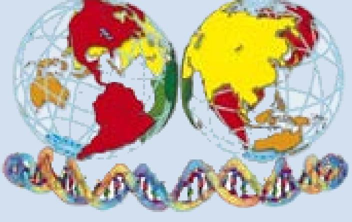
Biotechnology Summit 2014

Santa María Huatulco, Oaxaca, Mexico



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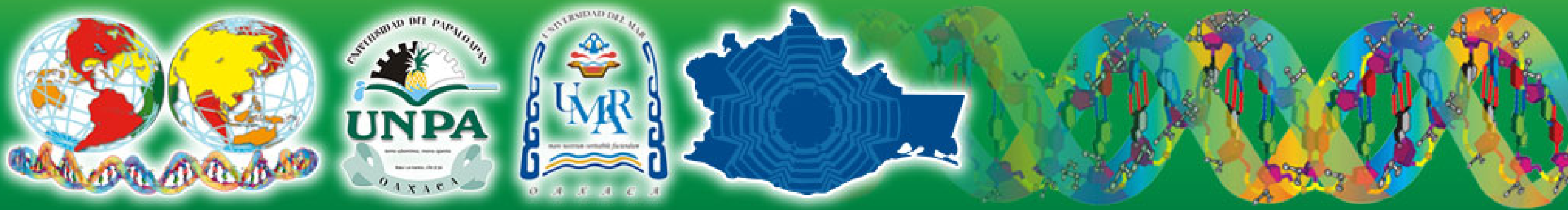

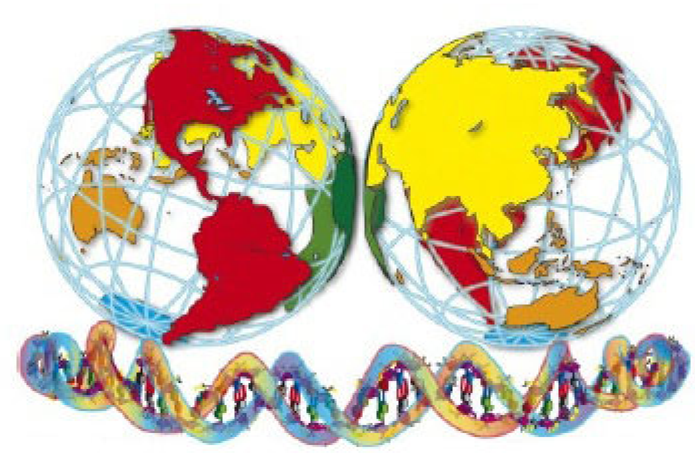

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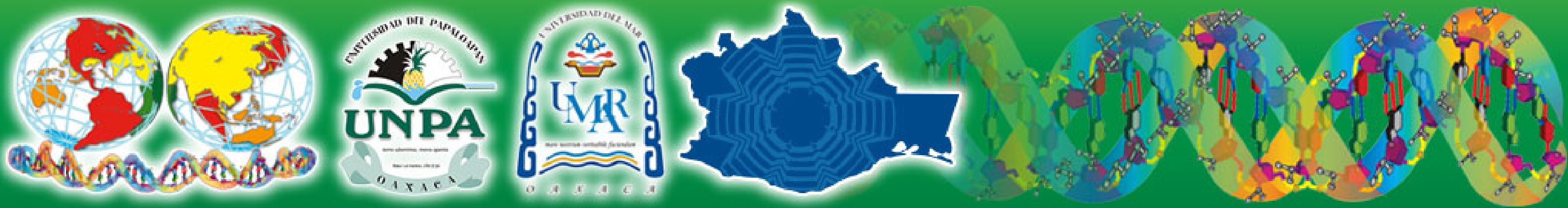

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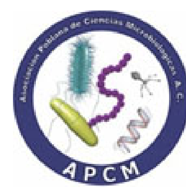

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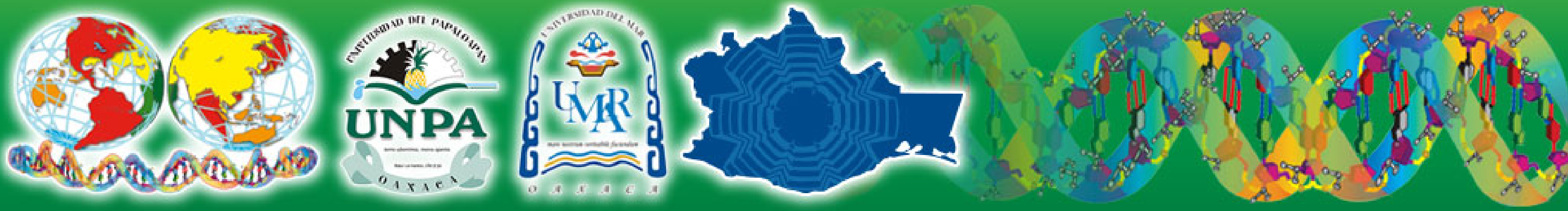

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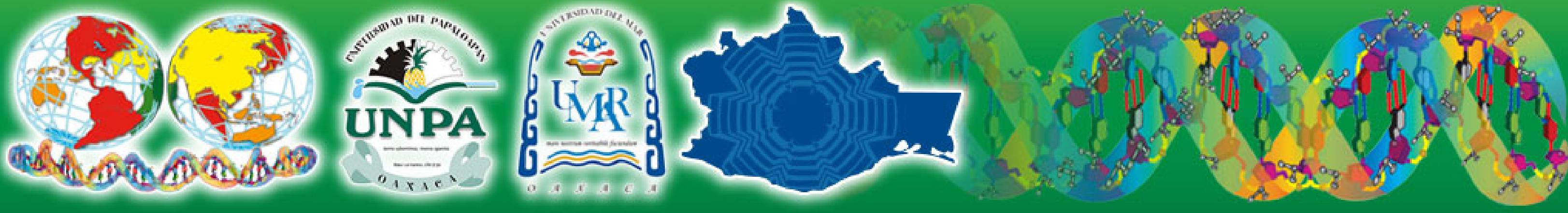

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BLUE Biotechnology: *Aquaculture, Coasts and sea, Fish health and nutrition, Aquatic animals reproduction, Cloning and genetic modifications, Aquaculture and fisheries pest and Disease control.*

BROWN Biotechnology: *Space and geomicrobiology, Arid Zone and Desert Biotechnology.*

DARK Biotechnology*: Human and animal and pest control, Bioterrorism, Biowarfare, Biocrimes and Anticrop warfare.*

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.*

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.*

PURPLE Biotechnology: *Strategy for the intellectual property protection, Patents, Publications, Inventions.*

RED Biotechnology: *Human Health & disease, Medical, Diagnostics and Tissue engineering.*

WHITE Biotechnology: *Gene-based Industrial biotechnologies.*

YELLOW Biotechnology: *Food, Nutrition Science and Nutraceuticals.*

PLATINUM Biotechnology: *Synthetic Biology.*

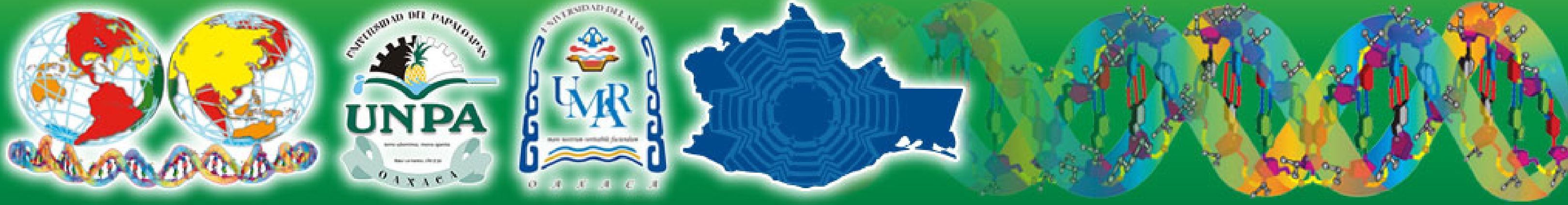
SILVER Biotechnology: *Biobusiness, BioEntrepreneurship & Marketing, Development Economics, Biobusiness and Marketing.*

IRIS Biotechnology: *Multidisciplinary Area on Biochemistry, Molecular Biology & Biotechnology and Applications based on omic's.*

TRANSPARENT Biotechnology: *Bioethics, Biotechnology and Society: tools for asses the support to the scientific sector, including its biotechnological potential and human resources.*

GOLD Biotechnology: *Bioinformatics, Nanobiotechnology, Microelectronic and Microelectromechanical systems (MEMS), Micro Systems Technology (MST), Nano Electro Mechanical Systems (NEMS) and micromachines.*

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.*



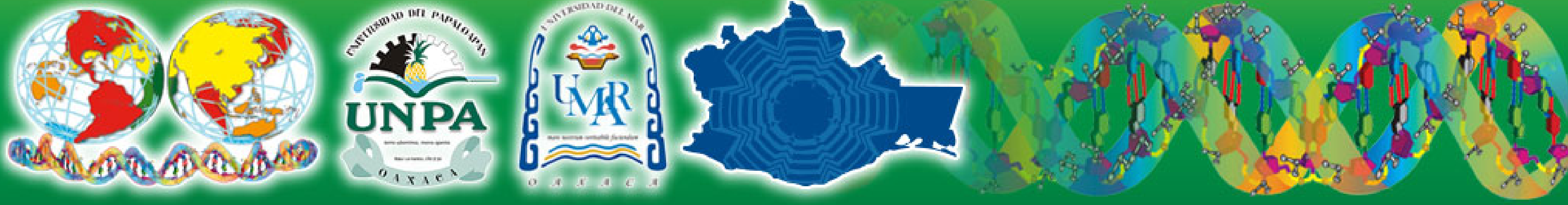
Welcome to the Biotechnology Summit 2014

Dear Professors, scientific leaders, businesspersons & entrepreneurs, public servants, government employees & young students:

The main theme of Biotechnology Summit 2014 (BS14) are the challenges of health, food, sea, agricultural, arid zone, bioterrorism, copyrights, classic industrial biotechnology & gene-based, bioinformatics & nanobiotechnology, bioethics, synthetic biology, bio-business, entrepreneurship and marketing, multidisciplinary, education & ICT. We do believe all of us can make contribution in some area of Biotechnology.

On behalf of BS14 Organizing Committee, it is a great pleasure for us to welcome you to attend the Biotechnology Summit 2014 (BS14) which will from October 8th to 10th, 2014 & hosted by the Universidad del Mar (UMAR), Campus Huatulco of Oaxaca & co-sponsored / co-organized by several Research Centres in Biotechnology of Mexico and/or various organizations as Universidad del Papaloapan, Sistema de Universidades del Estado de Oaxaca, International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society A.C., Facultad de Ciencias Biológicas - Universidad Autónoma de Nuevo León, Secretaría de Medio Ambiente y Recursos Naturales, Instituto Tecnológico de Mérida, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Centro de Investigación en Biotecnología Aplicada del Instituto Politécnico Nacional, 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, Yucatán and Oaxaca delegations, Instituto Nacional de Medicina Genomica, Illumina, Life Science, Programa de Apoyo al Desarrollo de la Educación Superior - Secretaría de Educación Pública, AgroBio Mexico, Quimlab, Instituto Potosino de Investigaciones Científicas Y Tecnológicas, Escuela Nacional de Ciencias Biológicas - Instituto Politécnico Nacional, Colegio de Posgraduados. Centro de Investigación en Biotecnología - Universidad Autónoma del Estado de Morelos, Southern Regional Research Center - Agricultural Research Service - United States Department of Agriculture & Santander.

The conference covers a wide range of active research areas, in particular featuring 25 invited plenary lectures presented by leading specialists. In addition, there will be two simposium with 28 plenary lectures presented by leading specialists. First, Massive sequencing of DNA & current and future strategies for the analysis of the results and success stories using these methodologies, Organized by Alfredo Mendoza, Instituto Nacional de Medicina Genómica (INMEGEN), México, D.F. and Enrique Morett, Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, México, Sponsored by: Illumine & Life Science. Second, Monitoring Resistance to *Bacillus thuringiensis* and New Approaches to Control Targeted Insects Organized by: Patricia Tamez-Guerra & Cristina Rodríguez-Padilla (FCB-UANL, México) & Carlos A. Blanco (University of New Mexico, USA), Sponsored by: AgroBIO México, A.C. & Cotton Inc. Four preconference workshops, oral and posters contribution sessions.



We give special thanks: Víctor Toledo-López, ITM; Mónica Lozano-Contreras, INIFAP; José Juan Zúñiga-Aguilar, CICY; Rogelio Rodríguez-Sotres, UNAM; Fabián Fernández-Luqueño, CINVESTAV-IPN; Fernando López-Valdez, CIBA-IPN; Patricia Tamez-Guerra, UANL; Carlos Blanco, USDA; Deepak Bhatnagar, USDA; Enrique Morett, UNAM; Alfredo Mendoza, INMEGEN; Obdulia Morales-Contreras, STYDE; Jose Antonio Hernandez-Contreras, UNPA; Monica Galicia-Jimenez, UMAR; Jacqueline Capataz-Tafur, UNPA; Hector López-Arjona, UNPA; Modesto Seara-Vázquez, SUNEO; and Mtra. Norma Munguía-Aldaraca, General Director of prime sectors and renewable resources at SEMARNAT. In addition, we want to give special thanks to everyone that helped us make this event possible. Finally, thank you for your patience and commitment.

Over the past time, the scientific fields of biotechnology have had a considerable advance, but we still need to develop new biotechnology approaches for utilization the resources available. The BS12 has been, and now BS14 continues to be, a major contributor to developments in the biotechnology & sciences. We invite you to join us; BS14 will bring together scientific leaders, young students & businesspersons and entrepreneurs. We hope this 3 days Congress; all participants will be inspired and will receive novel ideas and multidisciplinary knowledge. In addition, Huatulco is located in the south part of Oaxaca, Mexico, with a lot of beaches, and archaeological & cultural places to visit. We look forward to seeing you in Huatulco, Oaxaca, Mexico!

Sincerely yours,

Susan Lozano

Susana Lozano-Muñiz.
President, BS14 Organizing Committee,
President, International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society A.C., and President, Mexican Society for Biotechnology & Bioengineering Oaxaca Delegation.

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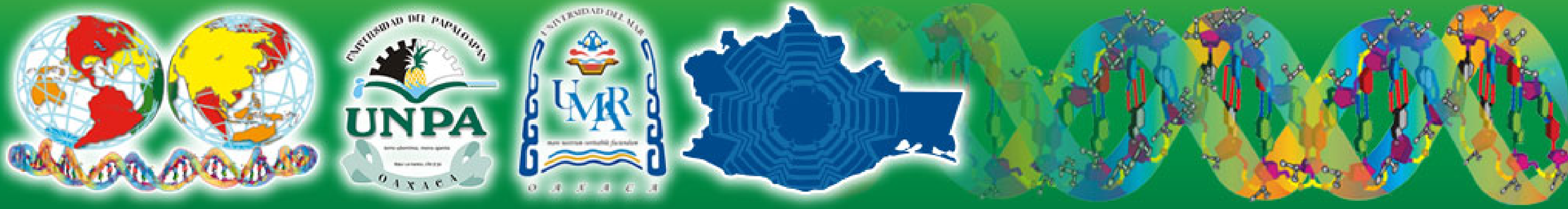

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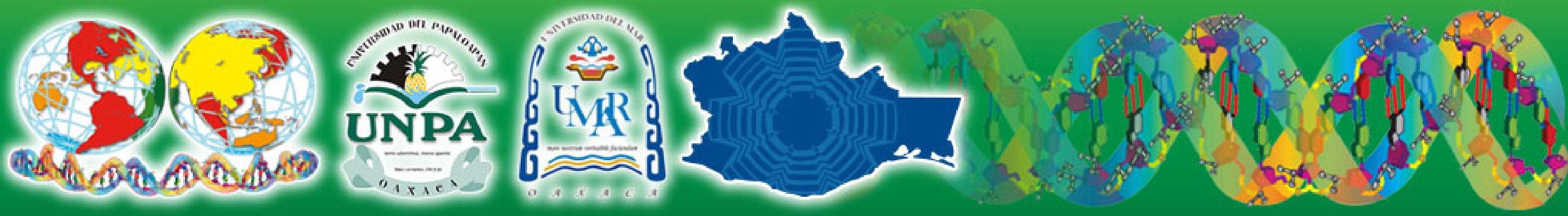

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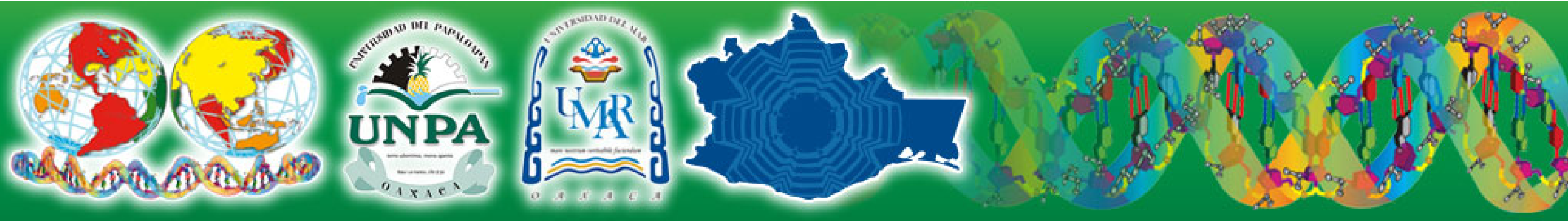

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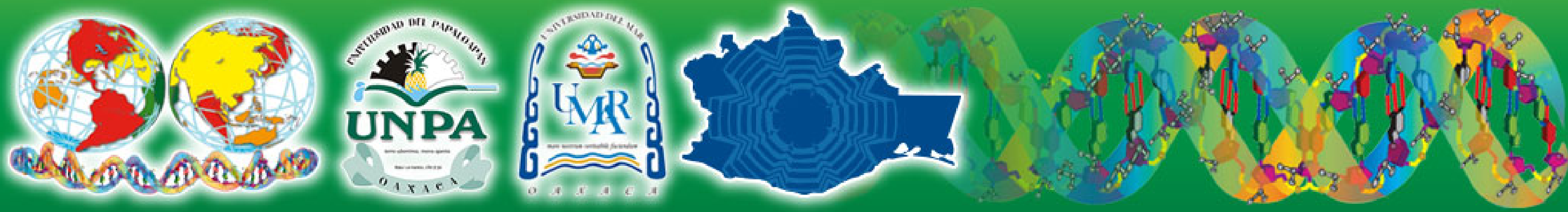

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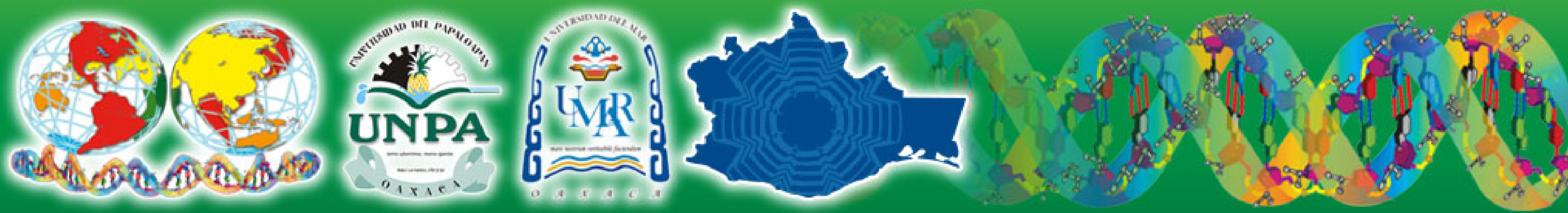

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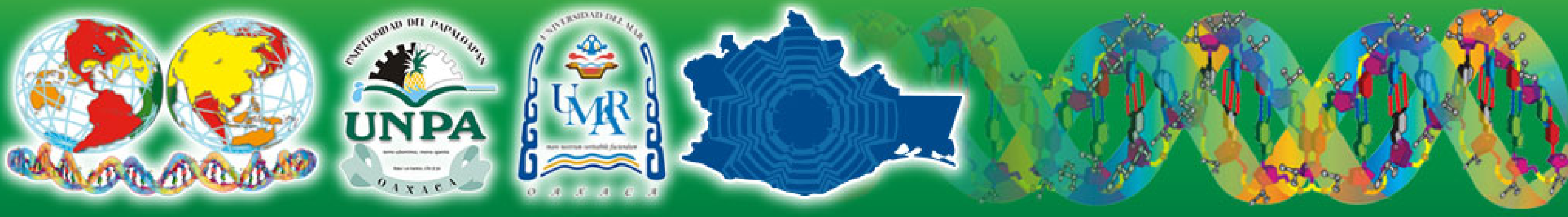

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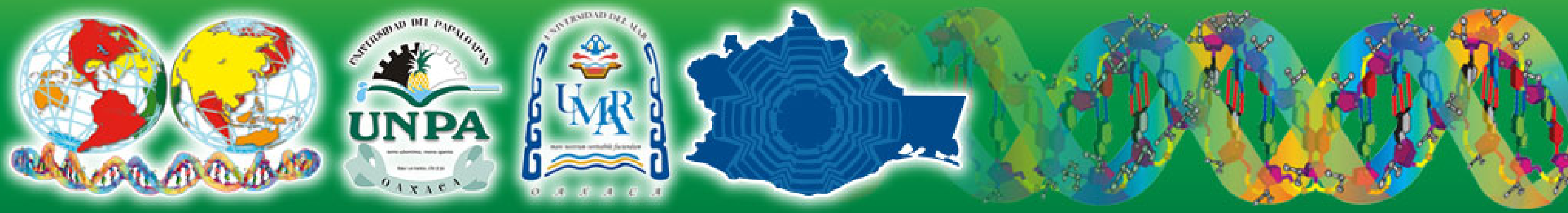

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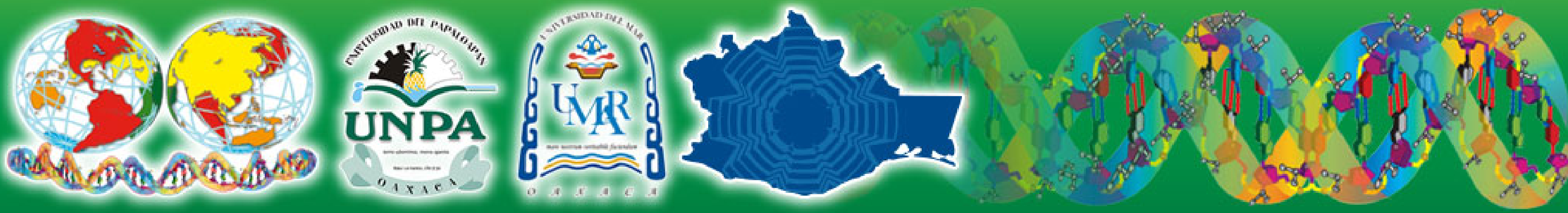

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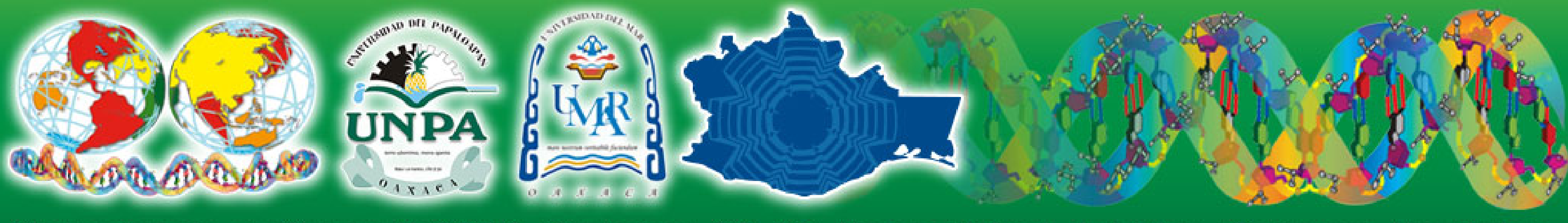

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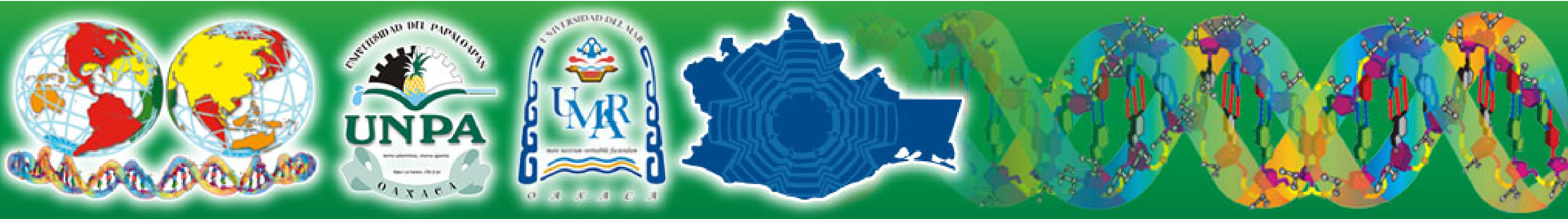

