which have been included in agricultural practices focused on diminishing the use of chemical fertilizer and pesticide to achieve higher yield. However, current agricultural practices still depend on a wide use of chemical fertilizer and pesticide for crop production,

i.g. the Yaqui Valley, Sonora. This region, 225,000 ha characterized by a semiarid climate contributes with about 40% of the national wheat production, by intensive agricultural practices (Matson *et al*., 2005). However, the wheat yield in this region has been affected by the high fertilizer dependency, increasing the cost of the production, soil salinization, and the disruption of soil microbial community, affecting its eco-functional role such as: nutrient cycling, soil organic matter decomposition, and soil formation (Matson *et al*., 2005). Thus the aim of this work was to isolate and characterize the genetic diversity of the genus *Bacillus* associated to the bulk and rhizosphere soil used for the wheat production in the Yaqui Valley, Sonora.

Material and methods

Sampling site: Bulk and rhizosphere soil samples were collected at three commercial wheat fields located in the Yaqui Valley, Sonora (108° 53' y 110° 37' W longitude and 26° 53' y 28° 37' N latitude), following the methodology reported by SAGARPA and Servicio Nacional de Sanidad (2015).

Isolation and identification of bacteria: The bacterial isolation and characterization was done applying the zig-zag methodology in a 1 ha. of each study site, and total of 25 samples were taken at a 30 cm deep. All bacterial isolates were deposited in the Native Endophytes and Soil Microorganisms Collection (COLMENA) (www.itson.mx/COLMENA), belongs to Laboratorio de Biotecnologia del Recurso Microbiano (LBRM) (http://www.itson.mx/lbrm) at the Instituto Tecnológico de Sonora (ITSON) Obregón City, México. These bacterial strains were characterized by macro and microscopic characteristics related to the genus *Bacillus*.

Molecular identification: Genomic DNA from each isolate sharing macro and microscopic traits with the genus *Bacillus* was extracted, according to Reader and Broda (1985). The 16S rRNA gene was amplified using the primers FD1 and RD1. The Polymerase Chain Reaction (PCR) technique consisted of an initial denaturation step at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 40 s at 57 °C and 2 min at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were verified by 2% agarose gel electrophoresis, purified and sequenced by Sanger platform. Sequences were compared and deposited in the NCBI Genbank. 16S rRNA gene sequences of isolates were aligned with software Clustal W and analyzed by the Neighbour-joining method, using MEGA 6. 16S rRNA of *Micrococcuss luteus* (KU707915.1) was used as outgroup. Stability of clades was assessed with 1000 bootstrap replications.

Results and discussion

The total cultivable bacterial population in the bulk soil was higher than rhizosphere soil, 5.7 $X10^5$ Colony-forming unit $(CFU)g^{-1}$ dry soil *vs*. 7.1 $X10^4$ CFU g^{-1} dry soil, probably due to the plant genotype, plant developmental stage, and bare soil characteristic have major impact driving the composition of rhizosphere microbial communities (Tkacz *et al*., 2015).

Sixty-six bacterial strains (40% of the total bacteria population) were selected based on distinct macroscopic and microscopic traits belonging to the genus *Bacillus*, such as: large, flat and dry colonies, with lobate margins (Figure 1A), as well as Gram positive staining, Rods often arranged in pairs or chains with rounded or square ends and usually have a single endospore, size (0.5 – 1.2 by 2.5 -10 µm), peritrichous flagella (Figure 1B) (Hold *et al*., 1994).

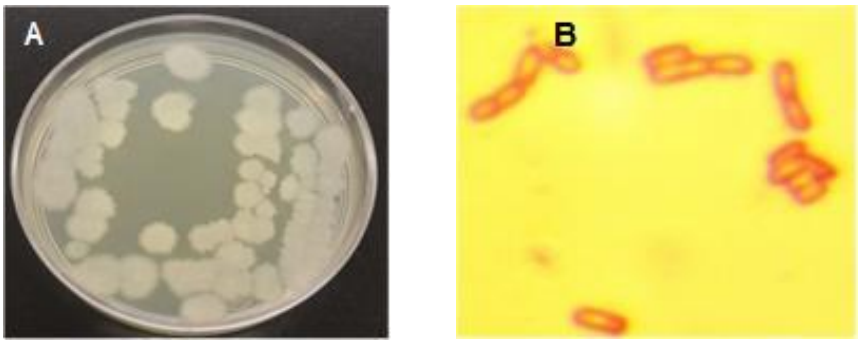

Figure 1. Macroscopic (A) and Microscopic (B) of a strain of the genus *Bacillus* isolated in the Yaqui Valle, Sonora.

The 16S rRNA gene analysis showed that these strains isolated in the Yaqui Valley associated to wheat are grouped into nine species: 1) *B. subtilis,* 2) *B. amyloliquefaciens,* 3) *B. licheniformis*, 4) *B. sonorensis*, 5) *B. pumilus*, 6) *B. endophyticus*, 7) *B.* cereus, 8) *B. thuringiensis,* and 8) *B. megaterium* (Figure 2). These species were isolated in both types of samples, except *B. licheniformis*, *B. sonorensis* and *B. endophyticus* which were found only in the wheat rhizosphere. The genus *Bacillus* is naturally present in bulk or cultivated soil, however fifty-five percent of *Bacillus* strains isolated in this work were identified in bulk soil, and 44 % in the wheat rhizosphere. This *Bacillus* community is regulated (positively or negatively) by the "rhizosphere effect", which consist in the stimulation of microbial communities in the rhizosphere soil by root exudates (Dotaniya and Meena, 2015). In addition, plants may modulate the rhizosphere microbiome to their benefit by selectively stimulating microorganisms with traits that are beneficial to their growth and health (Cook *et al*., 1995). Thus, the *Bacillus* species isolated in the rhizosphere might be able to exert a beneficial effect in the wheat, *i.g.*, the plant-selected *B. licheniformis* have been reported as PGP bacteria through the production and release of stimulatory metabolites such gibberellins (Gutierrez-Mañanero *et al*. 2001), exopolysaccharides and siderophore (Dan *et al*., 2012), and act as a potent biological control agent against *Fusarium moniliforme* in maize (Dan et al., 2012). While *B. endophyticus,* isolated from chilli rhizosphere soil was positive for various PGP characteristics, such as inorganic phosphate solubilization, indole acetic acid, ammonia, HCN, catalase, and siderophore production (Kummar and Audipudi, 2014).

Conclusion

The wheat rhizosphere, under edaphoclimatic conditions of the Yaqui Valley, had a negative effect on the B*acillus* population in comparison to the bulk soil, however the response was opposite in terms of the *Bacillus* species diversity. Thus, several studies are needed to understand the ecological role of the *Bacillus* diversity associated to wheat, as well as their potential utilization as a sustainable agricultural alternative for crop production in this region.

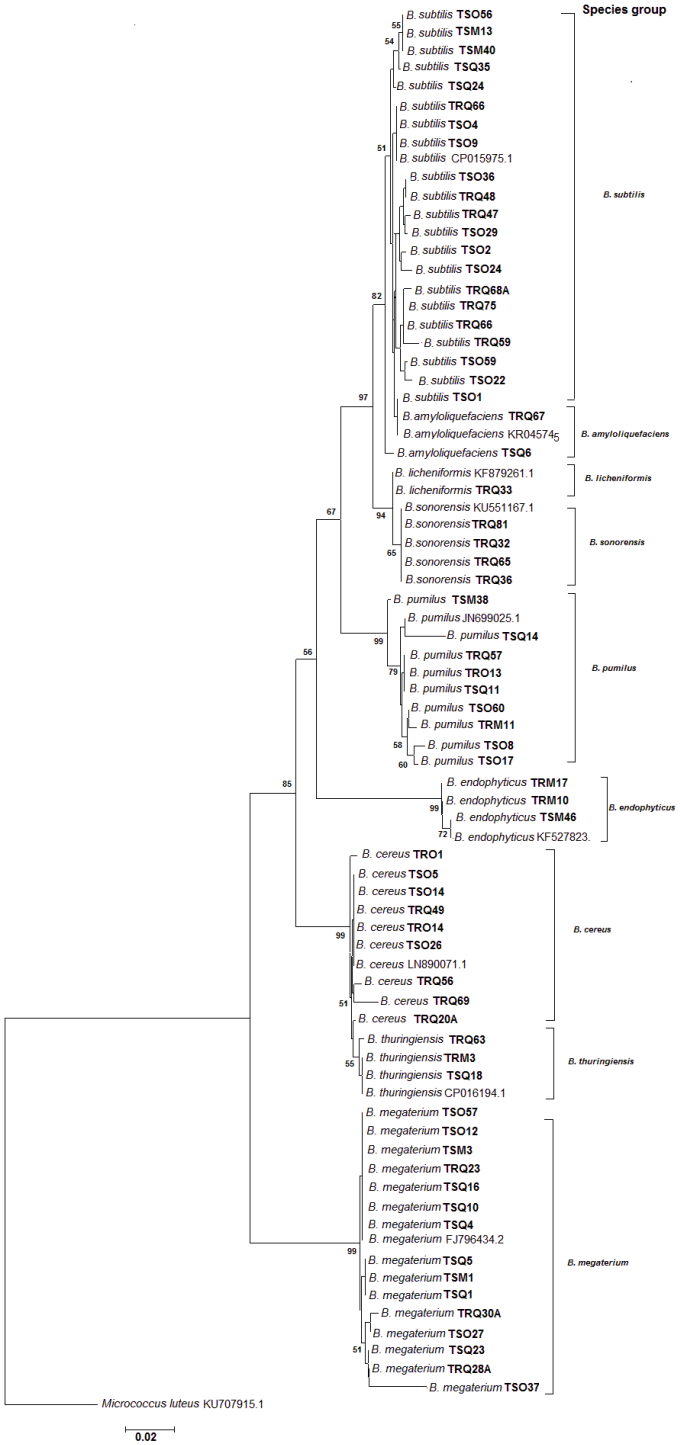

Figure 2. Phylogenetic tree (16S rRNA gene) of 66 *Bacillus* strains isolated in the Yaqui Valley. The tree was constructed using the Neighbor-Joining method. *Micrococcus luteus* (KU707915.1) was included as outgroup. Only Bootstrap percentages above 50% are shown (based on 1000 replications). The second letter in the strain codes refers to the isolate source where **S** means bulk soil and **R** rhizosphere, *i.e*. TSQ corresponds to bulk soil strains, whereas TRM, corresponds to rhizosphere strains.

Acknowledgements The authors acknowledge support by the Cátedras CONACyT Program through Project 1774 and Posdoctoral Program through Project 290941, "Alternativas agrobiotecnológicas para incrementar la competitividad del cultivo de trigo en el Valle del Yaqui: desde su ecología microbiana hasta su adaptabilidad al cambio climático", and "Estudio de la diversidad genética y funcional de las comunidades microbianas edáficas asociadas al cultivo de trigo en el Valle del Yaqui, Sonora", respectively.

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Agroecological production system: Voisin's rational grazing modified with a unique dose of asphaltic emulsion

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Abstract

An agroecological Voisin's Rational Grazing (VRG) production system was established in El Curay production unit, at Barinas state, Venezuela. In the year 2013, this lot of land had only 12 paddocks division, with 32 buffalos, a 32 L d^{-1} low milk production which barely covered production costs. The division of the area in 60 paddocks with the VRG management, produced a three-fold rise in pasture and milk production. The application of only one dose experimental treatments of: 1.5 Mg ha^{-1} asphaltic emulsion (AE) diluted 1:6 parts of water; 440 kg ha^{-1} of phosphoric rock (PR); Asphaltic emulsion and phosphoric rock (AE+PR) in the same amounts as the individual doses, and a VRG control without amendments (VRG-Control) were implemented. Four replicates of the treatments were established in 16 randomly chosen paddocks. The best yield dry matter of *U. brizantha* and *U. ruziziensis* pastures in kg ha^{-1} yr^{-1} corresponds to: AE+PR (48,119-36,056), EA (29,990-50,453) in comparison with VRG-Control (19,799-26,635) and PR (22,454-23,326) treatments, for years 2015 and 2016, respectively. The strategy of applying a sole dose of AE to accelerate the regular increases in time and achieve a sustained production in acid soils, yielded a 53% upsurge in comparison with the VRG-Control.

Key words: Voisin's rational grazing●*acid soils*●*asphaltic emulsion*●*phosphoric rock*● *biofertility.*

Introduction

In the last 200 years, the intensive food production in the world has been increased based on genetic improvement, the use of agroindustrial supplies (soluble fertilizers and agrotoxics) and plough and harrows in great extensions of agricultural soils. This is associated to local and global environmental degradation, many human health issues and an increase in cost production, all very thoroughly documented in international specialized literature (Howard 1943; Voisin 1994; Chaboussou 1987; Widdowson 1993; Faulkner 1945). Voisin's Rational Grazing (VRG) is an intensive agroecological technology for production of clean food. It is based on the universal laws of grazing (Voisin 1994) and an integral agroecological management (Pinheiro-Machado 2011; Pinheiro-Machado and Pinheiro-Machado Filho 2014), without the use of agrotoxics, soluble fertilizers and without breaking down soils with plough and harrows. Figure 1 shows the benefits of VRG over the agroindustrial dependence, climate change, soil fertility, water economy, cost production and sustained yields the throughout the year.

The differentiated response between intensive agroindustrial and agroecological VRG systems in acid-sandy soils of very low natural fertility, widely distributed in the tropics, is that the former offers a fast response with very short effective duration, great environmental impact with high and expensive agroindustrial dependence; where the latter offers a slow nondependent response, low production costs and sustained increases in biofertility and yields. Thus, the main goal of this work was to determine the effect of asphaltic emulsion (EA) and phosphoric rock (PR), applied in sole dose at the beginning of the VRG management, as accelerators of production changes in acidic sandy soils.

Figure 1. Benefits of Voisin's Rational Grazing Management

Material and Methods

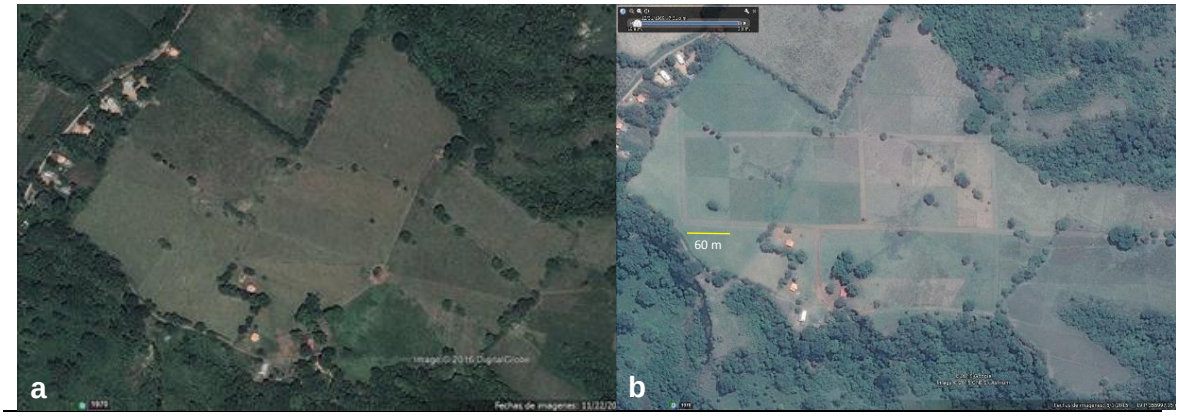
The production unit of El Curay, devoted to the production of livestock buffalo milk, crossbreed Murrah and Mediterranean, is located at Barinas state, in the municipality of Barinas, between the Santo Domingo and the Calderas rivers, 8° 43" North and 70° 18" West, to the east of Quebrada Seca the village. The terrace of El Curay has a particular landscape due to the intensity of the fluvial erosion, which has produced unlevelled terraces of up to 200 m, between the current courses of the rivers and the accumulation planes of rocky alluvial sediments of torrential characteristics belonging to the Inferior Quaternary.

The climate, as classified by Köppen, corresponds to tropical rainy moozonic forest (Amw´gi); subtype tropical rainy forest, characterized by a short dry season and annual isotherms of 24 and 26°C. The annual precipitation records (2,100 mm) of the meteorological station of Quebrada Seca (1950-1998) show that 93.4% of the rainfalls occur between April and November, and only 6.6% occurs between December and March. In the first 20-30 cm the soils show franc sandy and franc texture, with prevalence of the former

and accumulation of alluvial clay at the sub-surface which determines a slow permeability of the profile, moderately acid pH, very low phosphorous and magnesium and medium contents of calcium, nitrates and ammonium (Ruiz-Tirado 2000). Voisin's Rational Grazing management (VRG) involves the division of the total area into 60 paddocks of 3,600 m^2 each. Each paddock is grazed only when it has reached the optimal rest point and the timing of rotation is adjusted to the growth stage of the forage, generating a chess board effect. The paddocks are occupied during one day with instantaneous high livestock unit per hectare, and the laws of universal grazing established by Voisin are accomplished. The asphaltic emulsions (AE) oil in water (o/w) is of low cost in oil producing countries and represents a sub product of the oil industry. It contains 60% water which is very useful for its cold application, since it does not require any previous thermal treatment that could generate noxious volatile substances into the atmosphere. Thus, the AE can be easily applied, in vast agricultural areas, with simple manual aspersion or industrial equipment.
The AE application in soils is not recurrent, only one dose, at less than 0.08%, application to activate the biogenesis of the soil is considered necessary, for a soil layer 20 cm deep. The use of these substances in slope agriculture can significantly reduce hydric erosion processes; contribute to metal chelating and reduce the potential acidity of soils as well as the phosphorous specific adsorption which is so important in tropical soils due to the high oxi-hydroxides of iron and aluminium (Ojeda-Falcón, *et al.* 2012). The experimental design involved four treatments, with the same number of replicates, randomly distributed in 16 production paddocks. The treatments: 1.5 Mg ha^{-1} cationic asphaltic emulsions (AE) diluted 1:6 parts of water; 440 kg ha^{-1} of Riecito phosphoric rock (PR); joint application of AE and PR in the same dose as stated before (AE+PR) and an experimental VRG control without amendments (VRG-Control). The AE, PR and AE+PR were applied on a single dose in mid-February 2015. The soil and vegetation sampling took place during a year, in the periods of maximum and minimum precipitation, in three random places of a universe of 81 possible places of each treatment. In each sampling place a metallic 1m^2 frame was used. The botanical composition of the vegetation was determined. The forage was sheared on a level with the soil, it was weighed and, a subsample was taken weighed and placed, in a previously identified, brown bag which was oven dried at 60°C in order to obtain the dry weight. The results are expressed as yield averages (n = 12) in kilograms of dry matter (DM) with the respective standard deviation shown in brackets. Low case letters show statistically differences in the treatments, according to LSD test at ($P \leq 0.05$).

Results and discussion

Figure 2a, shows previous management of the production unit, with lot sizes of 3-4 ha, a uniform color of the pastures, which is related to the inadequate handling of the rest points of the pastures. In consequence, the production unit maintained only 32 milking buffalos with a 38.5 L d^{-1} yield, to produce 7 kg of cheese daily, and very low livestock units per hectare of 1.2 LU ha^{-1}. In contrast, to the original situation, Figure 2b shows the division changes of the 27 ha into 60 paddocks of 3,600m^2 each.



November 22nd 2013
A cartwheel design is seen with reduce number of lots (10-12) without chess board effect of continuous grazing.

January 5th 2015
14 months after the Voisin's rational grazing technique, the chess board effect on 60 paddocks can be seen.

Figure 2. GoogleEarth images of the El Curay production unit.

With the agroecologial VRG, it is possible to see the chess board effect, which is an indicator of well managed pastures with different rest times, from freshly pastured paddocks with pale color, to paddocks that are in their optimum rest time and ready to be pastured in dark green. This allowed a milk yield of 130 L d^{-1} for the manufacture of 24 kg of cheese daily from 32 milking buffaloes and livestock units of 3.7 LU ha^{-1} (Owner's, personal communication 2016). Table 1 shows the mean yield of DM (kg ha^{-1} and kg ha^{-1} yr^{-1}) for the different treatments: AE, PR, AE+PR and the VRG-Control, during the year 2015, for periods of minimum and maximum precipitations and for 2016 only the maximum precipitation period. During both years, the maximum yields were attained with the AE and AE+PR treatment for both periods measured.

Table 1. Mean yields for dry matter (DM) with Voisin's Rational Grazing (VRG) agroecological management Barinas State, Venezuela

Treatments	**Precipitation Periods**			**2015**	**2016**
	minimum 2015	**maximum 2015**	**maximum 2016**		
	kg ha^{-1}			**kg ha^{-1} yr^{-1}**	
Asphaltic Emulsions (AE)	1,615**c** (±136)	3,131**b** (±1,189)	6,054**a** (±2,877)	29,990	50,453
phosphoric rock (PR)	231**d** (±124)	3,043**b** (±1,285)	3,167**b** (±994)	22,454	23,326
AE+PR	1,376**c** (±433)	5,891**a** (±2,679)	4,168**ab** (±2,552)	48,119	36,056
VRG-Control	646**d** (±448)	2,367**bc** (±959)	3,344**b** (±1,214)	19,799	26,635

The yields, during the minimum precipitation period, were low (Table 1) in comparison with DM, using soluble fertilizers, which yield equivalent of 3,755 kg ha^{-1} for *Urochloa brizantha* cv. Toledo in acid soils (Rao *et al.* 2002); and equivalent of 1,700 and 7,000 kg ha^{-1} for minimum and maximum precipitation periods respectively, in well drained savannah acid soils (Lascano *et al*. 2002). The DM production determined by INIAP in 1991 (Instituto Nacional de Investigaciones Agropecuarias) for *Urochloa ruziziensis* was between 7,023-8,704 and 25,711-38,235 kg ha^{-1} yr^{-1}, for minimum and maximum precipitation periods, respectively. In this work, the yields of DM for both pastures (*U. brizantha* and *U. ruziziensis*) for PR, VRG-Control, AE+PR and AE treatments 1,157-22,169, 3,229-23,408, 6,882-29,176 and 8,073-42,378 kg ha^{-1} for minimum and maximum precipitation periods respectively. The VRG-Control and AE showed sustained DM (kg ha^{-1} yr^{-1}) for years 2015 and 2016 respectively. These organic source treatments were the ones that showed the highest annual increase of 25.7% for VRG-Control and 40.6% for AE; while the treatments that included inorganic sources showed small increase in the case of PR (3.7%), and a sharp annual decrease (-33.5%) with the AE+PR treatment. A year later, the VRG-Control and the organic treatment AE showed high and sustained yields without the use of agrotoxics, NPK or plough. These results substantiate the importance of the single dose concept, which does not pretend to substitute inputs of NPK and herbicides for others such as AE and PR, but to attain a sustained production with regular increases in time, without any aggressions to the soil structure and live. The sustained increases in yields awarded a larger carrying capacity to this pastures, and the exceeding forage for haymaking to be used during the periods of less precipitation, preserving a high and sustained yield the whole year around. The suppression of agroindustrial inputs and the low cost production generated a clear cost effectiveness evolution. The agroecological modified Voisin's Rational Grazing altered the structure of the herbaceous community. The pastures occupied the largest percentage (33.4-79.3%), while the weeds occupied between 1.1 and 11.1% and the leguminous 1.3 and 5.6%. The presence of weeds was reduced in 2016 only in 2.7%, where the leguminous emerged in a sustained manner, improving the protein offer and thus the milk production.

Conclusions

The agroecological modified Voisin's Rational Grazing, achieves sustained increases in pasture production, since the first year of application, without the use of soluble fertilizers such as NPK, suppressing plough and harrowing and the application of agrotoxics. This is particularly important since it breaks the dependence with agroindustrial inputs, reduces production costs and contributes to the savings of national economy. The application of a single dose of asphaltic emulsion, as a strategy to accelerate changes in the Voisin's Rational Grazing management, allowed the increase in yields of 53% in comparison with the VRG-Control. With this management a sustained production with regular increases in time was obtained in acid sandy soils of low fertility. In one year, Voisin's Rational Grazing agroecological management, showed a threefold increase in carrying capacity (from 1.2 to 3.7 LU ha^{-1}) and a significant increase of 5.6% in leguminous. This constitutes a clear indicator of sustained soil fertility and quality improvement of the pasture, which in turn induced the reduction of weeds and the space without emerging vegetation. Thus the modified Voisin's Rational Grazing agroecological management with a unique dosage of asphaltic emulsion constitutes a viable alternative for a wide agricultural frontier of the acid sandy soils within the Tropics.

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Combining EDXRF and DRIFT-MIRS to distinguish sedimentary sources in an agricultural catchment of Argentina: Identification of suitable fingerprint elements

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Abstract

Sediment transport and the associated deposition process are key environmental problems in the semi-arid center of Argentina where the agricultural frontier and new land uses grow at the expense of native vegetation. Using sediment source fingerprinting techniques could be a way of identifying hot spots critiques of land degradation. In this research we explore the use of natural elements in soil to identify sedimentary sources in a small basin in the province of San Luis, Argentina. Soil samples were collected in different points of the basin, representing different land use (source samples) and sediments samples were taken along the stream (mixed samples). Concentrations of possible fingerprint elements were measured using EDXRF technique. Additionally, multivariate PLS analysis was applied to the infrared spectral data of the samples. With the aim of validate the procedure, two artificial mixtures were made up with known proportions of source samples. Fingerprints elements were identified obtaining with them a very good reconstruction of the source proportions in the artificial mixtures. DRIFT-MIRS PLS analysis shows a good correlation between MIRS spectra and the identified fingerprint elements.

Keywords: Soil Sediment•Fingerprints•EDXFR•MIRS•Mixing Models

Introduction

Soil erosion is recognized as one of the most critical environmental issues. This problem becomes even more critical in arid and semiarid zones, such as the west-central region of Argentina, primarily for two reasons: *i)* natural forest area declined at expenses of agricultural expansion and *ii)* the increased rainfall events in the region (in frequency and intensity) associated with the climate change (Barros *et al*., 2015). In order to implement strategies to control the flow of sediment, it is necessary to establish both the nature and location of the main sources of sediments within the watershed. Thus, recent research shows that sediment fingerprinting can be an effective approach for assembling information on suspended sediment sources (Blake *et al*., 2012; Collins and Walling, 2002). This technique has been successfully applied in different ecosystems using as fingerprints stable and radioactive isotopes (Schuller *et al.,* 2013), biomarkers, soil properties or trace elements (Gibbs, 2007; Walling, 2005). In addition, in recent years there has been growing interest in the use of Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFT-MIRS) combined with chemometrics multivariate statistical methods for the accurate prediction of soil properties at low costs. Among the multivariate methods used, PLS analysis is the most widely used because of its ability to address multicollinearity of spectral data (Stenberg *et al*., 2010; Viscarra Rossel *et al*., 2006). The performance of this model relies on the ability to extract important spectral characteristic features (e.g., electron transitions, overtones and combination of fundamental vibrations in the mid infrared frequencies) relevant to the soil attributes of interest (Viscarra Rossel and Lark, 2009; Viscarra Rossel *et al*., 2006. PLS is based on the assumption of a linear relationship between the dependent variable of interest (e.g. soil concentration of chemical elements) and a predictor variable (e.g. absorbance peaks in the MIRS spectra). Nevertheless, most models have been validated with traditional wet analytical techniques. This study presents preliminary results on the feasibility of using natural elements in soil as fingerprint to identify sedimentary sources in a small basin in the province of San Luis, Argentina. In addition, we have used a non destructive technique such as Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF) to calibrate and validate partial least square (PLS) analysis of DRIFT-MIRS spectral data.

Materials and methods

Study site. The Estancia Grande sub-catchment (Fig. 1) is located in central Argentina (S 33°10'; W 66°08'), 23 km north-east of San Luis City (Province of San Luis) at 1100 meters above sea level. The explored area is about 6 km^2, being part of the Rio Volcán catchment. The average annual temperature is 17 ºC, while in summer (December–March) the mean temperature is 23 ºC. Annual rainfall ranges from 600 mm to 800 mm, increasing in the last few years Rainfall varies seasonally, with a dry season (from May to October) and a rainy season (from November to April). Rains in the dry season are scarce and sporadic, with occasional drizzles.

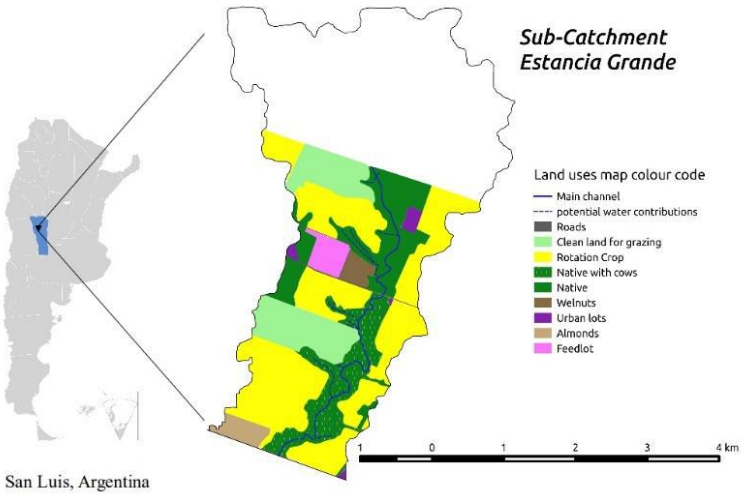

Figure 1: Sub-catchment Estancia Grande (San Luis, Argentina) with the land uses map.

The sub-catchment soils are mainly Xerosols Calcic with Solonetz inclusions, can also be formed by Lithosols. Soils in this series have as originating material a loamy loess, they are well supplied with organic matter in the upper 25 cm. The area is currently being used mostly for agriculture (rotation crop) and livestock (free grazing and feedlots), nevertheless there are some plots intended for growing nuts trees (walnuts and almonds).

Sampling. Soil and sediment sampling were conducted in April 2015. Source samples from surface soil layers (about 2 kg each one) were collected from the different land uses following standard procedures. Sediment samples (mixture samples) were collected from the deposition zone for the watershed sources (top 20 mm) on river banks or little flood plains where deposition has occurred. In laboratory, two artificial mixed samples were composed, using identified source samples.

Analytical methods. All the 29 collected soil and sediment samples and the two artificial mixed samples were initially prepared at the GEA-IMASL Laboratory. The sample preparation consisted of drying at 50° C and sieved to 2 mm. For Energy Dispersive X-Ray Spectrometry (EDXRF) analysis were the samples were ground to a fine powder which was used to make pellets of 25 mm diameter and 2.5 g weight. The pellets were measured in SPECTRO X-LAB 2000, a heavy-duty, fully software controlled EDXRF spectrometer (Pd-anode X-ray tube) utilizing 5 secondary targets, with the technical support of the Nuclear Science and Instrumentation Laboratory (IAEA Laboratories, Seibersdorf, Austria. Mid-Infrared Spectrometry -diffuse reflectance mode- (MIRS-DRIFT) were carried out at the Applied Chemistry Research Laboratory of the Central University of Venezuela. The samples were milled for 45 secs in a micromill. The spectral data was obtained in a Nicolet Si10 DRIFT-MIRS, finely grinded dry KBr was used as background. The spectra were collected between 4000 and 400 cm^{-1} with 4 cm^{-1} of resolution and 64 scans, the analyses were run in triplicates. The TQ analyst 9.4.45 software of Nicolet was used to perform the PLS of the EDFRX analysis in combination with the DRIFT-MIRS.

Normal spectral data as well as first and second derivative of the spectral were applied for developing prediction models that provides insight into a rapid and inexpensive estimate of the fingerprint elements in soils and sediments compared to conventional chemical procedures.

Mixing model. The CSSIAR v2.00 software (de los Santos-Villalobos *et al*., 2015) was used to study our catchment. The software has a friendly environment and is written in R language (free programming language). It provides the analysis of larger sets of data and gives more detailed statistical information (i.e. uncertainty).

Results and discussions

The CSSIAR v2.00 software was applied considering as input the concentration of the elements obtained from EDXRF analysis of the four soil sources forming part of the artificial mixed samples (MIX 1 and MIX 2). As it is known the real source proportions in the artificial mixtures, it is possible to find those tracers (constituent elements in this case) that can rebuild the mixture acceptably. Analyzing the X-Y plots of the elements concentrations it is possible to find which tracers can generate a polygon of sources that contain the 2 mixture's signatures. Once chosen an accurate sub set of elements, using the software, the model is executed using these fingerprints. The following 5 elements behave as effective tracers: Fe, Ca, Na, P and V. Figure 2 shows comparatively real and calculated proportion for MIX 1 and MIX 2.

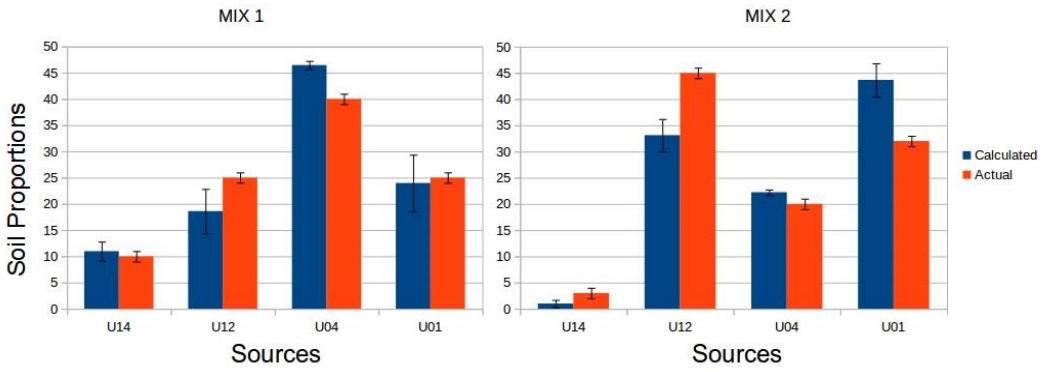

Figure 2. Comparision between actual and calculated soil proportion in the studied (mixed) samples.

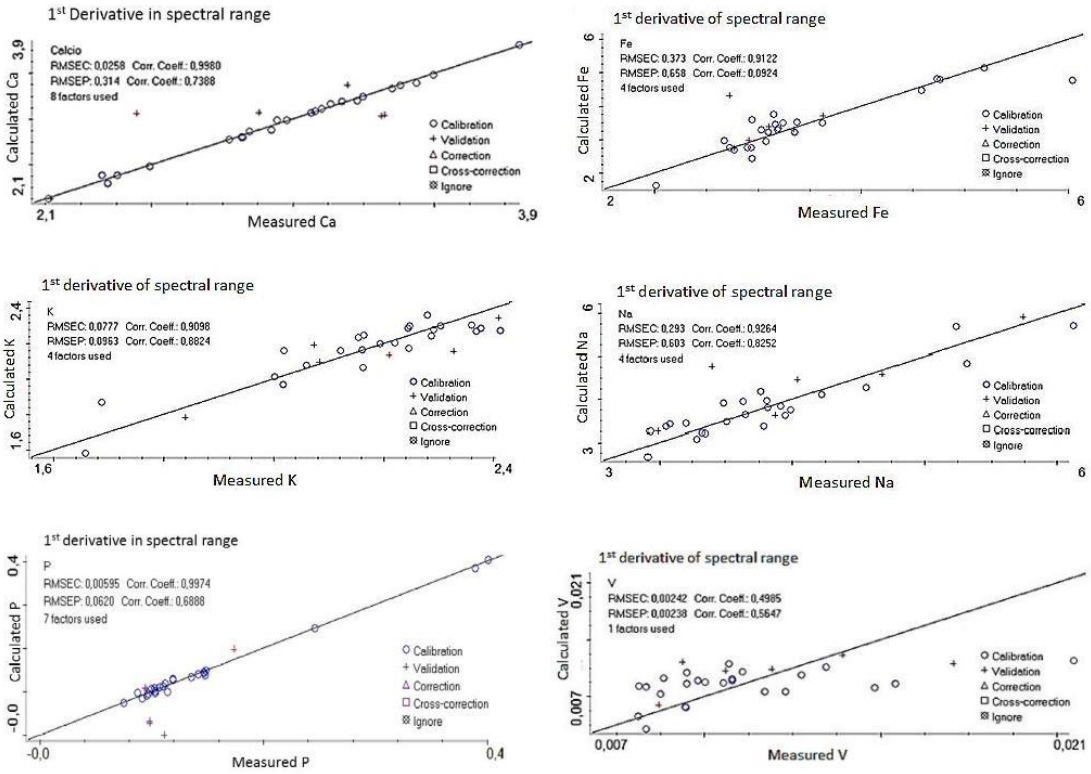

Figure 3. PLS Analysis. Predictive models using the 1st derivative of the spectral data.

A second point of this research was focused in determining if DRIFT-MIRS spectra combined with PLS (normal, first and second derivative) could generate good prediction models of the content of the identified fingerprint elements, across the different concentration ranges of them in all the soil/sediment samples. The cross-validation plots for the PLS of the 1st derivative using the DRIFT-MIRS spectra data for all samples (sources and mixed) are shown in Figure 3. The results for the model were obtained using P, Ca, Na, V, Fe and K to build the model (Table 1).

Conclusions

Fe, Ca, Na, P and V were suitable fingerprints for the deconstruction of mixed samples. In addition, the DRIFT-MIRS PLS analysis shows a good predictive model using the 1st derivative of the spectral data. This study demonstrated that it is possible to predict the parameters of the quality of P, Ca, K and Na as fingerprint elements, especially P which has been difficult to predict using DRIFT-MIRS PLS analysis with wet analytical techniques.

Table 1. Results of root mean square error for calibration and prediction of predictive model using the 1st derivative of the spectral data.

Results	Ca	K	P	Fe	V	Na
N° factors used	8	4	7	4	1	4
RMSEC	0.0258	0.0777	0.00595	0.373	0.00242	0.293
Correlation Coeff RMSEC	0.998	0.9098	0.9974	0.9122	0.4985	0.9264
RMSEP	0.314	0.0963	0.062	0.658	0.00238	0.603
Correlation Coeff RMSEC	0.7388	0.8824	0.6888	0.0924	0.5647	0.8252
% RMSEC	0.87	3.59	4.33	11.05	0.003	7.54

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BIOTECHNOLOGY SUMMIT

Photoperiod sensitivity of short aged Sri Lankan rice

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Abstract
Unawareness of photoperiodic sensitivity of rice is a disadvantage in breeding rice for adaptation to changing climatic conditions. This experiment was conducted to address the above problem through determining variation in days to flowering (DF) and morphological traits of selected twenty short aged Sri Lankan rice accessions under different photoperiod conditions: short day (SD), day neutral (DN) and long day (LD). Twelve accessions flowered early under LD. Five accessions did not respond to photoperiod for DF while 11 accessions flowered early under SD. LD condition produced significantly higher plant height irrespective of photoperiod responsiveness for DF. Variation in response to photoperiod for DF in short aged Sri Lankan rice indicates the potential diversity of genetic factors for photoperiod response.

Keywords Days to flowering * Photoperiod sensitivity * Short aged Sri Lankan rice accessions.

Introduction

Sri Lanka possesses a wide gene pool of traditional rice with higher environmental adaptability. Wider morphological variation of part of the above collection had been recorded (Team of NRC Research Project 12-129 2015). Despite potential advantageous, Sri Lankan rice cultivation is confined to new improved varieties since last few decades (Central Bank Report 2014). Rice (*Oryza sativa* L.) is a short day plant. Sri Lankan rice exhibits a wide variation in sensitivity to photoperiod, which controls rice plant growth and flowering leading to difficulties in cultivation (Chandraratna 1964). Identification of genetic factors controlling flowering time in response to photoperiod would be useful in future breeding programs on manipulation of cropping season for different agro-ecological zones (Kim *et al*. 2008 and Higashi and Izawa 2011). Rice plant architecture is affected by flowering time in response to photoperiod (Wei 2010). According to Team of NRC research project 12-129 (2015), different rice varieties differed markedly in flowering initiation during one short day season and several short aged accessions were recorded. In this experiment, selected short aged accessions were tested for their photoperiodic responses. Determination of photoperiod sensitivity in early flowering rice will be useful in developing early-flowering rice with desired plant architecture.

Materials and Methods

Plant material selection.

Twenty-eight accessions (of 70 -75 DF) were chosen (Table 1) from Team of NRC Research Project 12-129 (2015). Three improved rice varieties of Bg 300, Bg 379-2 and At 308, were included as control.

Table 1. Selected traditional rice accessions.

No.	Accession	Variety name	No.	Accession	Variety name
1	2088	*Suduheenati*	15	4144	*Mudukiri al*
2	2091	*Suduheenati*	16	4217	*Ballawala*
3	2979	*Heenmurunga*	17	4220	*Batapolayal*
4	3450	*Andaragaha wee*	18	4223	*Dhemas wee*
5	3457	*Ittikulama*	19	4237	*Hathe pas davase wee*
6	3677	*Herathbanda*	20	4245	*Iri wee*
7	3693	*Herathbanda*	21	4358	*Sudumada al*
8	3738	*Mudukiri al*	22	4387	*Baana wee*
9	3845	*Kahamalan*	23	4390	*Chellanayagam*
10	3883	*Japan heenati*	24	4513	*Galu wee*
11	3884	*Japan heenati*	25	4615	*Driver wee*
12	3943	*Cheenadi*	26	4734	*Inthiankaruppa*
13	3970	*Mudukiri al*	27	6305	*Enawakka*
14	4042	*Gam podi wee*	28	6741	*Kahasinanayam*

Site description

The experimental site for photoperiodic experiment was located in Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna (6° 17' 0" of North and 81° 17' 0" of East, 24 m above mean sea level). The experiment was conducted in a photoperiodic chamber (where SD, LD and DN conditions were imposed) from July, 2014 to January, 2015. Average monthly temperatures of 29.5 ^{0}C in July, 28.6 ^{0}C in August, 29.2 ^{0}C in September, 28.5 ^{0}C in October, 28.4 ^{0}C in November, 28.1 ^{0}C in December, 2014 and 29 ^{0}C in January, 2015 were recorded.

Experimental design and management practices

Nursery grown seedlings were maintained for 14 days under natural photoperiod prevailed at the time until transferring to the mud pots of 20 cm diameter. Plants were grown in three photoperiod conditions according in a Completely Randomized Design (CRD) in 3 replicates: short day (SD) condition with 8 hours of light and 16 hours of darkness, day neutral (DN) condition with 12 hours of light and 12 hours of darkness and long day (LD) condition with 14 hours of light and 10 hours of darkness. All plants were exposed to 8 hours of natural light while the plants in DN and LD chambers were exposed to light from white florescent bulbs. Fertilizer was added at two weeks, six weeks and ten weeks after plant

establishment, according to the recommendation of Department of Agriculture, Sri Lanka: Muriate of Potash (MOP) 0.157 g per plant and Triple Super Phosphate (TSP) 0.196 g per plant and Urea 0.157 g per plant (Urea 50 kg/ha, TSP 62.5 kg/ha, MOP 50 kg/ha) were applied. Plants were watered regularly.

Plant measurements and Data Analysis

Data were taken at flowering stage. Days to flowering (DF), plant height (PH) and culm number (CN) were measured according to the guidelines of Standard Evaluation System for Rice (PGRC 1999). Two-way analyses of variance (ANOVA) and analysis of means (ANOM) were conducted using Minitab® (Version 15.1.0.0.) statistical software and $p < 0.05$ was used to determine significance. Mean separation was carried out through Duncan Multiple Range Test (DMRT) using SAS software (9.1 version, USA) to investigate the most responsive accessions for selected traits.

Results and discussion

Effect of photoperiod on days to flowering and morphological traits

Under LD, the minimum DF (60 ± 0.706 days, CV = 1.66 %) was from accession 2088 (*Suduheenati*) and the maximum DF (113 ± 1.767, CV = 2.22 %) was from accession 4144 (*Mudukiri al*). The lowest and highest PHs were from At 308 (88.7± 8.96 cm, CV = 17.5 %) and accession 6305 of *Enawakka* (197.0 ± 0.707 cm, CV = 0.51 %) respectively. The CN varied in LD condition giving the lowest value by accession 2088 (2 ± 0.0, CV % = 0.0 %) and highest value as 10 ± 1.06 (CV % = 15.78 %) by accession 3970 (*Mudukiri al*). Under DN condition, accession 2088 gave the lowest DF as 60 ± 0.0 days (CV = 0.0 %) while accession 4387 (*Baana wee*) gave the highest DF of 107 ± 3.68 days (CV = 5.95 %). The lowest and highest PH was recorded in Bg 379-2 (93 ± 3.53 cm, CV = 5.37 %) and in accession 6305 (184.5 ± 2.47; CV % = 1.89 %) respectively. Accession 4513 (*Galu wee*) gave the lowest CN as 2 ± 0.353 (CV % = 33.3 %) and accession 3884 (*Japan wee*) gave the highest CN as 7 ± 2.49 (CV % = 3.63 %).

Under SD treatment also accession 2088 gave the lowest DF (68 ± 0.35 days, CV % = 0.74 %). Accession 3970 (*Mudukiri al*) recorded the highest DF in SD condition (110 ± 0.707, CV % = 0.909 %). The minimum and maximum PH were given by accession 3943 (*Cheenadi*), 67.5 ± 2.82 cm (CV %= 3.22 %) and accession 6305 (134.5 ± 9.54, CV %= 10.03 %) respectively. The lowest CN given by accession 3677 (2 ± 2.82, CV %= 4.65 %) and highest CN was given by accession 3884 (12 ± 3.74, CV %= 6.05 %).

Twelve accessions were identified as LD sensitive: 2088 (*Suduheenati*), 2091(*Suduheenati*), 2979 (*Heenmurunga*), 3450 (*Andaragaha wee*), 3883 (*Japan heenati*), 4217 (*Ellawala*), 4223 (*Dhemas wee*), 4245 (*Iri wee*), 4390 (*Chellanayagam*), 4615 (*Driver wee*), 3970 (*Mudukiri al*) and 6741 (*Kahasinanayam*). Ten accessions were sensitive to SD condition: 3693 (*Herathbanda*), 3943 (*Cheenadi*), 4042 (*Gam podi wee*), 4220 (*Batapola al*), 4237 (*Hathe pas dawase wee*), 4358 (*Sudu madal*), 4387 (*Baana wee*), 4513 (*Galu wee*), 4734 (*Inthiankaruppa*) and 4144 (*Mudukiri al*). Five accessions were identified as photoperiod insensitive: 3457 (*Ittikulama*), 3677 (*Herathbanda*), 3738 (*Mudukiri al*), 3884 (*Japan wee*) and 6305 (*Enawakka*) and three improved rice varieties of Bg 300, Bg 379-2 and At 308. Irrespective of photoperiod sensitivity, LD, SD and DN groups produced significantly higher PH under LD. CN was not affected by photoperiod for tested short aged

accessions (Table 2). However, increased CN had been previously reported when long aged Sri Lankan rice was grown under SD, DN and LD (Geekiyanage et al. 2012).
Vergara and Chang in 1985 and Chandraratne in 1964 wrote that many of the reported LD sensitive accessions were found to be SD sensitive in subsequent testing. Therefore, these accessions must be tested for further confirmation for photoperiod sensitivity. Exploration of photoperiodic effect on Sri Lankan traditional rice germplasm and flowering time gene expression analysis will be useful to identify genetic factors responsible for photoperiodic flowering, yield and plant structure manipulations in the future.

Table 2. Comparison between average values of DF, PH and CN of LD sensitive, SD sensitive and photoperiod insensitive plants in LD, DN and SD conditions.

Photoperiod sensitivity	Photoperiod condition	Days to flowering	Plant height (cm)	Culm number
LD sensitive	LD	70	123.9	5
	DN	78	126.3	4
	SD	80	94.1	5
SD sensitive	LD	94	143.3	3
	DN	91	137.9	3
	SD	75	90.7	4
Photoperiod insensitive (traditional accessions)	LD	83	151.1	5
	DN	81	144.8	4
	SD	84	103.7	6
Photoperiod insensitive (Improved varieties)	LD	91	92.1	5
	DN	90	100.5	5
	SD	90	81.3	4

Conclusion

There were different photoperiod responses for days to flowering from the tested short aged Sri Lankan traditional rice accessions. Accessions 3693 (*Herathbanda*), 3943 (*Cheenadi*), 4042 (*Gam podi wee*), 4220 (*Batapola al*), 4237 (*Hathe pas dawase wee*), 4358 (*Sudu madal*), 4387 (*Baana wee*), 4513 (*Galu wee*), 4734 (*Inthiankaruppa*) and 4144 (*Mudukiri al*) were short day sensitive. Accessions 2088 (*Suduheenati*), 2091(*Suduheenati*), 2979 (*Heenmurunga*), 3450 (*Andaragaha wee*), 3883 (*Japan heenati*), 4217 (*Ellawala*), 4223 (*Dhemas wee*), 4245 (*Iri wee*), 4390 (*Chellanayagam*), 4615 (*Driver wee*), 3970 (*Mudukiri al*) and 6741 (*Kahasinanayam*) were long day sensitive while accessions 3457 (*Ittikulama*), 3677 (*Herathbanda*), 3738 (*Mudukiri al*), 3884 (*Japan wee*) and 6305 (*Enawakka*) were photoperiod insensitive. Long days increased plant height in all accessions.

Acknowledgement Authors are thankful to National Research Council for funding through NRC 12-129 grant and Plant Genetic Resources Centre, Sri Lanka for traditional rice seeds.

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Determination of soil erosion sources in forest catchments in central Chile using Compound-Specific Stable Isotope technique (CSSI)

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Abstract:
There are several conventional methods to determine soil erosion rates and sources. However, nuclear techniques have become increasingly important due to its simplicity and effectiveness. Compound-Specific Stable Isotope technique (CSSI) has become an effective tool to determine soil erosion sources. This method employs biomarkers such as fatty acids to match soil sources with land uses within a catchment. To identify sources and measure sediment contribution rates this method match $\delta^{13}C$ of fatty acids with bulk $\delta^{13}C$ data using a mixing model. The study sites were located in central Chile. They present several potential soil erosion sources such as slopes (with pinus, native forest and eucalyptus plantations), roads, buffer zone (mainly comprised by native forest) and stream banks. Sediment traps were placed in V notched weirs at the output of watercourse and by CSSI technique were determined which of the land uses was the main contributor of sediments. The results indicated that roads were the main sediment contributor to the catchments (more than 70% of total source contribution). Finally, it is possible to conclude that the technique is capable to discriminate between different land uses and estimate sediment contribution in percentage according to total sediment load within the catchments.

Keywords: Soil erosion • Fatty acids • Soil apportionment • delta^{13}C • CSSI.

Introduction

Soil degradation is a major problem around the world (Dotterweich 2013). This event has negative impacts on the future of soil fertility, water quality, food safety, etc. (Dercon *et al.* 2012). With the aim of understanding the real impact of soil erosion, it is relevant to determine the proportions of each potential source of sediment contributing to the affected areas (Walling 2013). This process not only implies soil loss, as well the transport of soluble compounds or chemicals that are bounded to soil particles, such as: pesticides, heavy metals, fertilisers, etc. Furthermore, the process of identifying sources became essential to track down the hot spots of land degradation in order to implement mitigation actions. At

the moment, different techniques have been applied to determine the “hot spots” of land degradation. Within the techniques used are: direct measurements, GIS methods, geological interpretations, modelling, satellite imaging, etc. (Bayramov, Buchroithner, and Bayramov 2016). Nowadays, the isotopic techniques are becoming a powerful tool to assess soil erosion, including fallout radionuclides such as ^{7}Be, ^{137}Cs and ^{210}Pb. These tracers are able to determine the erosion and deposition rates at different geographical scales (Smith and Blake 2014, Dercon *et al.* 2012, Mabit *et al.* 2013). However, their application in fingerprinting to track down the sources of soil erosion in forest areas has not been extensively used (Schuller *et al.* 2013). The assessment of soil apportionment using Compound Specific Stable Isotopes (CSSI) of δ^{13}C for fatty acids was firstly introduced to study the origin of estuarine sediments (Gibbs 2008). The technique is based on the signature that is produced by different types of vegetation primarily through the roots onto the soil, that is different according to the different isotopic values of a suit of fatty acids from diverse land uses (*e.g.* forest, agricultural, etc.) (Gibbs 2008). These fatty acids are linked to the soil and when erosion occurs, the soils travels together with its signature. Previous studies have determined that CSSI technique using fatty acids was able to discriminate different type of land uses including forest catchments (Hancock and Revill 2013). The Chilean forest activity is principally based on exotic plantations that involve fast growing species such as Pinus radiata and Eucalyptus *spp*. This activity has increased significantly during the last 15 years (CORMA, 2012), and consequently the increment of sediment loads, especially during activities post-harvesting as well the construction and intensive use of roads by heavy machinery. Particularly in areas with steep slopes, that generates the greatest potential for sediment mobilisation (Schuller *et al.* 2013, Litschert and MacDonald 2009). To accomplish forest certification requirements, Chilean forest companies are required to protect catchments from the off-site impacts of forest operations. Careful location and layout of roads and logging operations and proper planning and use of best management practices can reduce the magnitude of erosion and related sedimentation effects (Stringer and Thompson 2000, Schuller *et al.* 2013). The aim of this research is introduce for the first time the use of the CSSI technique, using data of δ^{13}C values of fatty acids for soil fingerprinting in Chile, and specifically in forest catchments with no harvesting operations and to determine if it is possible to differentiate within the possible identified sources using the software CSSIAR v.2 to determine the temporal and spatial source contribution of soil from the forest catchment.

Material and Methods:

Study sites. Three study sites were selected from forest catchment sites in south-central Chile. These catchments were all with no harvesting operation in order to investigate the sources of sediments under normal conditions and then compare (in the future) with post-harvesting operations. Within the catchments, the potential sources of sediments were identified (roads, native forests, buffer zones, watersheds and forest plantations).

Sampling collection. Collection of samples for each source material as well as mixtures was done by multiple composite surface sampling (2 cm depth using metal spatulas and PVC disks) to characterise the spatial variability of the fingerprint properties (small branches, pine needles, roots, leaves and other major objects were removed before taking the samples). The samples were collected using a grid pattern (samples taken on the borders of a 10x10 m square and a central point) then mixed; whereas for roads and stream bank the samples

were collected from representative points along these sites. The individual samples of watersheds material comprised composite samples of material from the bottom and from the full vertical extent of the bank profile. Figure 1 shows where the samples were collected at each study catchment.

Collection of bulk suspended sediment samples at the catchment outlets for fingerprint analyses were obtained during the rainy season (from 2009 to 2014) by collecting the suspended sediment from the v-notched weir pools. The weir was emptied and cleaned after collection of the suspended sediment sample and prior to the collection of the subsequent bulk sample. The sediments trapped were washed with the same stream water and collected in plastic containers, then let the sediment to settle and pour the water.

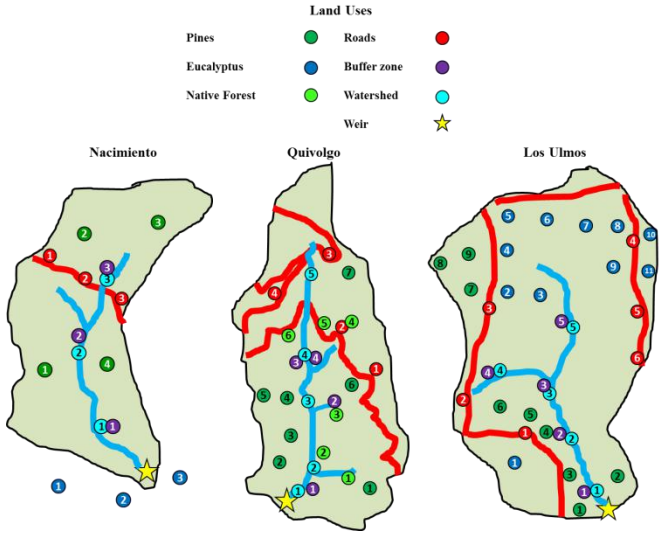

Figure 1. Sampling sites at the different catchments according to the land uses.

Sample preparation and analysis. The samples were oven dried at 60°C for 48 hrs, then sieved through 2 mm mesh. The extraction procedure was performed using an accelerated solvent extraction (ASE) equipment with dichloromethane (Nacimiento Catchment). For Los Ulmos and Quivolgo catchments the following extraction methodology was applied: 20 grams of dry sediments were placed into glass flasks with dichloromethane and sodium sulphate anhydrous, and then they were placed in a shaker at 200 rpm for 24 hrs. The extracts were poured into a rounded flask and then more solvent was added to the same sediments to perform a second extraction using an ultrasonic bath for 2 hours. The extracts were combined, concentrated using a rotoevaporator and then dried with pure nitrogen. Posteriously, the dry extracts were derivatized with a mixture of BF_3/Methanol 5% solution (for 20 min at 70°C) to obtain the fatty acid methyl esters (FAMEs) and further extracted with a vortex with a mixture of dichloromethane:hexane 1:4 and water, the organic phase was placed into a 2 mL amber vials and dried with pure nitrogen. The bulk $\delta^{13}C$, carbon % and the isotopic values were determined at the Stable Isotope Facilities UC-Davis, California using a GC-c-IRMS equipment. The $\delta^{13}C$ values of different FAMEs were used to describe different land uses in the forest catchment. The methyl group added from the derivatisation process of the fatty acids using BF_3/MetOH was corrected using the following expression (Gibbs 2008):

$$\delta^{13}C = \frac{\delta^{13}C_{FAMEs} - (1-X)\delta^{13}C_{Methanol}}{X}$$

Where X is calculated using the total number of carbon of the original fatty acid divided by the number of carbon of the derivatized molecule (FAME). These fingerprint properties were used to discriminate between the primary potential fine sediment sources in the catchment: pines, eucalyptus, roads, buffer zones, native vegetation and watersheds (bank erosion). For fingerprinting, the data was analysed using the software CSSIARv.2, this software is a mixing model that was adapted from SIAR (Parnell *et al.* 2010) to work with isotopic values, but exclusively for soil erosion studies.

Results and discussion

The results indicated that in all catchments (with no harvest) the average contributions of fine sediments to the weir were: Los Ulmos (roads: 8.9%, buffer zone: 25.2%, stream banks: 59.5%, eucalyptus: 2.4% and pines: 4.0%); Quivolgo: (roads: 41.6%, buffer zone: 4.8%, stream banks: 39.4%, native forest: 6.3% and pines: 5.6%) and in Nacimiento (roads: 21.5%, buffer zone: 19.3%, stream banks: 45.3%, Eucalyptus: 5.7% and pines: 8.2%). These show that roads and stream banks were the main sources. However, it is important to highlight that calculation in intermediate points of the catchments (before the weir) presented that roads were the main source of sediment to the stream banks, and consequently roads are the main source of sediment for all studied catchments. This is expected due to with no harvesting conditions roads are used by machinery that operates within the catchments making the soil able to be eroded. Also rain impacts directly onto the bare soil, and in forested areas the canopy and leaves on top the soil prevent the soil to be eroded. Nevertheless, this situation may drastically change when clear cut operations take action within the catchments.

Conclusions

CSSI technique is a good tool to determine soil erosion sources within a catchment. When no harvesting operation take place, roads are the main source of sediment (more than 70% of total source contribution).

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BIOTECHNOLOGY SUMMIT

Molecular diversity of soil bacteria tolerant to Chlorothalonil, associated with wheat in the Yaqui Valley, Sonora

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Abstract
Tolerance to the fungicide Coraza® 720 S (52% Chlorothalonil) of bacteria isolated from soils destinations wheat production in the Yaqui Valley, was evaluated. This fungicide has been reported as a growth inhibitor fungus, ignoring their impact on soil bacterial communities. Thus, 127 native bacterial strains (preserved in the Colección de Microorganismos Edáficos y Endófitos Nativos, COLMENA) were inoculated (1 x 10^3 UFC) in duplicate on agar, supplemented with 4.3 g L^{-1} of Coraza® 720 S, and incubated at 28 °C for 48 hours, then the growth of these strains in presence of Chlorothalonil was observed. A total of 67.7% of the tested bacterial strains were able to grow at tested concentration. The taxonomic identification of these strains was done by partial sequencing of the 16S rRNA gene. The results showed that *Bacillus*, *Pseudomonas* and *Stenotrophomonas* were the most predominantly genera, indicating that not all bacteria have the ability to grow under these conditions, thus, compatibility tests between bacterial inoculants and this fungicide are necessary as additional control point to increase the chances of success of these inoculants in agricultural systems in the region for production of wheat.

Key words: wheat • tolerance • Chlorothalonil • inhibition.

Introduction

Wheat (*Triticum spp.*) is one of the three most produced and consumed grains worldwide (IBCE, 2012). The national production of wheat is about 3.7 x 10^6 tons, where 50% is produced in Sonora (SAGARPA, 2011). However, wheat production is diminished by several plant pathogens, being the most important those caused by fungi, such as: *Fusarium graminearum*, *Bipolaris sorokiniana*, *Aspergillus spp.*, *Penicillium spp. and Tilletia indica,* causal agents of Fusarium head blight, black point, seed rot, powdery mildew and karnal bunt, respectively (Warham *et al.* 1998; Agromeat, 2010). In order to protect wheat grains against fungal infections, these are commonly treated with fungicides before their storage. Several fungicides are currently used for the seed wheat treatment, some of them act by contact (captan, mancozeb, maneb, thiram), or have a systemic action (difenconazole, metalaxyl, tebuconazole, thiabendazole, triadimenol) (Lozano *et al.* 2006). In Mexico, the NOM-001-FITO-1995 states that wheat seeds should be exposed to the fungicide Chlorothalonil, at a dose of 750 grams per ton of seeds, in order to mainly control karnal bunt of wheat (*Tilletia indica* Mitra). Chlorothalonil, is a contact fungicide, with broad spectrum, has a half-life of up to 90 days, and it is listed as a carcinogenic compound

(Greene y Pohanish, 2005). Chlorothalonil inhibits respiration of fungal cells, *i.e.* the transformation of carbohydrates into energy. Since Chlorothalonil molecules bind to sulfhydryl groups of some amino acids, blocking the enzyme production, being negatively affected the Krebs cycle (no ATP is produced), which stops the growth and eventually results in the death of the fungus (De Liñan, 2015). Despite the beneficial effects of chemical treatment, negative side effects have been reported, such as alterations in the seed and ecosystem due to its high toxicity and permanence. Besides the target pathogens these chemicals indiscriminately affect all organisms, including the human (Juarez *et al*. 2014; Lozano *et al.* 2006). In this context, the objective of this work was to evaluate the tolerance of soil bacteria isolated from wheat filed in the Yaqui Valley, Sonora to the fungicide Coraza® 720 S (52% Chlorothalonil). This fungicide has been reported as a fungi growth inhibitor; however, its impact on soil bacterial communities has not been yet studied.

Materials and Methods

Bacterial strains and selected fungicide. The bacteria studied (127 strains) were isolated from soil used for the wheat cultivation in the Yaqui Valley, and belonging to the Colección de Microorganismos Edáficos y Endófitos Nativos (http://www.itson.mx/COLMENA), in Laboratorio de Biotecnología del Recurso Microbiano (LBRM), placed at the Instituto Tecnológico de Sonora. The fungicide used was Coraza® 720 S (Chlorothalonil 52%), according to the manufacture instruction.

Bacteria-Chlorothalonil tolerance test. The ability of the strains to tolerate the fungicide Coraza® 720 S, was determined qualitatively according to the presence of the strain growth, which were inoculated into Petri dishes containing nutrient agar as culture medium, supplemented with 4.3 g L^{-1} of the fungicide, equivalent to the double dose the commonly used to treat wheat seed (3 L/ton of seed) according to NOM-001-FITO-1995. Inoculated Petri dishes were incubated at 28°C for 48 hours.

Molecular Identification. Genomic DNA from each strain was extracted according to Reader and Broda (1985). The 16S rRNA gene was amplified using the primers FD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG -3') and RD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC -3'), the Polymerase Chain Reaction (PCR) technique consisted of an initial denaturation step at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 40 s at 57 °C and 2 min at 72 °C, and a final elongation step of 5 min at 72 °C. PCR products were verified by 2% agarose gel electrophoresis, purified and sequenced by Sanger platform. Sequences were compared and deposited in the NCBI Genbank. 16S rRNA gene sequences of isolates were aligned with software Clustal W. The phylogenetic tree was constructed using the Neighbor-Joining method, using the software MEGA 6. 16S rRNA of *Micrococcuss luteus* (KU707915.1) was used as outgroup. Stability of clades was assessed with 1000 bootstrap replications, only Bootstrap percentages above 50% are shown.

Results and discussion

A total of 127 bacterial strains were evaluated, where 67.7% - 86 strains- were able to grow in Petri dishes containing nutrient agar supplemented with 4.3 g L^{-1} Chlorothalonil. Based on the 16S rRNA sequence, these strains were identified belonging to 20 genera (Figure 1), being the dominant genera *Bacillus* (25 strains), *Pseudomonas* (11 strains), and *Stretophomonas* (10 strains) (Figure 2).

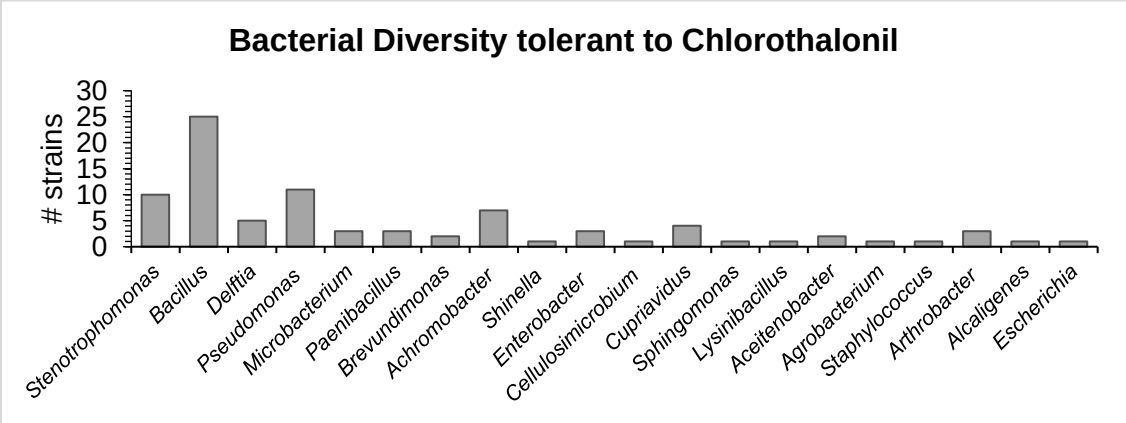

Figure 1. Bacteria genera tolerant to fungicide Coraza® 720 S (Chlorothalonil 52%).

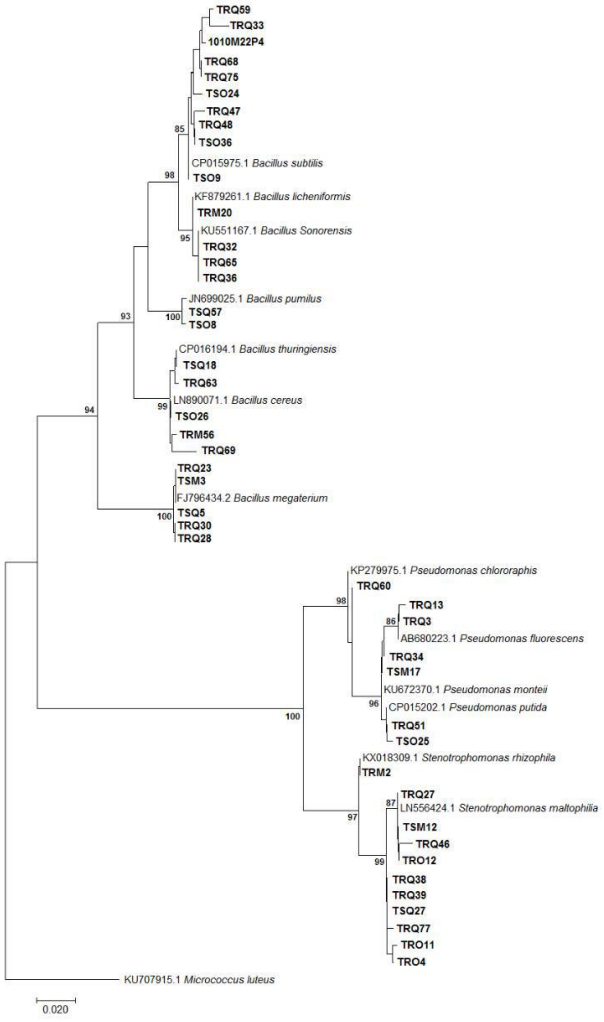

Figure 2. Phylogenetic tree (16S rRNA gene) of 46 Chlorothalonil tolerant strains isolated in the Yaqui Valley. The tree was constructed using the Neighbor-Joining method. *Micrococcus luteus* (KU707915.1) was included as outgroup. Only Bootstrap percentages above 50% are shown (based on 1000 replications).

Thirteen strains were identified as *Pseudomonas* among them, 84% tolerated the fungicide at the concentration evaluated. Macroscopically, *Pseudomonas* strains showed higher growth showing any kind of inhibition by Chlorothalonil (Figure 3a). Alanis and Guerrero (2014), reported *Pseudomonas* strains with the intrinsic ability to tolerate toxic agents, often supported by genes contained in plasmids, known as resistance factors. Even when the *Bacillus* genera were the most abundant (55 strains), only 45% tolerated the fungicide at the concentration evaluated. The *Bacillus* strains showed lower growth compared to other genera strains. Also, 90% of the strains identified as *Stenotrophomonas*, present fungicide tolerance, which development were greater than *Bacillus*, but smaller than the strains identified as *Pseudomonas* (Figure 3b).

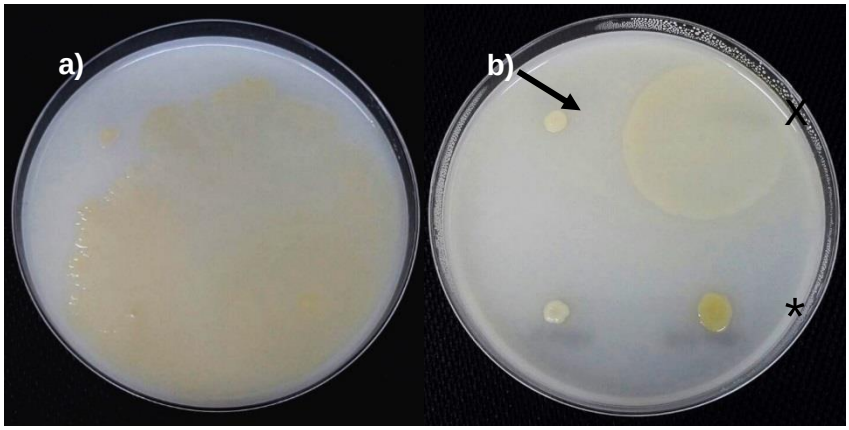

Figure 3. a) Testing of tolerance of the strain *Pseudomonas putida* TSM17 to Chlorothalonil at a concentration of 4.3 g L^{-1}, with no inhibition by the fungicide. b) Growth comparison of *Bacillus megaterium* (TRQ28) (arrow), *Pseudomonas sp* (TRQ52) (cross) and *Stenotrophomonas maltophilia* TRQ39 (asterisk) in nutrient agar media supplemented with 4.3 g L^{-1} of Chlorothalonil at 48 hours.

Moreover, 32.3% of the strains were inhibited by Chlorothalonil, the taxonomic identification of these showed that 73% belong to the genus *Bacillus*, 7% *Paenibacillus*, and 20% to *Pseudomonas*, *Lysinibacillus*, *Stenotrophomonas*, *Enterobacter*, *Staphylococcus* and *Streptomyces*. Hence, there was no genera selectivity by Chlorothalonil, therefore, the tolerance capacity of the fungicide Coraza® 720 S (Chlorothalonil 52%) is specific of each strain.

Conclusions

In assessing bacterial isolates of soil for wheat production in the Yaqui Valley, Sonora, Mexico, it was found that Chlorothalonil besides inhibiting the growth of fungi also impacts negatively several bacterial genera, which may have specific ecological niches on the soil of the Yaqui Valley for wheat production. Modifying the native microbial communities with potential metabolic characteristics involved in plant growth promoting, induction of tolerance to various types of stress and / or biological control agents. However, the bacteria that can tolerate fungicides and also have some metabolic characteristics can be considered for

potential use in combination with chemicals present in the soil without some adverse effect in their microbial development and increase the plant growth, such as microbial inoculants. The use of microbial inoculants for promoting plant growth and biocontrol pathogen, favors increased production performing sustainable agriculture, however, as demonstrated in this assay conventional agrochemicals like fungicides are not completely selective, and these diminish the beneficial microbial diversity. Therefore, this work allowed the identification of microorganisms tolerant to the fungicide Coraza® 720 S, which will be evaluated to determine whether they are plant growth promoters or perform functions as biological control agents to be implemented in the field to encourage grain production while reducing the use of chemical agents applied to agricultural soil.

Acknowledge The authors acknowledge support by the Cátedras CONACyT Program through Project 1774 “Alternativas agrobiotecnológicas para incrementar la competitividad del cultivo de trigo en el Valle del Yaqui: desde su ecología microbiana hasta su adaptabilidad al cambio climático”, and the CONACyT Project 253663 “Fortalecimiento de la infraestructura del Laboratorio de Biotecnología del Recurso Microbiano del ITSON para la creación de COLMENA: COLección de Microrganismos Edáficos y Endófitos NAtivos, para contribuir a la seguridad alimentaria regional y nacional”.

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BIOTECHNOLOGY SUMMIT

Adequacy of a DNA extraction method for agaves obtained by *in vitro* culture

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Abstract

The agaves are endemic to the American continent and are present majorly in abundance in Mexico. The more important are those that provide an economic benefit for society, however there exist copies that don't get their due attention. There are biotechnological alternatives for the multiplication of these copies, but it should be assured that the seedling would aim to spread, because there exists species of agaves that cannot be identified adequately with morphological characteristics. It's because of them that it could be identified through molecular identification to make sure, being the most important thing to have the best quality of DNA. This method initially reported in cacti suggests remove el supernatant and leave the pellet, however in this work the technique to preserve the supernatant which contained the appropriate DNA for molecular analysis and sequencing is appropriate. This method is reproducible and simple for DNA extraction routine of Agavaceae.

Keywords: Agavaceae ●Maturase K gen ●DNA ●protocol ●supernatant.

Introduction

The agaves are endemic to the American continent. Of approximately 200 species, 150 are found on Mexican territory, they are more abundant is arid and semiarid according to García-Mendoza (2007). Agaves are perennial plants, herbaceous or trees, with fibrous leaves, seated baselines are locations at the end of the branches where rosettes arranged spirally, and occasionally with the margin spiky y succulent. Verhoek, S. *et al.* (2003). Much of the wide diversity of species in México it consists only of species that inhabit our country. Some are restricted to certain areas, these species are endemic, have restricted distribution to a particular territory (CONABIO, 2012). Which makes them endemic to many, but some are considered by the Norma Oficial Mexicana NOM-059-SEMARNAT-2010, to be endangered, risk or subject to special protection, such as the *Agave titanota,* Gentry (1982) one of the authors with more input on the subject, they describe it in their book Agaves of Continental North America Having found a large population in the canyon walls close Rancho Tambor, Oaxaca, Mexico. Species identification and knowledge of their biological cycles are very slow to *Agave*, since most of its species growth is slow and a large number of species have only bloom and then die, they live on an average of 15 to 20 years in the wild (Granados-Sánchez D. 1993). In order to contribute to the micropropagation of agaves obtained by culture *in vitro,* we should make sure that these correspond to initially established reports to

the molecular identification, standardizing a method of extracting DNA suitable to meet the initial purpose.

Materials and methods

Seedlings used for standardization of the extraction method were obtained from the Laboratory of Cultivo de Tejidos Vegetales, Facultad de Biología, Universidad Veracruzana Xalapa, Ver. Two methods for DNA extraction are compared. The first consisted of a protocol used in Agavaceae reported by Keb-Llanes *et. al.* (2002). To this, were collected approximately 300 mg of leaf tissue, which were placed in sterile mortar to maceration and 300 µL of buffer A (2% Hexadecyltrimethylammonium bromine (CTAB) (w/v), 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 4% polyvinylpyrrolidone (PVP-40) (w/v), 0.1% ascorbic acid (w/v), and 10 mM β-mercaptoethanol) and 900 µl of buffer B (100 mM Tris-HCl (pH 8), 50 mM EDTA, 100 mM NaCl and 10 mM β-mercaptoethanol) and 100 µL SDS 20% were added. After maceration, the solution was transferred to 1.5 mL sterilized tube and vortex the mixture and incubated in a water bath at 65°C for 10 min. Next were added 410 µL of cold potassium acetate (5 M at -20°C) mixed thoroughly and centrifuged for 15 min at 15,300 *g*. The supernatant was taken and transferred to a clean 1.5 mL tube and added 540 µL of cold isopropanol the solution was incubated at -20°C for 20 min. Subsequently was centrifuged for 10 min at 9600 g. The supernatant was discarded and the pellet was washed with 500 µL 70% ethanol and let dry. The pellet was resuspended in 600 µL of TE buffer (10 mM Tris, 1 mM EDTA (pH 8)) and added 60 µL 3 M sodium acetate (pH 5.2) and 360 µL cold isopropanol. This solution was incubated on ice for 20 min and centrifuged at 9600 *g* for 10 min, (repeat 5-7 twice). Finally, the pellet was resuspended in 50 µL of TE buffer. The second protocol used in pitahaya, was a modification to Tel-Zur *et al*. (1999) proposed by Hernández-de la Cruz *et. al.* (2015) work successfully tested in agavaceas. To this, were collected approximately 500 mg fresh roots with distilled water to remove foreign material, which were placed in sterile mortar to maceration with liquid nitrogen, the frozen powered tissue was transferred in to a 1.5 ml centrifuge tube, added 600 µl extraction buffer (100 mM Tris-HCl pH 8, 0.35 M sorbitol, 5 mM EDTA pH 8 and 1% β-mercaptoethanol (added just before use) and it was kept on ice and centrifuged for 10 min at 10,000 *g*. The supernatant was discarded and the pellet dissolved with 150 µl extraction buffer, 100 µl high-salt CTAB buffer (50 mM Tris-HCl pH 8, containing 4 M NaCl, 1.8% CTAB and 25 mM EDTA pH 8) and 10 µL of SDS 30%. Next were incubated for 60-90 min at 55°C and added 250 µl de chloroform:isoamyl alcohol (24:1, v/v) and centrifuged for 10 min at 10,000 *g*. The supernatant was transferred to 1.5 mL centrifuge tube and added 160 µl of cold absolute isopropanol mixed with 35 µl sodium acetate solution (3M, pH 5.2) and centrifuged at 10,000 *g* for 20 min. The supernatant was discarded and the pellet was washed with 500 µl 75% cold ethanol. Next the ethanol was discarded and the samples were air-dried. The pellet was dissolved in 200 µL TE buffer (10 mM Tris pH 8, 1 mM EDTA pH 8) and added 10 µL of RNase stock solution and incubate at 37°C for 40 min. Added 250 µl phenol:chloroform (1:1 v/v). Subsequently was centrifuged at 10,000 *g* for 10 min. The supernatant was transferred in to a 1.5 ml centrifuge tube and added 250 µl cold chloroform. Then was centrifuged for 10 min at 9600 *g.* The upper aqueous phase was removed and the DNA precipitated with 200 µl of absolute cold 100% ethanol mixed with 30 µl of sodium acetate solution and kept at -20°C for 30 min. Finally, the tube was centrifuged al 1000 *g* for

15 min. The pellet was rinsed with cold 75% ethanol and air-dry the samples and was dissolved with 50-100 µL of TE buffer.
To assess the integrity degree of the DNA was carried out an agarose gel electrophoresis 0.8% (0.5 X TBE) in a horizontal chamber (BioRad) and 100 V were applied consistently. Gels were stained in 100 mL of 1X TBE solution added with 2 mL of ethidium bromide (10 mg mL-1) for 20 min and visualized with photodocumentation system (GelDoc BioRad).
PCR amplification. Maturase K (matK) was amplified using the matkF (5´-TTGGTCTCAACCGTACAGGA-3´) y matkR (5´-ATTGGCCCAGATCGGCTTAC-3´) oligonucleotides obtained from GenBank database, No. Accession AM884165. The reaction was using a reaction volume of 25 µl composed of 1X PCR buffer, 2.5 mM MgCl2, 1 U of Taq DNA pol (Promega), 1 mM dNTPs, 0.25 mM of each primer and ~ 50-100 ng of genomic DNA. Amplifications were performed in a thermocycler (BioRad) under the following thermal cycling program: an initial denaturation for 2 min at 95 ° C; 40 cycles of denaturation at 94 ° C for 45 s, 55°C for 1 min, and extension at 72 ° C for 1 min; followed by a final extension at 72 ° C for 5 min. Modifications to the protocol described above to obtain a single amplification product of the expected size were performed.

Results and discussion

The proposed method Keb-Llanes *et al*. (2002) they report that the success of the extraction method is the repetition of the steps where the pellet is washed several times, however in the experience of this work it was not possible to obtain DNA, so it makes a matching, eliminating repetition washing, obtaining DNA but not of good quality (Figure 1).

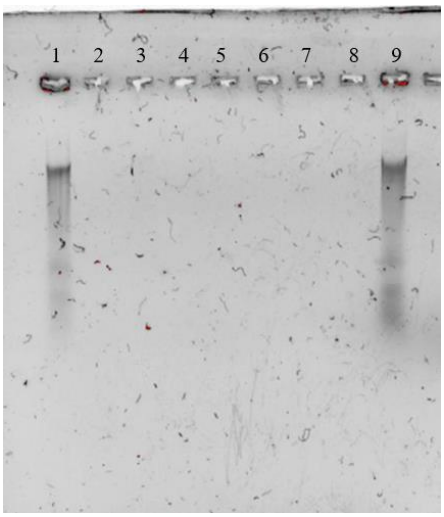

Figure 1. Agarose gel (0.8%) of genomic DNA extracted with Keb-Llanes *et. al.* (2002) method.

In contrast, the method modified from that proposed by Tel-Zur *et al*. (1999), proved efficient for extracting DNA of agave, achieving obtain material degraded and best quality in all the samples (Figure 2).

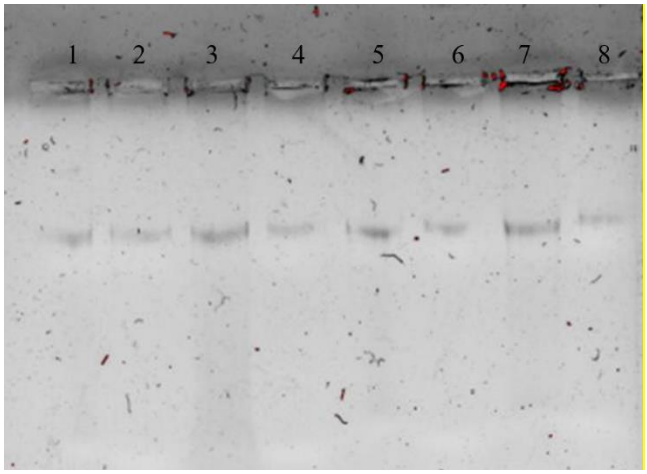

Figure 2. Agarose gel (0.8%) of genomic DNA extracted with Tel-Zur *et. al.* (2002) method.

This might be attributable to that among the modifications made to the original extraction method is the incubation temperature it was modified of 55°C to 80°C for 75 min. The original protocol suggests removing the supernatant and retaining the pellet, however it decided to retain this supernatant and it was used directly in molecular biology protocols (matK amplification) omitting the step of precipitation with ethanol or isopropanol, which reduces the risk of material loss during manual decantation alcohol. The matK gene amplification was performed under thermal reaction conditions and as indicated by the proposed procedure, with satisfactory results. By separating the amplification products on agarose gel (1.8%) allowing amplify only products of ~350 bp well-defined and reproducible (Figure 3).

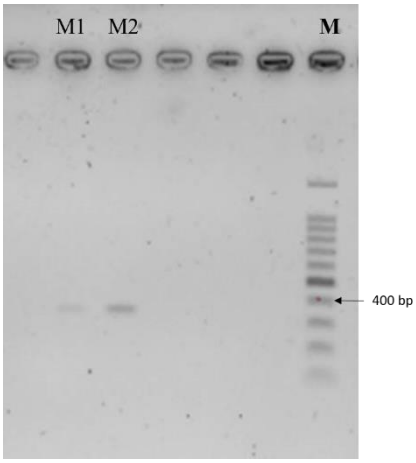

Figure 3. Agarose gel (1.8%), where well defined band of ~350 bp. M=Molecular weight marker.

Conclusions

The proposed method Tel-Zur *et al* (1999) not only effective for cactuses, but agaves as well, with the adjustments mentioned above provides a better result in obtaining DNA, as to make the pellet and the supernatant, a larger amount of genetic material is obtained, stating that better sample quality for the supernatant was obtained.

Acknowledgements To the Laboratorio de Cultivo de Tejidos Vegetales, Facultad de Biología, Universidad Veracruzana en Xalapa, Veracruz by providing plants to develop this study.

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BIOTECHNOLOGY SUMMIT

Improved gold phytoextraction of transgenic and wild plants of *Brassica juncea* using chemical chelators in silica sand

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Abstract

Transgenic indian mustard *Brassica juncea* plants overproducing the enzymes ATP sulfurylase, γ-glutamylcysteine synthetase (ECS) and glutathione synthetase (GS) were shown previously to have increased levels of the metal-binding thiol peptides phytochelatins and glutathione, and enhanced heavy metals tolerance and accumulation. To examine the gold phytomining potential, APS8, cpECS2, and cytGS2 transgenic lines were utilized in a greenhouse experiment in which the transgenics were grown on silica sand. The influence of ammonium thiocyanate and thiourea as chelators were determined to induce gold hyperaccumulation in four treatments. The ECS and GS transgenics plants accumulated significantly ($P< 0.05$) more gold in their tissues than wild type plants while the cytGS2 plants in treatment with ammonium thiocyanate did not.In all cases, presence of gen determining overexpression of these enzymes, increase activity of absorption of gold in transgenic plants, compared with wild type plants. So, overexpression of γ-glutamylcysteine synthetase, ATP sulfurylase and glutathione synthetase in transgenic plants appears to be a promising strategy to enhancing the efficiency of heavy metal phytoextraction from metal contaminated sites and the innovation to phytoextract precious metals like gold.

Keywords: Phytotechnologies • Enzymes • Phytomining.

Introduction

It has been recognized that some plants are able to hyperaccumulate metals to a concentration much higher than the substrate concentration (Sheoran *et al*. 2013). Previous studies, have demonstrated that plants can be used to recovery gold from mine tailings, originating phytomining. This technology is based on the ability of the plants to extract and translocate valuable metals from the roots to the above ground plant parts through phytoextraction mechanisms (Gardea *et al.* 2005). Phytomining is considered to be less intrusive, requires reduced energy than traditional mining techniques which are energy and resource intensive, and require substantial site remediation at the end-of-life of the mine (Sheoran *et al*, 2013). Besides the existence of current technology for engineering plants utilized as hyperaccumulators of heavy metals, another new development for phytoremediation purposes is the use of transgenic plants (Pilon-Smits, 2005). Genetic engineering of plants offer a low cost mechanism to enhance capacity of plants to accumulate and tolerate heavy metals to remediate environmental pollutants, genetic engineering has the advantage that is relatively fast and it is possible to introduce genes

from another species, and has already been used successfully to enhance plant metal tolerance and accumulation (Bennett *et al.* 2003). In a different approach to enhance metal tolerance and accumulation, the metal-binding peptides phytochelatins (PCs) were overproduced via expression of enzymes involved in their biosynthesis. Transgenic mustard (*B. juncea*) plants with higher levels of glutathione and phytochelatins, which play a role in homeostasis of heavy metals in plants, that is the mechanism that regulates the availability of metal ions in the plant cell (Zenk 1996), were created through the overexpression of either of three glutathione synthesizing enzymes, ATP sulfurylase, γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS), these types of transgenics plants showed enhanced cadmium and selenium tolerance and accumulation (Zhu, Pilon-Smits *et al (a)*,1999; (Zhu, Pilon-Smits *et al (b) 1999;* Pilon-Smits, Hwang et al. 1999). Since glutathione (GSH), is a substrate for synthesis of phytochelatins, transgenic plants with increased GSH content provide a promising system to test the phytoremediation of heavy metals form contaminated sites (Koprivova, Kopriva *et al*. 2001). In other experiments, have been demonstrated that poplar transgenic plants overexpressing γ-glutamylcysteine synthetase (γ-ECS), *Arabidopsis thaliana* transgenic plants with *AsPCS1* gene and *Oenothera odorata* respectively, accumulated more cadmium that wild plants (Koprivova, Kopriva *et al*. 2001; Guo, Xu et al. 2012; Kyung, Dae et al. 2012). Other transgenic lines of *B. juncea* overexpressing genes encoding the enzymes selenocysteine lyase (cpSL) and selenocysteine methyltransferase (SMT) and cystathionine-γ-synthase (CGS) were test to evaluate the ability to hyperaccumulate selenium under field conditions. These results demonstrate that cpSL and SMT and cystathionine-γ-synthase (CGS) transgenic lines have significantly greater selenium phytoremediation potential than wildtype of Indian mustard (Van Huysen, Abdel Ghany *et al*. 2003; Van Huysen, Terry, *et al*. 2004, Bañuelos, LeDuck *et al*. 2007). In all cases, genetic manipulation is an example of genetic engineering may improve the ability of plants to phytoremediate metal polluted soils Karenlampi, Schat et al. 2000), Those studies confirms the importance of testing transgenic lines for evaluating transgenic plants for gold phytomining, however to our knowledge no studies have been reported where the efficacy of genetically modified plants for gold phytomining were proved, therefore, the development of a new technology to recovery of gold from mine tailings is required. The aim of this work was determine the ability of ATP sulfurylase, γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS) lines of transgenic *B. juncea* plants to hyperaccumulate gold compared with wildtype plants of the same species enhanced with ammonium thiocyanate (AT) and thiourea (TU) as chelators in silica sand as artificial substrate. To our knowledge, this is the first report of the use of transgenic plants for gold phytomining in greenhouse level in silica sand as substrate.

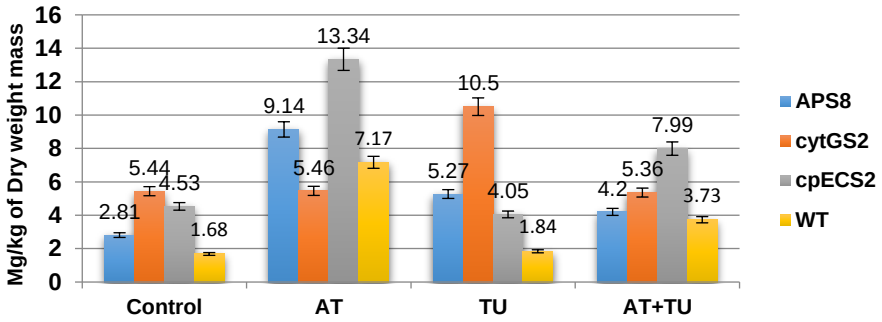
Materials and methods

To evaluate the ability of gold hyperaccumulation of APS8, cpECS2 y cytGS2 transgenic lines overexpressing ATP sulfurylase, γ-glutamylcysteine synthetase and glutathione synthetase respectively and wild type plants of *B. juncea* enhanced with AT and TU as chelators an experiment was conducted. To realize this experiment, seeds of APS8, cpECS2 y cytGS2 transgenic lines and seeds of wildtype plants of *B. juncea* were utilized. Transgenic and untransformed (WT) seeds were obtained from Pilon-Smits Lab (Biology Lab, Colorado State University, Fort Collins, Colorado, U.S.A.). Seeds were sown in

substrate commonly used in the greenhouse and two weeks after emergence, seedlings were transplanted into silica sand in pots containing 1 kg of substrate. Two weeks after transplant, nitrogen, phosphorus, potassium, as chemical fertilizer was applied. After six weeks of growing, the influence of AT and TU determined to induce gold hyperaccumulation in treatments. The treatments including three replicates to each transgenic and WT plants were as follow: (1) Control, (2) AT, in a dose of 1.5 mg/kg of substrate (3) TU, in a dose of 0.0608 g/kg of substrate and (4) a mixture of AT, in a dose of 1.5 mg/kg of substrate mixed with TU, in a dose of 0.0608 g/kg of substrate. The source of gold for all transgenic and WT plants was 3 mg/kg gold per pot where gold chloride was applied to silica sand. One week after treatments were applied, plants were harvested. Once harvested, plants were washed with running water to remove debris of metals and other substances in surface of tissues. The plants were oven dried at 70^{o}C for 48 hours in a muffle and a sample of 1.2 g of each transgenic and WT line was choosen and samples were ashed at 550^{0}C in borosilicate test tubes and acid digested. The digestion was carried out using microwave-assited digestion (Perkin Elmer Microwave Reaction System Multiwave 3000) with aqua regia (12 mL of nitric acid and hydrochloric acid reactive grade 1/3 v/v). The digestion temperature and pressure were set to 150ºC and 20 bars, respectively during 45 minutes. After acid digestion, the solution was filtered in Whatman paper 1002-125 and tubes containing solution digested were filled to 25 mL with tridistilled water. A Perkin Elmer AAnalist 800 Flame Atomic Absorption Spectrometer was used to determine the gold content in samples. The analytical wavelenght used for this metal was 242.8 nm. Periodically (each twelve samples) known gold standard (Perkin Elmer Life and Analytical Sciences Shelton CT U.S.A.) were used to check the instrument response. Correlation curves were made and a correlation coefficient of 0.997916 was obtained. The samples were analyzed in triplicate. The experiment is a triple-factored experiment in a completely randomized design.

Results

The gold concentration for all transgenic and a WT lines for all treatments indicates that transgenic lines, in all cases, were more effective to concentrate gold, compared with untransformed plants (Figure 1). The gold concentration with cpECS2 and cytGS2 transgenic lines, are considered the most important results in this experiment. The cpECS2 transgenic line was more effective in gold concentration wit AT and AT+TU mixture treatments. In AT treatment, cpECS2 plants absorb 13.34 mg/kg of gold in DWM, a difference of 46.26% compared with WT line. In AT+TU mixture treatment, cpECS2 line absorb 7.99 mg/kg of gold, a difference of 53.32% compared with untransformed plants. In the case of cytGS2 line, these plants absorb 10.5 mg/kg of gold in DWM, a difference of 82.48% in contrast with gold absorption in untransformed plants. In all cases, there are significant differences ($P< 0.05$). It's too noticeable that against expected, plants of WT lines concentrate numerically more gold than cytGS2 plants in treatment with AT. The induced hyperaccumulation of gold in treatments indicate that all chelators (AT, TU and a mixture of AT+TU) were effective to improve the gold absorption in all transgenic and untransformed plants. In control treatment, cytGS2 transgenic plants concentrate 69.12% more gold compared with WT plants, this difference was significantly different ($P<0.05$). The application of AT for improve the gold absorption, was significantly different in cpECS2 transgenic line compared with APS8 cytGS2 and WT plants.



Discussion

As expected, experimental results of this work support hypothesis that *B. juncea* transgenic plants overexpressing γ-glutamylcysteine synthetase, glutathione synthetase and ATP sulfurylase enzymes, are feasible plants to be used in gold phytomining due it capacity of hyperaccumulate gold. This difference in gold concentration between transgenic and untransformed plants, could be due transgenic Indian mustard (*B. juncea*) cytGS2 plants were developed encoding *gshII* gene from *E. coli* and overexpress glutathione synthetase (GS), cpECS2 transgenic line was genetically engineered to overexpress the *E. coli gshI* gene encoding γ-glutamylcysteine synthetase targeted to the plastids, and the APS8 plants was modified using Arabidopsis *APS1* gene, encoding a plastidic ATP sulfurylase (Zhu, Pilon-Smits *et al (b)*,1999; Pilon-Smits, Hwang et al. 1999). Our results, where cpECS2 transgenic line overexpresses γ-glutamylcysteine synthetase, are comparable with other ones. *Populus tremula* x *Populus alba* transgenic plants overexpressing γ-glutamylcysteine synthetase was studied (Koprivova, Kopriva *et al*. 2001), and accumulate two-fold cadmium in roots than wild type plants. The same coincidence can be expressed with results of Zhu, *et al*. (1999), who found that transgenic plants of *B. juncea* overexpressing γ-glutamylcysteine synthetase, exhibit great capacity of accumulate and tolerate cadmium in his tissue. In other experiment Noctor *et al.* (1998) with same transgenic plant species, it was demonstrated that *Populus tremula* x *Populus alba*, increase production of glutathione in chloroplasts and cytosol, increasing quantity and activity of γ-glutamylcysteine synthetase. The most likely explanation for the increased gold concentration of the transgenic line plants used in this experiment, is that these plants produced more phytochelatins compared with WT plants, which are enzymatically synthesized peptides in plants that usually contains three amino acids, glutamic acid (Glu), cysteine (Cys), and glycine (Gly) (Grill, *et al*. 1985. So is expected to lead to a greater capacity to concentrate more gold, because PCs bind heavy metals, followed by sequestration and storage in the vacuole (Zenk 1996).

Conclusion

This work has provided better insight into the gold concentration using *B. juncea* transgenic plants overexpressing γ-glutamylcysteine synthetase, ATP sulfurylase and glutathione synthetase and these plants offer great promise for increasing the efficiency of phytoextraction of gold.

Acknowledgment We thank Dr. Elizabeth A.H. Pilon-Smits (University of Colorado, Fort Collins Colorado, U.S.A.) for his generous gift of seeds of the cpECS2, APS8 and cytGS2 transgenic and WT lines of *Brassica juncea* utilized in this experiment.

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BIOTECHNOLOGY SUMMIT

Efficient technological platform development for the identification of PGPR in tomato and their potential use in bio-fertilizers

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Abstract

In this job, a total of 1105 isolates were evaluated in a Technological platform that allows the rapid and effective assessment of a large number of bacteria. This is based on the *in vitro* evaluation of the effect of bacteria on the overall growth of Arabidopsis seedlings, with particular emphasis on the development of the root. Through this system, it was able to identify about 170 isolates with PGPR activity by contacting the bacterial inoculum and 32 though volatile compounds effect. Then these isolates are being evaluated in tomato seed at different nutritional levels (fertilizer concentration), in greenhouse, and the 32 VOCs producers were evaluated in tomato plants in an *in vitro* effective system showing interesting results. Currently, we are working in the identification of the volatile compounds produced by the bacterial isolates.

Keywords: PGPR• bacteria• technological platform• bio-fertilizers• plant growth.

Introduction

The idea of implementing a sustainable agriculture, leads to leads to decreasing or eliminating the use of agrochemicals in both fertilization and combating plant diseases or pests. The terms of biofertilization and biocontrol agents, have become more important in recent years. Additionally, it is known as rhizosphere to the contact area of the root of the plant with soil and rhizosphere microorganisms, mostly heterotrophic and able to promote or stimulate plant growth, known as PGPR (Plant Growth-Promoting Rhizobacteria). The mechanisms by which PGPR manifest their activity are numerous and not quite fully known. Linked to promoting growth hormone production appears, in relation to protection against pathogens, antagonisms can be detected, production of antibiotics, enzymes release that act on the walls of fungi or insects' convers, etc., besides the induction of systemic resistance of the entire plant, to virus, bacteria and fungi. And more directly related to nutrition, nitrogen fixation, phosphate solubilization and other nutrients, as well as the mobilizing of them, etc. Most soil bacteria contribute directly or indirectly to plant growth-promoting by the excretion of metabolites, which can be liquid or solid, but a few is known about those that are volatiles. In this job, a total of 1105 isolates were evaluated in a Technological platform that allows the rapid and effective assessment of a large number of

bacteria. This is based on the *in vitro* evaluation of the effect of bacteria on the overall growth of Arabidopsis seedlings, with particular emphasis on the development of the root, which allows rapid evaluation of the potential of promoting the growth of plants, of hundreds or thousands of bacterial strains at a low cost.

Materials and methods

For the evaluation of the 1105 bacterial isolates of corn, lime and soil rhizhospere, an *A. thaliana in vitro* system was used according to Ortiz-Castro et al. (2013) (Figure 1). *A. thaliana* seeds were surface sterilized. Then, were grown in MS 0.2X media and subsequently plants with 6 days of germination, were transferred to MS 0.2X media, using the system described Ortiz-Castro et al., (2013). Through this system, it was able to identify about 170 isolates with PGPR activity by contacting the bacterial inoculum and 30 though volatile compounds effect, using split plates. As scrutiny controls *Pseudomonas aeroginosa* mutant LasI was used.

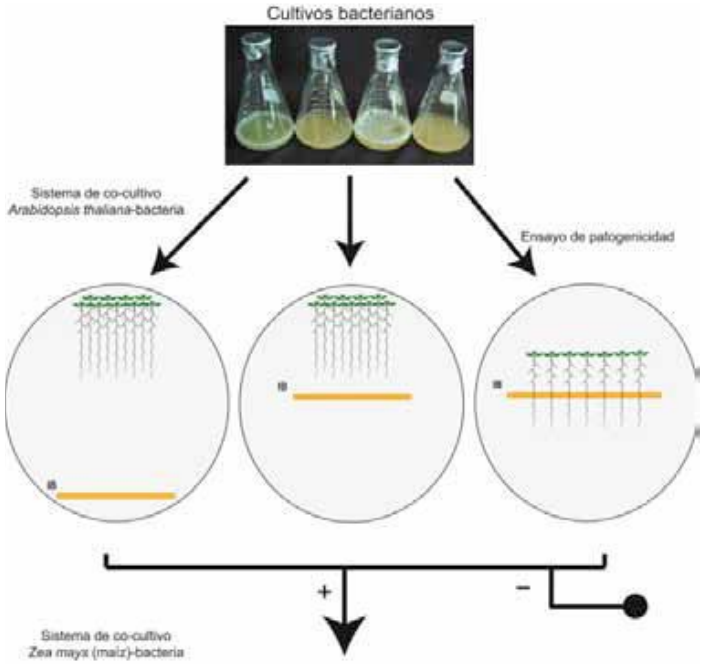

Figure 1. *A. thaliana* – bacteria different *in vitro* inoculation systems, by Ortiz-Castro et al. (2013).

To identify the MVOC´s responsible of these effects, the atmosphere of the plant-bacteria interaction was sampled using solid-phase micro-extraction and then analysed by gas chromatography–mass spectrometry (GC-MS). Also, the growth promoting effect of the candidate MVOC´s identified in tomato plants, were evaluated. For this experiment tomato seed were disinfected and germinated in plates with MS 0.2X pH 7.0 medium using the Mohamed-Hasan et. al protocol (2010). Then 3 dag tomato plants were transferred to 150 x 15 mm plates with MS 0.2X pH 7.0 medium containing 60 x 15 mm plates with the same medium. In the smaller plates the isolate of interest was inoculated. One of the principal

objects of this job, is to take the *in vitro* results to greenhouse, so then an inoculation system in greenhouse were standardized using tomato plants as culture of commercial interest. Also, three levels of fertilization in soil were evaluated in order to evaluate the effect of bacteria in the presence of different concentrations of micronutrients. For this, tomato seeds were disinfected and planted in plastic cups with sand: perlite as substrate, previously fertilized with a solution of the 0%, 25% or 100% of the stablished concentration, and finally seeds were inoculated with the appropriate volume of bacteria to an optical density of 0.8 at a wavelength of 600 nm.

Results and discussion

For the evaluation of the 1105 bacterial isolates an *A. thaliana in vitro* system was used according to Ortiz-Castro et al. (2013) (Figure 1). Through this system, it was able to identify about 170 isolates with PGPR activity by contacting the bacterial inoculum and 30 though volatile compounds effect, using split plates. As scrutiny controls *Pseudomonas aeroginosa* mutant LasI was used. To summarize the scrutiny, we can see that the system in *A. thaliana* vitro - isolated bacteria allows those discriminate with certain bacterial pathogenic activity or negative growth plant *A. thaliana* those promoting activity plant growth (Figure 2). Isolates were grown in LB media previous to inoculation with plants. Then they were striated in MS 0.2X media plates to get contact with *A. thaliana* plants (6 dag).

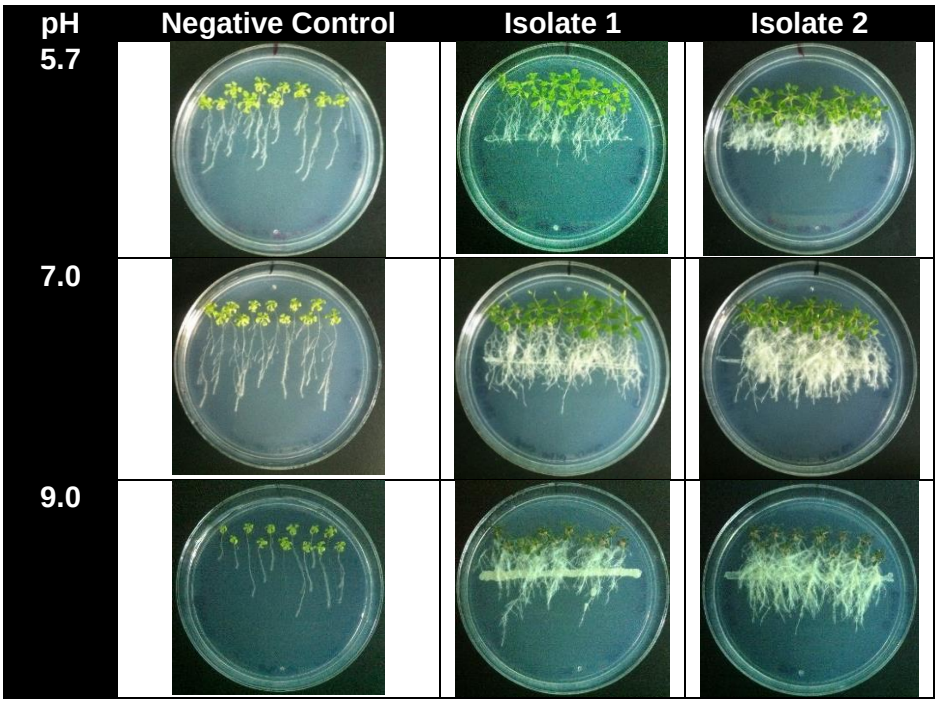

Figure 2. Inoculation effect in Plant Growth - Promotion of *A. thaliana*.

Since one determinant factor in the nutrient availability in soil is the pH, three different conditions of pH (pH 5.0, pH 7.0 and pH 9.0), were evaluated to all bacterial isolates (Figure 2). The system *in vitro A. thaliana* - bacteria, allowed to discriminate those isolated with high

potential in promoting plant growth under different pH conditions. The *in vitro* results were scaled to greenhouse, using three levels of fertilization in soil (0%, 25% and 100%), in order to evaluate the effect of bacteria in the presence of different concentrations of micronutrients, the tomato seeds were planted in plastic cups with sand : perlite as substrate. As measurement parameter fresh weight and dry weight of the biomass of the aerial part of the plants was evaluated. In graphic 1, the effect of the activity of 14 bacterial isolates can be observed compared with negative control, and commercial control bacteria (C.B.), (n=10).

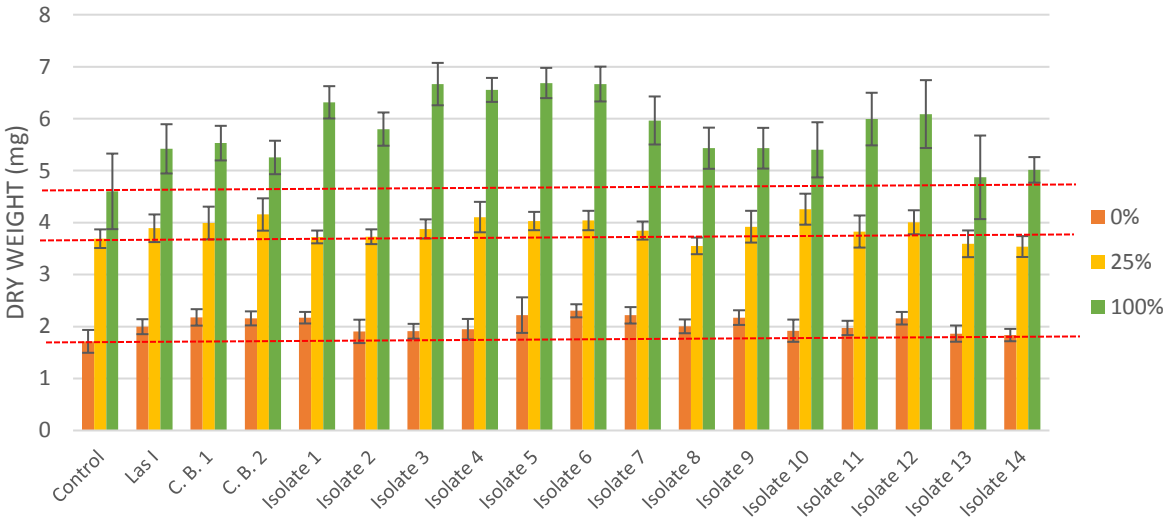

Figure 1. Effect of the inoculation of some isolates compared with negative control and commercial bacteria.

As shown in Figure 1, Isolates 4, 5, 6 and 10 were the best in growth-promotion than other isolates and even commercial control bacteria, at a 25% level of fertilization, and isolates 2, 3, 4, 5 and 6 were the best in growth-promotion at a complete fertilization level. Until now, approximately 75 of 170 isolates have been evaluated in greenhouse, but many more assays are necessary to select an efficient consortium system. The 30 isolates identified to have promoting-growth activity by volatile compounds (VOCs) were inoculated in split plates with 6 dag *A. thaliana* plants. To identify the MVOC´s responsible of these effects, the atmosphere of the plant-bacteria interaction was sampled using solid-phase micro-extraction and then analysed by gas chromatography–mass spectrometry (GC-MS). We have found that some PGPR release a blend of volatile components that drive the plasticity of postembryonic developmental program of *Arabidopsis.* Also, the growth promoting effect of the candidate MVOCs identified in tomato plants, were evaluated. In Figure 3, some results of the use of VOCs Bacteria are demonstrated.

Figure 3. Effect of the VOCs in tomato growth, a) Control, b) Isolate 1, c) Isolate 2.

In the Figure 3, evident results of growth-promotion due to the volatile compounds produced by some isolates are shown in comparison of negative control, but a few bacteria are better than the others in tomato growth-promotion in an *in vitro* system. It is necessary to identify all volatile compounds involved in the accelerated development of these plants in an *in vitro* system in order to evaluate the possibility of their individual use in the same system or a greenhouse level with crops of commercial interest.

Conclusions

Approximately 75 of 170 isolates have been evaluated in greenhouse, but many more assays are necessary in order to select an efficient group of PGPR or a consortium of them, to promote plants growth, not only in tomatoes but in other crops of commercial interest. Parallel, we are working in the identification of the volatile compounds produced by the isolates that showed significant results in the plant-growth promotion. It is necessary to identify all VOCs involved in the accelerated development of *A. thaliana* and tomato plants in an *in vitro* system in order to evaluate the possibility of their individual use in the same system or a greenhouse level with crops of commercial interest. Also, it is necessary to complete the molecular identification of all isolates to have a better outlook of their use in future assays.

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BIOTECHNOLOGY SUMMIT

Phenotyping tools and physiological breeding: optimizing biomass distribution within the plant to increase harvest index in wheat cultivars

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Abstract

Conversion of intercepted radiation into biomass is a key major process determining productivity under yield potential environments. While allocation of carbon to the developing wheat spike determines grain sink strength, concurrent growth of other plant organs competes for carbon. Therefore, it is crucial to identify traits and markers enabling breeders to maximize assimilate partitioning to the grains. Genetic variation in dry matter (DM) partitioning to the stem, sheath and spike at seven days after flowering was observed in 26 CIMMYT spring wheat cultivars and advanced lines under high radiation, irrigated conditions in NW Mexico, during 2011-12 and 2012-13 growing cycles. Furthermore, results showed that lower DM partitioned to the stems just after flowering was associated with higher spike growth at the same stage. Moreover, the negative association between stem and spike growth was found to be linked to greater DM partitioned to stem internode 2 and 3, but not to the peduncle or lower stem internodes. Results indicated associations between spike morphological characteristics at harvest and fruiting efficiency (FE; grains per g of spike DM at flowering). According to our results, a value of HI >0.6 could be potentially achieved in CIMMYT spring wheat by combining the biggest expression for 'useful' biomass traits.

Keywords Phenotyping●yield potential●harvest index●wheat breeding●partitioning.

Introduction

Yield progress through conventional breeding during the last century was achieved by empirical selection for yield *per se* (Loss and Siddique 1994) but yield gains appear to be slowing down in the last decades (Fischer 2007). To accelerate rates of genetic gains in the near future, complex physiological traits must be incorporated as additional criteria in breeding programs. The use of physiological selection criteria plays an important role by making empirical selection more efficient by defining useful traits and enabling the identification of the most suitable parental germplasm (Richards 1996). Furthermore, physiological breeding has a higher probability of achieving the benefits of cumulative gene action for yield than crossing (physiologically uncharacterized) elite by elite lines. Based on traits identified in conceptual models and systematic screening of genetic resources, phenotyping tools are employed to identify complementary parental sources for adaptive traits (Reynolds and Langridge 2016). These approaches have achieved significant genetic gains over conventional approaches at International Maize and Wheat Improvement Center (CIMMYT) for yield potential as well as for improving adaptation to abiotic stress and

moreover, new lines and phenotyping methods have been adopted by national wheat programs (Pask *et al.* 2014). Under yield potential environments (with non-limiting inputs and the absence of biotic and abiotic stress), three major processes are determining productivity: i) interception of incident solar radiation by the canopy, ii) conversion of intercepted radiation into biomass and iii) optimized partitioning of assimilate among plant organs (Hay and Walker 1989; Araus *et al.* 2001). It is predicted that future increases in yield potential will rely largely on improved biomass production boosted by higher radiation use efficiency (RUE) (Long *et al.* 2006). However, it is important to optimize biomass distribution within the plant to increase harvest index in order to fully exploit current and further increases in biomass. An example of this is the recent study of Aisawi *et al*. (2015), where genetic progress in yield potential of CIMMYT spring wheat lines are reported to be associated mainly with above-ground biomass and grain weight with decreases in harvest index (HI). In this scenario, it seems that biomass will be an essential determinant of genetic gains in wheat yield potential, however it will be crucial to identify traits and markers enabling breeders to maximize assimilate partitioning to the grains. In this context, boosting spike fertility and an optimal assimilate partitioning among organs are two promising avenues to overcome the physiological limits in biomass conversion into grain yield. The main idea behind this approach is to decrease assimilate partitioning to alternative sinks, such as stem biomass to favour spike growth around flowering (Foulkes *et al.* 2011), a key stage determining grain number (Brooking and Kirby 1981). On the other hand, increasing spike fertility is considered in this project as a complementary strategy to increase grain number and harvest index; mainly through targeting the fruiting efficiency (ratio between grains at harvest per gram of spike dry matter at flowering; FE). This integrative trait is an indicator of spike fertility and a good predictor of grain number m^{-2}, from a plant to a crop scale basis, for which there is clear variability among modern cultivars (González *et al*. 2011; Lázaro and Abbate 2011). Furthermore, although there is a general assumption regarding limited genetic diversity for traits contributing to yield potential in modern wheat, high genetic variation in traits conferring improved harvest index and higher photosynthetic capacity have been found recently in CIMMYT elite spring wheat material (Molero *et al*. 2016). Within this context, the present work aimed to i) identify novel genetic variation in dry matter partitioning among plant organs at seven days after flowering and ii) examine associations with spike growth and fertility in 26 CIMMYT high biomass elite wheat cultivars and advanced lines in the Yaqui Valley. High-throughput and precision phenotyping has been applied to evaluate these specific traits in the representative set of elite lines.

Methods

A set of 26 CIMMYT elite spring wheat (*T. aestivum* L.) cultivars and advanced lines were selected from the CIMMYT Mexico Core Germplasm (CIMCOG) panel (Table 1), and grown in two seasons (2011-12 and 2012-13) at CIMMYT experimental station near Ciudad Obregon, NW Mexico. The experimental design was an alpha-lattice with three replications under raised beds. In each experiment, plots were irrigated using a gravity-based system with flood irrigations 5–6 times during the crop cycle will a full prophylactic control of weeds and pests. The growth scale used to determine flowering date (GS65) was the Zadoks decimal scale for cereals (Zadoks *et al*. 1974). The 26 wheat cultivars were evaluated for growth analyses at seven days after flowering (GS65 +7 days) and at initiation of booting

(GS41) from 0.8 m^2 field sample, including above-ground dry-matter (AGDM; g m^{-2}), dry matter (DM) partitioning among plant organs (spike, stem, sheaths and leaf lamina) and fruiting efficiency (FE). The DM partitioning among organs was represented as indices or proportions of single shoot AGDM (e.g. spike partitioning index; SPI; spike DM per shoot/AGDM per shoot), measured from 10 randomly selected shoots from the quadrat biomass sample. FE was calculated as a ratio between the number of grains at harvest (m^2) per gram of spike dry matter at GS65 +7 days (g m^{-2}). Harvest index (HI) and grains m^{-2} were estimated from 100-fertile shoot samples at harvest. Grain yield was measured in a plot area of 5 m^2 at harvest. For a subset of nine cultivars with a restricted range of flowering date but representing the full range of DM partitioning at flowering, further DM partitioning analyses were carried out of stem internode DM partitioning (peduncle, internode, 2 internode 3 and internode remaining) at GS65 +7 days (Figure 1a). For stem internodes, internode DM as a proportion of AGDM per shoot was calculated (e.g. peduncle DM/AGDM DM per shoot). In addition, DM partitioning analyses were performed for 10 randomly selected shoots from the biomass quadrat sample at initiation of booting (GS41) for the determination of stem DM per unit area at this stage. Finally, for seven of the nine cultivars in 2011-12, non-grain spike DM partitioning was assessed ta harvest for each morphological component of the spike (glume, palea, lemma, rachis and lemma) (Figure 1b), reported as morphological component DM/non-grain spike DM (e.g. glume DM/non-grain DM per spike). Rachis length was also measured in the same seven CIMCOG cultivars, allowing calculations of rachis specific weight (RSW; mg cm^{-1}).

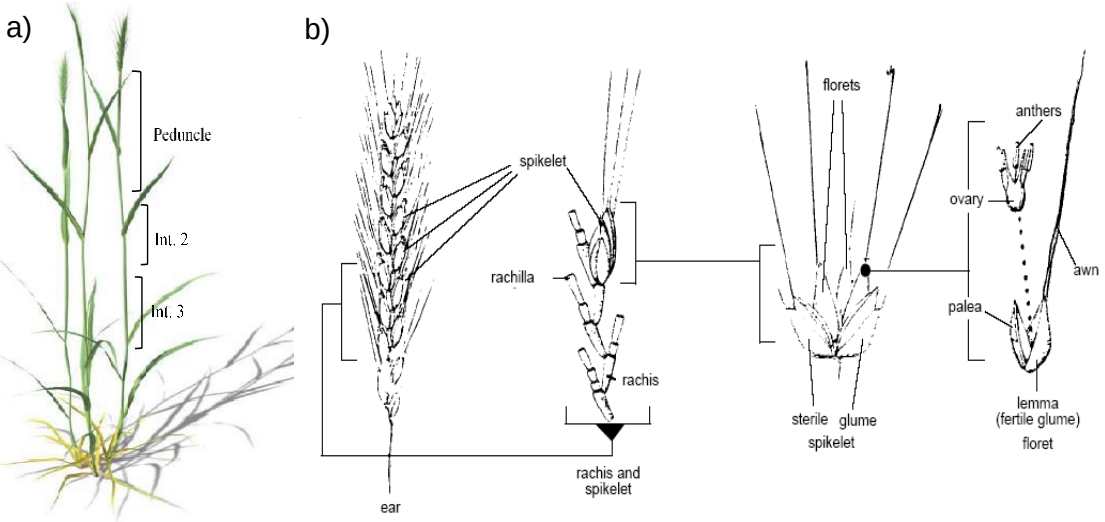

Figure 1 Diagrams of a) stem internodes in wheat and b) wheat spike structures (Setter & Carlton 2000).

Table 1 Entry number and cultivar name of the spring wheat CIMMYT material sown in 2011-12 and 2012-13 in experimental fields, NW Mexico. Sub-set of nine cultivars is shaded.

Entry	Cultivar name
1	BABAX/LR42//BABAX/3/VORB
2	BACANORA T 88
3	BCN/RIALTO
4	BECARD/5/KAUZ//ALTAR 84
5	BRBT1*2/KIRITATI
6	CAL/NH//H567.71/3/SERI/4_1
7	CAL/NH//H567.71/3/SERI/4_2
8	CMH79A.955/4/AGA
10	CNO79//PF70354/MUS/3
11	CROC_1/AE.SQUARROSA
12	KBIRD//INQALAB 91*2
13	MILAN/KAUZ//PRINIA
14	PAVON F 76
15	PBW343*2/KUKUNA*2
16	PFAU/SERI.1B//AMAD/3
17	SERI M 82
18	SIETE CERROS T66
19	SOKOLL//PBW343*2
20	TACUPETO F2001/7/CAL/NH
21	TACUPETO F2001/BRAMBLING
22	TC870344/GUI
23	TRAP#1/BOW/3/VEE/PJN
24	UP2338*2/4/SNI/TRAP#1/3
25	WBLL1*2/ KIRITATI
26	WBLL1*2/KURUKU*2/5/REH
27	YAV_3/SCO//JO69/CRA/3

Results and discussion

Relationships between grain yield, harvest index and above-ground biomass at harvest. Results showed HI was positively associated with grain yield amongst genotypes (R^2 = 0.30; P< 0.001; Figure 2a) with yield increasing up to ca. 0.49 and then levelling off. Overall HI ranged amongst the 26 genotypes from 0.45-0.51 (P< 0.001), indicating significant scope for raising HI towards the theoretical maximum of ca. 0.65 (Austin et al. 1980; Foulkes et al. 2011). Although grain yield was strongly related to above-ground biomass (R^2 = 0.59, P< 0.001) among the 26 cultivars and the nine cultivars (R^2 = 0.57, P< 0.001) (Figure 2b and 2c, respectively), this was not observed when just considering the 10 cultivars showing the highest biomass values (not shown). Moreover, there was a trend for a negative linear relationship among the 26 cultivars between HI and above-ground biomass (R^2 = 0.17; P< 0.05; Figure 2d). These findings indicated that the highest biomass cultivars were less efficient at partitioning assimilate to grain; therefore, novel traits to maximize partitioning to grain must be identified. Overall, there was a stronger relationship between grains m^{-2} and fruiting efficiency (FE) (R^2 = 0.40, P< 0.001) than for spike partitioning index (SPI) (data not shown).

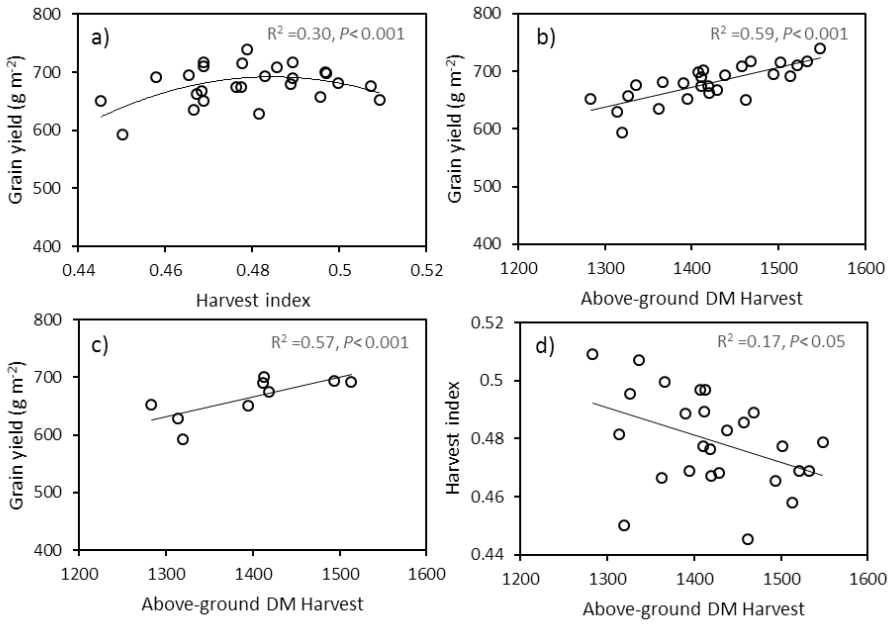

Figure 2 Regressions between key harvest traits for 26 and subset of nine CIMMYT spring wheat cultivars. Values represent means in 2011-12 and 2012-13.

Genetic variation in DM partitioning among plant organs and associations with spike growth at seven days after anthesis. The genetic variation in DM partitioning at GS65 +7 days for the stem, sheaths, spike and leaf lamina ($P< 0.001$) is shown in Figure 3. There was significant genetic variation for DM partitioning to all plant organs components. Stem DM partitioning (StPI) ranged from 24.7 to 36.3% ($P< 0.01$). Spike partitioning index (SPI) was negatively correlated with StPI at GS65 +7 days ($R^2 = 0.50$, $P< 0.05$) but was not associated with sheath DM partitioning index (ShPI) (Figure 4a). Stem DM partitioning was also negatively correlated with spike DM per unit area at GS65 +7 days ($R^2 = 0.51$, $P< 0.05$; Figure 4b). Moreover, there was a trend for a lineal negative association between the amount of stem DM accumulated during the stem elongation period and the amount of spike DM amount at GS65 + 7 days ($R^2 = 0.36$, $P= 0.08$; Figure 5), as well as with SPI (data not shown). These results indicated that, in modern high yield potential CIMMYT cultivars, the stem (presumably structural DM) component is the main target to decrease DM partitioning to enhance spike growth around flowering time.

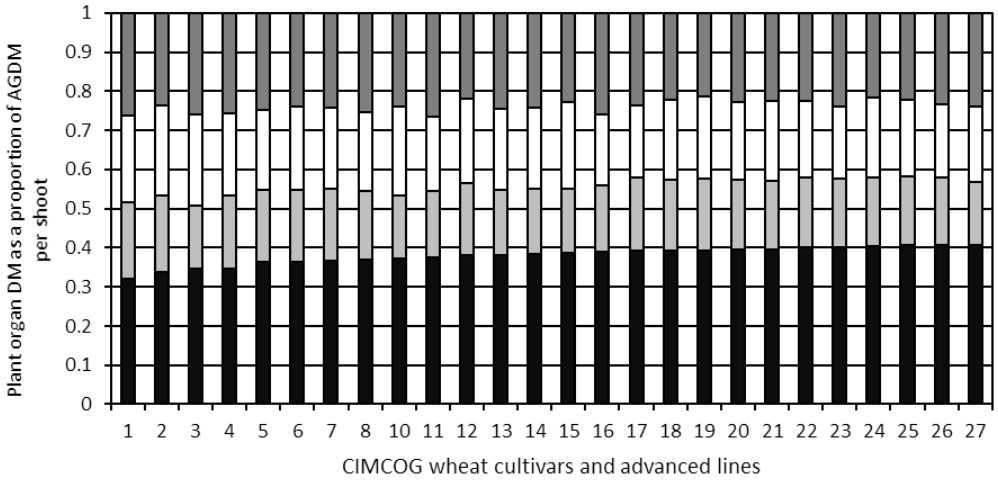

Figure 3 Dry-matter partitioning (proportion of plant organ DM to AGDM per shoot) for each of the 26 cultivars (averaging 2011-12 and 2012-13). Stem, leaf-sheath, lamina and spike in ascending order.

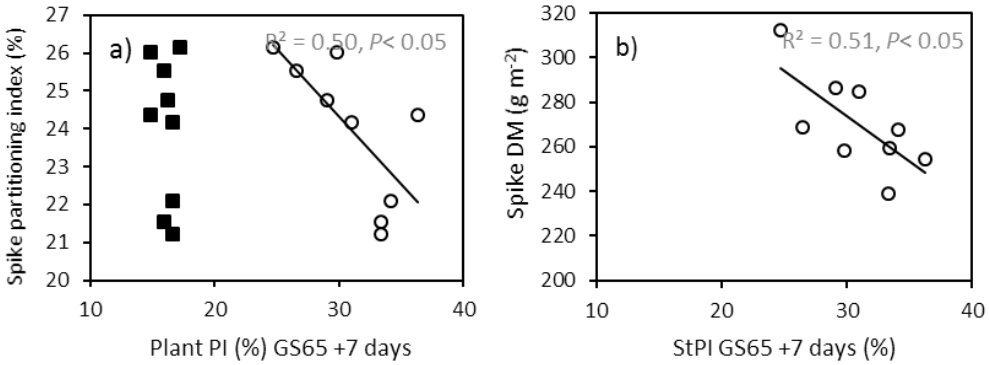

Figure 4 Regression of a) SPI on StPI (○) and ShPI (■) at GS65 +7 days and b) spike DM per unit area at GS65 +7 days on StPI at GS65+7d amongst the subset of nine cultivars. Values represent means in 2011-12 and 2012-13.

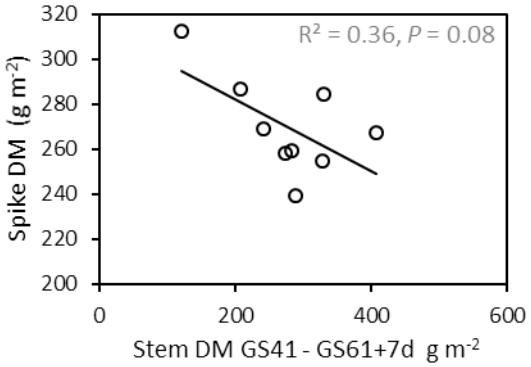

Figure 5 Regression of Stem DM accumulated from GS41 to GS65 +7 days amongst the subset of nine cultivars. Values represent means in 2011-12 and 2012-13.

In order to reduce stem DM partitioning in the most effective way to benefit spike growth, it is necessary to identify those stem internodes which compete most strongly with spike growth during stem elongation. Our results showed that internode 2 and internode 3 had the strongest negative association with SPI (Figure 6) and spike DM per unit area (GS65 + 7 days) of the four internode classes assessed (not shown).

Fruiting efficiency and association with spike morphological partitioning.
Non-grain spike DM at harvest was dissected into its morphological components in seven of the nine cultivars in 2011-12, as well as rachis specific weight (RSW; mg cm^{-1}) was calculated for these seven cultivars. There were differences in RSW in the range 11.0 to 15.1 mg cm^{-1} (P <0.01). The FE was negatively associated with RSW (R^2 =0.66, P <0.05) (Figure 7a) and with glumes DM (% of non-grain spike DM; R^2 =0.72, P <0.05) (Figure 7b). Interestingly, a trend for a positive association between paleas DM (% of spike DM) and the FE was also observed (R^2 =0.51, P =0.07) (Figure 7c).

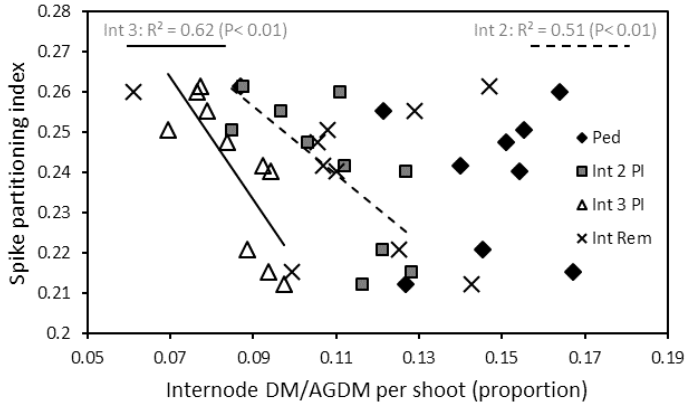

Figure 6. Regression of spike DM partitioning index (SPI) at GS65 +7 days on stem internode DM proportions (internode DM/AGDM per shoot) among the subset of nine cultivars. Values represent means in 2011-12 and 2012-13.

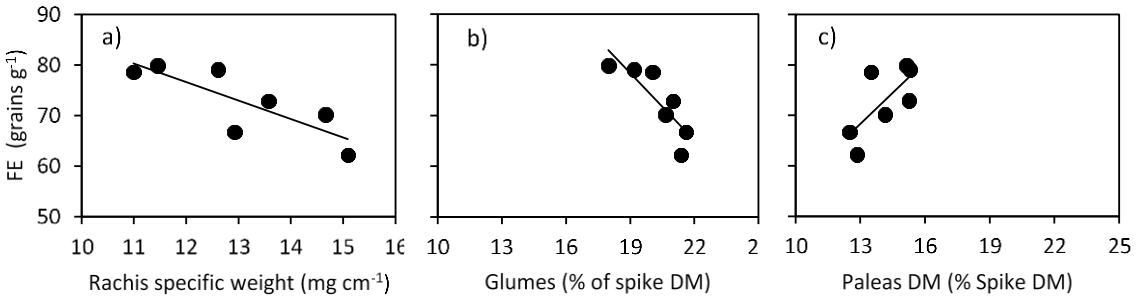

Figure 7 Linear regression of fruiting efficiency on a) rachis SW (R^2 =0.66, *P* <0.05), b) Glumes % of DM (R^2 =0.72, *P* <0.05 and c) paleas % of DM (R^2 =0.51, *P* =0.07) (as a proportion of non-grain spike DM at harvest) among seven cultivars in 2011-12.

Conclusions

According to our results, a value of HI >0.6 can be achieved in CIMMYT spring wheat by combining the biggest expression for 'useful' biomass traits: including, decreased partitioning to the lower internodes 2 and 3 (0.206; Entry 1) to enhance spike growth and decreased rachis specific weight (11.0 mg cm^{-1}; Entry 4) and increased lemma fraction (0.256; Entry 14) to enhance FE. Stepwise regression analysis shows 51% of variance in spike partitioning and 53% in FE is accounted for by these traits. A Genome Wide Association Study (GWAS) is ongoing in to identify genetic markers for the key target traits including of structural stem DM partitioning to internodes 2 and 3 and rachis specific weight determining SPI and FE in the High Biomass Association panel at CIMMYT Ciudad Obregon 2015-2016 as part of the PhD study of Aleyda Sierra-Gonzalez.

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Microbial fuel cells using complex substrates from corn tortilla industry

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Abstract

Microbial fuel cells (MFC) have emerged recently as a promising yet challenging technology that convert biomass into electricity through the metabolic activity of the microorganisms. This technology is considered to meet increasing energy needs, specially using wastewaters as substrates,which can generate electricity and accomplish wastewater treatment simultaneously. In this work, we used a substrate from process nixtamalization (nejayote) for obtain electricity with endogenous microorganisms. Different conditions were used: 1) nejayote dilutions with sodium acetate for stimulate electron transfer, 2) nejayote crude without initial treatmentand, 3) nejayote dilutions with biomass added. The maximum current density was determinate. In condition 3, 50% reduction in chemical oxygen demand and a maximum current value were obtained. A sample of final substrate used in MFC was obtained for isolation of microorganism and its morphological and characterization.

Keywords: microbial fuel cells•wastewater industrial•wastewater treatment•bioelectricity.

Introduction

México have serious environmental problems due to minimal treatment applied to industrial effluents (Romero-Aguilar *et al*. 2009). Especially food industry effluents containing high levels of organic material that can be used as substrate for biological reactions. In this sense, corn tortilla industry generates large quantities of wastewater during process nixtamalization (Rojas-Molina *et al*. 2008). The cooking liquid generated in tortilla production plants is known as nejayote (Reyes-Vidal *et al.* 2012). This effluent has characteristics such as alkalinity (pH between 7 and 11), high chemical oxygen demand (10,000 to 30,000 mg/L) and high temperature (over 70 °C), which make the nejayoteis considered a pollutant effluent (Salmerón-Alcocer*et al*. 2003). For these high values in parameters and high volume of nejayote generated, it is necessary to find a solution for its treatment. Currently new technologies are developing such as microbial fuel cells (MFC). These devices use microorganisms to convert chemical energy present in a substrate to electrical energy (Pant *et al*. 2010). MFCs are considered a promising and sustainable technology to meet the growing energy needs, especially in the use of wastewater as substrates because they can generate electricity and simultaneously carry out wastewater treatment. The substrate is considered one of the most important factors for the operation of the MFC. The use of complex substrates (industrial effluents) in a MFC is of great interest because, in addition to being sources of energy, these can degrade before discharge to the environment (Pant *et*

al. 2010). Another most important factors for a MFC generates a current of electrons are microorganisms that are responsible for carrying out the process of degradation of organic matter to compounds such as CO_2 and H_2O and the release of electrons to the system (Bond *et al.* 2003).

Materials and methods

Nejayote crude was obtained from local maize tortilla mill. Six conditions were used, using a phosphate buffer:

1) Nejayote dilution 50:50 (A)
2) Nejayote dilution 25:75 (B)
3) Nejayote crude (C)
4) Nejayote dilution 50:50 with biomass added (AB)
5) Nejayote dilution 25:75 with biomass added (BB)
6) Nejayote crude with biomass added (CB)

Biomass added was obtained from a culture (24 h, 100 rpm, 30°C) using isolate microorganism and LB broth. Culture broth was centrifuged at 4000 rpm for 15 min at 25 °C to recover pellet. All experiments were carried out using a simplified single-chamber and not adjust pH. The anode electrode dimensions were 2 x 2.5 cm of felt carbon and cathode electrode were made of titanium metal mesh (2.5 x 2.5 cm). Titanium wire was used to connect the circuit and the fuel cell was placed under temperature controlled at 35 °C. The MFC was operated in a batch mode over a full cycle, where the duration for each batch cycle was 7 days. Chronoamperometry technique was determinated using a potenciostat/galvanost (Biologic Science Instrument). In all experiments were determinated total solids (Mexican Norm, NMX-AA-034-SCFI-2001) and chemical oxygen demand (COD) using Hach digital reactor DRB200 and Hach DR6000 spectrometer. A sample of nejayote crude used in a MFC was obtained for microbiology isolation of endogenous microorganisms and biochemical identification using Test API20E (Biomerieux). LB agar medium was used for isolation.

Results and discussion

Results showed a reduction of total solids and COD in all conditions tested. In treatments without biomass added, the minimum value of COD removed was recorded from nejayote crude (Table 1). The COD values decreased in all three experimental conditions (A, B and C) from the initial value down to a final value and the maximum value of COD removed was in 25:75 dilution condition. The progress of current density curve showed a maximum value of 52 mA/m^2 in 25:75 dilution (Figure 1) with an acclimatization period (phase lag) approximately of 2 days (biofilm developed) and two exponential increase of current density production. The first peak could have been due to utilization of biodisposable compounds while the secondary peak might be a result of complex organic utilization. It is interesting to interpret that in this condition was consumed the maximum substrate (maximum COD removed) using only endogenous microorganisms. In condition A (50:50 dilution) an acclimatization period was observed during six days (Figure 1), related with minimum amount of cells present in MFC available for endogenous microorganisms.

Table 1. Parameters evaluated in different conditions of MFC.

	Substrate A		Substrate B		Substrate C	
PARAMETERS	**INITIAL**	**END**	**INITIAL**	**END**	**INITIAL**	**END**
Total solids (Reduction)	100%	18%	100%	6%	100%	20%
COD (Reduction)	100%	10%	100%	5%	100%	52%

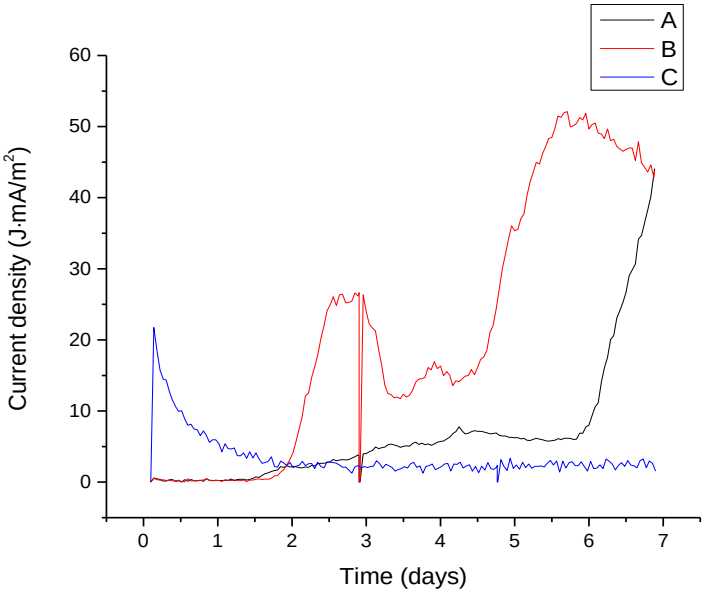

Figure 1. Chronoamperometry technique analysis in a MFC. Line black: condition A; red line: condition B; blue line: condition C.

In order to have a better understanding of microbiome present in MFC for to analyze the current output, we isolated a strain from a sample of MFC. Staining of the cells isolated showed clearly that the isolate was Gramm negative rod shaped bacterium. Initial identification using biochemical test (API20E) indicate that belong to *Pseudomonas* genus. Actually, we identified strain isolated using molecular techniques. A lot of research has been done by using different bacterial species such as *Shewanella oneidensis, Geobacter sulfurreducens, Bacillus subtilis* and recently *Pseudomonas*, with respect to current generation with different wastewaters. In conditions AB, BB y CB, reduction of total solids and COD were different to experiments without biomass added (Table 3). The major value of total solid reduction was in AB condition but the highest COD removed was in nejayote crude (condition CB). Notably, in condition BB was removed 63% of COD (the minimum

value in all conditions) but also the maximum current value was obtained (118 mA/m^2). This results could indicate that biomass added not assimilated all compounds present in the substrate but is capable to generate a significant current value (Figure 2). Otherwise, in the conditions AB and CB showed values lower than BB in current density (52 mA/m^2 and 55 mA/m^2, respectively) but good values of COD removed (Table 2).

Table 2. Parameters evaluated in the MFC with substrate and aggregate biomass.

	Substrate AB		Substrate BB		Substrate CB	
PARAMETERS	**INITIAL**	**END**	**INITIAL**	**END**	**INITIAL**	**END**
Total solids (Reduction)	100%	8%	100%	15%	100%	48%
COD (Reduction)	100%	10%	100%	37%	100%	5%

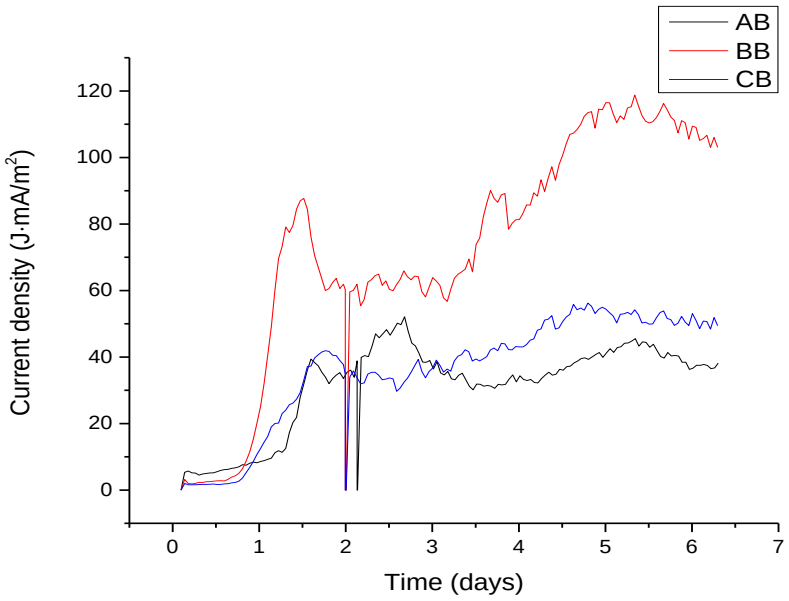

Figure 2. Chronoamperometry technique analysis in a MFC. Line black: condition AB; red line: condition BB; blue line: condition CB.

Conclusions

We have isolated *Pseudonomas* sp. from an industrial effluent. Conditions A and B showed good values of power generation with 44 and 52 mA/m^2, respectively. We found that nejayote dilution conditions were a suitable substrate to exploit in a MFC and that the substrate dilution BB showed high values of power generation with 118 mA/m^2. We found that the substrate with aggregate biomass (BB) of *Pseudomonas* sp. has the best potential

to generate electricity.MFC are an optimal system for treating the effluent of corn tortilla industry (nejayote) because observed decrease in COD.