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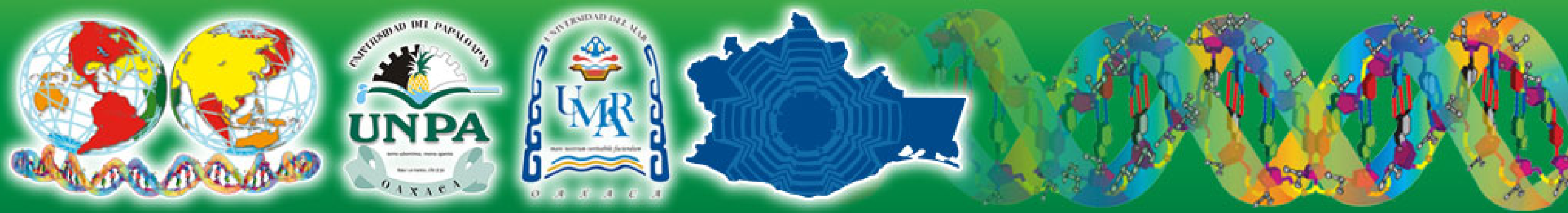

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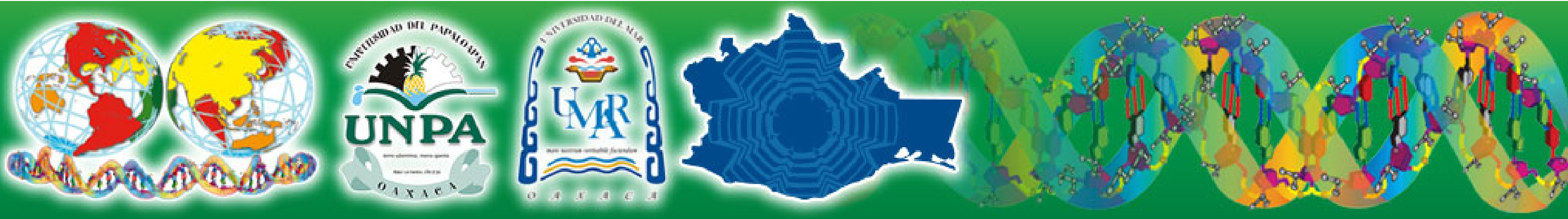

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1. Articles *in extenso*

1.1 RED AREA

Antineoplastic potential of bioactive fractions of *Rhus trilobata* on colon cancer cells CaCo-2

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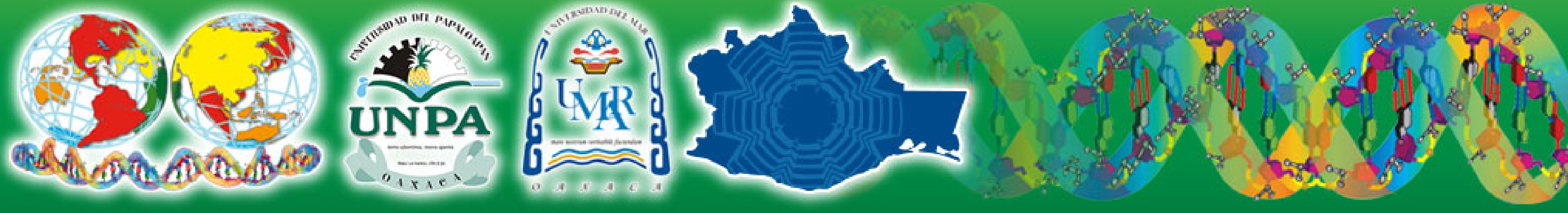
Abstract: Colorectal cancer (CaCo) incidence has increased considerably in recent years, as well the number of drugs available to treat it. People to treat malignant diseases such as leukemia and gastric cancers have traditionally used *Rhus trilobata*, a skunbush that grows in the state of Chihuahua. Previous studies have demonstrated the cytotoxic and antiproliferative effects of aqueous and methanolic extracts of *R. trilobata* on CaCo-2 and SKOV-3 cells. The objective of this work was to demonstrate the antineoplastic effect of solid phase C-18 obtained fractions from both extracts of *R. trilobata* on CaCo-2 cells. Additionally, fractions were partially characterized by high-pressure liquid chromatography. Results showed that fractions eluted with 1% acidified water in both extracts, had the highest inhibitory effect on the growth of CaCo-2 cells, followed by the ethyl acetate eluted fraction. Chromatograms at 284 nm displayed that compounds present in crude extracts eluted mainly in fraction 3 and 4 in both extracts, thus suggesting that compound nature could be related with polyphenols, which haves been shown to have some anticancer activity.

Keywords: *Rhus* ● Antineoplastic ● Cancer ● Plant extracts ● Polyphenols

Introduction: Colorectal cancer (CaCo) is the most common malignancy of the gastrointestinal tract; the third cause of cancer and the fourth leading cause of death from this disease in the world (Vilches, 2010). In 2008, it was reported that the state of Chihuahua occupies the second place in mortality by CaCo, with a rate of 3.1 per 100,000 habitants (Tirado, 2008). The aggressiveness of the currently available cancer treatments has given importance to the development of new chemical drugs derived from plants, less toxic but retaining high antitumor activity (Avila, 2008). Traditionally, the people of the state of Chihuahua haves been using the infusion of *R. trilobata* for the treatment of cancer such as leukemias and CaCo. Studies about the nature of antineoplastic compounds present in *R. trilobata* are scarce (Petit, 1978). Recently, studies done by our research group demonstrated that aqueous and 70% methanol extracts decrease the proliferation of both CaCo-2 cells and ovarian cancer cells SKOV-3, decreasing the number of viable cells by an apoptotic-dependent pathway (Varela-Rodriguez, 2013; Saenzpardo-Reyes, 2014). Solid phase extraction (SPE) using Bakerbond® cartridges C-18 is one of the techniques used for fractionation of antioxidant compounds in plant extracts (Hock, 2012). The objective of this work was to demonstrate the antineoplastic effect of solid phase C-18 obtained fractions from both extracts of *R. trilobata* on CaCo-2 cells. Additionally, fractions were partially characterized by HPLC. The identification of the active compounds present in *R. trilobata* could promote the apparition of new drugs derived from natural products, with antineoplastic capabilities and represent a new option in the treatment of various cancers, including CaCo.



Materials and Methods:

Preparation of extracts. The aqueous infusion (AE) of *R. trilobata* was prepared using 25 g of stems in 500 mL of deionized water; mixture was boiled at 100 °C for 30 min. The methanol extract (ME) was prepared by maceration of 25 g of *R. trilobata* stems in 500 mL of 70% methanol; vegetal material was soaked at room temperature with constant stirring in absence of light for 48 h. Both extracts were filtered through Whatman #1 filter paper and concentrated under negative pressure with agitation of 100 to 150 rpm and a temperature of 37 to 40 °C to avoid degradation of thermolabile compounds.

Fractionation of extracts. A volume of 100 mL of crude extract (AE or ME) was centrifuged at 3,500 rpm during 15 min at 4 °C; the obtained pellet was considered fraction 1, the supernatant (Fraction 2) was passed through a previously activated BAKERBOND C-18 column. Fractions 3 to 6 were eluted using 1% acetic acid-acidified water, ethyl acetate, ethyl ether and methanol, respectively. All fractions were concentrated using a rotavapor Buschi, and resuspended in 2 mL of 50% methanol for HPLC assays and saline phosphate buffer for viability assays.

Characterization of chemical compounds. The fractions obtained from both extracts were subjected to HPLC analysis using a gradient of acidic water 1% with acetonitrile, with a flow of 2.5 mL min^{-1}. The samples were prepared at 10,000 ppm (10 mg min^{-1}), filtered through 0.22 μm Restek filters. A volume of 20 mL of each sample was injected into the chromatograph at 30 °C, the wavelength used for compounds UVvis detection were 254 and 280 nm, the signal acquisition was 65 min.

Cell viability assays. The antineoplastic activity of *R. trilobata* crude extracts and fractions were determined using MTT assays (Mossman, 1983). CaCo-2 cells were cultured in DMEM-high glucose medium complemented with 10% inactivated fetal calf serum, 1% penicillin-streptomicin, and 1% L-glutamine. The cellular density used was 10,000 cells per well, these, were incubated at 37 °C under 5% CO_2. 24 hours later the crude extracts and fractions were added to a final concentration of 5 μg mL^{-1}. The fractions were in contact with the cells for 20 hours, at this time the MTT solution (5 mg mL^{-1}) was added, cells were lysed and formazan crystals were solubilized in isopropanol acid. The absorbance was determined at 590 nm using a Varioskan microplate reader; the absorbance is proportional to the number of viable cells in culture.

Results and Discussion:

Chromatograms at 254 and 284 nm were obtained for both crude extract and their fractions; main differences were detected at 284 nm for both AE and ME (Figure 1 and 2, respectively). The HPLC chromatogram of crude extract displayed seven peaks: six peaks between 1.5 and 3.5 min, and one at 7.3 min. A similar pattern was obtained for A2 and A3 fractions; signal intensity was minor in all fractions compared with crude extract. As is depicted in Figure 2, crude ME and their fractions displayed similar peaks, however higher intensities were detected, which suggests a higher concentration of compounds in ME. Clearly, fractions obtained with polar and slightly polar solvents, such as acidified water and ethyl acetate contained a greater number of compounds. While the remaining fractions, 5 and 6, did not show compounds at this wavelength.

Cell viability was determined by the MTT assay, which was reported as percentage of inhibition taking as 100% the growth of untreated cells (Figure 3). Results showed that M2, M3 and M4 had the higher percentage of inhibition by a decrease of 25% at 5 μg mL^{-1}. In the same way, fractions A2 and A3 had the higher percentage of inhibition. These results agree with the presence of a higher concentration of compounds found by HPLC in these fractions. Additionally, controls treated with gallic acid (5 μg mL^{-1}), tannic acid (5 μg mL^{-1}), and curcumin (5 μg mL^{-1}) had lower percentages of inhibition.

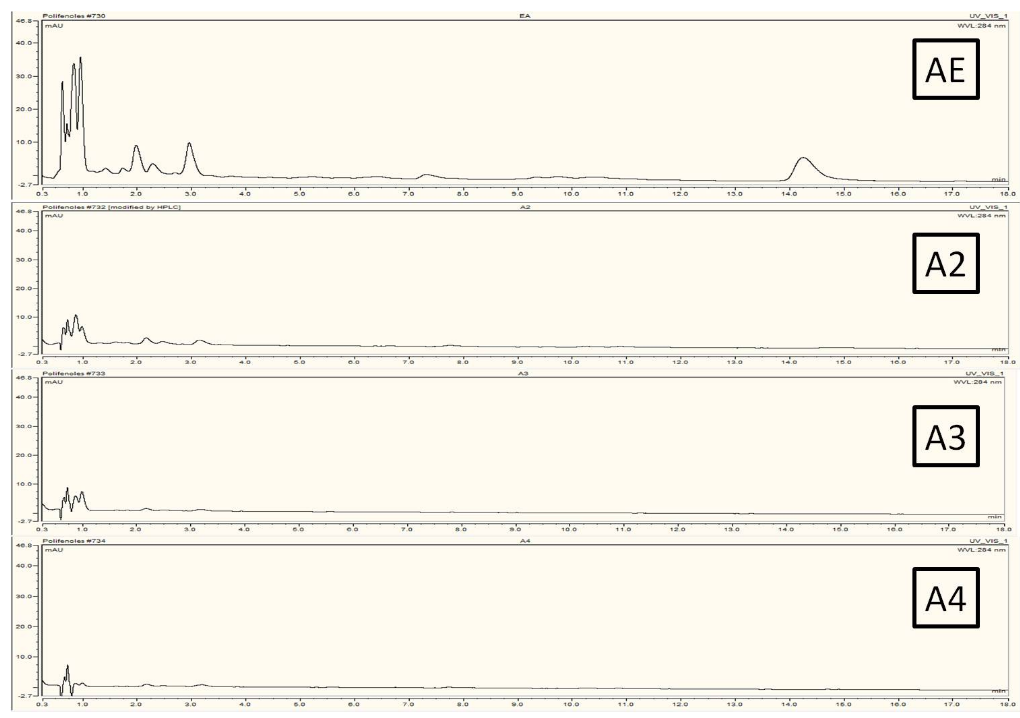


Figure 1. HPLC chromatograms at 284 nm of crude AE and fractions A2, A3, and A4.

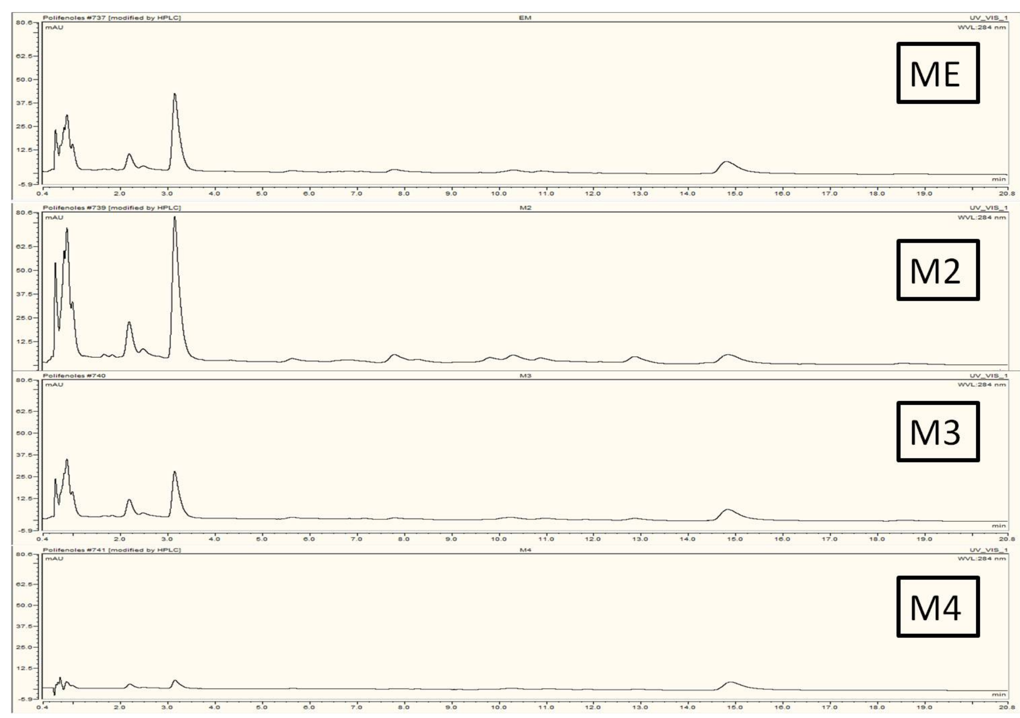


Figure 2. HPLC chromatograms at 284 nm of crude ME and fractions M2, M3, and M4.

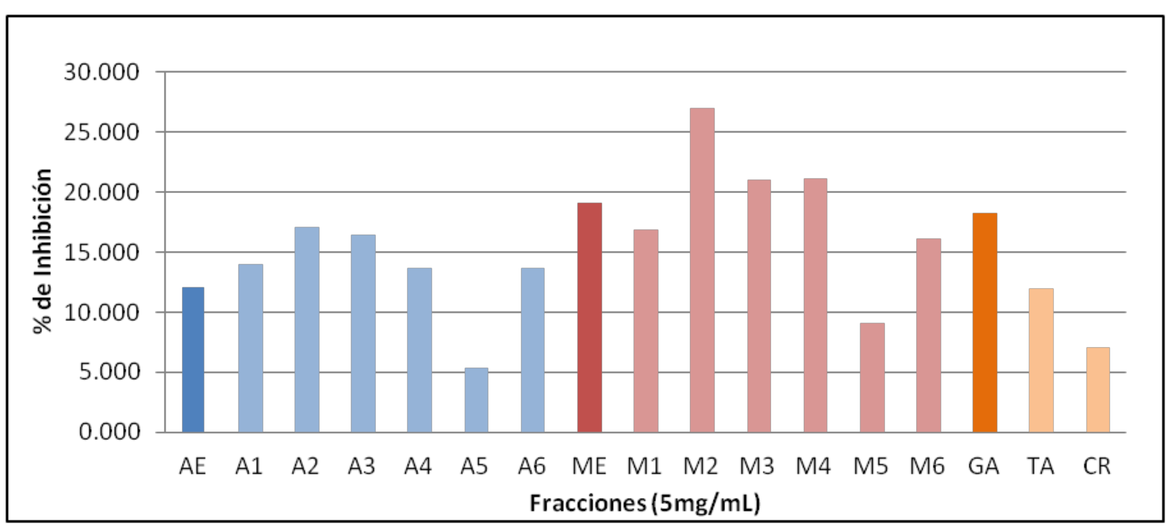
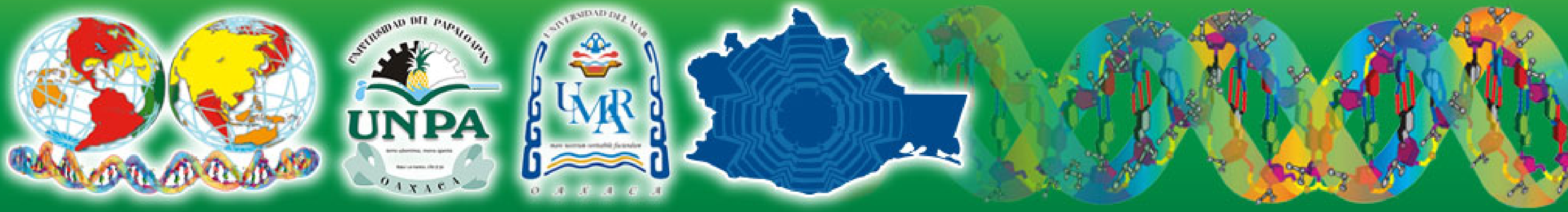


Figure 3. Percentage of growth inhibition by *Rhus trilobata* aqueous (AE), methanol (ME) crude extracts and their fractions. Controls with gallic acid (GA), tannic acid (TA) and curcumin (CR) were done.



Conclusions: The results show that methanol extraction achieved a higher concentration of the components also present in the crude extract. Likewise, it was found that fractions 2, 3 and 4, which contain compounds with a higher polarity, have a greater potential antineoplastic activity on CaCo-2 cells. For this reason, isolation of compounds present in these fractions is needed to identify the main principles responsible for the antiproliferative activity of *R. trilobata.* In our team, we continue working with antineoplastic activity of *R. trilobata*, the next step is to conduct experiments *in vivo*, which will carry out in nude mice, this for demonstrate the capacity of the activity components presents in *R. trilobata* to reduce the number of cancer cells *in vivo* models.

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Antioxidant activity of *Rhus trilobata* plant extracts

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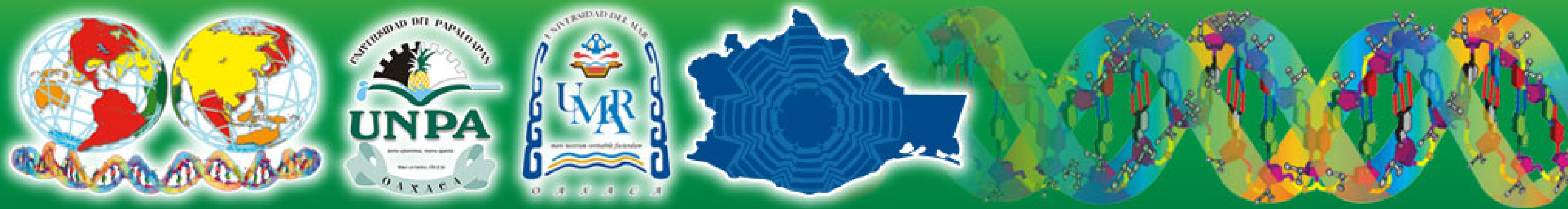
Abstract: Frequently, plant extracts have been used in herbal medicine, and they have proven to be rich in antioxidants. The extract of the regional plant *Rhus trilobata* has been used in the treatment of some types of cancer such as gastric cancer. To evaluate the antioxidant activity of *Rhus trilobata*, aqueous (AE) and 70% methanol (ME) extracts were prepared from stems. Fractions of both extracts were obtained by solid phase C-18 columns and different solvents. The total polyphenol content and antioxidant activity were analyzed by Folin-Ciocalteau method, oxygen radical absorption capacity (ORAC), and superoxide scavenging activity by the NBT assay; and reported in mg of gallic acid equivalents (GAE) per gram of dried sample (mgGAE g^{-1}), micromoles of Trolox equivalents (TE) per gram of dried sample (µmol TE g^{-1}), and percentage of inhibition, respectively. ME had the highest polyphenol concentration (107.6 mg GAE g^{-1}), followed by 02 and 03 ME fractions (19.37 and 16.04 mg GAE g^{-1}, respectively). Regarding antioxidant activity, ME and 02 and 03 fractions had the higher activity (1,819.19, 454.40 and 194.35 µmol TE g^{-1}), that agree with the content of polyphenols. Similar results were found in the NBT assay, where the ME reached an inhibition by 46.34% of the xanthine oxidase. The fractions 02 and 03 had an inhibition percent between 26 and 57%, respectively. The high antioxidant activity found in *Rhus trilobata* extracts suggests a potential use in the treatment against degenerative diseases associated with oxidative stress.

Keywords: *Rhus* • antioxidant • polyphenols • oxidative stress

Introduction: Excess of free radicals in the human being contributes to the oxidation of biomolecules such as lipids, proteins and nucleic acids, altering the cellular functions and promoting degenerative diseases (Bafna, 2005; Céspedes-Cabrera, 2000; Lu, 2010). Nowadays, a great amount of plants whose extracts have antioxidant activity have been used as a medicinal source in traditional herbal infusions. A particular case is the use of the aqueous extract from the regional plant *Rhus trilobata* as an alternative treatment for some types of cancer such as leukemia, breast and gastric cancer (Estrada-Castillón, 2010). *R. trilobata* is a plant of the Anacardiaceae family, also known as skunkbush, stinking sumac, and ill-scented sumac (Rose, 2005). *R. trilobata* is a deciduous, flowering native shrub that grows in arid high lands; is distributed from south of Canada, reaching Texas and north of Mexico. In the northern state of Chihuahua it grows in the region of the High Babícora (1,600-3,100 m.a.s.l.) (Sánchez-Ramírez, 1985). Studies performed in 1985 with *R. trilobata* stems showed the presence of a substance with a molecular weight below 500 Da, soluble at room temperature, with an antimitotic activity equivalent to 10^{-4} M of vincristine (Sánchez-Ramírez, 1985). Varela-Rodríguez (2013), worked with extracts obtained from *R. trilobata* stems, which had the presence of several polyphenol compounds that presented cytotoxic activity. The analysis of the antioxidant activity of this extracts could help to understand the basis of their potentially therapeutic uses.



Materials and Methods:

Plant samples. *R. trilobata* stems were collected from 29°15´01” latitude and 107°24´33” longitude, in May 2013. Plant specimens were identified by Ph.D. Toutcha Lebgue Keleng from the Natural Resources Management Department, and deposited in the herbarium of the Autonomous University of Chihuahua property (Reg. P5/000451). The stems were treated as described by Varela-Rodríguez (2013). Once the plant stems were completely dried, they were milled to a size sieve of 0.5 mm. Finally, the plant material was lyophilized, and kept in refrigeration at 4°C until use.

Preparation of plant extracts. Twenty five grams of dried powders were extracted and fractionated as established by Varela-Rodríguez (2013).

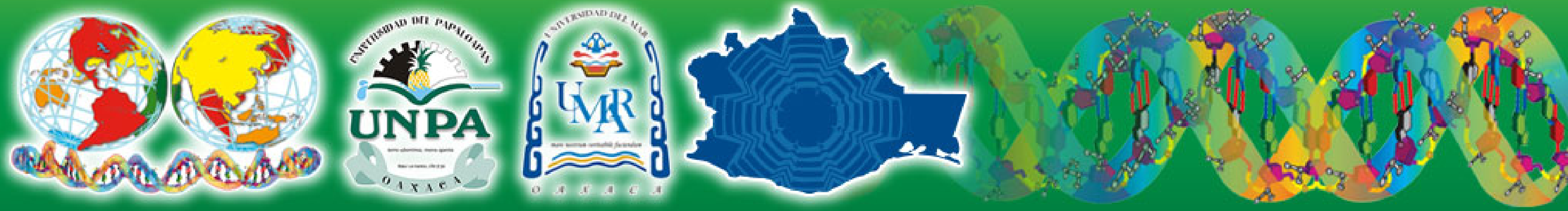
a) Aqueous Extract (AE): boiling in 500 mL of distilled water for 30 min.
b) Methanol Extract (ME): maceration by stirring for 48 h.

Samples were rotary evaporated at 40 °C, lyophilized and stored at -20 °C.

Total polyphenol content. Total phenolic content was determined by the Folin-Ciocalteau method with modifications proposed by Varela-Rodríguez (2013), using gallic acid as standard. The mixture contained 2.5 µL of samples at a concentration of 10 mg mL^{-1}, 10 µl of 2 N Folin-Ciocalteau´s reagent and 150 µL of distilled water was mixed and allowed to stand at room temperature for 10 min. Then, 30 µL of 20% sodium carbonate was added, mixed and incubated 10 min at room temperature, and 15 min at 37 °C in dark. Absorbance was measured in a microplate reader (Thermo Scientific Varioskan) at 755 nm. Results were reported in mg of gallic acid equivalents (GAE) per gram of dried sample (mg GAE g^{-1}). All measurements were taken in triplicate.