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Exploring wheat biodiversity for next generation climate-ready cultivar development

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Abstract

Variation among commercial wheat varieties and farming systems can play an important role in alleviating heat and/or drought stress and increasing grain yields in rain fed environments. The genetic diversity kept on-farm and in germplasm banks need to be mobilized into breeding programs through advanced genomics application for a precision introgression. Mexican government funded CIMMYT's- Seeds of discovery project has made significant progress to characterize and mobilize under-utilized genetic variation to deliver new promising germplasm for target environments. Through large scale genetic (80,000 accessions) and phenotypic (100,000 accessions) characterization we have established working collections which are being used in pre-breeding. Over 1000 exotic germplasms, including synthetics and landraces, are being mobilized through SeeD's wheat pre-breeding pipeline for use by breeders. An initial set of 500 bridging germplasm lines and/or wheat landrace core sets have been evaluated for use by scientists in different parts of Mexico. Genomic knowledge generated in the project has been integrated in the pre breeding activities; as an example, a core set of wheat landraces was developed using both genomic (genotype by sequencing, or GBS) and phenotypic data, finally capturing 89% of the rare allelic variations. Genetic/genomic knowledge and large-scale data integration for wheat improvement through pre-breeding, presents a unique resource base for wheat scientists.

Keywords: wheat● pre-breeding● genotyping-by-sequencing● abiotic stress● biotic stress.

Introduction

Wheat, world's most important calorie source is a staple food crop and in coming decades, its demand is projected to increase by 60%. Increasing population pressure, climate change, shrinking farm resources and reduced genetic diversity are the major challenges for ensuring global food security that too in the highly populous regions of world such as South Asia and Africa. Diversification among cultivated varieties and farming systems will play a key role in mitigating stresses and maximizing yields in the stress prone wheat areas.

Genetic resources in gene banks are the repository of gene(s) or gene combinations harboring useful genetic variation. This variation need to be dug out for current and future varietal improvement programs. However, the complexity of wheat genome, up to five times larger than the human genome, has presented a major challenge in applying new methods and technology to solve problems that face the wheat productivity. In addition, because of its recent origin, bread wheat has a narrow genetic base. A systematic and focused effort with clear vision in mining genes/alleles from the available genetic stocks can be an alternative approach. CIMMYT's initiative, - Seeds of Discovery (SeeD; http://seedsofdiscovery.org) has initiated efforts in mobilizing under-utilized genetic variation in to wheat breeding pipelines and deliver new promising germplasm for target environments. The SeeD-Wheat project of CIMMYT aims to harness the potential of wheat gene bank through a comprehensive approach involving (1) large scale phenotypic characterization of wheat germplasm bank accessions; (2) high throughput genotype-by-sequencing (GBS) characterization of wheat gene bank and finally (3) capture the value of germplasm bank through systematic pre breeding efforts. Logistics have been discussed in subsequent sections in this article.

Materials and methods

Genotypic and phenotypic analysis: Genomic DNA extraction was carried out by a modified CTAB (cetyltrimethylammonium bromide) method. Genotyping was performed through DArT-seq GBS technology (called DArTseq™) at DArT Pyt Ltd, Canberra, Australia (Li *et al*. 2015, Vikram *et al*. 2016). Phenotypic characterization of wheat GeneBank accessions was carried out in three different environments: well irrigated, drought stress and heat stress. For abiotic stress experiments trials were conducted at the CIMMYT Experiment Station near Ciudad Obregon, Mexico (27 20° N, 109 54° W, 38 m ASL) and for biotic stress evaluation materials were planted in CIMMYT's experimental station at El batan, Texcoco as well as in Toluca, Mexico. Experiments for heat stress evaluation were planted in March – April so as to get exposed to high temperature stress at anthesis. Different trials was conducted following augmented/alpha lattice designs with 0.3 m^2 – 1m^2 plot sizes along with check varieties- Vorbey and Baj. Standard measures (CIMMYT's SOP) were used to fertilize and control weeds, diseases, and pests (Vikram *et al*. 2016). In the well-irrigated trial, approximately 600 mm of water were applied during the complete wheat cycle and irrigation was provided to plots whenever approximately 50% of available soil moisture was depleted. For drought treatment approximately 200 mm of total soil moisture was provided during the growing season. For the grain quality analysis, grain morphological characteristics were evaluated with the digital image system SeedCount SC5000 (Next Instruments, Australia) and thousand-kernel weight, test weight, average grain length and width, as well as percentage of grains affected by yellow berry (%) were determined. Grain size distribution was measured using sieves of 2.8 mm, 2.5 mm and 2.2 mm. For grain hardness (GH, %) and grain protein content (GP, %) determination Near-infrared spectroscopy (NIRS, Antaris II FT-Analyzer, Thermo Scientific, USA) was used. Whole-meal flour samples were obtained using a UDY Cyclone mill (0.5 mm sieve) and only one gram of whole-meal flour was used to perform the SDS-sedimentation (SDS, ml) test (Pena *et al*. 1990). The Mexican wheat landrace core set was evaluated in CIMMYT, Mexico and PAU, Ludhiana, India for yellow rust disease score following the methodology explained by (Hao *et al*. 2011).

Core set development and pre breeding: In order to select the core reference sets, landraces were classified using the data matrix containing genotypic and phenotypic data sets. PCA was done first of all to reduce the dimensions of the marker data explaining at least 80% of the total variance. Scores of the PCA components and phenotypic variables were used for hierarchical multiple-factor analysis (HMFA). Principal axes were selected from HMFA in such a way that genotypic and phenotypic contributions to the total variance explained by the six axes were 75% and 25%, respectively. Gower's distances were estimated to categorize the samples in to different groups and from each group representatives were taken to form core set using D-method (Vikram *et al*. 2016). Linked top cross population (LTP) panels were developed in SeeD-Wheat project to mobilize genetic variation from exotics to elites. Each panel has a series of top cross populations in which each top cross was linked with another one via common elite parent and therefore top crosses were linked making a panel called, LTP. Landraces, wild relatives and other germplasms with adaptive features (harboring beneficial alleles) were used as exotics and popular farmer varieties as elites.

Results and discussions:

Genetic resources in gene banks are the repository of gene(s) harboring useful genetic variation which need to be explored and exploited for current and future varietal improvement programs (FAO, 2013). A systematic and focused effort with clear vision in mining genes/alleles from the available genetic stocks can be the most feasible and safest approach for development of climate resilient wheat cultivars. With advent of new cost effective and feasible genomics technologies it has been possible to explore and exploit the value of GeneBank in a more efficient way. Trait donor accessions for heat tolerance, grain quality and yellow rust have been identified in the preliminary analyses. Further analysis for validating the obtained results is in process. These trait donor accessions are currently being used to develop pre breeding germplasm sets tolerant to heat and/or drought stresses (CIMMYT, Unpublished). Tables 1, Figures 1-2 present the preliminary results of phenotypic evaluation of the GeneBank accessions. As compared to CIMMYT's best elite checks (Kachu#1 & Reedling#1) Mexican landraces HGO94.9.1.25 and OAX93.4.6 showed quite higher grain weight, grain length, grain width and protein content as well. One of the Iranian landrace IWA8613600 performed extremely well as compared to checks (Table 1).

Table 1: Table presents the list of Potential grain quality donors

Pedigree	1000 Grain Weight	Grain Length	Grain Width	Grain Protein
HGO94.9.1.25	57	6.83	3.67	14.2
OAX93.4.6	57.6	7.1	3.7	15.2
IWA 8606442	57.1	9.2	3.1	15.8
IWA8613600	58.1	7.1	3.7	15.6
IWA8613617	57	7	3.7	15.8
IWA8613630	57.5	7.1	3.7	15.2
KACHU #1 (check)	38	6.5	3.2	12.9
REEDLING #1 (check)	42.6	6.8	3.3	12.4

In addition to the grain quality evaluation, large scale Mexican and Iranian landrace screening for heat stress revealed the genetic similarity of accessions showing tolerance (Figure 1). Also, some of the Mexican bread wheat landraces have been identified as rust resistant sources, for example-CHIH95.5.18, CHIH95.5.23, DGO95.3.8 and OAX93.1.1.1. These landraces showed disease severity score of 5-10% and the susceptible check showed 100% (Figure 2).

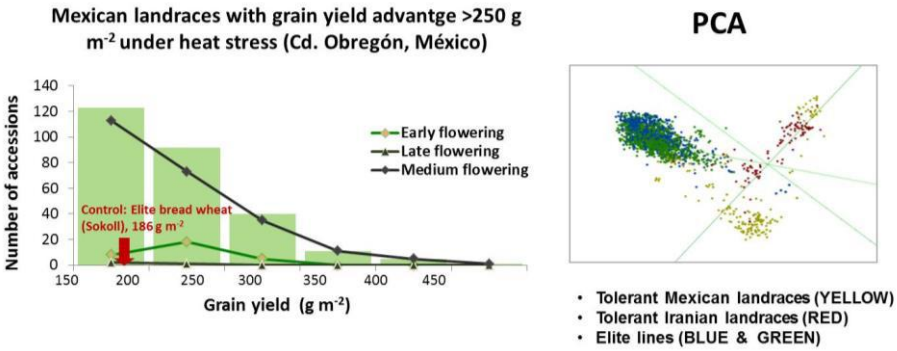

Figure 1: Figure presents the principal component analyses of wheat landraces to identify accessions for heat tolerance

Yellow rust resistant wheat landraces

Accession / Pedigree	YR (%) in Mexico	YR (%) in India
CHIH95.5.18	20	10
CHIH95.5.23	10	10
DGO95.3.8	20	10
OAX93.1.1.1	20	5
Check	100	100

Figure 2: Figure presents the susceptible and resistant yellow rust wheat accessions along with potential donors for use in breeding

These germplasm sets (or trait donors) can be very efficiently utilized by Mexican wheat breeders in their varietal improvement programs. Breeders from different parts of Mexico have been involved and trait donors will be provided to them upon request. Core set strategy of SeeD-Wheat follows the phenotypic as well as high throughput GBS information has been illustrated in Figure 3. This strategy comprises the simultaneous use of phenotypic and genotypic informations making core set a useful one for the wheat breeders (Vikram *et al*. 2016).

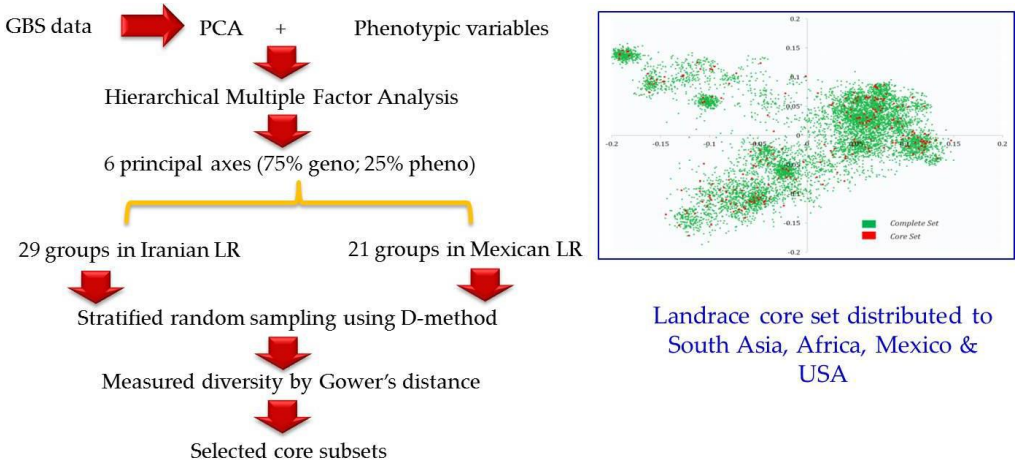

Figure 3: Figure presents the core set development strategy in Mexican and Iranian wheat landraces as well as principal component analysis graph showing complete as well as core set accessions of the Mexican landraces. Core set of Mexican and Iranian landraces have been distributed to breeders in South Asia, Africa, Mexico and USA

On the delivery part two complimentary strategies are being followed: (1) incorporating general diversity to elite genetic backgrounds & (2) using trait donors in breeding as the source for useful diversity. Genetic information generated in the study was used for development of an array of pre-breeding germplasm involving 1000 exotic wheat accessions using above mentioned both strategies. Population panels developed in the project are being maintained for breeding selections, genetic analysis as well as genomics assisted breeding. A set of 500 pre breeding genotypes are currently being evaluated in Tlaxcala, Texcoco, Acamilpa, Guanajuato and Cd Obregon. In nutshell, a comprehensive strategy is being followed in SeeD-wheat project for leveraging GeneBank and derived resources for achieving short term, medium term and long term impacts. Efforts made in SeeD-wheat project are largescale, unique and being welcomed by the global wheat community. We encourage and welcome to Mexican wheat breeders to joining us, leverage resource and deliver impact.

Conclusion

CIMMYT's Seeds of discovery-wheat project is leading wheat pre breeding at global level. Nearly 40% of CIMMYT's wheat GeneBank have been sequenced by GBS technology, around 100K wheat accessions have been phenotyped for one of the priority traits (heat-drought, diseases, quality) and an array of bridging germplasm (exotic x elite) have been developed in the program. These resources are currently being used by wheat researchers in Mexico as well as abroad. Genetic knowledge, trait donors and pre breeding resource have potential to bring a paradigm shift in wheat breeding in Mexico through an intensive follow up of the efforts with Mexican NARS partners. We here welcome Mexican wheat scientists to involve more closely, leverage these resources and deliver impact.

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BIOTECHNOLOGY SUMMIT

Agroindustrial waste for biosurfactants producers endogenous bacteria

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Abstract

The agro- industrial waste among them Waste cooking oil (WCO) and Coffee wastewater (CW) haves been used as carbon source to produce biotechnological products due to its low cost and high availability. In this work, WCO and CW were used to produce biosurfactants (BS). Bacterial strains that were able to grow and showed halos around the colonies, by the formation of a complex with Cetyl Trimethyl Ammonium Bromide agar (CTAB) with methylene blue as indicator, were selected to produce biosurfactants. The selected strains were evaluated in liquid medium with 2% (v/v) of WCO as carbon source. The assay was conducted in Erlenmeyer flask containing 300 ml aliquots of mineral salt media (MSM) + residue and incubated at 100 rpm at environmental temperature for 96 hours. The biosurfactants produced in the samples reduced the surface tension from 50 to 30-29 mN/m. Strains A and 83 showed the maximum emulsification index at 58-59%. Strain A showed the highest biosurfactant yield with a production of 3.7 g/l in comparison with strains B, 83 and *Pseudomonas aeuroginosa* ATCC27853. Our results suggest that the biosurfactant has a great potential in wastewater treatment and soils contaminated by insoluble compounds.

Key words: Waste cooking oil•, Coffee wastewater•, endogenous bacteria•, biosurfactants

Introduction.

Biosurfactants (BS) are naturally occurring molecules produced by microorganisms, are amphiphilic molecules, i.e., have a hydrophilic part and a hydrophobic, this gives them the characteristics of reducing the surface tension between two phases, form emulsions or micelles, this increases the bioavailability of insoluble carbon sources to be used as a carbon source for microorganisms that produce (Singh *et al*. 2007; Rahman and Gakpe 2008). The BS are produced by microorganisms such as yeasts, fungi and bacteria during growth (Cortés and Barragan 2013). The strains studied in this paper are native to the state of Chiapas, were investigated by previous studies reporting its ability to produce BS using various sources of renewable carbon (Vazquez *et al.* 2014). One problem faced by BS are the high costs, compared to the production of synthetic surfactants, since 10% to 30% of the resources in this process are directed to the substrate (Rocha e Silva *et al*. 2013), hence the important to use carbon sources inexpensive to reduce production costs, an alternative

is to use agro-industrial waste as a carbon source. The water used for pulping coffee is known as Coffee wastewater (CW) and contains high concentrations of organic pollutants (Haddis and Devi 2008) such as caffeine, tannin and polyphenols; waste from the coffee industry pose an environmental problem for coffee producing countries due to improper disposal, causing water pollution and soil (Murthy and Madhava 2012). Cooking oil is vegetable fat usually remain liquid at room temperature and is edible, it is used for cooking, the oil is heated to high temperatures (150-190 °C) which generates compound harmful to health (Alfadhl *et al*. 2015), once that has been used is considered a residue which is known as Waste cooking oil (WCO), is considered one of the main waste that causes pollution of urban wastewater because it is insoluble in water, washed into sewers directly without pretreatment, this hurts water bodies where wastewater is discharged, it affects the process of biological treatment of waste water and can clog the sewers in winter, low temperatures (Lan *et al*. 2015). The aim of this work is to select endogenus bacteria of the state of Chiapas, producing biosurfactants from Waste cooking oil and Coffee wastewater and determine the best waste and or endogenus bacteria with the best production performance BS.

Materials and methods.

Waste cooking oil (WCO) as sole carbon source for culturing bacteria was used. Which it was provided by the cafeteria of Polytechnic University of Chiapas, in the municipality of Suchiapa, and Coffee wastewater (CW) was obtained from the coffee zone in the city of Tapachula, Chiapas. Assessment of bacterial strains were taken from a laboratory strain collection Environmental Technology Engineering isolated from an anaerobic digestion process and a culture collection of PhD. Arnoldo Wong Villarreal of Agrifood Division Technological University of the Forest, Ocosingo, Chiapas. The culture media used was the DIBICO®; reagents used were, K_2HPO_4, $CaCl_2$. $2H_2O$, $MgSO_4$, $CHCl_3$, iodo, ninhydrin brand GOLDEN BELL®, Na_2HPO_4 y $NaNO_3$, CH_3OH, CH_3COOH, CH_3CH_2OH, HCl, brand of FERMONT®, methylene blue, bromothymol blue, the mark LABESSA®, 3,5 dinitrosalicylic SIGMA® and NaOH brand MEYER®.

Reactivatión Strains. Solid medium was prepared which consists of: casein peptone 5 g/l, yeast extract 3 g/l, $CaCl_2$ 0.1 g/l and 18 g/l bacteriological agar (PY). The medium was boiled for 2 minutes and then autoclaved at 121 ° C at a pressure of 1 Kg/cm^2 for 15 minutes, then poured in Petri dishes and incubated for 24 hours. The strains were sown by rifling in the PY solid medium and incubated for 24 hours.

Strains adaptation. Solid mineral salts medium and methylene blue (MSM + MB) composition was prepared, K_2HPO_4 0.7 g/l, $NaHPO_4$ 0.9 g/l, $NaNO_3$ 2 g/l, $MgSO_4$. $7H_2O$ 0.4 g/l, $CaCl_2$. $2H_2O$ 0.2 g/l, methylene blue 0.03 g/l, agar bacteriological 15 g/l and add waste cooking oil (MSM + MB + WCO) at concentrations of 2, 6 y 10 % (v/v) and coffee wastewater (MSM +MB + CW) at concentrations of 2, 6, 10, 14 y 18 % (v/v). The medium plus the residue was boiled for 5 minutes and autoclaved at 121 ° C at a pressure of 1 Kg/cm^2.He emptied to solidify in petri dishes and incubated for 72 hours. At 30 °C, checking the petri dishes at 24 and 72 hours. For the presence of halos, indicating the presence of BS.

Inoculum production. For the production of inoculum once confirmed the production of BS by strains that showed halo of growth, these were planted in the medium PY liquid in test tubes with 10 ml of medium, planting was performed in duplicate plus a negative control and they kept under stirring at 100 rpm, at room temperature for 24 hours. Subsequently the medium was centrifuged at 15000 x g a 4°C for 15 min to separate the biomass, the

precipitate was washed 2 times with a NaCl saline 2% and left at an optical density of 0.1 ABS at a wavelength of 600 nm using a spectrophotometer VE-5000V a 600nm λ.

Bacterial growth kinetics. The kinetic study was performed in Erlenmeyer flasks 500 ml with 300 ml of mineral salts medium described previously, removing bacteriological agar and methylene blue and waste cooking oil and coffee wastewater to concentration was added 2% and 18% (v/v) respectively, the flasks were inoculated with 1% v/v inoculum; assays were performed in duplicate and maintained at 100 rpm, at room temperature for 96 hours. During the kinetic 8 ml of sample was collected and every 24 hours with the cell-free supernatant (CFS) surface tension (ST), index emulsification (EI_{24}), reducing sugars by the DNS method.

Surface tension. The surface tension measurements were performed with a tensiometer Easy Dine KRÜSS K20®, by the method Wilhelmy Plate, which consists in measuring the surface tension between a liquid and a solid, measuring the force acting on a submerged vertical plate.

Emulsion Index (EI_{24}). For quantification index emulsion 2 ml of cell free supernatant were collected and 2 ml of diesel is added. The mixture was stirred in a vortex for one minute and allowed to stand 24 hours. To calculate EI_{24}, the height of the formed emulsion and the total height of the liquid column was measured and the following formula was used: $EI_{24}= (H_E/H_T)*100$. Where: EI_{24}: Emulsion index, H_E: Total height of the formed emulsion, H_T: Total height of the liquid column

Reducing sugars by the DNS method. For the development of the reaction DNS in glass tubes of 10 ml 0.5 ml sample CFS and 0.5 ml DNS reagent of they were added. The tubes were placed in water bath at 100 ° C for 5 minutes, cooled to room temperature and was added 5 ml of distilled water; stirred and reading at 540 nm was performed in the spectrophotometer VE-5000V.

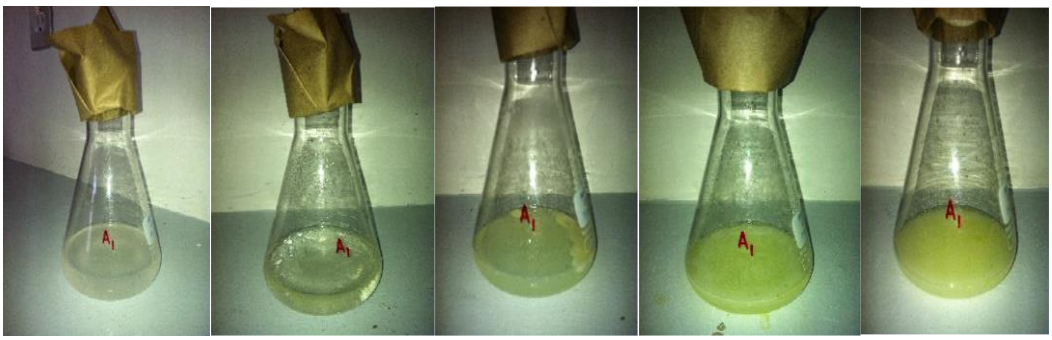
Results and discussion.

Adaptation and selection of strains with agro-industrial waste. The strains that were activated in PY medium showed abundant growth in the medium mentioned at 24 hours. The strains were inoculated in salts and minerals medium with methylene blue and the residue (MSM+MB+R) to confirm the BS production. The results are presented in Table 1. Where (xx) presence of abundant halo at 24 h; (x) presence of halo at 72 h; (-) there is not growth.; (Δ) growth without presence of halo. It is observed in Table 1. that the strains A, B, C, D presented a halo formation at 24 hours on 2% WCO*, Pseudomonas aeruginosa* ATCC 27853,101, 89 and 83 which were selected to their culture in liquid medium at concentrations of 2 % of WCO. The selection process was made with wastewater coffee as medium and the result was that to the concentration of 18 % (v/v) the strain A and the reference strain *Pseudomonas aeruginosa* ATCC 27853 presented an indicative halo of production of BS at 24 hours and the strains B, C,D,98, 89 presented indicative halo until 72 hours . The strains 101 and 83 presented growth without causing halo. In the culture medium and the strain 97 did not grow up the culture medium.

Table.1. Solid selective medium assay with bacterial strains, forming of halo as indirect evidence of biosurfactant production.

STRAINS	MSM+MB+R							
	COFFEE WASTEWATER					WASTE COOKING OIL		
	CW 2%	CW 6%	CW 10%	CW 14%	CW 18%	WCO 2%	WCO 6%	WCO 10%
A	Δ	Δ	x	x	xx	xx	xx	xx
B	Δ	x	Δ	x	x	xx	x	x
C	Δ	x	x	x	x	xx	xx	xx
D	Δ	x	x	x	x	xx	xx	xx
Pseudomonas aeruginosa **ATCC27853**	Δ	Δ	xx	x	xx	Δ	xx	xx
101	Δ	x	x	Δ	Δ	xx	Δ	-
98	Δ	x	x	x	x	x	Δ	Δ
97	Δ	Δ	-	-	-	Δ	-	-
89	Δ	x	x	x	x	xx	-	Δ
83	Δ	Δ	Δ	Δ	Δ	x	Δ	-

Kinetics growth and production of liquid medium. During the kinetic growth and the production of BS in MSM liquid several parameters were monitoring, the ST, IE_{24} were monitoring every 24 hours, and the DNS method was monitoring every 48 hours. Lan *et al*., (2015), mention the shake flasks have a milky appearance in the medium an indicates the production of BS. Observed tis result in figure 1.



0 hours 24 hours 48 hours 72 hours 96 hours

Figure 1. Alteration in the SMM aspect during the BS production of the strain A.

From the strains selected, the measurements were taken from the surfactant properties of ST, IE_{24} and the bacterial growth in liquid medium of MSM + WCO in concentration of 2% and the MSM +CW in concentration of 18%. In the determination of bacterial growth with WCO in 2% they reach their exponential phase at 40-96 hours in culture, during this period is recording the tension surface decrease and rate emulsion increase; relative to CW in 18% they grow up reaching the exponential phase at 48 hours, but they do not have the ability to

decrease the surface tension and they do not present emulsifying activity. The determination of the ST with WCO in 2% was observed that the strains were decrease the surface tension 54mN/m to values of 30-28mN/m at 96 hours and in the CW medium in 18 % at the end of the kinetic the strains did not reduce the surface tension. An analysis of variance was realized with a confidence level of 95 %, in order to establish the differences between the strains, the surface tension and the emulsion rate were used as response variables to stablish the best kinetic conditions, and the strains with best response according to their surfactant properties. This analysis gave us that when some native bacteria is in culture in medium MSM+WCO in 2%, the optimal time of the kinetic process is 96 hours and the strains with better performance were the strains A, B and 83 and the same strains were used to make a new kinetic to stablish the best strain with more production.

Surface tension. The surface tension was measured during every 24 hours, can be observed that the conduct of the strain A, B and 83 during the kinetic is similar, the exponential phase of ST decrease at 48 hours. All the strains reduce from 50 to 30mN/m the surface tension as the positive control. See figure 2. Rocha e Silva *et al*., 2013 got the surface tension in culture medium was decrease until 27.7 mN/m using *P. cepacia* in soy oil corn steep liquor as carbon source. However, some authors report reduction values in surface tension lower than those obtained in this study, like (Lan *et al*., 2015) in which was obtained a minimum value in surface tension of 24.1 mN/m using WCO as carbon source for BS production.

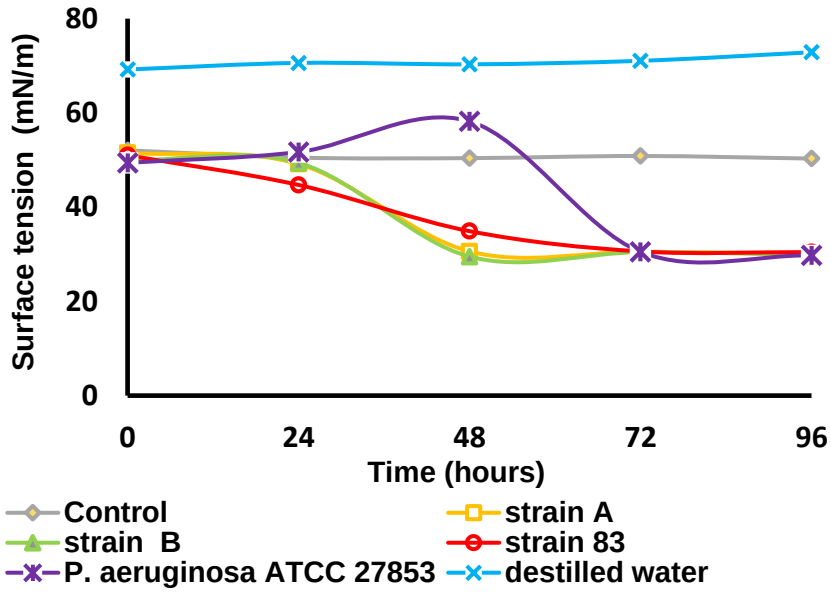


Figure 2.Surface tension of strains in MSM liquid and WCO 2%.

Emulsion index. In figure 3. are presented the emulsions generated from the strains studied, the strains A and 83 started the emulsion to emulsify at 24 hours, presenting and index of emulsions between 58-9 % the hydrophobic compound used (diesel) at 96 hours the strain B started its emulsion at 24 hours at the end of the 96 hours presented an emulsion of 30 % and the *Pseudomonas aeruginosa* do not have emulsion. The values obtained are similar to the results reported by Gudiña *et al*. (2014), and Lan *et al*. (2015) who obtained an

emulsion index of 58-59 % using hexadecane. The most emulsion in this research is presented with the strain 83 and A as is mentioned before this indicates the produced BS has the ability of emulsify insoluble, similar with other bacteria sit the same carbon source, even better that the *Pseudomonas aeruginosa* used as positive control.

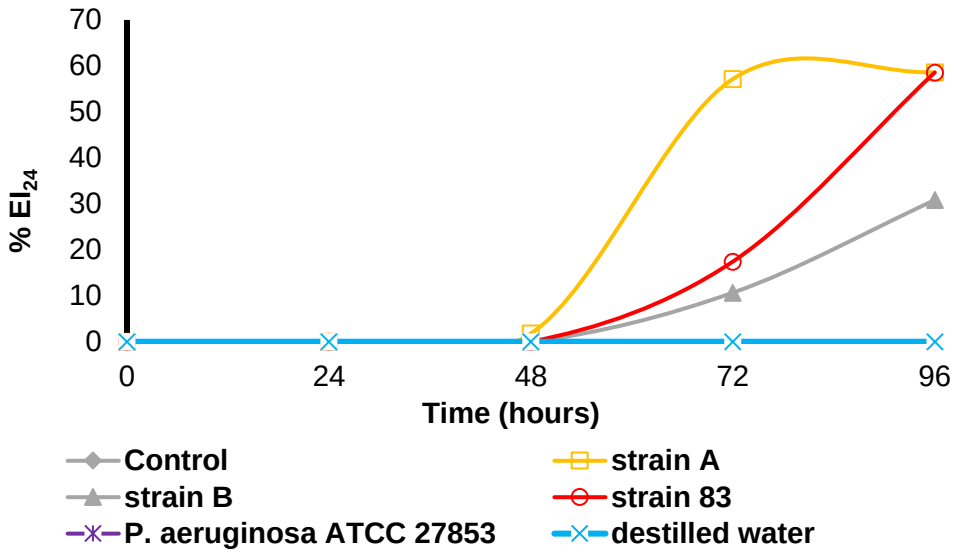


Figure 3. Emulsion Index from the strains cultured in MSM liquid and WCO 2%.

Reducing sugars in DNS method. The DNS method is a quantitative test and indicates the presence of reducing sugars in a sample. In Table 2 are showed results of initial and final reducing sugars concentration. The bacterial strains A, B,83 and *Pseudomonas aeruginosa* ATCC27853 increase the concentration of grams per litre of reducing sugars, the rhamnolipids may contain one or two molecules of rhamnose , this molecule is evidenced with this method.

Table 2. Results of DNS method to detection reducing sugars.

Strain	Concentration initial (g/l)	Concentration final(g/l)
A	2.422±0.01	2.516±0.01
B	2.333±0.01	2.605±0.02
83	2.039±0.003	2.140±0.04
P. aeruginosa **ATCC27853**	2.167±0.005	2.535±0.02
Control	1.968±0.001	1.972±0.002

Conclusions

The selected strains showed BS production in selective medium added with WCO in 2% at 24 hours, however in the kinetic of growing where strains 83, B an A which showed the best results. All strains reduced the surface tension from 50 mN/m to 30-29 mN/m, respect to the emulsion index, the strains presented emulsifying properties, but the strains A and 83 were which presented an superior emulsion index in 58%, except for the positive control that didn't present emulsion. According to the results obtained for surface tension and emulsion index,

the strains A and 83 showed the ability to be potentially exploited for the treatment of contaminated soil and water, with insoluble compounds.

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BIOTECHNOLOGY SUMMIT

Genetic diversity analysis of Sri Lankan traditional rice varieties "pachchaperumal" and "suduru samba"

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Abstract

Sri Lankan traditional rice varieties exhibit a wide variation in morphological characters, flowering time and yield. They are conserved at Plant Genetic Recourses Center, Gannoruwa, Sri Lanka. The objective of this study was to evaluate the diversity of two Sri Lankan traditional rice varieties *Pachchaperumal and Suduru samba* comprising 13 and 7 accessions respectively based on days to flowering (DF), 12 morphological and 2 physiological characters. DF among 13 and 7 accessions of *Pachchaperumal* and *Suduru samba* varied from 60 – 72 and 79 – 99 days respectively. Leaf temperature at flowering (LeT) and Leaf temperature difference at flowering (LeTD), vegetative morphological characters and yield components also varied between *Pachchaperumal and Suduru samba* accessions. Pericarp colour and grain width and length were distinct characters between two varieties. According to the hierarchical cluster analysis, 2 major clusters were identified at the rescale distance of 25 separating accessions of *Pachchaperumal* and *Suduru samba* except for accession 3136 of *Pachchaperumal*, which located in *Sudura samba* cluster. Seven clusters were derived at rescaled distance of 5 where accessions of similar quantitative and qualitative morphological characters were clustered together. Our results would be useful in determination of identity of accessions belonging to same variety, which could be further supported by molecular analysis.

Keywords: morphology, *Pachchaperumal*, Sri Lankan rice, *Suduru samba*, yield

Introduction

Sri Lanka is self-sufficient with rice production. Unfavourable climatic and environmental conditions and reduction of land use could adversely affect the rice production (Bambaradeniya and Amerasinghe, 2004). Therefore, it is a necessity of increase the production by enhancing the genetic diversity though breeding new varieties to face future challenges of climate change (Yoshida, 1983). Several environmental factors affect the plant architecture, vigour and yield of rice plant; photoperiod and temperature are the main factors among them. Approximately 2000 accessions of Sri Lankan traditional rice (*Oryza sativa indica*) germplasm from 46 agro-ecological sub regions of Sri Lanka are conserved at Plant Genetic Resources Centre (PGRC), Sri Lanka, which may contribute to the genetic diversity on days to flowering (DF), plant architecture, yield and photoperiod responsiveness (Irangani and Shiratake, 2013). There are around 500 farmer varieties in the collection while most of the varieties contain more than one accession from different agro-ecological sub regions of Sri Lanka. Team of NRC research project 12-129 (2014; 2015a: 2015b)

characterized 755 accessions belonging to different varieties. A mini core collection was developed for flowering time variation (Rathnathunga et al, 2016a). Sri Lankan traditional rice cultivation was eliminated due to the introduction of new improved rice varieties. Current Sri Lankan traditional rice cultivation is around 0.1% from total cultivated land area, which produces about 1000 ha annually (Wang et al., 2012). Sri Lankan Government is attempting the re-introduction of traditional rice to farmer field as a component in organic agriculture mainly due to its nutritive value, *Ayurvedic* value and resistant ability to ipests, diseases and adverse climatic conditions (Berger, 2009; Dharmasena, 2010; Fisher and Lucy, 2012; Vanlanka Community Foundation, 2014). *Pachchaperumal* is a red rice variety, which was considered as the divine rice in traditional *Sinhalese* culture. *Suduru samba* is a white rice variety of very small, palatable grain considered to be comparatively resistant to brown plant hoppers and thrips. *Pachchaperumal is* known to be a good diet for diabetes and cardiovascular diseases and *Suduru samba* as an aphrodisiac in traditional Sri Lankan medicine. *Pachchaperumal and Suduru samba* are short aged varieties in farmer fields. Characterization of *Pachchaperumal and Suduru samba* accessions would be useful to assess the genetic diversity among accessions on morphological variation and to determine the DF variation within variety.

Materials and methods

Rice Accessions. Thirteen and seven accessions of Sri Lankan traditional rice varieties *Pachchaperumal* (Accession numbers: 8827, 9049, 3136, 3752, 3408, 5549, 5550, 5548, 5547, 5546, 6834, 5383, 3946) and *Suduru samba* (Accession numbers: 4362, 5402, 2202, 3333, 3572, 3594, 3671) were obtained from Plant Genetic Resources Center (PGRC), Sri Lanka.

Field experiment. Pachchaperumal and *Suduru samba* accessions were grown at Rice Research and Development Institute, Batalagoda, Sri Lanka. Average temperature during the cropping season was around 32 °C. Each replicate was a plot of three rows with 9 plants: The spacing was 20 cm x 20 cm within and between rows and 40 cm between plots in a Complete Randomized Design (CRD). There were 4 replicates. The experiment was carried out in late short day season (*Maha*), from December 2012 to long day season till July 2013. Fertilizer application, pest and disease management and weed control were according to the recommendation by Department of Agriculture, Sri Lanka.

Evaluation of morphological traits. Following quantitative morphological characters were measured from each replicate from each accession: Days to Flowering (DF), Plant height at maturity (PH), Culm length at maturity (CL), Culm number at maturity (CN), Culm diameter at maturity (CD), Panicle `length at maturity (PL) Grain length at maturity (GL), Grain width at maturity (GW), Leaf number at maturity (LN), Leaf length at maturity (LL), Leaf width at maturity (LW), Leaf temperature at heading (LeT), Leaf temperature difference at heading (LeTD), Shoot weight at maturity (SW) and Panicle weight at maturity (PW). Panical type, Awn presence, Lemma and palea colour, Seed shape and Pericarp colour were evaluated as qualitative morphological characters. With respective to each character in a given accession, average value of replicates was considered for analysis. Measurement techniques were according to modified descriptors of rice published by the Team of NRC research project 12-129 (2014; 2015a; 2015b).

Statistical analysis. Data were analyzed using PCA with correlation matrix through SPSS software (version 20), IBM, USA to define the patterns of variation between all explanatory

variables. Measure of dissimilarity was given by the Euclidean distance and the clustering method was Ward's linkage. The number of clusters was determined at the rescaled distance of 25 and 5.

Results and discussion

Variation between accessions of Sri Lankan traditional rice variety Pachchaperumal and Suduru samba. DF among 13 and 7 *Pachchaperumal and Suduru samba* accessions varied from 60 – 72 and 79 – 99 days respectively (Table 1). DF variation in traditional rice *Sudu wee* and *Hondarawala* were comparatively wider with 62 to 200 days+ and 57 to 200 days+ (Rathnathunga and Geekiyanage, 2015 and Rathnathunga et al., 2016b). LeTD, LL, LW, PH, CL, CN, CD, GL, GW, PL, SW and PW varied differently among *Pachchaperumal and Suduru samba* accessions, which differentiated *Pachchaperumal and Suduru samba* varieties in to 2 different clusters at the rescale distance of 25 according to the hierarchical cluster analysis (Figure 1). Grain length and width of all accessions of *Suduru samba* were lesser making a small seed shape while in all *Pachchaperumal* seed shape was larger (Table 2 and Table 3).

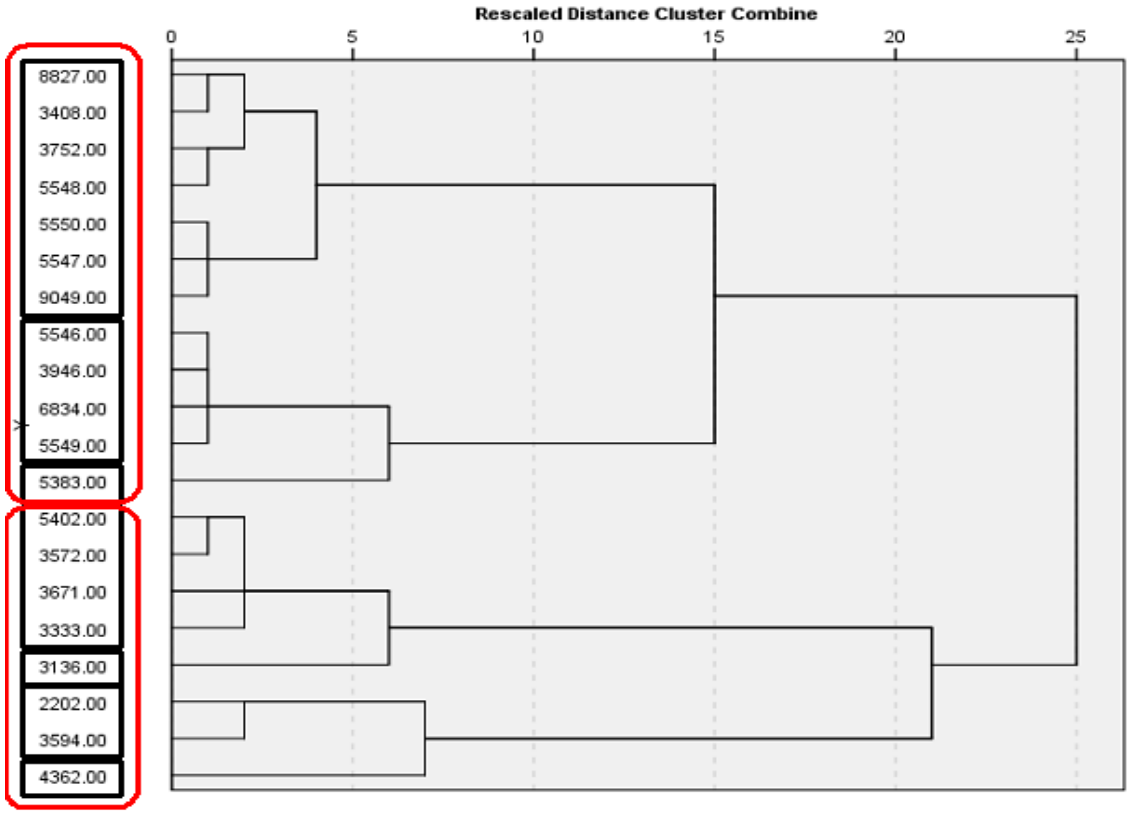

Figure 1. Dendogram of *Pachchaperumal* and *Suduru samba* accessions derived through Ward's linkage method of Cluster Analysis based on days to flowering, 12 morphological and 2 physiological characters.

Table 1. Descriptive statistical explanation of the variation of quantitative characters among the accessions of *Pachchaperumal* and *Suduru samba*

		Variety *Pachchaperumal*			Variety *Suduru samba*		
Character	Unit	Range	Average	Standard deviation	Range	Average	Standard deviation
DF	days	60 - 72	64.8	3.8	79 - 99	89.1	8.5
LeT	^{0}C	23.67 - 27	25.9	1.0	22.67 - 25.67	23.6	1.0
LeTD	^{0}C	3.1 - 6.4	4.2	1.0	6.4 - 9.4	8.5	1.0
LN	number	25.3 - 127	67.3	31.8	25.7 - 107	57.8	28.9
LL	cm	41.5 - 54.9	46.4	3.7	52.3 - 71.6	61.6	7.2
LW	cm	9 - 13	10.8	1.1	6.2 - 12.6	9.8	1.9
PH	cm	116.5 - 151.5	131.7	10.6	141.5 - 164.3	155.3	8.0
CL	cm	87 - 129.5	108.7	11.0	120.7 - 149.7	130.5	9.7
CN	number	9 - 27	14.1	4.9	13.7 - 37.3	24.7	8.6
CD	cm	0.32 - 0.65	0.4	0.1	0.35 - 0.8	0.6	0.2
GL	cm	0.7 - 1.1	0.8	0.1	0.5 - 0.6	0.5	0.0
GW	cm	0.3 - 0.35	0.3	0.0	0.2 - 0.3	0.2	0.0
PL	cm	11.75 - 19.75	15.6	2.4	12.4 - 28.4	21.7	5.7
SW	g	65.63 - 138.18	97.2	24.2	108.83 - 177	139.9	22.2
PW	g	11.83 - 47.08	24.4	8.9	4.2 - 32.98	19.5	10.3

Principal Component Analysis and Cluster Analysis revealed the variation in DF among Pachchaperumal and Suduru samba accessions. First four Principal components (PC) explained 78.9% of total observed variation. PH, CL, LL and SW included in PC1. DF, LL, PL and -PW were included in PC2 while LeT, LeTD, LW and GW were included in PC3. CN, LN LW and SW were included in PC4. The PC1, PC2, PC3 and PC4 explained 21.3%, 20.5%, 20.1% and 17.0% of variance respectively. According to the hierarchical cluster analysis, 2 major clusters were identified at the rescale distance of 25, which were further separated in to 7 clusters at rescaled distance of 5 indicating similar DF groups in most clusters (Figure: 1 and Table 2). *Pachchaperumal* accessions of 8827, 3408, 3752, 5548, 5550, 5547 and 9047 of^t cluster one, 5546, 3946, 6834 and 5549 of cluster two, accession of 5383 of *Pachchaperumal* cluster three were grouped in to one major cluster at rescale distance 25 indicating the genetic similarity of *Pachchaperumal* accessions. Only accession of 3136 of *Pachchaperumal* clustered with *Suduru samba* accessions. Accessions of 5402, 3572, 3671 and 3333 of *Suduru samba* were included in the cluster 4, accessions of 2202 and 3594 of *Suduru samba accessions in* cluster six and 4362 of *Suduru samba* in cluster 7 suggesting the genetic similarity between all *Suduru samba* accessions. Variation of DF and other quantitative characters was evident among accessions among different clusters. The qualitative morphological background of accessions of each cluster indicated significantly similar lemma and palea colour, and similar pericarp colour variation within 4, 6 and 7 clusters of *Suduru samba* accessions and cluster 1, 3 and 5 of *Pachchaperumal*

accessions (Table 3). Chandrarathna (1964) explained the gene interactions involved in pericarp colour as very important to determine the genetic variation of similar accessions of similar varieties. Genetic basis for morphologically similar accessions of *Pachchaperumal* and *Suduru samba* with different DF would provide more precise information on pleiotropic effect of flowering time on vegetative growth and yield. Our results would be useful in future breeding for manipulating flowering time and yield within similar genetic backgrounds.

Conclusions

Suduru samba variety was distinguishable from *Pachchaperumal* variety due to seed shape and pericarp colour. DF among 13 and 7 *Pachchaperumal* and *Suduru samba* accessions varied from 60 – 72 and 79 – 99 days respectively. According to the hierarchical cluster analysis, 2 major clusters were identified at the rescale distance of 25 separating *Pachchaperumal* and *Suduru samba.*

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Identification of conserved salt responsive miRNAs from Halophyte *Atriplex canescens*

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Abstract

Atriplex canescens, a halophyte wild plant, is often found in arid soil and desert environments in México. MicroRNAs (miRNAs) are non-coding RNAs that play important roles in growth and development and maintenance of genome integrity, miRNAs are also important components in plant stress responses. There are very few reports on the discovery of salt-responsive miRNAs from halophytes. In this study, two small RNA libraries, one each from the control and salt-treated (250 mM NaCl for 24 h) whole plant of *A. canescens*, were sequenced, which yielded 107 know miRNAs. Additionally, we used publicly available transcriptomics data of *Beta vulgaris* which led to the discovery of additional 563 predicted miRNAs along with their pre-miRNA sequences through in silico analysis. In total, three frequent known miRNAs were up-regulated whereas, two frequent known miRNAs were down-regulated under salinity stress. Furthermore, 14 known miRNAs seems to regulate 141 target genes. These targets with the help of gene ontology analysis were found to be involved in several important biological processes that could be involved in salinity tolerance. Relative expression of the miRNAs was detected by Illumina sequencing and the results were found to be coherent with literature. Thus, the present study provides an account of miRNA-target networking that is involved in salinity adaption of *A. canescens*.

Keywords: miRNAs, salt stress, Atriplex, AGO1

Introduction

Plants have developed intricate mechanisms for sensing and responding to environmental changes (Wani *et al*., 2016). To turn on protective mechanisms, plants trigger a network of genetic, transcriptional and/or translational regulations, including altered expression of large proportion of genes by different pathways. Plant resistance to salt stress is controlled by multiple genes and is regulated at multiple levels. At the molecular level, various stress-responsive genes are involved (Zhu 2003; De Costa *et al*. 2007). MicroRNAs (miRNAs) are a series of endogenous small non-coding RNA molecules that, based on sequence complementarity with their target(s), can negatively regulate gene expression at transcriptional, post-transcriptional and translational levels by modulating mRNA degradation and suppression of ribosomal activity (Sunkar *et al.* 2012). Aside from the roles in modulating a wide range of essential biochemical, molecular and physiological processes, many studies reported that miRNAs were involved in plant responses to a variety of abiotic stresses such as salt (Liu *et al.* 2008; Ren *et al*. 2013), drought [Li *et al.* 2011; Eldem *et al.* 2012), heat (Yu *et al*. 2011; Chen *et al*. 2012), cold (Liu *et al*. 2008; Zhang *et al*. 2014] and oxidative stress (Sunkar *et al.* 2006). More important, a growing body of evidences

suggested that miRNA-guided gene regulation could play a vital role in plant response to salt stress. Understanding the miRNA-mediated regulatory network of salt stress response will lay the foundation for unraveling the complex molecular genetic mechanism of salt-stress tolerance and perhaps reproduce such adaptation in different models. The halophyte *Atriplex canescens*, a member of the Chenopodiaceae, has been extensively used in physiological studies to explore stress-related mechanisms. Tolerance to salinity is the main characteristics of Atriplex species as model of study. Yet, no research focused on identification of miRNAs and their target genes related to salt stress has been reported to date. In this study, two libraries of small RNA, each one from the control and salt-treated (250 mM NaCl for 24 h) whole plant of *A. canescens* were constructed and sequenced using NGS technology with the aim to detect salt responsive miRNAs and explore their roles in plant response to salt stress. The results of this study could provide valuable information for further validating the regulatory roles of salt-responsive miRNAs in *A. canescens*, and facilitate the description of molecular mechanism underlying plant adaptive response to salt stress.

Materials and methods

Plant growth conditions. The *A. canescens* plants used for RNA preparation were grown under controlled environmental conditions: 21 to 23°C, 100 µmol·photons.m^{-2}·s^{-1}, 60% relative humidity, 14h light/10h dark. Plants were spotted with peat and vermiculite (1:1), and watered with Hoagland solution twice per week prior to treatment. After 50 days of growth, plants were watered with 250 mM NaCl. After 24 h treatment, the harvested samples (young leaves, stems and roots) were immediately frozen with liquid nitrogen and kept at −80 °C until total RNA extraction protocol.

Small RNA library construction and deep sequencing. Total RNA was extracted from *A. canescens* samples mixture using a mirVana™ miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA). RNA extraction quality was checked by spectrophotometry and gel. The library of small RNA was generated from the total RNA samples using the Illumina Truseq™ Small RNA Preparation kit according to Illumina's TruSeq™ Small RNA Sample Preparation Guide. The purified cDNA library was used for cluster generation on Illumina's Cluster Station and subsequently sequenced on Illumina GAIIx (Illumina, Inc., Santa Clara, CA) following the manufacturer's instruction. Raw sequencing reads (40 nts) were obtained using Illumina's Sequencing Control Studio software version 2.8 (SCS v2.8) following real-time sequencing image analysis and base-calling by Illumina's Real-Time Analysis version 1.8.70 (RTA v1.8.70). The ACGT101- miR program (version 4.2; LC Sciences) was used for standard sequencing data analysis (Li *et al* 2011; Wei *et al.* 2010; Meyer *et al.* 2010).

Identification of conserved miRNAs. First, the sequences were blasted to the RFam database, repeat sequences, and mRNAs. Matched sequences were discarded. In absence of *Atriplex canescens* genome, the sequences then were compared with *Beta vulgaris* genome. Sequences downloaded from the *Beta vulgaris* database (http://bvseq.molgen.mpg.de/Genome/Download/RefBeet-1.1/). The unmatched sequences were removed. Finally, the remaining sequences were mapped to all known plant miRNAs sequences to identify the conserved miRNAs in *Atriplex canescens* from the miRBase database (version 21.0, http://www.mirbase. org/).

To identify conserved miRNAs in *Atriplex canescens*, conserved candidate miRNAs sequences were blasted against *Beta vulgaris* genome sequences, and their flanking sequences in the genome were used to predict their secondary structures by using the mfold Web server (http://mfold.rna. albany.edu/?q=mfold/download-mfold) (Zuker 2003).

Results and discussion:

Deep Sequencing Results of Small RNAs from A. canescens. Deep sequencing generated 15,472,639 and 11,323,212 raw reads in two libraries. After removal of low-quality and corrupted adapter sequences, 12,773,736 and 9,870,100 reads remained in the two libraries, which were considered as mappable. The size distribution of the remaining reads was assessed. The data showed that 24 nt small RNA is the major size class, followed by 21, 22, 23, and 19 nt. Similar results were reported in other plant species, such as *Arabidopsis thaliana* (Rajagopalan *et al.* 2006; Fahlgren *et al.* 2007), *Medicago truncatula* (Szittya *et al.* 2008), *Oryza sativa* (Morin *et al.* 2008), *Arachis hypogaea* (Chi *et al*. 2011) and *Cucumis sativus* (Martínez *et al.* 2011)]. From the analysis performed with the genome assembly of *Beta vulgaris*, we observed that 20.3% of reads are mapped to known plant pre-miRNA in miRbase. Also, 9% of reads are mapped to plant repeats, mRNA, and other RNAs including tRNA, rRNA, snRNA and snoRNA. In addition, some of the reads that could not be mapped to pre-miRNA in miRbase and other RNA were mapped to *Beta vulgaris* genome sequences, and a fraction of them showed high probabilities to form hairpins. Moreover, nearly half of these reads had no mapping information (75%).

Conserved miRNAs in A. canescens and their relative abundance. To identify the conserved miRNA in *A. canescens*, small RNA sequences were mapped to miRNA in miRBase. Based on sequence homology, 107 known miRNA were found. The majority of the identified miRNA were 20–22 nt long. We were able to classify these identified miRNAs into 14 different families (Table 1). The number of miRNA members in each known family shows significant divergence. The miR166 family is the largest one with 7 members, and for the miR319, miR159, and miR167 family each of them has 3, 3, and 5 members respectively. Five families, including miR396, miR156, miR394, miR168, and miR172, contained four members, and the remaining 5 miRNA families contain one to two members of our identified miRNA (http://pmted.agrinome.org/by_mirna.jsp). With the scientific evidence available on the mirBase 21.0, we were able to determinate that these 14 families of conserved miRNA regulate at least 141 genes, including mainly transcription factors and DNA interaction proteins. Interesting, each one of these genes are reported to regulate a number of other genes involved in plant response to abiotic stress, probably activating or inhibiting signaling pathways. These secondary regulated genes are not shown on this work.

Table 1. Conserved miRNA from *A. canescens* with their respective targets.

miR name	miR_seq	Reference miR	Target description
aca-miR166a-3p	TTCGGACCAGGCTTCATTCCC	ath-miR166a-3p	Homeobox-leucine zipper protein ATHB8, ATHB9, ATHB14, ATHB15, FL
aca-miR319a	CTTGGACTGAAGGGAGCTCCCT	ath-miR319a	MYB DOMAIN PROTEIN MYB104, MYB33, ATMYB65
aca-MIR159a-p5	AGCTCCTTTTGATCCAAAAAC	mdm-MIR159a-p5	MYB proteins
aca-miR167d-3p	GATCATGTGGTAGCTTCACC	stu-miR167d-3p	Unknow
aca-miR396a-5p	TTCCACAGCTTTCTTGAACTG	ath-miR396a-5p	Growth Regulating Factor (GRF), rhodenase-like proteins, and kinesin-like protein B
aca-miR156a-3p	GCTCACTGCTCTTTCTGTCATC	ath-miR156a-3p	Squamosa-promoter Binding Protein (SBP) box
aca-miR394a	TTGGCATTCTGTCCACCTCC	ath-miR394a	F-box proteins
aca-miR167a-5p	TGAAGCTGCCAGCATGATCTC	ath-miR167a-5p	Auxin response factors
aca-miR157d	CTGACAGAAGATAGAGAGCAC	ath-miR157d	Squamosa-promoter Binding Protein (SBP) box
aca-miR894	CACGTCGGGTTCACCA	ppt-miR894	unknow
aca-miR168a	TCGCTTGGTGCAGGTCGGGAA	lus-miR168a	unknow
aca-miR172a	CGAATCTTGATGATGCTGCAT	ath-miR172a	APETALA2-like transcription factors
aca-miR535	TGACGATGAGAGAGAGCACGC	cpa-miR535	unknow
aca-miR168a-3p	CCCGCCTTGCATCAACTGAAT	ath-miR168a-3p	AGO1, SUVR4

Relative abundance and salt responsive miRNA in A. canescens. The read counts of miRNA in sequencing libraries can be used as an index to estimate their relative abundance. With this focus, miRNA reads revealed that 22 out of 107 known miRNA were represented by more than 1000 reads in both libraries, and 5 of them, aca-miR396a (142,662 reads), aca-miR166a (94,169 reads), aca-miR159a (36,245 reads), aca-miR535 (10,548 reads), and aca-miR168a (3,131 reads) were among the most frequent. Interesting, the changes in the frequency of miRNA between the salt treated and control libraries might indicate that their expression is regulated in response to NaCl stress. As criteria, miRNA with relative expression > 0.80 and $p < 0.05$ were designated as up-regulate and miRNA with relative expression < −0.80 and $p< 0.05$ were designated as down-regulated. Under the stress of NaCl treatment, 2 miRNAs (Log2 -1.0) belonging to miR166a family are down-regulated, and three miRNAs families miR168a (Log2 1.56), miR532 (Log2 1.19) and miR172a (Log2 0.96) are up-regulated. To identify the potential functions of NaCl-responsive miRNA, target genes for these 14 conserved miRNA are predicted, and the representative results are listed in Table 1. These genes were reported to be involved in many plant physiological processes. As *A. canescens* is salt-tolerant, these salt responsive miRNA may play an important role for high salinity adaptation. Some miRNAs, such as *Zea mays* miR166, miR159, miR156 and miR319, and *Arabidopsis sp* miR393, miR397b, and miR402, have been reported to show altered expression profile under salt stress (Ding *et al.* 2009; Sunkar *et al*. 2004). In our study, the most important up-regulated known miRNA (Table 1), aca-miR168a, is predicted to target ARGONAUTE 1 (AGO1) that interestingly regulates the expression of MIR genes, repressing DICER and it has been linked to salt tolerance in plants (Dong *et al.* 2009). These findings suggest that the found miRNA may play a role in main adaptations of *A. canescens* to salinity. However, further investigations are needed to confirm the above hypothesis.

Conclusion

By using high-throughput sequencing and the genome information of other relative plants, 107 known miRNA were discovered in *A. canescens*, with 14 of them showing response to salt stress and reported interaction with 141 gene targets. Further investigation for the function and interaction of potential target needs to be performed.

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DNA polymorphisms in blue maize hybrids

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Abstract

The objective of this work was to identify DNA polymorphisms in four blue maize hybrids and including their respective parental lines, all of them genotypes contained high anthocyanin levels, this pigments are favorable because their nutraceutical properties and because their intake decreases cardio - vascular problems, degenerative - chronic diseases and cancer. It was developed new varieties and hybrids for to contribute to satisfy the blue maize demand in the Central Highlands Valleys of México, and it was important to develop the varietal description of them. Then, fingerprinting patterns were obtained by the RAMPD technique in our study, it was used five primers (ten bp of longitude) and the $(GATA)_4$ microsatellite. Results showed that in the hybrid genetic combination, one of both progenitors genetic heritage prevailed. Particularly, in the D hybrid (a three-lineal hybrid), the male genetic background of the male progenitor it was preserved. According to these data, it will be possible to select the highest anthocyanin content hybrids via the Molecular Marker Assisted Selection.

Keywords: RAMPD, DNA polymorphisms, molecular markers.

Introduction: In Mexico is wide the genetic variability of maize, which includes different colors in the grain, for example, white, yellow red and even blue; these colors have different intensity levels. Yellow grains contain carotenoids, red and black grains have anthocyanins, and white grains do not have those pigments (Arellano *et al.*, 2003). In the Central Highlands of Mexico, 150 thousand hectares of blue corn that produce 300 thousand grain tons are planted under rainfed conditions and this production is used to prepare traditional foods, such as, tortillas, tlacoyos, quesadillas, etc. (Arellano, 2014). It is estimated that the current demand for blue corn at the Mexican Central Region is about 450 thousand tons for traditional use and additionally five thousand for nixtamalized and flour industry. New varieties or hybrids that duplicate current performance and have good agronomic characteristics are required to satisfy the demand. The blue pigments have an important use in the cosmetic industry, moreover has recently been shown that their intake decreases cardio - vascular problems, degenerative - chronic diseases and cancer (Guzmán-Maldonado, 1999). Particularly in blue corn grain, the pigments responsible for the color are

anthocyanins, which are found in the pericarp, the aleurone layer or both structures (González *et al.*, 2009).

The anthocyanins of blue corn are derived from cyanidine (Salinas *et al.*, 1999). In order to implement a high-anthocyanin content maize improvement program, it is necessary to know the genetic effects and the variance components of the possible basic populations in order to generate, from these populations, suitable progenitors for obtaining better hybrids or varieties. In this sense, it is important to develop the varietal description for the new genotypes, and for it the genomic fingerprinting is a crucial aspect, by this reason in our investigation team realized the study present. The objective of this study was to identify DNA polymorphisms obtained by RAMPD in 4 blue maize hybrids, each one in relation to their respective parental lines (L1, L2, L3, L4, L5, L6, and L7).

Materials and methods

The genotypes selected were: Four three-lineal hybrids (A, B, C, and D), three single-cross hybrids (E, F, and G) and their respective parental lines (L1, L2, L3, L4, L5, L6, and L7). It was used embryonic axis of maize seeds from each genotype of blue maize for the DNA extraction. This procedure was apply according to CIMMYT (2006). Then DNA was quantified by 260 nm of wavelength. It was charged 100 ng / µL of the DNA dilutions in agarose gel (0.7 % p/v) in order to estimate their quality. Molecular fingerprints were obtained through random amplification anchored with microsatellite of the polymorphic DNA (RAMPD) (Gutiérrez *et al.* 2011), which consists of combining a microsatellite (DNA short sequences across the entire genome) in addition to a fragment of 10 base pairs with random sequence in the same reaction mixture (Table 1).

Table 1. Mix reaction components for the random amplification anchored of the DNA polymorphic (RAMPD).

Reactive	**[Final]**	**Volumen 1X (µL)**
Taq Jump Start	2.5 U/reaction	12.5
Primer RAPD (10 pM/µL)	30 pM/µL	3
Microsatellite (20 ng/µL)	30 ng/µL	1.5
DNA (20 ng/µL)	80 ng/µL	8
Total		25

It was used five primers (Table 2) and the microsatellite 5'-GATAGATAGATAGATA-'3. The amplification reaction was carried out in a final reaction volume of 25 µL, with the following program: a) denaturation, 1 min at 94 °C, b) 38 cycles of 30 s at 94 °C, 30 s at 48 °C and 1.5 min at 72 °C, and finally c) 2.5 min at 72 °C, for a final extension. The produced amplicons were resolved on agarose gels (1.2 %, w/v) in a horizontal electrophoresis Chamber (Thermo EC Classic CSSU911, 9 x 11 cm), the DNA was stained with Texas Red (2 %, v/v). The images of the gels were documented (KODAK Digital Science D 2.0), by Molecular Imaging program (v 4.0.3).

Table 2. Primers and microsatellites utilized for the random amplification anchored of the DNA polymorphic (RAMPD).

Primer	Sequence (5' - 3')	Microsatellite	Sequence (5' - 3')
1	GAGTCTCAGG	1	$(GATA)_4$
2	ACGCACAACC	1	$(GATA)_4$
3	GGTGCGGGAA	1	$(GATA)_4$
4	TTATCGCCCC	1	$(GATA)_4$
5	CCCAAGGTCC	1	$(GATA)_4$

Results and discussion

A genomic profile specific for each genetic material was founded in our study (Figure 1), this fact was agree with other studies about to obtain the molecular description by RAMPD (Gutiérrez *et al.*, 2011). Banding patterns obtained on 1.2 % agarose gels for the D blue maize hybrid, located join to its respective female (A) and male (C) progenitors, using RAMPD technique, with 5 primers and combined with the 5'-GATAGATAGATAGATA-'3 microsatellite and the DNA template it is showed below.

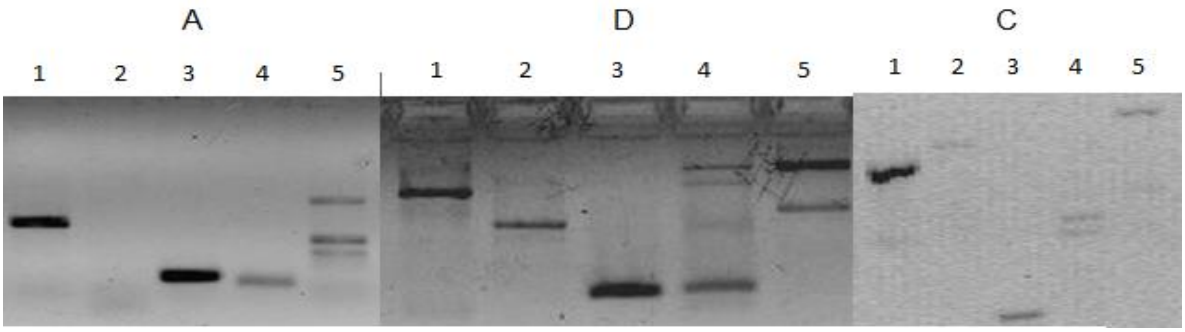

Figure 1. Banding patterns obtained in 1.2 % agarose gels in blue maize genotypes by RAMPD technique, with 5 primers. Hybrid D: A = Female parent, C = Male parent in each lane (1 - 5), microsatellite-primer-DNA combinations were loaded.

It was obtained that D hybrid had a proportion greater of male progenitor heritage (C) compared to the female progenitor (A). This can be determined by checking the banding pattern generated by each hybrid along the different lanes. This fact implies that the genetic relations among the parental lines which made the hybrid were important and determined the genetic pool for each maize hybrid. This interaction will be the heterosis expression in accord to McRobert *et al* (2014), and our investigation team is advocated to analyze the heterosis in particular for anthocyanin level.

Conclusions

It was generated a genetic profile specific for each genetic material by RAMP. D Hybrid banding pattern showed higher male progenitor heritage than female progenitor. DNA Polymorphism level in hybrids was bigger than in progenitors respective.

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Genomic and protein quality expression in QPM hybrids

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Abstract

At the Campo Experimental Valle de México (CEVAMEX) (Chapingo, México) were developed 15 simple experimental hybrids of quality protein maize, derived from six lines (L1 to L6) contained the *opaque2* gen. Lysine, tryptophan, quantity and quality of protein were quantified. It was used a randomized completely design with two replicates of 100 seeds each. It was carried out an analysis of variance, means test (Tukey, 0.05), and principal component analysis. L2, L3, and L6 were detected as quality protein maize, as well as, the L2 x L4 hybrid. On the other hand, the protein quality index did not correlate with protein (r = 0.2) but it did with the essential amino acids tryptophan (r = 0.9) and lysine (r = 0.9). The hybrids fingerprinting showed a close similarity among them however all of them were discerned in the dendrogram. It was founded a close relation among quality protein parameters (lysine, tryptophan, total protein, and quality protein index) and the genetic characteristics, all of them was showed by analysis of component principals.

Keywords: Quality protein maize•lysine•opaque2•qpm•tryptophan

Introduction

After wheat and rice, corn is one of the crops of greatest importance in the world since it is an essential part of the diet of Latin-Americans and Africans (OECD/FAO, 2011). Meanwhile the inhabitants of Mexico consume an average 330 g/day per capita, corn has an outstanding importance to economic, social and culture (Fernandez *et al.*, 2013); mainly for people with limited financial resources who live in marginal areas of the country (*Mendoza et al*., 2006). The corn contains vitamins such as niacin, β -carotene (pro-vitamin A), α -tocopherol, in addition it is rich in carbohydrates (58 to 72 %); but its level of lipids and proteins is significantly low (5 % and 10 %, respectively). Also their proteins are deficient in tryptophan and lysine (Serna *et al.*, 2008), which are considered essential because their levels are directly proportional to the biological quality of the proteins (amount of assimilated N_2) and are not synthesized in monogastric animals (Ufaz and Galili, 2008) by what should be part of people daily intake. In this sense, it has been documented the existence of maize germplasm with high content of essential amino acids as a product of mutant gene opaque2, which enables the generation of genotypes with grain protein quality through conventional breeding, molecular markers-assisted or by OGM (Ufaz and Galili, 2008;) Manicacci *et al.*, 2009). These maize varieties with quality protein are also called modified or QPM (quality

protein maize). The objectives of this study were: i) To obtain the genomic fingerprinting of six inbred lines and fifteen of *o2* maize simple hybrids, and ii) To establish the protein quality of the all experimental o2 genotypes (parental lines and hybrids), in terms of the content of lysine, tryptophan, total protein, and protein quality index.

Materials and methods

It was studied six maize experimental lines of protein quality high (QPM), 15 single hybrids F1, 15 F2 crosses and three controls, one of them possessed high expression of the quality protein (CML503B), another one with middle quality (CMLBA2192503 HEW2), and the H70, commercial hybrid with normal protein (unmodified) (Table 1).

Table 1. Lines and F1 and F2 generations of quality protein maize and controls employed (CEVAMEX, 2010).

Genotype	Description	Genotype	Description
Line			Cruzas
L1	CH 46 (CML 525)	C1	CH46 x CH47
L2	CH 47 (CML 528)	C2	CH46 x CH48
L3	CH 48 (CML 524)	C3	CH46 x CH49
L4	CH 49 (CML 526)	C4	CH46 x CH50
L5	CH 50 (CML 527)	C5	CH46 x CH51
L6	CH 51 (CML 529)	C6	CH47 x CH48
Controls		C7	CH47 x CH49
T1	CMLBA2192503 HEW2	C8	CH47 x CH50
T2	CML503B	C9	CH47 x CH51
T3	H70	C10	CH48 x CH49
		C11	CH48 x CH50
		C12	CH48 x CH51
		C13	CH49 x CH50
		C14	CH49 x CH51
		C15	CH50 x CH51

The mentioned lines had more than 90 % of inbreeding level and were generated from material with adaptation to the High Central Valleys of Mexico (2200-2800 masl). The original opaque2 genotype was the CML176, a line developed by the International Center of Maize and Wheat Improvement (CIMMYT), this genetic material was the donor of the recessive gene opaque2 (o2) The 15 F1 generation were obtained by controlled pollination among these six lines and these crosses were advanced to the F2 generation by open pollination. Quantifications chemical: Full seeds were powdered and lipids were eliminated (hexane for 6 h); with 100 mg of the obtained flour the amino acid colorimetric determination was made. For lysine (LYS) the protocol was based on the reaction of the 2-chloro, 3, 5-dinitropiridine (390 nm) and tryptophan (TRP) in the of glyoxylic acid (560 nm) (Galicia *et al.*, 2009). Total protein (PT) was calculated by multiplying the total nitrogen (Galicia *et al.* 2009), by the factor 6.25 (Salinas and Vázquez, 2006). Protein quality index (ICP) was calculated respect to the amino acid tryptophan, which is a smaller amount than the lysine, it was employed the procedure: ICP = [tryptophan (%) x 10^2] [protein (%)]$^{-1}$ (Galicia *et al.*, 2009). The results were expressed as a percentage on dry basis.

Fingerprinting determination: DNA extraction was performed according to CIMMYT (2006) from de embryonic axis of seed from each genotype. DNA was then quantified at 260 nm of wavelength. It was charged 10 ng / µL of the DNA dilutions in agarose gel (0.7% p/v) (CIMMYT, 2006) in order to estimate their quality. Molecular fingerprints were obtained through random amplification anchored with microsatellite of the polymorphic DNA (RAMPD), which consists of combining a microsatellite (short DNA sequences across the entire genome) in addition to an initiator of 10 base pairs with random sequence in the same reaction mixture (Table 2). It was used ten primers and the microsatellite 5'-GATAGATAGATAGATA-'3. The amplification reaction was carried out in a final reaction volume of 25 µL, with the following program: a) denaturation, 1 min at 94 ° C, b) 38 cycles of 30 s at 94 ° C, 30 s to 48 ° C and 1.5 min at 72 ° C, and c) 2.5 min at 72 ° C, for a final extension. The produced amplicons were resolved on agarose gels (1.2 %, w / v) in a horizontal electrophoresis Chamber (Thermo EC Classic CSSU911, 9 x 11 cm), the DNA was stained with Red Texas (2 %, v / v). The images of the gels were documented (KODAK Digital Science D 2.0), with Molecular Imaging program (v 4.0.3).

Table 2. Primers and microsatellites utilized for the random amplification anchored of the DNA polymorphic (RAMPD).

Primer	Sequence (5’ - 3’)	Microsatellite	Sequence (5’ - 3’)
1	GAGTCTCAGG	1	$(GATA)_4$
2	ACGCACAACC	1	$(GATA)_4$
3	GGTGCGGGAA	1	$(GATA)_4$
4	TTATCGCCCC	1	$(GATA)_4$
5	CCCAAGGTCC	1	$(GATA)_4$
6	TCAGGGAGGT	2	$(GACA)_4$
7	CACCAGGCTA	2	$(GACA)_4$
8	ACGCACATCC	2	$(GACA)_4$
9	AAGACCCTCC	2	$(GACA)_4$
10	GCTGACTGTG	2	$(GACA)_4$

Analysis of genomic data: It was constructed the basic matrix of data database (MBD) which consisted of 187 rows and 21 columns, in correspondence with the bands obtained and genotypes (6 lines and 15 crosses F_1, respectively) under study; all the amplicons were codified for each treatment by assigning the number 1 to the presence of the band and 0 for its absence. Statistical analysis: The variables tryptophan, lysine, protein content and protein quality index was analyzed according to the random completely design with two replications and the results were submitted to analysis of variance and means comparison by the method of Tukey ($P \leq 0.05$). The principal components analysis was made (Peña, 2002) for to relate the molecular groupings of the genotypes *o2* in the dendrogram with their respective levels of protein quality. Statistical data processing was done with the SAS program (SAS Institute, 2002), for the analysis of major components was the PROC PRINCOMP.

Results and discussion

It was founded that L2, L3 and L6 lines were progenitors of the best hybrids (α ≤ 0.05) for PT (L1 x L2, L1 x L3, L2 x L3, L2 x L4, L2 x L5, L2 x L6, and L3 x L6), of which only three stood out significantly also in TRP, LIS and ICP (L2 x L4, L2 x L6 and L3 x L6) (data no shown). The general average of LYS (0.36) was five times higher than that of TRP (0.072). Among cultivars LIS content unchanged (α ≤ 0.05) in lines neither crosses F_1 and F_2, even their proportion increased about 60% in F_2, generation, which remained significantly higher than the control. In this paper, we be obtained an eventual relationship between the clusters developed by RAMPD technique and the groups obtained by the analysis of principal components (Figure 2), *i. e.* the joining among genotypes were similar in both procedures. This fact is very important because it was possible to establish a genotype conglomerate for the higher or lower quality protein expression.

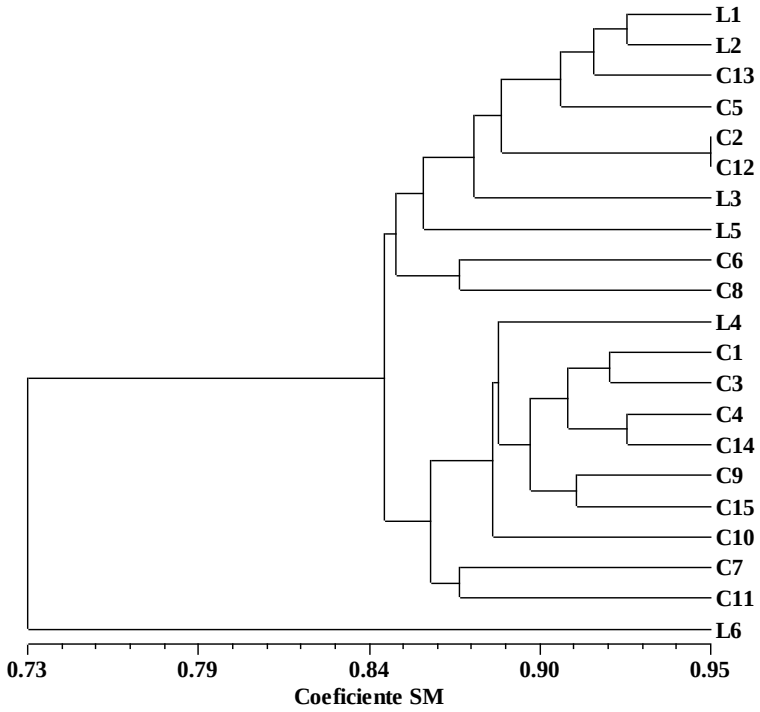

Figure 1. Dendrogram obtained for the genotypes studied. Dendrogram generated from basic database (MBD) obtained for anchored amplification of polymorphic DNA (RAMPD) of parental lines and crosses F_1, using the coefficient of concordance simple (SM) and the average cluster method (UPGMA.

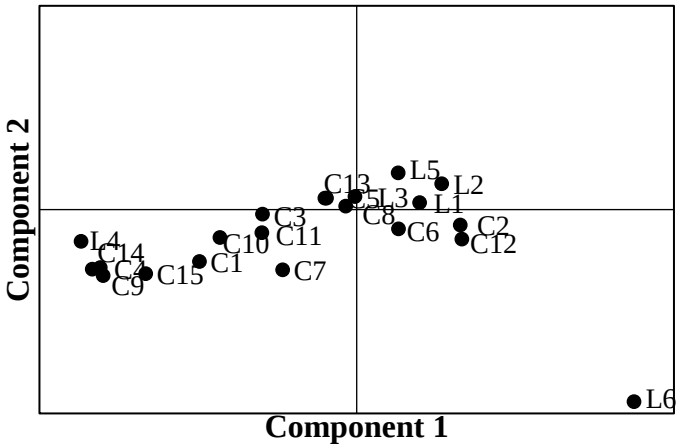

Figure 2. Principal component analysis projection from the covariance matrix for anchored amplification of polymorphic DNA (RAMPD), for parental lines and F1 crosses.

Conclusions

It was founded a close relation among quality protein parameters (lysine, tryptophan, total protein, and quality protein index) and the genetic characteristics, all of them was showed by analysis of component principals.

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BIOTECHNOLOGY SUMMIT

Assessment of abiotic stress on microorganisms isolated from the Yaqui Valley

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Abstract

The purpose of the study was analyze the response of microbial activities in two different soil samples, evaluating the effect of agricultural activities and environmental parameters on soil capabilities. One sample corresponds to soil designated to oregano cultivation and the other soil, from the Yaqui Valley, Sonora. The objective of this study was estimate the microbial species diversity of both soil samples, evaluate those under three scenarios: temperature, salinity and pesticide resistance, and determine their potential as plant growth promotion bacteria (PGPB) by metabolic test. The bacteria population was $5.9x10^6$ and $3.8x10^6$ CFU/g dry soil, native and oregano soil respectively. A total of twenty-four bacteria and one actinobacteria, were isolated. Native soil microorganisms presented better results in the fungicide tolerance and indole acetic acid (IAA) production assays; however temperature stress, salinity resistance, siderophores production and phosphates solubilization agricultural soil microorganisms showed better results. This prove the impact of changing the land use from native to agriculture, in ecofunctional terms.

Keywords: Soil microbiology ●land change● agriculture.

Introduction

The Yaqui Valley is located in the northwest coast of Mexico, in Sonora State. It consists of a 225, 000 hectares (ha), approximately 49% is dedicated to agriculture. The principal crops cultivated in Yaqui Valley are: wheat (177,719 ha), maize (13,734 ha), safflower (13,272), chickpea (9,103), and a small area dedicated to spices, such as oregano with a 3.4 ha. (Villasenor, 2012). Agriculture practices affect the base of its own future through soil's disrepair, salinization, water extraction excess and agricultural genetic diversity reduction (FAO, 2004). Additionally, there are different abiotic parameters such as: a) temperature raise, b) salinity and c) pesticide resistance, which directly rely on the crop production and maintenance. Ashour and Al-Najar (2012) evaluate the effect of temperature increasing on olive, palm, grape, citrus and guava determining that a higher temperature water and soil salinity increase and this could be associated with a higher irrigation demand. It is estimated that temperature could raise about three degrees by 2050 Rowlands *et al.*, (2012). Furthermore, if salinity raise in agricultural soils, the productivity will decreases in a 35% approximately (Lavado, 2008).

Moreover fungicide resistance of plant pathogens can be an important factor in the disease management (Forbes, 2001) and also could affect roots colonization and growth (Channabasaya *et al*., 2015). Under these scenario, the use of plant growth promotion microorganism (PGPM) could be a promising alternative to diminish the fungicide dependency and reduce the environmental impact and enhance crop production (Garcia 2012). The objective of this study was to estimate the microbial species diversity in two soil samples: oregano cultivation and native soil, evaluate them under three scenarios: temperature, salinity and pesticide resistance, and then determinate their potential as plant growth-promoting bacteria (PGPB) by metabolic test.

Materials and methods

Sampling site. Oregano cultivation and native soil samples, were collected at two different sites: a) Cocorit, Sonora (27.578837N,-109.958887W) and b) Corral station in Cajeme, Sonora (27.623670N, -109.965773W), respectively. Following the methodology reported by Ferraris (2016). The samples were kept in bags for less than 24 hours before their use.

Bacteria isolation. In order to assess the bacteria diversity, the microbial communities isolation was conducted using the serial dilutions (1:10) method until 10^{-6}. Then .1 mL of the 10^{-3}, 10^{-4} and 10^{-5} dilutions were inoculated on petri dishes containing nutrient agar (NA). Inoculated petri dishes were incubated for 24 h at 28 °C. Then colonies were isolated based on their micro and macroscopic traits characterized (colony morphology, color, growth and gram stain).

Temperature stress assay. The isolated microorganisms were inoculated in petri dishes with NA and incubated at 28 °C and 32 °C for 24 hours. The assay was performed using two independent replicates.

Fungicide resistance assay. The ability of the isolated strains to tolerate fungicide active ingredient chlorothalonil 720 was evaluated on NA petri dishes supplemented with the fungicide at a concentration of 5.4 gr/L of NA, microorganisms were inoculated in the medium then petri dishes were incubated at 28 °C for seven days.

Salinity resistance assay. The isolated microorganisms were inoculated on NA petri dishes supplemented with sodium chloride (30% equivalent to 40.8 dS/m). Petri dishes were incubated at 28 °C for six days.

Siderophores production. Microorganisms isolated were inoculated in Chrome Azurol S (CAS) petri dishes (Schwyn and Neilands, 1987) and incubated at 28 °C for 5 days.

Phosphate solubilization. The microorganisms were inoculated in Pikovskaya (PVK) medium and incubated at 28 °C for 5 days (Alvarez and Garcia, 2012).

Indole acetic acid (IAA) production. The microorganisms were inoculated in 25 mL of nutrient broth supplemented with L-tryptophan (100ppm) and incubated at 28 °C for six days in constant agitation 180 rpm. The determination of indole acetic acid production was done following the methodology reported by Lara *et al.* (2011).

Results and discussion

The bacteria population was $5.9x10^6$ and $3.8x10^6$ CFU/g dry soil, for the native and oregano soil respectively. A total of twenty-four bacteria and one actinobacteria, strains were isolated. 50% of the bacteria strains were gram-positive with coccoid and bacillary morphology. Strains showed an optimal growth at 28 °C and 32 °C, however at 32 °C, the strains grew faster and colony color variation were presented (Table 1), Ramos and Zuniga (2007) found similar results with lima beans in a temperature stress assay, microorganisms showed better growth at higher temperature. Only one strain from agricultural soil, OSM12, from the agricultural soil, did not resist the temperature stress.

Only the strains isolated from native soil (16%) were able to grow in culture medium supplemented with 5.4 g L Chlorothalonil (Table 1). Šantavec and Kocjan (2011) showed different results in crops of wheat, demonstrating the lack of variation in organic and conventional farms. Currently it is estimated that the environmental degradation processes and climate change, will raise the salinity levels in agricultural soils (IPCC, 2007). Maas and Hoffman (1997) suggested an alternative to deal with this threat by pursuing salinity tolerant crops, these include: barley, cotton, beet, wheat, etc. In this study, eight microorganisms were tolerant to 30% of NaCl (Table 1) becoming an attractive alternative to future agriculture application in crops based on their salinity concentrations. In strains isolated from native soil, only one strain produced siderophores (VNS14) however they showed a great efficiency in the phosphates solubilization, nonetheless microorganisms from the agricultural soil showed excellent results in both tests (Table 1). The IAA production were higher for the native soil microorganisms with a IAA production range from 40 and 65 ppm (Figure 1) while in agricultural soil the IAA production were lower (01 and 15 ppm). The VNS8 strain produce 65.346 ppm, being the higher concentration of IAA obtained. The results from the metabolic assays proof that the microorganisms collected from different soils, present the capacity of solubilize phosphates, produce siderophores and Indole acetic acid, becoming in excellent microorganisms to be used in biotechnological researches and use them to improve the agriculture.

Conclusion

The results presented in this study were diverse, native soil microorganisms presented better results in the fungicide tolerance and IAA production assays than the agricultural soil; however in temperature stress, salinity resistance, siderophores production and phosphates solubilization the agricultural soil microorganisms showed better results. Demonstrating that microorganisms isolated from the agricultural soil showed a better response to the abiotic stress and the metabolic assays, becoming a great potential growth-promoting bacteria.

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Table 1. Results to the tests applied to the 25 microorganisms found in both samples of soil.

Strain	Gram stain	T Effect (32°C)	Salinity test	Siderophores production	Phosphates solubilization	Fungicide test	IAA production
OSM1	-	NG	+	-	+	-	Low
OSM10	+	NG	-	-	+	-	Low
OSM11	+	NG	+	-	-	-	Low
OSM12	-	LG	-	-	-	-	Low
OSM13	-	NG	-	-	+	-	Low
OSM14	+	NG	+	+	+	-	Low
OSM2	+	NG	-	+	+	-	Low
OSM4	+	NG	-	-	-	-	Low
OSM8	+	NG	-	-	+	-	Low
OSM9	+	NG	+	-	+	-	Low
VNS1	-	FG	+	-	-	-	Low
VNS10	-	NG	+	-	-	-	Low
VNS11	+	NG	-	-	+	-	Low
VNS12	+	NG	-	-	-	-	Low
VNS13	-	NG	-	-	-	+	High
VNS14	-	NG	-	+	-	-	Low
VNS15	+	FG	+	-	-	-	Low
VNS2	-	NG	-	-	-	-	Low
VNS4	-	NG	-	-	-	+	High
VNS5	-	NG	-	-	-	-	Low
VNS6	-	NG	+	-	+	-	Low
VNS7	+	NG	-	-	+	-	Low
VNS8	+	NG	-	-	+	-	High
CSGE12	-	NG	-	-	-	+	High

(+) Showed resistance to the assay. (-) Didn't show resistance to the assay. (NG) similar growth at 28°C. (FG) faster growth than at 28°C (LG) didn't resist the stress. ● Microorganisms from the agricultural soil, ● Microorganisms from the native soil. (Low) ≥ 30ppm, (High) < 30ppm.

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BIOTECHNOLOGY SUMMIT

Population genetics of two cattle pests, *Haematobia irritans* and *Musca domestica* in Mexico

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Abstract

We sequenced 650 base pairs of the mitochondrial cytochrome oxidase I gene in two species of cattle pests in Sonora, Mexico. A sample of 24 *Musca domestica*, a common pest at pig farms showed considerable nucleotide sequence variability for this gene, while 16 *Haematobia irritans*, the cattle horn fly, exhibited no variation. Additional localities are being sampled to determine levels of gene flow at different geographic scales for these two pests.

Keywords: Population genetics●mitochondrial CO1●*Haemotabia irritans,* ● *Musca domestica*● Sonora.

Introduction

Two species of Diptera are serious pests in the cattle industry in Mexico. The horn fly *Haematobia irritans* causes serious blood loss and reduction in milk production in beef and dairy cattle, while *Musca domestica* is a major pest of pigs and vector of diseases such as infectious anemia (Prullage *et al.* 1993) and hemoplasmosis (Hornok *et al*. 2011). While considerable information exists about the reproductive biology of the two species, virtually nothing is known about their effective population sizes, their levels of genetic variability, or their dispersal and gene flow between ranches and farms located at various distances from each other. This basic knowledge is fundamental to the development of successful eradication or control programs for these two pests. We have undertaken an investigation of genetic variability in the two species and report here preliminary results.

Materials and methods

Collection of specimens for sequencing: Horn flies were collected directly from cows, using nets and aspirators from Rancho Los Cuatro, Navojoa, Sonora. The same methods were used to collect houseflies from pigs at a neighboring farm in Navojoa. Flies were placed in 95% ethanol and transported to the laboratory at LANGEBIO-CINVESTAV, Irapuato, Guanajuato.

DNA sequencing: Total genomic DNA was isolated from individual adult flies using the procedure described in the DNeasy® Blood and Tissue Kit (Qiagen). Whole flies were used for DNA extraction. The mitochondrial gene mtCOI (cytochrome oxidase subunit 1) was used for polymerase chain reaction (PCR) and sequencing: Primers are shown in Table 1. PCR amplifications for mtCOI were performed using the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30sec, 52°C for 45 sec, 72°C for 1 min and 72°C for 7 min. Purified PCR products (QIAquick® PCR Purification Kit, Qiagen) were sequenced (Sanger sequencing, Genomic Service, LANGEBIO) and obtained sequences were aligned and

corrected with Geneious® software. Calculations of genetic diversity indices were performed in DnaSP version 5.10.01 (Librado and Rozas 2009).

Table 1. Forward and reverse primers for mtCO1. The amplified COI fragment (658 bp) (Ratnasingham and Hebert 2007).

Gene	Primer	Sequence (5´- 3')	Reference
mtCOI	LCO1490-F HCO2198-R	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAAT	Folmer *et al*. 1994

Results and discussion

The two species from the same locality in Sonora differ in their levels of variability in mtCO1 (Table 2). While *M. domestica* shows considerable diversity, with four haplotypes in 24 individuals, only one haplotype was found in 16 *H. irritans.* A larger sample from the same locality may reveal additional haplotypes in *H. irritans*, but it is unlikely that there is sufficient variability in mtCO1 gene for studies of population differentiation and gene flow. Our next step is to sequence different loci to identify those that will be more informative to examine demographic history and gene flow. An earlier study in Brazil using RAPIDs, an older resolution method, suggested that gene flow and thus dispersal is limited in *H. irritans*. If this is the case in Mexico, *H. irritans* may be suitable for more local control or eradication strategies. Only by identifying more informative loci and sequencing them across various spatial scales, will we know if this is true for *H. irritans* in Mexico.

Table 2. Genetic variability in mtCO1 in *M. domestica* and *H. irritans* from Navojoa, Sonora, Mexico.

Species	*n*	*k*	*K*	*h*	*π*
M. domestica	24	4	4	0.598 ± 0.057	0.001 ± 0.000
H. irritans	16	0	1	-	-

n, number of individuals; *k*, number of variable sites; *K*, number of haplotypes; *h*, haplotype diversity; *π*, nucleotide diversity.

For *M. domestica*, the variability revealed in our sequences of mtCO1 demonstrate that this locus will be sufficiently informative to examine gene flow and dispersal within Mexico at contrasting spatial scales. We expect to see high levels of genetic variability in *M. domestica* as it is a host generalist. Previous studies of genetic differentiation in *M. domestica* have been conducted on a global scale, but we will be the first to examine population structure using more modern molecular approaches. We also will obtain the first data on genetic variability and gene flow within Mexico. For both fly species, we will obtain samples from more distant ranches in Sonora as well as from states in the central, eastern and southern Mexico.

Conclusions

Mitochondrial CO1 is more variable in *M. domestica* than in *H. irritans* in a population in Sonora, Mexico.

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Litter decomposition of an ecological succession site in a tropical dry forest

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Abstract:
Litter decomposition is a key process in the carbon cycle, this process is accomplished by macro- and microorganisms. The decomposition rate depends upon the microbial population and is controlled by soil conditions (e.g. soil temperature and soil moisture). The decomposition process is modified by anthropogenic activities. Therefore, we determined the litter mass loss, decomposition rate, and microbial population associated with this process, also, we measured soil temperature and moisture for an ecological succession in a tropical dry forest (TDF). The four sites are: a mature TDF (Md), a secondary TDF (Sc), a cultivated site that has been abandoned (Ab), and a site dominated by *Acacia cochilacantha* (Ac) located in the northwest of México. The litter mass loss in the mature and abandoned forest was significantly larger than the secondary and Acacia cochilacantha sites [54.35±7% (Md), 61.88±10 % (A) vs 42.4±15% (S), 42.34±9 %(Ac)]. The decomposition rate was significantly different for each of the study sites, where the abandoned site had the highest [0.002036±6.5x10-5 (Md), 0.001430±8.3x10-5 (Sc), 0.003186±1x10-4(Ab) and 0.001526±6.6x10-5 (Ac)], probably due to its better litter quality and higher microbial population. The microbial population increased when the soil moisture was higher and this increase continued through the early-dry season (November-January).

Keywords: decomposition rate ● microbial population ● carbon cycle ● sierra de Alamos

Introduction:

Tropical dry forest (TDF) constitutes about 42% of tropical forests worldwide and approximately 11.26% of all Mexican land cover (Becknell, 2012; CONABIO, 2016). This ecosystem can store and incorporate significant amounts of carbon (C), having a great influence on the global C cycle (Jaramillo et al. 2011; Bejarano et al. 2014). The C dynamics within TDF are controlled by two factors: seasonality of rain and disturbances associated with human activities (Jaramillo *et al.* 2011; Anaya *et al.* 2012; Becknell, 2012).

Decomposition of plant material (where senescent plant material is degraded and consequently incorporated) and nutrient release of leaf litter, are key processes to ensure the proper functioning of the C cycle and to improve the condition of the soil (i.e. physical, chemical and biological) (Yahdjian *et al.* 2006; Pérez-Suárez *et al.* 2012; Austin *et al.* 2014). Three factors control the litter decomposition: the climate (i.e. temperature, soil moisture, and precipitation), the leaf litter quality, and the abundance of decomposers (macro and microorganisms) (Rocha-Loredo and Ramírez-Marcial, 2009; Anaya *et al*. 2012), where microorganisms play an important role and are key elements in this process (Díaz-Raviña, 1993; Gomashe *et al.* 2013).

In México, the TDF is the ecosystem most threatened by anthropogenic activities (i.e population growth, agricultural activities, leading to the formation of successional sites within these ecosystems, modifying the dynamic and function of the C cycle in the soil (Álvarez-Yépiz *et al.* 2008; García-Oliva and Jaramillo, 2011; Anaya *et al.* 2012).

In this context, with the purpose of understanding the C cycle in the soil of successional sites within a TDF in northwest México, we have the following objectives: i) to estimate the mass loss of leaf litter, ii) to calculate the decomposition rate, iii) t) to quantify the microbial population associated with the litter decomposition process at each site.

Materials and Methods:

Study area

The study was conducted in a tropical dry forest (TDF) in "Guayabo " ranch, within the Area de Protección de Flora y Fauna Sierra de Álamos-Río Cuchujaqui (APFF-SARC) localized in southwest Sonora, Mexico (26° 52' 48'' to 27° 09' 00'' N, 108° 34' 12'' to 109° 04' 48''W). The landscape is composed of plains, mountains, and valleys (Arriaga, 2000). The soil has been classified as lithic leptosols and haplic phaeozem (CONABIO, 2016). Mean annual temperature is 24.3°C, and the mean annual rainfall is 600 to 700 mm (Álvarez-Yépiz *et al.* 2008). Rainfall is strongly seasonal with two seasons: wet (June – October) and dry (November – May) (Anaya *et al.* 2007).

The study sites were selected according to the pattern of ecological succession within the TDF. We selected a mature TDF (TDF-Md) that has remained devoid of human activities, conserving representative vegetation of TDF: *Lysiloma divaricatum Jacq*, *Croton flavescens Greenm*, *Pachyecereus pecten-aboroginum briton* and *rose. The litter production* is 4.03 Mg ha-1 year-1 and the soil organic matter (SOM) content is 5.5±0.2%. The second site we selected is a secondary TDF (TDF-Sc) that has 30 years of succession; the litter production is 2.34 Mg ha-1 year-1. Our third study site is dominated by *Acacia cochliacantha* (TDF-Ac) and is considered an early succession. The litter production at the Acacia site is 3.66 Mg ha-1 year-1 and the SOM content is 4.2±0.1%. The final site is a site impacted recently, (~7 years) and abandoned (TDF-Ab), the litter production is 3.66 Mg ha-1 year-1 and SOM is 2.8±0%. In the final two sites (Ac and Ab), the abundant species are *Mimosa spp*., *Croton flavesens Greenm*, *Desmanthus bicornutus S. Watson and Lysiloma divaricatum* (Sandoval-Aguilar *et al.* 2014; Villanueva-Hernández, 2015; Nevescanin-Moreno *et al.* 2016).

Field design

A central point was determined for each site; four 5 m x 5 m plots were located at each site, 60 m distance from the central point in each cardinal direction N, S, E and W. At each plot, 24 litterbags were buried for a total of 96 per site. The collection of bags was performed after 86, 207, 297 and 365 days of being buried, in each collection six bags were taken.

Litterbag preparation

Leaf litter was collected at each site and was dried at approximately 65°C for 72 hours in a convection oven. Subsequently, 10±0.5 g of litter was placed in a 20 x 20 cm bag (polyester fabrics; 0.5 mm mesh size) and each bag was closed and labeled.

Mass loss

After each litterbag was collected, they were transported to the laboratory and we used a 2 mm-mesh size to remove foreign materials from the litter (i.e. soil particles, rocks). A sample of 1±0.5 g of litter was separated, and the rest of the litter was dried at ±65°C for 72 hours to determine the dry mass this process was repeated for each collection.
The mass loss was determined based on the following equation:

$$ML\% = \frac{X_t}{X_o}\ x\ 100 \qquad \text{Equation 1}$$

where Xt= the leaf-litter mass remaining at time t (g); X0= the initial leaf-litter mass (g).

Decomposition rate

The decomposition rate was estimated by a simple negative exponential model (Olsen, 1963).

$$\frac{X_t}{X_0} = e^{-kt} \qquad \text{Equation 2}$$

Where: t= time (days); k= decomposition rate.

Microorganisms

The cultivatable microbial population associated with the decomposition process was determined through colony-forming units (CFU). We used the litter extracted (1±0.5 g; 30 g per site each collected) for the serial dilutions. The medium used for the growth and isolation of microorganisms was that proposed for Gomashe *et al*. (2013). For this study, we replaced the synthetic carbon source (peptone and carboxymethylcellulosa) with litter from each site. The serial dilutions used were 10^{-3}.

Soil moisture, soil temperature and precipitation

The soil moisture (%) and soil temperature (°C) were measured monthly (March 2015 to March 2016) with a Theta Probe ML2x, Delta Device (Cambrige, U.K.) soil moisture sensor and a Thermocouple Thermometer (Barnant Co., Barrington, IL, USA). The precipitation was measured at a meteorological station located in TDF-Sc.

Statistical analysis

To test for differences in soil moisture, soil temperature, cultivatable microbial population, and mass loss, a Kruskal-Wallis model was applied using site and month as parameters. A one-way ANOVA model was used to test for differences in decomposition rate between sites. Post hoc multiple mean comparisons were carried out with a Tukey's test using a significance level of $\rho<0.05$. All statistical analyses were conducted using SigmaPlot 12.0 (Systat Software, Inc., San Jose, California, USA) and Statgraphics plus 5.0 (Statpoint Tecnologies, Inc., Warrenton, Virginia, USA).

Results and Discussion:

Soil moisture range sites was: 13.40±0.80 to 18.42±1.4% (TDF-Md), 4.11±0.9 to 15.25±1.7 % (TDF-Sc), 4.11±0.9 to 14 ±1.63 % (TDF-Ac) and 5.74±0.8 to 22.19±1.9% (TDF-Ab), while the ranges for soil temperature were: 24±0.2 to 34.49 ±0.5 °C (TDF-Md), 18.35±1.8 to 46.9±1.7°C (TDF-Sc), 21.26±0.7 to 40.49±0.7 °C (TDF-Ac) and 13.38±1.3 to 32.13±0.6 °C (TDF-Ab). The behavior for soil moisture is descended in the months where rainfall is scarce or absent and increases in the months with the presence of rain, a

reverse pattern is presented for the soil temperature increases in the months with little or no rainfall and decreases in the months where rains.

By the end of our study period, leaf litter was not totally decomposed (table 1). In all sites, the mass loss process was negligible throughout the first 86 days, but increased significantly after the beginning of the wet season. TDF-Sc y TDF-Ac show a similar pattern of mass loss: the mass loss increases during the rainy period (87-207 days), changes to a resting state after the rain (208-297 days), and finally increases again (298-365 days). At the other two sites, there is no difference in the final loss, but there is in their behavior. The TDF-Md had constant decomposition from the first to the last day, under all environmental conditions; however, in the TDF-Ab the mass loss was activated after the first rain, but once started, had a higher rate.

Table 1. Litter decomposition in each site of sucesional ecology in a tropical dry forest of the Sierra de Álamos, Sonora, México.

	TDF-Md	TDF-Sc	TDF-Ac	TDF-Ab
Mass loss (%)	46±1.99 a	57±4.12 b	58±2.25 b	41±2.29 a
Decomposition rate (*k* year^{-1})	0.002036±6.5x10^{-5} b	0.001430±8.3x10^{-5} d	0.001526±6.6x10^{-5} c	0.003186±1x10^{-4}a
Microbial population (UFC g^{-1} leaf litter)				
dry season (March 2016)	5.92x10^{5}±3.76x10^{4}	5.88x10^{5}±1.60x10^{5}	4.62x10^{5}±1.82x10^{5}	2.80x10^{5}±1.63x10^{4}
wet season (October 2015)	4.55x10^{5}±8.39x10^{4}	1.34x10^{6}±1.54x10^{5}	4.48x10^{5}±2.25x10^{4}	4.72x10^{5}±4.36x10^{4}

Notes: Values are means ± SE. Lowercase letters indicate significant difference among site ($\rho<0.05$).

In all sites, soil temperature decreases and soil moisture increases when rainfall occurs. The total rain was 673 mm year^{-1} with 95% of that (638 mm) occurring during the rainy season (June to October). Across all sites, the microbial population associated with the litter decomposition rose during the wet season, but the TDF-Sc presented distinct differences in the quantity and the trend (table 1).

According to Petersen and Cummins (1974) classification, decomposition rates for all sites are slow (k less than 0.005). The k value in this study are less at the range of 0.008 to 0.615 values reported for TDF (Berg and McClaugherty 2008).

The climate has a significant effect on the decomposition and can be a limiting factor on microbial activity (Cuevas *et al.* 2013). In this study, precipitation was a factor controlling the decomposition process by creating favorable conditions of temperature and soil moisture where microbial decomposition is carried out, this result is similar to other studies of this process (Castellanos-Barliza and León, 2010; Celantano *et al.* 2011; Schilling *et al.* 2016).

Microbial population and activity are key in controlling litter decomposition (Austin *et al.* 2014). In this study, the microbial population was highest in the TDF-Sc site for both seasons; however, this site did not have the greatest mass loss and the decomposition rate was the lowest. In contrast, TDF-Ab had greater mass loss and decomposition rate was higher, this can indicate that microorganisms' ability to decompose is different among sites. In previous work, Sandoval *et al.* (2014) determined a high ability to degrade carbon (i.e. cellulolytic activity) by TDF-Ab, while the TDF-Md had lower cellulolytic activity; it presented different methods of degrading complex forms of carbon. This result suggests that the quality of litter is important and can be different in different study sites; in sites that

are located in a successional gradient the quality litter changes in accordance with forest age, where at an earlier stage the litter can be more labile and less diverse (Schilling *et al.* 2016).

Conclusion:

Decomposition process are different in diferent sucessional stages, not lose the capacity to respond to functional process. This result demonstrates that climate, microorganisms, and availability of litter exert direct or indirect control on the decomposition dynamics. Microorganisms obtained are fundamental to begin further research and identification of specific functional groups.

It is important to continue with studies of the factors influencing the ecosystem functional processes in order to understand and create proposals that benefit these ecosystems (i.e. bioremediation, reforestation).

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A new collection of native soil microorganisms with potential agro-biotechnological uses: COLMENA

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Abstract:
COLMENA (www.itson.mx/COLMENA) is a culture collection specialized in the preservation, classification, characterization, and transferring of native microorganisms isolated from different agro-systems, and other habitats. The aim of this collection is to decrease the loss of microbial diversity associated to land-use changes. So far we have isolated soil microorganisms from different agro-systems, the Yaqui Valley and the Fuerte Valley, Mexico. Until now, COLMENA preserves about 1,464 soil microbial strains associated to several crops, such as: wheat (448), maize (313), bean (35), and others. Additionally, we have developed the taxonomic classification of 353 strains, by molecular techniques, being *Bacillus* (27%) and *Aspergillus* (8%) the most abundant genera of bacteria and fungi, respectively. Three percent of total microbial collection produced high level of indole acetic acid (>5 mg/L), while siderophore production and phosphorus solubilization was observed in 61% and 36% of 396 tested strains, respectively. Three percent of the whole collection has been identified as lytic enzymes producers and 3% showed β-hemolysis. These results show the versatility of these microbial strains as cost-effective alternatives in agro-industrial practices, focused on contributing to the global food safety, through establishing successful collaborations among scientist and industrial sectors.

Keywords: COLMENA ● agriculture ● culture collection

Introduction:
Microorganisms provide critical ecosystem services that keep our planet habitable, and their economic potential is limitless, including: biomolecules production, bioremediation, bioenergy and biofertilizers (Prakash *et al*. 2013). The total microbial diversity in soil comprise up to 10^5 species (Dohrmann *et al*. 2013), however, only a minor fraction (1 – 10%) has been cultured (Prakash *et al*. 2013). Nevertheless, the metabolic potential can only be truly verified in studies with cultivated organisms, avoiding changes in their phenotypic and genotypic traits (Prakash *et al*. 2013; Overman *et al*. 2015) These strains can be used to generate goods and services for the agro-system. Thus, the isolation, characterization, and preservation of soil microbial strains is mandatory for future references, researches and new discoveries.
In this way, culture collections -genetic stock centers- have several goals in common: i) obtaining microbes with scientific, ecological, commercial values, or other, iii) validating their taxonomic, genetic, phenotypic, morphological properties, or other, iii) preserving

microbes under appropriate conditions to maintain them for future generations, and iv) distributing strains to researchers (Boundy-Mills *et al*. 2015). It is estimated that only a very small fraction (approximately 1%) of strains obtained in research laboratories are preserved in public collections (Overmann *et al*., 2015). According the World Federation for Culture Collection (WFCC, http://www.wfcc.info/), there are 708 culture collection around the world; approximately 2.5 million of strains are preserved, of which, the 15% are associated to soil and plants. México represents only 0.4% of total microorganisms preserved worldwide, thus is necessary the development and establishment of new culture collections that allow the storage and study of native strains at the national level.

In this context, emerges COLMENA (Native Endophytes and Soil Microorganisms Collection, www.itson.mx/COLMENA), with the purpose to create a microbial collection specialized in the preservation, classification, characterization, and transferring of native microorganisms, with the aim of diminish the microbial soil degradation. These preserved strains could be used to enhance plant health and promote plant growth through different mechanisms, such as, i) enhance plant nutrient uptake, ii) production of phytohormones and iii) biological control agents (Vejan *et al*. 2016; Trabelsi and Mhamdi, 2013).

Materials and methods:

Sampling and microbial isolation. The most important agricultural zones in northeast México were sampled in this first stage: the Yaqui Valley in the State of Sonora (principal producer of wheat in México (Ahrens *et al.*, 2008)), and the Fuerte Valley in the State of Sinaloa. Sixty seven soil samples (0 - 30 cm) associated to different crops were collected in these places according to SAGARPA (2013). The strain isolation was realized by serial dilutions technique, the culture media used was Nutritive Agar with 80 ppm of terbinafine for the isolation of bacteria and Potato Dextrose Agar with 80 ppm of nalidixic acid for isolation of fungi. Pure cultures of each strain were cryopreserved with 70 % glycerol to -80° C.

Molecular characterization. Genomic DNA from the isolated microorganisms was extracted according to Reader and Broda (1985). Bacterial diversity was estimated by 16S rRNA gene sequencing and 5.8S rRNA for fungal diversity. For bacteria the amplification was done using the primers FD1 and RD1, the PCR consisted of an initial denaturation step at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 40 s at 57 °C and 2 min at 72 °C, and a final elongation step of 5 min at 72 °C. For fungus, the primers ITS1F and ITS4R was amplified, the PCR consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and a final elongation step of 10 min at 72 °C. PCR products were verified by 2% agarose gel electrophoresis, purified and sequenced by Sanger platform. Sequences were compared and deposited in the NCBI Genbank.

Metabolic characterization. The isolates were characterized under several biochemical traits, focused on promoting plant growth, and biocontrol, *i.e.* production of indoles (de los Santos *et al*. 2013), phosphate solubilization (Onyia *et al*. 2013), siderophores production (Alexander *et al*. 1991), and cellulolytic activity (Gomashe *et al.* 2013). Additionally, members of this microbial collection were tested for their capacity to produce hemolysis (Figueroa-Lopez *et al*. 2016). Strains were positive for indoles production when concentration was > 5 mg/L AIA, siderophores production, phosphate solubilization, and cellulolytic activity were positive when the halo was >0.5 cm, and the hemolysis indicates β-hemolysis.

Results and discussion:

Until now, COLMENA preserves about 1,464 soil microbial strains associated to several crops, such as: wheat (448), maize (313), bean (35), broccoli (51), alfalfa (54) and others. The molecular identification of 24% of COLMENA strains, shows that 28 bacterial genera were found, being the most abundant: *Bacillus* (27%), *Pseudomonas* (8%), and *Stenotrophomonas* (6%) (Figure 1). While the predominant fungi genera (24 genera found) were *Aspergillus (8%)*, *Penicillium* (3%), and *Myrothecium* (3%) (Figure 2).

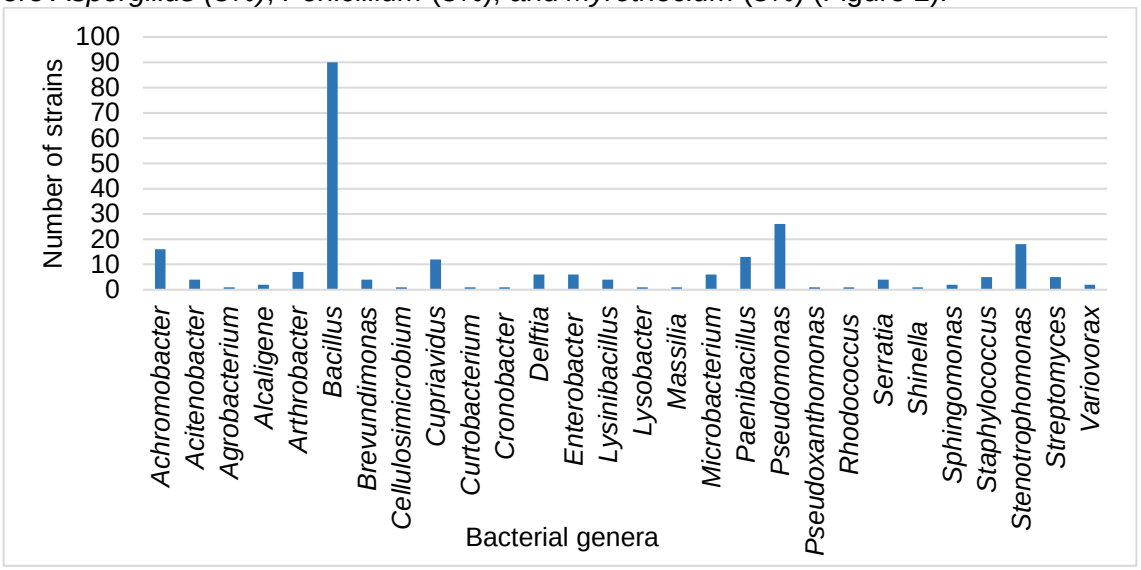

Figure 1. Bacterial diversity preserved in COLMENA.

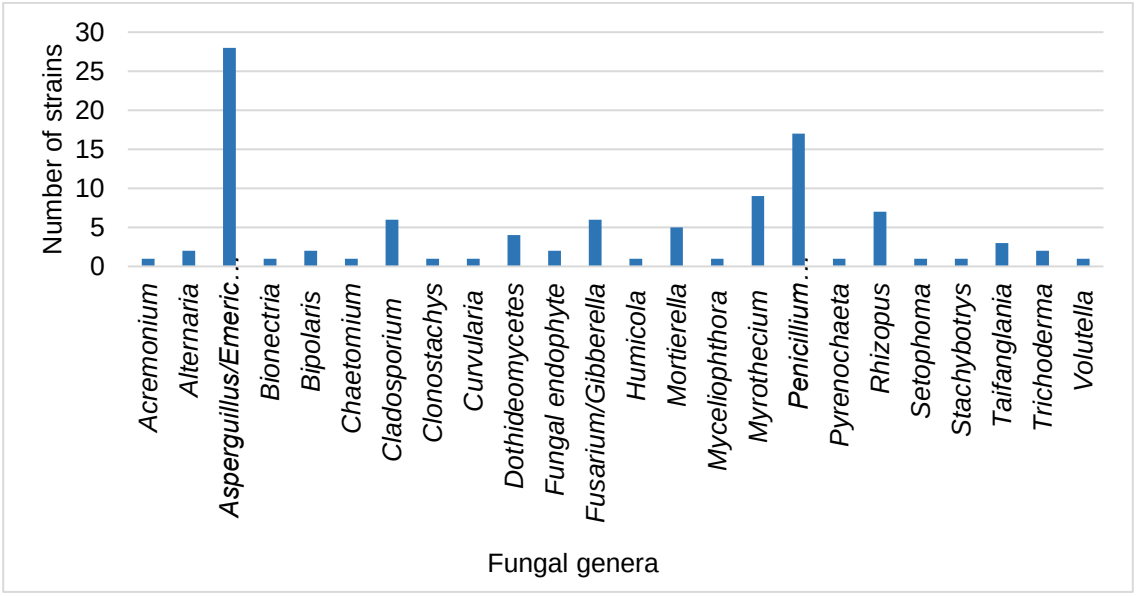

Figure 2. Fungal diversity preserved in COLMENA.

COLMENA preserves some genera strains previously identified like promote plant growth microorganisms, such as, *Bacillus*, *Pseudomonas*, *Stenotrophomonas* and *Trichoderma* (Mahmood *et al*. 2014), it suggests the potential on the native strains of Northeast of México to be included in strategies to implement the sustainable agricultural in the area. Moreover, the microbial collection included genera such as *Aspergillus, Bipolaris,* and *Fusarium,* which mostly have been reported as plant pathogens (Pal and McSpadden, 2006), the research about these strains allows the strategies development for biocontrol, and the potential reduction of pesticides in the field.

According to the metabolic characterization (Figure 3), the phosphorus solubilization and siderophore production, mechanisms of plant growth promotion and biocontrol, were observed in 36% and 61% of the tested strains, respectively. In addition, only 3% of this microbial collection produced high level of indoles (>5 mg/L), these phytohormones are related with the increased root length (Grover *et al*. 2011). So far, 3% of the whole collection has been identified as lytic enzymes producers, suggesting these strains like potential biocontrol agents (Ahmad *et al*. 2008) and 3% of strains the total collection showed β-hemolysis, therefore, is important evaluate its pathogenicity in humans (Figueroa-Lopez *et al*. 2016).

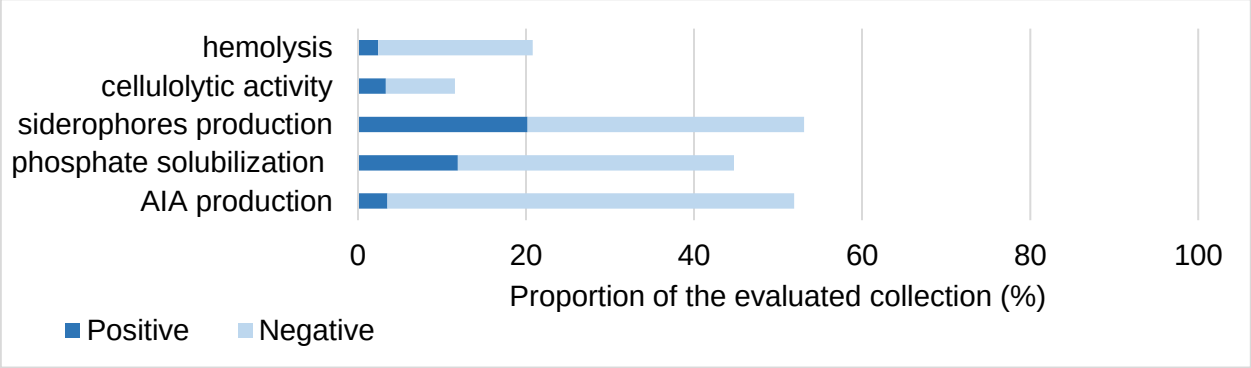

Figure 3. Metabolic characterization of COLMENA members.

In addition, we have identified three promising bacterial strains against *S. sclerotinia,* causal agent of white mold in common bean, nineteen against *Fusarium verticillioides,* causal agent of ear rot in maize, and fourteen against *Bipolaris sorokiniana,* causal agent of spot blotch in wheat.

Conclusions:

These results show the potential use of these microbial strains as agro-biotechnology alternatives focused on contributing to the global food safety, due to their high production on indoles, siderophores production, phosphate solubilization, and cellulolytic activity. However, it is necessary to complete the metabolic characterization of these strains in order to explore the whole application of them. Now, we are evaluating the entire microbial collection looking for other metabolic traits, such as: antibiotic production, stress tolerance (heat, hydric, salt), among others.

Acknowledge:
The autors acknowledge support by the Cátedras CONACyT Program through Project 1774 “Alternativas agrobiotecnológicas para incrementar la competitividad del cultivo de trigo en el Valle del Yaqui: desde su ecología microbiana hasta su adaptabilidad al cambio climático”, and the CONACyT Project 253663 “Fortalecimiento de la infraestructura del Laboratorio de Biotecnología del Recurso Microbiano del ITSON para la creación de COLMENA: COLección de Microrganismos Edáficos y Endófitos NAtivos, para contribuir a la seguridad alimentaria regional y nacional”.

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Research perspectives vs biosecurity in tropical and subtropical zone

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Abstract:
Biosecurity is defined as the set of preventive measures to maintain control of risk factors from biological, physical or chemical agents, achieving the prevention of harmful impacts on living beings and the environment, CDC 2016. The aim of this work is show some statistical disease caused from virus in tropical and subtropical region of Mexico and comparing two nonstructural proteins (NS3-NS2B) that have relevant importance in the replication of some virus such as, Chikungunya, Dengue, West Nile Fever and Zika.

Keywords: safety • preventions • virus • protease.

Introduction:
There is a lot of virus transmitted by vectors in tropical and subtropical zone, and other disease affecting the state of Oaxaca Mexico. We show some statistical data about the occurrence of diseases caused by these and other microorganisms, and the comparison of the sequence alignment to identify potential sites that are repeated in several cases of virus.

Materials and methods:
We were looking information about some diseases at Weekly Epidemiological Bulletin of Mexico (WEB, 2016) & GEDEON Informatics, Inc. 2016 and sequences of some virus at PubMed, Protein Data Bank and UniProt: Dengue Virus serotype 1 (DENV-1) protease 3L6P (Chandramouli *et al.* 2010), Dengue Virus serotype 2 (DENV-2) protease 2FOM (Erbel, *et al.* 2006) (Lei, *et al.* 2016), Dengue Virus serotype 3 (DENV 3) protease 3U1J (Noble, *et al.* 2012), Dengue Virus serotype 4 (DENV 4) protease 2WHX (Luo, *et al.* 2010), (Lavanya, *et al.* 2016), West Nile Virus (WNV) protease 2FP7 (Erbel, *et al.* 2006) (Lei, *et al.* 2016), Zika Virus ZIKV 5LC0 (Lei, *et al.* 2016), Chikungunya virus GenBank: EU372006 (Togaviridae, 2016). Code from UniProt: P17763, Q91H74, Q5UB51, Q2YHF0, P06935, Q1H8W5, A0A141ZVF6. We use MSA Viewer, JalView 2.9.0b2 program and UniProt viewer to analyze the sequence of proteins.

Results and discussion: Clinical findings by viral infection vectors in tropical and subtropical zone are shown in Table 1, we can observe the comparison in percentage.

Table 1. Comparison of clinical findings of some virus GEDEON Informatics, Inc. 2016.

Clinical findings	Chikungunya	Dengue	West Nile Fever	Zika
Neutrophilia	-	-	V	-
Relative bradycardia	-	V	-	-
Liver dysfunction	-	V	-	-
Arthritis	V	-	-	V
GI and intraabdominal - disease or dysfunction	-	-	V	-
Fever	+	+	+	V
Thrombocytopenia	-	V	-	-
Macules and / or papules	V	V	V	+
Skeletal muscle - muscles, bones and joints	+	+	+	V
Back pain	-	V	-	V
Neurological - headache, meningitis, etc.	-	+	+	V
Lung, chest, chest wall or heart	-	-	-	V
Neutropenia	V	V	V	-
Myalgia; muscle mass or swelling	V	+	+	V

+ >80%, V 20%-80%, — < 20%

In the figures 1 to 4 we can observe the global distribution of Chikungunya, West Nile Fever, Zika & Dengue disease. Data were downloading from GEDEON Informatics, Inc. 2016.

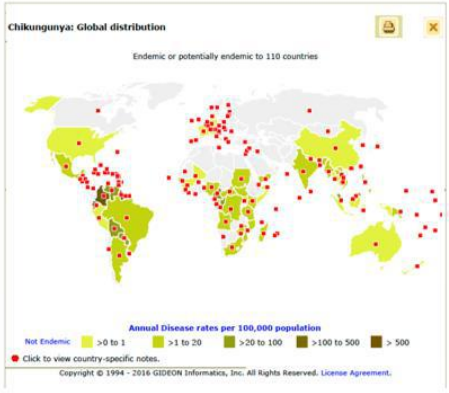


Figure 1. Global distribution of Chikungunya. We can see 110 endemic or potentially endemic countries.

Figure 2. Global distribution of West Nile Virus. We can see 91 endemic or potentially endemic countries.

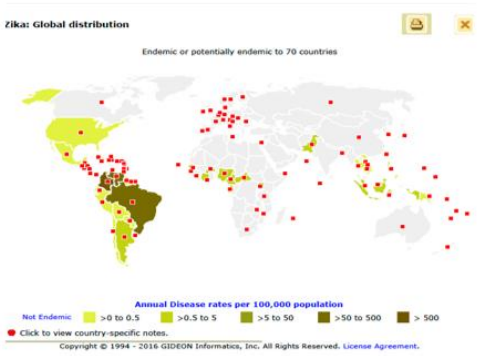


Figure 3. Global distribution Zika. We can see 70 endemic or potentially endemic countries.

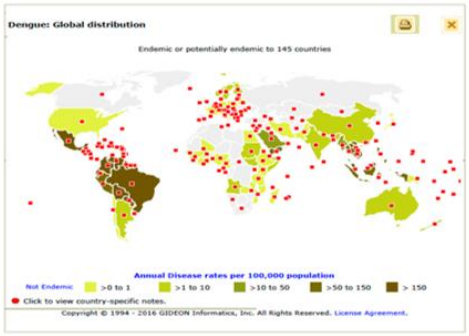


Figure 4. Global Distribution of Dengue. We can see 145 endemic or potentially endemic countries.

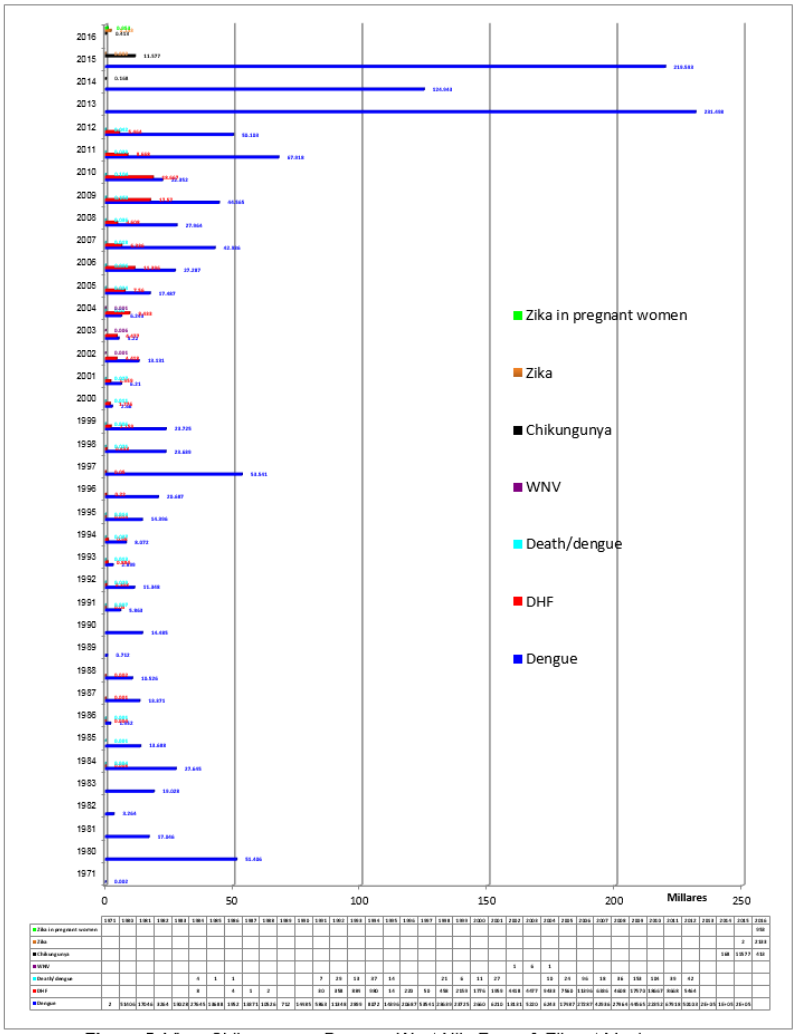
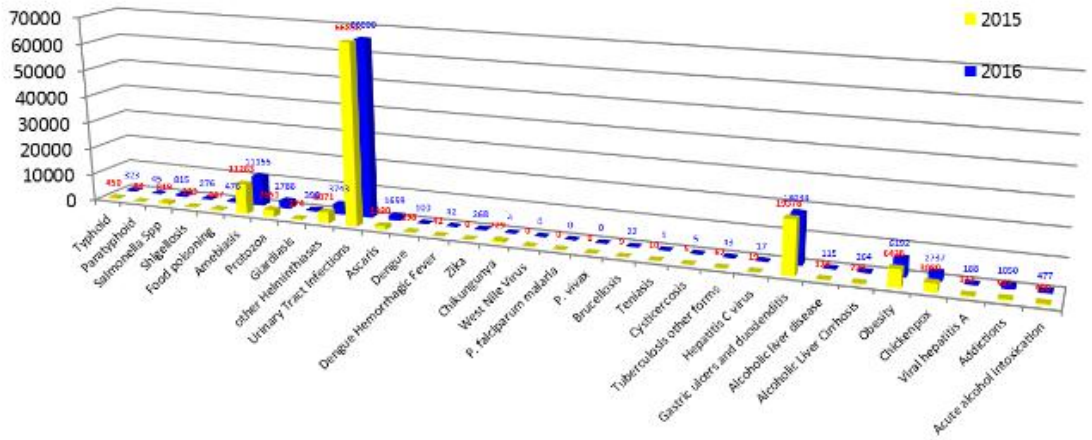


Figure 5. Virus Chikungunya, Dengue, West Nile Fever & Zika at Mexico.

In Figure 5 we can observe the cases of Chikungunya, West Nile Fever, Zika, Zika in pregnant women, Dengue, Dengue Hemorrhagic Fever (DHF) & Death by Dengue in Mexico since 1971 to 2015-2016, data were download GEDEON Informatics, Inc. 2016.

At Figure 6 we can see different disease at Oaxaca Mexico at the year of 2015 and 2016, data were download from Weekly Epidemiological Bulletin from Mexico accessed in April 2016. At Figure 7 we can observe the topological distribution of proteins in flavivirus take it from Almeida 2013. There are no vaccines or antiviral treatments for dengue fever, the possible targets to fight dengue fever are the viral NS3 protease (NS3PRO), which is in part responsible for viral processing and replication. It is now widely recognized that virtual screening campaigns should consider the flexibility of target protein by using multiple active conformational states.



	Typhoid	Paratyphoid	Salmonella Spp	Shigellosis	Food poisoning	Amebiasis	Protozoa	Giardiasis	other Helminthiasis	Urinary Tract Infections	Ascaris	Dengue	Dengue Hemorrhagic Fever	Zika	Chikungunya	West Nile Virus	P. falciparum malaria	P. vivax	Brucellosis	Teniasis	Cysticercosis	Tuberculosis other forms	Hepatitis C virus	Gastric ulcers and duodenditis	Alcoholic liver disease	Alcoholic Liver Cirrhosis	Obesity	Chickenpox	Viral hepatitis A	Addictions	Acute alcohol intoxication
2015	450	84	819	233	670	11283	2251	276	4071	66858	1123	153	42	0	723	0	0	0	5	10	5	62	15	15378	124	225	6438	3000	312	543	450
2016	333	45	815	275	676	11155	2768	306	3741	66800	1659	100	42	268	4	0	0	0	22	1	5	43	17	18034	115	264	6152	2737	188	1050	477

Figure 6. Other diseases at Oaxaca Mexico (Accumulation per year)

The flexibility of the DENV NS3PRO could explain the relatively low success of previous virtual screening studies. They explore the DENV NS3PRO conformational states obtained from molecular dynamics (MD) simulations to take into account protease flexibility during the virtual screening/docking process. They build NS3PRO complete model by multiple template homology modeling and this study allowed the use of protein flexibility in the virtual screening technic against the dengue virus NS3 protease.

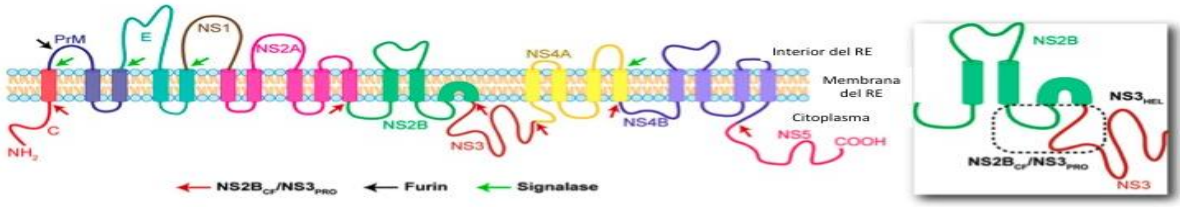


Figure 7. Topological distribution of proteins in flavivirus from Almeida 2013.

Pathogenic members of the flavivirus family, including West Nile Virus (WNV) and Dengue Virus (DV), are growing global threats for which there are no specific treatments. The two-component flaviviral enzyme NS2B-NS3 cleaves the viral polyprotein precursor within the host cell, a process that is required for viral replication. Aleshin in 2007 report the crystal structure of WNV NS2B-NS3 protease both in a substrate-free form and in complex with the trypsin inhibitor aprotinin/BPTI. They show that aprotinin binds in a substrate-mimetic fashion in which the productive conformation of the protease is fully formed, providing evidence for an "induced fit" mechanism of catalysis and allowing they to rationalize the distinct substrate specificities of WNV and DV proteases.

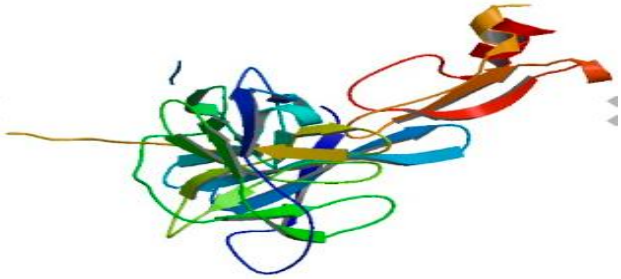


Figure 8. Crystal Structure of the West Nile virus NS2B-NS3 protease complexed with bovine pancreatic trypsin inhibitor, protein data bank: 2IJO from Aleshin 2007.

They also show that the NS2B cofactor of WNV can adopt two very distinct conformations and probable can be a general feature of flaviviral proteases, providing further opportunities for regulation. Finally, by comparing the flaviviral proteases with the more distantly related Hepatitis C virus, they provide insights into the evolution of the Flaviviridae fold. They work should expedite the design of protease inhibitors to treat a range of flaviviral infections.

We made alignment of DENV1 3L6P, DENV2 2FOM, DENV3 3U1J, DENV4 2WHX, WNV 2FP7, CHIKV 3N41, ZIKV 5LC0 sequences at MSA Viewer, JalView 2.9.0b2 as Figure 9.

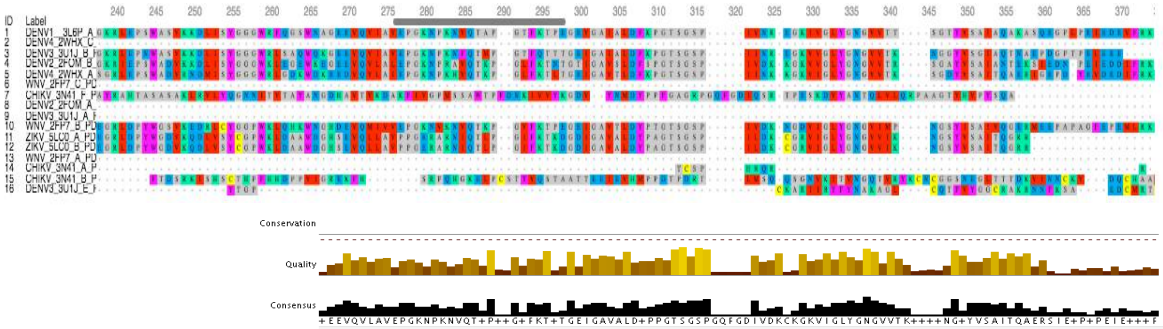


Figure 9. Alignment of CHIKV, DENV1-4, WNV, ZIKV at MSA Viewer and JalView 2.9.0b2 program.

In Figure 10 we can observe the phylogenetic tree for the sequences of P17763, Q91H74, Q5UB51, Q2YHF0 P06935, Q1H8W5, A0A141ZVF6 of CHIKV, DENV1-4, WNV, ZIKV, in UniProt. Checked with Phylo.io too.

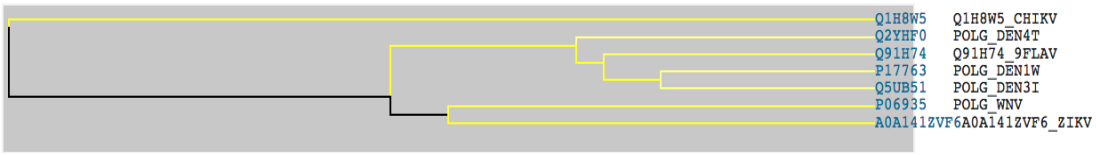


Figure 10. Phylogenetic tree of CHIKV, DENV1-4, WNV, ZIKV at UniProt Version 1.0k.

Conclusions: There is no culture of paying taxes much less obey safety standards in the vending food businesses in the town. Also there is no assurance of safety of water in the water bodies available as river or canals that are polluted. The population without access to water in their houses, bathes in these polluted river, wash their clothes and fishing to eat. The population has access to health services, but there is no culture of prevention.it is necessary that we obey the standard on food safety assurance. It is necessary to teach food handlers to process food and to ensure food safety. It is necessary to purify water bodies from rivers and canals. it is necessary avoid the growth of mosquitoes in water bodies. We need conduct research to prevent expansion of viral diseases transmitted by mosquitoes. The molecular dynamics (MD) simulations can be used to find some kind of virus NS2B-NS3 protease complex inhibitor to avoid the ensemble of virus and disperse of this disease.

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3rd BIOTECHNOLOGY SUMMIT
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Area VII
PURPLE

Patents, IPR: Strategy for Intellectual Property Protection, Patents, Publications, Inventions.

BIOTECHNOLOGY SUMMIT

Patent protection strategy of R&D results: A Cuban experience from the Center for Genetic Engineering and Biotechnology, since 1990

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Abstract:
The introduction of the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPs Agreements) on January 1, 1995, turned Patents of Invention into one of the mainstays of international commerce between member countries of the World Trade Organization (WTO). Patent of Inventions are designed to protect any invention, regardless of a particular field of technology, which satisfies the patent requirements of novelty, inventiveness and industrial applicability. After the signing of TRIPs, the number of applications for Patents of Invention rose significantly. This was especially true for applications intended to protect products of the pharmaceutical and biotechnological industries, owing to the fact that second uses of already known substances and new combinations of known compounds, or even new dosage regimes producing a particular effect in patients, started to be regarded as patentable matter by patent offices. Taking into account their importance for protecting the results of research and development projects and ensuring the success of subsequent commercialization effort, the Center for Genetic Engineering and Biotechnology (CIGB) has systematically protected its results by means of Patents of Invention in different countries. This work, then, presents the patent management experience of CIGB from 1990 to the present, discussing a protection case study as granted under USA, EPO and Cuban legislations.

Keywords: TRIPS • Biotechnology Patent • Patent Eligibility US • EPO

Introduction:

The Uruguay Round of 1994, conducted amidst strong pressures from developed countries and transnational corporations to uphold the protection of intellectual property rights –including those pertaining to biotechnological inventions- as one of the mainstays of modern international trade, eventually led to the creation of the WTO and a number of binding multilateral trade agreements for its members. Among the latter sits the TRIPs Agreement, which not only reinforced the existing international order regarding industrial property rights, but undermined the independence of developing countries for the management of their patent legislation and the implementation of protectionist systems catering to their development goals and other social and/or economic needs.

The CIGB, part of the network of scientific centers at western Havana, is considered the leading biotechnological institution of the Republic of Cuba. It operates on the basis of a closed cycle of research-development-production and marketing of biotechnological products, whose applications range from biomedicine to agriculture. Although the center itself was officially created on July 1st of 1986, a core research group had already been operating since 1981, when it achieved, for the first time in the country, the production of leukocyte interferon through genetic engineering.

The present work summarizes the experience of CIGB on the protection of its intellectual property rights on its products, taking into account the existing legal framework and new legislation relevant to this field in the countries where the institution intends to or is currently marketing its products.

Materials and Methods:

CIGB patent data were acquired from the institutional database, which has been constantly updated from 1990 onwards as new patent applications are submitted and registered at national offices and international patent organizations, such as the Cuban Industrial Property Office (OCPI), the European Patent Office (EPO), the United States Patent and Trademark Office (USPTO) and the World Intellectual Property Organization (WIPO). The data were cross- checked against patent information services such as Qpat, Patentscope and Espacenet, and then analyzed as frequency measures and statistically compared in percentage as relative measure. Additionally, documentation regarding the TRIPS Agreement and patent legislations from Cuba, Europe and USA were analyzed, and a case study of a patent granted under each of these legislations is presented.

Results and discussion:

The existing international order and the globalization of world economy have required that the scientific staff of CIGB and its workforce, in general, be well versed on matters of intellectual property rights, which cannot be ignored if the products of the institution are to be internationally marketed.

Cuban patent law from 1983 to 2012, embodied by Decree-Law 68, had a markedly protectionist nature that enabled the development of the country's biotechnological and pharmaceutical industry. Such a development would have been impossible in the current regulatory context.

Article 27 of the TRIPs Agreement establishes that patentable subject matter includes…"any inventions, whether products or processes, in all fields of technology, provided that they are new, involve an inventive step and are capable of industrial application" (Art.27 TRIPs Agreement, 1994). However, it also offers the possibility of excluding from patentability a number of objects such as diagnostic, therapeutic and surgical methods for the treatment of humans, or plants and animals other than micro-organisms. Furthermore, the TRIPs Agreement does not enforce the implementation of a unified patent examination procedure. A single, unified international patent system does not exist, providing some maneuvering room for adjusting the national legislations of developing countries in order to curb the proliferation of never-ending patent protections ("evergreening") on specific products through patents on different forms or applications of said product (Correa CM, 2010).

Many of the patent legislations enacted after the TRIPS Agreement exclude from patentability or do not consider inventions several objects such as new uses, methods of treatment and diagnosis, natural products and its replicates, and others. Table 1 shows what subject matter is considered patentable in Cuba in comparison with current US and European (EPO) legislation.

The system for the protection of industrial property rights implemented at CIGB starts operating from the very moment that a research project is designed to the moment it is negotiated with a second party or becomes a marketed product.

The novelty and progression of research projects are continuously assessed, so as to avoid incurring unnecessary patent maintenance costs if the expected goals are not met.
CIGB's patent coverage is broad, consisting on Cuban patent applications for 135 inventions, 117 of which also have pending patents abroad. Up to December 2015, CIGB held 1130 patent applications worldwide, of which 496 had been granted (70% of these are being exploited). CIGB has also licensed the rights to a patent from the University of Havana and Ottawa University on a vaccine against *Haemophilus influenzae*. The distribution of this patent portfolio per research field is shown in Table 2.
Figure 1 shows the geographical distribution of current patents and patent applications from CIGB.

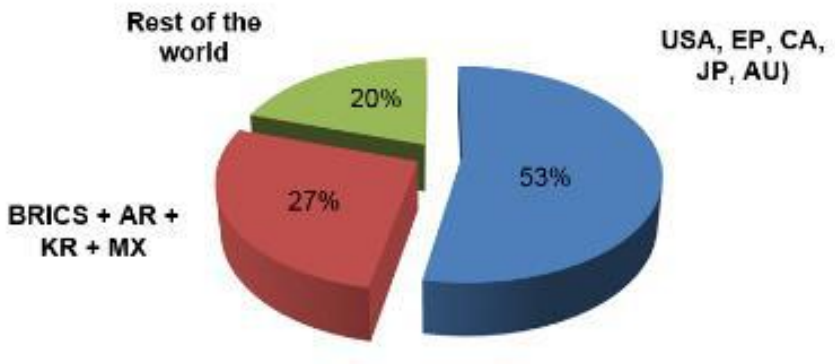

Figure 1. Distribution of current CIGB patent applications per geographical area.

A patent application may include a thorough description of the subject matter that is not necessarily circumscribed to what is considered patentable in the specific country where the application is being submitted, including in its scope all objects of invention that are properly defined in the description. This offers the possibility of claiming them as novel and worthy of patent protection in other countries if they are held as patentable subject matter by the corresponding national patent law. An international application (PCT) provides an expert report on compliance with patentability requirements within a relatively short time, constituting a valuable tool to assess the status of a patent or project.

Table 1. Patentable subject matter in Cuba, EPO and USA (Patent Eligibility in USA).

Territory	Inventions:	Exclusions
Cuba D-L 290	Products Procedures (Art.21)	The following are not considered inventions: Uses. Plans, methods, rules. Scientific discoveries, principles and theories. Subject matter already existing in nature and its replicates (excepting genetically modified microorganisms). Procedures, which are essentially biological. The human body and/or its parts. Inventions that go against public order or morality, human or animal health or the environment (Art.21.2) The following cannot be patented: Animals and plants. Therapeutic, diagnostic and surgical procedures. Cloning of human beings, processes for modifying the germline genetic identity of human beings, etc. (Art.22)
EPO European Patent Convention 15th Edition, 2013	European patents shall be granted for any inventions, in all fields of technology, provided that they are new, involve an inventive step and are susceptible of industrial application. (Art.52 (1))	The following are not considered inventions: (a) discoveries, scientific theories and mathematical Methods b) aesthetic creations; c) schemes, rules and methods for performing mental acts, playing games or doing business, and programs for computers; d) presentations of information. (Art.52 (2)) Exceptions to patentability: a) inventions the commercial exploitation of which would be contrary to "public order" or morality; b) plant or animal varieties or essentially biological processes for the production of plants or animals; c) methods for treatment of the human or animal body by surgery or therapy and diagnostic methods practiced on the human or animal body (Art.53)
USA Appendix L Patent Law United States Code Title 35 – Patents	The invention should be directed to one of the four patent-eligible subject matter categories: process (includes treatments and method), machine, manufacture (product), composition of matter (all compositions of two or more substances and all composite articles) 35 U.S.C.101	Abstract ideas (such as mathematical algorithms), natural phenomena, and laws of nature, inventions directed to or encompassing a human organism (AIA § 33 (Related to 35 U.S.C. 101) * *However, methods and products employing abstract ideas, natural phenomena, and laws of nature to perform a real-world function may well be eligible for patenting.

Table 2. CIGB Patents for inventions per research field.