Antioxidant activity. Antioxidant activity was assessed with the ORAC assay according to Huang (2002) in a 96-well plate, using Trolox as standard. Samples were prepared at 10 µg mL^{-1} in 75 µM phosphate buffer (pH 7.4). Forty microliters of Trolox standards, 40 µL of samples, 40 µL of blank and 200 µL of a 1.4 µM fluorescein solution were added to each well and then incubated at 37 °C for 20 min in a microplate reader (Thermo Scientific Varioskan). After incubation, the first fluorescence reading was made (time= 0). After that, 35 µL of 2,2-azobis (2-amidino-propane) dihydrochloride (AAPH) solution was added to each well. Excitation wavelength of 485 nm and an emission wavelength of 535 nm were used. A total of 60 readings were taken for each sample (1 h). ORAC was expressed as micromoles of Trolox equivalents per gram of dried sample (µmol TE g^{-1}). This expression was obtained comparing the net area under curve (AUC) of the samples against the Trolox calibration curve. All measurements were taken in triplicate.

Superoxide scavenging activity. The conditions of the NBT assay were adapted from Kikkeri (2013). Xanthine oxidase was separated from the ammonium sulfate by centrifugation at 10,000 x g for 10 min to form a pellet, from which the supernatant was removed. The pellet was taken up in buffer (50 mm KH_2PO_4/KOH, pH 7.4, in deionized water) placed in Eppendorf tubes and stored at -20 °C.The reaction system contained 150 µL of samples at a concentration of 10 µg mL^{-1}, 30 µL of 0.2 mM hypoxanthine in 50 mM KOH, 50 µL of 0.6 mM NBT in buffer and 50 µL of xanthine oxidase solution in buffer (0.066 units per mL). The extent of NBT reduction was followed spectrophotometrically by measuring the increase of absorbance at 560 nm against a positive control of gallic acid. All experiments were replicated three times.



Results and Discussion:

The total polyphenols content in samples is reported in Table 1. Total polyphenols content was higher in ME (107.61 ± 0.73 mg GAE g^{-1}) and 02 and 03 fractions of both methanol and aqueous extracts (19.37 ± 0.28, 16.04 ± 0.31, 18.29 ± 0.06 y 7.13 ± 0.05 mg GAE g^{-1}, respectively). However, there was no significant difference between the solvents used for the extraction ($P>0.05$).These results are comparable with those obtained by Carloni *et al*. (2012), who analyzed the total polyphenol content and the antioxidant capacity present in samples of green, white and black tea, finding that black tea had the higher polyphenol content. From this it can be observed that extracts and fractions of *R. trilobata* stems have comparable polyphenol content to black tea.

Table 1. Total polyphenol content in *Rhus trilobata* crude extracts and fractions.

SAMPLE	mg GAE g^{-1}	SAMPLE	mg GAE g^{-1}
AE	87.89 ± 0.19 ^{A}	ME	107.61 ± 0.73 ^{A}
AE01	0.49 ± 0.02 ^{B}	ME01	0.14 ± 0.02 ^{B}
AE02	18.29 ± 0.06 ^{A}	ME02	19.37 ± 0.28 ^{A}
AE03	7.13 ± 0.05 ^{A}	ME03	16.04 ± 0.31 ^{A}
AE04	N.D.	ME04	N.D.
AE05	N.D.	ME05	N.D.
AE06	N.D.	ME06	N.D.

Aqueous extract (AE), methanol extract (ME), precipitate fraction (01), supernatant fraction (02), acid water 1% fraction (03), ethyl acetate fraction (04), ethyl ether fraction (05), methanol fraction (06). All values are mean ± standard deviation of triplicates. Values in columns with different letters are significantly different ($p<0.05$).

The results obtained in the ORAC assay are described in Table 2. Crude ME, and ME02 and ME03 fractions had higher antioxidant activity compared with crude AE and their fractions. There was no significant difference between the solvent used for the extraction. These results suggest that the antioxidant capacity is probably due to their polyphenol content. Similar results have been reported for another herbal extracts such as green tea (Carloni *et al*., 2012).

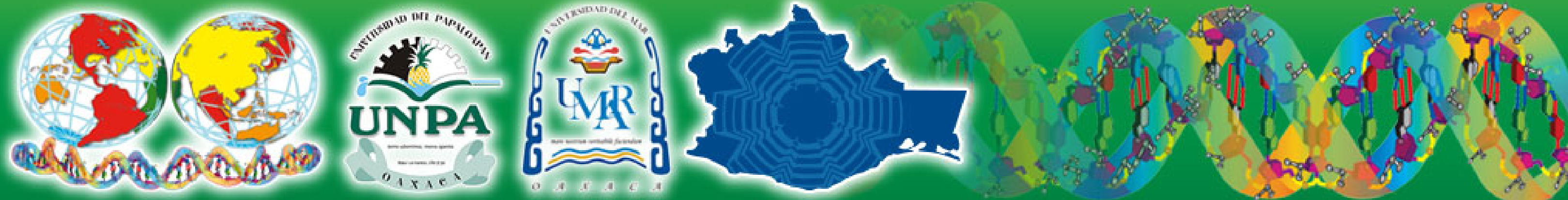


Table 2. Antioxidant activity of crude extracts and fractions from *R. trilobata*.

SAMPLE	ORAC (µmol TE g^{-1})	SAMPLE	ORAC (µmol TE g^{-1}).
AE	1389.09 ^{A}	ME	1819.19 ^{A}
AE01	0.0685 ^{B}	ME01	0.0063 ^{B}
AE02	454.40 ^{A}	ME02	517.89 ^{A}
AE03	194.35 ^{A}	ME03	364.62 ^{A}
AE04	N.D.	ME04	N.D.
AE05	N.D.	ME05	N.D.
AE06	N.D.	ME06	N.D.

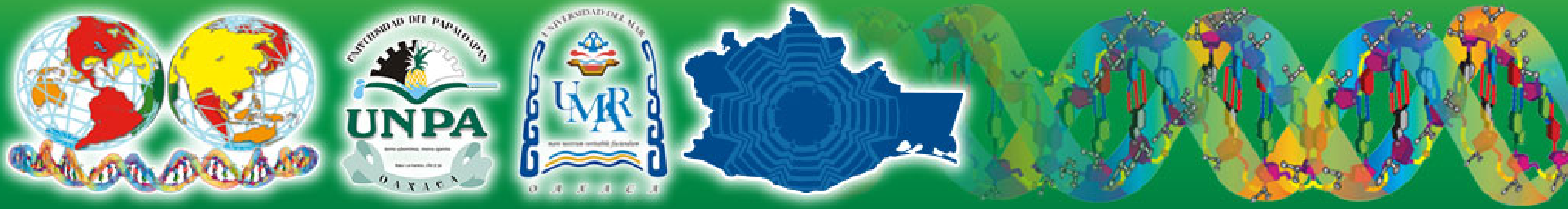
Aqueous extract (AE), methanol extract (ME), precipitate fraction (01), supernatant fraction (02), acid water 1% fraction (03), ethyl acetate fraction (04), ethyl ether fraction (05), methanol fraction (06). ORAC, antioxidant capacity expressed as Trolox equivalents (TE). Values in columns with different letters are significantly different ($p<0.05$).

The superoxide radical scavenging was estimated by the Nitroblue tetrazolium (NBT) method. Both extracts showed a high scavenging activity of free radicals (Table 3), and from these fractions ME02 and ME03 had the highest scavenging activity. Fractions 02 and 03 from both extracts probed to be as effective superoxide scavengers as gallic acid ($P>0.05$).

Table 3. Superoxide scavenging activity found in extracts and fractions from *R. trilobata*.

SAMPLE	%I	SAMPLE	%I
AE	40.74 ^{A}	ME	46.34 ^{A}
AE01	N.D	ME01	N.D
AE02	34.48 ^{A}	ME02	56.92 ^{A}
AE03	26.48 ^{B}	ME03	41.22 ^{A}
AE04	N.D	ME04	N.D
AE05	N.D	ME05	N.D
EAE06	N.D	ME06	N.D

Aqueous extract (AE), methanol extract (ME), precipitate fraction (01), supernatant fraction (02), acid water 1% fraction (03), ethyl acetate fraction (04), ethyl ether fraction (05), methanol fraction (06). %I, Percentage of inhibition of NBT. Results with different letters are significantly different compared to the positive control of gallic acid (59.82%).



Conclusions: Our results indicate that the extracts and fractions obtained from *R. trilobata* stems have an important antioxidant activity, which can be explained due the total polyphenol content. In addition, the methodology used for fractionation allowed us to obtain two fractions with high polyphenol and antioxidant activity. These results justify, the use of this extracts as an alternative treatment in the prevention of diseases associated to oxidative stress.

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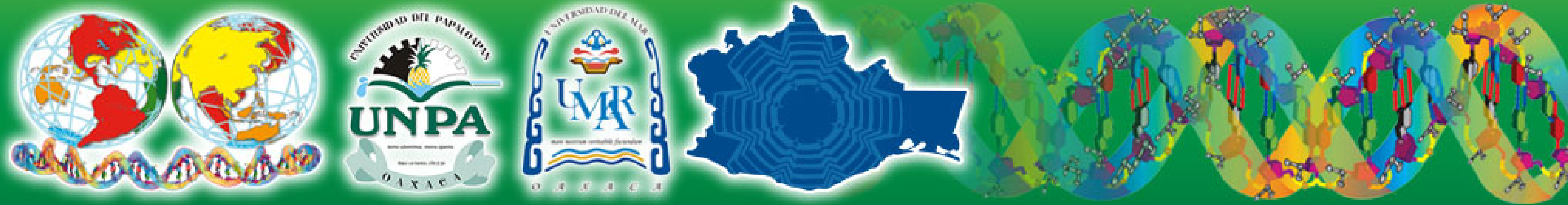

Effects of dietary flavonoids added to pharmacological antihypertensive therapy on PPARγ and PGC-1α gene expression

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Abstract: The peroxisome-proliferating activator receptors gamma (PPARγ) regulate processes that involve lipid and carbohydrate metabolism, anti-inflammatory factors and blood pressure (BP) regulation. Its partner, the cofactor that coactivates PPARγ (PGC-1α) has shown to increase the PPARγ activation. The effects of dietary flavonoids (DF) added to antihypertensive drugs as captopril (inhibitor of ACE) or telmisartan (AT1 blocker) on PPARγ and PGC-1α gene expression have not been studied. Twentyfour hypertensive patients (n=6 each group) that received captopril (Cpr), Cpr+DF, telmisartan (Tms) and Tms+DF during six months were analyzed for: BP, lipid and inflammatory profile. The PPARγ and PGC-1α gene expression was analyzed by standard methods. The data were analyzed by non-parametric statistics; gene expression levels were corrected by b-actin gene expression and correlated to biochemical parameters by Pearson's test. The PPARγ and gene expression was significantly higher for Cpr+DF and for Tms groups vs. Cpr alone ($P<0.01$). PGC-1α gene expression was raised for all the groups except Cpr alone ($P<0.05$). DF reduced triglyceride and LDL levels ($P<0.05$); a significant negative correlation was shown for leptin levels ($P<0.03$). DF added to pharmacological antihypertensive therapy based on Cpr or Tms increase PPARγ and PGC-1α gene expression.

Keywords: Hypertension ● dietary flavonoids ● PPAR gamma ● PGC-1alpha

Introduction: Flavonoids have shown reduce blood pressure and to ameliorate inflammation associated to metabolic disease (Grassi, 2005; Wolfram, 2006; Wang-Polagruto, 2006; Hodgson 2012; Moreno-Luna, 2012). Recently has been demonstrated that DF added to pharmacological antihypertensive therapy (AHT) based on captopril (Cpr) or telmisartan (Tms) are able to reduce blood pressure, body mass index (BMI), inflammation and to modify lipid profile in young hypertensive people (Romero-Prado 2014). The monocyte-macrophage (Mn-Mf) system plays a very important role on the pro-inflammatory condition derived from other chronic complications as hypertension. Some of the principal pathways that act on Mn-Mf system involve the peroxisome-proliferating activator receptors (PPARs). The PPAR gamma (PPARγ) participates in processes that involve lipid and carbohydrate metabolism, anti-inflammatory factors and blood pressure (BP) regulation. Its partner, the cofactor that coactivates PPARγ (PGC-1α) has shown to increase the PPARγ activation. The aim of this work was to analyze the effects of dietary flavonoids DF added to antihypertensive drugs as Captopril (inhibitor of ACE) or Telmisartan (AT1 blocker) on PPARγ and PGC1α gene expression in the Mn-Mf system.

Materials and Methods:

Total blood samples were taken from 24 hypertensive patients of both sexes, from 20 to 50 years old, non diabetic, neither presenting hepatic, kidney nor endocrinology diseases, diagnosed to have slight or moderate hypertension according WHO criteria. The patients were under antihypertensive treatment supplemented with a dietary flavonoids (DF) (see below). The samples were taken at 0, three and six months (Romero-Prado, 2014). The pharmacological therapy was telmisartan 40 mg/24 h, or captopril 25 mg/12 h.

Dietary Flavonoids (DF). They were composed of dark chocolate 30 mg (Alpezzi Chocolates, Zapopan, Jalisco, México), dehydrated red delicious apple 30 mg (Deshidratadora Nacional de Alimentos, México) and brewed green tea 4 mg (Therbal, México) that summarized 997.33 ± 23.73 mg gallic acid equivalents (GAE) of polyphenols and 425.8 ± 20.63 mg cathechin equivalents (CE) of flavonoids per day (Romero-Prado, 2014).

Lipid and inflammatory analysis. The lipid profile was determined by standard final point assays; the hsCRP was determined by turbidimetric assay (BioSystems). Plasma leptin was analyzed by ELISA method according the instructions by the provider (R&D).

PBMC isolation. The procedure to obtain peripheral blood monocyte cells (PBMC) was made in Histopaque gradient according to instructions from provider (Histopaque 1077, Sigma Aldrich México) as follows: 1 vol of PBS:BSA (1:0.01%) was added to 1 vol of heparinized total blood obtained by vein punction. The mix was added very carefully on the top of 1 vol of Histopaque 1077, centrifuged at 1,500 rpm during 30 min. The cell pellet was washed twice with PBS BSA (1:0.01%), and stored at -80°C until the nucleic acids isolation was done.

RNA isolation and RT-PCR. RNA was obtained by the Trizol method according the instructions by the provider. The RT reaction was made using the AMVL retro-transcriptase enzyme by a standard protocol (Epicentre). The PCRs for PPARγ and PGC1α as well as for a constitutive gene (β-actin) were made by standard protocols. Statistical analysis for parametric and non-parametric variables was made; Pearson's test correlation was made between lipid and inflammatory profile vs. gene expression levels.

Results and Discussion:

At this date, this is the first work that analyzes the gene expression of PPARγ and PGC1α in samples from patients that received an AHT based on Cpr or Tms supplemented with DF. Tms is known as a partial agonist of PPARγ in cardiomyocite and monocyte cells. Previous work by our group demonstrated that DF added to an AHT based on Cpr or Tms is able to reduce significantly blood pressure and BMI, and to modify lipid and inflammatory profile (Romero-Prado, 2014). Additionally, we found that the combination Cpr+DF produced an important reduction on cholesterol and triglycerides. In the present work, we considered the Mn-Mf system as a metabolic monitor for subtle cell changes that have been produced in hypertension, dyslipidemic or pro-inflammatory conditions that could give cellular and molecular information about the progress or contention of the disease under the applied treatments.

In a preliminary sampling, we selected 6 randomly patients to represent the effects of DF on AHT. The samples were analyzed at 0, 3 and 6 months for PPARγ (Figure 1) and PGC1α (Figure 2) gene expression, both normalized by β-actin.

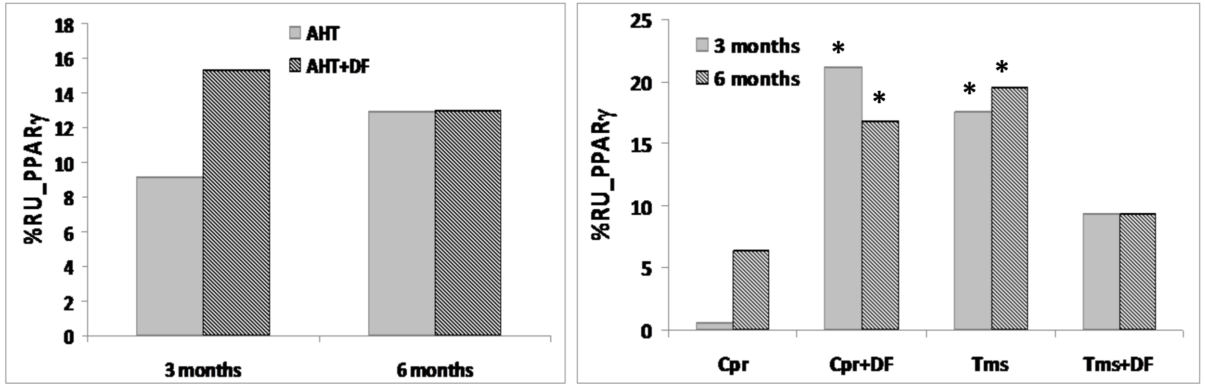


Figure 1. Total mRNA was analyzed by RT-PCR for PPARγ transcript and normalized by b-actin. RU, relative units in %; * P<0.05 vs Cpr.

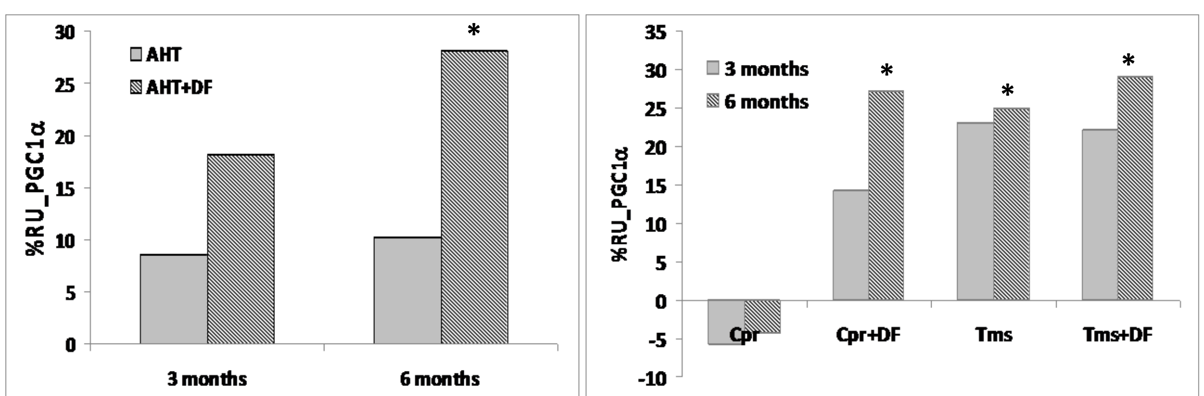
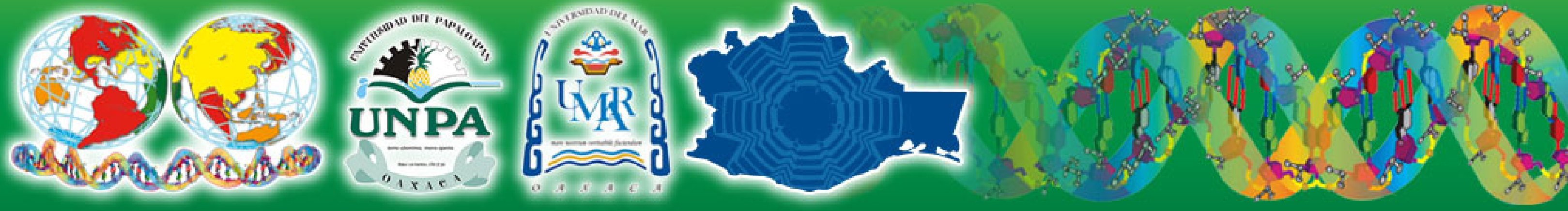


Figure 2. PGC-1α gene expression. Total mRNA was analyzed by RT-PCR for PGC-1α transcript and normalized by b-actin. RU, relative units in %; *, P<0.05.

We found a significant increase in both PPARγ and PGC-1α gene expression comparing the basal values vs 3- and 6-month periods (P<0.05). The Cpr group was the only that did not show an increase in PPARγ and PGC-1α gene expression levels. Previously has been reported that PBMCs express PPARγ that acts as anti-inflammatory molecule down-regulating the NFkB system (Jiang 1998), so we think the DF are able to act on these cells contributing to an anti-inflammatory and systemic effect. PPARγ is a transcription factor that has a principal role in adipocyte and trophoblast differentiation as well as an important regulator of genes involved in lipids and glucose metabolism from tissues from liver, fatty mass, bone, muscle and pancreas (Barak 1999; Shalom-Barak 2012).



By other hand PGC-1α, as coactivator of PPARγ, has an important role in brown fat differentiation and as an important factor for mitochondrial biogenesis in developing heart (Puigserver & Spiegelman, 2003). A recent experimental study in the spontaneously hypertensive rat showed that the molecular clock expression in skeletal muscle is disrupted in an age-associated way that results in alterations in PGC-1α expression and other genes (Miyazaki 2011).

Our results point to that DF are able to modify in an important manner the expression of both genes, being more notorious for PGC-1α expression. Importantly, there is no reports of PGC-1α in the Mn-Mf system, neither the role of flavonoids on its gene regulation so are needed more studies to elucidate the participation and biological role of PGC-1α in the immunological system under normal and pathological metabolic conditions.

Finally, we looked for the correlation between biochemical variables and gene expression levels of PPARγ and PGC1α. We found a significant negative correlation between both genes and leptin levels independently from the treatment. The negative correlation between leptin and PPARγ has been described for other tissues as pancreas (Mahmoodzadeh 2013) and osteoblast (Wang 2012).

Conclusions: The DF added to a pharmacological antihypertensive therapy are able to increase the gene expression of PPARγ and PGC1α. There is a negative correlation between plasma leptin levels and PPARγ expression in the Mn-Mf system.

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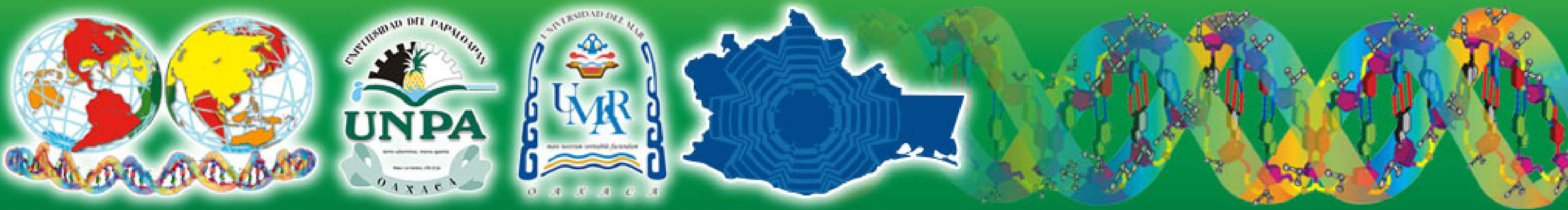

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Pharmacokinetics of IgY in rabbits

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Abstract: In this work, we report the pharmacokinetics of IgY used as a antivenom. IgY is an immunoglobulin isolated from chickens. To perform the pharmacokinetics analysis we used rabbits as a mammalian model as it is the model most used, previous to test in humans. The dosage application to each rabbit was intramuscular and subcutaneously. The results of the pharmacokinetics parameters show that IgY remains a shorter time in the body system than others previously reported for immunoglobulin such as horse IgG, Fab_2 or Fab.

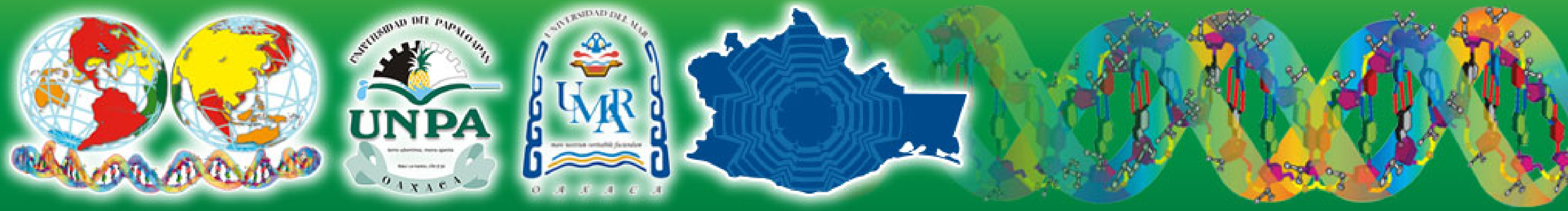
Keywords: Pharmacokinetics ● IgY Immunoglobulins ● antivenom

Introduction: Animal poisoning in humans has been successfully treated with specific polyclonal antibodies. Antivenoms are prepared by immunizing large animals. Some animal species have been used on antivenom production such as horses, sheep, donkeys, goats, rabbits and for experimental purposes camelids, dogs or hens. Animal selection is based on economical and practical consideration (Theakston *et al*., 2003).