Field of Research	Area	Patents In Cuba	Project
Biomedicine	Wound Healing	7	Diabetic foot
		1	Antifibrotic
	Autoimmunity	4	Peptides against RA[1] and other autoimmune diseases
	Neurology	2	Therapy against MS[2].
			Brain Infarct
	AMD[3]/ Cancer	1	Anti-VEGF antibody
	Gastroenterology	1	Acute Hemorrhoidal Disease
	Cancer	13	Antitumoral peptides against HPV, prostate cancer, cervix cancer Formulation against non-melanoma skin cancer, gliomas and others. Anticancer vaccines.
	Infectious diseases	13	Vaccine against HCV, HBV, VD. Anti-HIV Therapy
	Cardiology	1	Cytoprotective agent
Agro-biotechnology	Aquaculture	5	Growth and health improvement
	Agriculture	5	Control of nematodes and pathogens
	Functional food	1	Biocatalyst for production of fructooligosaccharides (FOS)
	Animal health	4	Antiparasitic antigen Vaccine against CSFV Vaccine against sea lice. Antimicrobial peptides

[1] RA Rheumatoid Arthritis. [2] MS-Multiple Sclerosis. [3] AMD- Age-related Macular Degeneration

Conclusions:

The results of the work presented here have afforded CIGB a broad patent coverage in several regions of the world, placing the institution in a strong position for the negotiation of its projects and for continuing the development of novel solutions meeting the health and agricultural needs of Cuban people. Patent protection is an efficient tool for ensuring that there is a return of investment on research activities, and for that reason it has become imperative that developing countries acquire expertise and skills on the principles, international rules and national legislation concerning industrial property.

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Area VIII
WHITE

Industrial Biotechnology (gene-based).

Translational fusion GFP-phasin from *Azospirillum brasilense*

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Abstract

Phasins are a family of small amphipathic proteins that are found forming a layer coating granules of biopolymers. Phasins allow to the hydrophobic biopolymer be stable in hydrophilic environment of cytoplasm. These proteins also prevents coalescence of granules and the binding of others no GAPs proteins to it. The aim of this project was generate a chimeric protein of phasin from *Azospirillum brasilense* and green fluorescent protein. A plasmidic construction with phasin and GFP fused was obtained to evaluate, in future assays, how these proteins interact with the granules *in vivo* and if the number and size of them are affected.

Keywords: *Azospirillum brasilense* ● Phasin ● PHB-proteins interaction.

Introduction

Poly-β-hidroxybutyrate (PHB) is a polymer produced by several species of microorganisms in which is used as storage of carbon and energy. PHB is synthesized and accumulated in cytoplasm when the cell is exposed to stress occasioned by the lack of an essential nutrient but an excess of carbon source (Anderson *et al*. 1990). PHB is a polyester of industrial interest because of its biodegradability and similar characteristics to polypropylene so, it can be used as an alternative source of plastics derivate from petroleum (Byrom *et al*. 1987).

The genes, enzymes and pathways for the biosynthesis of PHB have been already studied; this polihydroxyalcanoate is synthesized from acetyl-CoA in three steps: first, the condensation of two acetyl-CoA molecules by a β-ketothiolase (*phbA*), then a reduction catalyzed by a NADPH-dependent acetoacetyl-CoA reductase (*phbB*) and finally a PHB synthase (*phbC*) that polymerizes β-hidroxybutyrate (3HB) to PHB. On the other hand, the degradation pathway of the polymer starts with the depolymerization by the enzyme PHB depolimerase (*phbZ*) that break down the polymer into monomers of D-β-hidroxybutyrate which is then oxidated to acetoacetate by a NAD^+ dependent D-β-hidroxybutyrate dehydrogenease. The product of this reaction is then converted into acetoacetyl-CoA by acetoacetyl-CoA synthase, in the following reaction, acetoacetyl-CoA is break down into two molecules of acetyl-CoA which can enter to the Krebs Cycle (Aneja and Charles, 1999).

Gene mutation studies of the PHB synthesis and degradation pathway have been carried out; Martinez *et al.* (2015) cloned and overexpressed PHB synthase (*phbC)* gene, as a result, PHB production was increased. On the other hand, strains mutated on *phaZ* are unable to degrade the polymer, leading to a PHB accumulation (Kadouri *et al*. 2003)

Most of the microorganisms produce and accumulate PHB as intracellular granules coated with a layer of proteins referred as granule-associated proteins (GAPs) and phospholipids. These proteins include enzymes involved in PHB synthesis and degradation, small amphipathic proteins -phasins- and others (Furst *et al*. 1995).

Phasins are a class of low molecular mass amphipathic proteins that form a layer on the surface of PHB granules and are the most dominant all GAPs in natural producers of this type of polymers. Phasins provide the interface between the hydrophobic core of granules and the hydrophilic cytoplasm; they also have influence on the size and number of granules and have been reported to regulate the synthesis of the polymer (York *et al*. 2001).

The regulation of these proteins -phasins- is important preventing the accumulation of PhaP in cells when they are not producing the polymer. Regulation of these proteins have been studied in *Paracoccus denitrificans* concluding that there is a protein (PhaR) that interacts directly with the polymer and DNA, regulating itself and the transcription of phasin gene when there is a production of PHB (Maehara *et al*. 1999).

Phasins may also be used for numerous biotechnological applications, so it is important to understand the binding of these proteins to the polymer granule and their interaction with other proteins. There is not any previous study of granule-associated proteins in *Azospirillum brasilense*, so, this work is focused on the study of a phasin from this bacterium, our group is trying to understand better the nature of this family of proteins.

Materials and methods

Bacterial strains and growth conditions. Azospirillum brasilense Sp. 7 cells were batch cultivated in minimal media at 30°C. *Escherichia coli* strains XL 1-Blue and DH5-α were grown in LB at 37°C.

Induction. The AMK58-17045 (*pha*) gene from *Azospirillum brasilense* was cloned in pCR 2.1 TOPO resulting pCR 2.1 TOPO::AMK58-17045. After that, phasin gene was subcloned into *Bam*HI/*Hind*III sites of pQE-31, obtained pEQ-31-AMK17045. Cells of *E. coli* XL1-Blue were transformed and a single colony was growth and induced by adding 0.5 and 1.0 mM of IPTG at OD_{600} 0.5. Culture flasks were incubated at 37°C and samples were taken each 1, 3 and 5 hours after induction. Samples were centrifuged, washed and sonicated. A SDS-PAGE was performed to separate proteins expressed during the induction with IPTG.

Subclonation. The AMK58-17045 gene was subcloned in plasmid pGLO resulting pGLO-AMK58-17045 (*pha*), double digestion with *Mfe*I and *Xma*I was performed to obtain the fragment of interest. Similarly, pGLO was digested with *Eco*RI and *Xma*I. A ligation reaction with the vector and the insert were performed with T4 DNA ligase.

Primers design. An *in silico* analysis of pGLO-AMK58-17045 (*pha*) was carried out to design the primers for remove the stop codon of GFP, taking care that both proteins were in frame to obtain a translational fusion with Phasin. Restriction site for the *Bgl*II (underline in the primers) was designed in both primers: forward: 5´-GATAAGATCTAATTGCATGGCCAAGCAGAC-3´ and reverse: 5´-GATAAGATCTTTTGTAGAGCTCATCCATGCC-3´.

Clonation. Polimerase chain reaction was performed with *Pfu* polymerase at an melting temperature of 57°C and an extension time of 12 minutes. After the reaction, DNA was doubly digested with *Bgl*II and *Dpn*I. DNA was then ligated in a reaction with T4 DNA ligase. *Escherichia coli* DH5-α were transformed with the ligation mix and transformants were selected on LB plates with Amp.

Digestions. Using the site *Bgl*II, double digestions with *Eco*RV-*Bgl*II and *Hind*III-*Bgl*II were carried out.

Expression of GFP-AMK-17045 translational fusion. pGLO-AMK58-17045 (*pha*) was induced by adding 1.5mM of L-arabinose to a previous culture in LB broth with an OD_{600} 0.5. Culture flasks were incubated at 37°C and samples were taken each 1, 3 and 5 hours after induction.

Results and discussion

Induction of pEQ-31-AMK17045 was successful, as observed in SDS-PAGE of figure 1: lanes 1 and 5 at 0 h, lanes 2 and 6 at 1 h, lanes 3 and 7 at 3 h, lanes 4 and 8 at 5 h. Lanes 1 to 4 induced at 0.5 mM of IPTG and lanes 5 to 8 at 1 mM of IPTG. The best conditions for Phasin induction were 0.5 mM during 5 hours.

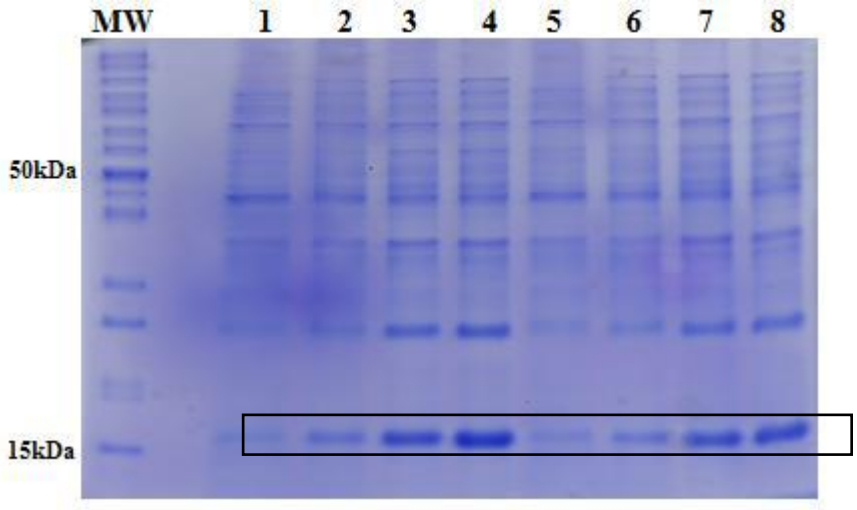

Figure 1. SDS-PAGE of Phasin induction.

The figure 2a shows a construction scheme. To verify the transformant cells, digestions were carried out, in figure 2b electrophoresis gel: lanes 1, molecular weight; lines 2 to 5, four clones digested with *Bgl*II and *Eco*RV; lines 6 to 9, same clones digested with *Bgl*II and *Hind*III. In the first case are observed two fragments of 4130 and 1661 bp, in the second assay three bands of 4720, 580 and 491 bp were obtained.

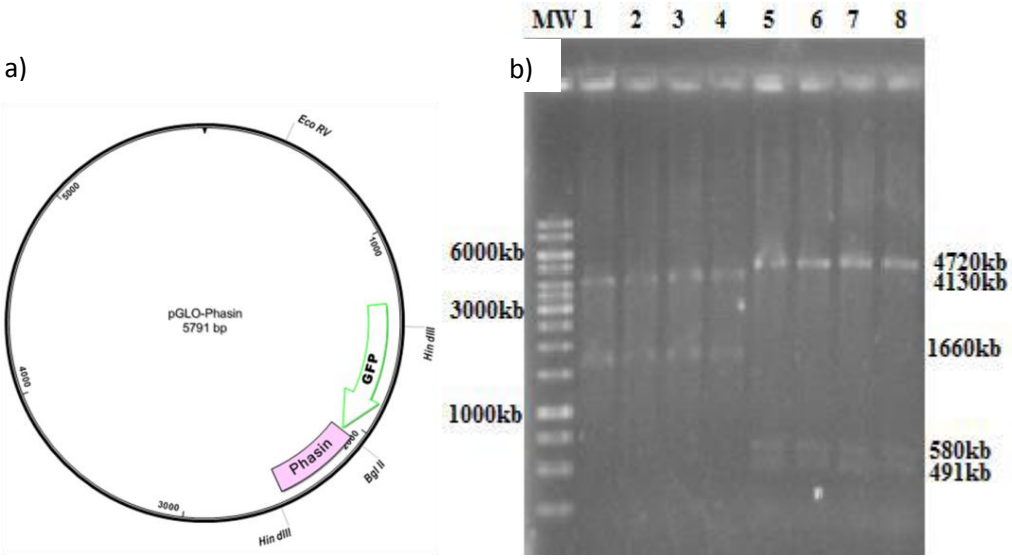

Figure 2. a) Construction map. B) Digestions *Eco*RV-*Bgl*II and *Hind*III-*Bgl*II.

The pGLO-AMK58-17045 (*pha*) was induced by adding 1.5 mM of L-arabinose to a previous culture in LB broth with an OD_{600} 0.5. Culture flasks were incubated at 37°C and samples were taken each 1, 3 and 5 hours after induction.

A construction containing a translational fusion of green fluorescent protein and the phasin from *Azospirillum brasilense* was obtained. The pGLO-AMK58-17045 (*pha*) plasmid has the gene of interes in frame without stop codons, resulting in the production of a single protein of a phasin attached to a green fluorescent protein.

In this particular project it is essential to investigate the binding capacity of fusion GFP-phasin and analyze if there are any changes in the secondary structure because it has been found that predicting the structure with bioinformatic models, phasins have a high α-helix content because of the presence of hydrophobic aminoacids (Neumann *et al.* 2008).

Conclusion

A construction containing the *pha* gene from *Azospirillum brasilense* was obtained (pEQ-31-AMK17045) This plasmid showed a clear induction. A better induction is observed after 5 hours.

A construction containing a translational fusion of green fluorescent protein and the phasin from *Azospirillum brasilense* was obtained pGLO-*pha*, shows different induction to pEQ-31-AMK17045.

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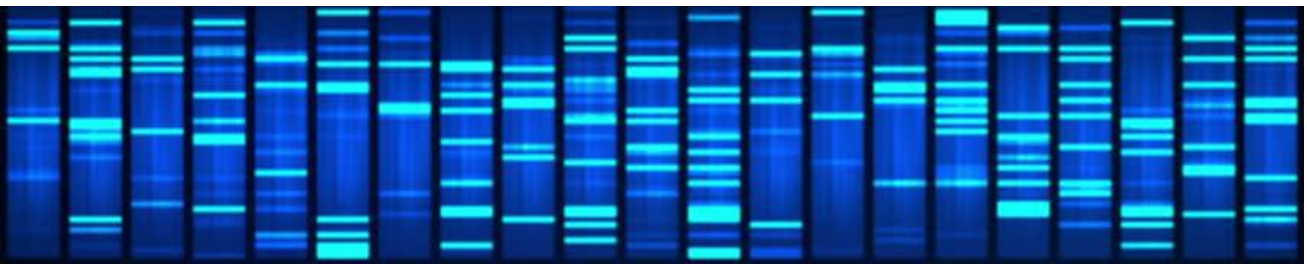
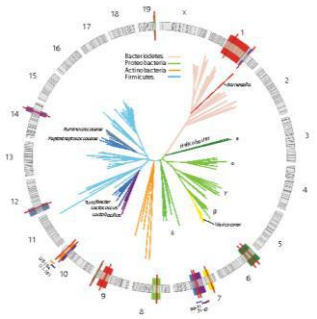
Area IX
GOLD

Bioinformatics: Nanobiotechnology, Microelectronic and Microelectromechanical Systems (MEMS), Microsystems technology (MST), Nanoelectromechanical Systems (NEMS) & Micromachines.





Manuscripts regarding this knowledge area were not received

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Area X
GREY

Classical Biotechnology (Fermentation): Industrial Biotechnology; Classical Fermentation & Bioprocess/Bioengineering Technology; Engineering & Technological Equipment for Bioproduction; Output of Science-Intensive Bioproducts.

Synthesis of medium chain length Polyhydroxyalkanoates from *Cupriavidus necator* with beeswax hydrolyzates as carbon source

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Abstract
This study demonstrates that using beeswax hydrolyzates (Hw), short and medium chain length Polyhydroxyalkanoates (scl-mcl-PHAs) can be obtained, by a three stages fed-batch fermentation system. The fed batch fermentations with a *Cupriavidus necator* strain were conducted. Glucose and ammonium sulphate were used as carbon and nitrogen sources respectively, in a first and second culture stages. In the third stage (production of scl-mcl-PHA) a fresh medium with Hw as a carbon source at a concentration of 5 g/L was fed, obtaining 8.91 g/L of intracellular scl-mcl-PHA and 13.64 g/L of biomass, resulting in 65.3 % w/w of scl-mcl-PHA. With 1H and ^{13}C NMR, was determined presence of scl and mcl monomers, 3-hydroxybutyrate (3HB), 3-hydroxyvaletare (3HV), 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HDD). The percentage of comonomer incorporation different to 3HB was 4.6 mol %. *Cupriavidus necator* was able to accumulate mcl-PHAs.

Keywords: Biopolymer • GC • mcl-PHA • NMR • Fed-batch fermentation.

Introduction

The polyhydroxyalkanoates (PHA) are stereospecific isotactic polymers synthesized by many microorganisms. They are classified according to the length of the side chain of their monomers: short chain (scl-PHA), with 3-5 carbon atoms and medium chain length (mcl-PHA), 6 to 14 carbon atoms (Liu et al. 2011). The composition and amount of intracellular PHA are related to the carbon source, which microorganisms use in the process of synthesis (Jiang 2010). Several studies have evaluated various carbon sources such as, canola oil (Rathinasabathy et al. 2013) and used cooking oil and it has been demonstrated great biotechnological potential for the production of mcl-PHAs (Cruz et al. 2015). During the biosynthesis of PHA by *Cupriavidus necator* (formerly *Wautersia eutropha* > *Ralstonia eutropha* > *Alcaligenes eutrophus*) from fatty acids, Lopez-Cuellar et al. (2011), Rathinasabapathy et al. (2013) and Inomata et al. (2014) showed that *C. necator* was able to synthesize mcl-PHAs using as substrates fructose and canola oil in a three stages fed-batch system, although it has been reported that the PHA synthase of *C. necator* is strictly specific for scl-PHA monomers (Slater et al. 1992). The synthesized mcl-PHA presented three medium chain length comonomers (3HV, 3HO, 3HDD) bound to 3HB. Like vegetable oils, beeswax has a great demand on the world market. Beeswax has a wide range of uses

in cosmetics manufacturing, candle processing, pharmaceutical, agriculture and food industry (Zhang and Xiao 2013).
Beeswax is a mixture of wax acid esters; which to date its use has not been reported as a carbon source for PHAs synthesis. When is methanolyzed with alkali, beeswax generates hydrolyzates, rich in fractions of fatty alcohols and acids (Maia and Nunes 2013). Some studies suggest that fatty alcohols can be converted to fatty acids providing precursors for synthesis of mcl-PHAs by β-oxidation (Jiang 2010). In this study, the effect of Hw in the culture medium on the chemical properties of scl-mcl-PHA obtained during the accumulation phase of *C. necator* in a 3-stages fed-batch system was evaluated.

Materials and methods

Microorganism and medium: *C. necator* was propagated in Luria Bertani broth (LB) and incubated at 30 °C. The inoculum for all assays was prepared by transferring 100 μL of seed flask cultivation with LB medium, incubated for 36 h at 150 rpm and 30 °C. The fermentation medium and fed-batch culture (stage 2 and 3) contained respectively (g/L): Glucose (10, 20, 30, 0); $(NH_4)SO_4$ (3.3, 4, 0.35, 0); $MgSO_4(7H_2O)$ (0.2, 1.2, 1.2, 1.2); KH_2PO_4 (1.5, 4.5, 4.5, 4.5); Citric acid (1, 1.7, 1.7, 1.7); Trace Elements Solution (mL/L) (1, 10, 10, 10); Hydrolyzed beeswax (0, 0, 0, 5). The trace elements solution concentration was descrited by Shang et al. 2004.

Fermentation: The cultures were done in triplicate in a 5 L fermentation unit. The content of the seed flask (10 % v/v) was transferred to fermentation medium under the same conditions described above, pH 7.0 controlled with 2M NaOH and 0.47 M HCl. Samples were taken every 3 h to quantify glucose, ammonium sulphate, biomass and PHA. The fermentation was done in three stages (modified method from López-Cuellar et al. 2011): Stage 1: Initial batch cultivation. Stage 2: Biomass production by a fed batch system. Stage 3: Synthesis of PHA, using Hw as a carbon source fed at a rate of 0.33 g_{Hw}/L.h in presence of 0.43 g/L Triton X-100 (similar to Budde et al. 2011).

Analytical techniques: Determination of dry weight (X) by gravimetry; residual biomass (rX) was defined as the concentration of X without PHA (Budde et al. 2011). Glucose concentration was determined by DNS method (Barbosa et al. 2005). Quantification of ammonium sulphate was carried out by Weatherburn method (López-Cuellar et al. 2011). The amount of residual hydrolyzed wax (rHw) was determined by the modified equation described by Budde et al. (2011).

PHA purification: The PHA was purified by chloroform extraction (modified method of Nurbas et al. 2004). The procedure was repeated three times to carry out the purification of the polymer and avoid the presence of cell debris and possible residual metabolites present in beeswax.

PHAs characterization: NMR was carried out to elucidate the structure of PHAs. Deuterated chloroform ($CDCl_3$) was used as solvent. The spectra corresponding to ^{1}H and ^{13}C were analyzed with specialized software (MestReNova version 6.0). GC system was used, equipped with an AllTech AT-FLAME Serial-508315 capillary column (30 m x 0.32 mm x 1 μm; carrier gas He, 0.45 mL/min) and a flame ionization detector.

Results and Discussion

scl-mcl-PHA synthesis: In fed-batch fermentation, Figure 1 shows the profile of biomass and PHA production. *C. necator* produced 13.64 g/L of total biomass at the end of the fermentation, when 0.43 and 5 g/L of Triton X-100 and Hw were respectively aggregated and ammonium sulphate was exhausted. The initial ammonium sulphate concentration was 3.1 g/L, with a consumption rate of 0.52 g/h for 21 hours (first stage of fermentation). Then after 21 hours of culture, 0.3 g/L of ammonium sulphate concentration was simultaneously fed with the carbon source at a consumption rate of 0.13 g/h. This procedure was followed in order to maintain the metabolism of *C. necator* and stress conditions necessary for the intracellular accumulation of PHA. At this stage fed of fresh culture medium with Hw was started. As a consequence, an increase of the intracellular PHA concentration from 1.25 g/L to 8.91 g/L was observed (Figure 1), which is attributed to the inhibition of cell growth and accumulation of the waxy carbon source. The Hw concentration decreased in the culture medium 3.35 g/L during the last 21 h of culture, with a consumption rate of 0.45 g/L h. The $Y_{x/s}$ obtained in this work was higher (0.225 g_x/g_s), this indicates that Hw is a substrate that accumulates PHA quickly under stress conditions (Figure 1). The amount of synthesized PHA obtained at 45 h in the third stage of fed-batch culture, corresponded to a 65.3 % w/w of the total biomass generated (13.64 g/L).

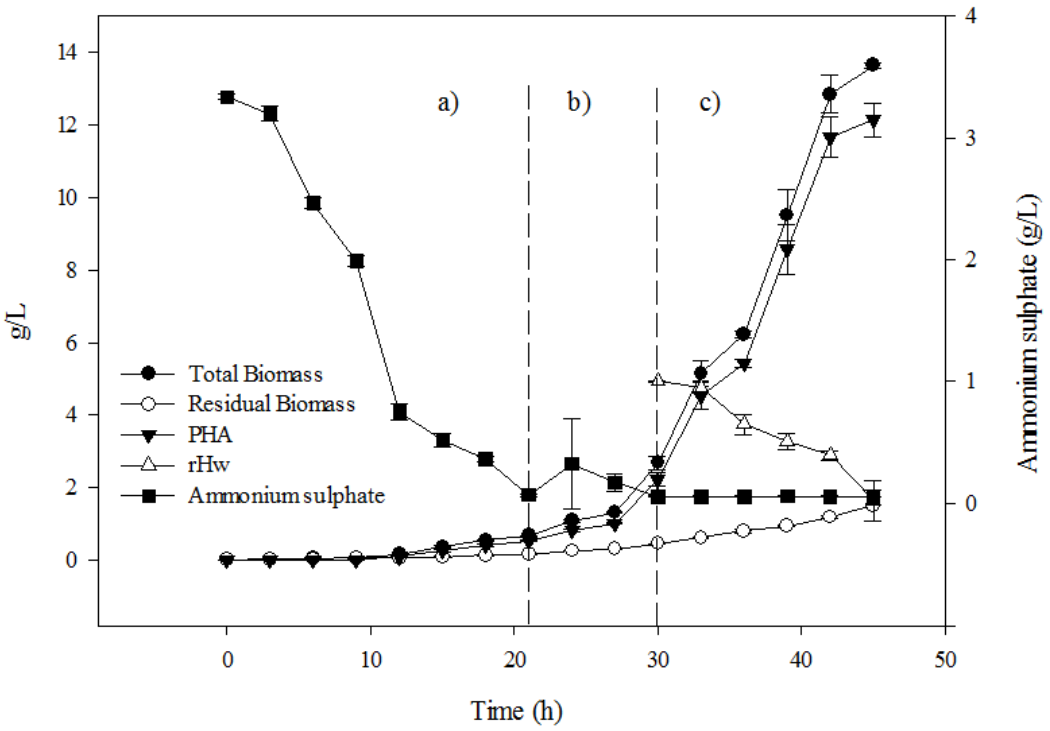

Figure 1. Profile of total biomass (●), residual biomass (○) polyhydroxyalkanoate (▼) ammonium sulphate concentration (■) and Hw consumption (△), observed in (a) batch culture, (b) fed-batch and (c) scl-mcl-PHA production step, during fermentation of *C. necator* with glucose and Hw as carbon sources.

Nuclear magnetic resonance (NMR): Figure 2a shows ^{1}H NMR spectra of PHA isolated from *C. necator* when grown on Hw as carbon source (third stage fed-batch culture). The corresponding signal to methyne (-CH-) of the monomeric fraction of 3HB is observed as a sextet at 5.24 ppm, which is a stereogenic center bonded to oxygen, to methyl and methylene of PHA (Impallomeni et al. 2011). However, it should be seen as a quintuple resonance, for links with both methylene groups of the main chain and the aliphatic chain. The characteristics peaks of the 3HB and mcl-PHAs monomers methylene groups (-CH_2-) are observed as an ABX system. The center of the resonance peaks for both monomers is observed at 2.50 ppm (López-Cuellar et al. 2011). An additional signal was observed in the aliphatic region of the spectra with a chemical shift of 0.88 ppm. This shift is characteristic for terminal methyl groups of mcl-PHAs monomers (López-Cuellar et al. 2011). The resonance peak of the 3HB methyl group observed at 1.32 ppm appears as a triplet due to signal overlap with the signals corresponding to the methylene group of mcl-PHAs monomers (Liu et al. 2011). The resonance peak with a chemical shift of 1.56 ppm corresponds to the methylene moiety of the aliphatic chain attached to the stereogenic center of mcl-PHAs monomers (Liu et al. 2011).
The ^{13}C NMR spectra of the obtained biopolymer is shown in Figure 2b. The peaks corresponding to the carbonyl group, the methine and the methylene were observed at 169.0, 67.6 and 40.7 ppm respectively for the monomers. The chemical shift for the methyl group was observed at 19.8 ppm for HB while mcl-PHAs monomers were observed in 14.1. ppm. The remaining signals of the methylene groups of the aliphatic chain suggest the presence of 3HDD monomer, observed between 22 and 35 ppm. The determination of the integration values for the resonance peaks of the ^{1}H NMR spectra for the methyl, methylene and methine groups was applied to the determination of the proportion of mcl-PHAs monomers of 4.6 mol % over the 3HB.

Characterization of PHA obtained by GC: The same samples of PHAs were used to determine the monomeric composition by both techniques, NMR and gas chromatography (GC). The similarity between the retention times of the standards and methanolyzed samples were observed for 12 and 14 C (determined by ^{1}H NMR), showing the incorporation of medium chain length monomers corresponding to 3HV (2.38 mol %), 3HO (0.63 mol %) and 3HDD (0.31 mol %) in relation to the proportion of 3HB (96.48 mol %) (Table 1). The proportion of mcl-PHA monomers determined by GC (3.32 mol%) varies with respect to that obtained by NMR (4.6 mol%). This difference could be explaind as a result of degradation of the sample during the methanolysis. The presence of medium chain length monomers showed that Hw is a suitable substrate that can be assimilated by *C. necator* to produce scl-mcl-PHA.

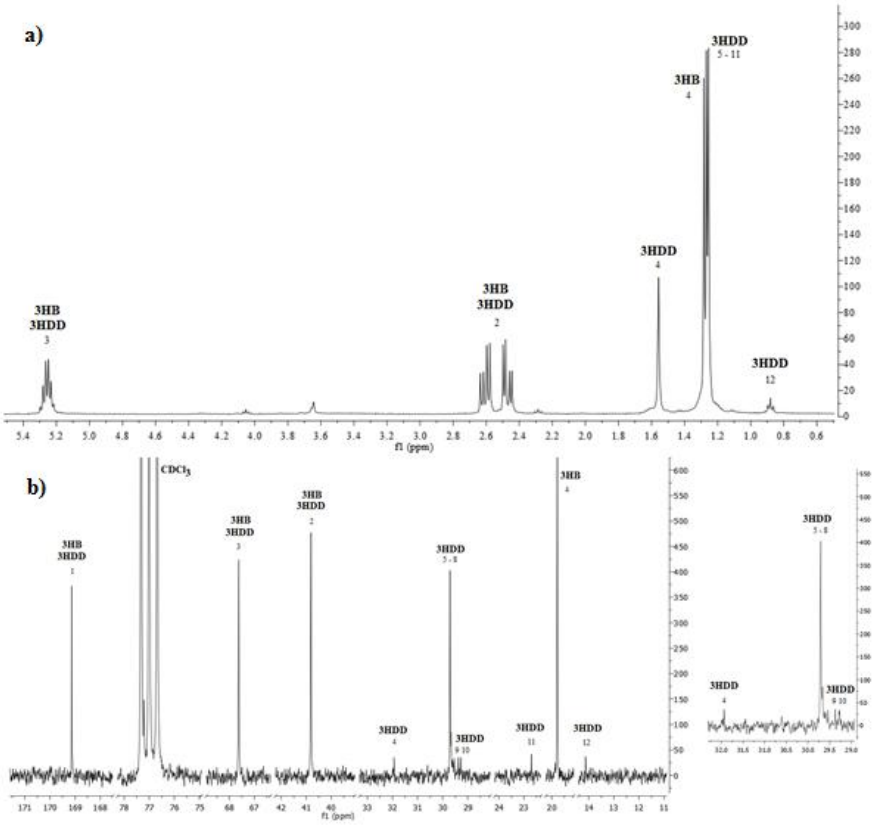

Figure 2. NMR spectra at 300 MHz of scl-mcl-PHA obtained by Hw fermentation of *C. necator* a) ^{1}H spectra b) ^{13}C spectra.

Table 1. Retention times for GC analysis of standards of fatty acids against scl-mcl-PHAs produced by *C. necator* using Hw as carbon source.

3-Hydroxyalkanoic acid methyl esters	Retention time (min)		Heigth
	Standard	Sample	(pA)
3-hydroxybutyrate	2.5	2.57	33744.2
3-hydroxyvalerate	6.25	6.68	832.27
3-hydroxyoctanoate	9.4	9.34	221.87
Unidentified 3-hydroxyalkanoate	-----	10.89	68.53

Conclusions

C. necator was able to synthesize scl-mcl-PHAs using Hw as carbon source under nitrogen limiting conditions in a 3 stages fed batch fermentation system. The increase in the consumption rate of Hw showed that the carbon source is assimilated by *C. necator*, favoring the production of scl-mcl-PHA inclusions. Adding hydrolyzed beeswax in the presence of Triton X-100 did not affect the growth of *C. necator*. The ^{1}H and ^{13}C RMN analysis was used to determine that the obtained polymer has a relationship of 4.6 mol % of mcl-PHAs monomers due to the incorporation of Hw fractions during biosynthesis. The monomeric composition of scl-mcl-PHA determined by GC confirms the presence of 3HV, 3HO and 3HDD monomers.

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Alkaline extracellular amylase production by moderately halophilic bacteria

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Abstract

Three moderately halophilic bacteria with extracellular amylolitic activity where isolated from saline soils in southern Sonora, Mexico. Maximum enzyme activity was obtained at 50 ºC and pH 11.0 for all isolates. The amylase activity was induced by starch. The highest amylase activity for strains BLANE2AM1042 (148.21 IU/L), PPAPAM1094 (98.44 IU/L) and PPSE3AM1065 (43.48 IU/L) was obtained after 240, 120 and 216 h of incubation respectively at shake flask level. The medium used for amylase production was marine broth adjusted to 10% NaCl containing starch 2% (w/v), while the culture condition for amylase production were pH 7.35, temperature 37.5 ºC, agitation speed 180 rpm, medium volume 70 ml in 125 ml flask, and incubation time 240 h.

Keywords

Moderately halophilic ● Halophilic hydrolase ● Alkaline ● Amylase ● Starch hydrolysis

Introduction

Hypersaline environments usually have between 8 and 10 times the salt concentrations found in sea water, thus they are considered extreme. These environments vary in terms of salt, ionic composition, temperature, pH and nutrients; influenced by geographic zone, climate, and altitude, among others. Nevertheless, even though these conditions are adversary for life, these ecosystems are dynamic and show a great microbial diversity (Bell, 2012). Microorganisms that inhabit these environments are capable of producing compounds such as metabolites and extracellular enzymes with great industrial potential (Delgado-García *et al*. 2012; Enache and Kamekura, 2010). Many of these enzymes can be stable and active in more than one extreme condition, such as high salinity and temperature and/or a wide range of pH (Delgado-García *et al*. 2012). Since halophilic bacteria belong to the extremophile group of microorganisms, they offer plenty of

application in many different fields with the production of hydrolytic enzymes such as amylase, lipase, protease, glucosidase, among others.

In this study, we focused on amylase. Amylase hydrolyzes starch, producing dextrin and small polymers made up of glucose units (Gupta *et al.* 2003). These enzymes have a wide range of uses in food, paper and detergent industries among others (Aiyer, 2005; Pandey *et al.* 2000). Because of this, most of the amylase producing bacteria belong to the genus *Bacillus*, including: *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* (Prakash and Jaiswal, 2010). Some halophilic bacteria with amylolytic activity has also been reported such as *Chromohalobacter sp.* (Prakash *et al.* 2009), *Halobacillus sp.* (Amoozegar *et al.* 2003), *Halomonas meridiana* (Coronado *et al.* 2000), *Bacillus dipsosauri* (Deutch 2002) and *Micrococcus sp.* (Khire 1994).

Materials and Methods

Screening of hydrolase producing bacteria and culture medium

Saline soil samples were collected from salterns at different locations in Sonora, México. Isolates were taken from soil samples and streaked in marine agar (Difco) adjusted to 10% NaCL pH 7.35 and incubated at 37.5 ºC for 48 h to obtain bacterial growth. After incubation, they were restreaked in the same medium containing different specific substrates to induce the production of extracellular hydrolases as described by Rhoban *et al.* (2009). A hydrolytic clear halo around bacterial growth indicated positive hydrolytic activity from the isolates.

Amylase production and amylase activity

Amylase production was done in marine broth (Difco) adjusted to 10% NaCl, starch 2% (w/v), and pH 7.35, agitation 180 rpm, medium volume 70 ml in 125 ml flask, and incubation time 168 h. One ml samples were taken every 24 h and centrifuged for 10 minutes at 10,000 g. Supernatant was extracted and stored in micro tubes at -20 ºC.

Amylolytic activity of the enzymatic extracts was evaluated via reducing sugars using the DNS method as described by Miller (1969). One hundred µL of the enzymatic extract was mixed with 100 µL of a 2% starch phosphate (pH 6 – 8) and glycine – NaOH (pH 9 – 12) buffer solution. Solutions were incubated at 50 ºC in a water bath for 30 minute to initiate enzymatic activity. The amount of liberated reducing sugars was later determined by using 3, 5 dinitrosalicylic acid. Each sample was measured all by triplicate at 540 nm with a Biorad IMARK microplate reader. Quantification of protein in the extracts was done via Bradford reagent (Bradford, 1976).

Results and Discussion

Screening of hydrolase producing bacteria

The hydrolytic profile of five moderately halophilic bacteria from saline soils was evaluated. Optical microscopy and Gram stain revealed 4 Gram negative and 1 Gram positive short rods, and one Gram negative cocci. All five isolates showed combined hydrolytic behavior including amylase, xylanase, pectinase, inulinase and pullulanase (Table 1).

Table 1. Morphologic and hydrolytic profile of moderately halophilic isolates.

Isolate	Gram	Cell shape	Colony color	Protease	Amylase	Cellulase	Dnase	Lipase	Chitinase	Xylanase	Pectinase	Inulinase	Pullulanase	Esterase
PPAPAM1094	-	Rod	white	-	+++	-	-	-	-	++	-	+++	+	-
BLANEAM1041	+	Rod	white	-	++	-	-	-	-	+	+++	+++	+	-
PPSE3AM1065	-	Cocci	white	-	+	-	-	-	-	+	+	+	++	-
BLLS1AM1063	-	Rod	white	-	-	-	-	-	-	+	-	-	+	+
GRAM1043	-	Rod	white	-	-	-	-	-	-	+	-	-	+	-

All evaluated isolates showed positive behavior for the hydrolysis of at least 2 different substrates. Similar studies on both moderately and extremely halophilic bacteria also showed combined hydrolytic activities across a large number of isolates (Flores *et al*. 2010; Rhoban *et al.* 2009). However, in contrast to their results which showed a larger amylolytic, proteolytic and inulinolytic activity across the gram-positive isolates, our findings were that the highest amylolitic activities were from a Gram-negative rod, followed by a Gram-positive rod and lastly a Gram-negative coccus. Strains PPAPAM1094, BLANE2AM1041 and PPSE3AM1065 were selected for evaluation of their amylolytic properties.

Amylase activity

Amylolitic activity among the three isolates was found to be significantly different. The highest amylase activity for strains BLANE2AM1042 (148.21 IU/L), PPAPAM1094 (98.44 IU/L) and PPSE3AM1065 (43.48 IU/L) was obtained after 240, 120 and 216 h of incubation respectively (Figure 1). The highest activity of the enzymatic extracts was found to be at pH 11 across all isolates. Optimum pH was evaluated once the time of maximum enzymatic activity was determined via the DNS reagent method. Extracts at the time of

maximum activity were incubated for 30 minutes at 50 ºC with buffer solutions ranging from pH 6.0 to 12.0. After comparing results, it was determined that pH 11 would be the optimum for the quantification of amylolytic activity.

Mai *et al.* (1993) evaluated amylolytic activity in *Bacillus stearothermofilos* and *Bacillus licheniformis* and found that these microorganisms show activities of 190 and 150 U/mL respectively. Likewise, Niziolek (1998) evaluated extracellular amylase production from *Bacillus cereus*, *Bacillus megaterium* and *Bacillus polymyxa*, where maximum yield for each microorganism was 36, 93 and 204 IU/L respectively.

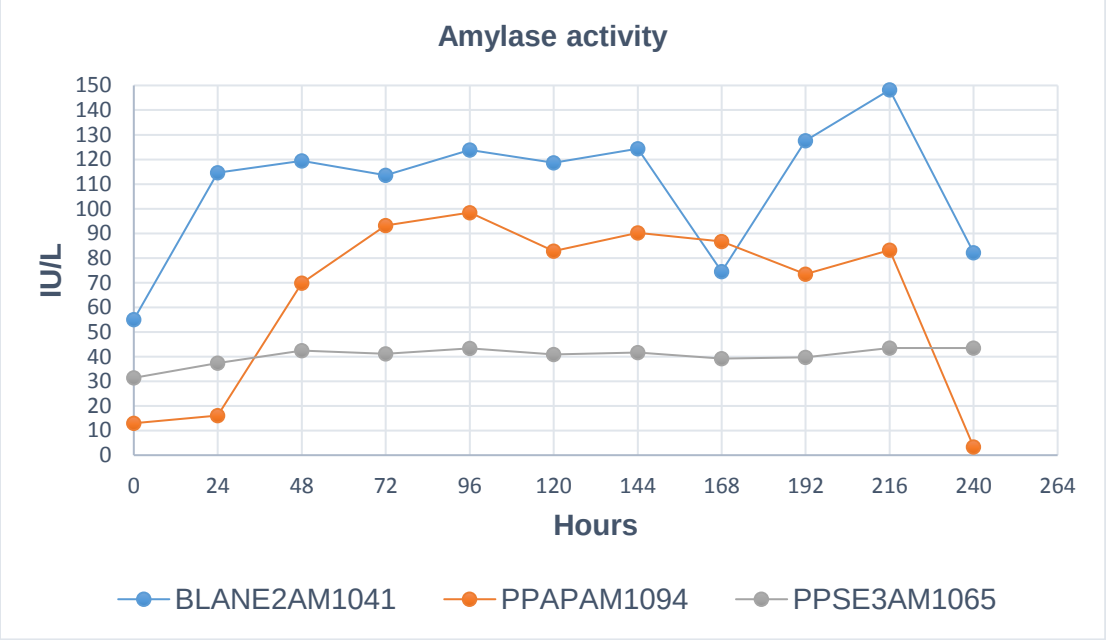

Figure 1. Kinetic of extracellular amylase activity from moderately halophilic bacteria.

Coronado *et al.* (1999) and Amoozegar *et al.* (2003) in their studies with moderately halophilic bacteria also found amylolytic activity at high temperatures and slightly alkaline pH of 7 to 8-5 and 50 ºC. Our findings show an optimal pH of 11 for maximum activity at 50 ºC which is much higher than those reported by the authors previously cited. Li *et al.* (2012) found an optimal pH and temperature of 9 and 55 ºC respectively while studying the inulinase production of the moderately halophilic *Marinimicrobium sp*. LS-A18. Based on the findings by the formerly cited studies, it suggests that the characteristics of the environments that these microorganisms live on has a big impact in how their metabolisms adapt and develop. Hypersaline environments have been proven to cover a wide range of pH, so it is possible that the soils from which these isolates were taken are highly alkaline. However, as Vidyasagar *et al.* (2006) and Annamalai *et al.* (2011) described in their studies of thermostable haloalkaline enzymes, optimal NaCl, pH and temperature values

for growth are not always the same as the optimal conditions for maximum enzymatic activity.

Conclusion

The present work reports the production of extracellular alkaline amylase by three moderately halophilic bacteria. Amylase produced by BLANE2AM1041 showed the highest activity, followed by PPAPAM1094 and finally PPSE3AM1065 at 50ºC and pH 11. Under optimal conditions, maximum amylase activity (148.21, 98.44 and 43.48 IU/L respectively) was obtained after 240, 120 and 216 h respectively. The high yield of exoamylase coupled with unique properties make these isolates promising candidates for production of alkaline amylases in novel biocatalytic bioprocesses; however, purification and further analysis of both the isolates and amylase described in this work is necessary and highly recommended.

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Selection of native fungi isolated from Yaqui Valley, Sonora, Mexico using enzymatic index for their application in biopulping process

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Abstract

Lignocellulose is the most abundant organic matter in nature, it is composed by three components, cellulose, hemicellulose and lignin. Biopulping is the pretreatment for delignifying lignocellulose using microorganisms, like fungi, as they have an enzyme complex with lignin peroxidase, laccase and manganese peroxidase. The native fungi may have the ability to delignifying lignocellulosic material such as soft wood, agroindustrial wastes and others. The selection of strains was performed by measuring enzyme activity of lignin peroxidase and xylanases for 14 days incubation and determination of enzymatic index for selection of strains producing such enzymes. From ten strains evaluated, the best strain was 623M23P1 with a value of 3.8351 ± 0.2913 as enzymatic index for Lignin peroxidase and strain 1010M31P2 with 1.38361 ± 0.00026 as enzymatic index for xylanases. This study has determined the potential for incorporate native fungi of Yaqui Valley in a pretreatment process of delignification by biopulping.

Keywords: lignocellulose ● lignin peroxidase ● xylanase.

Introduction

Lignocellulose is the most abundant organic matter in nature. It is a complex of three polymers: cellulose (35-50%), hemicellulose (25-30%) and lignin (25-30%) (Wang *et al.* 2011). For the extraction of cellulose it is necessary depolymerize or solubilize lignin which is the compound that gives recalcitrance to lignocellulosic waste. Thermal and chemical treatments for delignifying this waste are the most used, but they are expensive, they need an extensive spending of water for washing treated solids and they generate environmental pollutants. Biopulping is defined as the pretreatment of lignocellulosic

material (hard wood, soft wood, agroindustrial wastes, grasses, etc.) before physical-chemical processes and it is based in the ability of white rot fungi to degrade lignin (Ferraz *et al.* 2008). White rot fungi are widely known for their ability to degrade lignocellulosic material as they have a complex of specific enzymes such as laccases, manganese peroxidases and lignin peroxidases. The aim of this work is to test several native fungi strains from Yaqui Valley, Sonora, Mexico, by analyzing their enzymatic index to determine their potential for enhancing physicochemical treatment of lignocellulosic waste throw biopulping.

Materials and methods

Microorganisms

The native fungal strains (623M14P1, 623M23P1, 1010M31P2, 1010M24P1, 623M44P1H, 623M33P1H, 1010M42P1, 1010M11P10, 1010M41P1, 623M21P3) evaluated here were collected by COLMENA laboratory of Technologic Institute of Sonora in Yaqui Valley. All fungi were routinely maintained on 2% Malt extract agar (MEA).

Xylanolytic activity

The screening of the fungi looking for xylanolytic (hemi cellulolytic) activity was evaluated on MEA containing 0.1% (w/v) birch wood xylan (Sasidhara and Thirunalasundari, 2014). The plates were inoculated in the center with $2x10^6$ spores of fungal strains and they were incubated at 23°C for 14 days. Three replicates were maintained for each set of observations. Positive xylanolytic isolates were selected based on the clear zones of hydrolysis after flooding the plates with a 0.1% aqueous Congo red solution for 15 minutes, followed by repeated washing with 1M NaCl (Koyani *et al.* 2014).

Ligninolytic activity

The screening of the fungi for lignin peroxidases activity was evaluated on MEA containing 0.1 g/L of Azure B (Jing-Hua *et al.* 2015). The plates were inoculated in the center with $2x10^6$ spores of fungal strains and incubated at 23°C for 14 days. Three replicates were maintained for each set of observations. Positive lignin peroxidase activity isolates were selected based on change of color from purple to light blue.

Enzymatic Potential

Xylanases production were indicated by the appearance of a pale halo with orange edges, they were hydrolysis areas. Lignin peroxidases production were indicated by areas with color change from purple to light blue. These halos were measured with Image J 1.44

software for subsequent calculation of the enzymatic index (EI) using the expression (Florencio *et al.* 2012):

$$EI = \frac{\text{diameter of hydrolysis zone}}{\text{diameter of colony}}$$

The results were reported as mean ± standard deviation (SD) of triplicate samples. ANOVA simple-test was performed to compare mean the best enzymatic index between strains. The significance of differences among treatment means was determined using Statgraphics Plus 5.1 with P-values <0.05.

Results and discussion

The Table 1 presents the results of the experiments, where -* indicates non detectable enzymatic activity.

Table 1. Enzymatic index results

Strain	E.I. LiP	E.I. Xylanase
623M14P1	1.1410 ±0.0189	1.03128 ±0.00019
623M23P1	3.8351 ±0.2913	1.2931 ±0.00098
1010M31P2	1.2002 ±0.1317	1.38361 ±0.00026
1010M24P1	1 ±0.0	-*
623M44P1H	-*	-*
623M33PIH	-*	1.09609 ±0.00226
10101442P1	-*	-*
1010M11P10	1.4838 ±0.2822	-*
1010M41P1	-*	-*
623M21P3	-*	-*

In order to select the strains, enzymatic index calculation were performed. The strains were more desirable or selectable if the value of EI was higher, which means that the relative enzymatic activity was higher. The study was conducted for 14 days (figure 1). In a descending order of E.I., the strains were ordered for LiP activity as follows, 1010M41P1>623M23P1>1010M11P10>623M14P1 and for Xylanases, as 1010M31P2> 623M23P1>623M14P1.

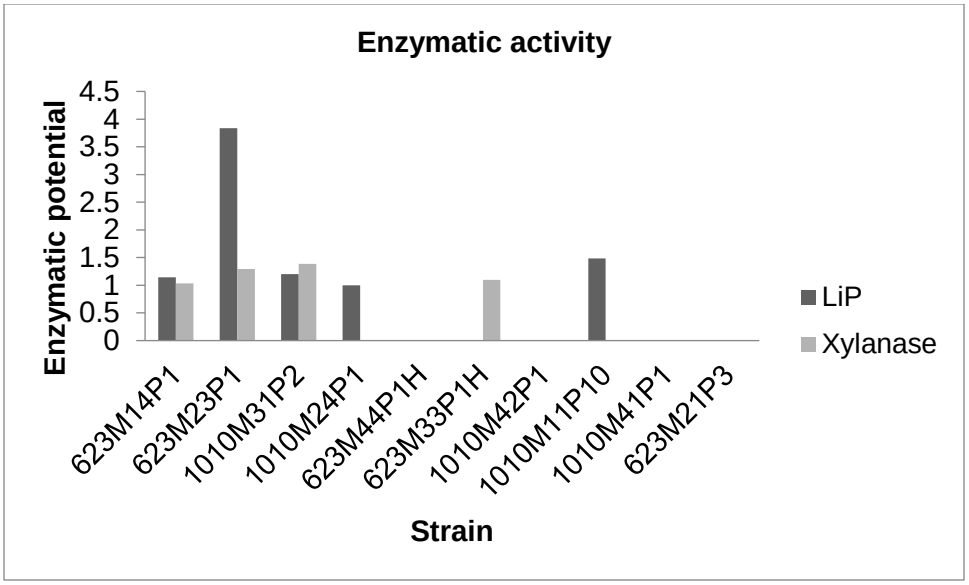

Figure 1. Enzymatic Index by enzymatic halo formation in 14 days of incubation

De la Rosa-Hernández *et al.* (2011) assessed 20 native strains finding 19 strains with xylanolytic capabilities as Lugol-iodine halo indicator of xylanolytic enzyme activity. Garcia-Gomez *et al.* (2003), selected fungal strains by enzyme index value for producing proteases strains.

Conclusions

Strains with lignin peroxidases and xylanases activity were obtained from Yaqui Valley strains. 623M23P1 strain presented the best activity for LiP and 1010M31P2 strain presented the best activity for xylanases. They have potential for their use in a process of delignification of lignocellulosic biomass.

Acknowledgments

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Search of halophilic microorganisms with proteolytic activity and identification of type of protease of extract enzymatic

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Abstract

Microbial life can be found over a wide range of extreme conditions (salinity, pH, temperature, pressure, light intensity, oxygen and nutrient conditions). Halophilic microorganisms are a good example of extreme microorganisms that are a good source of enzymes of industrial interest. There were studied 13 strains isolated from high-salt environments in the state of Sonora and three of them presented a significant production of proteases and a kinetic of protease production was carry out. Determination of protein concentration and specific enzymatic activity confirms the production of proteases by three strains and an enzymatic inhibition by PMSF and Chymostatin indicates a presumptive identification of serine proteases produced by two of those strains.

Keywords: halophiles●enzymes●proteases●serine protease●inhibitors.

Introduction

Microbial life can be found over a wide range of extreme conditions (salinity, pH, temperature, pressure, light intensity, oxygen and nutrient conditions). Hypersaline environments constitute typical examples of environments with extreme conditions due to their high salinity, exposure to high and low temperatures, low oxygen conditions and in some cases, high pH values. Bacteria and Archaea are the most widely distributed organisms in these environments (Moreno *et al.* 2013). The world of halophilic microorganisms is highly diverse. The brines of saltern crystallizer ponds worldwide are colored pink-red by Archaea (*Haloquadratum* and other representatives of the *Halobacteriales*), Bacteria (*Salinibacter*), and Eucarya (*Dunaliella salina*) (Ma *et al.* 2010).

To adapt to saline conditions, bacteria have developed various strategies to maintain cell structure and function. Studies of such bacteria are of great importance, as they may produce compounds of industrial interest, such as extracellular, hydrolytic enzymes that have diverse potential usage in biomedical science and chemical industries. Most

industrial process are carried out under specific physicochemical conditions which may not be definitively adjusted to the optimal points required for the activity of the available enzymes; thus, it would be of great importance to have enzymes that exhibit optimal activities at various ranges of salt concentration, pH and temperature (Rohban *et al.* 2009).

Material and methods

The selected strains were spread in Marine Agar adjusted to 10% of NaCl incubated at 37ºC for 48 h. A Gram staining was performed for each strain and for the enzymatic screening the strains were spread in Ventosa Agar (Ventosa, 1982) with inducers 1-2% (w/v) and incubated at 37ºC. After 48 h the petri dishes with hydrolytic halos were selected. In some inducers it was needed a hydrolysis reveal with lugol, congo red 0.1% or chlorhydric acid 1 N. As inducers were used skim milk, starch, carboxymethyl cellulose, DNA, olive oil, chitin, xylan, pectin, inulin from agave and chicory, chitin deacetylase, pullulan, tween 80 and keratin.

A kinetic of protease production were performed with the selected strains ($1x10^6$ cells/mL) in marine broth adjusted to 10% of NaCl and 2% of skim milk incubated at 37ºC and 180 rpm for 6 days with samples collected every 24 h. Protein concentration (Bradford, 1976) and enzymatic activity (Iversen *et al.* 1995) were determined to obtain the specific enzymatic activity. Electrophoresis was performed with 12% SDS-PAGE (Laemmli, 1970) at 15 mA per gel for 90 minutes. Silver stain (Wray *et al.* 1995) and zimogram (Lemos *et al.* 1999) of casein hidrolysis were carry out for each gel.

For enzymatic characterization there were used enzymatic inhibitors: EDTA (20 mM), Pepstatin A (1.4 mM), E64 (10 uM), TPCK (5mM), TLCK (10 mM), PMSF (100 mM), Tripsyn inhibitor (100 µM) and chymostatin (100 µM). Inhibitors were tested in the method of enzimatic activity determination (Iversen *et al.* 1995) with the addition of 10 µL of each inhibitor before the incubation at 37ºC.

Results and discussion

Table 1 shows the morphological characteristics of the strains obtained by Gram staining and observation of colonies.

In Figure 1 is shown the protein concentration and specific enzymatic activity of the three strains selected from the enzymatic screening: PPSE3AM1057, BLLSAM1058 and BLLS-3. In the three graphs there is a decreasing in protein concentration that confirms the production of proteases that hydrolyses the casein from skim milk and also there is an increasing of the specific enzymatic activity that corresponds to the protein concentration decreasing. In BLLS-3 graph there is an increasing of the specific enzymatic activity after 120 h that could have been caused by cell lysis.

Table 1. Morphological characteristics of the strains.

	Strains											
	YPE2AM1034	**YIEAM10610**	**YRAM1062**	**PPPCAM1063**	**PPPCAM1064**	**PPSE3AM1057**	**BLRMAM1065**	**BLCCAM1061**	**BLLSAM1058**	**BLCCAM1062**	**GRAM1062**	**C5**
Gram	-	-	-	-	-	-	-	-	-	-	-	-
Shape	Rod	Cocci	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Colony color	Yellow	White	White	White	White	White	White	White	White	White	White	White

Enzymatic screening of the strains is shown in Table 2. As can be seen in the table, there is significant positive results for skim milk or proteases production. The strains with the best results of proteases production were selected for a kinetic.

Table 2. Enzymatic screening. (+): < 1 mm. (++): > 1 mm.

		Strains											
		YPE2AM1034	YIEAM10610	YRAM1062	PPPCAM1063	PPPCAM1064	PPSE3AM1057	BLRMAM1065	BLCCAM1061	BLLSAM1058	BLCCAM1062	GRAM1062	C5
Inducers	Skim milk	-	-	++	++	++	++	++	-	++	-	-	+
	Starch	-	-	+	+	+	++	++	-	++	+	+	+
	Carboxymethyl Cellulose	-	+	-	-	-	-	+	-	+	-	-	-
	DNA	-	-	+	+	+	-	++	-	++	-	+	-
	Olive Oil	+	-	+	-	-	+	-	+	-	-	-	-
	Chitin	-	-	-	+	+	-	+	-	+	-	-	+
	Xylan	+	+	+	+	+	+	++	+	++	+	+	-
	Pectin	+	+	+	+	+	+	+	+	-	-	+	+
	Inulin from agave	-	+	-	++	++	++	++	-	++	-	+	+
	Inulin from chicory	+	+	+	+	+	++	+	+	++	+	-	+
	Chitin deacetylase	-	-	-	-	-	-	-	-	-	-	-	-
	Pullulan	+	+	+	+	+	+	+	+	+	+	+	+
	Tween 80	-	+	+	-	-	+	-	-	-	-	-	-
	Keratin	-	-	+	+	+	+	+	-	+	-	-	-

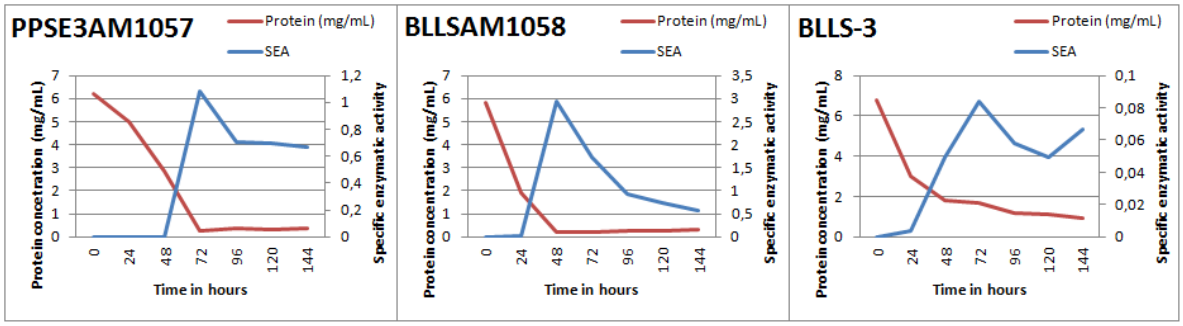

Figure 1. Protein concentration and specific enzymatic activity from kinetic of protease production of the strains PPSE3AM1057, BLLSAM1058 and BLLS-3.

Electrophoresis was developed to confirm the results obtained from the kinetic of protease production and to detect which proteins had proteolytic activity. The results of electrophoresis in SDS-PAGE gels are shown in Figure 2. In D (PPSE3AM1057) there appear to be two proteins that caused the casein hydrolysis, while in E (BLLSAM1058) and F (BLLS-3) there are several proteins that caused the hydrolysis.

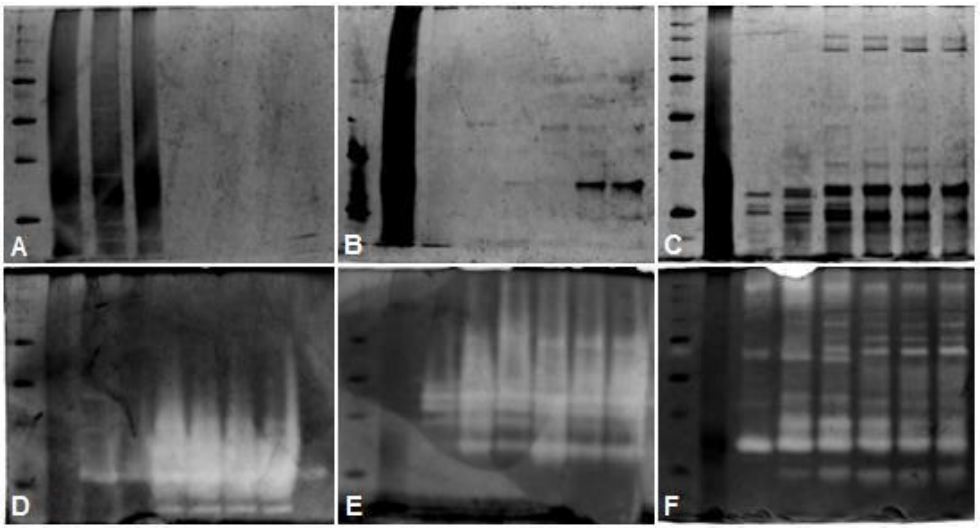

Figure 2. SDS-PAGE protein gels (A,B and C) and zimograms (D,E and F). PPSE3AM1057: A and D. BLLSAM1058: B and E. BLLS-3: C and F.

In Figure 3, there is not apparent effect in enzymatic activity for the proteases of strain BLLSAM1058 but for the proteases of the strains PPSE3AM1057 and BLLS-3 there is a presumptive identification of serine proteases.

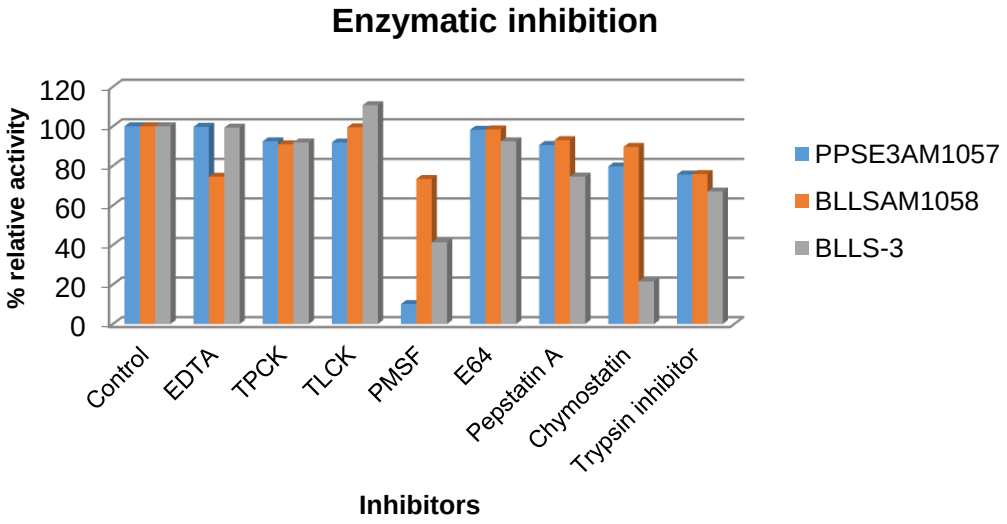

Figure 3. Enzymatic inhibition of enzymatic extracts of the strains PPSEAM1057, BLLSAM1058 and BLLS-3.

Conclusions

There were successfully produced extracellular proteases from halophilic microorganisms isolated from the state of Sonora. The enzymatic screening indicates that halophilic microorganisms are a good source of enzymes. The results from the determination of protein concentration were confirmed by the SDS-PAGE electrophoresis. It was also shown a presumptive identification of serine proteases produced by the strains PPSE3AM1057 and BLLSAM1058. There may be needed a concentration and possibly a purification of the enzymatic extracts to confirm the presumption.

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BIOTECHNOLOGY SUMMIT

Isolation of moderately halophilic microorganisms searching of proteolytic enzymes

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Abstract

Proteases are enzymes that hydrolyze the peptide bonds of proteins, represent one of the three groups of industrial enzymes. In this researching of halophilic microorganisms producing proteases from different saline soils of Sonora State, this study was divided into two parts: the first part will include screening of moderately halophiles bacteria collected from various regions with a total of 210 moderately halophilic strains were isolated, relying on the isolation of strains with potential proteolytic activity such as BLRMAM1066, BLCLAM1064, PPSE3AM1053; while the second part will consist the enzyme production by fermentation in liquid medium both at flask. As a result of this research, it should be a collection of halophiles with proteolytic activity.

Key words: Isolation●Screening●Moderately halophiles●Saline areas●Proteases

Introduction

The proteases are the only class of enzymes that occupy an important position with respect to its application in the commercial and biological fields (Banik and Prakash, 2004). These enzymes catalyze the breakdown of peptide bonds in other proteins, are degradative enzymes which catalyze the complete hydrolysis of proteins and represent one of the three largest groups of industrial enzymes (Oren, 2010). Microorganisms are an excellent source of enzymes, these accounts for 40% of global sales of enzymes (Litchfiel, 2011). In addition to its physiological importance, this group of enzymes has enormous industrial interest being widely used in the detergent industry, food, beverage, textile and paper (Margesin and Schinner, 2011). On the other hand, although the proteases have a very specific action, it is a very diverse group of enzymes, making it very attractive for biotechnological exploitation (Nigam, 2013).

Estimates worldwide sales of industrial enzymes are very high, of which 75% have hydrolytic activities (Prabhavathy et al. 2012). Proteases represent one of the three main

groups of industrial enzymes (Li et al. 2013). This domain of proteases in the industrial market is expected to continue to increase (Sánchez et al. 2003). Demand for enzymes with strict requirements of various biotechnological processes have led to investigations of proteolytic enzymes that satisfy both market needs and environmental preservation. Proteases produced today have proved; years biotechnological tool useful, not only to improve the efficiency of industrial processes, which have surpassed traditional methods (detergents, textiles, silver recovery, etc.). Therefore, there is a need for novel enzymes having properties with greater tolerance to factors such as temperature, pH, salinity; current can be coupled to the extreme conditions of industrial processes (Margesin and Schinner, 2011).

Materials and methods

Isolation and identification of protease producing bacterial strain

Diverse bacterial strains were isolated from soil samples of different saline areas of Sonora State such as Bahia de Lobos, Yavaros, Guaymas and Puerto Peñasco. Pure bacterial isolates were grown at 37°C for 48 h onto the marine agar medium containing 10% NaCl and 2% skim milk. The clear zone of hydrolysis around the colony confirmed the protease production. Bacterial strain was selected on the basis of the maximum diameter hydrolysis.

Screening of strains for extracellular hydrolytic activities

In order to obtain the production of extracellular enzymes, screening of strains of a total of 210 moderately halophilic strains were isolated from different saline areas from Sonora State. Enzymatic agar plate assays were performed. The pH of all media was 7.35 and 10% NaCl were added for detecting hydrolytic activities for moderately halophilic bacteria. Twenty-two halophilic bacteria were screened for production of different extracellular enzymes like amylase, lipase, protease, cellulase, inulinase, xylanase, pectinase, quitinase, pullulanase, esterase and DNAase. Halophiles were isolated by salt (100.0 g/L, NaCl) and substrate enrichment. The marine agar supplemented with respective substrates was used for production of these enzymes.

Individual colonies were transferred to marine agar plates. All isolates, which formed a remarkable clearing zone on 2% skim milk marine broth such as BLRMAM1064, PPSE3AM1053, and BLCLAM1069. The cultures were incubated at 37°C with rotary shaking at 120 rpm and adjusted pH 7.35. The concentration of protease production was checked every 24 h during five days. The culture was centrifuged at 10,000×g for 10 min at 4°C.

Protein determination

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard. Protein concentration was estimated by observing the absorbance at 595 nm. All experiments were done in triplicate.

Protease assay

The activity of protease was measured by a slightly modification of Iversen and Jorgesen (1995). The crude enzyme solution was incubated with azocasein solution. The supernatant was harvested; one milliliter of supernatant was read at 366 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of azo group in 1 min at 37°C. The specific activity is expressed in units of enzyme activity/mg of protein.

Molecular weight determination by SDS-PAGE

Electrophoresis of the enzyme extract samples taken at different time intervals was performed according to the method of Laemmli (1970). Protein electrophoresis was carried out in 12% polyacrylamide gel using a Mini Protean Tetra Cell Plus (Bio-Rad, USA). Molecular weight of protease was estimated by comparing the relative mobility of proteins of different molecular size using a Precision Plus Protein Standards molecular weight marker (Bio-Rad USA). Gels were fixed and stained with silver nitrate as described by Blum et al. (1987).

Results and discussion

Qualitative evaluation of production of proteases

Definitely, the strains producing proteolytic enzymes showed extracellular form, the marine agar medium supplemented with skim milk 2% and incubated at 37 ° C was used. That is, the release of proteases and the activity of these same will be evidence by Alquicira (2003). Studies by Sánchez et a. (2004) reported that 26 strains isolated from fish effluent and grown in culture medium containing 1% casein. Sharmin et al. (2005) obtained between the inmates bacteria, Bacillus amovovirus, exhibited proteolytic capacity in the hydrolysis of casein agar skim milk; Vishwanatha et al. (2010) in the search for substrates for the production of proteases from Bacillus licheniformis observed hydrolysis medium casein (1% w/v) milk powder 1%, use similarly, Hindhumathi et al. (2011) with Bacillus sp GPA4 agar incubated skim milk provided a clear zone of hydrolysis due to proteolysis after 24 hours of incubation.

Clearly sources of carbon and nitrogen are transformed as microorganisms metabolized them, and in any case the nutrients contain all molecules a cell requires, so the breakdown of nutrients in compounds useful for the maintenance of the microorganism is carried out by performing biochemical reactions enzymes. Therefore, based on the ability of these strains to produce proteases such as: BLRMAM1066, PPSE3AM1053, and BLCLAM1064.

Screening bacteria from saline soil, mud, brine and salt sediments of Bahia de Lobos, Yavaros, Guaymas and Puerto Penasco led to the isolation of 22 moderately halophilic bacteria which there were 7 Gram-positive (5 rods and 2 cocci), 15 Gram-negative (7 rods, 6 Gram-positive coccobacilli, 1 coccus and 1 vibrio).

Combined hydrolytic activities have been detected in a number of strains. Only four strains presented the eleven hydrolytic activities tested (amylase, DNase, protease, pectinase and xylanase). Besides, 14 strains showed three combined hydrolase activities and 5 strains were able to produce two extracellular enzymes (Table 1).

Table 1. Cellular morphology and hydrolytic activity of moderately halophilic strains from Bahia de Lobos, Yavaros, Guaymas and Puerto Penasco.

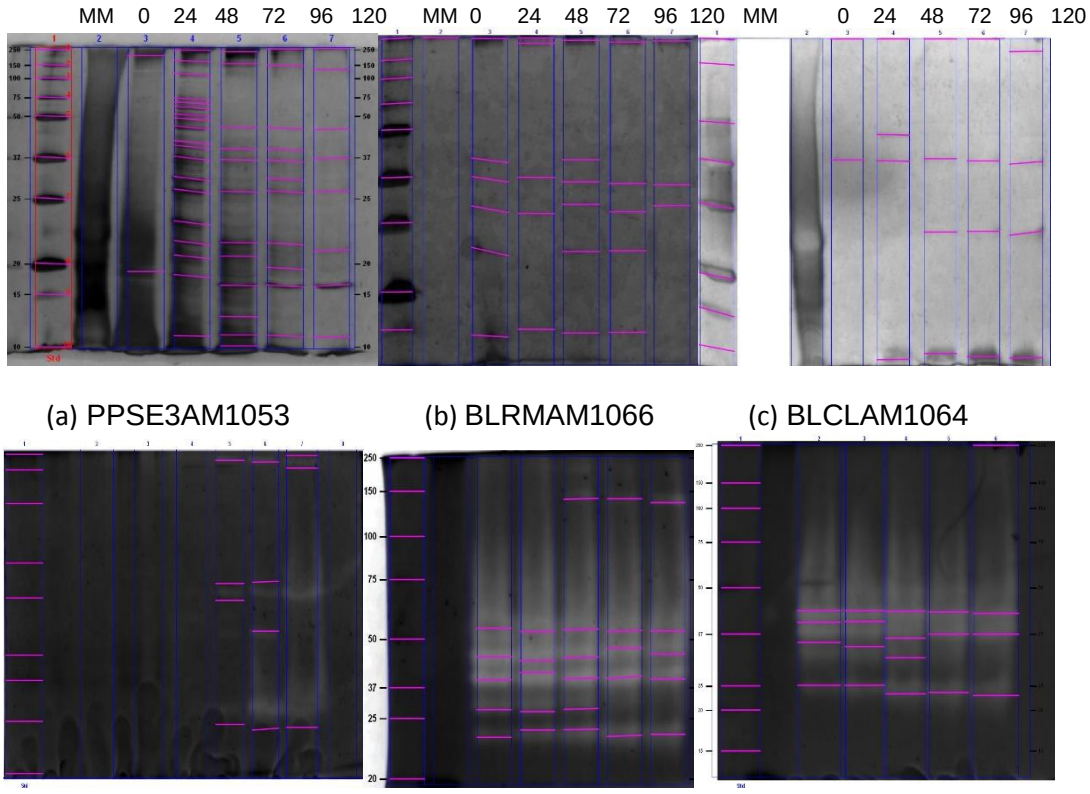
			Hydrolitic activities										
Strain	Gram	Celular Morphology	Skim milk	Starch	CMC	DNAase	Olive oil	Chitin	Xylan	Pectin	Inulin	Pullulan	Tween 80
BLCCAM1063	-	bacillus	-	-	-	-	-	-	+	-	+	+	-
BLCLAM1063	-	coccobacillus	+++	-	-	-	-	+	+	-	-	+	-
BLCLAM1064	-	bacillus	+++	-	-	++	-	+	+++	+		+	-
BLLSAM1054	-	coccobacillus	-	-	-	-	-	-	+	-	-	+	-
BLLSAM1067	-	coccobacillus	+++	-	-	-	-	+	+	-	-	+	-
BLLSRAM1069	-	coccobacillus	-	-	-	-	-	-	+	-	-	+	-
BLNM30AM1064	-	bacillus	-	-	-	-	-	-	+	-	+	+	-
BLRAM1067	-	vibrio	-	-	-	-	-	-	+++	++	+	+	-
BLRMAM1062	-	bacillus	-	-	-	-	-	-	-	-	-	+	+
BLRMAM1066	-	coccobacillus	+++	-	-	++	-	+	+++	+	+	+	-
GRAM1041	-	bacillus	-	-	-	-	-	-	+	+	+	+	-
GRAM1063	-	bacillus	-	+	-	-	-	-	+	-	-	+	-
PPAPAM1042	-	coccus	-	-	-	-	-	-	+	-	-	+	+
PPAPAM1051	-	bacillus	-	-	-	+	-	-	+	-	+	+	-
PPPCAM1062	+	bacillus	-	-	-	-	-	-	+	+	+	+	-
PSE3AM1053	+	bacillus	+	+	-	-	-	-	+	+	-	+	-
YIEAM10610	+	bacillus	-	-	-	+	-	-	+	+	+	+	-
YPE2AM1031	+	bacillus	-	-	-	-	-	-	+	-	+	+	-
YPE2AM1036	+	coccus	-	-	-	-	-	-	+	-	-	+	-
YRAM1064	+	bacillus	+	-	-	-	-	-	+	-	-	+	-
YRAM1068	+	coccus	-	-	-	-	-	-	+	-	-	+	-
YRAM1069	-	coccobacillus	+++	-	-	++	-	+	+++	++	+	+	-

Determination of concentration of soluble protein and proteolytic activity in marine agar with skim milk 2%

To stimulate the enzymatic activity of these microorganisms and in order to study the ability to metabolize protein substrates as sole carbon source and nitrogen kinetics fermentation was carried out for five days in culture. The strains BLRMAM1066, PPSE3AM1053 and BLCLAM1064 to grow and develop their activities, should have the nutrients that provide energy and materials for the biosynthesis of the culture medium.

Polyacrylamide gel electrophoresis and determining the molecular weight.

The enzyme found from crude extract fermentation kinetics appeared several bands in SDS-PAGE gel 12%, some of them constantly, from 193.6 kDa to 19.15 kDa in all evaluated times (Figure 3a, 3c and 3e) and analysis zymogram with casein as substrate proteolytic bands were obtained, being the most important the 96 hours (Figure 3b, 3d and 3f).



(a) PPSE3AM1053 (b) BLRMAM1066 (c) BLCLAM1064

(d) PPSE3AM1053 (e) BLRMAM1066 (f) BLCLAM1064

Figure 3. (a,c,e) Electrophoresis of enzymatic extract SDS-PAGE gel at 12%, using silver staining by Chevallet *et al*. (2006). MM lane molecular weight marker. Figure (b, d, e) zymogram, using 1.5% casein as substrate.

The apparent molecular weight of these proteases were obtained from 25 kDa. Reports Rao *et al.* (2009) the appearance of a single band on zymography with molecular mass of 39.5 kDa. Hernandez-Martinez *et al.* (2011) cited that can be found in the literature, different molecular weights to proteases and serine proteases reports 32 kDa and 124. In comparison, with regard to estimates and research Annamalaia *et al.* (2014) of *Bacillus firmus* purified proteases CAS 7; molecular masses ranging from 21 kDa *Bacillus firmus* Tap in 5 of 34 kDa and *Bacillus cereus* TKU006 33 kDa.

Conclusions

210 strains were isolated from soil samples of different saline areas of Sonora, but these remarkable strains moderately halophilic bacillus Gram (-) BLCLAM1064, PPSE3AM1053 and coccobacillus Gram (-) BLRMAM1066 have been found to be able to secrete extracellular proteases. The molecular masses of proteases were found in the range of 19 to 193 kDa. Also it highly recommended to characterized biochemically and purified these enzymes, suggested that they have may potential for use as biocatalyst in industry.

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BIOTECHNOLOGY SUMMIT

Evaluation of protease activity from two brewing malts

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Abstract

Alcoholic fermentation is a set of biochemical transformations in which, certain yeast strains are responsible for transforming the carbohydrates present in wort, in a liquid containing alcohol and carbon dioxide. Therefore, processing of wort is a high-impact process. In this, proteases play a vital role because its hydrolytic activity generates worts rich in nutrients such as amino acids and peptides, which are essential for fermentation. The role of these biomolecules is well known, but there are still fundamental questions about the variation of the content and type of these proteins. In this study we analyze the protease activity of two malts using zymograms and we found differences in the profile and level of activity of protease with cysteinyl-type activity.

Keywords: Brewing malt ● zymogram ● proteases ● proteome.

Introduction

Biotechnology has been part of preparation of beer since the beginning, the study of brewing process has contributed to increase the knowledge of important features of beer, mainly in areas like raw material quality, taste, aroma, appearance and microbiological quality, which has given greater durability, increase in production and demand.

The need to combine agronomic aspects with industrial quality makes to malting barley a complex crop to work on genetic improvement; therefore, the failure to have a reliable marker of malt quality, could cost to the beer company substantial gains per year in expenses process adjustments to compensate for the lack of quality of the raw material. Malt proteases play an important role because they determine the type and concentration of amino acids in the wort, which are metabolized by yeast and generate ester type flavors and set the flavor profile of beer. Because we do not have a full knowledge and control about the synthesis of compounds that affect the characteristics of beer, proper characterization of malt is necessary to know the levels of expression of yeast genes involved in the production of volatile compounds in the beer and to study the relationship

between proteases and amino acids present in the wort. Because of this, we decided to study the protease activity in malt to help to brewer to accept, reject or combine malt types using specific molecular markers as selection criteria to control the characteristics of final beer.

Materials and methods

Two samples of brewing malt named M1 and M2, were provided by Cervecería Cuauhtémoc-Moctezuma. The extraction of soluble proteins was carried out using two buffer conditions, total protein was quantified by Bradford technique and protein profiles were–separated by SDS-PAGE and proteome analysis was performed with 2D-PAGE. Protease-like activity was studied using zymograms with native and reducing conditions incorporating gelatin as substrate, in these tests we evaluated the optimum pH and the concentration at which enzyme activity is stable for both malts, finally specific inhibitors were employed, 10 mM PMSF and 10 μM E64, to determine whether the samples had protease activity cysteinyl-type.

Results and Discussion

Protein profiling malt

In this paper we investigate the profile of proteases into two brewing malts. The soluble protein fraction of each malt was extracted with 5 mM Tris-HCl at pH 7.5 and acetate buffer 50 mM pH 5.0: 50 μg of sample was applied to each lane (Wrobel and Jones (1992); Ostergaard *et al.* (2004)). The profile and soluble protein concentration, showed no effect of pH on the extraction (Figure 1). Only a few bands in both malts show an increase in the concentration with pH 5.0.

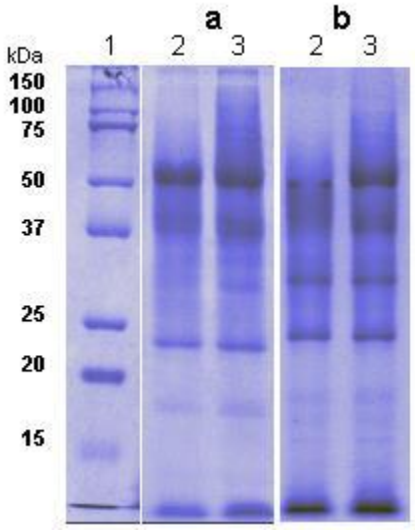

Figure 1. Malt samples protein profile. **(a)** pH 7.5 **(b)** pH 5.0 **(b)**. Lanes: **1.** Molecular weight marker Kaleidoscope (Bio Rad); **2.** Malt M1. **3**. Malt M2.

Native zymograms condition

Proteolytic activity assays showed that there are differences between the two malts. To characterize proteases presents in each malt, zymograms gels were performed using 12% polyacrylamide and 1 % gelatin copolymerized in native conditions. 50 µg of sample was applied to each lane. After separations, gels were incubated at different pH. We identified several bands with protease activity in each malt as shown in Figure 2, these results are very similar to those reported by Jones and Marinac (2002).

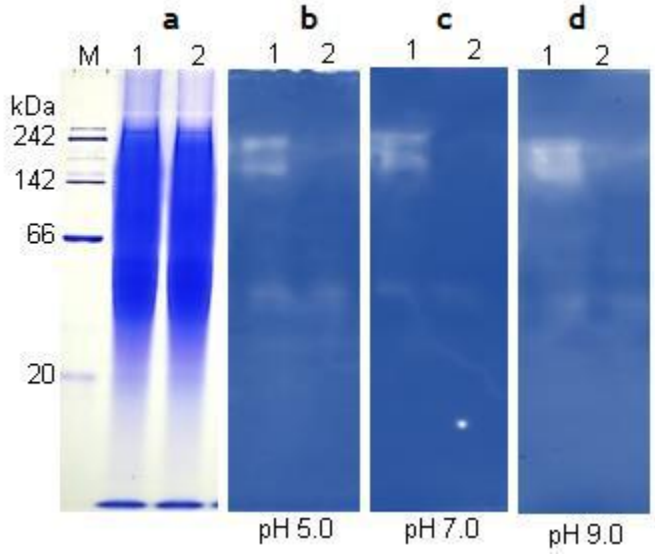

Figure 2. Effect of pH on the proteolytic activity in zymograms sandwich-type. **a)** protein profile of malts. **b), c)** and **d)** show the proteolytic activity in different pH values. **M.** Native molecular weight marker (Invitrogen). **1.** Malt M1. **2.** Malt M2.

2D-Zymograms

Protease activity of both malts was analyzed by 2D-zymograms in in 12% polyacrylamide with 0.2% gelatin copolymerized in reducing conditions. 200 µg of sample was applied to each lane. After separations, gels were incubated in 0.05 M Acetate buffer pH 5.0. We observed differences between malts as shown in Figure 3.

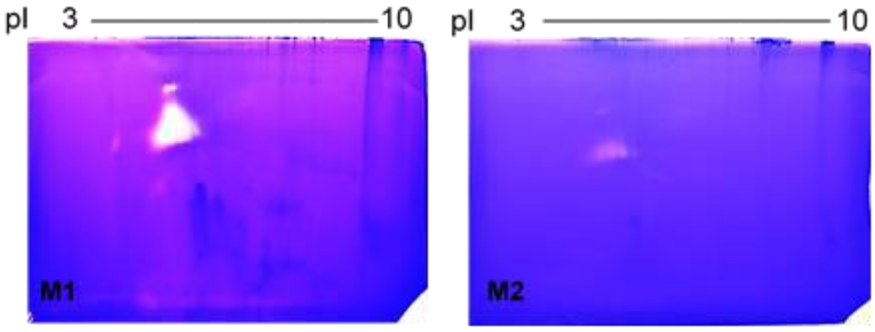

Figure 3. Effect of pH on the proteolytic activity in 2D-zymograms.

Biochemical characterization of catalytic molecules

Zymogram gels for protease activity in polyacrylamide gel 10% with 0.1 % gelatin copolymerized in reducing conditions. Gels were incubated in phosphate buffer 0.1M pH 6.0, to determine the type of protease present in each malt we used specific protease inhibitors for cysteinyl-type activity, E-64 [10 µM] and PMSF [10mM] (Figure 4).

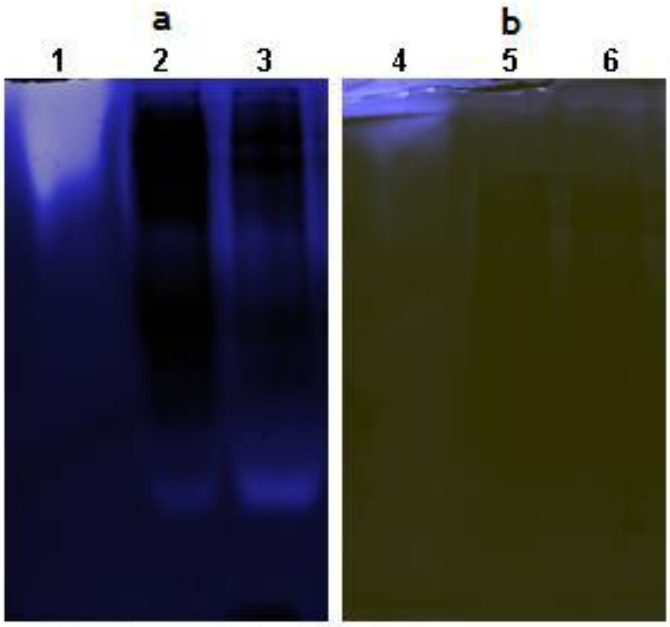

Figure 4. Effect of specific inhibitors on the proteolytic cysteinyl-type activity **a)** No inhibitors, **b)** 10 mM PMSF and 10 µM E64: **1, 4.** Trypsin 0.02 µg/ml. **2, 5.** Malt M2. **3, 6.** Malt M1.

Both malts were inhibited completely, so we assume that proteases present in both malts are cysteinyl-type mostly with different levels of activity. We are now in the process of studying other types of protease activity to characterize the "proteasome" of both malts to identify which could serve as quality markers.

Conclusions

We were able to determine the protein profile of malt samples extracted at pH 5.0 and pH 7.5. We found that M1 has more proteolytic activity at different pH as it has more bands with activity, while M2 has only 1 band of approximately 30-50 kDa. Using 2D-zymograms, we observed activity in a pH range of 4.0-5.0, being the M1 which showed greater proteolytic activity. We determined cysteinyl-type protease activity using zymograms under reducing conditions in the presence of E-64 10 µM and PMSF 10 mM, which are specific inhibitors for this type of protease. The M2 has greater cysteinyl-type activity since inhibition is clearly seen to be in contact with the inhibitors. We were able to detect at least 4 bands of cysteinyl-type proteases.

Acknowledgements

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Tolerance to ammonium of *Chlorella vulgaris* under laboratory conditions

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Abstract
The microalgae have the potential of consume high amounts of nitrogen, phosphorus from wastewater avoiding the risk of eutrophication of the water bodies. The wastewaters have different nitrogen species but the ammonium usually has the higher concentrations and can inhibit the growth of microalgae. Tolerance to ammonium is specific of each strain and the presence of this compound can causes different effects; so the study of the tolerance of every microalgae strain is the first step in the development of tertiary wastewater treatment proposals employing microalgae. *C. vulgaris* reached 0.49 g L^{-1} of DW at 66 ppm N-NH_4 with complete depletion of ammonium in the medium; also phosphorus consumption rate was not affected by the addition of N-NH_4 and was close to 2 mg $L^{-1}d^{-1}$ in all the experiments. This allows to propose this strain of *Chlorella* for the removal of nitrogen and phosphorus from tertiary wastewater and the biomass obtained in the process can be used for the production of high value products as protein, carbohydrate or used for animal feed.

Keywords
Wastewater • Microalgae • Ammonium • *Chlorella vulgaris*.

Introduction

In the last years the microalgae has been proposed as an alternative treatment for wastewater; this because they have the capacity to consume high quantities of nitrogen and phosphorus, to remove heavy metals and because its oxygen production improves the inactivation of pathogens (Cañizares-Villanueva *et al*. 2001; González *et al*. 1997). For that, wastewater with high amount of nutrients is a good culturing media for the microalgae.

Also there is another advantage, the microalgae can use different nitrogen sources as ammonium, nitrate, nitrite, urea, etc. (Barsanti and Gualtieri, 2006). The nitrogen from ammonium (N-NH_4) is the best; this because does not need enzymatic modifications for its use (González *et al*., 1997). The nitrogen consumption rate is high because the nitrogen constitutes an important fraction of the microalgae biomass in some conditions can be even 10% of the dry weight (DW). The value always depends of the availability, specie used and culturing conditions (Isleten-Hosoglu *et al*. 2012).

In the wastewater, the availability of the N-NH_4 depends directly of chemical conditions as temperature, pH, etc. (Cornet *et al*. 1992); this is an inconvenient because can affect the growth of the microalgae especially when the concentration is elevated. The N-NH_4 is toxic at high concentrations for microalgae because inhibits the photosynthetic apparatus specifically (Cornet *et al*. 1992; Soletto *et al*. 2005) the electronic transport in the Electronic Transport Chain (ETC).

Chlorella, is a genus reported as an efficient consumer of nitrogen and phosphorus from its culturing media and also when is used for the treatment of wastewater. Nevertheless, some strains have sensibility to high concentrations of N-NH_4; so, its tolerance to ammonium has to be investigated previously to propose it for the tertiary treatment of wastewater (Rehman and Shakoori, 2001).

Due to this particular characteristic this project has the objective to determinate the tolerance of *Chlorella vulgaris* to different N-NH_4 concentrations to know its potential to remove nitrogen and phosphorus from wastewater.

Materials and Methods

Chlorella vulgaris was provided by Pof. Hugo V. Perales Vela from the Facultad de Estudios Superiores from UNAM. To evaluate the growth and productivity of *C. vulgaris* several experiments in the lab were carried out. Five different concentrations of N-NH4 corresponding to 22, 44, 66, 88 y 110 ppm of N-NH_4 and the BBM as a control were assayed using. The experimental units consisted in 1 L Kimax® bottles constantly bubbled with air illuminated using cool white light at ~ 300 µEm-$2s^{-1}$ and at 25°C. The reported results are the mean of three independents experiments. The analytical determination were: DW, nitrogen and phosphorus concentrations (N-NH_4 and Pi respectively), Chlorophyll (Total) (ChlT) and Carotenoids (Car).

The determination of Pi and N-NH_4 was done according to APHA, 2012 to the supernatant. For the DW a volume of culture was filtered using a Fiberglass filter and dried in over until constant weight (Martínez-Roldán *et al*. 2014). For the ChlT and Car extraction we used a solution of acetone (80%) and the quantification was done according to (Wellburn, 1994).

Results and discussion

All the cultures grew exponentially until the first 6 days achieving final biomass concentrations from 0.3 to 0.49 g L^{-1} (Table 1). Using the DW data and an exponential fit the values of specific growth rate were obtained (Fig. 1). The curve has a Gauss curve shape with a maximal value of 0.205 d^{-1} for the culture exposed to 66 ppm N-NH_4; while the minimal values of µ were the obtained at 20 and 110 ppm N-NH_4 and were 25 and 40% lower than the maximal value.

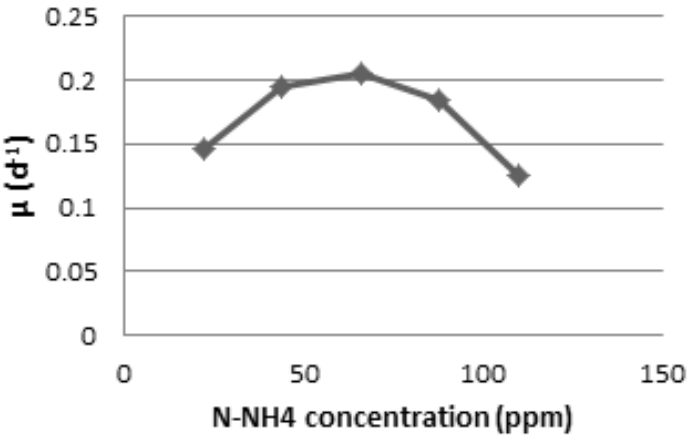

Figure 1. Specific growth rate of *C. vulgaris* growing at different N-NH_4 concentrations.

There are three points where the μ does not change significantly and these can be considered as the "optimal" interval for N-NH_4 concentration. This interval goes from 44 to 88 ppm N-NH_4. This is similar with the values obtained by who report and optimal growth of *C. vulgaris* at ppm of N-NH_4.

Table 1. Kinetic parameters for *C. vulgaris* growing at different N-NH_4 concentrations.

PARAMETER	**Treatment**					
	MBB	22 ppm N-NH_4	44 ppm N-NH_4	66 ppm N-NH_4	88 ppm N-NH_4	110 ppm N-NH_4
Final DW (gL^{-1})	0.34	0.3	0.31	0.32	0.49	0.36
μ (d^{-1})	0.138	0.145	0.195	0.205	0.185	0.1261
Vel. NH4 ($mg_{N\text{-}NH4}$ $L^{-1}d^{-1}$)	nd	-2.35	-4.63	-8.49	-9.43	-7.94
Vel. Pi (mg_{Pi} $L^{-1}d^{-1}$)	-1.66	-2.27	-0.552	-2.143	-0.786	-0.296
Specific content of ChlT (mg_{NH4} g_{DW}^{-1})	8.152	25.20	28.16	25.42	29.85	24.19

Nd Not determined

The final DW in all the treatments was directly proportional with the nitrogen consumption rate (Table 1). In the treatment with 88 ppm, the final DW was 0.49 and higher than the value obtained for the culture exposed to 22 ppm. This behavior is also observed for the nitrogen consumption; while the culture at 66 ppm reached 8.49 mg $L^{-1}d^{-1}$, the experiment at 22 ppm just consumed 2.27 mg $L^{-1}d^{-1}$ and this value is 73% lower compared with the value at 66 ppm. This increase is due because the rate of nitrogen consumption is directly proportional with the initial nitrogen concentration (Isleten-Hosoglu *et al*. 2012).

The concentration of nitrogen in the culturing media is observed in Figure 2. All the experiments have the same tendency; the nitrogen concentration decrease until reach the completely depletion of the ammonium. The day when the nitrogen concentration was lower than the detection limit of the method depended of the initial concentration; while in the culture at 22 ppm occurred at 4d there, the cultures at 44, 66, 88 y 110 reached the completely consumption of nitrogen at 7, 8, 8 and 9 days respectively.

On the other hand, the consumption rate of Pi (Table 1) did not present significantly differences between the treatments. This can be caused by the fact that the microalgae consume high amount of phosphorus accumulating it in the cytoplasm and for that reason generally there is not directly related with the growth Cañizares *et al*. 1994; González *et al*. 1997).

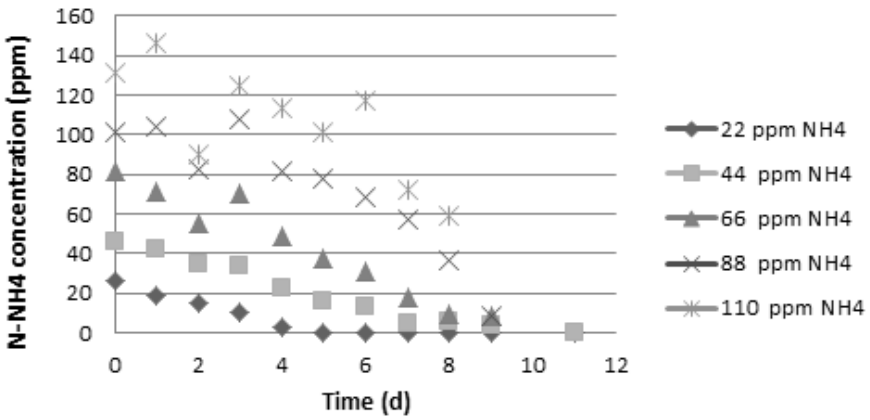

Figure 2. Residual nitrogen concentration in culturing media.

The specific content of chlorophyll is an indicative of the physiological status of the culture (Martínez-Roldán *et al*. 2014). For all the experiments exposed to N-NH4, the specific content of chlorophyll was higher than 24 mg g^{-1}; different authors reported values from 15 to 30 for this parameter indicating a good status and at exponential growth phase (Barsanti and Gualtieri, 2006; González *et al.* 1997; Martínez-Roldán *et al*. 2014)

Conclusions

C. vulgaris has a great potential to consume nitrogen and phosphorus from its culturing medium and this can be useful for its proposal for the tertiary treatment of wastewater coupled with the obtaining of high value products as protein, animal feed, etc.

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Design and construction of a bubbled column for microalgae culture

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Abstract

The microalgae are photosynthetic microorganisms capable to carry out photosynthesis (light to fix CO_2) and also to produce very important compounds as proteins, carotenoids, carbohydrates, etc. For its massive production a special type of bioreactors is normally used, made with transparent materials to allow the light transfer. The design of this bioreactors uses specific parameters as the ratio between the diameter and the height, the productivity, final biomass concentration, etc. In the present work a Photobioreactor (PBR) with configuration of bubbled column was designed and constructed. The prototype was evaluated trough the cultivation of *Spirulina* and *Chlorella*. Both microalgae were capable to growth in the PBR but did not reach the maximal reported by some author. The final biomass concentrations were 0.437±0.005 g L^{-1} and 0.895±0.120 g L^{-1} (8 and 7 d respectively) for *Chlorella* and *Spirulina*; these values corresponding with a biomass productivity of 54.6 and 127.8 mg L^{-1} d^{-1}. Final concentration and biomass productivity resulted low according with the values reported for both microalgae. An optimization of the operational conditions is necessary to ensure high concentration cultures.

Keywords: Photobioreactor●microalgae●design●prototype.

Introduction

The microalgae are unicellular or colonial microorganisms who has the capacity of do photosynthesis converting the light in chemical energy (Barsanti and Gualtieri 2006). They are the primary producers in the aquatic ecosystems and has the capability to produce many product with high value; some of this products include carotenoids, poliunsaturated fatty acids, proteins, polysaccharides, etc. (Leu and Boussiba 2014). Nevertheless, is necessary to design process with high cellular concentrations and productivities. In order to improve this is necessary to design Photobioreactor (PBR) to provide the specific conditions of every strain and process (Martínez-Roldán and Cañizares-Villanueva 2015).

Today there are many different configurations of PBR's that permits to reach concentrated cultures; but there are other operational parameters that can be modified permitting to reach optimal conditions (Martínez-Roldán and Cañizares-Villanueva 2015).

By the above, this investigation had the objective of design and construct a PBR with configuration of bubbled column using the productivity, final concentration of biomass and the diameter/height ratio (D/H) as design parameters. The prototype was evaluated cultivating *Chlorella* and *Spirulina* in autotrophic conditions.

Materials and Methods

Chlorella vulgaris and *Spriluna platensis* were provided by Prof. Hugo V. Perales Vela from the Facultad de Estudios Superiores from UNAM.

To evaluate the growth and productivity of *C. vulgaris* and *S. platensis* experiments in the lab were carried out. For *Spirulina* a modified Zarrouk's medium was used while *Chlorella* was cultivated in Bold medium. Once built the PBR a growth curve was carried out for every strain some analytical determinations were done. The analytical determination were: Dry Weight (DW), nitrogen and phosphorus concentrations ($N\text{-}NH_4$ and Pi respectively), Total Chlorophyll (ChlT). The DW was determined according to (Martínez-Roldán *et al.* 2014); while the nitrogen and phosphorus was quantified according to (APHA *et al.* 1999). The Chlorophyll was extracted employing an acetone solution at 80% and the quantifications was done using the formulas reported by Wellburn (1994).

For the design, a biomass productivity of 100 mg L^{-1} d^{-1} and a biomass production of 5.5 g were considered; also tree diameter were used (10.16, 12.7, 15.24 cm). According to literature, the D/H ratio from 3 to 10 (Jacob-Lopes *et al.* 2008). The following equations were used to obtain the operational volume.

$$BP = (P)(V_{OP})(t) \qquad V_{OP} = \frac{\pi H D^2}{4} \qquad Vo_p = [0.7 \text{ a } 0.8]V_T$$

Where: BP: Biomass production; P=Biomass productivity; VOP= Operational volume; t= time; H: Height D= Internal diameter; VT= Total volume

Results

Sizing and construction of the PBR. Employing the equations mentioned above and the values of productivity and final production, we calculate the dimensions of the PBR considering the tree diameters available (10.16, 12.7, 15.24 cm); the result obtained can be observed in Table 1.

Many author reported very different D/H ratio for the construction of PBR's; the values are in function of the PBR configuration, the microorganism, the biomass concentration, etc. (Carvalho *et al*. 2006; Martínez-Roldán and Cañizares-Villanueva 2015). Particularly, for bubbled columns this ratio is 2–10; this because permits a good superficial area (for light transfer) and because the residence time of the gas is enough to ensure the mass transfer (Jácome-Pilco *et al.* 2009; Jacob-Lopes *et al.* 2008).

The design parameters are enlisted in the Table 1. In all the cases the D/H ratio it is inside the reported interval (2-6). Nevertheless, the tubing with an internal diameter of 15.24 cm because a PBR with that diameter and 60 cm of height is more stable.

Illumination system. The illumination system consists in an array of 6 fluorescent lamps of 30W (Havells®, Mexico) evenly distributed along the PBR. This permitted a supply around 300-350 µE m^{-2} s^{-1}.

Insufflation system. The system was selected considering a volumetric flux of at least 3 L min^{-1} (corresponding to 0.4 vvm). A Resun® air compressor was selected because has the capability to pump until 20 L min^{-1}; this equipment has an energy consumption of 10.5 W and its dimensions are 23x18x13 cm. In bubbled-PBR the air supply is very important because permits a correct mixing and an efficient CO_2 diffusion into the liquid (Martínez-Roldán and Cañizares-Villanueva, 2015).

Table 1. Design parameters obtained for the tree internal diameters.

Design Parameter	Internal diameter (cm)		
	10.16	12.7	15.24
Operational Volume (L)	7	7	7
Total Volume (L)	13	11	9
Headspace Volume (L)	6	4	2
Total height (cm)	87.37	71.16	60
Liquid height (cm)	68	55	48
Headspace height (cm)	19.37	16.16	12
Internal Diameter (cm)	10.16	12.7	15.24
External Diameter (cm)	10.1	12.64	15.18
Thickness (cm)	0.06	0.06	0.06
D/H Ratio	8.59	5.62	3.93

In many cases the airlift systems are selected above other type of (paddle wheel, pump, etc.) because are very simple and its operation is easy; also the shear stress caused is very low and this is useful when is cultivated a flagellate or filamentous strain (Martínez-Roldán *et al.* 2014; Martínez-Roldán and Cañizares-Villanueva 2015).

Growth of microalgae. The evaluation of the prototype's performance was carried out by the culture of two microalgae *Chlorella vulgaris* (eukaryotic green alga) and *Spirulina platensis* (prokaryotic cyanobacteria). In the figure 1 the behavior of the DW for both species can be observed. *C. vulgaris* started with a biomass concentration of 0.080±0.028 0.080 g L^{-1}. The microalgae grew until the 8 d when the concentration was 0.437±0.005 g L^{-1}. While for *S. platensis*, the initial biomass concentration was 0.238±0.067 g L^{-1} and at the end of the experiment was 0.895±0.120 g L^{-1} (7 d).

According to Ugwu (2005), in the closed PBR it can be reached very concentrated cultures with biomass concentrations above 1 g L^{-1}. In this study, the final biomass concentrations were 0.437±0.005 g L^{-1} and 0.895±0.120 g L^{-1} (8 and 7 d respectively) for *Chlorella* and *Spirulina*. These values are far from the theoretical value considered for the design and the diminution is 56 y 11% lower than the reported for this microalgae. The above proves that the operational conditions chosen were not the optima; some of the conditions that can be optimized are the insufflation with CO2-enriched air, the increase in the amount of light supplied. There are configurationally modification that can be done in the PBR; the simplest is the addition of an internal pipe to produce an airlift effect and increase the mixing and mass transfer (Martínez-Roldán and Cañizares-Villanueva 2015).

Using the DW data and employing an exponential fit the specific growth rate was calculated. The cultures reached a μ of 0.207 and 0.209 d^{-1} for *Spirulina* and *Chlorella* respectively. This values are similar that the obtained for other authors to cultures of microalgae (González *et al.* 1997; Martínez-Roldán *et al.* 2014) but are lower than the reported for *Spirulina* and *Chlorella* in similar culture conditions (Cañizares *et al.* 1994; Ugwu *et al.* 2005).

The behavior of the nitrogen and phosphorus concentration in the culturing media is shown in the Fig 2A and 2B respectively. The reduction in the value of final DW reached by both cultures has not been caused by a nitrogen or phosphorus starvation. This because the complete depletion of nitrogen (Fig. 1A) occurred at 4 and 7 d for *Spirulina* and Chlorella respectively. In the case of P_i only for *Chlorella* a total consumption occurred at 7 d while for *Spirulina* there was a final concentration of Pi was 45.673±0.259 mg L^{-1}. The amounts consumed and its velocity was according with the values reported for these two strains of microalgae (Cañizares *et al.* 1994; Ugwu *et al.* 2005)

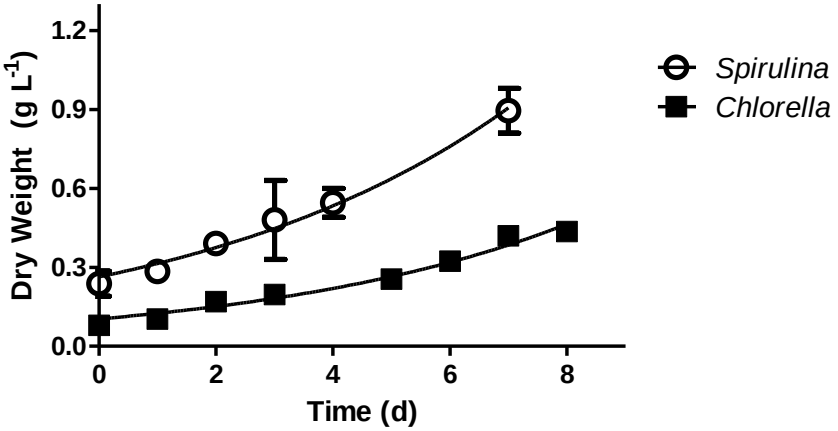

Figure 1. Dry weight obtained during the experiments.

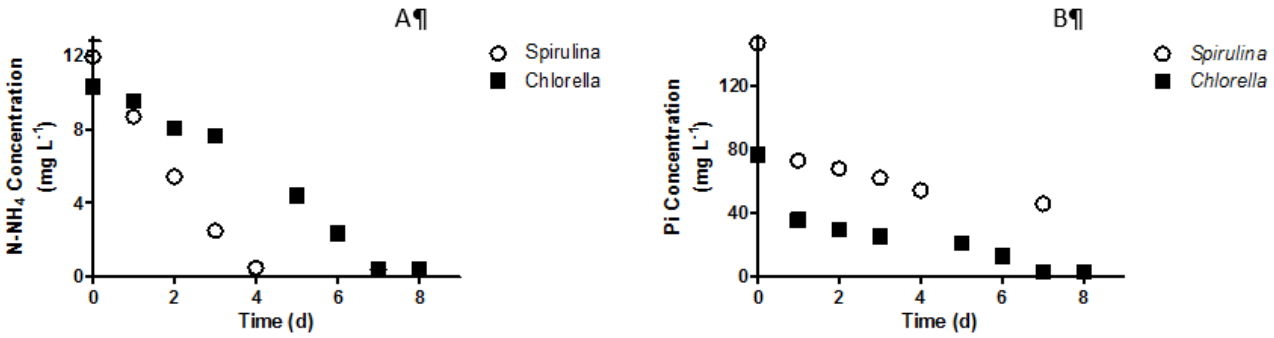

Figure 2. (A) Nitrogen and (B) Phosphorus concentrations in the culturing media.

Conclusions

The PBR built with methacrylate had the enough transparency to allow of the light; this was evidenced in the growth of both microalgae. Nevertheless, the final biomass concentration and biomass productivity were lower that the reported by some author; this could be caused by an inefficient mixing o by the auto-shading effect. If the cause was a poor mixing the major solution would be to transform this bubbled column in an airlift PBR by the addition of a concentrically internal tube; this will improve the mixing and the mass transfer inside the PBR and could cause increases in the biomass productivity

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A toxicological ranking regarding Silver Nanoparticles (AgNP) in the environment

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Abstract
The continuous efforts of different health and environmental protection agencies to understand the consequences existing on silver nanoparticles (AgNP), coupled with the actual regulatory policies, still do not allow an adequate consideration of the exposition levels and repercussions that these materials might trigger. AgNP rank secondly among the most commonly nanomaterial used, only after carbon nanotubes, while they are considered by the Environmental Protection Agency (EPA) as a pesticide. Due to the absence of proper knowledge about their toxicity mechanisms, in addition to the wide variety of processes and manufactured products which are increasing the market introduction with a clear potential exposure to workers, consumers, and environment, a ranking system is proposed. In this ranking system, most current toxicological factors were scored, providing a scale that could assist and supply a classification system, considering the priorities and concerns in the regulatory and standardization of the NP-polluted environment as well as the occupational health and safety issues on nanomaterials.

Keywords: ● AgNP ● Silver nanoparticles ● Ranking system ● Regulatory framework ● Human and environmental health.

Introduction

The use of silver nanoparticles (AgNP), due to their physicochemical properties as highest thermal and electrical conductivities, catalytic activity, antibacterial and antifungal effects among others (Lide, 2000), impulse their commercial applications on household and electrical appliances, such as batteries, superconductors, coatings, water purification systems, disinfectants, cleaning products, automotive devices, food storage containers, kitchen appliances, curling irons, hair dryers, make-up, burn creams, sprays, soaps, detergents, and medical products (Kulinowski and Lippy, 2011). The expanding use of AgNP in consumer markets suggests that, depending on their concentration, background conditions and environmental final media, they could increase the exposure adding long-term disposals and thus, producing adverse effects to humans and the environment.

A regulatory framework for AgNP risk assessment, based on their toxicological properties, developed from recent exposure and toxicity studies, is presented. The properties we identified might help advice and toughen development parameters, giving some tentative steps towards a new regulatory approach on which this technology may operate. They should not be understood as the unique factors to be considered in a safety regulation, several factors can be developed and included in an overall human risk assessment; rather this system ought to be regard as a ranking scheme for interpreting toxicological data to improve health risk assessments and regulatory policies. In addition, regardless of the regulatory approach that is applied, there should be a commitment among regulators and the regulated community to ensure that a nanomaterial (NM) is properly evaluated, to determine whether it poses a risk to human or environmental health. This evaluation should be based not only on the intrinsic hazard potential of the materials but also on the consideration of exposure potential during manufacturing, use, and final disposal. Finally, we want to emphasize that for a complete and proper regulation it is indispensable to analyze the environment (air, water, and soil) under various conditions (physical, chemical, biological, economical, or social), in which any or all of these factors could be determinant in the design of a complex scheme which represents the numerous linkages, transfers, and feedback loops on AgNP.

Materials and Methods

The factors we used to score AgNP were chosen through the latest research available on genotoxicity and cytotoxicity; the system is composed of eight factors (Table 1). These factors can vary qualitatively and quantitatively among the differences between conditions, but diverse investigations show common aftermath effects, indicating the implication of an inherent toxicological property; because each factor is as important as the others to determine toxicity, we assign the same maximum value to each one (12.5 pts.), being in a worst case scenario a total of 100 pts.

For the (A) factor (Table 1), a relationship between the AgNP size and their toxic effects was studied by Choi and Hu (2008), who investigated the size variation effects of 5, 10, 15, 20 nm AgNP, concluding that 5 nm AgNP were more toxic than other fractions; furthermore, Carlson et al. (2008) also found a size-dependent toxicity and a toxicity mechanism mediated through oxidative stress produced by AgNP. Additionally, Filon et al. (2015) observed that AgNP smaller than 40 nm show skin penetrations with a hazard potential, suggesting as critical size of 70 nm for AgNP. Based on Carlson et al. (2008) data, we observed 4.8x proportional concentration rate between the smaller and bigger AgNP sizes and a 0.9x rate between intermediate and smaller sizes, taking this into consideration, we establish 12.5 pts. to NP smaller than 20 nm, 11.3 pts. sizes between 20-70 nm and 2.6 pts. to NP bigger than 70 nm. Moreover, another property considered was the factor B (shape; Table 1). Pal et al. (2008) investigated the antibacterial properties of different AgNP shapes, concluding that truncated triangular nanoplates have a higher reactivity than spherical or rod-shaped particles. However, Favi et al. (2015) and Gorka et al. (2015) also studied the shape influence of NM in toxicity finding that nanorods and nanocubes shapes show to be less toxic than spherical particles. Furthermore, Awasthi et al. (2013) reported the toxicity of 5.0 +/- 1.0 nm AgNP with spherical shape, inferring that AgNP induced oxidative stress, damaging DNA, leading to apoptosis. Pal et al. (2008) investigation led to establish a 1.1x proportional rate based on their basal plane facets, leading with 12.5 pts. for triangular shapes, 11.3 pts. for spherical ones, 10.2 pts. in case

of wires, 9.3 pts. for cube shapes and finally 8.4 pts. to the rest forms. Another physical property that determinates the behavior of the AgNP is the concentration (Factor D) on final media; this factor was investigated by Tripathy et al. (2014) who observed an elevated concentration result from higher aggregation rates, slowing down their dissolution and affecting directly their toxicity. Moreover Braydich-Stolle et al. (2005) studied *in vitro* mouse spermatogonial cell line, and inferred that cytotoxicity in mitochondrial function and membrane leakage increase, as the concentration increases as well. For our ranking, we consider Tripathy et al. (2014) proportional rate of 10x between the highest and lowest concentrations on cell viability after 24 hours, and a 2.5x between the lowest and the intermediate concentrations; establishing as a low concentration level less than 20 mg L^{-1} grading it with 12.5 pts., a medium level between 20-40 mg L^{-1} with 5 pts. and a high level over 40 mg L^{-1} scoring 1.3 pts.

The concentration is tightly related with the superficial charge (C) as well as for the stability (E), for the superficial charge section El Badawy et al. (2010) shows a direct correlation between the toxicity of AgNP and their surface charge, demonstrating that negative Citrate-AgNP were least toxic than positively charged branched polyethyleneimine coated AgNP; Additionally the agglomeration of NP can influence the cellular viability and cytotoxicity. Furthermore, Huk et al. (2015) studies the interactions of AgNP with different charges, they found that positive AgNP had greater impact on cytotoxicity and genotoxicity than neutral or negative charge particles. We used Huk et al. (2015) data between surface charges and cellular responses, for a proportional rate of 1.3x among positive and negative, and 2x between positive and neutral; leading with 12.5 pts. positive charged, 9.6 pts. for negative charged, and 6.3 pts. for neutral charged.

On stability case (Factor E), Pinto et al. (2010) showed that stabilized AgNP solutions reduced the rate of changes in terms of size and shape. Furthermore, El Badawy et al. (2010) determines that the stability of NP is a function of several factors, that includes the type of capping agent and environmental conditions like pH, ionic strength, and the background electrolyte composition. This section brings the possibility to consider the surrounding media in the evaluation; using Pinto et al. (2010) absorbance data, inferring a proportional ratio of 2x between the highest and medium stability and 1.2x rate among the highest and lowest; rating with 12.5 pts. a low stability, 10.4 a medium and 6.3 pts. for a high stability. Another parameter that modifies the toxicity of the AgNP is the surface chemistry (Factor F); Caballero et al. (2013) studied the effect of different AgNP surface coatings finding that the surface chemistry is correlated with the agglomeration and their uptake by cells which in turn influences their toxicity. Additionally, Ahamed et al. (2008) found that different surface chemistry of AgNP induce different DNA damage responses, being the coated AgNP more toxic than the uncoated, on an approximately proportion rate of 2x after 72 hours; taking this into account we divided the section into coated and uncoated, assigning with 12.5 pts. to the first one and 6.3 pts. to the second. In addition, to the above factors, the storage time (Factor G) also influences the toxicity of AgNP, Pinto et al. (2010) concluded that citrate stabilized Ag NP solutions proved to be fairly stable after 1 year, changing their size distribution and morphology; moreover, Kittler et al. (2010) inferred that “aged” AgNP are more toxic to cells than freshly prepared NP. Furthermore, Izak-Nau et al. (2015), studied the impact of time on AgNP at 0, 3 and 6 months; deducing that significantly changes on size, agglomeration, surface charge and dissolution occurs after 6 months. Founded on Izak-Nau et al. (2015) we classified the storage time on <6

months and >6 months; to weigh each class we obtain the average of the zeta potential measurements to establish a rate of change of 1.5x, with this we confer with 12.5 pts. to >6 months and 8.3 to <6 months. Finally, the last criterion in consideration refers to the previous studies available about the toxicity of AgNP (Factor H); gathering points if existing research demonstrates their genotoxicity, cytotoxicity or harmful effects.

Table1. Proposed system for ranking AgNP toxicological impacts.

Factor		Score
A	<20	12.5
Size / nm	20-70	11.3
	>70	2.6
B	Triangular particles	12.5
Shape	Spherical particles	11.3
	Wires particles	10.2
	Cube particles	9.3
	Others	8.4
C	Positive	12.5
Superficial charge	Negative	9.6
	Neutral	6.3
D	High <40	12.5
Concentration / mg L^{-1}	Medium 20-40	5
	Low >20	1.3
E	Low	12.5
Stability	Medium	10.4
	High	6.3
F	Coated	12.5
Surface chemistry	Uncoated	6.3
G	>6 months	12.5
Storage time	<1 month	8.3
H	Positive	12.5
Suggest toxicity	Possible	6.3
	Negative	1

Results and Discussion

The categories we proposed to classify allow an adequate glance for a several regulatory assessment (Table 2). As shown before, once the scoring system is applied, total scores will vary from 40.5 to 100. In this system, a score higher than 88 pts. is ranked into Class I, which represents the widest potential hazard, i.e. taking into account its use, it should be forbidden. Classes I and II should be priority relevant in regulation, while materials in Class III, IV or V can allow several actions such as labeling, limited use, or restricted sales. Nevertheless, the development of a definite ranking system would require the coordinated effort of a multidisciplinary committee. The regulatory options would be influenced by the nature or intended use of a NM, the estimated level of exposure, and the environmental media.

Table 2. Ranking AgNP classes according to total factor score.

Total Factor Score	AgNP Class	Possible Effect
88-100	I	Acute
75-87.9	II	Severe
62-74.9	III	Medium
49-61.9	IV	Unobtrusive
>48.9	V	Slight

The adequate detection of AgNP effects on public and environment health is subject for long-term exposure, with a proper design and evaluation of complex procedural experiments in real conditions, to determine the real effects of using and releasing NM in common applications (León-Silva et al., 2016). This framework ought to rank the potential exposure and toxicity of AgNP, although it is applicable to other NM as well, in order to get a first approach to the interpretation of toxicological data required for the assessment and improvement of a regulatory policy, impacting as few as possible with regard to human health and the environment.

Conclusion

The proposed ranking system considers some aspects of risk assessment on AgNP, by weighting the toxic effects caused as the result of the variation of certain properties. Nevertheless, because most of the research was made *in vitro* with controlled conditions, appropriate tests that ensure system validity are lacking; further toxicological research and hazard protocols might provide higher profits. Additionally, new regulations and policies from government and scientific community focused on public safety and environmental control should be expanded. This ranking system is based on available data of AgNP toxicology mechanisms; however, inclusive studies about environmental factors and their perspectives should be considered for an appropriate system of NM regulation.

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A Bioethical approach to Biotechnology

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Abstract

There is a vast amount of resources on ethics and/or bioethics; a lot of that information is related to the clinical setting, in particular issues regarding patients and medical tasks. However, bioethics is also concerned with human beings and their interaction with other living beings and the environment, in this sense, Biotechnology, the use of living systems or organisms to develop technologies and products, reveals itself as a source of bioethical conflicts but also as a solution. This paper aims to philosophically reflect on the possible implications that biotechnology could have for living beings, the environment and its impact on human society from a bioethical standpoint.

Keywords: • ethics • biotechnology • Deontology • Utilitarianism.

Introduction:

In moral philosophy there are two currents: on one side the consequentialists for whom 'the correct moral action is determined by the production of good consequences' (Hume, Bentham/Mill: the moral criterion of the greatest good for the greatest number). On the other hand we find the deontologists for whom the concepts of duty and justice are independent of happiness or the good that is achieved and all moral action depends on the goodwill (impartiality and universality of the principle from which it is judged. The main proponent of this thesis is Kant (Alcoberro, 2016). These two world views can be harnessed to try to understand human actions in the context of technological advancement.

Materials and methods:

For this work we consulted investigations and reports related to the use of biotechnology available in online portals, educational materials for teachers, consortia and virtual universities. First, we identify the four ethical principles and briefly discuss bioethical issues derived from biotechnology developments.

Results and discussion:

Four ethical principles inform decisions in Bioethics (Alcoberro, 2016):

1- Principle of autonomy: Two very different philosophers have defended the modern concept of 'autonomy': Kant (deontologist) and Stuart Mill (utilitarian). At the core their difference relies in while Mill understands autonomy as 'non-interference', in Kant dominates the idea of "respect" for the person. The principle of autonomy can negatively formulate as: 'the autonomous actions cannot be subject to limitation or permit another'. And positively as: 'I am the only subject of my actions and my responsibility'. A bioethics realization of autonomy is the patient's informed consent.

2. Principle of beneficence ('Doing good'). Both utilitarians and Kant derive beneficence from utility, kantians also recognize that doing well is an a priori requirement of the action. But the principle of beneficence depends on autonomy (do good to the individual requests). Beneficence is not to be confused with piety, kindness or charity, because it includes all forms of action on behalf of individuals who have the right to decide. It originates in the Hippocratic oath, but goes beyond philanthropy while recognizing the patient as a subject of rights (not charity).

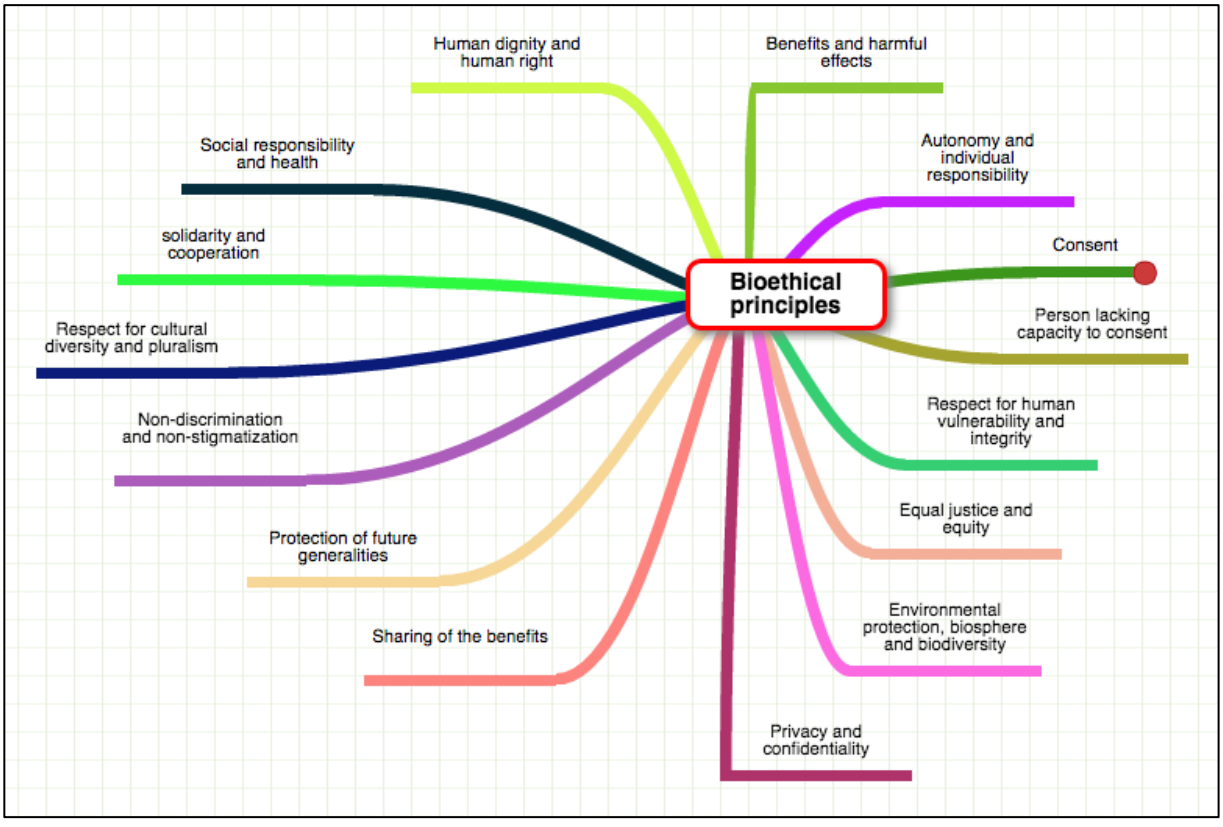
3. The principle of non-maleficence ('Primum non nocere') is not easy to distinguish from the above but is essential in the case of evaluation of treatments. Realistic and with a strong anti-utopian component (not always know or can do good) and acts preventively. It is not in the 'Corpus' Hippocratic to the letter, but derives from it.

4. Principle of Justice: Justice is a difficult requirement to define, but essential. In principle (according to the Aristotelian tradition) it requires the equal treatment of what is equal and unequally what is unequal. This is what we mean by 'fairness'. It can also be understood as a principle of fairness, but the difficulty of justice lies in the distribution of scarce resources. Without an impartial justice criterion, confidence in the system collapses.

With the large-scale introduction of techniques for genetic intervention, a new era of bio techno scientific knowledge was inaugurated, with the potential for positive but also negative effects, exceeding the spatial limits known to this day. On one hand, this creates the illusion that gaining new technical tools will conduct to an improved adaptation of human beings, and living beings in general, and the environment, on the other hand, it also creates irrational fears of irreversible risks of incalculable dimensions that will be passed onto future generations (Roland & Kottow 2001). Bioethics as a discipline emerged in the second half of the twentieth century as a result of the concerns of many scientists and thinkers for the reckless advancement of science perceived by many as a threat to the future of humanity; biotechnological advances continue to emerge raising questions about their impact on our concept of life and its dignity. Bioethics strives to counteract Scientism; a school of thought that excessively values scientific advances and manages some nonconforming respect for other bioethical models. This makes urgent and necessary the inclusion of bioethics as a part of higher education (Arango 2011). Olivé (2006) divides the bioethical perspective in two fronts: An American bioethics with based on right and duty, as opposed to a European bioethics heavily influenced by Greek philosophy traits of the binomial vice-virtue, while the American legacy to bioethics is the crucial concept of

informed consent, European bioethics has made a significant contribution by philosophical research on the idea of personhood (Olivé 2006). From the point of view of bioethics, we are on the verge of complex and substantial changes in global culture, one that does not recognize territorial boundaries or national legislation, because the whole planet Earth is interconnected through internet and to the slightest movement of a click and find sites dedicated to “expose” threats arising from biotechnology such as the "Frankenstein syndrome" a common denominator for food improved by biotechnology tools creating the so-called “Frankenfood” (Castro *et al.* 2004). We live in a time where professionals of any walk of life commit illegal activities for personal gain or the company they work, science and technology are not immune to this situation, and the lay person becomes suspicious of that he cannot understand. This leaves us clearly with the perception of the barrier between right and wrong becoming increasingly invisible (Barajas 2012). Despite all of this, it is undeniable the biomedicine, nanoscience, pharmacogenomics, and nanomedicine have made a lot of contributions to human society. Biotechnologies should serve every human being (Carrera 2016).

Figure 1. Conceptual Map of bioethics (Mind 2016)



Conclusions:

There are many opportunities in basic or applied biotechnology regulation for bioethical discussion and analysis; we can use the principles of bioethics on this endeavor. Taking these principles as guide, one cans easily advice against the use of biotechnology as a tool for weapon development in bioterrorism. However, for other issues, resolution may not

come easily, as can be exemplified by the issues raised by the development of transgenic plants and animals and research involving stem cells, promising technologies that have yet to deliver in part due to social conflicts surrounding their use by humans.

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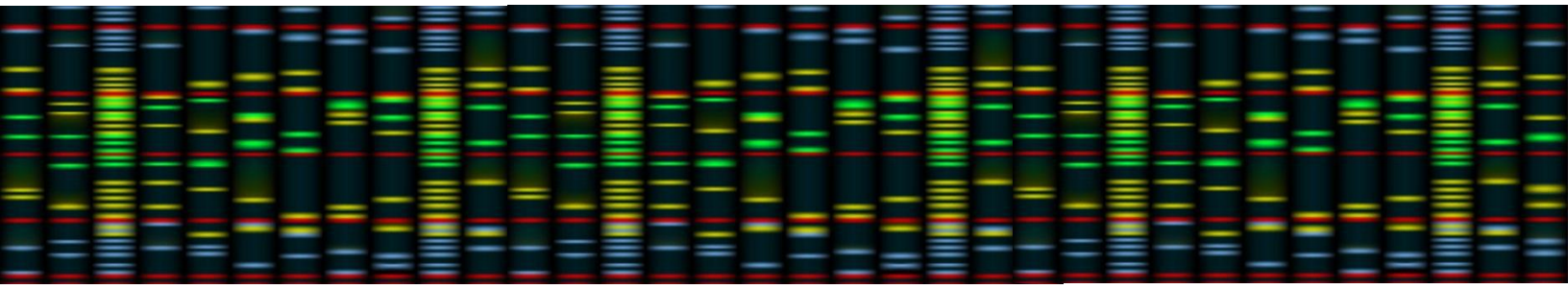

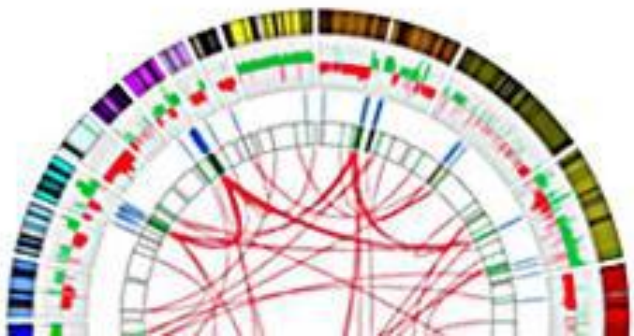

BIOTECHNOLOGY SUMMIT

Quantitative contribution of the beta-adrenergic receptors in the vasorelaxation effect

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Abstract

The aim of this study was to investigate the quantitative contribution in the vasodilation response elicited by the binding of each β-adrenergic receptor. Cumulative concentration-response curves (CCRCs) to isoproterenol were experimentally obtained on rat aorta in the presence and absence of selective β-adrenergic antagonists. A mathematical model based on the law of mass action was proposed which simultaneously fitted several experimental results. A set of optimal parameters was obtained. We found that the contribution of β_1-, β_2- and β_3-adrenoceptors to the aorta vasorelaxation effect elicited by one ligand were 20%, 68%, and 12%, respectively.

Keywords: β-adrenoceptors subtypes • Mathematical model • Vasorelaxation response

Introduction

The binding of a molecule to an enzyme, transporter, ion channel receptor is commonly described as a mathematical expression based on the law of mass action (Bylund and Toews 2014). This mathematical expression is used to describe biochemical reactions when there is an interaction between a ligand and a single type of response. However, the majority of the physiological functions came from the interaction of only one ligand with more than one type of receptor. This is the case of the adrenergic system (Lands et al. 1967). This system has three β-adrenergic receptors (Adrb), which share a common physiological effect, vasodilation (Guimaraes and Moura 2001). Although the contribution of the Adrb in the relaxation effect is qualitatively accepted, little attention has been paid to the quantitative contribution in the vasorelaxation effect. The present paper presents a mathematical model optimally fitted to quantify the contribution of each beta-adrenergic receptor in the vasorelaxation effect.

Materials and Methods

Tissue preparation. Adult male Sprague-Dawley rats (225-300 g) were anesthetized using an isoflurane anesthesia chamber (EZ- Anesthesia 108SA, Pennsylvania, USA). The thoracic aorta was quickly excised from the animal. It was placed onto cold (4°C) Krebs solution of the following (mM) composition: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.7 and EDTA 0.026, pH 7.4, aerated continuously with a mix 95:5 %; O_2:CO_2 %. Shortly, the thoracic aorta is cleaned from adipose tissue and cutting into ring segments.

Functional Experiments. Thoracic aortic rings segments (2.5mm) were set-up in an isometric organ bath (MLT0201 of ADINTRUMENTS, USA) filled with Krebs solution, thermostatically controlled at 37°C, pH 7.4 and gassed composition as describe above (Stollak and Furchgott 1983). Rings were stabilized for 5 min at a resting tension of 1.5 g. The aortic rings functionality was verified using a α_1 -adrenoceptor agonist phenylephrine (1µM), contracting the rings until 80% of maximal contraction. Functional endothelium was tested with a bolus of acetylcholine (ACh) (10µM). Only the aortic rings that relaxed more than 60% to Ach were considered endothelium-intact. To determine the functionality of the Adrb subtypes stimulated by isoproterenol, the aortic rings were contracted with phenylephrine (1µM) then CCRCs of relaxation were performed in the presence and absence of β_1-, β_2- and β_3-adrenoceptors antagonists CGP-20712A (1µM), ICI 118, 551 (1µM) and L 748337 (1µM), respectively. The antagonists were added 5 min before start to relax the aortic rings with isoproterenol. CCRCs were constructed with sequential increments of 0.5 log units until a steady state was observed. The contact time for every single concentration of agonist was 3 min.

Mathematical model. A set of algebraic equations to describe the binding of one ligand to three different receptors are developed applying the law of mass action (Bylund and Toews 2014).

Statistical analysis. Experimental data are presented as the mean ± SEM. The aortic rings were obtained from different animals, n=30.

Results and Discussion

Adrb has differential vasorelaxation responses to isoproterenol

To evaluate the vasorelaxation response to isoproterenol of each Adrb, two Adrb were pharmacologically blockaded and it was assumed that the remaining Adrb is an antagonist-free receptor. As can be seen in Figure 1A, when only the β_2-adrenergic receptor (Adrb2) was activated, CCRCs to isoproterenol show that the tissue relaxes in a concentration-dependent manner describing a similar behavior in comparison with the control curve. Figure 1B shows that a significant isoproterenol concentration -6.5 to -6.0M (around 100 to 1000nM) was necessary to relax the tissue at the same level of the control curve, when only the Adrb1 receptor was activated. However, when only the Adrb3 receptor was activated and stimulated with increasing doses of isoproterenol, the tissue hardly relaxes and remains unaffected, needed a higher isoproterenol concentration to generate a vasorelaxation response at the same level as the control group (Fig. 1C). Similar results were found by Flacco et al. (2013) and Baker et al. (2005), who using a selective Adrb agonists for each receptor found distinct vasorelaxation responses elicited by each Adrb.

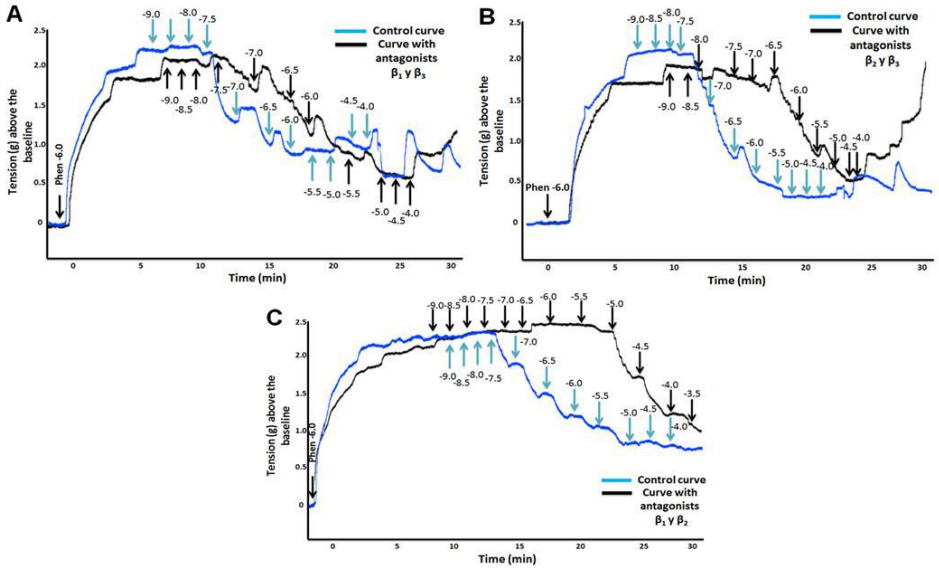

Figure 1. Representative traces showing relaxant responses of thoracic aortic rings to isoproterenol.

Mathematical model development of one ligand to three receptors

The activation of the three Adrb receptors through their binding to isoproterenol, triggering a series of molecular events culminating in the activation of protein kinase G (PKG) cGMP-dependent pathway (Bond Richard et al. 2013). To build a mathematical model we assume that each Adrb is represented by a Michaelis-Menten behavior in steady state (Equation 1). Where, [L] means the extracellular isoproterenol concentration, K_{D1}, K_{D2}, K_{D3} are the dissociation constants of Adrb1, Adrb2, and Adrb3, respectively. Each receptor generates a constant individual production of PKG represented by the parameter β. Where, β_1, β_2, β_3 belongs to Adrb1, Adrb2, and Adrb3, respectively. The total [PKG] is the sum of the [PKG] of each Adrb. Furthermore, when this [PKG] increases, activate calcium release proteins embedded in the sarcoplasmic reticulum membrane eliciting a vasorelaxation effect. If we assume that this vasorelaxation effect follows a Michaelis-Menten behavior, the increasing vasorelaxant effect (r_T) observed is related to the increasing [PKG]. This equation takes into account the participation of the Adrb on the final vasorelaxation effect.

Equation 1

$$[r_T] = \frac{1}{1 + \frac{1}{\beta_1 \frac{[L]}{K_{D1} + [L]} + \beta_2 \frac{[L]}{K_{D2} + [L]} + \beta_3 \frac{[L]}{K_{D3} + [L]}}}$$

Modeling the individual response of each Adrb to the final vasorelaxation response to isoproterenol

To elucidate, in a quantitative manner, the contribution of each Adrb on the vasorelaxation effect, we developed a mathematical model for each Adrb response. Each model incorporates, the participation of selective antagonists derived from the law of mass action theory where an

antagonist competes with isoproterenol to its selective Adrb in a reversible manner. In consequence, equations 2, 3 and 4 denote the vasorelaxation response due to Adrb1 ([r1]), Adrb2 ([r2]) and Adrb3 ([r3]), respectively. Where the parameters A_1, A_2, A_3 and K_{I1}, K_{I2}, K_{I3} represents the experimental antagonist concentration used (1000 nM) and the dissociation constant affinities of the antagonists, respectively. The values of the parameters are described in Table 1.

Equation 2

$$[r_1] = \frac{1}{1 + \frac{1}{\beta_1 \frac{[L]}{k_{D1} + [L]} + \beta_2 \frac{[L]}{k_{D2}\left(1 + \frac{[A_2]}{k_{I2}}\right) + [L]} + \beta_3 \frac{[L]}{k_{D3}\left(1 + \frac{[A_3]}{k_{I3}}\right) + [L]}}}$$

Equation 3

$$[r_2] = \frac{1}{1 + \frac{1}{\beta_1 \frac{[L]}{k_{D1}\left(1 + \frac{[A_1]}{k_{I1}}\right) + [L]} + \beta_2 \frac{[L]}{k_{D2} + [L]} + \beta_3 \frac{[L]}{k_{D3}\left(1 + \frac{[A_3]}{k_{I3}}\right) + [L]}}}$$

Equation 4

$$[r_3] = \frac{1}{1 + \frac{1}{\beta_1 \frac{[L]}{k_{D1}\left(1 + \frac{[A_1]}{k_{I1}}\right) + [L]} + \beta_2 \frac{[L]}{k_{D2}\left(1 + \frac{[A_2]}{k_{I2}}\right) + [L]} + \beta_3 \frac{[L]}{k_{D3} + [L]}}}$$

Quantitative contribution of each Adrb through minimum least squares fitting

All the previously describe equations (1, 2, 3 and 4) have three parameters β_1, β_2, β_3 that represent the participation of each receptor. To calculate the best value of these parameters that fit all the experimental data at the same time, we used the least squares method, equation 5. Where, *y0, y1, y2* and *y3* represent the relaxation response percentage of every experimental data group and the functions F_0, F_1, F_2 and F_3 denote the equations 1, 2, 3 and 4. *X* is a constant that delimitate the range (0.1-10^5nM) of experimental isoproterenol concentration used in each CCRCs.

Equation 5

$$g(\beta_1,\beta_2,\beta_3) = \min \sum_i (F_0((\beta_1,\beta_2,\beta_3),x_i) - y0_i)^2 + \sum_i \left(F_1\left((\beta_1,\beta_2,\beta_3),x_i\right) - y1_i\right)^2 + \sum_i \left(F_2\left((\beta_1,\beta_2,\beta_3),x_i\right) - y2_i\right)^2 + \sum_i \left(F_3\left((\beta_1,\beta_2,\beta_3),x_i\right) - y3_i\right)^2$$

Table 1. Parameter values.

Parameter	Range (nM)	Value used (nM)	Reference
k_{D1}	100-250	8,000	Bond Richard et al.
k_{D2}	390	10,000	Bond Richard et al.
k_{D3}	631-63,100	40,000	Bond Richard et al.
k_{I1}	0.630-3.16	3.16	Baker, J.G.
k_{I2}	0.316-0.631	0.631	Baker, J.G.
k_{I3}	4	4	Baker, J.G.
$[A_1]$	1000	1000	This study
$[A_2]$	1000	1000	This study
$[A_3]$	1000	1000	This study

The change in relaxation as a function of isoproterenol concentration for every experimental group is shown in Figure 2 (Control n=20, 5/rats; Antagonists β_1 y β_3 n=20, 5/rats; Antagonists β_2 y β_3 n=30, 6/rats; Antagonists β_2 y β_1 n=30, 6/rats), where in presence with β-adrenergic antagonists results in a markedly different behavior in comparison with the control group. The continuous lines denote the simultaneous fitting for each experimental group. The calculation of the optimum β_1, β_2, β_3 parameters were β_1 = 24.81, β_2 =84.70, β_3 =14.94. To analyze the contribution of the Adrb, we isolated the equations that simulate the behavior of every receptor times their respective β parameter using the value calculated previously. The equation 6 represents the maximum vasorelaxation effect elicited, lineally, by the three receptors. Additionally, equations 7, 8 and 9 show the independent contribution of each receptor. Simulations of these equations are shown in Figure 3. As can be seen, the contribution of the three receptors reaches the maximum percentage of relaxation (100%) in a high isoproterenol concentration. In low isoproterenol concentration, the behavior of the Adrb1 and Adrb2 were the same. When isoproterenol concentration increases, the maximum percentage of relaxation eliciting by the Adrb2 (68%) was major than the Adrb1 (20%). Furthermore, the Adrb3 had the minor response (12%). In addition, a delay in the response was found in low isoproterenol concentrations (1-10nM) in comparison with the other receptors. Our results evidenced the quantitative contribution of the Adrb on the final vasorelaxant effect elicited by one ligand (isoproterenol).

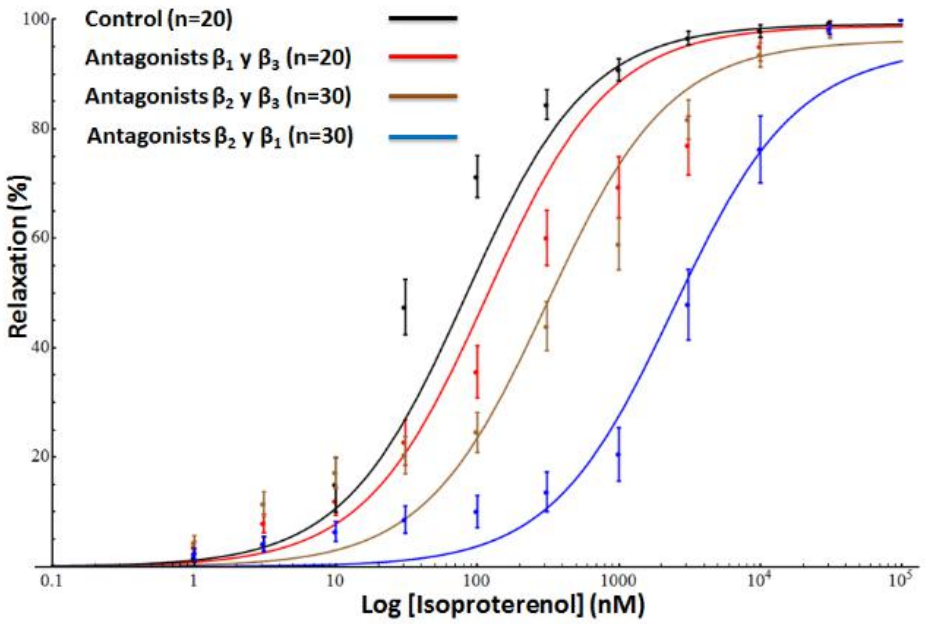

Figure 2. Minimum least squares simultaneous fitting to all experimental groups.