Horses are commonly used in commercial antivenom production because they have some advantages, they are docile, adaptable to climate changes and they produce large volumes of antisera for driven to high yield. The most important disadvantage on antivenom production is the presence of heavily glycosylated IgGT with high capabilities to promote immunogenic responses (Theakston *et al*., 2003). Sheep have also been used to produce antivenom and the most important advantages are economical and technical management. They can tolerate oil-based adjuvant like horses and their antibodies can be applied in patients with hypersensitivity to equine proteins (WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins).

Animal production is immunized with crude venoms to promote an immune response, such producers add adjuvants as Freund's complete and incomplete, aluminum hydroxide or alginate (Theakston *et al*., 2003). Serum titre of the immunized animals is followed by EIA during the immunization phase, and efficacy measurements are done as neutralization potency assays of lethality when the immunization is completed or before blood collection. Plasma for fractionation can be obtained either from the collection of whole blood or by the apheresis procedure (WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins).

Blood collection is an invasive method therefore other alternatives are suggested on antivenom production. Chicken antibodies can be used to prevent invasive practices and bleeding. IgY is contained on bird eggs and some isolation methods have been developed. IgY isolation is fast, simple and economically feasible; low quantities of antigens are required to obtain high specific IgY immunoglobulins in the yolk from immunized hens (Tini *et al*., 2002).



Several experimental IgY antivenom have been developed (Aguilar *et al*., 2014, Almeida *et al*., 2008, Andrade *et al.,* 2013, Alvarez *et al*., 2013, Araújo *et al*., 2010, Mendoza *et al.,* 2012), and have shown an interesting neutralizing potency for commercial antivenom. Another important advantage of IgY arises from genetic differences between birds and mammals, this allows the production of antibodies against highly conserved mammalian proteins. Additionaly IgY does not activate mammalian complement or interact with mammalian Fc receptors that could mediate an inflammatory response (Dias da Silva and Tambourgi, 2010). Neutralizing potency and pharmacokinetics abilities are prompt characteristics to get a clinical success.
The present report describes a Pharmacokinetics study of IgY, an inmunonglobulin reported as an effective antivenom, when it is administered intramuscularly and subcutaneously in rabbits on a single dose.

Materials and Methods:

IgY Purification. Isolation of IgY from yolks of non-immunized hens was done with modifications of Polson protocols (Polson, 1980; Polson *et al*., 1990). Eggs were cracked and transferred to a paper filter to remove white egg, and yolk membrane was cut; the content was recovered and mixed with 3 volumes of 4.7% of PEG-600 (W/W). IgY was separated from fatty and non-soluble compounds by centrifugation at 4 °C for 20 min (13,000 x g), and filtered. Soluble fraction was precipitated adding 35% ammonium sulfate, mixing and separated by centrifugation at 4 °C for 20 min (13,000 x g). Precipitated IgY was dissolved on PBS and dialyzed against PBS buffer, the IgY extract was liofilizated for storage. Final preparations were analyzed by SDS-PAGE.

Animal experimentation. The experimental protocol was approved by the ethical committee of the Instituto de Biotecnología (IBT), Universidad Nacional Autónoma de México (UNAM), in Cuernavaca, Morelos, México. Four rabbits of the white New Zealand breed were used for all the assays. 300 g kg^{-1} of IgY was administered to each rabbit. To measure serum drug concentration, blood samples were drawn at 10 min intervals from 0 to 60 min, then at 2 h, 4 h, 8 h, then at 24h and finally on days 3, 5, 7, 9, and 11. Experimental animals were immobilized with a harness, and catheterized in the right ear marginal vein with a butterfly needle (winged Infusion Sets, 21G×3/4" ultra thin wall needle, Terumo). A blank sample at t=0 (t0) was drawn from the catheterized ear. After the injection, samples were drawn through the catheter at each designated time. Blood samples were incubated for 2 h at 37 °C, and centrifuged for 8 min at 25,000 g (Eppendorf, 5417R). Serum was separated and kept frozen at −20 °C until quantification of the immunotherapeutic.

ELISA method. Maxisorp (Nunc Inc, USA) plates were coated with 100 µL/well of 5 µg/mL polyclonal anti-chicken anti-rabbit antibodies diluted in 100 mM carbonate/bicarbonate buffer, pH 9.5 and incubated overnight at 4 °C. The plates were then washed 3 times with 250 µL/well with washing buffer (50 mM Tris/HCl, 150 mM NaCl, 0.05%, Tween 20 and pH 8). The remaining binding sites were blocked with 200 µL/well of blocking buffer (50 mM Tris/HCL, 5 mg/mL gelatin, 0.2%, Tween 20, pH 8) and incubated for 2 h at 37 °C. The plates were then washed 3 times with 250 µL/well of washing buffer. The standards were diluted in a mixture of untreated rabbit serum and vehicle buffer (50 mM Tris/HCl, 500 mM NaCl, 1 mg/mL gelatin, 0.05% Tween 20, pH 8). For this purpose 100 µL/well from IgY standard (10 µg/mL) were placed in the first well of the plate and serially diluted 1:3 from well 2 to 11 with vehicle buffer, Well 12 contained just vehicle buffer as control, the plates were incubated 1 h at 37 °C. Plates were washed 3 times with washing buffer

(250 µL/well), after this 100 µL/well rabbit anti-chicken IgY antibody HRP conjugate (Millipore) diluted in vehicle buffer (1:2000) were added and incubated 1 h at 37 °C. Plates were then rinsed 5 times with 250 µL/well rinsing buffer. Finally, 100 µL/well of peroxidase were added with peroxydase chromogenic substrate (ABTS solution Roche) and incubated 15 min at 25 °C; after this time, the reaction was stopped with 25 µL/well of 20% sodium dodecyl sulfate. Absorbance was read at 405 nm. The immunotherapeutic concentration in each plasma sample was determined by triplicate. The calibration curves were done by duplicate on each plate, antivenom concentrations ranging from 200 ng mL^{-1} to 0.78 ng mL^{-1}. The dilutions of the samples were adjusted by trial and error until they fell on the lineal range of the standard curve. The results were plotted using Prism 4.0 graphic package (Graph Pad software) with a non-lineal analysis of regression.

Statistics procedures. Nonparametric statistical procedures for small size samples were used. Data is presented as medians and their 95% confidence interval calculated with the procedure of Hodges and Lehmann. Statistical significance of differences was decided with the Mann–Whitney (Wilcoxon) test, and multiple comparisons were done using the Kruskall–Wallis nonparametric analysis of variance.

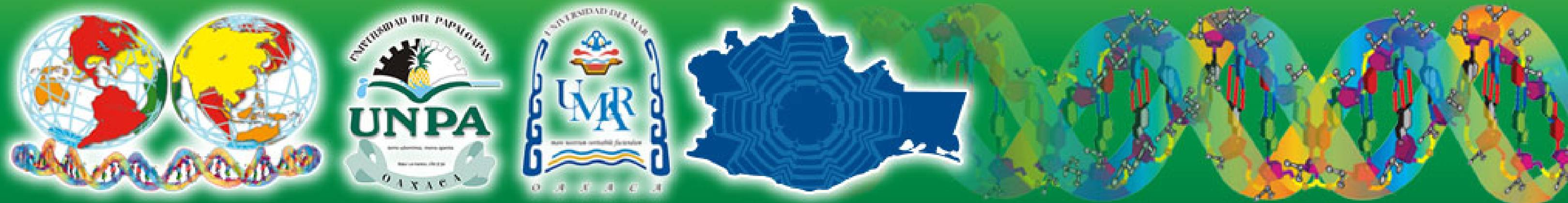
Determination pK constants. The plasmatic concentrations of antivenom (IgY) vs time were adjusted to z exponential equation as it is described in Vazquez *et al*. (2010).

Results and Discussion:

The IgY concentrations were determined by quantitative ELISA, from samples of rabbit serum using rabbit anti-IgY as captor. PK parameter was obtained with z = 3, fitted to the concentration vs time plasma curves. The pharmacokinetic parameters were derived by fitting data to a 3 exponential equation. The values of Ci and ti obtained for the immunotherapeutics were used to calculate the PK parameters shown in Table 1.

Table 1. Pharmacokinetic parameters of IgY derived from tri-exponential component fittings to concentration vs time curves.

	IgY	Units
AUC∞	5234827.7	ng/mL/min
AUMC∞	16540194706	$ng/mL/min^2$
C1	5990.6	ng/mL
Vs	122.6	mL
Vz	312.4	mL
Vss	175692.2	mL
CL	0.19	mL/min
MRT	2893.1	min
t1/2,1	4.9	min
t1/2,2	19.4	min
t1/2,3	494.2	min



Our results were compared with previous results in Vázquez *et al*. (2010), it may be appreciated that the mean retention time (MRT) of chicken IgY is as small as horse IgG, in the meantime a molecule of drug stays in the body, the lower MRT indicates that IgY lifespan in the body is shorter than IgG's. The total clearance of drug was compared and the larger value observed in the value suggests that IgY is eliminated faster from the body than horse IgG.

Conclusions: It has been reported that IgY inmunogloblines, extracted from chicken, are considered and effective antivenom molecule. In order determine the effectiveness of this immunoglobulin as antivenom in mammalians, we have administrated IgY to rabbits to perform the pharmacokinetics and pharmacodynamics. Rabbits are a common preclinical pharmacokinetic and pharmacodynamics model for many drugs prior to clinical trials in humans. The results indicate that IgY remains less time in the body, reaching a smaller volume of distribution in steady state and is eliminated faster. These characteristics are important in pharmacokinetic antivenom.

Acknowledgement: To Felipe Olvera Rodriguez for assistance with the blood sampling procedure.

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PCR typing of *Brucella* species-specific strains isolated from livestock and humans and differentiation of vaccine strains S19 and RB51

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Abstract: Members of the genus *Brucella* are causal agents of brucellosis in humans and domestic and wild animals. Brucellosis remains a major worldwide zoonosis with negative effects on public health. In humans, it is usually a zoonotic disease, and its control represents a challenge. Because of this, it is important to implement rapid and sensitive diagnostic techniques. Bruce-ladder PCR assay is capable of differentiating between all species and vaccine strains of *Brucella*. In this study, reference, vaccine, and human and livestock isolated strains of the genus *Brucella* were analyzed by its molecular type using the modified Bruce-ladder assay to corroborate correspondence of this method with microbiological analysis. Genomic DNA was extracted from pure cultures by heat lysis of bacteria; the amplicons obtained were of 1682, 1071, 587, 272, 218, and 152 bp from the *wboA*, *omp31*, *eryC*, TBP, *rpsL,* and CRP genes, respectively. The modification of the assay consisted in adding 18.75 pmol from each oligonucleotide to each reaction mixture and the reaction was performed in two test-tubes. It was possible to reproduce the characteristic pattern of each strain, and there was complete correlation with microbiological analysis for all the strains studied. Two control bacteria (*E. coli, S. typhi*) tested negative.

Keywords: *Brucella* ● identification ● Bruce-ladder ● PCR

Introduction: Brucellosis is a re-emerging zoonosis that preceded humans but continues to cause significant medical, veterinary and socioeconomic problems, because it remains underestimated and neglected. Although it has been present for hundreds of thousands of years, it has been ignored because it is a human and animal disease of poverty. Currently, there are ten species of *Brucella* described on the basis of host specificity: *B. abortus* and *B. melitensis* (bovine and small ruminant, respectively), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep), *B. neotomae* (rodents), *B. pinnipedialis* (seals), *B. ceti* (dolphins, porpoises), *B. microti* (voles) (Whatmore, 2009), and *B. inopinata*. However, its ecology and epidemiology have evolved rapidly in recent years, and it is now clear that *Brucella* knows no borders (Pappas, 2010). Different species may act as hosts or vectors of *Brucella* spp. and humans can acquire a debilitating febrile illness, known as 'Mediterranean or undulant fever', as the result of contact with infected animals or consumption of their products (Pappas *et al*., 2006; Godfroid *et al*., 2011). This background highlights the importance of developing effective surveillance and control systems for emerging infectious zoonotic diseases with pandemic potential, as the World Bank has declared (Godfroid, 2013). Because of this, DNA-based techniques have been developed to identify and differentiate between the various *Brucella* species and strains, a task that has represented a great challenge because of the high degree of genetic homology of the genus *Brucella* (DelVecchio *et al*., 2002; Paulsen *et al*., 2002; Halling *et al*., 2005). Among these techniques, which offer advantages over traditional microbiological testing, one can mention the AMOS-PCR assay (Bricker *et al*., 1994;

Bricker *et al*., 1995) and the Bruce-ladder PCR assay (García-Yoldi *et al*., 2006). The latter has shown efficiency, rapidity and facility of performance and interpretation, it is useful for molecular typing of all *Brucella* species, including vaccine strains, and it has been evaluated in seven different European laboratories (López-Goñi *et al*., 2008). It is important to establish these protocols in diagnostic labs and to evaluate its efficiency against microbiological testing.

Materials and Methods:

The strains examined in this study were procured from the Laboratory of Microbial Pathogenicity from the Instituto de Ciencias-BUAP (CICM-ICUAP), Mexico. The collection included reference strains: *B. melitensis* M16 (INDRE. Mexico), *B. suis* 1330 (Institut national de la santé et de la recherche médicale, INSERM, Marseille, France), vaccine strains: *B. abortus* S19 (INDRE, Mexico), *B. abortus* RB51, and *B. melitensis* Rev1 (Tornel Labs), and field strain isolates from human blood, dog, fetus, and bovine cultures. Gram negative strains were *E. coli* O157:H7 and *S. typhi* CT18 (CICM-ICUAP). *Brucella* isolates were typed using the standard microbiological procedures described by Alton *et al*., 1998. This work was performed in biosafety level 2 containment facilities. Genomic DNA extraction was prepared from pure cultures by heat lysis of bacterial cell cultures (Seal *et al*., 1999). Bacteria were heated at 99 °C for 15 min and centrifuged for 10 min at 13,000 x g. The supernatant obtained was then used in PCR. The modification of original multiplex Bruce-ladder (García-Yoldi *et al*., 2006; López-Goñi *et al*., 2008) consisted in adding 0.8-1.0 μg of template DNA, and 18.75 pmol from each oligonucleotide to mix each reaction. This was 3 times the amount used in the original technique. Besides, we performed the PCR reaction separately in two-test-tube assays (the original technique uses a single-test-tube assay), in one mix reaction, primers were added to amplify fragments of 1682, 587, and 152 bp from the *wboA*, *eryC*, and CRP genes, respectively, according to the DNA sample analyzed; In another mix reaction, primers were added to amplify fragments of 1071, 272, and 218 bp from *omp31*, ABC transporter binding protein (TBP), and *rpsL* genes, respectively, according to the DNA sample analyzed. GoTaq Green Master Mix (Promega) was employed for the reaction mixture. The thermocycler used was a Mastercycler Personal (Eppendorf). And the molecular marker was the 1 kb Plus DNA ladder. PCR products were analyzed by standard 1.5% agarose electrophoresis and documented.

Results and Discussion:

All reference and vaccine strains and livestock and human isolates analyzed in this study showed the characteristic patterns of the amplicons reported by García-Yoldi *et al*. (2006). In Figure 1, one may see fragments of *B. abortus* S19: 1682 and 152 bp (lane 2) amplified from genes *wboA* and CRP, respectively. The last fragment is present in all genomes of the genus *Brucella,* except in *B. neotomae,* which was not part of the strain collection studied. No fragment was amplified in the second mixture reaction (lane 3); the same for lanes 6 and 7, which show the results for a control reaction lacking DNA. In lane 4, it is possible to identify two characteristic bands; this pattern is similar to that *B. abortus,* except that the DNA of *B. abortus* vaccine strain RB51 does not amplify the 1682 bp fragment because of a *wboA* gene disruption by an IS711 element (Vemulapalli *et al*., 1999). Thus, for *B. abortus* RB51, one amplicon was of 587 bp from the *eryC gene*, and another one was of 152 bp, from TBP (García-Yoldi *et al*., 2006; López-Goñi *et al*., 2008).

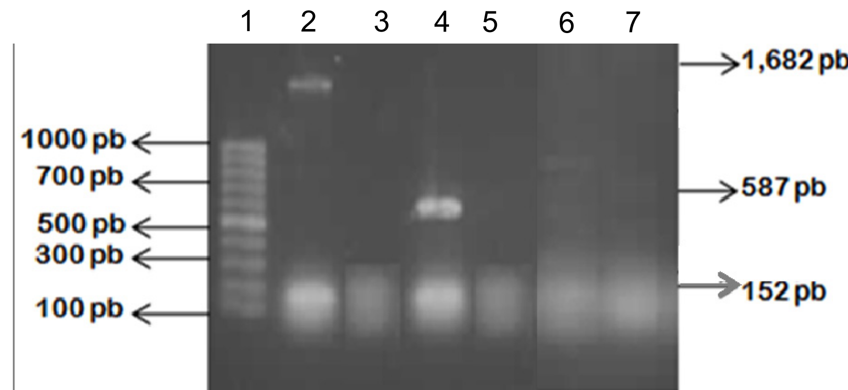

Figure 1. Identification of *Brucella* vaccine strains by amplified products obtained of mutilpex PCR.

Multiplex PCR assay with DNA from human blood cultures was used to identify several strains of the genus *Brucella*, Figure 2 shows a representative example of the results obtained for two samples identified as *B. abortus* (first sample in lanes 2 and 3, and second sample in lanes 10 and 11) where three fragments of 1682, 587, and 152 bp, from *wboA*, *eryC,* and CRP genes, respectively, were amplified. The absence of the 1071-bp fragment is characteristic of all *B. abortus* strains because of the loss of the *omp31* gene causing a 25-kb DNA deletion. It was interesting for us to identify the vaccine strain *B. abortus* RB51 from a hospitalized human patient (lanes 8 and 9), which presents a pattern similar to *B. abortus*, except for the 1682-bp band from the *wboA* gene that is disrupted in this vaccine strain and hence the fragment is not amplified (Vemulapalli *et al*., 1999). Identification of this strain might be useful for epidemiological studies.

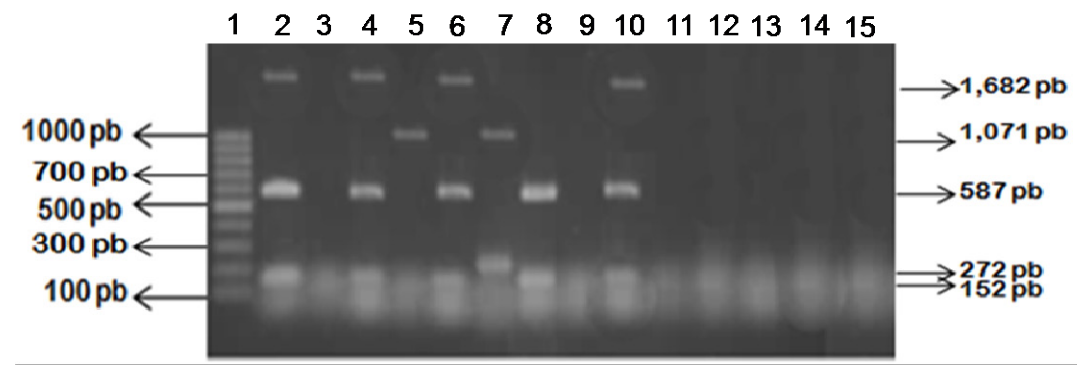

Figure 2. Identification of field strains isolates from human by multiplex PCR.

Brucella melitensis genomic DNA extracted from human blood cultures showed 1682-, 587-, 152- (lane 4), and 1071-bp (lane 5) fragments, as it may be seen in Figure 2. The fragment of 587 bp was amplified from the *ery* operon (Sangari *et al*., 1994) present in all *Brucella* strains, except in *B. abortus* S19. Another characteristic pattern for *B. melitensis* is the absence of the 272- and

218-bp fragments (Cloeckaert *et al*., 2002). The only difference between the patterns of *B. melitensis* and *B. suis* is the presence of an additional 272-bp fragment in *B. suis* (lane 7) amplified from the ABC transporter binding protein (TBP) target gene (Halling *et al*., 2005; Paulsen *et al*., 2002). The specificity of this multiplex PCR assay was shown by means of genomic DNA from Gram-negative strains of *E. coli* and *S. typhi* (lanes12-15, Figure 2, and Figure 3) which did not amplify any of the 6 fragments.

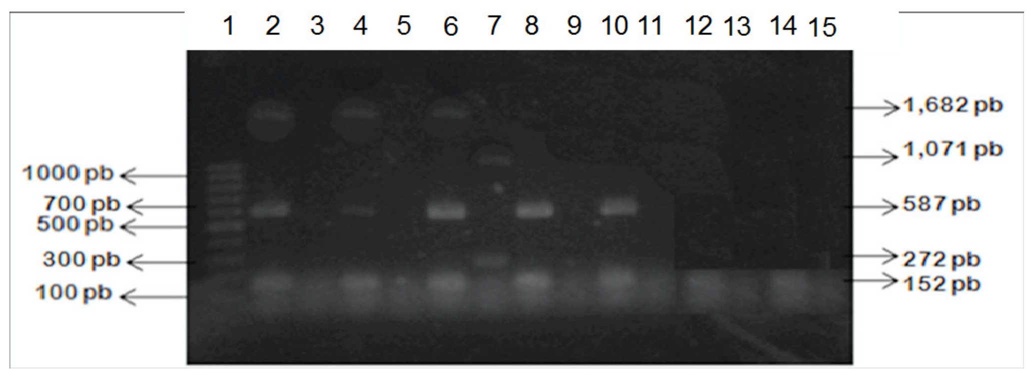

Figure 3. Identification of field strains from livestock by multiplex PCR.

Figure 3 shows the amplicons obtained from genus *Brucella* genomes extracted from several animal samples: aborted bovine fetus (lanes 2 and 3), dog (lanes 4 and 5), and cows (lanes 6 and 7, 8 and 9, 10 and 11). *B. abortus*, *B. suis*, and *B. abortus* isolates from tissues were identified. These results highlight the importance of identifying vaccine strains in cattle. The molecular typing of all the strains analyzed showed correlation with microbiologic standard techniques. These findings show that this assay could be a great tool in cattle trade because it provides a fast and reliable way to differentiate between vaccine and pathogen strains in bovine cattle, which may be important information for the movement and release of sale animals, and it could contribute to the control of disease in livestock and humans.

Conclusions: This study proves the utility of the modified Bruce-ladder assay for PCR typing of livestock and human isolates of the genus *Brucella*, as well as of vaccine strains S19 and RB51. Using this method is a reliable, rapid and specific way to identify all *Brucella* strains. Its results agree with those previously obtained by microbiological standard techniques. Another advantage of this technique is the minimal sample preparation required, which limits the manipulation of live *Brucellae* and reduces the risks thereby involved, since most of the *Brucella* strains are highly pathogenic for humans. It is important to establish this assay as a common routine practice to differentiate *Brucella* species in diagnostic labs, principally in low-income countries.

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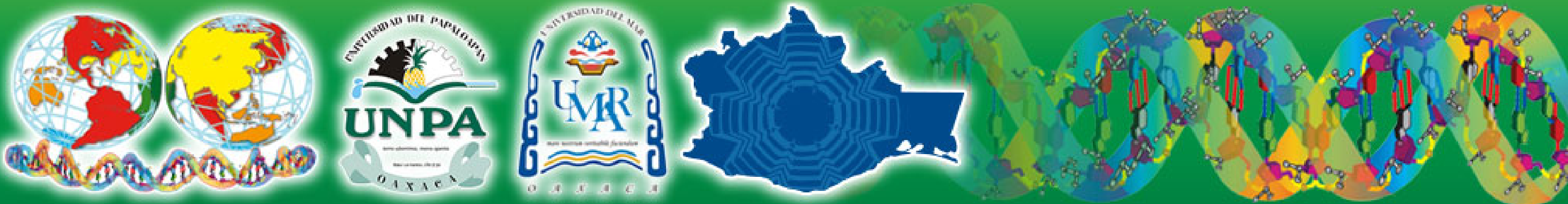

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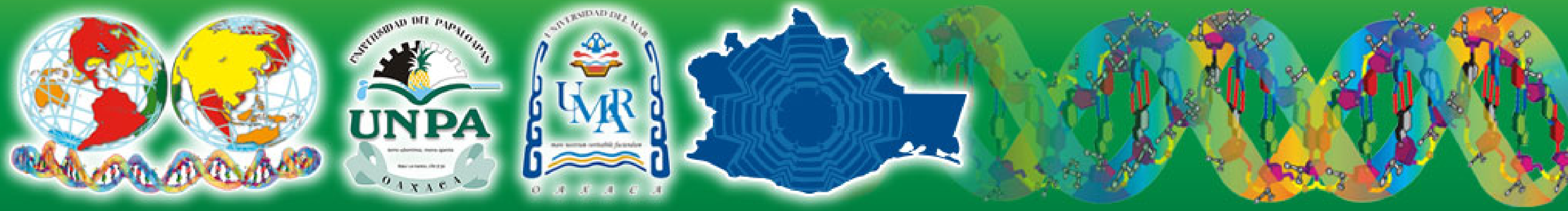

Proteomic analyses for detection of pathogens in water bodies in Mexico City

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Abstract: Water pollution, an inevitable consequence of urbanization and industrialization has major public health and socio-economic impact. Animals are often carriers of human pathogenic bacteria excreted in the feces usually through feces and urine; consequently these microorganisms can contaminate water bodies such as rivers, lakes, and streams. Then, the objective of this study was to identify pathogen in the water of rivers, lakes, and streams by proteomic analysis. This will be useful to develop a microarray test in the near future. Firstly, we collected water samples in Mexico City and the surrounding areas in January-May and September-October 2012. These samples were analysed in the proteomic equipment MALDI-TOF to investigate the most frequent pathogens. In the collected water samples we found bacterial pathogens that cause diarrheas and enteric illness, such as *Proteus mirabilis*, *Salmonella enterica* and *Shigella flexneri*, *Vibrio*s, Clostridium and viruses. Identification of these pathogenic bacteria will be use to design and develop a microarray test to detect pathogens in water samples from rivers, lakes and streams.

Keywords: Proteomics ● *Salmonella sp*. ● *Shigella sp.* ● Enterobacteriaceae family

Introduction: The contamination of rivers, lakes, and stream water by pathogens causing diarrhoeal disease is the most important aspect of health. The problem arises as a consequence of contamination of water by faecal matter, particularly human or animal faecal matter, containing pathogenic organisms. The water-related pathogens can be a threat to aquatic environments and this contamination can occur from a wide range of sources including wastewater effluents, the application of animal manure to agricultural lands, wildlife and urban areas. Increase in globalization of trade and human movement facilitates urban crowding, favoring host-to-host contact and the dissemination of emerging infectious diseases. The potential for bacteria present in human and animal wastes to contaminate water in nearby rivers and lakes needs special attention because the acute microbial diarrheal diseases are a major public health problem in developing countries.

Microbial pathogen detection in water by enzymatic methods is much less time consuming than traditional techniques, however, in very low contaminated waters, enzymatic methods might not be able to detect them. The identification of bacterial and viral contaminants in water through proteomic analysis is a molecular method that is rapidly evolving in the field has a wide range of applications for monitoring and rapidly diagnoses microbial pathogens in the sea, lakes or rivers and even in drinking water. We demonstrate direct evidence of water contamination by using this method and displayed a wide variety of pathogens in the collected water samples. These pathogens will be use as specific biomarkers to design a microarray able to detect pathogens. In addition we will include pathogens such as Escherichia coli described previously as indicator of faecal pollution. In addition, we also identified this bacteria in collections made in 2011.

Materials and Methods:

Water samples were collected in January-May and September-October 2012 in Mexico City and surrounding areas. The collection sites (Xochimilco, Milpa Alta and Dinamos waters; Guadalupe, Los Reyes and Zumpango lakes) were selected because they are major recreational and irrigation water reservoirs in the area. Xochimilco located in Mexico City has the lakes Huetzalin and Acitlalin; the lake of Guadalupe is located in the state of Mexico inside the metropolitan area close to Mexico City; Laguna de Los Reyes Aztecas is located in Tláhuac in Mexico, City and lake of Zumpango is also located close to Mexico City. All this areas are surrounded by trees and vegetation and are considered with semiurban landscape. The water collected were stored at 0 °C after collection and stored at -20 °C until processing. Most of the samples were processed for proteomic analyses as has been described previously the next day of collection.
Proteomics processing was performed to confirm the presence of the target organisms in the water samples collected. Total protein extracts was obtained by the Trizol method as recommended by the supplier. Subsequently, purification, solubilisation and digestion with trypsin were made and finally desalting peptides was done by Zip Tips. These peptides were analyzed by MALDI-TOF equipment to identify proteins from pathogens that are contaminating the collected water from the different sources.

Results and Discussion:

Rivers, lakes, and stream can become contaminated with faecal pathogens that threaten the health of people who recreate in the polluted water. Total coliforms and E.coli are used as indicators to measure the degree of pollution and sanitary quality of well water, because testing for all known pathogens is a complicated and expensive process. The main source of pathogens in water from Recreation Rivers, Lakes, and Stream is through recent contamination from human or animal waste. The developments of new rapid test methods are needed to increase the ability to identify these pathogens.
Pathogens of diarrheas and enteric illness in water collected in 2012 were identified in this study. We found peptides of *Proteus mirabilis*, *Salmonella enterica* and *Shigella flexneri. Proteus mirabilis* has been detected in food and contaminated water. Salmonella do not multiply (out of digestive tracts) significantly in the natural environment, but they can survive several weeks in water and in soil if temperature, humidity, and pH are favorable. Shigellosis infection is transmitted from person to person by the faecal-oral route, contaminated food where the transmission vector are flies, or because the use of contaminated water. *Clostridium tetanic* and *Clostridium cellulolyticum* also detected, although they probably do not growth in surface waters, the high resistance of the spores makes their presence ubiquitous in environmental waters. Vibrios are primarily aquatic bacteria and some species with a low sodium requirement are also found in freshwater habitats. In this study, peptides of *Vibrio vulnificus* and *Vibrio flexneri* were identified. *V. vulnificus* is an important cause of septicemia and wound infections and also may be fatal. Other vibrios, namely *Allivibrio fischeri* (*Vibrio fischeri*) has no relation with humans but infect bivalves.
Peptides of other important pathogens such as *Streptococcus pneumoniae*, *Brucella suis* (Brucellosis) and *Francisella tularensis* (tularemia) and some viruses like *Bat coronavirus*, *Human herpesvirus 2*, *(strain HG52)*, *Pigeon circovirus* and African horse sickness virus *4* (pulmonary infection) were also identified. Results are shown in Table 1-4. These results are very valuable no

only taking in account the human health but also because economically is very important the health of some animals like horses, bovine among others. Consequently, the next step in this work will be to design and fabricate the microarray base on these results.

Table 1: Proteins of pathogen organisms found in water collected in January-May, 2012.

Protein names	Organism	Location of sample
Holliday junction ATP- DNA helicase	*Clostridium tetani*	Zumpango lake
DNA-directed RNA polymerase subunit beta'	*Pseudomonas putida (strain W619)*	Dinamos
Deoxyguanosinetriphosphate triphosphohydrolasa	*Vibrio vulnificus (strain YJ016)*	Dinamos
Inheritance of peroxisomes protein 2	*Candida glabrata (strain ATCC 2001)*	Xochimilco
ATP-dependent Clp protease ATP-binding	*Proteus mirabilis (strain HI4320)*	Xochimilco
Non-structural protein 3	*Bat coronavirus 512/2005 (BtCoV)*	Guadalupe lake
Transcriptional regulatory protein M	*Mycoplasma synoviae (strain 53)*	Guadalupe lake
UvrABC system protein C	*Fusobacterium nucleatum*	Guadalupe lake
DNA polymerase catalytic subunit	*Human herpesvirus 2 (strain HG52)*	Guadalupe lake
Capsid protein	*Pigeon circovirus (PiCV)*	Guadalupe lake
Serine/threonine-protein kinase ndrD	*Dictyostelium discoideum*	Guadalupe lake

Table 2: Proteins of contaminants found in water collected in January-May, 2012.

Protein names	Organism	Location of sample
FAS1 domain-prot KLLA0E16841g	*Kluyveromyces lactis (milk-derived products)*	Zumpango lake
NEDD8-conjugating enzyme ubc12	*Schizosaccharomyces pombe (strain 972)*	Dinamos
50S ribosomal protein L4	*Rhodopseudomonas palustris (swine waste lagoons)*	Dinamos
Small nuclear ribonucleoprotein	*Caenorhabditis elegans (nematode feeds on bacterias)*	Dinamos
Vomeronasal type-1 receptor A16	*Rattus norvegicus (Rat pheromone receptor)*	Xochimilco
Cobalt-precorrin-6A synthase	*Clostridium cellulolyticum (strain ATCC 35319*	Xochimilco
40S ribosomal protein S1-B	*Saccharomyces cerevisiae (strain ATCC 204508*	Guadalupe lake
ATP synthase subunit delta	*Prosthecochloris aestuarii (strain DSM 271)*	Guadalupe lake
Coatomer subunit alpha-2	*Arabidopsis thaliana (Mouse-ear cress)*	Guadalupe lake

Table 3: Proteins of pathogen organisms found in collected in September-October, 2012.

Protein name	Organism	Collection site
Putative coenzyme A transferase	*Shigella flexneri serotype 5b (strain 8401)*	Reyes lake
Phase 2 flagellin	*Salmonella enterica subsp. enterica*	Reyes lake
Autophagy-related protein 13	*Phaeosphaeria nodorum* (pathogen of wheat)	Reyes lake
Elongation factor Ts	*Streptococcus pneumoniae* (opportun. pathogen)	Reyes lake
Dihydroorotate dehydrogenase	*Stenotrophomonas maltophilia* (opportun. pathog)	Reyes lake
Core protein Vp4	*African horse sickness virus 4* (pulmonary infec)	Reyes lake
Carbamoyl-phosphate synthase	*Brucella suis biovar 1* (brucellosis)	Reyes lake
Isoleucine-tRNA ligase	*Burkholderia vietnamiensis* (opportun. pathogen)	Milpa Alta
Acetate non-utilizing protein 9	*Ustilago maydis* (Parasitic on maize)	Milpa Alta
Sulfite reductase [NADPH]	*Vibrio fischeri (symbiont of squids)*	Reyes lake

Table 4: Proteins of contaminants found in water collected in September-October, 2012.

Protein names	Organism	Location of sample
Serpentine receptor class delta-28	*Caenorhabditis elegans*	Reyes lake
Hermansky-Pudlak syndrome 5	*Drosophila melanogaster* (Fruit fly)	Reyes lake
Unconventional myosin heavy chain 6	*Caenorhabditis briggsae* (nematode)	Reyes lake
Vacuolar protein sorting-associated	*Gallus gallus* (Chicken)	Reyes lake
Mitochondrial carrier C29A3.1	*Schizosaccharomyces pombe* (yeast)	Milpa Alta
Selenate reductase subunit alpha	*Thauera selenatis* (polluted freshwater)	Milpa Alta
Immunoglobulin-binding protein 1	*Rattus norvegicus* (Rat)	Milpa Alta
Formyl peptide receptor	*Mus musculus* (Mouse)	Milpa Alta

Conclusions: Globally surface, ground and potable water are receiving pollutants mainly from faecal origin. The monitoring and identification of a pathogen from water samples remain difficult due to presence of large numbers of harmless background micro-flora and several contaminants rather than target microorganisms. We are developing a method to determinate pathogens and contaminants in recreational and drinking waters by proteomics analyses. Our results reinforce the need for studies of *Salmonella sp*. and *Shigella sp*. and members of Enterobacteriaceae family in order to minimize the risk of infection of the susceptible population.

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Inhibition of *Helicobacter pylori* growth by an Asteraceae family plant methanol extract

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Abstract: *Helicobacter pylori* is a spiral Gram-negative bacterium associated with inflammation of the gastric mucosa, peptic ulcer, and gastric adenocarcinoma; it is one of the leading causes of cancer death worldwide. Treatment prescribed to patients with gastric ulcer has failed in many cases mainly due to antibiotic resistance and important side effects such as taste disturbances, vaginal candidiasis, and pseudomembranous colitis. Interest in botanical medicine is increasing as a viable alternative to the traditional one. Plant leaves of the Asteraceae family are used to treat diarrhea, ulcers and rheumatism. The aim of this study was to evaluate the activity of aqueous and methanolic extracts from plants of the Asteraceae family against *H. pylori* growth *in vitro*, using the colorimetric tetrazolium bromide (MTT) reduction assay. We observed that methanolic extracts from plants of the Asteraceae family showed up to 82% *H. pylori* growth inhibition (MIC at 500 µg mL^{-1}).The results of the present study contribute to the body of knowledge of medicinal plants with antimicrobial potential, particularly against *H. pylori*.

Keywords: *Helicobacter pylori* ● Asteraceae family plant ● Methanolic extracts● Aqueous extracts

Introduction: *H. pylori* infects and colonizes the human stomach in 50% of the world's population (Hongying *et al*., 2014). Chronic infection in the human stomach is characterized by chronic inflammation. The development of gastric adenocarcinoma, particularly of the intestinal type, is preceded by the development of chronic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia. In developing countries, 70 to 90% of the population becomes infected before 10 years of age and more than 80% of adults and 50% of children are colonized by *H. pylori* compared with 30% of adults and 10% of children in developed countries (Dunn *et al*., 1997, Dye *et al*., 2002). In México, seroprevalence of *H. pylori* is higher than 50% (Alvarado-Esquivel *et al*., 2013). The first-line treatment option for *H. pylori* consists of a 7 to 10 days regimen with proton-pump inhibitor (PPI), plus amoxicillin, and clarithromycin (Alahdab *et al*., 2014).The use of clarithromycin increases resistance to *H. pylori* (Wu *et al*., 2014). Treatment prescribed to patients with gastric ulcer fail in many cases mainly due to antibiotic resistance, in addition to induced side effects. Interest in botanical medicine has increased in recent years, physicians and people seem to show preference for products that contain "natural extracts" instead of products based on "synthetic" substances (Borchers *et al*., 2000, Torrado-Truiti *et al*., 2003) The first report of plants against *H. pylori* was made in 1991 (Cassel-Beraud *et al.,*1991). There are reports of methanol and aqueous extracts of Mexican plants that have inhibitory effect against *H. pylori* (Castillo-Juárez *et al*., 2009).The plant compounds including polyphenols, flavonoids, quinones, coumarins, terpenoids and alkaloids. The anti-*H. pylori* action mechanism, including inhibition of enzymatic(urease, DNA

gyrase, dihydrofolate reductase, N-acetyltransferase, and myeloperoxidase) and anti-adhesion activities, high redox potential and hydrophilic/hydrophobic natures of compounds(Wang, 2014). The aim of this study was to evaluate aqueous and methanol plants extracts using the MTT tetrazolium reduction assay as a viability test to assess direct effects of these plant extracts against *H. pylori* growth *in vitro*.

Materials and Methods:

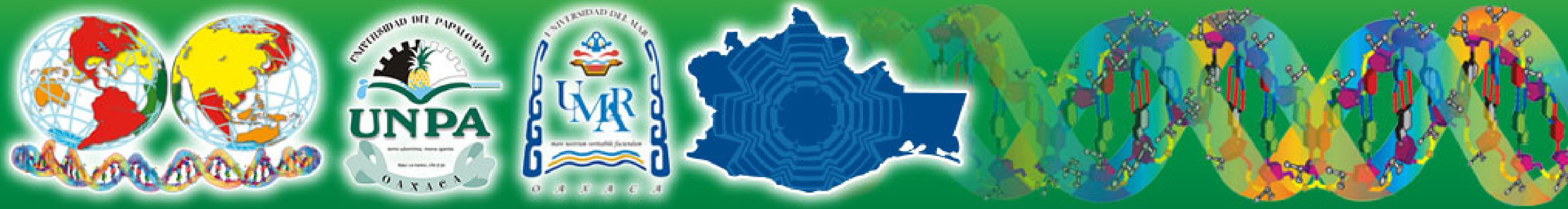
Five grams of each sample were used to prepare the extracts. For aqueous extracts, the plants were boiled for 10 min with 80 mL of purified water filtered and then lyophilized in freeze dry system (LABCONCO Corp., Kansas City, MI) for about four hours. Once the sample was obtained the amount of extracts was calculated. In the case of methanol extracts the material was extracted for 72 hours in 80 mL of methanol. After filtration the extracts were evaporated in a Speed Vac (Milford, MA). The plants screened in the present study were *Persea americana* Mill (Lauraceae), *Pachycereus marginatus* (DC.) Britton & Rose, a plant of the Asteraceae family (its use is in the process of patenting), *Ibervillea sonorae Green,* and *Phoenix dactylifera Linn*.

Bacterial strain and culture conditions. *H. pylori* standard strain ATCC 43504 was grown on *Brucella* broth for a day at 37 °C. The strain was identified by Gram staining morphology and biochemical positive tests for catalase and urease.

Minimum inhibitory concentration (MIC) determinations. The aqueous and methanol extracts were tested by MTT tetrazolium reduction assay in 96-flat well microplates. The extracts were dissolved in *Brucella* broth to obtain a final concentration 7.8, 15.6, 31.2, 62.5,125, 250, and 500 µg mL^{-1}. A volume of 50 µL of *H. pylori* (2.5×10^5 bacteria mL^{-1}) was placed in the plate wells and incubated. The MIC was determined using the MTT reduction assay, being the lowest concentration of the extracts in the plate with no bacterial growth. Before extracts were incubated, we added 15 µL of MTT and incubated for 15 min, then 80 µL of DMSO were added to dissolve the formazan crystals. The absorbances were measured in a microplate reader at 570 nm (DTX 800/880 Multimode Detectors, Fullerton, CA). All the experiments were performed in triplicate and repeated at least three times. Tetracyclin was used as a positive control.

Results and Discussion:

Methanolic and aqueous extracts of 5 different plants were tested *in vitro* for their anti *H. pylori* activity. No aqueous extract of the 5 plants showed *in vitro* effect against H.*pylori* ATCC 43504. Only methanolic extracts from plants of the Asteraceae family showed inhibitory effect against H. *pylori.* Such results are shown in Figure 1. The vehicle control did not affect *H. pylori* viability.



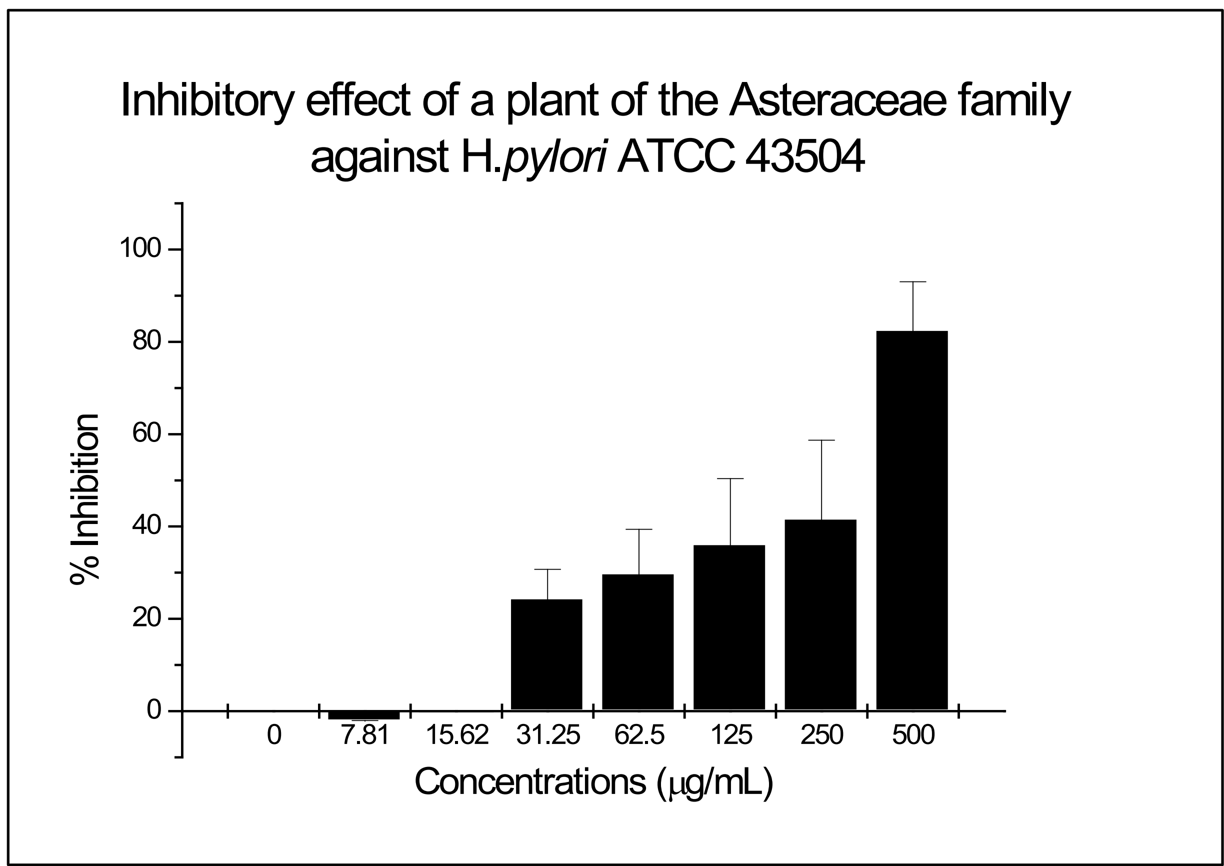


Figure 1. Inhibitory effect of methanol plant leaves extract of a plant of the Asteraceae family.

As seen in Figure 1, the Asteraceae plant methanolic extracts showed up to 82.3% growth inhibition of *H. pylori* ATCC 43504 at a concentration of 500 µg mL^{-1} ($p<0.05$), and the growth inhibition activity was in a concentration-dependent manner.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, which are synthesized and deposited in specific parts or in all parts of the plant (Parekh *et al.,* 2005).

Although our aqueous extracts of *Persea americana* Mill (Lauraceae), *Pachycereus marginatus* (DC.) Britton and Rose*,* a plant of the Asteraceae family, *Ibervillea sonorae Green,* and *Phoenix dactylifera Linn* did not affect *H. pylori* viability, authors such as Castillo *et al*. (2013), reported anti-*Helicobacter pylori* activity from aqueous extracts of *Persea americana* Mill with a minimum inhibitory concentration (MIC) > 1000 µg mL^{-1} tested with the agar dilution method.

Methanolic extracts of plants used in this work have previously been tested against *H.pylori.* The MIC of *Ibervillea sonorae Green* against *H.pylori* is reported of 200 to 400 µg mL^{-1} (Robles-Zepeda *et al*., 2011). Also, methanol extract of *Persea americana* Mill has been reported as active with a MIC <7.5 µg mL^{-1} tested with the broth dilution method (Castillo *et al*., 2013).

Asteraceae plants have been reported to contain essential oils, flavonoids (Wollenweber *et al*., 1981), among other substances, and possess antimicrobial and antitumor activities.

Our Asteraceae plant methanolic extracts showed a growth inhibition of *H. pylori* ATCC 43504 at a concentration of 500 µg mL^{-1}, and the activity was in a concentration-dependent manner. However the results obtained in this research can not be compared with others due to the bioassay employed. The active concentrations used in the present study can be considered high, nevertheless there are active components which can inhibit the growth of the bacteria.

The MTT reduction assay was standardized in order to test the activity anti-*Helicobacter pylori* of plants. It is possible that during the extraction method or when the plants were boiled some trace amounts of compounds evaporated.

Conclusions: The methanolic extracts from a plant of the Asteraceae family showed up to 82.3% *H. pylori* growth inhibition at a concentration of 500 µg mL^{-1}, which warrants further studies on diverse *in vitro* and *in vivo* parameters.

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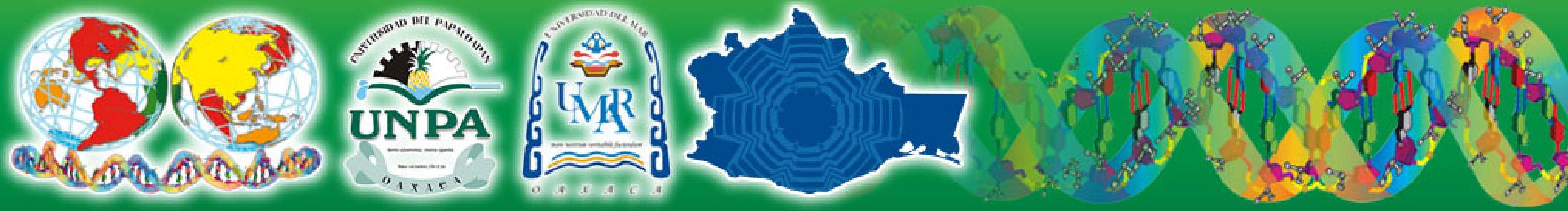

Downregulation of human $Na_V1.6$ channel by an inducible shRNA

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Abstract: Functional expression of voltage-gated sodium channels (VGSCs) in cancer cells promote invasion and other cellular properties related to the metastatic behavior. We have previously reported that $Na_V1.6$ is the major VGSC expressed in cervical cancer (CeCa). The purpose of this work was to knock down the $Na_V1.6$ expression in CeCa cells by lentiviral vector-mediated transduction with a doxycycline-inducible shRNA. Two constructs were generated using the same bicistronic backbone that also encodes the green fluorescent protein, as a useful marker to identify the positively transduced cells. Constructs were packaged into lentiviral particles, and then tested on a stably-transfected HEK-293 cell line expressing the $Na_V1.6$ channel. To test the effectiveness of the shRNAs we carried out whole-cell patch-clamp experiments on transduced HEK-293 and CeCa cells. Our preliminary results show a reduction in the sodium current amplitude in transduced cells compared to controls. This observation suggests a decrease in the amount of functional channels in the cell membrane of transduced cells. Therefore, this shRNA-lentiviral system could be used to investigate the role of $Na_V1.6$ channels in the biology of CeCa and other cancer cells.