Equation 6

$$R_T = \beta_1 \frac{L}{k_{D1} + L} + \beta_2 \frac{L}{k_{D2} + L} + \beta_3 \frac{L}{k_{D3} + L}$$

Equation 7

$$R_1 = \beta_1 \frac{L}{k_{D1} + L}$$

Equation 8

$$R_2 = \beta_2 \frac{L}{k_{D2} + L}$$

Equation 9

$$R_3 = \beta_3 \frac{L}{k_{D3} + L}$$

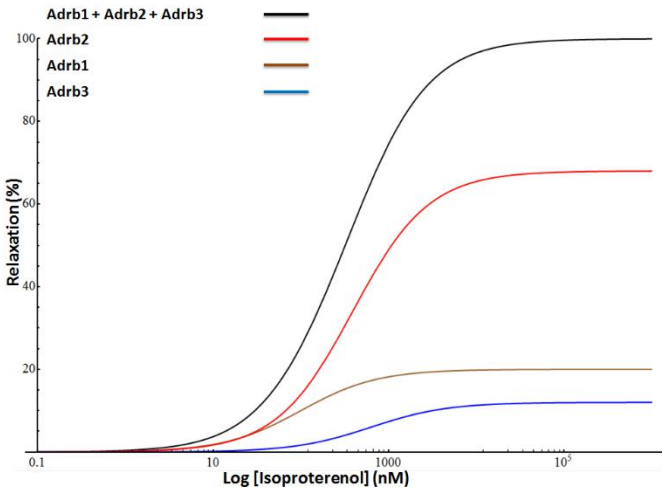

Figure 3. Vasorelaxation contribution of each Adrb as a function of isoproterenol concentration.

Conclusions

In this paper, the contribution of the beta-adrenergic receptors in the vasorelaxation effect was quantitatively estimated. We found that the contribution of β_1-, β_2- and β_3-adrenoceptors to the aorta vasorelaxation effect elicited by one ligand were 20%, 68%, and 12%, respectively.

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Intracellular calcium response to mechanical stimulation of mouse cardiomyocytes

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Abstract

Mechanical stimulation of isolated cells can activate mechanosensitive ion currents, specifically in cardiac myocytes has been reported that cationic mechanosensitive channels are activated. The aim of this study was to determine the effect of locally deforming the plasma membrane on the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) of mouse cardiomyocytes. To do this we applied a jet of liquid perpendicular to the membrane surface and monitoring the fluorescence changes of an intracellular calcium fluorophore. The localized and non-invasive mechanical deformation of mouse cardiomyocytes produce an elevation of the $[Ca^{2+}]i$, which was not affected by nimodipine or thapsigargin, in contrast the apparent elastic module significantly decreased when the cells were incubated with any of this compounds. In conclusion, the mechanical stimulation of mouse cardiomyocytes produced an increase of the $[Ca^{2+}]i$ due to the activation of a Ca^{2+} plasma membrane conductance other than the voltage-activated Ca^{2+} channels, and that altering the intracellular calcium regulation affects the mechanical properties of these cells.

Keywords: ● Mechanical activated ion conductance ● Intracellular Ca^{2+} ● Cardiomyocytes.

Introduction

The heart function as a pump is possible due the electro-mechanical coupling that result in the contraction of the cardiomyocytes in response to an electrical stimulation originated at the sinoatrial node. The action potential generated in the pacemaker cells propagates through the whole heart resulting in an elevation of the intracellular Ca^{2+} ($[Ca^{2+}]i$) and the subsequence contraction of each cardiomyocyte. Mechanical stimulation has been known to increase automaticity and modify the action potential in whole hearts and isolated tissues. Changes in the mechanical properties of the heart may constitute a mechano-electrical feedback (Lab, 1982) that can affect the heart function, per example, changes in heart rhythm are been reported but their origins are still unclear (Kohl *et al*., 1999) or changes in mechanical load are thought to be involved in arrhythmias ranging from extrasystole to fibrillation and sudden death (Bett and Sachs, 1997). Mechanical stimulation of isolated embryonic chick heart cells, pressing on the membrane with a fire polished micropipette, activated Na^+ and K^+ mechanosensitive currents that were independent of external Ca^{2+} (Hu and Sachs 1996). In this work we test the effect of locally deforming the plasma membrane of mouse cardiomyocytes on the $[Ca^{2+}]i$, using a non-invasive mechanical stimulation by applying a jet of liquid perpendicular to the membrane

surface and monitoring the fluorescence changes of a calcium fluorophore located in the cytoplasm.

Materials and Methods

Cardiac myocytes preparation. CD1 male mice of 30 -40 grams were anesthetized with Xilocine-Ketamine (2:1) (2 ml/g of weight) and heparinized (5 ml/g of weight). The chest cavity was opened and the heart removed, then the aortic arch is cannulated and the heart was transferred to an isolated organ perfusion system, where is perfused with collagenase, trypsin and EDTA medium to dissociate the tissue; the dispersed cells are washed by centrifugation with culture medium.

Mechanical stimulation. Single cell mechanical stimulation were performed using a Scanning Ion Conductance Microscope (SICM) (Ionoscope Limited, London, UK) as described previously (Sanchez *et al*., 2008). Briefly, the SICM uses a pipette as a scanning probe arranged perpendicularly to the sample, mounted on a three-axis piezo translation stage. The SICM feedback control system keeps the ion current through the pipette tip constant to approach and scan over cells, while maintaining a constant separation distance from the cell surface of approximately the pipette internal radius. The SICM can be used to mechanically test the cell, applying hydrostatic pressure, the pipette was mounted in a patch-clamp electrode holder and positive or negative hydrostatic pressure can be locally applied through the pressure port of the holder (Figure 1A). The pressure jet (of a maximum of ~120 kPa) was applied manually and digitally recorded using a PM100D pressure manometer (WPI, Sarasota, FL, USA), along with corresponding changes in the pipette position that occur while the SICM distance feedback followed the resulting local deformation of the cell surface (Figure 1B). To calculate the elasticity module (E) (apparent Young's modulus) we use the gradient of applied pressure versus the deformation distance, as previously described (Sanchez *et al*., 2008). The pipette tip internal radius, based on the resistance of the pipette was about 500 nm. The position of the pipette and the applied pressure were digitized at 10 kHz using a computer equipped with a DigiData1440A and the data acquisition and analysis were performed with pCLAMP software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA).

Ca^{2+} Imaging. Cells were loaded with 5μM Fluo-4 acetoxymethyl ester (Fluo-4 AM; Molecular Probes, Eugene, Oregon) and 0.06% pluronic F-127 for 60 min at 37°C in the dark. Later, the fluo4-cardiomyocytes solution were placed on glass coverslips treated with 0.05% polylysine in the recording chamber and after 20 min the external solution was infused to wash the supernatant. Ca^{2+} fluctuations were imaged by exciting fluo-4 using a 75-W Xenon lamp with a filter of 488nm and detecting emitted fluorescence at 535nm using a CCD camera DS-Qi1 (Nikon Instruments Inc, Japan) coupled to an inverted microscope (Nikon TE2000-U) and controlled by NIS-Elements Software (Nikon Instruments Inc, Japan). Images were acquired at 5 Hz and recorded for 3 to 4 minutes before and after stimulus. The increase of $[Ca^{2+}]i$ were represented as $\Delta F/F0$ ratios after background subtraction close to the area of interest, where F was the maximum peak of fluorescence signal intensity, and F0 was the baseline calculated as the average of the first 15 seconds prior to stimulus application. Only cardiomyocytes that does not show intracellular Ca^{2+} oscillations were used and all experiments were performed at room temperature.

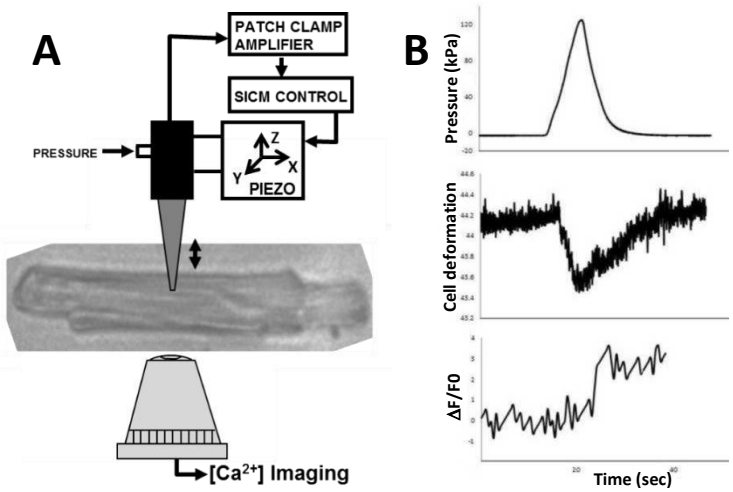

Figure 1. (A) Experimental setup for controlled mechanical stimulation using a SICM and simultaneous Ca^{2+} imaging in mouse cardiomyocytes. (B) Representative records of a typical experiment to mechanically stimulate the cell, top graph show the pressure ramp applied, middle graph show the cell surface deformation in response to the pressure ramp and bottom graph show the normalized changes in the Fluo-4 fluorescence in response to cell deformation.

Statistical Analysis. Student's t-test was applied to determine whether the data are significantly different ($p < 0.05$).

Results and Discussion

Local mechanical deformation of mouse cardiomyocytes surface induce an increment in the $[Ca^{2+}]i$ (Fig 2), these increases vary depending on the applied stimulus, and every cell responds differently (Fig 2B, black squares), however in average the $[Ca^{2+}]i$ increase in about 4 times in the non-treated cardiomyocytes. Previous reports of mechanical stimulation or cardiomyocytes using different methods have shown that Na^+ and K^+ conductance's in chicken cardiomyocytes (Hu and Sachs 1996) and a non-selective cation channels in human atrial myocytes (Kamkin et al. 2003) can be activated by mechanical stimulation, nevertheless this is the first report that show that calcium can be elevated due a mechanical stimulus in mouse cardiomyocytes. To determine if the increment in the $[Ca^{2+}]i$ is due to an effect on one of the two principal participants of the calcium regulation during the electro-mechanical coupling in the cardiomyocyte, we use Nimodipine (1 µM) in order to inhibit the L-type voltage-gated calcium channels that allow the influx of Ca^{2+}, and Thapsigargin (1µM) to inhibit the sarcoplasmic reticulum Ca^{2+} (SERCA) pump that recapture the free Ca^{2+} into the sarcoplasmic reticulum, thereby affecting the availability of this ion from this intracellular reservoir. Neither incubation with Nimodipine or Thapsigargin, was able to inhibit the Ca^{2+} increase due the mechanical stimulation, which indicates that Ca^{2+} comes from outside the cell, and that the mechanical deformation of the membrane is activating a cationic conductance different than the Cav channels. The response of the cells incubated with Nimodipine is very similar to control cells,

nevertheless, myocytes incubated with Thapsigargin shows a non-significant increase in the Ca^{2+} response (Fig. 2A). In addition, these cells were more easily deformed, reaching greater deformations with mechanical stimuli of similar magnitude than in the other experimental groups (Fig. 2B), perhaps this would explain the apparent greater response.

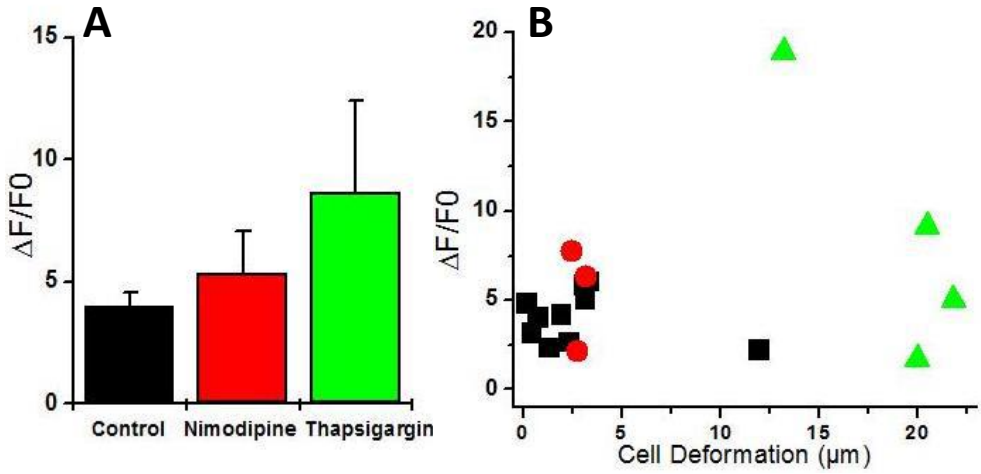

Figure 2. Effect of mechanical stimulation on the $[Ca^{2+}]i$ of mouse cardiomyocytes. **A.** Normalized changes in fluorescence of cardiomyocytes stimulated with a liquid jet produced by a ramp of pressure. Bars represent the average of the normalized maximum fluorescence change reach after the stimulation ± SEM for Control (n=10), Nimodipine (1μM) (n=3) and Thapsigargin (1μM) (n=4) treated cardiomyocytes. **B.** Relation between the maximum normalized fluorescence change and the magnitude of cell deformation of Control (■), Nimodipine (●) and Thapsigargin (▲) treated cardiomyocytes.

From these experiments we calculated the apparent Young's modulus (E) to characterize the mechanical properties of these type of cells. Figure 3 show the average elastic modules calculated for the non-treated cardiomyocytes (control), and for the Nimodipine and Thapsigargin treated cells; for each cell was possible to adjust two different modules, E1 corresponding to the initial deformation of the cell surface (plasma membrane) and E2 corresponding to the subsequent deformation. The mouse cardiomyocytes present average apparent elastic modules of E1 ~31.8 kPa and E2 of ~34.5 kPa, that are similar to the values of around 30 kPa reported previously for rat cardiomyocytes measured by ATF nanoindentation (Lieber *et al*. 2004). Notably, the elastic modules decrease significantly when the cardiomyocytes are incubated with Nimodipine (E1 ~11.4 kPa and E2 ~12.7 kPa), or Thapsigargin (E1 ~5.9 kPa E2 ~1.6 kPa). This reduction in the stiffness of the cells could help to understand why the Thapsigargin treated cells can be easily deformed in comparison with the non-treated cardiomyocytes, resulting in a larger mechanical affectation of the cell.

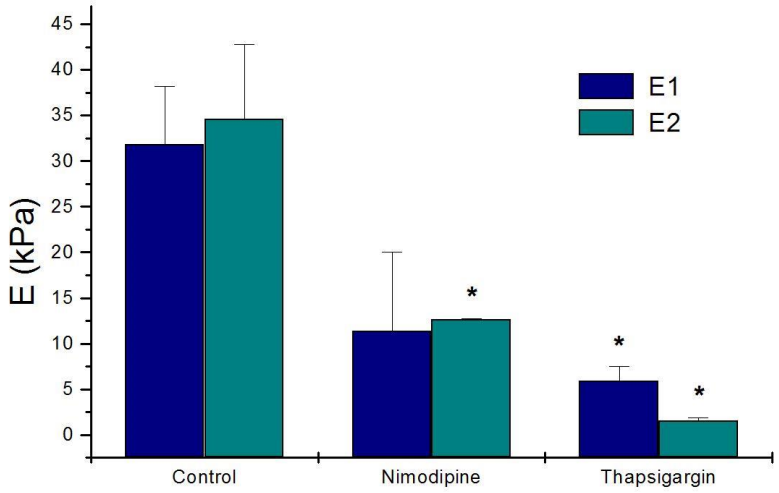

Figure 3. Mechanical properties of mouse cardiomyocytes. Bars represent the average ± SEM of the apparent Young's modulus (E) calculated for non-treated cardiomyocytes (Control, n=17) and Nimodipine (n=3) or Thapsigargin (n=6) treated cardiomyocytes. (*) denotes $P < 0.05$ against its corresponding pair of control group.

The activation of a Ca^{2+} permeability through mechanical stimulation in cardiomyocytes is relevant because changes in the regulation of this ion could alter the duration of the action potential in cardiomyocytes (Lab, 1980) probably resulting in positives or negative modifications of the heart rhythm, and also calcium can affect the function of the contraction machinery in this cells changing their properties.

Conclusions

Localized mechanical stimulation of mouse cardiomyocytes produced an increment in the intracellular Ca^{2+} concentration due to activation of a Ca^{2+} influx, different to the voltage-activated Ca^{2+} channels, into the cells.
The alteration of the intracellular calcium regulation affects the mechanical properties of the mouse cardiomyocytes.

Acknowledgments

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Mathematical modeling of the clearance and productive states in cells infected with dengue virus

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Abstract

The aim of this study was to investigate the interplay between the virus and the interferon stimulated genes encoded proteins (ISG proteins) in the productive and clearance cell phenotypes. A mathematical model was developed. It was validated with several experimental results from the literature. The model presents bistability. Our analysis and simulations suggest that the overexpression of ISG proteins was necessary to remove the productive infection state.

Keywords: • Dengue • Bistability • ISG proteins • Intracellular dynamics.

Introduction

Dengue virus is an arthropod-borne disease caused by any of the four-dengue virus serotypes (DENV 1-4), which are transmitted to non-human primates and humans primarily by the genus *Aedes*. Infection with one of the four serotypes of DENV can lead to dengue fever and sometimes to fatal dengue hemorrhagic fever or dengue shock syndrome. DENV is endemic in more than 100 countries, and its incidence has increased 30-fold over the last 50 years. Up to 50-100 million new infections are now estimated to occur annually, putting almost the half of the world's population at risk.

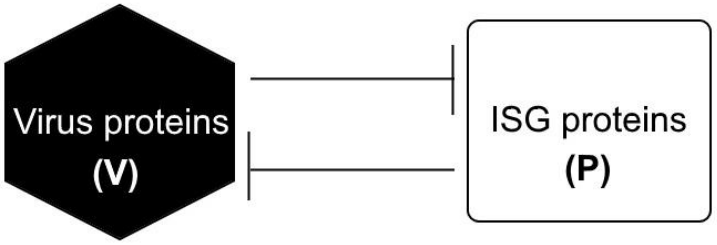
At the molecular level, innate immunity and type I interferon (IFN) response function are the first line of defense against viral infection. Their proteins inhibit the virus life cycle. Infection with dengue induces a high tittered neutralizing antibody response that can provide long-term immunity to the homologous DENV serotype, while the effect of the antibody on the heterologous serotypes is transient. During acute infection, innate immune response plays a key role in determining disease outcome. Differences in the level of dengue virus replication and its viral load may be a contributing factor in the development of dengue (Despre *et al. 2005*). The IFN inhibits virus infection by induction of IFN-stimulated genes (ISG) that affect distinct steps of the viral replication cycle (Schoggins, 2014). However, although IFN treatment of cells induces the expression of hundreds of cellular genes only a dozen ISGs have been experimentally demonstrated to generate an antiviral state against dengue virus (Jiang *et al.* 2010). Experimental evidence suggests that IFN system plays an important role in limiting dengue virus replication (Shresta et al. 2004). Also, the pretreatment of cultured cells with IFN reduces dengue virus replication (Diamond et al. 2001).

On the other hand, the virus inhibits the ISG proteins production. They affect several targets in the JAK-STAT signaling pathway. Understanding the dynamic regulation of antiviral immune responses is essential for predicting the manifestations and outcome of an infectious disease (Tan et al. 2012). Bistability is a recurrent motif in biology, and there are many examples of systems which can operate, in a stable manner, in two very distinct modes.

Here, we are interested in the dynamics of the interplay between the virus and ISG proteins in the productive and clearance cell phenotypes to bistable behavior. Can the bistability be removed in order to obtain cell in clearance state?

Materials and Methods

In this section, we developed a mathematical model for the interaction between innate immune responses and virus molecules described. The interaction of dengue virus and the ISGs proteins in a single cell is represented in a minimalist model. It is a two-dimensional differential equation model. This simplified model takes into account the dynamics of Viral RNA (V), which down regulates the ISGs proteins expression, and the dynamics of ISGs proteins (P) that inhibits the virus concentration. The dynamics of this network is determined by a system of nonlinear ordinary differential equations, Figure 1. The meaning of the functions, variables, and parameters in the below equations are given in Table 1.



$$\frac{dV}{dt} = \alpha \frac{1}{1 + \left(\frac{P}{k_1}\right)^{n_1}} - d_1 V$$

$$\frac{dP}{dt} = \beta \frac{1}{1 + \left(\frac{V}{k_2}\right)^{n_1}} - d_2 P$$

Figure 1: Schematic and equations for a minimal model of dengue. Left side: A double negative feedback loop between the dengue virus and the ISGs proteins is presented. The virus down regulates the ISGs protein transcription, besides the ISGs proteins inhibit the virus replication cycle. Right side: Mathematical model of chemical virus species (V) and ISG proteins (P) is presented.

Numerical simulations. All numerical simulations are implemented using MATLAB 2014b, the system of ordinary differential equations were numerically solved by the subroutine ode45 and ode23s.

Table1. Parameter values on the model described by equations in Figure 1 right.

Parameter	Description	Value	Reference
k_1	Repression coefficient	1400 molecules	This study
k_2	Repression coefficient	51.72 molecules	This study
d_1	Virus degradation rate	0.4/h	Schmid, 2015
d_2	Protein degradation rate	0.069 /h	Padmanabhan, 2014
V_c	Carrying capacity of a cell for DENV RNA	3600 molecules	Diamond,2000
P_c	ISGs proteins concentration of a cell with IFN=100UI	1×10^7 molecules	Padmanabhan, 2014
n_1, n_2	Hill coefficient	3	This study
α	Maximal production rate of DENV	1440 molecules/h	Diamond, 2000
β	Maximal production rate of ISGs proteins	$69x10^4$ molecules/h	Padmanabhan, 2014

Results and Discussion

To test the model feasibility, a couple of experiments by Diamond were simulated (Diamond, 2000). In these experiments the ISG proteins are measured under different IFN concentration. We mimicked these experiments by numerically solving the model equations with the following set of initial conditions: $V(0) = 0$ and $P(0) = 1$. The red line corresponds to experimental results of the ISGs proteins concentration in HuH-7.5 (Hepato cellular carcinoma) cell culture with IFN=100UI Padmanabhan *et al*. (2014). The ISG proteins dynamic is shown in blue line (Figure 2 left). Our numerical predictions are in good agreement with Padmanabhan *et al*. (2014). Furthermore, we numerically solved the system with the initial condition, $V(0) = 300$ and $P(0) = 0$. The numerically simulation result is presented in green line and experimental data obtained from a HepG2 (Human liver cancer cell line) in blue circles (Figure 2 right). It is evident that the steady state values for ISG proteins and virus obtained here are in good agreement with Diamod *et al*. (2000) results. The fact that our model reproduces the experimental results makes us

confident that it captures the necessary dynamic characteristics of the system to attempt further analysis.

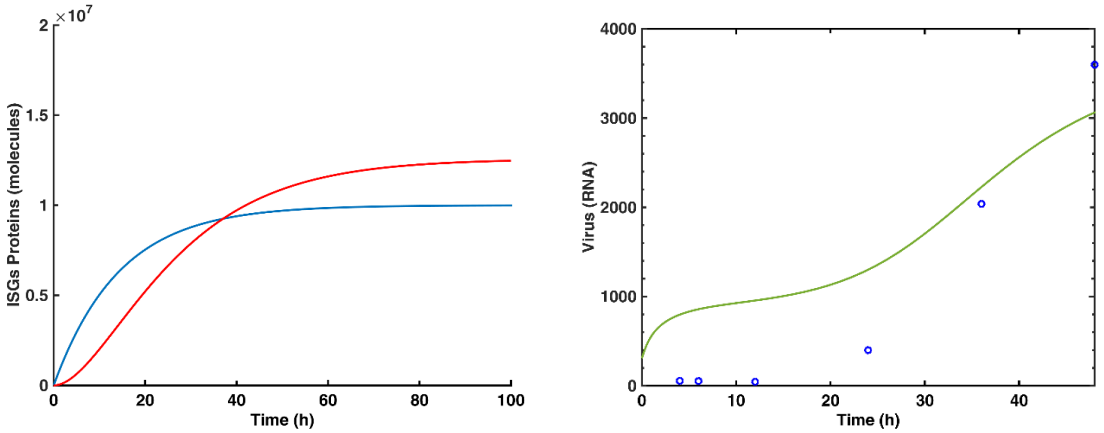


Figure 2: Left side: The time course of the ISGs proteins with absence of dengue virus, red line is an experimental approach and the blue line is model solution using the parameter in table 1 with $V(0) = 0$ and $P(0) = 1$. Right side: The time course of virus concentration without immune innate response, the blue circles are the experimental data from a HepG2 culture and the green line is the model solution with $V(0) = 300$ and $P(0) = 0$.

We further studied the bistability in the minimal model of dengue. The steady states of the system were calculated (using all the parameters in Table 1). Numerical values of two stable steady states are productive state, this state represents the virus replication in cell (V=3600, P=0 molecules) and clearance state, this state represents a no productive state in cell (V=0, P=1x10^7 molecules). The phase plane is shown in Figure 3. The viral dynamics depends on the initial viral load. The threshold was 130 molecules (initial conditions). The system exhibits a great separation and transversally which are two necessary conditions for an efficient biological switch model (Cherry *et al*. 2000).

To analyze the influence of the ISG proteins in the bistability of the minimal model of dengue, we modified the value of the k_2 parameter, which represents the strength of ISG proteins feedback loop. As illustrated by Figure 4 the clearance state is the only steady state present when k_2 parameter value is increased 1.2 times the nominal value reported in table 1. The productive steady state has been removed from the system. Only one stable steady state is presented, the clearance. This modulation would be experimentally compared as a transient overexpression in some of the ISG proteins as result of the exogenous addition of IFN or interfering RNA therapy, according to (Meng *et al*. 2013).

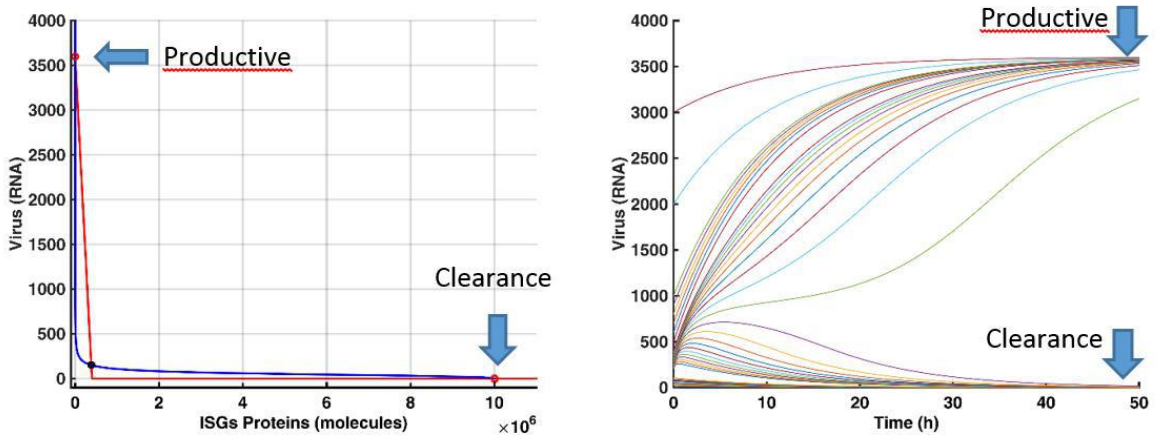


Figure 3: Bistability in the minimal model of dengue. Left side: Nullclines that show the multiple steady states, red circles are the stables and the blue circle the unstable steady states. Right side: Initial virus concentration *versus* time shows bifurcation and the existence of three steady states.

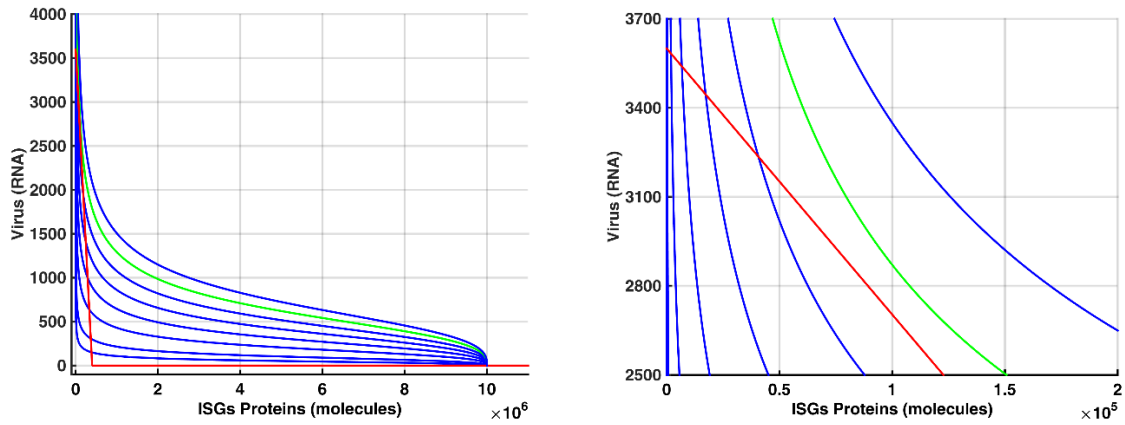


Figure 4: Virus behavior in the ISG inhibition-less dengue model. Nullclines in response to decreasing strength of the ISG inhibition loop. Left side: The multiple nullclines varying the k_2 parameter value in range of 0 to 1.4 fold changes and the nullclines in blue color. Nullclines have moved from left to right: Red nullcline was fixed. *Right side*. A zoom area when the productive steady state was removed as k_2 increased 1.2 fold in green color, and higher values.

Finally, to corroborate the above predictions, we performed again numerical simulations with k_2 increased 1.2 fold and different initial conditions in virus load. Figure 5 illustrates the simulations results. The productive steady state was removed.

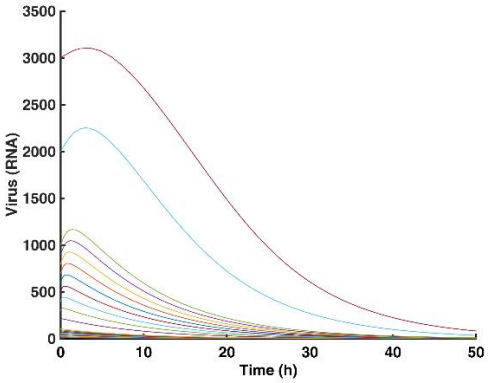


Figure 5: Time course of virus concentration with different initial conditions, after k_2 parameter was increased 1.2 fold change.

Conclusions

We developed a mathematical model based on the minimal model of dengue. Our model is a first approach to single cell interaction between dengue virus and innate immune response. It was validated by experimental data. We predicted bistability and that the overexpression of ISG proteins (k_2 parameter) was necessary to remove the productive steady state.

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BIOTECHNOLOGY SUMMIT

Label Free Molecular Interaction Studies for the Advancement in Human Disease Research

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Abstract

Surface plasmon resonance (SPR) spectroscopy is a phenomenon that occurs when polarized light hits a metal film at the interface of media with different refractive indices. This is a label-free and powerful technique that has been widely used for studying biomolecule kinetic processes in applications such as cancer and Alzheimer's research. In this paper, we will present a novel SPR design approach and its applications in the study of detecting aggregates/fibrils of amyloid-β (Aβ). Recently, electrochemical enhanced Surface Plasmon Resonance (EC-SPR) has been utilized to successfully evaluate the treatment efficiency of daunorubicin (DNR) and HepG2 cancer cell. Studies of a glucose biosensing detector using EC-SPR will be covered in this paper.

Keywords: surface plasmon resonance ● real-time molecular interactions ● cancer cell ● biosensor ● electrochemistry.

Introduction

When a light beam hits a metal-coated film at a resonance angle, the electrons in the film are set to resonate with the light wave. The resonance results in the absorption of light (Schasfoort *et al.* 2008). The focused light provides a range of incident angles, and the reflected beam will cover the same range of the angles, which its projection forms a rectangular shaped band of light. When the SPR angle occurs within the spread of angles, a dark line will appear in the band. An intensity profile of this band is plotted against the range of angles.

This profile is often used in a typical SPR experiment to indicate that the metal film matches the SPR resonance condition. However, the actual measurement of molecular adsorption kinetics is only related to the shift of the absorption dip in the intensity profile corresponding to the SPR angle change (Figure 1). One has to sacrifice the resolution of the measurement to acquire the whole intensity profile curves and compare them to find the shift of the dip.

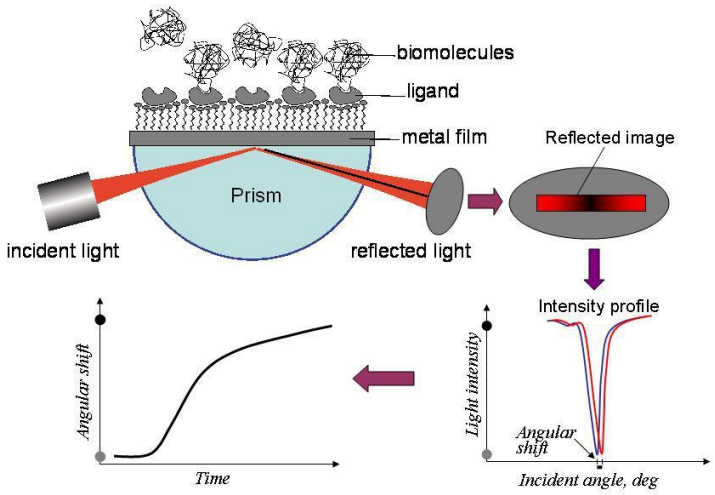

Figure 4 SPR architecture.

Biosensing Instrument (BI) uses a different approach to detect the SPR angle change by using an enhanced position-sensitive device with advanced algorithm to detect the SPR absorption dip shift. Since it only measures the position shift of the dip, it offers a highly sensitive detection scheme to measure extremely small angle changes of the SPR. As long as the SPR angle resides inside the angle spread, the system delivers exceptionally high angular resolution in its measurement.

With this SPR architecture, a study to quantify amyloid-β (Aβ) from cerebrospinal fluid samples collected from Alzheimer's disease patients and healthy donors was performed. Capture antibodies for each peptide was immobilized in two channels to measure the concentration of Aβ(1-40) and Aβ(1-42). The experimental data shows that the ratio of Aβ(1-40) concentration vs Aβ(1-42) concentration of Alzheimer's disease patients is almost twice as high as the values from healthy people (Xia *et al* 2010).

Voltametric technique in electrochemical (EC) detection is a very powerful analytical method that can be potentially be used for a wide range of applications. When combined with SPR technology, EC-SPR provides deeper insight and greater capability for studying molecular interactions and surface processes.

In EC-SPR, the thin metal film not only serves to excite surface plasmons, but also acts as a working electrode for electrochemical detection. A change in the electrode potential can

shift the resonance angle via different effects. Traditionally, EC-SPR has been used for developing biosensors for health, food and environmental applications. In here, we present a biosensor for glucose detection. A thin gold film modified with polypyrrole and glucose oxidase (PPy-GOx) as the sensor chip revealed that the enzymatic reaction between GOx and PPy in the presence of glucose can lead to distinct changes in the SPR signal (Tian *et al* 2010).

In a recent study, EC-SPR has been utilized to measure apoptosis of cancer cells (HepG2) after treatment of Daunorubicin (DNR), an anti-cancer drug commonly used to treat specific types of leukemia. This study shows that the redox activity was attributed to two electroactive groups (a *p*-diquinone and a *p*-diphenol) of DNR (Wu *et al* 2015).

Materials and Methods

Various BI SPR models with the enhanced position-sensitive architecture were utilized to carry out the experiments. For EC-SPR experiments, an electrochemical workstation (such as CH Instruments) and the BI's EC analysis module was used. Systems were calibrated using a standard method.

SPR Au standard chips (18mm x 18 mm, 47nm Au and 2nm Cr coated on BK7 glass) were utilized. Different preparations were done on the Au chip depending on the applications reported in this paper.

Results and Discussion

Table 1 shows the concentrations of Aβ(1−40) and Aβ(1−42) in the samples of five age-matched Alzheimer's patients. For comparison, samples of five healthy donors of similar ages were also analyzed.

As expected, the concentration of Aβ(1−40), the most abundant Aβ peptide, is higher than that of the Aβ(1−42) concentration. Perhaps the most interesting finding is that the Aβ(1−42) concentration in the Alzheimer patients' cerebrospinal fluid samples is much lower than that taken from the healthy controls. In fact, the Aβ(1−40)/Aβ(1−42) concentration ratio for the AD patients is about twice as high as that for the healthy controls. The cause of this decrease can be rationalized by deposition of the insoluble aggregates of Aβ(1−42), which reduces the soluble Aβ(1−42) monomers in the fluid.

Table 2 Clinical parameters and SPR results for Aβ(1−40) and Aβ(1−42).

Samples	Aβ(1−40)	Aβ(1−42)	Aβ(1−40)/ Aβ(1−42)
Healthy controls: 3 males and 2 females Ages of 78 years (3.2)*	2.87 +/- 0.043	3.14 +/- 0.13	3.91 +/- 1.05
AD patients: 3 males and 2 females Ages of 76.4 years (4.2)*	1.03 +/- 0.013	1.18 +/- 0.047	6.89 +/- 1.55

* Mean age (standard error of the mean) and matched (Mann-Whitney test $p = 0.402$)

In the polypyrrole and PPy-GOx EC-SPR experiments, a linear relationship was obtained in the range 1–100 μmol L^{-1} between glucose concentration and the rate of redox transformation of PPy shown in Figure 2. The detection limit was 0.5 μmol L^{-1} (S/N = 3) and recoveries were 95.2–102.7%.

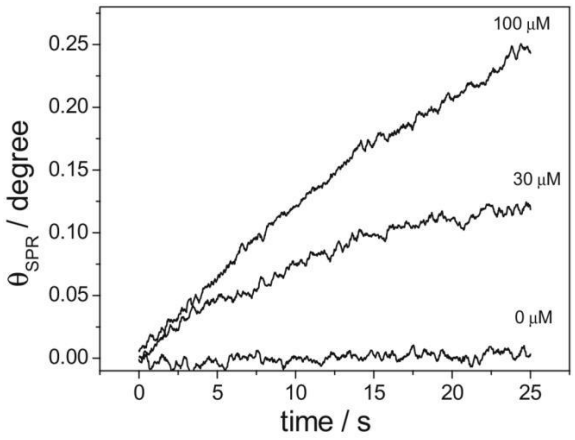

Figure 5 Glucose Detection.

For the EC-SPR experiment on the HepG2 and DNR, incubation of the cells with DNR led to apoptosis of the cells and detachment from the SPR chip surface. This exposed the Au film to the cell culture medium, and increased the electrochemical current. A further study of current change vs. DNR concentration and incubation times was performed. The SPR signal decreased as the potential cycled to negative direction due to removal of oxidized layer on the Au electrode (Lioubimov 2004).

Conclusions

SPR is a relatively new technique that determines the mass changes by measuring resonance on the surface of immobilized molecules as molecular interactions occur in real time. In this paper, we have shown that SPR can potentially serve as an alternative for clinical analysis of important biomarkers such as neurodegenerative diseases, research in health related biosensors and cancer studies.

Acknowledgments

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Whole Genome Sequencing, Annotation and Manual Curation of WSSV-MX08 isolated from *L. vannamei* cultivated in Sonora

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Abstract

It has been manually cured the first genome of white spot syndrome virus, sampled from white shrimp farming in Mexico. A pandemic virus that has devastated shrimp production for more than 10 years in our country. Availability databases of a well-annotated reference, isolated on another continent and several years after its first outbreak in Asia, will help to epidemiological studies, contributing to molecular diagnosis and disease prevention in shrimp farming.

Keyboards. Ion Torrent. ● Illumina. ● WSSV. ● manual curation.

Introduction

In Mexico, shrimp farming is the most important aquaculture economic activity. However, its sustainability has been at risk by viral outbreaks. Presently, White Spot Syndrome Virus (WSSV) is the most devastating shrimp viral pathogen worldwide, classified under a new genus *Whispovirus* in the family *Nimaviridae*, WSSV is extremely virulent and with a wide host tissue tropism (Marks *et al*. 2005). Currently there are nine WSSV complete reference genomes deposited in the public databases included the one presented here. Nevertheless, the information related to WSSV functional genomics is scarce, 90% of the predicted ORFs have no similarity to any known proteins, additionally there are evidences about the existence of strain variability and differences in pathogenicity among geographical isolates of WSSV (Gonzalez-Galaviz *et al*. 2013; Durán-Avelar *et al*. 2015).

Tabla 1. Information of WSSV reference genomes published in the genebank.

Strain	Host	Access	Genome Size (bp)	Sampling Date month/year	Country	Autors
WSSV-TW	*P. monodon*	AF440570	307,287	11/1994	Taiwan	Tsai *et al.,* 2000
WSSV-CN	*P. japonicus*	AF332093	305,119	10/1996	China	Yang *et al*., 2001
WSSV-TH	*P. monodon*	AF369029	292,967	05/1996	Thailand	Van Hulten *et al*., 2001
WSSV-KR	*L. vannamei*	JX515788	295,884	08/2011	Korea	Chai *et al*., 2013
WSSV-EG3	*No data*	KR083866	305,119	2014	Egypt	Megahed, 2015
WSSV-CN01	*M. japonicus*	KT995472	309,286	10/1994	China	Gao *et al*., 2015
WSSV-CN02	*P. clarkii*	KT995470	294,261	04/2010	China	Gao *et al*., 2015
WSSV-CN03	*L. vannamei*	KT995471	284,148	05/2010	China	Gao *et al*., 2015
WSSV-MX08	*L. vannamei*	KU216744	293,183	11/2008	Mexico	Rodriguez-Anaya *et al*., 2016

Due to advances in sequencing technologies, the number of genomes is constantly increasing allowing us to make analysis of genomic comparisons in search important mechanisms of evolution (Kulkarni-Kale *et al*. 2006) and variations to understand how the genetic sequence of the pathogen it is related to the mechanisms by which causes the disease (Liu *et al*. 2009). However, it is well known that in the absence of a cured and functional annotation, the utility of genomic data is minimal and the sequence remains as an entry in the database (Kulkarni-Kale *et al*. 2006). Therefore, the aim of this work, was to manually cure the WSSV-MX08 and thus increase its quality to identify regions of variation that would indicate differences in virulence and the design of an efficient and updated diagnosis of possible strains.

Material and Methods

Sample, DNA extraction and Sequencing: WSSV-infected shrimp stored at -80°C from the 2008 shrimp farming cycle in Sonora State was used for this work. Total DNA was extracted with the GeneJET genomic DNA purification kit according to the manufacturer's instruction. Genomic DNA was quantified with a NanoDrop 2000c spectrophotometer (Desjardins and Conklin, 2010) and the quality was determined via agarose gel electrophoresis. Whole genome sequencing was performed using an Illumina NextSeq500

and Ion Torrent PGM instruments at Unidad de Secuenciación Masiva y Bioinformática, Universidad Nacional Autonoma de Mexico (UNAM).

Mapping reads, Assembly and Genome annotation: Using Bowtie2 program (Langmead and Salzberg, 2012) we aligned the reads to four reference genomes (WSSV-TW, WSSV-CN, WSSV-TH and WSSV-KR) to separate those belonging to the virus from the shrimp genome. Subsequently assembled with MIRA4 (Chevreux *et al*. 2004) using WSSV-TH as a reference and RATT program (Otto *et al*. 2011) for transfer its annotation to WSSV-MX08. With Artemis Genome Browser v16.0.0 (Rutherford *et al*. 2000) we visualized the genome to identify possible changes in the genomic structure.

Manual curation and Sanger sequencing using forward and reverse primers: A manual curation was perform aligning the reads with CLC Genomics v8.5.1 (QIAGEN Company) and reviewing them with Artemis to evaluate discrepancies in our genome. Only the primers described by Marks *et al*. (2005) (ORF14/15) aligned in the correct region. So, with Primer3Plus (Untergasser *et al*. 2007) we design primers for the ORFs 125, 126 and 128. Table 2 show the PCR protocols, amplicons were analyzed by agarose gel and recovered DNA using the Zymoclean Gel DNA Recovery Kit (Humphreys *et al*. 2015). Both, primer synthesis and Sanger sequencing were realized at Unidad de Sintesis y Secuenciación de DNA, UNAM.

Table 2. Primers designed and amplification protocol.

Primer ID	Sequence (5'- 3')	Annealing temperature (˚C)/Elongation time (s)	Amplicon Size (bp)
ORF125-MX-F	AAGAGAAGATCGGACGCAAA	60/120	1990
ORF125-MX-R	CCCCTTTTCTAGTTCCATGTCTT		
ORF126-MX-F	CACATCTGTGAACATTTCTTGC	60/120	1700
ORF126-MX-R	GGGTGTTGCAAACAAACAAA		
ORF128-MX-F	ACAAATATCGGTGTCGCTGA	60/120	1350
ORF128-MX-R	TTATTTTTGTTGCACAAAGGACAT		

Results and Discussion

Of the MIRA4 assembly, it was obtained a genome with 293,183 bp before the manual curation, the G+G content was 41%. After to curing, the size was reduced to 292,589 bp. Analyzing the annotated genomic structure we found that the 184 ORFs transferred from WSSV-TH, 16 ORFs were rejected for our genome. Table 3 shows the differences between WSSV-MX08 and WSSV references where rearrangements such as indels and SNPs mainly truncated the annotation of those ORFs forming stop codons.

Table 3. Variations identified in the ORFs that needed manual curation.

ORF	Error	Cured
5.1	A insertion	Yes
6.1	SNP A-G	Yes
14.1	Due to a deletion in this region, many stop codons were generated	No*
33.1	T insertion	Yes
66.1	C insertion	Yes
70.1	This ORF is smaller now	Yes
75.1	C insertion	Yes
85.1	C insertion	Yes
122.1	Forced annotation from the reference, this ORF was removed	Yes
123.1	Forced annotation from the reference, this ORF was removed	Yes
125.1	AAAAA insertion, SNP G-A, A insertion, A deletion and SNP C-T	No*
126.1	7 SNPs along the ORF	No*
128.1	A deletion	No*
154.1	TGA deletion, TCC insertion, G AGG, AGG, AGG insertion.	Yes
156.1	T and A insertion	Yes
162.1	TA insertion	Yes

*These ORFs could not be cured with reads mapping analysis, they need a Sanger sequencing.

We corroborate that not only exist sequencing artifacts, also assembly errors. ORFs 122 and 123 originated a large number of “Ns”, because RATT program forced an entry in that region, however, these ORFs are no longer part of WSSV-MX08, the lack of these ORFs were reported for WSSV-KR (Chai *et al*. 2013), WSSV-CN01, WSSV-CN02 and WSSV-CN03 (Gao *et al*. 2015). Another example of forced annotation was ORF70.1 because could not be inserted into its region by changes in size, according to Gao *et al*. (2015) in WSSV-CN01 the wsv108 (ORF70) it is composed of 690 bp and 894 for WSSV-MX08 instead the 1219 bp for WSSV-TH (van Hulten *et al*. 2001). Some of the major challenges associated with technologies of next generation sequencing (NGS) are repetitive regions of DNA, ie, sequences that are similar or identical througout the genome, they can be in different shapes or sizes (Treangen and Salzberg, 2013). This problem was found in 10 ORFs not annotated, when we analyzed the reads we observed that there are mapping errors described as “incorrect overlap reads” due to repeated regions, as well, sequencing errors by the presence of homopolymers (Brown, 2008).

On the other hand, we need support the lack of four ORFs uncured because two of them have been widely used as molecular markers (ORF14 and ORF125) (Gonzalez-Galaviz *et al*. 2013; Duran-Alvear *et al*. 2015) besides that, ORF128 encode for a structural protein (VP52B) (Lin *et al*. 2015). All the PCR product were the expected, however, when we looking the annotation of start codon-stop codon, the ORF14.1 decreased its lenght to 294 bp due a deletion in this region, like the wsv461(ORF14) of WSSV-CN03 (Gao *et al*. 2015). The ORF125.1 and ORF126.1 showed sequencing artifacts for homopolymers and mapping errors for repeated sequences. Both ORFs also downsized (1347 bp ORF125.1 and 726 bp ORF126.1). Finally, in ORF128.1 sequence showed a deletion of adenine creanting stop codons. However, to realign all references to the Sanger sequence, it was observed that the deletion, was again a sequencing artifact for Adenine homopolymers.

In order to assess the possible sequencing errors with the NGS platforms, Quail *et al*. (2012) compared the results of PacBio, Illumina and IonTorrent PGM. One test was sequenced to *P. falciparum* to have a significant amount of repeated sequences, they found that mapping reads of PGM only covered 65% of the genome with good quality (> Q20) compared to 98-99% of the other platforms. With the antecedent of the study mentioned above Bragg *et al*. (2013) conducted an errors characterization of the Ion Torrent PGM data, describing the indels as the dominant errors, being the insertion more common than deletion and homopolymers are responsible for 96-97% of the total error. All these errors were found during the manual curation process of WSSV-MX08.

Conclusion

The manual curation of WSSV-MX08 confirmed several structural changes that will be important to understand the WSSV evolution. However, further studies are needed to characterize the genomic variations in a protein context and associate them to other variables such as the geographical distribution, virulence phenotypes and host-virus interactions.

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Potential production of melanin in two mutants Δ-Pma of *Ustilago maydis*

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Abstract

Ustilago maydis is the causal agent of the corn smut, known as "hutlacoche". In the diploid phase, during the infectious stage, the teliospores produced by the fungi generate galls of dark-gray color in the grains. *U. maydis* is capable of forming melanin that it's found mainly on the cell wall. In our work group two *U. maydis* mutants of the proton pump H+ATPAse were obtained and were morphologically characterized by transmission electron microscopy (TEM), presenting intracellular accumulation, suggesting the presence of melanin. The pigment extracted presents different properties that are characteristic of melanin, like insolubility at acid pH and a dark brown color. The pigment was partially purified and analyzed by UV-VIS and infrared. The spectrums obtained were compared with commercial synthetic melanin (Sigma) and the peaks presented were characteristic for the melanin pigment. In this work we have shown that mutants with H+ATPase deletion accumulate melanin. Pigment characteristics offer the possibility of using these mutants as melanin producers for biotechnological use.

Keywords: ● Melanin ● *Ustilago maydis* ● TEM ● proton pump H+ATPase.

Introduction

Melanin is a high molecular weight biopolymer produced from the oxidation and polymerization of phenolic compounds (Almeida-Paes *et al*. 2012). It´s found in a large group of organisms, from bacteria to humans. Most fungi produce melanin and the function of these pigments is related to the survival in unfavorable environments (Liu and Nizet 2009). In addition, melanin has antioxidant and antimicrobial properties (Dharnik and Gomashe 2013).

Ustilago maydis is a phytopathogenic dimorphic basidiomycete that causes the "corn smut" disease. During the infectious stage it produces galls on the grains with a dark grey or black color (Ruiz-Herrera *et al*. 1995). *U. maydis* is capable of forming melanin that its distributed in the cell wall, since in his life cycle during the teliospore form its highly melanised (Choi *et al*. 2015). In our work group two mutants with deletion in the H+ATPases (Pma1 and Pma2) were obtained and morphologically characterized by TEM, presenting large intracellular electrodense accumulations, suggesting the presence of melanin.

Currently, there is no information about melanin metabolism in *U. maydis*, therefore this study has as main objective the identification and characterization of the pigment produced in these mutants.

Materials and methods

Biological material: The commercial strain *U. maydis* FB2 ATTC 201384 and two mutants (ΔPma1 y ΔPma2) obtained from the collection of the biochemical and biotechnology of fungi laboratory from the National school of biological sciences - IPN were used.

Growth kinetic: 80 ODU/L were inoculated in Erlen-Meyer flasks with 30 mL of minimal medium (1% glucose, 0.3% $NaNO_3$, salt solution) and YPD (1% glucose, 0.15% NH_4NO_3, 0.25% peptone, 1% yeast extract, salt solution), incubated 60 hours, aliquots were taken every 12 hours to calculate growth by optical density, also, dry weight and pigment production was assessed.

Pigment extraction: Every 12 hours 25 mL of YPD and minimum medium were taken, centrifuged for 5 minutes at 2 000 rpm. The wet cell pellet was weighed and the lysis solution was added (150 mM NaCl, 0.5 % DOC, 0.1 % SDS, 50 mM Tris pH 8, 1% Tween 20) adding 2 mL of the lysis solution for each gram of the wet biomass. Cell breaking was performed by vortex with 10 cycles combined with pauses of 1 minute on ice. 10 minutes centrifugation at 3,000 rpm was used to remove cell debris, the supernatant was discarded and washed with 2 mL of distilled water and re-centrifuging. 1 mL NaOH 2M was added per gram of the pellet wet weight and allowed to rest 24 hours. After that time, a 10 minutes 3,000 rpm centrifugation was made and the supernatant was collected in a clean, labeled ependorf tube, adjusting it to pH 2 with HCl 2M. It was finally centrifuged 5 minutes 3 000 rpm and dried at 60° C for 48 hours.

Pigment purification: For partial purification of the pigment, it was washed with an alkaline solution and chloroform, centrifuged for 10 minutes at 2,000 rpm and adjust the pH to 2 with HCl 2M. It was dried at 60° C for 48 hours.

UV/Vis spectroscopy analysis and infrared: For the UV, 1 mg of dry sample was taken and mixed in a NaOH 0.1 M solution, the absorbance was measured with a continuous scan of wavelengths every 5 nm from 200 to 800 nm, a Biomate 3® spectrophotometer was used. For the infrared spectrum, 10 mg of dry sample were taken and analyzed in a mixture with KBr (1:10), the spectrum ran from 4000-400 cm^{-1}. Both U.V. spectroscopy and infrared spectrums obtained from the pigment was compared to the spectrums of the commercial synthetic melanin.

Results and discussion

In the growth kinetics, wild strain (FB2) and the both mutant strains reached stationary phase similarly in the first 24 hours, however, the growth rate on the exponential phase was higher of the mutants (Figure 1).

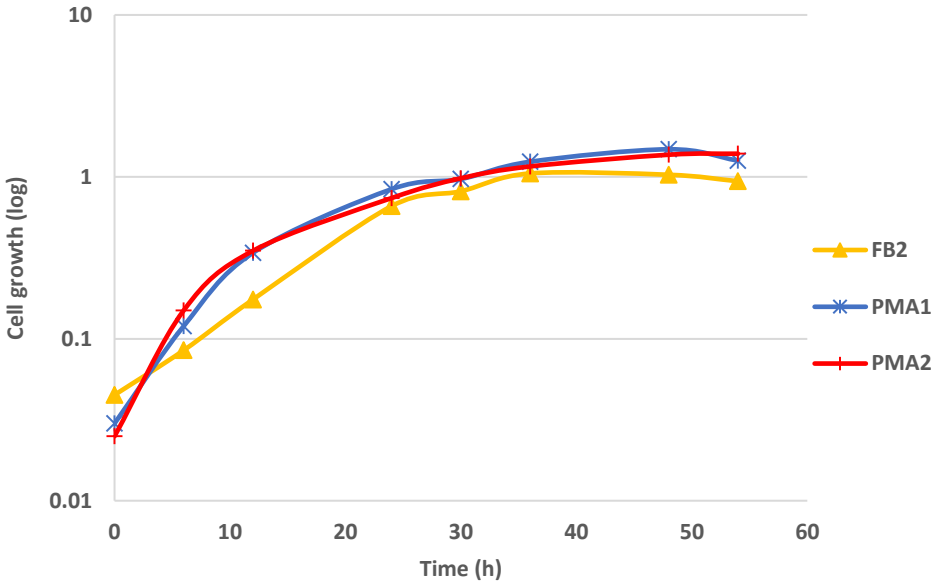

Figure 1. Growth kinetics of *U. maydis* on YPD medium.

The physical and chemical properties of the pigment produced by *U. maydis* were compared with a synthetic melanin (Sigma). The purified melanin showed typical features such as insolubility in water, solubility in NaOH, precipitation at pH below 2, and maintaining a dark brown color.

The absorption spectrum of melanin obtained from culture mediums showed similar patterns. Having a lower absorbance with the increase of the wavelength profile, typical for melanin absorption. The absorption spectrum shows a characteristic peak between 250 and 300 nm (Figure 2) due to the presence of conjugate complexes in the melanin molecule (Manivasagan *et al*. 2013).

The infrared spectrum of the pigment was similar to the synthetic melanin. The IR spectrum (Figure 3) showed a broad absorption band between 3200 and 3300 cm^{-1} relating to the presence of hydroxyl groups (O-H) and bounds (-NH) (Manivasagan *et al.* 2013; Selvakumar *et al*. 2008; Suryanarayanan *et al*. 2004). The peak of the 1718 cm^{-1} region was caused by the vibration of C=O or COOH groups and the peaks at 1620 cm^{-1} were assigned to the vibration of the aromatic groups C=C and COO^{-}. The small peak shown at 2300 cm^{-1} represents the N-H stretch.

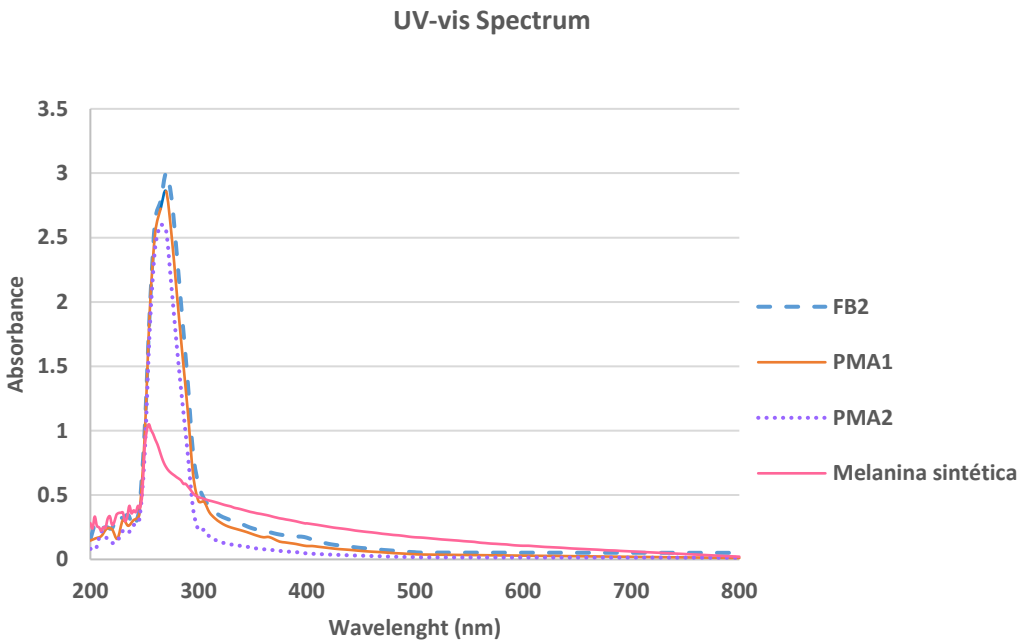

Figure 2. UV absorption spectrum of melanin pigment obtained from *U. maydis* and synthetic melanin (Sigma)

Conclusions

Ustilago maydis is a basidiomycete fungal that accumulates melanin in its teliospore form, in this work we have shown that mutants with deletion of proton pump H+ATPases from plasmatic membrane, accumulates melanin in form of melanosomes. This feature offers the possibility of using these mutants as major melanin producers for biotechnological use.

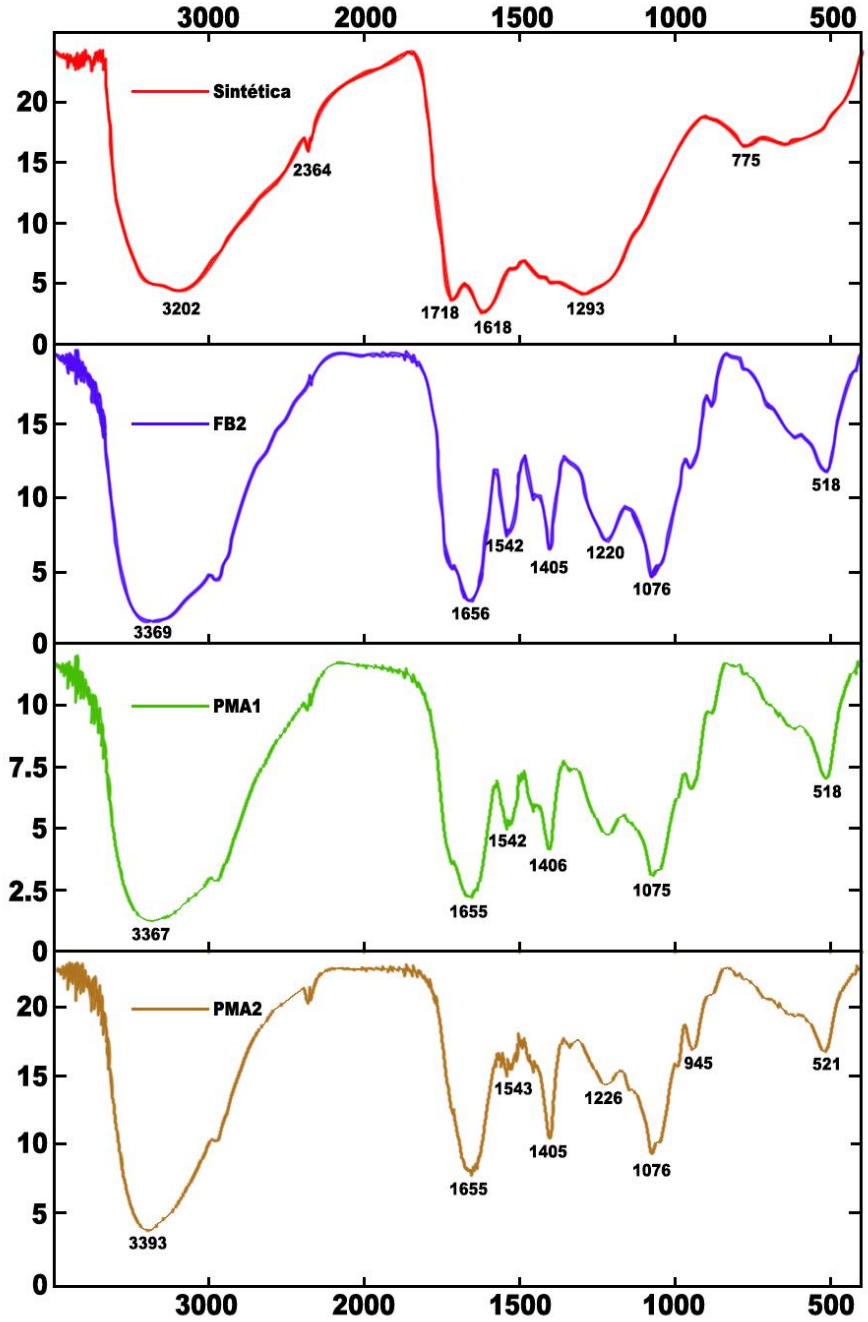

Figure 3. Infrared spectrum of melanin extracted from *U. maydis* compared to synthetic melanin (Sigma)

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Expression of sialyltransferases in the liver of tumor-bearing mice under chronic stress conditions

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Abstract

Chronic stress is now considered a significant threat to human health, and the link between psychogenic stress and a diverse array of mechanisms relevant to cancer initiation and progression has been proved. Although not considered a hallmark of cancer, it is accepted that aberrant glycosylation has an important role in promoting or facilitating cancer survival through its many roles in cell adhesion, recognition and signaling, and of the many types of alterations in glycosylation, the addition of sialyl groups seems to have an important role in cancer. In this work, to explore the potential influence of chronic stress in sialylation activity during tumor progression, we used an immobilization restraint paradigm to induce stress in mice bearing the L5178YR lymphoma. After ethical sacrifice, samples from blood, liver and tumor were obtained. To determine HPA and sympathetic activation, plasma levels of corticosterone and norepinephrine were measured by ELISA. Relative expression of the sialyltransferases ST3Gal1, ST3Gal2, ST6GalNac1 and ST8Sia1 genes was assessed by quantitative PCR. Our results provide evidence that while ST3Gal1 and ST3Gal2 remained unchanged under stress, expression of ST6GalNac1 and ST8Sia1 is modulated by both tumor presence and stress conditions.

Keywords: • Neuroimmunomodulation • Glycobiology • Sialylation • cancer • L5178Y

Introduction

One of the molecular mechanisms relevant for tumor progression and survival is the aberrant glycosylation of proteins and lipids in the cell membrane of tumor cells (Christiansen et al. 2014; Häuselmann and Borsig, 2014). Even if not included in the famous review “The Hallmarks of Cancer” (Hanahan and Weinberg, 2011), for many scientists aberrant glycosylation is a molecular signature of cancer. Among the various alterations in glycosylation, sialylation, the addition of sialyl acids to oligosaccharides in glycoproteins and glycolipids in the cell surface is one that has attracted a lot of attention due to its potential as biomarker in cancer (Büll et al. 2014). This posttranslational modification is performed by a group of enzymes collectively known as sialyltrasferases, their activity significantly increasing in various cancer types, such as myeloma (Glavey et al. 2014) and breast cancer (Ren et al. 2014), this role in cancer has been thoroughly

reviewed before (Dall'Olio and Chiricolo 2001; Harduin-Lepers 2012; Dall'Olio et al 2014). To this day, there is little evidence of the effects of stress on sialyltransferase activity, a couple of studies suggest a potential axis for stress modulation mediated by IL-6 (Dalziel et al. 1999), in addition, it has been observed that individuals with psychiatric disorders display abnormal levels of sialyltransferases (Maguire et al. 1997) suggesting a link between glycosylation activity and the HPA axis. In the most complete study to this day testing this hypothesis; it was observed that chronic stress was able to influence the activity of sialyltransferases in liver and spleen of rats (Dabelić et al. 2004). However, this potential relationship has not been explored during cancer progression, thus giving originality to our study.

Materials and Methods

Animals were kept in an animal care facility; water and food were provided *ad libitum*. All procedures performed in animals were in compliance with ethical standards on animal welfare. For this study, female BALB/c mice aged 10-12 weeks were used, from day 0 to 23 the animals were subjected to stress; two paradigms, immobilization (30') and forced swim (15'), were randomly applied to avoid habituation in the animals (Zamora-González et al. 2013), on day 1 the animals received an intramuscular injection of $1x10^6$ L5178Y-R cells in a total volume of 0.2 mL of PBS in the right posterior flank (Gomez-Flores et al. 2005). On day 23, the animals were sacrificed and blood, liver and tumor samples were collected and kept at -80°C until their analysis. Plasma levels of corticosterone and norepinephrine were assessed using the Corticosterone EIA Kit (Enzo Life Sciences) and Mouse Noradrenaline ELISA kit (MyBiosource). For gene expression analysis, RNA from liver was isolated using trizol (Life Technologies), quantified and cDNA was obtained. For Real-Time PCR, pre-validated TaqMan probes for mouse GAPDH, ST3Gal1, ST3Gal2, ST6GalNac1 and ST8Sia1 were acquired from Applied Biosystems (USA). Relative expression of the genes of interest was calculated using the $\Delta\Delta$Ct method, normalizing to GAPDH as reference gen. For the statistical analysis of data, we performed t-Test for independent samples using the Vassar Stats online website (Lowry,n.d.)

Results

To determine the activation of the hypothalamic-pituitary-adrenal axis (HPA), levels of corticosterone in plasma of control and tumor-bearing mice in resting conditions and subjected to stress were assessed by ELISA. We found that both stress and tumor presence were associated with changes in corticosterone plasma levels. When comparing control animals with tumor-bearing mice, corticosterone levels were found to be lower in tumor-bearing mice under resting conditions ($p<0.0001$). In control animals, corticosterone levels were lower in stressed animals ($p<0.01$). While in tumor-bearing mice, higher levels of corticosterone were observed in stressed animals ($p<0.05$). Regarding sympathetic activation, we found that for norepinephrine, tumor-bearing mice, regardless of stress experience, showed a statistically significant elevation in plasmatic levels ($p<0.05$), suggesting that tumor presence acts as a stressor activating the sympathetic nervous system.

Table 1. Neuroendocrine responses of BALB/c mice to chronic stress.

		Corticosterone	Norepinephrine
CONTROL	Resting	50802.41±2265.25	106.51±3.78
	Stress	35601±401392**	146.15±21.66
TUMOR	Resting	26459.30±2884.47***	845.37±67.23*
	Stress	38665.27±7861.48*	729.40±71.06*

The results of the analysis of relative expression of the genes ST3Gal1, ST3Gal2, ST6GalNac1 and ST8Sia1 can be seen in Figure 1. While no variations of importance were observed for ST3Gal1 and ST3Gal2; ST6GalNac1 and ST8Sia1 showed alterations in expression due to both tumor and stress. In the case of ST6GalNac1, stress and tumor, separately, downregulated ST6 expression, interestingly, tumor-bearing animals under stress showed expression levels close to normal, this pattern of expression mirrors the changes observed for corticosterone under the same conditions. For ST8, while the liver of stressed mice showed expression levels close to the reference gene, the liver of tumor-bearing mice showed and increment of more than 2-fold, however, when tumor-bearing mice are subjected to stress, the effect associated to tumor not only disappears, but expression levels are approximately half of reference gene.

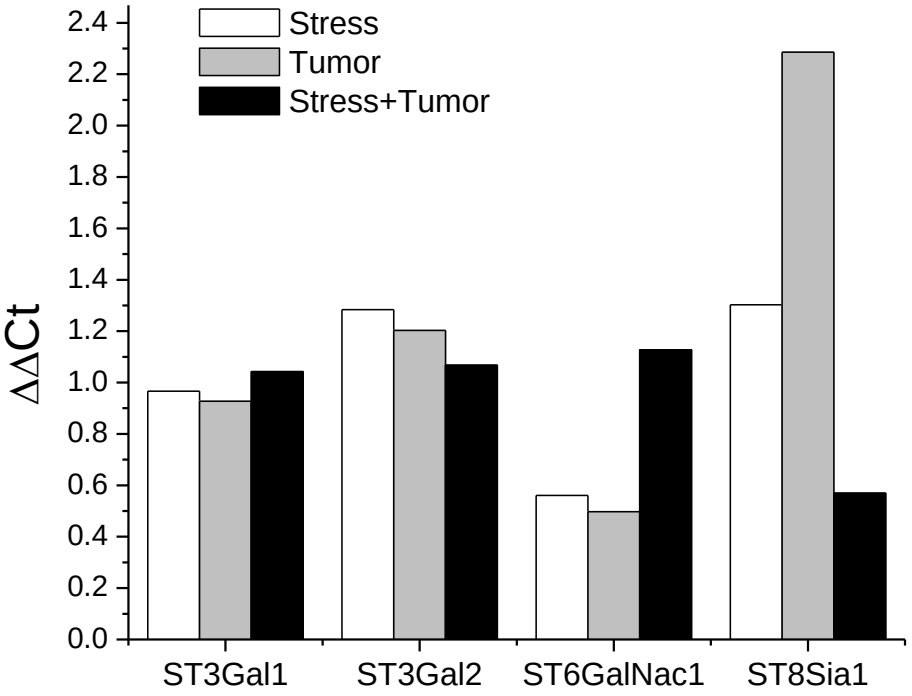

Figure 1. Relative expression of sialyltransferases genes in liver of BALB/c mice.

Discussion

This is the first work, to our knowledge, testing the hypothesis of modulation of sialylation by chronic stress by assessing the expression of genes coding for sialylation enzymes. Previously, Dabelić et al (2004) showed in rats that while acute stress increased total sialyltransferase activity in liver, chronic stress had the opposite effect. Interestingly, in the same study, corticosterone levels ran parallel to sialyltransferase activity, unsurprisingly, according to the authors, since sialyltransferase activity is transcriptionally regulated by corticosteroids (Wang et al, 1990). In this work we provide evidence of HPA activation in BALB/c mice subjected to a stress paradigm, as mentioned before, in our results the pattern of expression observed for the ST6 gene mirrors the changes observed for corticosterone under the same conditions, supporting the evidence of corticosteroid mediated stress effects on sialylation. Besides being in good agreement with the work of Dabelić et al, by targeting specific genes our work provides a clearer picture of the role of stress in the modulation of liver sialyltransferase activity, and additionally, their modulation in presence of tumor.

Conclusions

We found that chronic stress was able to activate the HPA axis in tumor-bearing mice increasing their plasmatic levels of corticosterone. Tumor alone was enough to activate the sympathetic nervous system with increased levels of plasmatic norepinephrine in tumor-bearing BALB/c mice. The analysis of gene expression showed that while ST3Gal1 and ST3Gal2 liver expression remained unchanged under stress in both control and tumor-bearing mice, expression of ST6GalNac1 and ST8Sia1 is modulated by both tumor presence and stress conditions.

Acknowledgments

We are grateful to the Laboratory of Immunology and Virology, FCB, UANL and the Secretariat of Public Education (PRODEP project UANL-PTC-835), Mexico for their support to this project. We also thank CONACYT for support for SMM.