Keywords: Voltage-gated sodium channel ● shRNA ● knock down ● cervical cancer

Introduction: Voltage-gated sodium channels (VGSCs) are transmembrane-protein complexes containing a pore-forming α-subunit (~230 kDa), formed by a single polypeptide chain; and two smaller regulatory β-subunits (~40 kDa). Hitherto, there have been cloned nine different VGSC α-subunits ($Na_V1.1$–$Na_V1.9$). All of them have been functionally expressed and characterized (Catterall *et al*., 2005). These channels are classically expressed in excitable cells, such as neurons, muscle and endocrine cells, where they are responsible for the generation and propagation of action potentials, neurite outgrowth, pathfinding, and migration. Additionally, VGSCs are functionally expressed in metastatic cancer cells where they promote invasion and other cellular properties related to the metastatic cascade (Brackenbury, 2012; Fraser *et al.*, 2005). The expression of VGSC in cancer cells seems to be primary-tumor dependent. We recently reported that $Na_V1.6$ is the major VGSC expressed in CeCa (Hernandez-Plata *et al.*, 2012). The mRNA levels of this channel are ~40 times higher in CeCa cells than in noncancerous cervical tissue. In addition, pharmacological experiments with the whole-cell patch-clamp technique demonstrated the functional expression of the channel in CeCa plasma membrane. More interestingly, the specific block of $Na_V1.6$ channel reduced the invasive capacity of CeCa cells in *in vitro* assays. A useful pharmacological tool to discriminate among VGSCs is based on their sensitivity to tetrodotoxin (TTX); $Na_V1.1$–$Na_V1.4$, $Na_V1.6$ and $Na_V1.7$ are blocked by nanomolar concentrations of TTX, whereas $Na_V1.5$, $Na_V1.8$ and $Na_V1.9$ require micromolar

concentrations of toxin for being blocked (Catterall *et al*., 2005). Although there are several pharmacological agents that can block the activity of these channels, none of them has exhibited a higher selectivity in blocking the activity of $Na_V1.6$ channel than the displayed by the TTX.
In this work we propose the use of a shRNA as a tool to knock down the functional expression of $Na_V1.6$, mediated by the respective mRNA silencing, taking advantage on the high specificity that can be accomplished with this method, in order to block the metastatic cellular processes promoted by this channel. The final goal is to deepen the knowledge on the role of $Na_V1.6$ channels in the biology of cancer cells, and to lay down the basis of an alternative gene therapy for CeCa by targeting this channel.

Materials and Methods:

Cloning of constructs coding for shRNAs. Molecular cloning techniques were used to insert double stranded oligonucleotides coding for shRNAs (Table 1) into the vector pLVUTHshGATA1-tTR-KRAB (Szulc *et al*., 2006), thereby generating Krab16H and Krab16S constructs (Fig. 1).

Table 1. Oligonucleotides coding for shRNAs.

shRNA	Oligonucleotide Sequence
16H	F: 5'- gcgacttctgaaatccgatttgaaactgtgaagccacagatgggtttcaaatcggatttcagaagctgc -3' R: 3'- cgctgaagactttaggctaaactttgacacttcggtgtctacccaaagtttagcctaaagtcttcgacg -5'
16S	F: 5' gcgaaggtaccgttaatcacactgtctgtgaagccacagatgggacagtgtgattaacggtacctctgc -3' R: 3'- cgcttccatggcaattagtgtgacagacacttcggtgtctaccctgtcacactaattgccatggagacg -5'

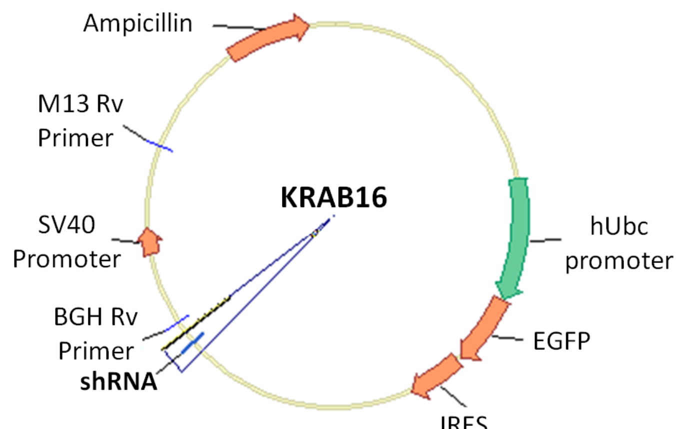
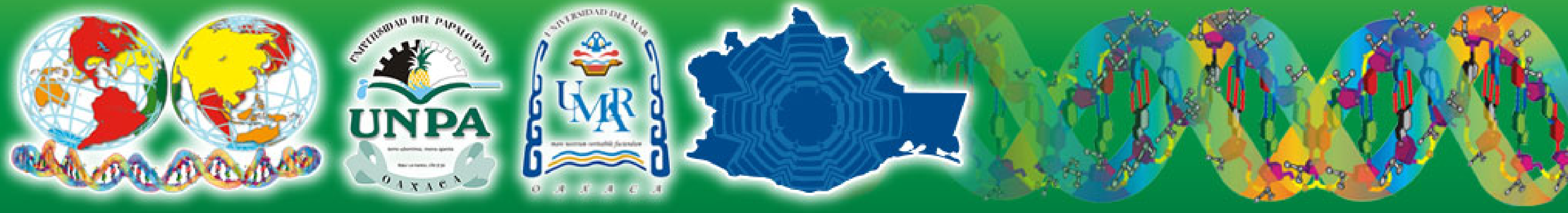


Figure 1. Map of the plasmids engineered for this study.

The illustrated structure was the same for Krab16H and Krab16S constructs. The identity and correct incorporation of shRNAs into the vector were corroborated by automatic sequencing. Validation of the shRNAs effectiveness was made by RT-qPCR assays measuring the mRNA levels of the $Na_V1.6$ channel in transiently transfected 293FT cells. Transfections were carried out by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), with each shRNA construct and a plasmid DNA coding for the corresponding target. Primers used for these purposes were as follow: F: 5'-



gctgctggtgtgtctcatct-3’, R: 5’-aaagcagtagtggtactttcccgc-3’, directed to Na_V1.6 channel (GenBank: AF225988).

Cell culture. Culture media employed for growing and maintaining cells were as follow: 293FT cells (human embryonic kidney; Life Technologies): Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 0.5 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}). HeLa cells: DMEM, 10% FBS and antibiotics. HEK-Na_V1.6 (HEK-293 cells stably expressing the human Na_V1.6 channel): DMEM-F12, 10% FBS and antibiotics. CeCa primary cultures: DMEM, 10% FBS, 1 mM sodium pyruvate, 25 mM HEPES and antibiotics. All cultures were kept into CO_2 incubators at 37 °C.

Lentiviral expression. Lentiviral particles packaging the KRAB16H or KRAB16S constructs were generated using the ViraPower™ lentiviral expression kit and 293FT cells. Lentivirus containing the shRNAs constructs were obtained and then titered in HeLa cells as described previously (Gonzalez-Buendia *et al*., 2014). The expression of constructs was induced by adding doxycycline (20 ng/ml) to the culture media, and starting 24 h after GFP (green fluorescence protein) was observed under the microscope. Then, in the same way HEK-Na_V1.6 cells were transduced for testing the effectiveness of the shRNAs on the Na_V1.6 channel sodium current with the use of the patch clamp technique. CeCa primary culture cells were also transduced to determine the contribution of Na_V1.6 to the total voltage-gated sodium current expressed in these cells.

Electrophysiology. General methods for whole-cell patch-clamp recordings of single CeCa cells were as specified by Díaz and coworkers (Diaz *et al.,* 2007) and are only summarized here. The peak voltage sodium current was obtained at -10 mV from a holding potential of -100 mV. Recordings were obtained 24, 48 and 72 h after doxycycline induction at 21–23 °C using the following solutions (in mM): external: 158 NaCl, 2 $CaCl_2$, 2 $MgCl_2$ and 10 HEPES-NaOH (pH 7.4); internal: 124 CsF, 30 NaCl, 1 CaCl2, 2 $MgCl_2$, 10 EGTA and 10 HEPES (pH 7.3). Data analysis and plotting was performed with Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism software (v.5, La Jolla, CA, USA).

Results and Discussion:

Whole-cell voltage-evocated sodium currents were recorded from transduced HEK-293-Na_V1.6 cells, after 24, 48 and 72 h of induction with doxycycline. The peak amplitude of each cell was averaged among each group of cells (i.e., KRAB-16S and KRAB-16H transduced cells). The maximum downregulation of the Na_V1.6 channels activity was observed after 72 h of induction, with a decrease of about 60% compared to the 16S-control cells. The respective current density values were -180 ± 71 pA/pF for KRAB-16S transduced cells, and -71 pA/pF for KRAB-16H transduced cells (Fig. 2). These results showed the utility and specificity of the lentivirus containing the shRNA-16H as an experimental strategy to knock down the functional expression of the Na_V1.6 channel. Then, following the same experimental procedures, we transduced CaCu354 cells, derived from a CeCa biopsy where the presence of functional Na_V1.6 channels has been previously demonstrated (Hernandez-Plata *et al.*, 2012).
Positively transduced cells represented ~70% of the population of CaCu354 cells, as estimated by observing the cells that exhibited the fluorescence of GFP. In all the cases, only green fluorescence cells were investigated.

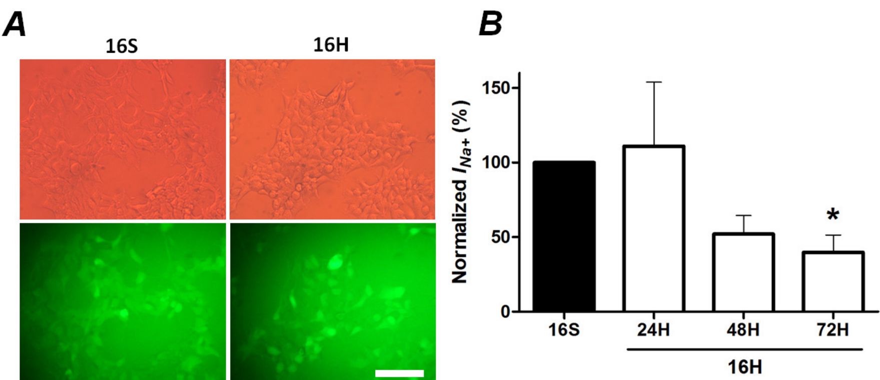


Figure 2. Knock down of $Na_V1.6$ channels mediated by a shRNA contained in lentiviral particles.

HEK-$Na_V1.6$ cells where transduced and treated with doxycycline to induce the KRAB16S (*A*, left) and KRAB16H (*B*, right) shRNAs expression. (***A***) Phase contrast (upper panels) and fluorescence (lower panels) photomicrographs. Green cells were recorded with the whole-cell patch-clamp technique after different times of exposure to doxycycline. (***B***) Columns are the averaged peak amplitude of sodium currents for each group. KRAB16H (n = 7) transduced cells are expressed as relative to the respective control, KRAB16S (n = 4) cells. Error bars indicate the standard error. *Statistically different after One-way ANOVA followed by a Tukey test ($p<0.05$). In addition, CeCa cells transduced with KRAB-16H where the shRNA expression was induced with doxycycline showed a 35% reduction of the peak sodium current amplitude in the current-voltage (*I-V)* curve (Fig. 3C and D), after an average of 36 h of induction. Density current values were -23 ± 3 pA/pF (n = 6) and -16 ± 2 pA/pF (n = 8) in control and doxycycline induced cells, respectively (Fig. 3D).

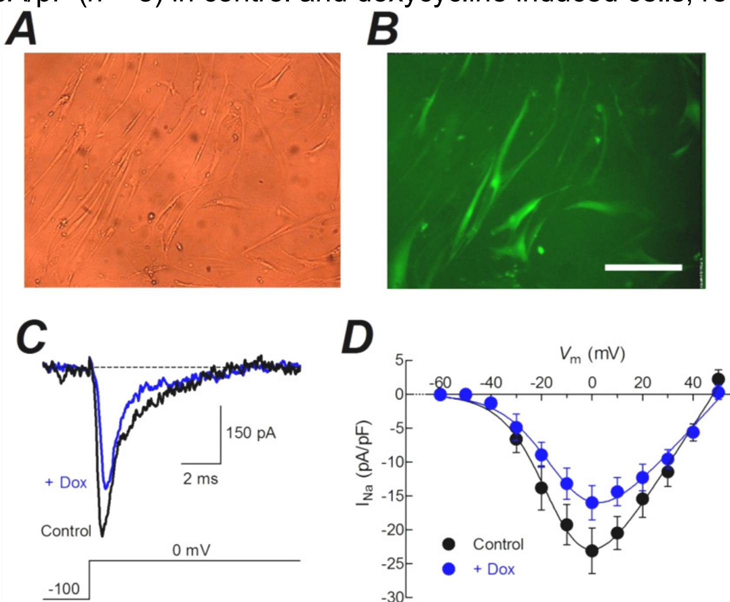
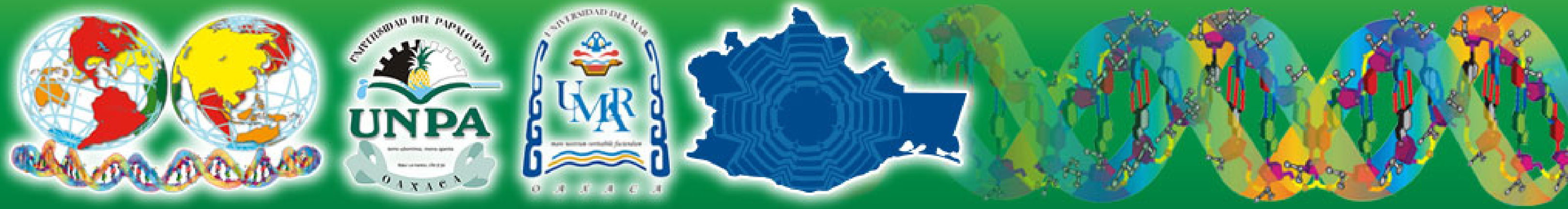


Figure 3. Reduction of sodium current transported by $Na_V1.6$ channels in CeCa cells by a shRNA.



Contrast phases (***A***), and fluorescence (***B***) photographs of 354 cells transduced with KRAB16H shRNA and induced with doxycycline. Scale bar = 100 µm. Electrophysiological recordings were made in induced (blue) and non induced (black) cells (***C***) to generate the *I-V* curves shown in (***D***). Smooth curved lines represent fits to the experimental data. These studies show that the KRAB16H shRNA induced a ~35% decrease of the maximum voltage-activated sodium current in CeCa cells. This level of knockdown agrees with our previous observations on blocking the $Na_V1.6$ channels using the Cn2 toxin. The doxycycline inducible expression of the shRNA allows to regulate specifically the expression of the channel and thereby to dissect its role in cancer cells.

Conclusion: KRAB16H shRNA represents a renewable and highly specific tool to perform studies to determine the role of the $Na_V1.6$ channel in the cancer biology, and establishes the basis for the treatment of CeCa with a gene therapy approach.

Acknowledgments: We thank to GlaxoSmithKline Laboratories (Hertfordshire, U.K.), for providing the HEK-293 cell line stably expressing the human $Na_V1.6$ channel, and Didier Trono for the lentiviral targeting plasmid (Addgene plasmid: 11650).

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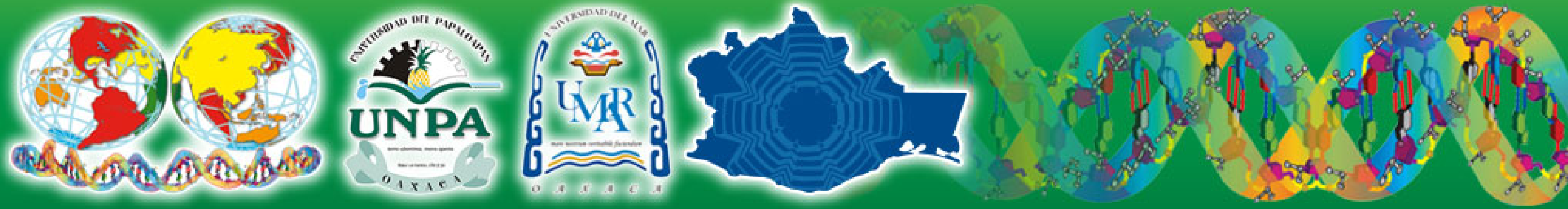

Role of SERCA on calcium waves propagation in rat ventricular myocytes

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Abstract: In Ca^{2+}-overloaded ventricular myocytes, spontaneous and self-regenerating, sarcoplasmic reticulum (SR) Ca^{2+} release waves propagate throughout the cell at uniform speed (ʋWave ~50-100 μm s^{-1}). According to the fire-diffuse-fire mechanism, released Ca^{2+} from active RyR clusters diffuses and activates adjacent sites by Ca^{2+}-induced Ca^{2+} release (CICR), allowing wave propagation. Nevertheless, it was suggested that rapid SERCA Ca^{2+} reuptake ahead of the wave front might further sensitize RyR. However, testing this hypothesis, based in results within limited SERCA activity range, is complicated because of changes in steady-state SR Ca^{2+}. We systematically altered SERCA steady-state activity and tested its role in Ca^{2+} waves, in normal (Ctrl) and hyperthyroid rat myocytes treated with low thapsigargin dose. Wave decay rate (ϰDecay; SERCA activity index) changed 24% in Ctrl. ʋWave (and wave time to peak; TPeak) vs. ϰDecay relations were described by saturating exponential functions, becoming asymptotic despite enhanced ϰDecay, an outcome incompatible with enhancement by rapid SERCA Ca^{2+} reuptake. ʋWave vs. ΔPeak (and TPeak) had steep dependency, suggesting that steady-state SR Ca^{2+} determines ʋWave.

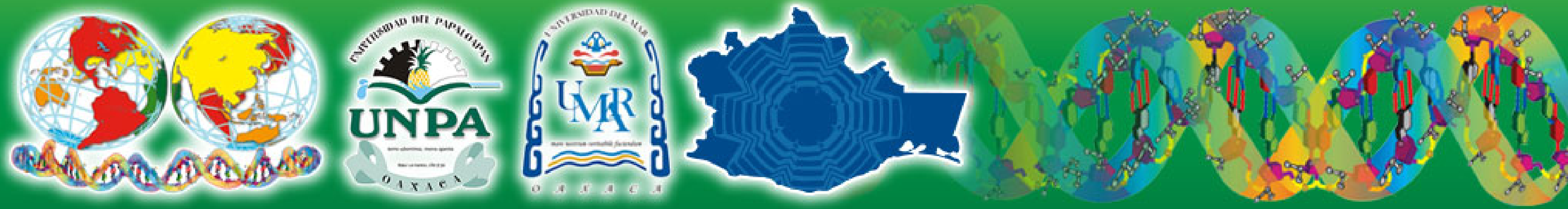
Keywords: calcium waves ● Serca ● Ryanodine receptors ● ventricular myocytes

Introduction: Thyroid hormones regulate long-term cardiac function (e.g. contractility, relaxation and rhythm) by controlling gene expression affecting contractile and Ca^{2+}-handling proteins. Hyperthyroidism (HT) enhances sarcoplasmic-reticulum (SR) Ca^{2+}-ATPase (SERCA) and Ryanodine-Receptors (RyR) expression, while decreasing phospholamban (PLB) and Na^{+}/Ca^{2+} exchanger. Increased SERCA/PLB-ratio (S/P-R) underlies enhanced cytosolic Ca^{2+} removal and SR-Ca^{2+}, explaining increased contractility and relaxation. However, high S/P-R and RyR should also affect diastolic Ca^{2+}-signaling. We tested whether increased S/P-R in HT-myocytes enhances Ca^{2+}-wave propagation (CWP).

Material and Methods:

Reagents. All chemical reagents were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), unless otherwise stated.

Murine model of hyperthyroidism. All studies were approved by the Internal Committee for Care and Handling of Laboratory Animals of the School of Medicine of the Tecnológico de Monterrey (Protocol 2012-Re-017), and were carried out according to the National Institutes of Health guidelines. Male Wistar rats of 250-350 g were provided (intraperitoneal; I.P.) with a daily dose of 3,3′,5-Triiodo- L -thyronine (30 μg/100 g body weight) dissolved in a sterile NaOH 0.1 N solution



(Kiss *et al*., 1994). Age-paired rats, injected with NaOH 0.1 N solution served as controls (Ctrl). Animals were fed standard rat chow and water ad libitum for two weeks.

Ventricular myocyte isolation. Myocytes were dissociated according to a method previously described (Hryshko *et al*., 1989). Briefly, rats were anaesthetized with sodium pentobarbital (80 mg kg^{-1} body weight, I.P.). Following thoracotomy hearts were quickly excised, mounted on a Langendorff apparatus and perfused with collagenase type II-containing solution (1 mg mL^{-1}; Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37 °C. Thereafter, ventricles were removed and mechanically disaggregated. Cells were kept in normal Tyrode (NT) solution at room temperature and used within 6 hours. NT solution contained (in mM): NaCl 140.0, KCl 4.0, MgCl2 1.0, HEPES 10.0, glucose 10.0, and CaCl2 1.0, pH. 7.4 adjusted with NaOH.

Confocal Ca^{2+} imaging. Ventricular myocytes were incubated for 30 min in NT solution containing Fluo-4 AM (10 µM; Life Technologies, Carlsbad, CA, USA). Confocal imaging was performed with a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with a HCX PL APO 40, 1.3 NA, oil objective. Fluo 4 was excited at 488 nm with an Argon laser and emission collected at 515-600 nm. For Ca^{2+} transients, myocytes were field-stimulated (MYP100 MyoPacer Field Stimulator, IonOptix LLC, Milton, MA, USA) with a train of 5 pulses at 0.5-1 Hz to attain steady state SR Ca^{2+} content, and the last paced event was recorded along with the subsequent 2-3 s for spontaneous Ca^{2+} sparks. To enhance Ca^{2+} wave development external Ca^{2+} was increased to 3-4 mM and line scan images were recorded during 10-15 s in the absence of electrical stimulation. SERCA activity was manipulated in one way: to slow SERCA Ca^{2+} removal rate, it was partially blocked by 5 min exposure with the irreversible inhibitor TG (100 nM (Landgraf *et al*., 2004)), thereafter the cells were washed with TG-free NT solution, and imaging was performed. Fluorescence data were normalized as F/F0, where F is fluorescence intensity and F0 is average fluorescence at rest. The SR Ca^{2+} steady state content was estimated as ΔF/F0 of the cytosolic Ca^{2+} signal in response to rapid caffeine (10 mM) application (Bassani *et al*., 1994). υwave (µm s^{-1}) was estimated in the confocal images from the slope of a linear regression to the wave front. Briefly, three regions of interest (ROI) with a width of 1 µm in equidistant positions within the recording were selected (see Figure 1A). Spatially averaged fluoresence from each ROI was plotted as a function of time to get TPeak (s) and ΔPeak (ΔF/F0). κDecay (1 s^{-1}) was assessed by fitting a single exponential to the decaying phase of the wave (Figure 1B) from each ROI trace. Values for each parameter from the three ROIs were averaged to have a representative description for each wave.

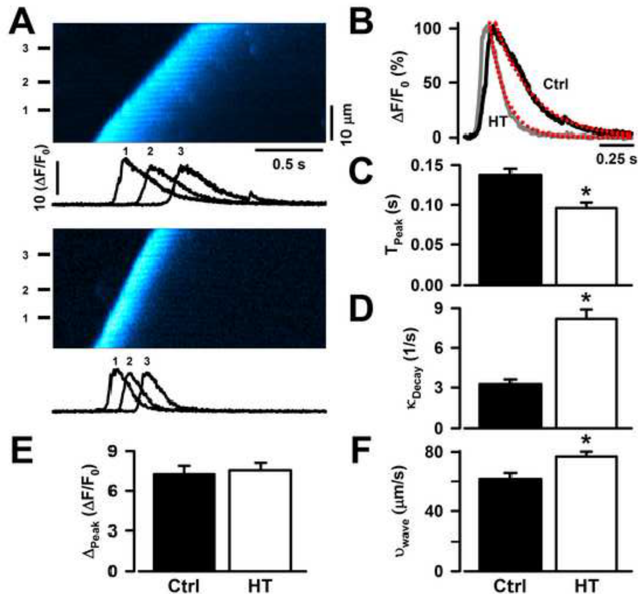


Figure 1. Calcium waves propagated and decayed faster in HT myocytes.

Statistics. Data were reported as mean ± standard error of the mean (SEM). *n* represents the number of cells studied. Unpaired Student's t-test and one-way ANOVA with Bonferroni adjustment were performed when appropriate to compare experimental groups. P <0.05 was considered significant. Data processing and statistical tests were carried out with Microsoft Excel (2007, Redmond, WA, USA) and GraphPad Prism (V. 2.0, La Jolla, CA, USA). Graphs were generated using Systat Sigma Plot (V. 12, San Jose, CA, USA).

Results and Discussion:

Ca^{2+} transient properties and steady state SR Ca^{2+} content in ventricular myocytes from hyperthyroid rats. We assessed Ca^{2+} dynamics in Ctrl and HT cells in basal conditions. For this purpose, we characterized field stimulated Ca^{2+} transients as well as spontaneous Ca^{2+} sparks in Fluo 4-loaded ventricular myocytes. Figure 2A shows representative line scan confocal images of systolic Ca^{2+} signals in Ctrl (Left) and HT (Right) ventricular myocytes, and the traces underneath represent the time course of the average fluorescence for each cell. As expected, for the increased SERCA/PLB ratio, the Ca^{2+} transients in HT myocytes were of higher amplitude and faster decay. Figure 2B shows Ca^{2+} transients for both cell types with normalized peak amplitude to emphasize the large increase in cytosolic Ca^{2+} removal rate in HT cells. Figure 2C and D show pooled data for the analysis of the Ca^{2+} transient peak amplitude (ΔF/F0) and time to 50% decay (t50%), for both, Ctrl and HT myocytes. Figure 2C shows that amplitude of the Ca^{2+} transient increased by ~20% (from 7.85 ± 0.37 to 9.37 ± 0.44, for Ctrl and HT, respectively; P<0.05). Figure 2D shows that t50% decrease by ~60% (0.39 ± 0.03 to 0.15 ± 0.01 s, for Ctrl and HT, respectively; P<0.05), suggesting a large increase in the rate of Ca^{2+} removal attributed to changes in SERCA/PLB expression ratio. Since the steady state SR Ca^{2+} content results from the balance between SERCA activity and diastolic SR Ca^{2+} leak (Shannon *et al*.,, 2002), and we found enhanced Ca^{2+} transient peak and rate of decay (Figure 2), we estimated the steady state SR Ca^{2+} content in these cells.

We measured the peak of the cytosolic Ca^{2+} signal in Fluo-4 loaded cells upon caffeine application (10 mM). We found that in HT cells, SR Ca^{2+} content increased from 6.47 ± 0.62 to 8.17 ± 0.39, (n= 13 and 24, for Ctrl and HT, respectively; P<0.05).

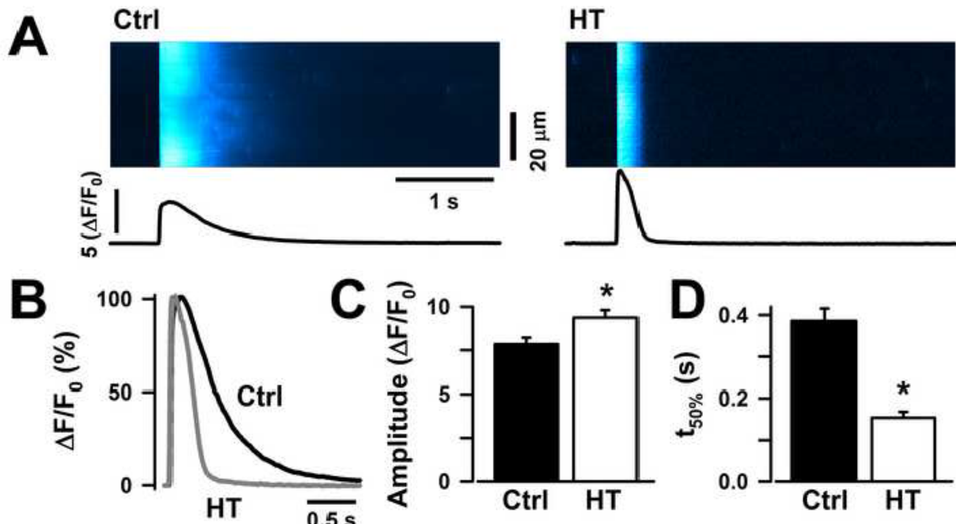


Figure 2. Field-stimulated Ca^{2+} transients in ventricular myocytes from HT rats displayed higher amplitude and faster decay.

Ca^{2+} waves in Ca^{2+} overloaded Ctrl and HT ventricular myocytes. We recorded at least one Ca^{2+} wave in 15 out of 39 and in 24 out of 49 cells studied, from Ctrl and HT hearts, respectively. The wave frequency in those cells was 0.070 ± 0.004 and 0.109 ± 0.016 waves/s (for Ctrl and HT myocytes, respectively; P<0.05). Figure 1A shows typical confocal recordings of Ca^{2+} waves in Ca^{2+}-overloaded Ctrl (upper image) and HT (lower image) isolated ventricular myocytes. The numbered traces underneath each image represent the time course of spatially averaged fluorescence for each narrow ROI marked in the images. The images show that Ca^{2+} waves in HT propagated faster along the cell and were narrower than in Ctrl cells. Figure 1B shows the average of each set of traces (shown in Figure 1A), after alignment, to emphasize the differences in the time course of Ca^{2+} waves in both cell types. The red dashed line on the decaying phase of each trace is a single exponential fit. Figure 1C-F show pooled data for Ca^{2+} waves TPeak, κDecay, ΔPeak and υwave, respectively, in both cell types. TPeak showed a ~22% decrease in HT (0.138 ± 0.008 to 0.108 ± 0.009 s, for Ctrl and HT myocytes, respectively; P<0.05), while κDecay, as predicted from the images and traces in Figure 1A and B, showed a large ~150% increase in HT (3.26 ± 0.35 and 8.18 ± 0.71 s^{-1}, for Ctrl and HT myocytes, respectively; P<0.05). ΔPeak showed no significant difference (7.37 ± 0.63 and 7.52 ± 0.52, for Ctrl and HT myocytes, respectively; P>0.05). Pooled data for υwave showed a significant increase of ~20% in HT (61.60 ± 4.17 and 73.36 ± 3.54 µm s^{-1}, for Ctrl and HT myocytes, respectively; P< 0.05).

Partial SERCA inhibition decreases Ca^{2+} wave propagation. We used a low dose of TG (100 nM for 5 min) in both cell types to partially decrease SERCA activity (Landgraf *et al.*, 2004; Ginsburg *et al.*, 1998). TG exposure largely decreased Ca^{2+} wave appearance; we recorded at

least one Ca^{2+} wave in 7 out of 25 and in 13 out 27 cells studied, from Ctrl and HT hearts, and a small decrease in frequency of waves in the cells studied (0.065 ± 0.008 and 0.077 ± 0.010 waves s^{-1}, for Ctrl and HT myocytes, respectively). Figure 3 shows normalized traces of the time course of Ca^{2+} waves in Ctrl (A) and HT (B) myocytes, respectively, in the presence (gray traces) and absence (black traces) of TG. Two clear changes are evident from those figures; first, there is a decrease in cytosolic Ca^{2+} removal rate due to the partial inhibition of SERCA, and second the TPeak increased. Figure 3C shows pooled data analysis of TPeak, demonstrating that TG exposure induced a significant increase in both cell types (0.213 ± 0.032 and 0.151 ± 0.009 s, for Ctrl+TG and HT+TG, respectively). As expected, Figure 3D shows a significant κDecay decrease caused by TG in both cell types (2.46 ± 0.44 and 3.06 ± 0.44 s^{-1}, for Ctrl+TG and HT+TG, respectively). Figure 3E shows that ΔPeak in Ctrl was slightly, but significantly, lower when TG was present (6.19 ± 0.63 ΔF/F0, Ctrl+TG), while HT did not show a significant decrease upon TG exposure (7.15 ± 0.51. HT+TG). Finally, Figure 3F shows a parallel decrease in υwave upon TG exposure in both cell types (51.21 ± 3.23 and 58.20 ± 3.08 μm s^{-1}, for Ctrl+TG and HT+TG, respectively).

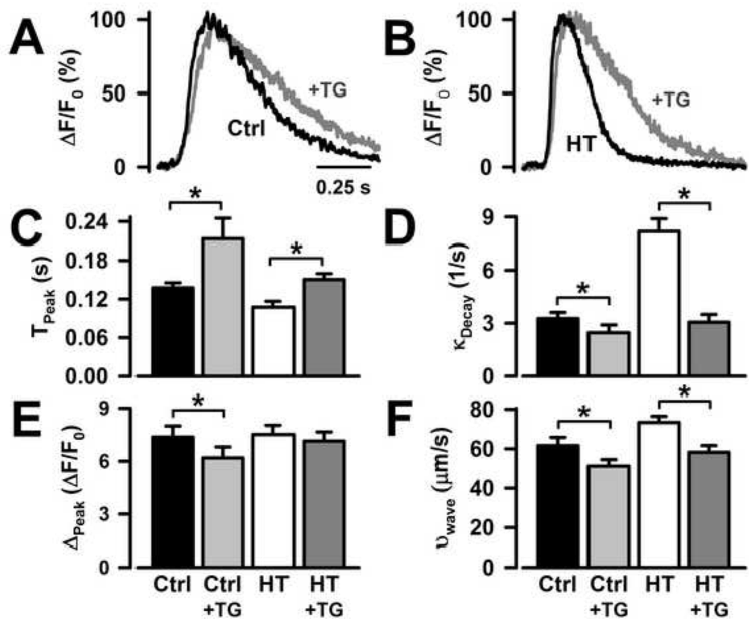


Figure 3. Partial SERCA inhibition with TG slowed Ca^{2+} rate of rise, decay and propagation.

Conclusion: In conclusion, the proposed Ca^{2+} reuptake by SERCA ahead of the wave front is incompatible with the saturation of the relation υwave vs. κDecay. While the steep dependency of υwave vs. ΔPeak and TPeak supports the hypothesis that the main role of SERCA in Ca^{2+} wave propagation is due to steady state effect on SR Ca^{2+} content, and subsequent RyR sensitization, and larger jSR Ca^{2+} available for release.

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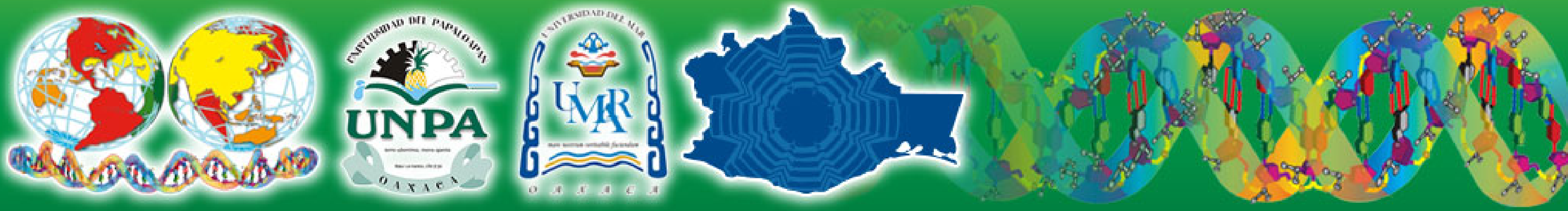

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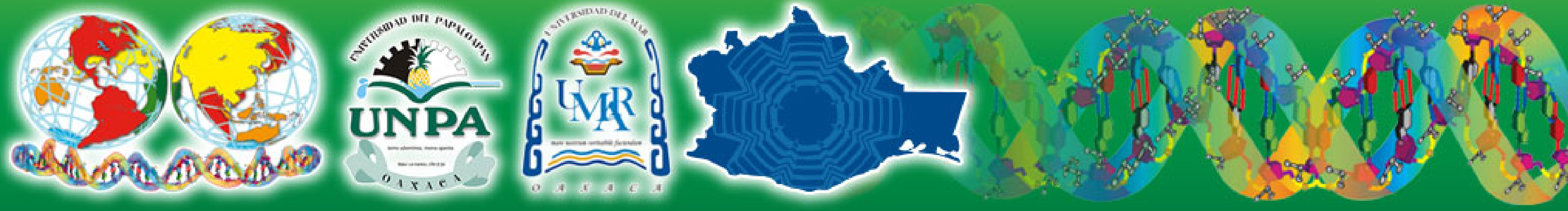
1.2 YELLOW AREA



Evaluation of heat stress tolerance in *Saccharomyces boulardii*

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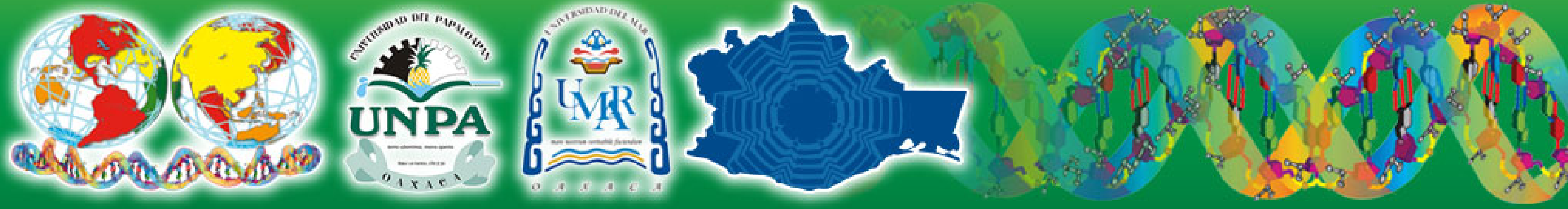
Abstract: Temperature resistance of a commercial strain of *Saccharomyces boulardii* at temperatures of 50, 55 and 60 °C was studied. Decimal reduction time (D_t values) were 27.85, 3.90 and 0.90 min respectively. Halo size of the colonies grown on YPD agar increased with the increase of the temperature in each treatment. Images by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) provided information about the damage to the organelles and the cell wall of *S. boulardii.*

Keywords: *Saccharomyces boulardii* ● microscopy ● heat stress ● probiotic

Introduction: The consumption of probiotic microorganisms is associated with health benefits, which have stimulated interest about this field to conduct research and develop products containing beneficial bacteria especially for children and high risk groups (FAO/WHO, 2002). Due to the above, the use of various food matrices as carriers of probiotic microorganisms, such as fermented products, meat, vegetables and traditional dairy products (Vernocchi *et al*., 2008) has increased. These processes involve drastic changes in pH, solute concentration or temperatures. Probiotic organisms must reach the gastrointestinal tract (GIT) alive and in high numbers (Gueimonde and Salminen, 2006), so that they not only must overcome the physiological barriers along the tract, but also survive the process and storage conditions of the food that serves as the vehicle. In order to exert the above health benefits, there must be at least 1×10^7 CFU mL^{-1} of live microorganisms in the product at the time of consumption (Ding and Shah, 2007).
Microorganisms have developed sophisticated systems and responses such as protein complexes and phosphorylation-dependent signal transduction system, to adapt and survive a variety of environmental changes and outlive under stress conditions (Serrazanetti *et al*., 2009). The growth or survival of the microorganisms under environmental conditions such as the ranges of temperature, pH, nutrient availability depend on the capacity of each organism to sense and respond to these varying conditions (Buck *et al.,* 2009; Serrazanetti *et al*., 2009). The nature of the response is reflected in the structure and composition of the bacteria, as well as in the properties of the final product (Guerzoni *et al*., 2007).
The aim of this study was to evaluate morphological changes and survival of the probiotic strain *Saccharomyces boulardii* under conditions of heat stress.

Materials and Methods:

Microorganism and medium. The probiotic yeast *Saccharomyces boulardii* (Hansen CBS 5926) (Floratil Dosage, Merk) was selected for this study due to its importance in functional food



products. The strain was kept refrigerated at 4 °C on YPD agar slants. It was reactivated by placing a loop of the slant in liquid YPD and replating every 2 months to keep it in a viable state. YPD broth (100 mL) was inoculated with *S. boulardii* and incubated for 24 h at 37 °C to obtain a population of 1×10^{8} CFU mL^{-1}. After yhe 24 h incubation period, the suspension was centrifuged at 10000 × g at 5 °C for 10 min (Model Marathon 21K/R, Fisher Scientific, USA). The supernatant was discarded and the pellet was washed by adding 10 mL of PBS (NaCl 8 g L^{-1}, KCl 0.2 g L^{-1}; $Na_2HPO_4.2H_20$ 1.44 g L^{-1}, KH_2PO_4 0.24 g L^{-1}, pH 7.2). This procedure was done twice (Collins, 1989).

Heat Treatment**.** Tolerance to heat stress was determined using the technique described by Gouesbet *et al*. (2001) with some modifications. The microorganism was suspended in 100 mL of PBS at room temperature and 500 µL aliquots were taken in 1 mL tubes. The tubes were immersed in a water bath with recirculation at temperatures of 50, 55 and 60 °C. The initial time (T0) was defined as the time to reach 1°C below the test temperature. Tubes with the cell suspension were removed from the hot water bath and immersed in ice water at various time intervals (0-30 min) and plated on YPD agar to perform the counts after an incubation time of 36 h at 37 °C.

D_t Value**.** Decimal reduction time (*D_t value)* was calculated by linear regression from the survival curve (log CFU mL^{-1} vs time) (Harrigan, 1998).

Scanning Electronic Microscopy (SEM)**.** To determine the morphological characteristics of *Saccharomyces boulardii*, a sample was placed on a cooper conductive tape and double carbon adhesion, which was previously fixed on an aluminium support SEM Zeiss EVO LS10. The sample was processed on environmental mode (87 Pa, air).

Transmission Electron Microscopy (TEM)**.** To study the possible changes at the level of ultrastructure of *Saccharomyces boulardii,* a transmission electron microscope JEOL 1010 (JEOL USA, Inc.) was used. Samples were fixed in glutaraldehyde and osmium tetraoxide and dehydrated with serial dilutions of ethanol and acetone. Finally, inclusión in resin to perform cuts were made for mounting them on a stand and observe the ultrastructure (Vázquez-Nin and Echeverria, 2000).

Results and Discussion:

Survival of S. boulardii and adaptation to heat stress**.** Figure 1 shows the survival to heat stress curve, corresponding to temperatures of 50, 55 and 60 °C, among which a significant log reduction for the yeast was obtained. It began with an average population of 5.0×10^{7} CFU mL^{-1} (7.6 Log) for the three temperatures test. At temperature of 50 °C, the yeast had a decrease in population of 1 log cycle after the 30 min of the assay. At 55 °C the D_{55} value was 3.90 min. The temperature of 60 °C was lethal to the microorganism by destroying the total population within the first 5 min. Garza *et al.* (1994) obtained values of D_{60} of 0.53, 0.20 and 0.1 min for three different of *S. cerevisiae* strain. We concluded that *S. boulardii* have greater thermotolerance than the *S. cerevisiae* strains used in that study.

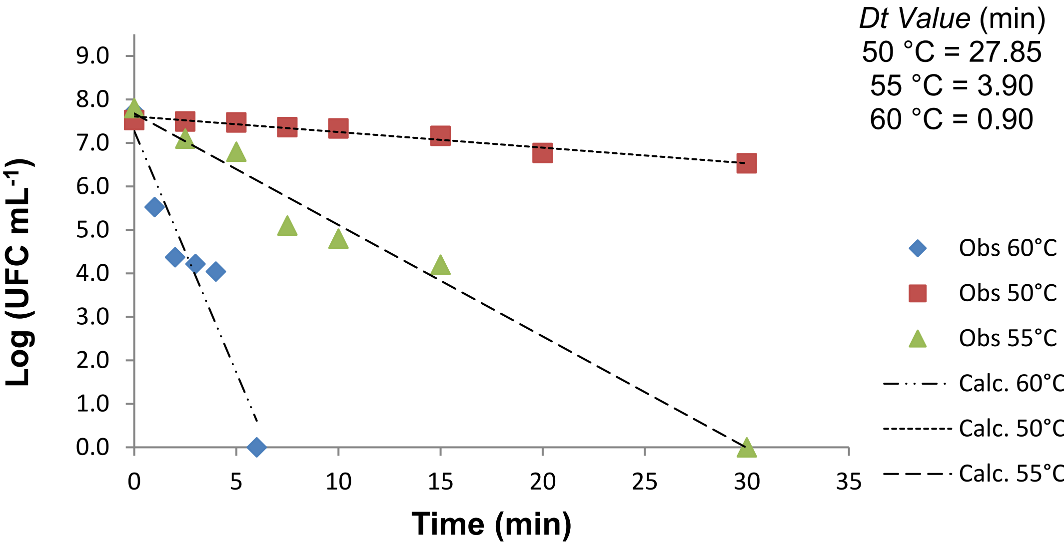


Figure 1. Survival curve of *S. boulardii* to heat stress.

Morphological evaluation of the colonies. Description of the yeast colonies after 48 hours of incubation was similar to the one reported in the literature, to: moist, smooth, shiny, creamy, and white to cream colored colonies. Colonies were grown for three more weeks after plating. Figure 2 shows the *S. boulardii* colonies which reveals that the increased size of the halo is proportional to the increasing treatment temperature. Cells regulate the fluidity of the membrane by varying the number of double bonds and the length of the fatty acids chains, as a way to adapt to the environment in which they are located. The fatty acid chains of the lipids may be in the bilayers and become rigid orderly or disorderly conformation (Guche form). Latter shape appears when the temperature exceeds their melting temperature (Madigan *et al.,* 1999), thus we can assume that the change in the conformation of the fatty acids could be the cause of the enlargement in the halo size of the *S. boulardii* colonies. It can be observed that at the temperature of 37 °C, the halo reached 1 mm of thickness (Figure 2a), while in the treatment at 55 and 60 °C this thickness achieved up to 3 and 7 mm respectively (Figures 2b and 2c).

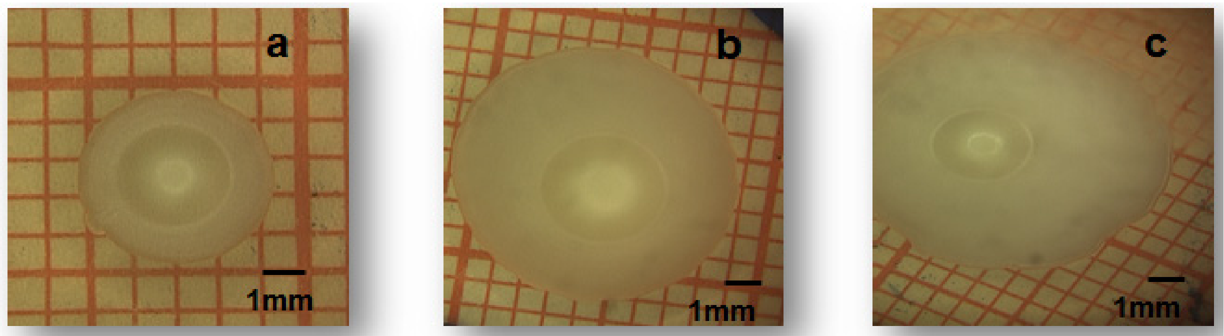


Figure 2. Colonies of *S. boulardii* on YPD solid medium after two weeks of incubation: (a) 37 °*C*, (b) 55 °C y (c) 60 °C.

Morphological evaluation of the yeast cells. Figure 3 shows TEM images obtained in untreated cells (Figure 3a) and after treatment at the temperature of 55 °C (Figure 3b). The presence of a large number of vacuoles, mitochondria and ribosomes can be observed. When the temperature reached 60 °C (Figure 3c) an absence of ribosomes and organelles was observed, along with damage in the cell membrane. In Figure 4, a gradual damage in the cell wall of *S. boulardii* was observed with the increase in temperature. The yeasts could survive even with that level of damage in their structure.

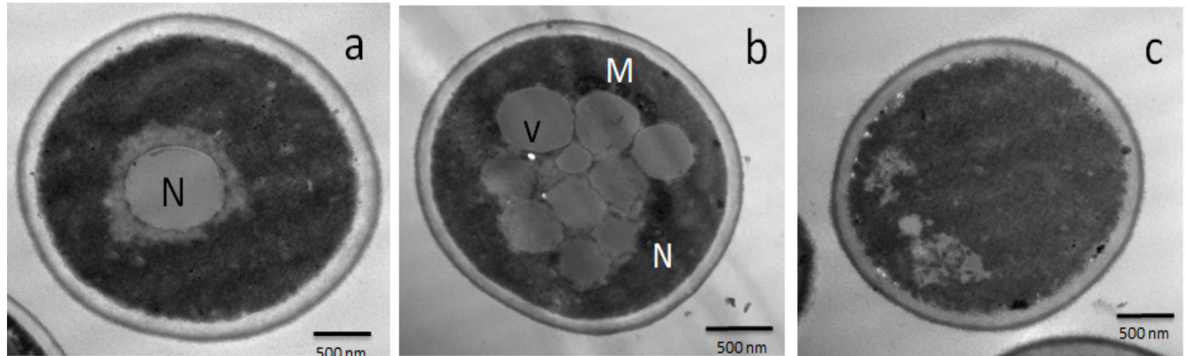


Figure 3. Images of *S. boulardii* by TEM: (a) untrated cells, (b) Treatment at 55 °C and (c) Treatment at 60 °C.

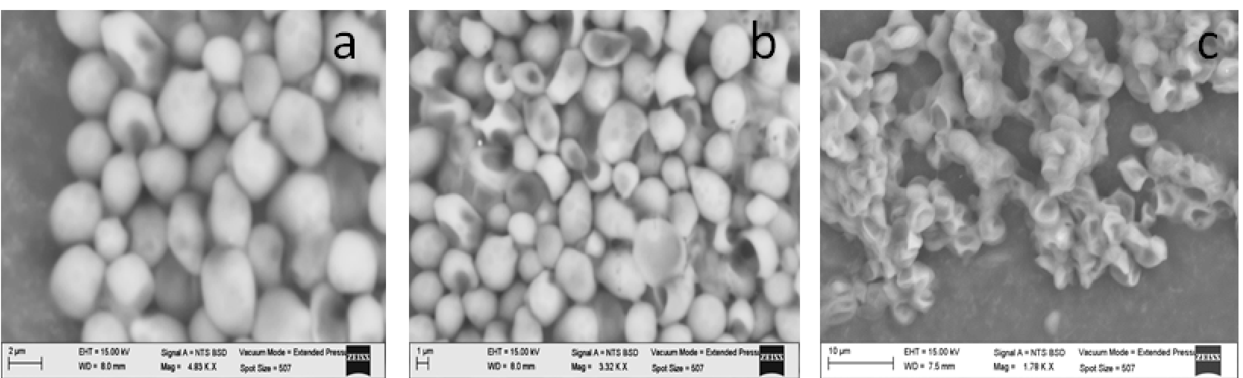


Figure 4. Images of *S. boulardii* by SEM: (a) untrated cells, (b) Treatment at 55 °C and (c) Treatment at 60 °C.