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Inhibition of urease enzyme by methanolic extracts from root of cholla (*Cylindropuntia cholla*)

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Abstract
This study analyzed the root of *Cylindropuntia cholla* as a possible source of potentially inhibitory substances of urease enzyme. *C. cholla* cactus is abundant in the arid regions of Mexico, traditionally used to treat kidney diseases, urinary tract infections, and in some cases, to combat the formation of kidney stones. Anti-urease action was evaluated by initial velocity methods. The methanolic extract of *C. cholla* showed an inhibition of urease activity. We calculated the IC_{50} using extract concentrations from 0 to 5 mg/mL, the optimal concentration was 2.04 mg/mL. To characterize the type of inhibition kinetics assays were performed increasing concentrations of the *C. cholla* extract (2 mg/mL). Data were fitted to Lineweaver-Burk model, a typical behavior of uncompetitive inhibition was obtained.

Keywords: Urease inhibition ● *Cylindropuntia cholla* ● Kidney stones

Introduction

Urease is an enzyme that catalyzes the hydrolysis of urea to ammonium ions and carbamic acid. Carbamic acid evolve into carbon dioxide and ammonia. Thus, high activity of urease causes an increase in the concentration of these compounds, which in turn leads a negative impact on health (Adil *et al*., 2011). A clinical complication, is the formation of struvite kidney, due to an increase in pH of urine caused by the presence of ammonia. It has been investigated different classes of compounds with inhibitory properties against urease, among them are (but not limited to): phosphoramidates, boric acid hydroxamic acids, and heavy metal ions (Modolo *et al*., 2016). However, most of these compounds are toxic or unstable. That is why, this study sought to analyze the root of *C. cholla* as a possible source of chemicals with potential inhibitory effects on urease, due to their ethnopharmacological uses in Northwest of Mexico. In traditional medicine has been reported that *C. cholla* is an adjuvant in the treatment of kidney ailments. The phytochemical characterization of this plant has not been widely studied, although the root of the plant has different uses in ethnomedicine as treatment of inflammation, flu, stomach pain and urinary tract infections (Andrade *et al*., 2006). The aim of this study was to evaluate the inhibition of urease activity by methanolic extracts of *C. cholla* roots.

Materials and methods

Plant extraction

Roots of four samples were processed. Firstly, roots of *C. cholla* were washed with distilled water to remove the soil residues, then, roots were stored in a plastic bag at −80 °C for 24 h. Thereafter, the samples were freeze-dried for 3 days at −48 °C and ground in a blender into fine particles. Extracts were obtained by weighing 10 g of plant material and 100 mL of methanol:water (80:20, v/v) were added. Mixture was sonicated for 30 minutes and centrifuged for 15 minutes at 3000 rpm. The supernatant was filtered and distilled at reduced pressure to remove the methanol. The residue was recovered, froze and lyophilized. The resulting extract was packed under vacuum and stored at −20 °C until later use (Núñez-Gastélum *et al*., 2015)

Inhibition test of urease by *C. cholla* extracts

A calibration curve was performed as follows: the assay mixtures were prepared in a microtiter plate 96 well of 300 µL. Increasing concentrations of urea (0−35 Mm) were added as a starting substrate to 150 µL of reaction mixture (MES 1 mM, NaCl 100 mM and phenol red 32 mM at pH 6.8), 10 µL of urease solution and 140 µL of water. The absorbance was followed on microplate reader at 558 nm. Same conditions were used to test the inhibition property of the extract, the reaction medium kept constant in urea (2 mM), volume (150 µL), and enzyme (3 µL), but different concentrations of the extract (0−5 mg/mL) were added. All experiments were made by triplicate.

Results and discussion

The methanol extract was yellowing with a yield of 2% in dry basis. The enzyme kinetic showed a K_m = 6.54 mM and V_{max} = 0.74 µmol ml^{-1} min^{-1}. As we can see, the enzyme fits to Michaelis-Menten equation (Figure 1).

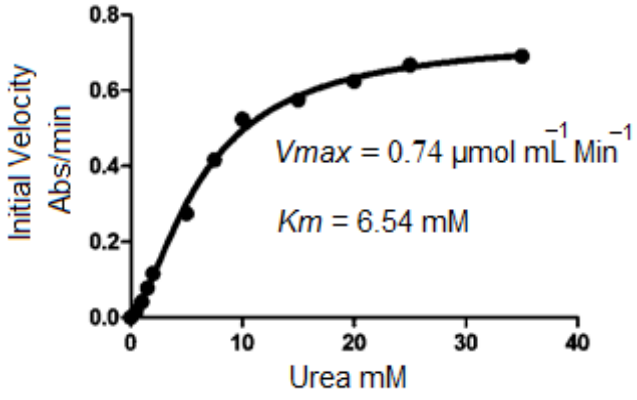

Figure 1. Effect of substrate concentration on urease activity.

Figure 2 shows the inhibition curve of the methanol extract at constant concentration of urea (2 mM) versus increasing concentrations (0−5 mg/ml) of *C. cholla* extract. The half inhibitory concentration (IC_{50}) of enzyme activity was 2.04 mg/mL. Kinetic analysis of urease inhibition by the methanolic extract from the root of *C. cholla* resulted in the reduction of V_{max} and increased K_m values. Up to our knowledge, there are no previous studies on the activity anti-ureasa by *C. cholla* extracts. Previous studies have been reported antiurease activity by different compounds, mainly; alkaloids, anthraquinones, saponins, cardiac glycosides, tannins, cyanogenic glycosides and flavonoid. Some of these compounds could be part of secondary metabolites of the plant used in this study (Modolo *et al*., 2016). Colchero (2009) reported the phytochemical analysis of the methanol extract in *Cylindropuntia imbricata*, and managed to identify the presence of alkaloids, flavonoids, coumarins, tannins, cardiac glycosides, steroidal compounds and aromatic derivatives associated with sugars.

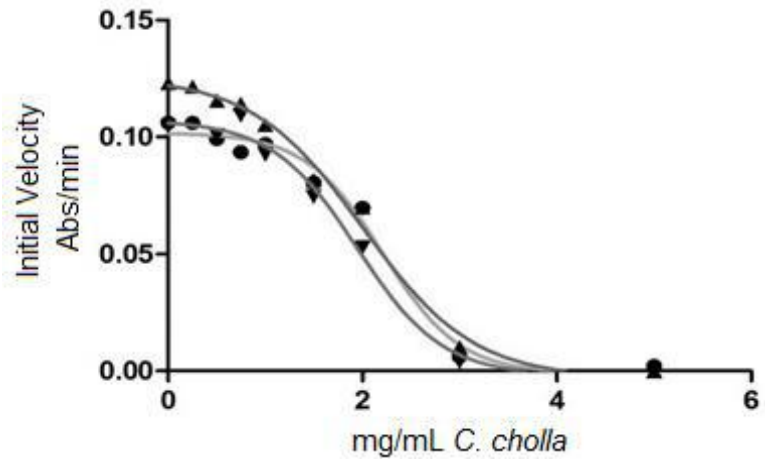

Figure 2. Inhibition curve of the urease activity by increasing extract concentrations keeping constant the concentration of urea (2 mM).

To determine the type of inhibition caused by the extract of *C. cholla* on the urease, kinetic inhibition experiments were performed. In this case, an increasing concentrations of urea, in the absence or presence of the extract (2 mg/mL), were evaluated (Figure 3). The equation of double reciprocals of Lineweaver-Burk was used (Figure 16B). Values without extract had a K_m = 3.1 mM and V_{max} = 0.14 µmol ml^{-1} min^{-1}; but with extract was 3.8 mM and 0.06 µmol ml^{-1} min^{-1}, respectively.

The set of parallel lines seen in the Figure 3B suggests an uncompetitive inhibition, which occurs when an inhibitor binds to an enzyme-substrate complex. This type of inhibition is typically observed with multisubstrate enzymes, which is not the case of urease whose only substrate is the urea besides the water. However, some uncompetitive inhibitors can interact with a hydrolytic enzyme in a place normally occupied by the catalytic water (Todd and Hausinger, 2000). In the urease structure, each nickel atom has roped to a water

molecule (Carter *et al*., 2009). This study gives clues to unravel the mechanism of phytopharmaceutical properties *of C. cholla*.

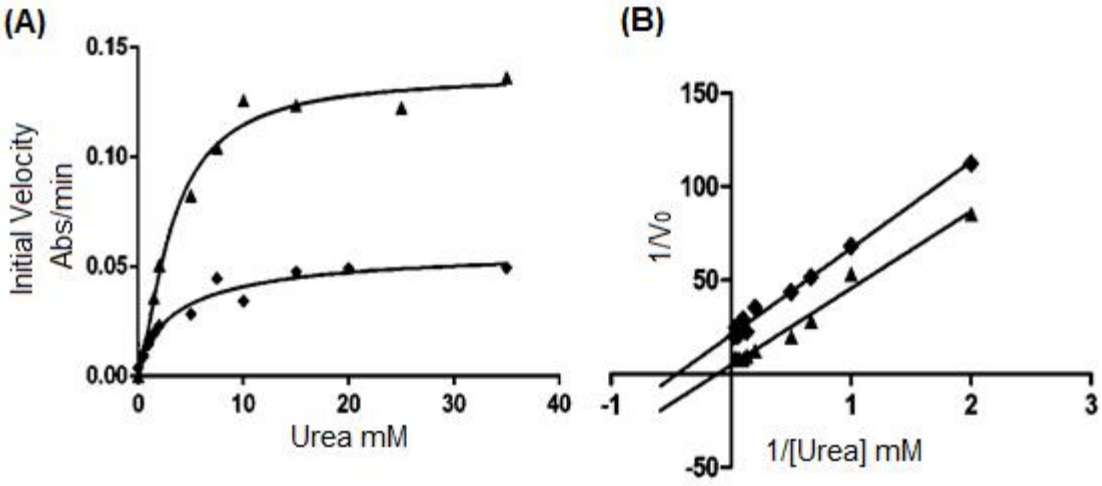

Figure 3. (A) Effect of substrate concentration on urease activity. (B) Lineweaver-Burk double reciprocal. ▲ No extract, ♦ with extract.

Conclusions

The methanolic extract of the root of *C. cholla* traditionally used in the treatment of kidney conditions presents substances with the capacity to inhibit urease activity. The extracts inhibits urease activity with an IC_{50} of 2.04 ± 0.08 mg/mL in an uncompetitive manner.

The authors declare no conflict of interests.

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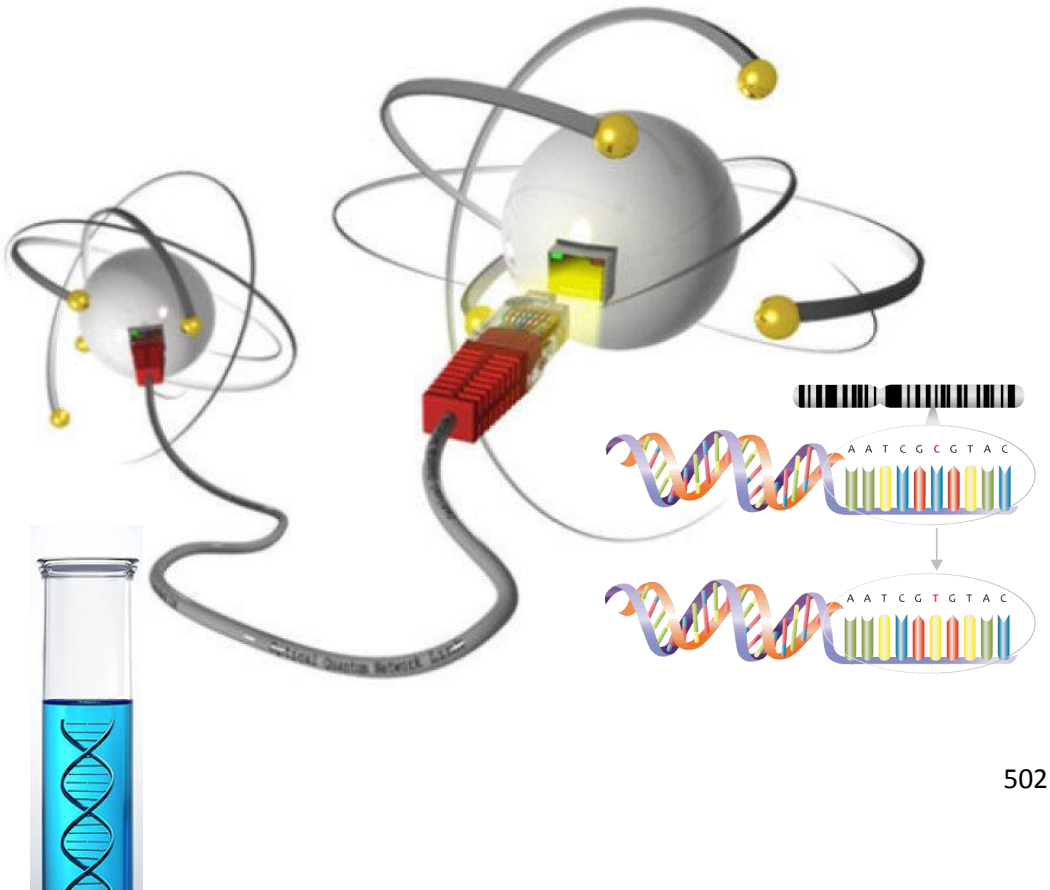

Science & biotechnology early stimulation: playing to do science with microbiology, biology and structural bioinformatics

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Abstract

As stated in the Declaration of the Rights of the Child, children have the right to go to school for free, to play and to have the same opportunity to develop and learn to be responsible and useful. In the past years, Mexico has made significant progress in basic education coverage to make significant progress in assessing the education system, through the annual implementation of the test called the National Assessment of Academic Achievement in Schools in Basic Education (ENLACE). According to Pérez (2004) we are in an "era of visual thinking that involves a new culture, a new era of knowledge and that the media today are a critical socialization stage" and the teacher profile and teaching processes in these same scenarios mentioned that we must resort to an educational model that promotes "reflective experiential learning" for students and teachers transmitting function, formulate problems, provokes questions, coordinate teams, systematizing experiences, facilitate searches, making more dynamic new situations communication. In the National Week of Science and Technology since 2007 we offer to children on basic education workshops for early stimulation on science and biotechnology knowing as playing to do science with: Structural bioinformatics of proteins and DNA, microbiology and/or biology.

Keywords: • ICT • empowerment •competition • virtual environments • software.

Introduction

In some studies are reported the ICT impacts positively on children education at an early age, because its impacts positively on children at an early age, and stimulates the minds of children, activating the imagination and attention span, by what today is an indispensable material for schools and social networks. Computers motivate students for their playfulness, using visual aids, and hearing colors and three-dimensional figures, teaching abstract concepts among other advantages (Domingo and Fuentes, 2010). Early stimulation is a set of actions that promote physical, mental and social aspects of any child, that help prevent child psychomotor retardation, cures and rehabilitates the motor, sensory deficits, mental retardation and language impairment, supporting integration into society, to feel useful and reduce negative conducts as aggression, indifference or rejection, and in the other hand increase solidarity, cooperation and hope (UNICEF, s/a). The educational backwardness and poverty are factors that affect in rural and indigenous communities, to involve ignorance of what they offer them health centers, the vicious circle of poverty leading to maintain and increase the high rate of persisting infant mortality,

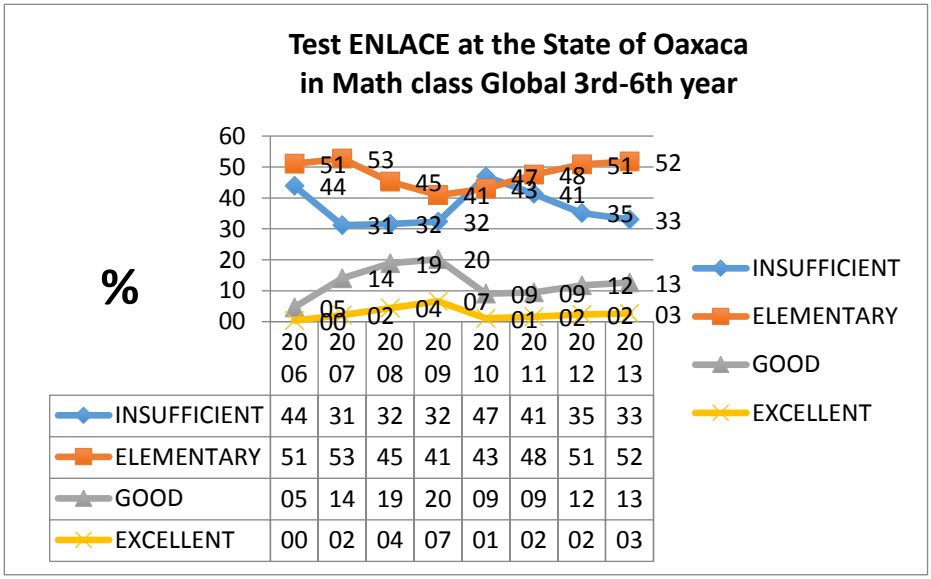
since a good education is directly proportional to maintain good health and vice versa. In some indigenous communities not exists internet access for kids teaching and learning.

Materials and methods

The aim to this work is approach kids in an indigenous community to scientific areas as microbiology and bioinformatics using TIC. For know the population, we begin analyze data of social, education and technology available in the Mexican indigenous population issues from National Institute of Statistics and Geography (INEGI), Ministry of Public Education (SEP), the National Population Council (CONAPO). Later, we applied two workshops in indigenous communities: one about bioinformatics and the second about microbiology. The workshops was offered to students from 2nd level of elementary school, secondary and high school education during SNCyT since 2007 to 2012 at the University of Papaloapan and in 2010 also in Monterrey.

Results and discussion

Figure No 1 we can see inpersonal the result of ENLACE. In Figure 2 we can see about how education gives women a new extensive social network, new reference groups and greater identification with the modern world of health institutions, reducing the education gap between women could be related to an increased interest in health services. Which it is possible with greater approval preventive behaviors and new remedies that help increase survival of less than one year. The percentage of women over 14 who have not completed the 9 degrees of compulsory basic education is expressed and located mainly in the states with the highest proportion of people in rural areas. In recent decades the gradual depletion of educational backwardness has a close relationship with decreasing mortality rates of children less than one year.



	2006	2007	2008	2009	2010	2011	2012	2013
INSUFFICIENT	44	31	32	32	47	41	35	33
ELEMENTARY	51	53	45	41	43	48	51	52
GOOD	05	14	19	20	09	09	12	13
EXCELLENT	00	02	04	07	01	02	02	03

Figure 1. Test results in ENLACE Global.

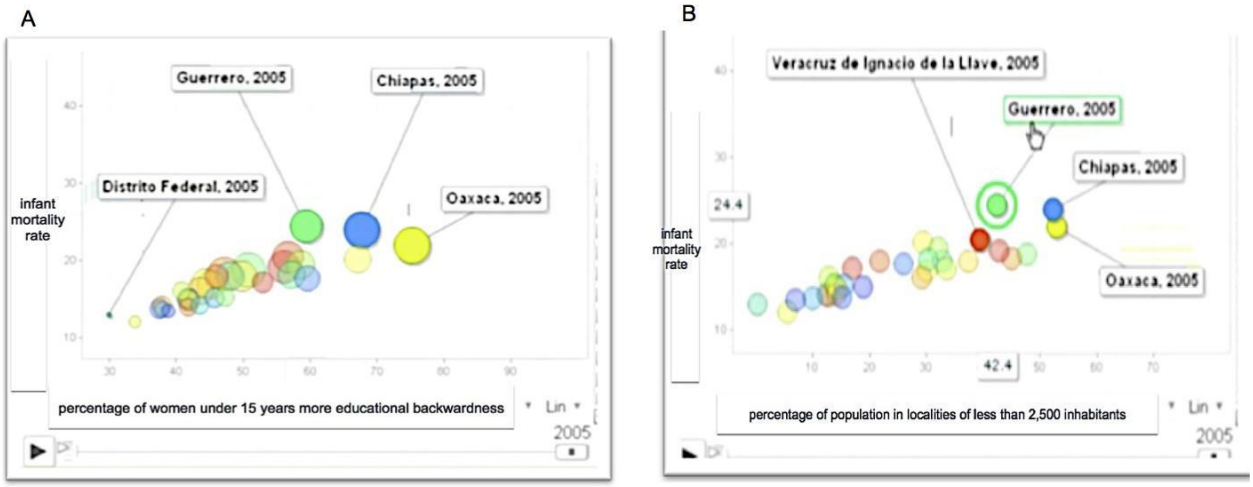


Figure 2. A Infant mortality & population. B Educational Backwardness in rural areas.

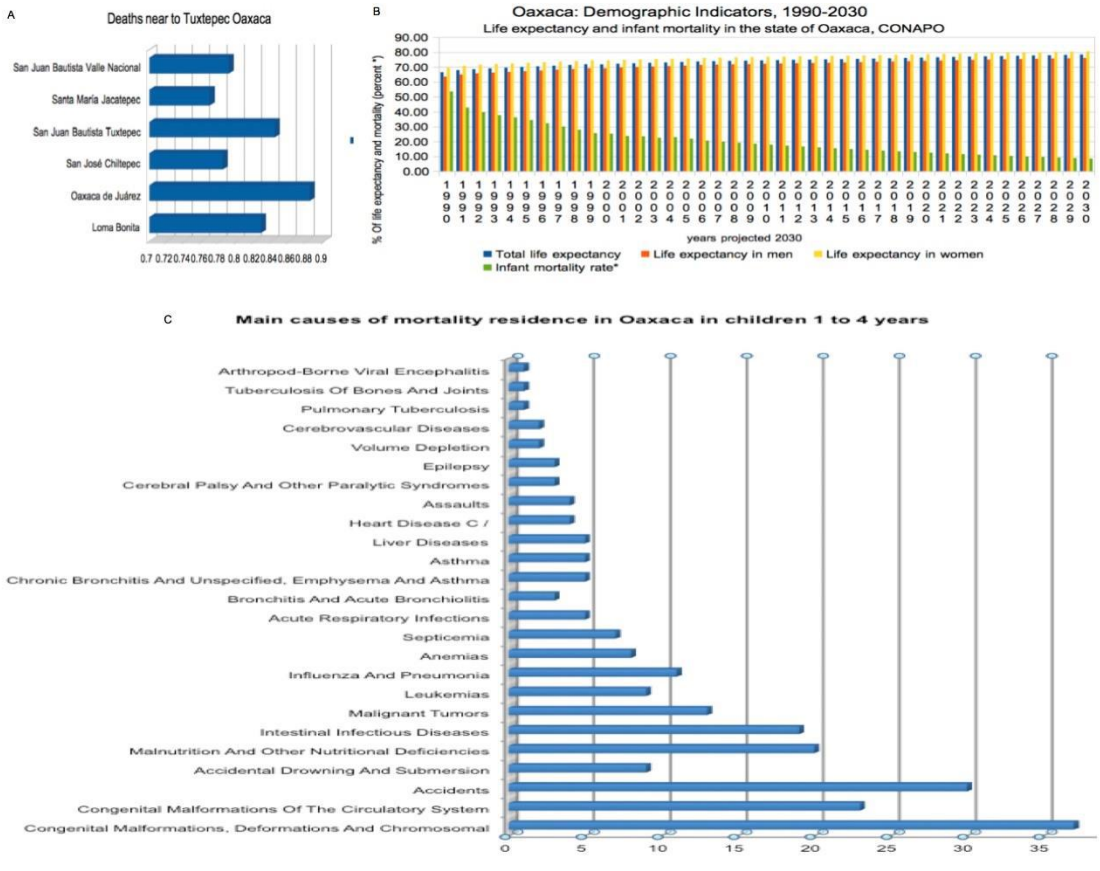


Figure 3. A is about deaths, B Life expectancy, C cause of mortality

In the next figure 4, we can see the use of the internet for education. In this graphic we can see the internet use has increased, but its use for education is approximately the 50%.

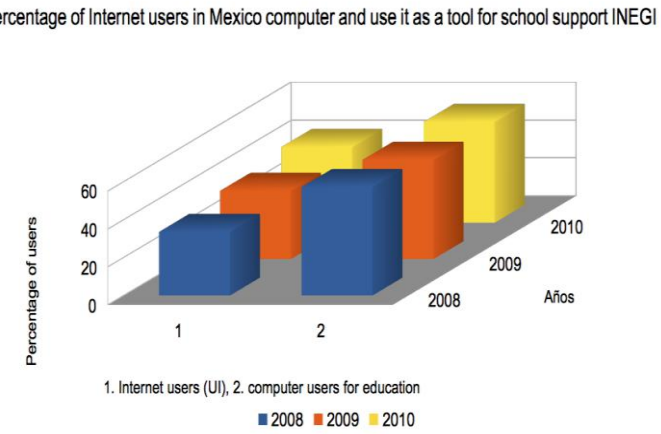


Figure 4. Households with a computer.

Workshop I: Playing to made Sc with structural bioinformatics of proteins and DNA:
The aim of this workshop is to present a methodology for the development of educational environments based on digital technology, Internet and free programs available on the web. It is divided into 5 sections: introduction to the topic, query database, download program & use of this bioinformatics tool and construction of a viral molecule. Requirements: PC computer, Internet access, RasTop program, students, social service support, slide projector.

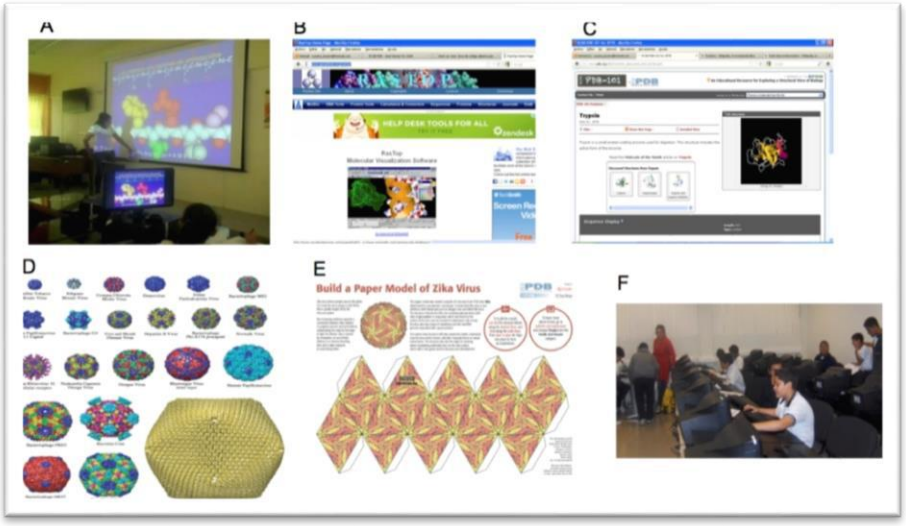


Figure 5. A Proteins structure, B PDB, C Ras Top, D-E Viral Capsid, F *in silico* analysis.

Workshop 2 Playing to made Sc with Microbiology:
The aim of this workshop is to present a methodology for growing *Saccharomyces cerevisiae* and microscopic observation, its use and application. It is divided into 3 sections: introduction to the topic, induction of strain, budding yeast observation under a microscope, conclusions and discussions. Basic requirements: yeast, warm iron, sugar, thermometer, beaker, microscope, cell iPone 6, making the microscope to television and projector slide.
The strain was activated at 37 ° C by adding sugar and leave for about 20 minutes to start the budding process. During this period the introduction to the topic is see the fungus growth. Subsequently the preparations made microscope to observe and take pictures. The material is followed and the method given in Lesaffre.com as virtual games for children.

Figure 6. A-C Students with microscope Workshop

Workshop III: Playing to made Sc with Biology
The aim of this workshop is to present a methodology for visualizing some biology structure by the stereoscope. It is divided into 3 sections: introduction to the topic, induction of biology organism, observation under a stereoscope, conclusions and discussions. Basic requirements: some part of biology structure or organisms, stereoscope, cell iPhone 6, making the stereoscope to television and projector slide. We can make preparations for stereoscope observations and take pictures.

Figure 7. Some pictures about biology structures representation.

Conclusions

Using this methodology as Early Stimulation on Science and Biotechnology we will be able to stimulate and intervene in the evolutionary development of children enrolled in basic education; promoting development in the scientific framework in the area of natural sciences positively impacting their curiosity to learn and take advantage of the free internet tool available. The network can be used to learn and not only for social networks. You can see specific structure represented in various biochemical ways, so that seeing them represented in different ways in their textbooks, may be exactly the same molecule and that the difference way of representing the molecule.

Acknowledgements

We special thanks to SNCyT, CONACYT, SUNEO, UNPA, UANL.

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3rd BIOTECHNOLOGY SUMMIT

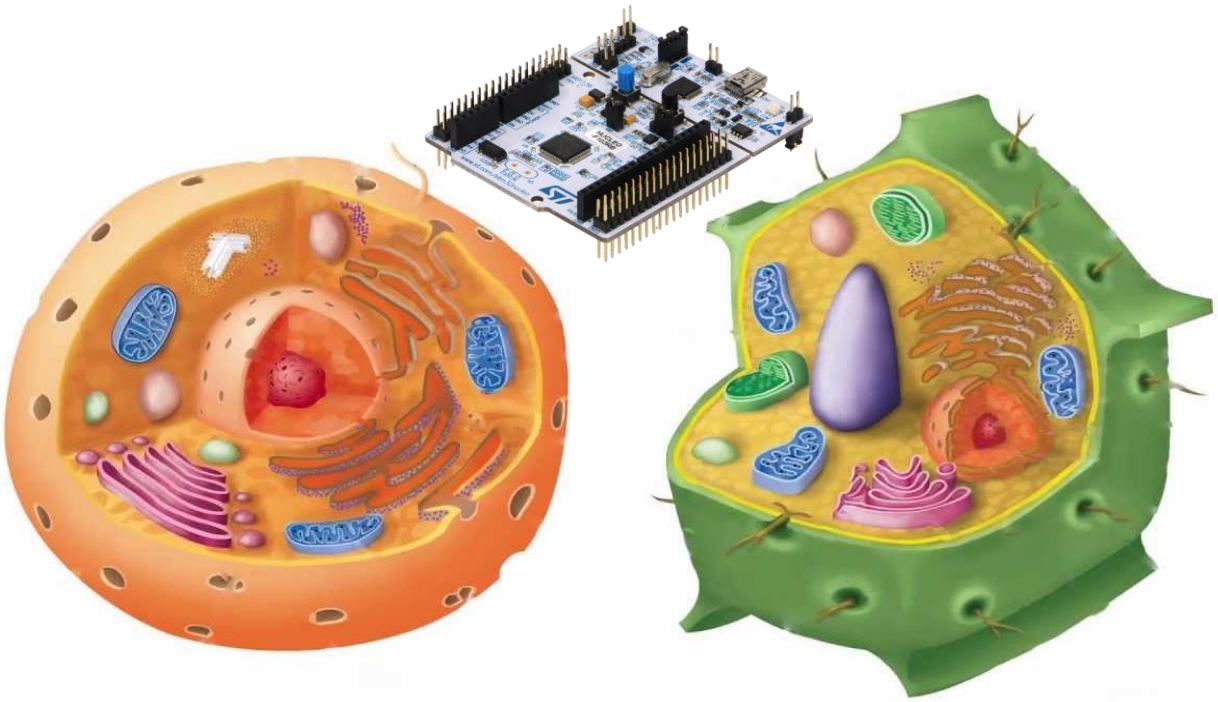
October 24-28, 2016

INSTITUTO TECNOLÓGICO DE SONORA

Ciudad Obregón, Sonora, México

Area XIV
PLATINUM

Synthetic Biology



Manuscripts regarding this knowledge area were not received

3rd BIOTECHNOLOGY SUMMIT

October 24-28, 2016

INSTITUTO TECNOLÓGICO DE SONORA

Ciudad Obregón, Sonora, México

Area XV
SILVER

Biobusiness, Bioentrepreneurship & Marketing: Strategy for Innovative Development of the National Economy, Improvement of the System of the S&T and Innovation Activities Management.

Report of the event, Biotechnology Summit 2014 (BS14)

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Abstract
The Biotechnology summit 2014 (BS14) was the second general meeting of the International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society, Civil Association, Nonprofit Organization (www.bio.edu.mx). The Biotechnology summit was scheduled in October 8-10, 2014 in Huatulco, Oaxaca Mexico (Universidad del Mar). The organizing committee developed an extensive program highlighting not only recent progress in biotechnology, but also the trends in biotechnology for future commercial applications. The Biotechnology summit is an inclusive event of all biotechnology-related topics, from issues of bioethics, biotechnology applications in basic and applied research. The areas were color-coded, following the color codes used by the Int. Biotechnol. Color J. ISSN 2226-0404. <http://www.cicy.mx/Sitios/Journal/home>. This summit was co-organized by Universidad del Mar (Huatulco) and Universidad del Papaloapan and included 38 lectures, 8 oral presentations, 56 posters, 2 symposia, 1 round table and 1 pre-Congress Workshop The numbers were, 150 national and international attendees, 67 different institutions and 8 countries.

Keywords: • Biotechnology summit 2014 • meetings • results • organizations • event.

Introduction

It is of utmost importance to promote biotechnology and recent findings specially for marginal communities, in particular for students from rural and indigenous communities. This idea was the motivation for the organization of the Biotechnology Summit 2014 in Huatulco, Mexico that hosted professors, scientific leaders, businessmen & entrepreneurs, public servants, government employees and young students. The main themes of the Biotechnology summit 2014 (BS14) were the challenges of health, food, sea, agricultural, arid zone, bioterrorism, copyrights, classic industrial biotechnology, gene-based biotechnology, bioinformatics, nanobiotechnology, bioethics, synthetic biology, bio-business, entrepreneurship, marketing, multidisciplinary education, and ICT.

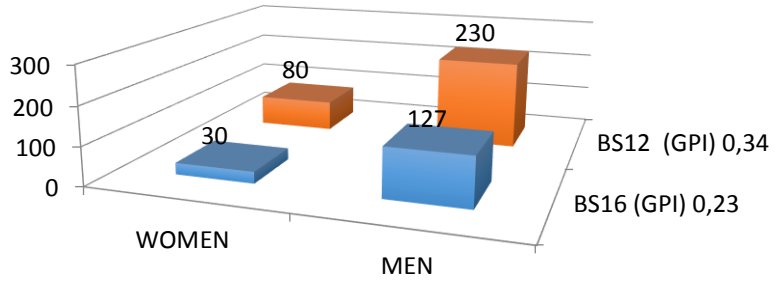
Materials and Methods

As every two years we publish a call for the conference via electronic media as Tab UNAM, Facebook, Twitter, Instagram, Linked In, Researchgate. The summit is sponsored by important mexican organizations: CONACYT, UNU BIOLAC, ICGEB, and PADES SEP.

Results and Discussion

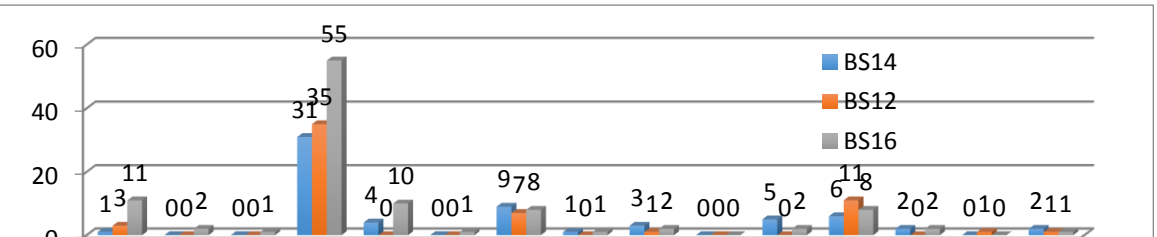
The 2nd Biotechnology Summit 2014 (BS14) took place on October 8-10, 2014 and it was hosted by the Universidad del Mar (UMAR), Campus Huatulco of Oaxaca and co-sponsored / co-organized by several Biotechnology-related research centers in Mexico and/or various organizations as: Universidad del Papaloapan (UNPA) - Sistema de Universidades del Estado de Oaxaca (SUNEO); Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, Secretaria de Medio Ambiente y Recursos Naturales (SEMARNAT); Instituto Tecnológico de Mérida; Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV); Secretaría de Turismo Y Desarrollo Económico; Consejo Oaxaqueño de Ciencia y Tecnología; Universidad Nacional Autónoma de Mexico; Centro de Investigación Científica de Yucatán A.C; Sociedad Mexicana de Biotecnología y Bioingeniería Nacional; Instituto Nacional de Medicina Genomica; Illumina; Life Science; AgroBio Mexico; Quimlab; Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional; Colegio de Posgraduados and Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos. One year prior to the summit, the organizing committee visited to identify the strengths, weaknesses, opportunities, and threats of this project/business venture.

The conference covered a wide range of active research areas, in particular featuring 38 invited plenary lectures presented by leading specialists. Two symposium were organized: Symposia # 1 Iris biotechnology area: Massive sequencing of DNA and current and future strategies for the analysis of the results and success stories using these methodologies. Lecturers Symposium commitee:Alfredo Mendoza (Instituto Nacional de Medicina Genómica, INMEGEN), México, D.F.; Enrique Morett (Instituto de Biotecnología, UNAM), Cuernavaca, Morelos, México. Sponsors: Illumina and & Life Science. Symposia # 2 Green Biotechnology area: Monitoring Resistance to *Bacillus thuringiensis* and New Approaches to Control Targeted Insects. Lecturers Symposium commitee:Patricia Tamez-Guerra, Cristina Rodríguez-Padilla (FCB-UANL, México) and Carlos A. Blanco (University of New Mexico, USA). Sponsors: AgroBIO México, A.C. and Cotton Inc. It was also organized a A roundtable: Bt Crops: Effect on NTO (beneficial insects) and Behavior (monitoring) of Nontarget pests. Lecturers Symposium commitee: Patricia Tamez-Guerra (FCB-UANL, México) and Carlos A. Blanco (University of New Mexico, USA). Sponsor: AgroBIO México, A.C. Finally Preconference workshop was lectured in Bioinformatics structural protein-taught by Rachid Charbel Maroun, Universitéd'Evry, France and oral and posters sessions.



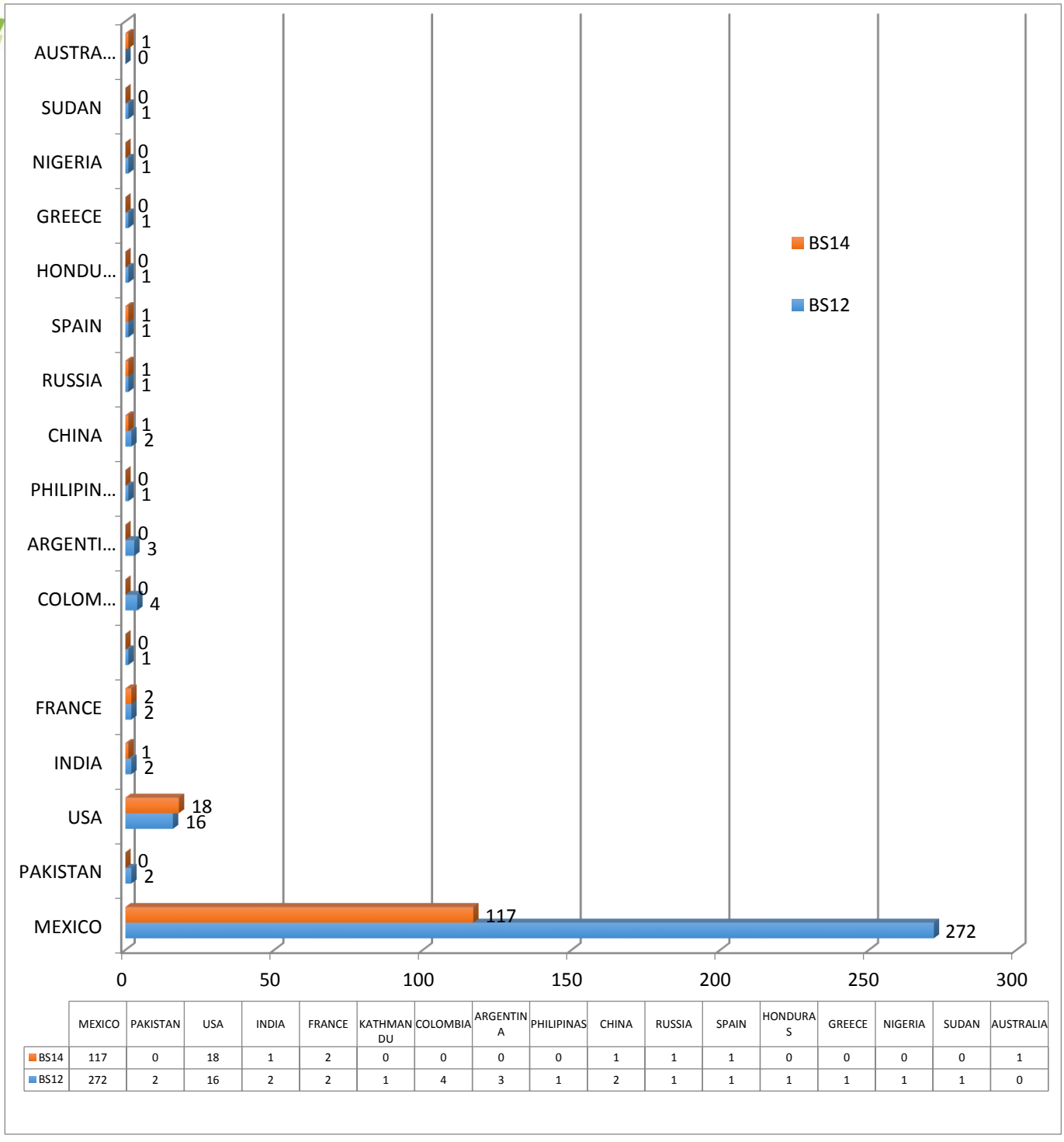
	WOMEN	MEN
BS16 (GPI) 0,23	30	127
BS12 (GPI) 0,34	80	230

Figure 1. Number of women and men assistants with Gender Parity Index (GPI) in BS12 and BS14.



	Blue	Brown	Black	Green	Gray	Purple	Red	White	Yellow	Platinum	Silver	Iris	Transparent	Gold	Indigo
BS14	1	0	0	31	4	0	9	1	3	0	5	6	2	0	2
BS12	3	0	0	35	0	0	7	0	1	0	0	11	0	1	1
BS16	11	2	1	55	10	1	8	1	2	0	2	8	2	0	1

Figure 2. Number of abstract contributions by biotechnology color area at BS12, BS14 and BS16.



	MEXICO	PAKISTAN	USA	INDIA	FRANCE	KATHMANDU	COLOMBIA	ARGENTINA	PHILIPINAS	CHINA	RUSSIA	SPAIN	HONDURAS	GREECE	NIGERIA	SUDAN	AUSTRALIA
BS14	117	0	18	1	2	0	0	0	0	1	1	1	0	0	0	0	1
BS12	272	2	16	2	2	1	4	3	1	2	1	1	1	1	1	1	0

Figure 3. Assistants' nationalities. BS12 and BS14.

The title of the pre Congress Workshop was “Bioinformatics structural protein-taught” lectured by Rachid Charbel Maroun, Universitéd'Evry, France.

Members of the presidium at the Summit’s opening ceremony were: Gerardo Leyte E. Morales, Academic ViceRector, Universidad del Mar; Jorge Vilar Llorens, Federal Delegate in the State of Oaxaca in the Ministry of Public Education; Pedro Macias Canales, Director of Services of Genetically Modified Organisms from National Service of Health, Food Safety and Agrifood Quality Control; Jaime E. Padilla Acero, Director of AgroBIO Mexico; Susana Lozano Muniz, Director of International Biotechnology Foundation; Beatriz Rodríguez Casasnovas, Federal Delegate of Economy State of Oaxaca and Patricia Tamez Guerra, BS14 Organizer from Universidad Autónoma de Nuevo León.

A poster competition was organized in order to prize remarkable scientific contribution and the winner were: “Calcium-activated potassium channel identification in *Strongylocentrotus purpuratus* sperm” in the first place; “Effect of alkaline treatment for inactivation of *Salmonella sp.* and coliphages in wastewater sludge” in the second place and "Bioinformatics approach for microRNAs targets prediction and analysis in papillary thyroid cancer” in the third place. An oral poster session contest was organized as well and the winners were: “Influence of thiamine and C/N ratio on production of pDNA of *E. coli* DH5α in a chemically defined culture medium”, in the first place; "Antioxidant activity of *Rhustrilobata plant extracts*" in the second place and “In silico design of multiple input genetic circuits using logic gates” in the third place.

Member of the Scientific Committee were:

María Soledad Córdova Agilar, Centro de Ciencias Aplicadas y Desarrollo Tecnológico, UNAM
Jagruti P. Gandhi, Raffaello Research Laboratories
Miguel Juan Beltrán García, Depto. de Química ICET. Universidad Autónoma de Guadalajara.
Porfirio Gómora Arrati, UAT-Cinvestav
Patricia Pavón Orozco, Facultad de ciencias químicas, Universidad Veracruzana
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Serafín Cruz-Izquierdo, Instituto de Recursos Genéticos, Colegio de Postgraduados
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Victor Manuel Ruíz-Valdiviezo, Instituto Tecnológico de Tuxtla-Gutierrez
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Federico Antonio Gutierrez Miceli, Instituto Tecnológico de Tuxtla-Gutierrez

Laura Grecia Fuentes Ponce, Universidad Politécnica del Estado de Morelos
Alicia Becerra Flora, IPICYT
Marina María de Jesús Romero Prado, INTEC, Universidad de Guadalajara
Ada María Rios Cortés & Minerva Rosas Morales, CIBA-IPN
Elba Cristina Villegas Villarreal, Alexis Joavany Rodríguez Solís, Centro de Investigación en Biotecnología; Universidad Autónoma del Estado de Morelos
Karla P García-Pelagio, University of Maryland at Baltimore

The staff and local organizers at UMAR: Eustace Ramirez Fuentes, María Nieves Trujillo Tapia, Darla Alejandra Torres Ariño, Ana Claudia Sánchez Espinoza, Mónica Alicia Calderón Oropeza, Laura Eugenia Hernández (student), Ana Esther Meléndez Patiño (student) Jesús Emmanuel Méndez González (student), Eduardo Gijón García(student), Ignacio Cid Oliva (student); staff and organizers from Universidad del Papaloapan, Campus Tuxtepec. Oaxaca Mexico: Jacqueline Capataz-Tafur, Paul Mauricio Sanchez Ocampo, Mónica Guadalupe Segura Ozuna, Isaac Machorro Cano, Izmael Ortiz Miguel; organizers from Instituto Tecnológico Superior de Tierra Blanca: Laura Inés Elvira Torales; organizers from Universidad Tecnológica de la Mixteca: Teresa Ivonne Castillo Diego. The national and international organizing committee appears as author of this document.

The top topics at the symposium "Monitoring Resistance to *Bacillus thuringiensis* and New Approaches to Control Targeted Insects" and the roundtable entitled "Bt Crops were the Effect in NTO (beneficial Insects) and behavior on Primary and Secondary none target pests".

The hot topic during the round table were Research interests include understanding various molecular, biochemical and physiological functions in insects, including Bt mode of action and receptor function, insecticide/Bt resistance, digestive biochemistry and physiology, immunity, and biotic/abiotic stress responses.

The hot topic during the symposium Information were:

Analysis of the complexity in the evaluation of adverse effects caused by GMOs in Mexico. Construction process of a non target organisms research network in Latin America. Environmental Interaction Studies in Support of Environmental Risk Assessment of Biotech Crops. Implementation of normalized procedures (biosafety measures, standards & technical guides) for the risk assessment of NTOs in Mexico

The scientific contribution of the symposium: Transgenic crops expressing one or more *Bacillus thuringiensis* (Bt) toxins are being commercialized worldwide, primarily because of their efficacy against several economically important lepidopteran and coleopteran pests. However, the risk of target insects developing resistance to Bt toxins has been an issue. The development of Bt resistance has concerned organic growers, farmers, and seed producers because most of these pests can migrate long distances, affecting their control with Bt elsewhere. Therefore, it was important to approach this potential issue proactively utilizing the best management programs.

Scientists from academic, research institutions and seed companies have organized symposia in Mexico since 2003. In Huatulco 2014, presentations in the sixth symposium included results not just on Bt topics, but new insect pest control strategies.

The application of recombinant DNA technology has led to various controversies on crops expressing pest insect resistance. Genetically modified (GM) crops expressing toxins from the bacterium Bacillus thuringiensis (Bt) have been planted on almost everyone and have proven to be an effective tool for pest control. However, it has questioned the potential negative effects that GM crops could have on non-target organisms (NTO) and / or ecological environment.

In Mexico, the main concerns about planting GM crops discussion was focused on: What is the effect of these GM crops on non-target insects? What about the population of the primary and secondary target pest, which is not GM crops? What is the experience in managing resistance in target insect pests GM crops?

Conclusions

It is undeniable that the scientific fields of biotechnology have had ~~a~~ considerable advances. Nevertheless there is a constant need for the development of new biotechnology approaches were available resources could be used. The BS12, BS14 have been, and now BS16 will represent, an important source of new, promising and useful knowledge in biotechnology and sciences. These summits have inspired novel ideas and have produced multidisciplinary knowledge. The number of attendees (150 registered) was less than expected, but the number of international and national speakers had ample representation. The results obtained in these prior summits were remarkable taking into consideration the reduced support from the most important scientific and educational organizations not as in 2012; at BS12 the sponsors were: ICGEB 20,000 euros, CINVESTAV IPN 250 thousand pesos and 75,000 pesos AgroBio Mexico, and at BS14 the sponsors were UNPA, UMAR, SUNEO, OCV, UAEM CEIB, UNAM, INMEGEN, Life Science, Cotton, AgroBio Mexico. The area with more research poster and oral session in both events was the color green, which corresponds to Agro biotechnology.

Acknowledgements

We give special thanks to all the participants and Tab UNAM for the trust, confidence and promotion of all of this event.

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Biotechnology summit 2016

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Mendieta RA 2015. The researcher SNI and women in Oaxaca: main challenges admission, retention and promotion. ¿Legitimacy or Recognition? Publisher: Ediciones La Biblioteca, S.A. de C.V. México, Withing the book: ¿Legitimidad o reconocimiento?

http://www.labiblioteca.com.mx/store/p13/%C2%BFLegitimidad_o_reconocimiento%3F_Las_investigadoras_del_SNI._Retos_y_propuestas.html DOI: 10.13140/RG.2.1.2933.4808 accessed 11 September 2016

Report of the event, I Biotechnology Meeting of Mexican Society of Biotechnology and Bioengineering, Oaxaca Delegation (I Biotech Meeting SMBBO)

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[1]Universidad del Papaloapan, [2]Universidad del Mar, [3]Sociedad Mexicana de Biotecnología y Bioingeniería, Delegación Oaxaca, [4]International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society.A.C. – Internaitional Biotechnology Foundation.

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Abstract

The I Biotechnology Meeting organized by the Oaxaca Delegation from the Mexican Society of Biotechnology and Bioengineering was the first general meeting of this Society <http://smbb.com.mx/congresooaxaca/index.html>. The I Biotechnology Meeting took place last July 7 and 8, 2016 and it was held by the Universidad del Papaloapan, campus Tuxtepec. The areas of biotechnology included in the discussions were: I. Food Biotechnology; II. Development of biotechnological processes; III. Environmental biotechnology; IV. Medical biotechnology and pharmaceutical; V. Aquaculture and livestock biotechnology; VI. Animal biotechnology; VII. Plant biotechnology; VIII. Biobusinesses; IX. Biotechnology and society; X. Other related topics as bionanotechnology, bioethics, etc. This first meeting had over 150 national and international attendees, 148-research papers submitted for oral and/or poster sessions, 11 lectures and 1 pre-Congress Workshop: "Introduction to Molecular Dynamics of Proteins", lectured by Dr. Francisco Noé Mendoza Ambrosio (Universidad del Papaloapan). The I Biotechnology Meeting was sponsored by: Sistema de Universidades Estatales del Estado de Oaxaca (SUNEO); Universidad Autónoma de Coahuila; Applikon Biotechnology; BIOMOLAB de México S.A. de C.V.; Biología Molecular para el Laboratorio; Proveedora diagnóstica, S.A. de C.V.; Sartorious de México, S.A. de C.V; Waters: The science of What´s possible; Regpa Ethanol; Bio-Rad Laboratories Inc.; TA Instruments; Sociedad Mexicana de Biotecnología y Bioingeniería; Universidad del Papaloapan; Universidad del Mar; and International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society Civil Asociation, Nonprofit Organization-International Biotechnology Foundation.

Keywords: • meetings • results • organizations • event.

Introduction

The Oaxaca Delegation (SMBBO) of the Mexican Society of Biotechnology and Bioengineering (SMBB), was officially established at the Universidad del Papaloapan on July 4, 2014. This significant event for the Biotechnology society and for the Oaxaca Biotechnology community was possible thanks to the generous support from the members of SMBB from different Universities and Organizations from the Papaloapan Region. One of the first duties SMBBO was the organization of the I General Meeting on Biotechnology known as "I Biotechnology Meeting organized by SMBBO" (I Biotech Meeting SMBBO), at Universidad del Papaloapan, in Tuxtepec, Oaxaca, Mexico. The event was organized for the vast community of professors, scientific leaders, businessmen and entrepreneurs,

graduated and post graduated students. Tuxtepec is a town located in the north part of the state of Oaxaca. Universidad del Papaloapan comprises two campus: Tuxtepec and Loma Bonita. Tuxtepec is the second most populous city of the Mexican state of Oaxaca and it is well connected with several regions in Oaxaca, Veracruz and Puebla states, which turns Tuxtepec into a strategic location for the reunion of scientist, student and businesspersons from both states.

Materials and Methods

It was published a call to sign the Oaxaca Delegation of Mexican Society of Biotechnology and Bioengineering (SMBBO) and/or the invitation to organize the I Meeting on Biotechnology of SMBBO in the official web page of Mexican Society of Biotechnology and Bioengineering <http://www.smbb.com.mx/index.php>. Diverse electronic media available, as Tab UNAM and social networks as Facebook, Twitter, Instagram, Linked In were also used in order to publish the national call. The SMBBO submitted a request for economical support to CONACYT & ASM. Gender Parity Index

Results and Discussion

The National SMBB official declaration was signed at June 01, 1992, in Mexico city at the notary no 129, Lic. Ignacio Soto Borja, book number 2307, statement 76,797. ~~As~~ Ignacio Magaña Plaza was the president of the society while Amelia Farraés González Sarabia as secretary and treasury; the tellers Armando Cahue and Amanda Gálvez, with 400 members. <http://www.smbb.com.mx/documentos_legales/Acta%20MDN%201992-1994.pdf>

The SMBBO official declaration was signed last July 04, 2014 at Universidad del Papaloapan. The society was presided Susana Lozano Muñiz (Universidad del Papaloapan); Jacqueline Capataz Tafur (Universidad del Papaloapan) as Vice president; Andrés Aguirre Cruz (Universidad del Papaloapan) as Mónica Marcela Galicia Jimenez (Universidad del Mar) as Secretary. Our society has 9 members so far. From Universidad del Papaloapan: Laura Patricia Ramirez Coutiño, Sandra Trinidad del Moral Ventura, Paul Mauricio Sanchez Ocampo, Nohemí Gabriela Cortés Lopez; From Universidad del Mar: Alejandra Torres Ariño, Eustacio Ramirez Fuentes, Nieves Trujillo Tapia; From Instituto Tecnológico Superior de Tierra Blanca: Laura Inés Elvira Torales: Ariana Alerne Huerta Heredia and David Paniagua Vega. As Vicepresident and after that as president of national SMBB Cristóbal Noé Aguilar González from Universidad Autónoma de Coahuila. <http://www.smbb.com.mx/delegaciones.html>

The I Biotechnology Meeting of the Mexican Society of Biotechnology and Bioengineering, Oaxaca Delegation (I Biotech Meeting of SMBBO) was scheduled from 7 to 8 of July of this year in Tuxtepec, Oaxaca, Mexico, at Universidad del Papaloapan.

The main goal of the pre Congress Workshop: “Introduction to Molecular Dynamics of Proteins" lectured by Dr. Francisco Noé Mendoza Ambrosio (Universidad del Papaloapan) was the discussion and training of computational tools used for chemistry, biology, physics and engineering analysis. This workshop was focused in the description of most important characteristics of the methods developed for the understanding of molecular dynamics (free software), which allow the calculation of thermodynamic properties of proteins in water. This knowledge is of utmost importance in the understanding of physical and chemical phenomena in interactions of proteins and ligands. The program of the workshop was: 1. Introduction to proteins lectured by Dra. Susana Lozano-Muñiz); 2. Introduction to

molecular dynamics; 3. Introduction to using Linux commands in terminal type; 4. Introduction to using command-line scripts for use with GROMACS; 5. Molecular dynamics of water at different thermodynamic conditions; 6. Getting thermophysical properties; 7. Construction and molecular dynamics of proteins in water; 8. structural and dynamic properties; 9. Molecular dynamics of proteins and ligands in water.

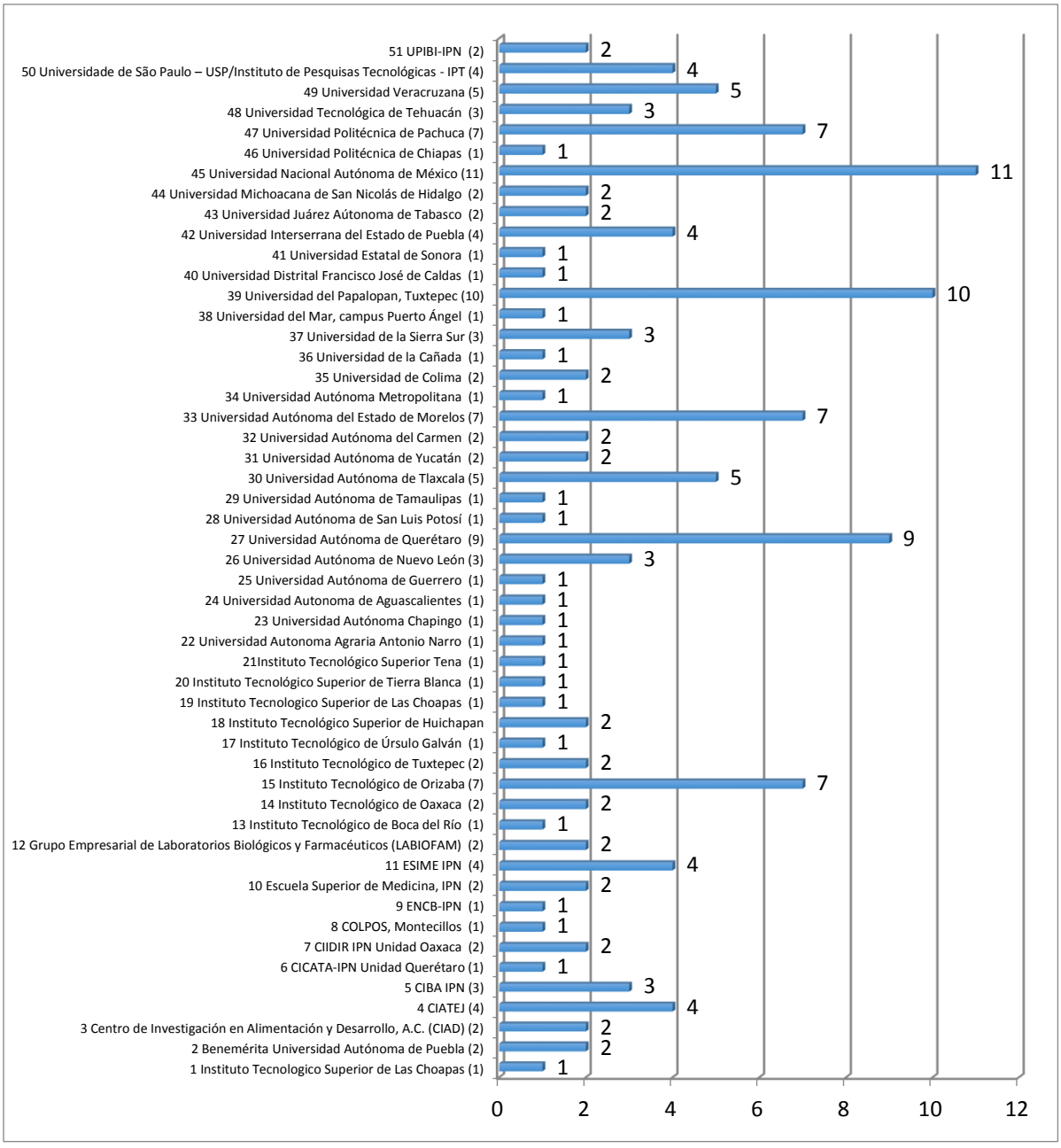

Figure 1. List of participant institutions.

The opening ceremony was moderated by Laura Patricia Ramirez Coutiño, Director of the Biotechnology Institute of Universidad del Papaloapan.

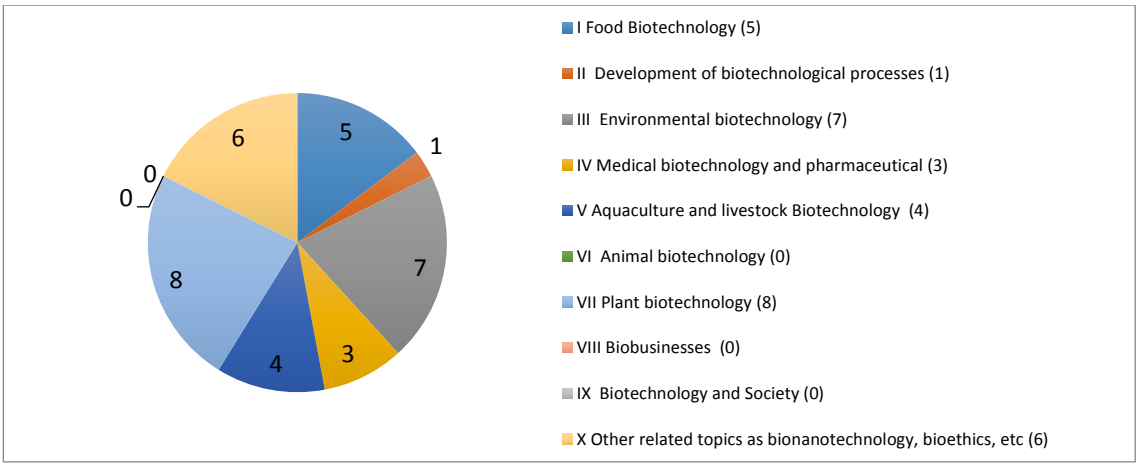

Figure 2. Areas and number of works submitted for oral session (34)

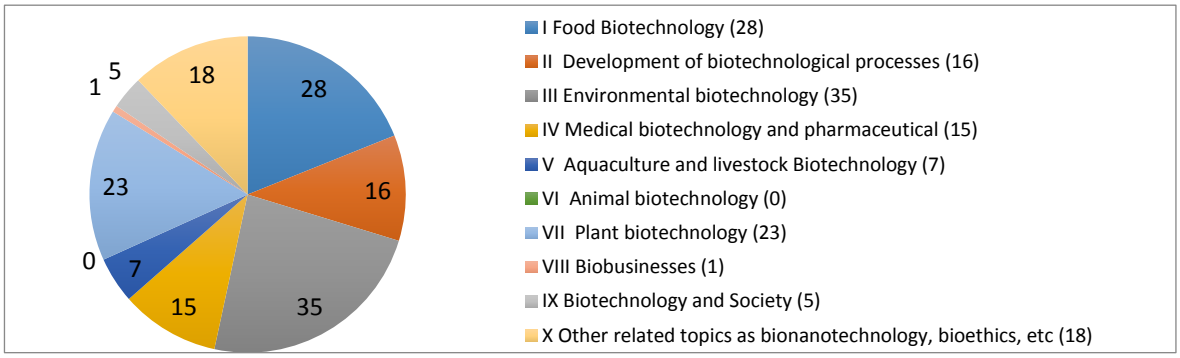

Figure 3. Areas and number of works submitted for oral/poster session (148)

In the poster session contest the First place was for: “Evaluation of a UASB reactor detoxification that was used to degrade trichlorethylene (TCE)” Vargas-Cano Felipe, Trajo-Espinosa Josué Eduardo, García-Solares Selene Montserrat, Guerrero-Barajas Claudia. Second: “The performance of the reverse transcriptase SMMLV in an RNA form esteructuras of guanine tetrads” Ramos-Alemán Fabiola, González-Jasso Eva, Pless C. Reynaldo. Third: “in vitro propagation of asparagus (*Asparagus officinalis L.*) by direct organogenesis from buds of rhizome” Millán Gabriela, Esqueda Martín, Robert Manuel, Gutiérrez Aldo. In the oral session contest the First place was for: “Process optimization spray drying cheese whey using the methodology response surface” Alfredo Domínguez-Niño, Calderón-Santoyo Montserrat, Andrade-González Isaac, Cantú-Lozano Denis, Luna-Solano Guadalupe. Second: " Development and evaluation of a simple protocol and inexpensive to build libraries of small RNAs from plants". Ríos-Villanueva Rogelio Antonio,

Ortiz-Yescas Amado, Pulido-Barajas José Francisco, Sepúlveda-García Edgar Baldemar, Peña-Castro Julián Mario, Barrera-Fuigueroa Blanca Estela. **Third**: "Evaluation sulphate reduction in a UASB for degradation of chlorinated compounds" Santana-Santos Mario Alberto, Trejo-Espinosa Josué Eduardo, García-Solares Selene Montserrat, Guerrero-Barajas Claudia.

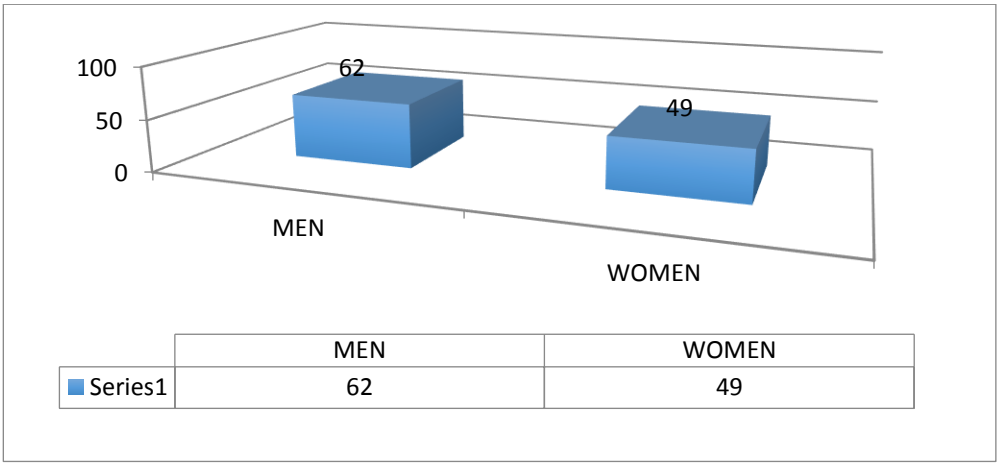

Figure 4. Number of abstract submitted to I Biotech Meeting SMBBO with Gender Parity Index (GPI) 0.79

The area with more research poster and oral session was the area VII: Plant Biotechnology.

The Scientific Committee was:

Alejandra Torres Ariño, Universidad del Mar, campus Puerto Ángel; Xenia Mena Espino, División de Ciencias Básicas e Ingeniería, Universidad Autónoma Metropolitana-I; Esther Ramírez Moreno, Área Académica de Nutrición de la Universidad Autónoma del Estado de Hidalgo; Reyna Lucero Camacho Morales, Universidad de Granada, España; Rosa Estela Quiroz Castañeda, CENID-Parasitología Veterinaria; Luis Alberto Hernández Osorio, Facultad de Ciencias Químicas, Universidad Autónoma Benito Juárez de Oax; Peggy Elizabeth Álvarez Gutiérrez, Tecnológico Nacional de México, Instituto Tecnológico de Tuxtla Gutiérrez; Graciela del Valle Leguizamón B, Facultad de Agronomía y Agroindustrias, Universidad Nacional de Santiago del Estero Santiago del Estero, Argentina; Karla Paola Garcia-Pelagio, Facultad de Ciencias de la UNAM; Rafael Barceló Hernández, Universidad Interserrana del Estado de Puebla-Ahuacatlán; Raúl A. Poutou-Piñales, Pontificia Universidad Javeriana, Facultad de Ciencias, Departamento de Microbiología; Ramón Batista, Centro de Investigación en Dinámica Celular, Universidad Autónoma del Estado de Morelos; Paula Figueroa-Arredondo, Maestría en Ciencias de la Salud Escuela Superior de Medicina del IPN; Anabel Copalcua Bello, UIEPA, Universidad Interserrana del Estado de Puebla – Ahuacatlán; Paul Mauricio Sánchez Ocampo, Catedratico CONACYT- Universidad del Papaloapan; Carolina Barrientos, Universidad Veracruzana; Pablo Augurio Hernández Romano, Centro Estatal de la Transfusión Sanguínea de Veracruz; Isaac Zepeda Jazo, Universidad de La Ciénega del Estado de Michoacán de Ocampo; Patricia Pavón Orozco, Facultad de Ciencias Químicas, UV Coatzacoalcos; From Instituto Tecnológico de Tuxtepec: Roselis Carmona García, Jesús Rodríguez Miranda, Cecilia E. Martínez Sánchez, Erasmo Herman y Lara. From Universidad del Papaloapan: Víctor Manuel Meza Villalvazo, Blanca Estela Barrera Figueroa, Susana Lozano

Muñiz, Julián Mario Peña Castro, Sandra del Moral Ventura, Leticia Guadalupe Navarro Moreno, Jacqueline Capataz Tafur, Hermenegilda Moreno Díaz, Jesús Carrillo Ahumada, Laura Patricia Ramírez; The Staff and local organizers: Walter Josué Hernández Santos, Luz del C. Quevedo Zarate, Jair Alexander García Ramón, Aurora Bautista Leyva, Berenice Madai Salcedo Cruz, Gabriela Elizabeth Hernández, Aurora Bautista Leyva, Macaria Martínez Morales, Nohemí Gabriela Cortés López, Mayra Santiago Velasco, María Magdalena Infante Jacobo, Laura Isabel Méndez Sánchez, Tito Adabel Pérez Felipe, Donaji Eugenio del Rivero, Elsi Yunuen Granados Bruno, Mercedes Pulido Galeana, Vianey Citlaly Carrasco José, Miriam Romero Lozano, Luis Donaldo Caamaño Sánchez, Marco Antonio Rodríguez Alejandro, Miguel Ángel Ramírez Merced, Abimael Martínez Sánchez. The Staff and organizers from Universidad Autónoma de Sinaloa Luis Armando Zamarripa-Lozano and from INOXIKA, S de RL de CV Juana María Lozano-Muñiz.

Conclusions

In México, the biotechnology researches have had a major improvement, but it is needed to develop new biotechnology approaches and spread knowledge, specialty among students from rural and indigenous communities. As in the previous events organized by part of the members of the society, Biotechnology summit 2012 (BS12), 2014 (BS14) and 2016 (BS16), the I Biotechnology Meeting of SMBBO was focused in the design, creation and sharing of novel Biotechnology ideas with the use of the resources available in order to increase the human quality of life. The number of attendees (150) and research abstracts for oral/poster session (148) was more than expected. This is a true image of the importance of Biotechnology for the society, research and student community. This evidence is confirming the need of more meetings and activities where Biotechnology is discussed and new ideas are created, and this is the driving force of our conformation as SMBBO.

Acknowledgements

We would like to give special thanks to all participants, research institutions, sponsors, researchers and students. SMBBO would also like to thank SMBB and Tab UNAM for the trust, confidence and promotion.

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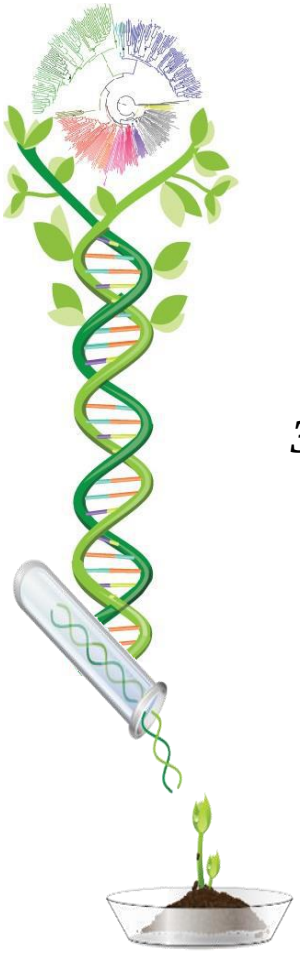

We thank you all for coming!

3rd Biotechnology Summit 2016