Conclusions: Results obtained in this study, showed that *S. boulardii* is a thermotolerant strain which can be used for technologies involving heat treatments for short exposure times. TEM observations showed that cells exposed to 60 °C treatment suffered cytological disruption in a large amount of the organelles, resulting in the death of the cell after a short period of exposure. Microscopy tools offer an alternative to study the strategies used by some microorganisms in order to survive in the presence of different types of stress.

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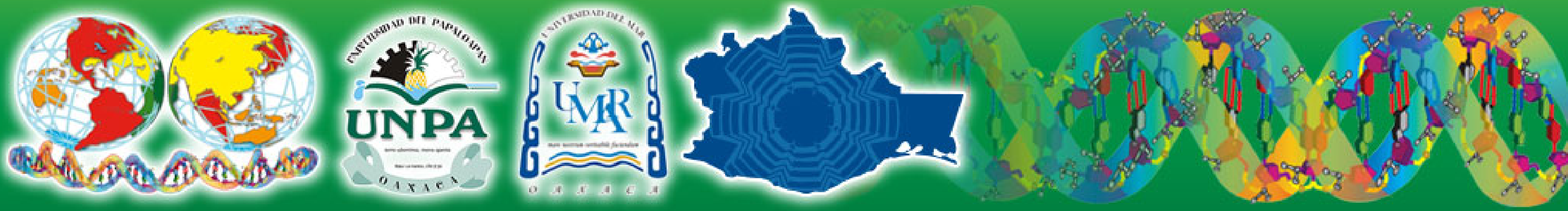

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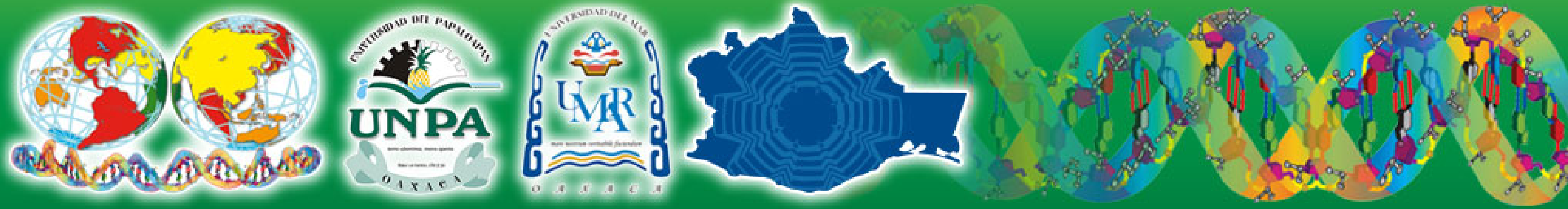

Current trends in functional foods: dietary fiber

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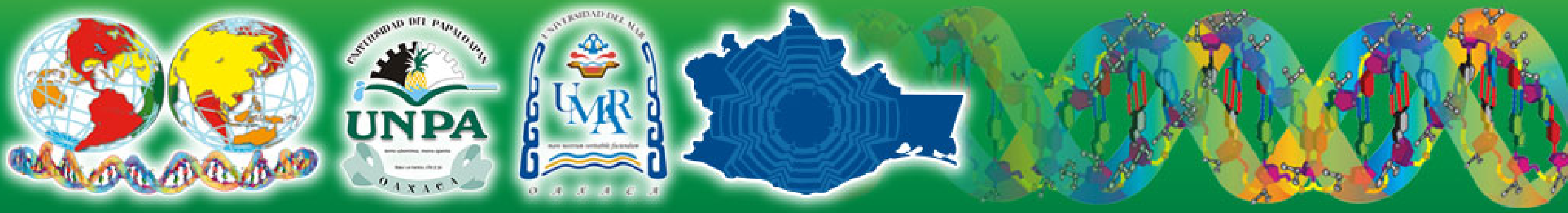
Abstract: Nowadays the importance of dietary fiber (DF) in health is well defined. Since the 90's the WHO and many national government bodies have written guidelines to increase the daily intake of foods rich in dietary fiber; but, the formulation of new high dietary fiber products is actually a challenge for the food industry. Dietary fiber fruit concentrates have a better nutritional quality than those from cereals due to the amount of associated antioxidant compounds and their balanced composition, such as: higher fiber content, soluble/insoluble DF ratio, water and fat holding capacities, lower energy value, and phytic acid content. On other hand, the food industry has large amounts of waste and byproducts that are a good source of dietary fiber and antioxidants and therefore could be used as food. In recent times, dietary fiber has been used as a functionalingredient in many commercial food products, either to increase the intake of dietary fiber and antioxidants or as a fat replacer in products with a high content of fat. This paper presents the latest research on dietary fiber as well as recent applications and its functions in different food products.

Keywords: Dietary fiber ● antioxidant ● fat replacer ● functional food

Introduction: American Association of Cereal Chemists (AACC) in 2000 defined dietary fiber as the edible parts of plant or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin and associated plant substances. Most appropriately, dietary fiber is classified into two categories the water insoluble and less fermented fibers: cellulose, hemicellulose, lignin; and the water soluble and well fermented fibers: pectin, gums and mucilages (Anita and Abraham, 1997). Insoluble fiber is found in plants, cereal grains, woody plants; this fiber is mainly the structural component in the plant cell wall but is also present in fruits and vegetable peels and seeds. Soluble fiber can be found in fruits, vegetables, legumes, tubers, seaweed, plants extracts and certain microbial fermentation products (Spiller, 2001). Many of functions and activities of fibers depend on their physicochemical properties, which are: particle size and bulk volume (controls intestinal transit time, fermentation and fecal excretion), surface area characteristics (controls fermentation and adsorption or binding of some organic molecules and ions) and hydration properties (induction of fermentation, swelling and water retention) (Raghavendra *et al.*, 2006; Guillon *et al.*, 1998; Renard *et al.*, 1994; Nassar *et al.*, 2008; Spiller 2001). Diets with a high content of fiber, from cereals, fruits and vegetables, have demonstrated a positive effect on health. The consumption of dietary fiber has been related to decreased incidences of several types of diseases. Some benefits of dietary fiber on human health has been investigated extensively since 70s and 80s such as reduction of appetite, decrease in blood sugar, cholesterol and triglycerides levels, reduction of risk for heart disease, possible reduction of symptoms of metabolic syndrome, diabetes and colorectal cancers. It is clear that dietary fiber has been widely used in various functional products (Kay, 1982; Takahashi *et al.*, 1993; Spiller, 2001). The importance of fibrous food has led to the development of a large market for fiber-rich products and ingredients and in recent years; and, there is a trend to find new sources of dietary fiber that can be used in the food industry. Supplementation has been focused on cookies,

crackers and other cereal-based products, enhancement of fiber content in snack foods, beverages, spices, imitation cheeses, sauces, frozen foods, canned meats, meat analogues and other foods has also been investigated (Hesser, 1994; Chau and Huang, 2003).

Antioxidant dietary fiber. Since the 90's it has been known as an antioxidant dietary fiber from fruits and vegetables that contain a considerable amount of antioxidants associated with the fiber matrix. Moreover, it has been demonstrated that fruit and vegetables posses a higher soluble/insoluble fiber ratio than cereals, thus is considered as a fiber with major nutritional quality (Saura-Calixto, 1998). Notwithstanding, the need for further research *in vivo* studies on antioxidants found in fruits, the data suggest that the consumption of phenolic-rich fruits increases the antioxidant capacity of blood, and when they are consumed with high fat and carbohydrate pro-oxidant food, they may counterbalance their negative effects (Burton Freeman, 2010). Dietary fiber and antioxidants are generally considered as two separate components in chemical and nutritional studies. However, it is a fact that a substantial proportion of antioxidant polyphenols and carotenoids contained in fruits are linked to dietary fiber matrix (Saura-Calixto *et al*., 2007), and some of the founded benefits of the fiber intake can be attributed to associated antioxidants. On this basis, dietary antioxidant can be divided into two groups: antioxidants accessible in the small intestine and antioxidants associated with fiber. In recent researches, it has been found that an important property of polyphenols is their bioavailability. Fiber of fruit and vegetables transports a significant amount of polyphenols and carotenoids (carotenoids, polymeric polyphenols and low molecular weight polyphenols linked to fiber matrix) through the human gut (Saura-Calixto *et al*., 2006, 2007). Polyphenols have aromatic rings and hydroxyl groups with the ability to bind to polysaccharides and proteins of dietary fiber. These compounds are called non-extractable polyphenols (NEPP) and they are mainly polymeric tannins and hydrolyzable polyphenols. (Hanlin *et al*., 2010). Because to this junction, antioxidants can reach the gut where human microflora hydrolyze, reduce, decarboxylate, demethylate and dehydroxylate NEPP, producing several antioxidants metabolites. (Gill *et al*., 2006). Only a few studies have considered a technique to determine NEPP, because it requires doing strong acidic hydrolysis treatments to disrupt and hydrolyze polysaccharides and proteins, allowing the release of hydrolysable polyphenols. There is also necessary extractions with polar solvents and strong acids to release proanthocyanidins and, if so, determinate NEPP profile and content by HPLC-MS and/or spectrophotometric methods. (Arranz *et al*., 2009; Perez-Jimenez *et al*., 2009). Reports have mentioned that 80-90% of whole polyphenolic compounds are linked to cell wall polysaccharides (Vitaglioni *et al*., 2008) and the highest concentrations of NEPP are found in fruits, legumes and nuts. With regard to amount of polyphenolics and their effects, the literature considers only available polyphenols but omits NEPP, until recent years. (Arranz *et al*., 2009; Arranz *et al*., 2010). The main antioxidant compounds found in vegetables and fruits arehydrobenzoic and cinnamic acids, flavanols, epicatechin, variety of flavonols in almonds; gallic, cafeic, coumaric, ferulic and sinapic acids in hazelnut, artichokes, cauliflower and lettuces. Flavonols as kaempferol and quercetin have been found in chicory, lettuce and mango. Flavonoids have been reported as hesperidin, narirutin, naringin, eriocitrin in different citrus species. Proanthocyanidins and anthocyanins compounds have found inapple, grape and banana as procyanidin B1 and B2, delphinidin, cyaniding, pelargonidin, malvidin and also flavanols (catechin), flavonols (quercetin-galactoside, quercetin-rhamnoside and quercetin-glucoside) However, there are still many non-traditional fruits and vegetables to research, cooca, chia seed, guava, starfruit, lime and roselle are some examples (Schieber *et al*., 2001; Llorach *et al*., 2003; Shahidi *et al*., 2007; Ajila *et al.,* 2010). Such antioxidants compounds have shown, *in vitro*, *in vivo* and epidemiological studies, reduction to



risk of coronary heart disease, enhancement of the excretion of lipids, proteins and water, positive effects on lipid metabolism, reducing lipid peroxidation, total cholesterol, LDL-cholesterol and triacylglycerides, and an increase of antioxidant activity in the large intestine.These effects are mainly attributed to the antioxidant and free radical scavenging capacity of compounds. Many products have presented antioxidants associated with dietary fiber as apple peel and pulp, grape pomace, cocoa fiber, artichokes, mango peel and other fruits with minor research (Bravo *et al*., 1992; Martín-Carrón *et al*., 1999; Goñi *et al*., 2005; Lecumberri *et al*., 2007; Brenes *et al*., 2008; Saura-Calixto *et al*., 2010). Saura-Calixto (1998) proposes that a vegetable material should contain certain requirements to be considered an antioxidant dietary fiber: 1) Dietary fiber content greater than 50% on a dry matter basis, 2) one gram should have a capacity to inhibit lipid oxidation equivalent to, at least, 200 mg of vitamin E and a free radical scavenging capacity equivalent to, at least, 50 mg of vitamin E and 3) the antioxidant capacity must be an intrinsic property, derived from natural constituents without chemical or enzymatic treatments.

***Dietary fiber as fat replacer*.** Fat replacers are substances that imitate organoleptic or physical properties of the triglycerides that are contained in foods. These replacers are common food constituents but may be chemically or physically modified to mimic the function of fat. Some carbohydrate-based fat replacers used in foods are employed as bulking agents to replace the volume lost when fat is reduced. Others applications of carbohydrate based fat replacement is as a texture modifier and emulsifier to substitute the functions of reduced fat in light products. Starch and cellulose are among the currently used fat replacers in food, however, other fat replaces, as inulin, fructooligosaccharides, pectin, carboxymethylcellulose, xanthan gum, carrageenan and others dietary fibers obtained to peel, pomace or waste of food industry (Akoh, 1994). In recent years, dietary fiber has been employed as thickener, gelling agent, stabilizer, binder and mouth-feel in researches that added it to ice cream, mayonnaise, yoghurt and meat products. Legumes flour, from blackeye bean, chickpea and lentil, were used in meatball formulations as extenders and increase of protein content (Serdaroglu *et al.*, 2005). Modi *et al.* (2003) reported that buffalo meat burgers containing different sources of fiber as soya bean, bengal gram, green gram or black gram dhal resulted in lower fat absorption and better sensory quality. However, dietary fiber from fruits has displaced cereal fiber in last years (Figuerola *et al*., 2005). Studies have demonstrated better benefits of fruit dietary than cereal fiber. Therefore, current reports in literature show a trend to use fruit fiber and its derivatives as a fat replacer ingredient. Verma *et al*. (2009) incorporated various dietary fibersources like pea hull flour, gram hull flour, apple pulp and bottle gourd in chicken nuggets. Fernandez-Gines *et al.* (2004) used lemon peel in sausages to increase moisture, fiber and protein contents as well as decrease fat content. Carrot dietary fiber was studied by Valeria *et al*. (2008) in four formulations of dry fermented sausage, known as sobrassada, improving nutritional and sensory attributes. Another widely employed fiber is sugarbeet fiber as a fat substitute alternative to offer high dietary fiber and low fat content in frankfurters (Vural *et al.,* 2004) and Turkish salami (Javidipour *et al.,* 2005). Has become popular, in recent years, the use of soluble fibers such oligofructosaccharides, barley beta-glucan, guar gum, xanthan, flaxseed mucilage and inulinas fat replacer in dairy products like yogurt and ice cream, meat products such as sausage, bologna, meatballs and meatloaf and mayonnaise. These fibers have improved texture and sensory properties, giving them preference in consumers, reducing syneresis, sugar and fat contents and improving emulsion capacity (Mendoza *et al*., 2001; Cáceres *et al.,* 2004; García *et al.,* 2006; Brennan and Tudorica, 2008; Yilmaz and Gege, 2009; Al-Sayed *et al*., 2012; Hoppert *et al.,* 2013; de Moraeset *et al*., 2013; Mahdian and

Karazhian, 2013; Damian, 2013). Furthermore, there are no studies on the prevalence of functional properties that the addition of fibers may confer to the final product.

Conclusions: Dietary fiber is a component of food that have been studied and shown its benefits to human health for forty years. Employment has diversified as a functional ingredient in many products fortifying its nutritional quality. In recent years, it has demonstrated its synergistic effect with other components such as antioxidants, improving the beneficent properties of both giving another possibility of development of high antioxidants contents foods. Moreover, the use of physicochemical properties of dietary fiber in the development of new food products with improved nutritional characteristics, decreasing contents of unhealthy sugars and fats; it gives us a new range of opportunities, which are the challenges ahead for food technologists and scientists.

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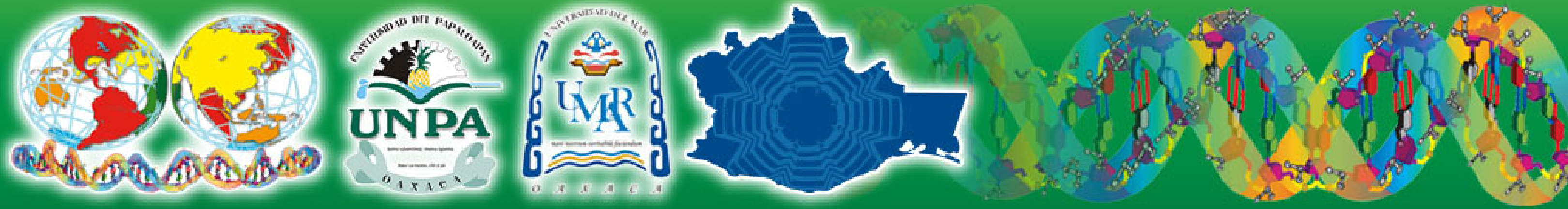

Skim milk permeate fermentation by *Lactobacillus helveticus*

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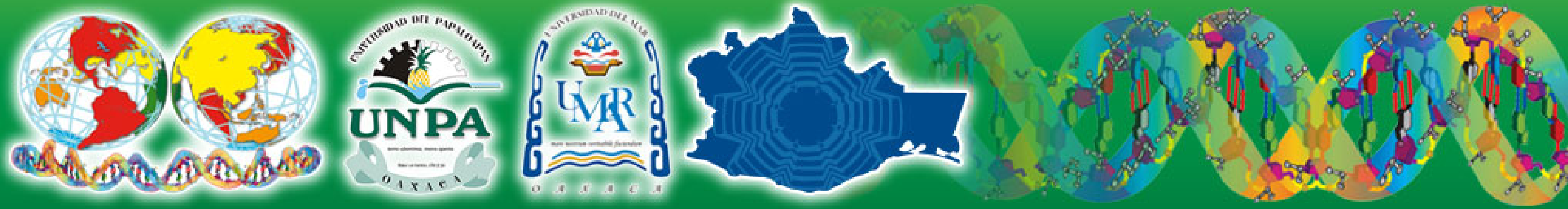
Abstract: *Lactobacillus helveticus* is a kind of lactic acid bacteria used to manufacture cheese and fermented milks, due to its higher production of lactic acid and its very active proteolytic system. Growth of *Lactobacillus helveticus* ATCC 15009 was evaluated using skim milk permeate as medium, as well as its capability to hydrolyze caseins during the fermentation time. Fermentation was carried out at 37°C for 24 hours. It was found that this strain grows at lower rates when permeate is used instead of MRS medium, which is possibly due to the medium deficiency in providing the essential nutrients required for the microorganism; however, it produces enough lactic acid to decrease pH of the medium to 3.67, that is an adequate pH for fermented beverages. A low degree of casein hydrolysis was observed too, and it could be related to its lower growth in the skim milk permeate.

Keywords: Fermentation ● skim milk permeate ● *Lactobacillus helveticus* ● casein hydrolysis

Introduction: Skim milk permeate (SMP) is obtained as a milk by-product when casein micelles are concentrated by microfiltration, it is a microbiologically sterile and clear liquid whose composition is close to sweet cheese whey (Fauquant *et al.,* 1988). This permeate does not contain casein macropeptides, cheese starters or chymosin, fat, bacteriophages or partially denatured whey proteins normally contained in the whey by produced in cheese making. Native whey proteins, which have good functional properties may produce a higher technological and economical value of the native whey than that of the standard or sweet cheese whey (Maubois, 2002). In order to give a use to SMP the most important step is the choice of a suitable culture of bacteria capable to produce a functional beverage with high nutrient value and acceptable sensory characteristics (Jeličić *et al*. 2008). *Lactobacillis helveticus* is one of the homo-fermentative, thermo- and acid-tolerant Lactic Acid Bacteria (LAB) capable to produce higher lactic acid titres from milk than other LAB (Roy *et al.,* 1986). It is used as an industrially important starter in the manufacture of hard cheese such as Grana and Provolone (Fortina *et al*., 1998), and in the manufacture of Mozzarella in combination with *Streptococcus thermophilus* (Broadbent *et al*., 2001; Perry *et al*., 1997). *L. helveticus* has a proteolytic system capable to hydrolyze milk proteins and there are many articles reporting the production of bioactive peptides generated from the hydrolysis of casein (Kunji *et al.,* 1996). To this day, there is no available information about the fermentation of SMP by *L. helveticus*, its capacity of acidification and non-casein protein degradation, so the aim of this work was to evaluate the growth, acidification and protein degradation capabilities of *L. helveticus* cultured in skim milk permeate as culture medium.

Materials and Methods:

Microorganism. *Lactobacillus helveticus* ATCC 15009 used throughout this research was obtained from the National Center of Agricultural Utilization Research (NCAUR). Stock cultures were maintained in MRS broth with glycerol (15% v/v) and frozen at -20 °C. Cultures were



reactivated by two sequential transfers in MRS broth at 37 °C by 24 h. Cell viability was determined by plating the appropriate dilutions of the culture in MRS Agar, plates were incubated at 37 °C for 48 hours and viability was expressed as log CFU mL^{-1}.

Medium. Skim milk Permeate was obtained by microfiltration of skim milk through a PVDF membrane of 0.3 µm pore size in a SEPA CF II (Osmonics) separation unit, at 45 °C and 0.75 bar. Average permeate composition was: 0.5% of protein and 2.5% of lactose.

Fermentation conditions. Skim milk permeate was pasteurized by heating at 60 °C for 30 minutes and cooling at 4 °C. Fermentation was performed in 15 mL sterile tubes containing 9 mL of SKP inoculated with 6, 7 or 8 log CFU mL^{-1} and incubated at 37 °C for 24 or 48 hours according to each experiment. Samples were aseptically withdrawn every two hours along culture time.

Analytical methods. Bacterial growth was monitored by optical density (OD) at 600 nm in a spectrophotometer (Bioscreen). pH measurements were determined with a digital pH meter (pH 210, Hanna Instruments). Protein degradation was analyzed by SDS-PAGE electrophoresis performed as follow: 25 µL of diluted culture medium was suspended in 25 µLof sample buffer (12.5% 0.5M Tris pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% mercaptoethanol) and incubated at boiling temperature for 5 minutes. SDS-PAGE was carried out in 15% (w/v) polyacrylamide gels on vertical slabs electrophoresis cells (BIO RAD Mini Protean® Tetra System) for 45 minutes at 200V. Coomassie brilliant blue G250 was used to stain the gels.

Results and Discussion:

Figure 1 shows the growth curves of *L. helveticus* inoculated at different concentrations in MRS broth. Lag phase duration was dependent of initial inoculum size going from 2 hours for the larger inoculum and increased to 6 hours with the lower inoculum, growth rate calculated as dX/dt was about 0.8 for 8 and 7 log CFU mL^{-1} inoculated cultures and 0.1 h^{-1} for 6 log CFU mL^{-1} with shorter period for the higher inoculated cultures; as a result, maximal growth was the same despite of initial inoculum. Those results demonstrate that *L. helveticus* ATCC15009 is viable and capable to grow efficiently in MRS broth and similar results can be obtained in this medium independently of the initial concentration of inoculum.
When skim milk permeate was used as fermentation medium (Figure 2), Lag phase increased when less microorganisms were inoculated; lag phase taking about 6, 10 and 14 hours for 6, 7, and 8 of CFU mL^{-1}. Maximal growth after 48 hours was much lower than using MRS broth as medium with OD values at $A\lambda 600$ nm between 0.7 for higher values of inoculum to 0.6 for the lower concentrated inoculum. Growth rate values calculated as previously are dependent of the initial inoculum with values of 0.039, 0.028 and 0.025 h^{-1} for 6, 7 and 8 log CFU mL^{-1} inoculums respectively. These results indicate that skim milk permeate is not the best culture medium for *L. helveticus* growth, but that it is capable to grow in this medium but at lower rate that is almost half comparing with its growth in MRS broth.

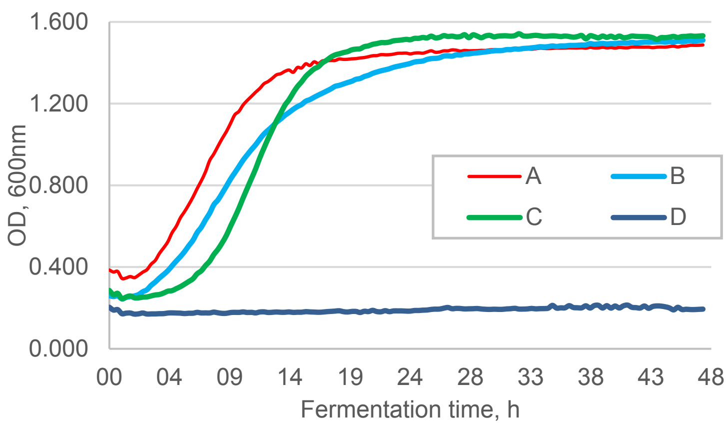


Figure 1. Growth of *L. helveticus* in MRS broth. Curve A: 8 log UFC mL^{-1}, curve B: 7 log CFU mL^{-1}, curve C: 6 log CFU mL^{-1}, and curve D: negative control.

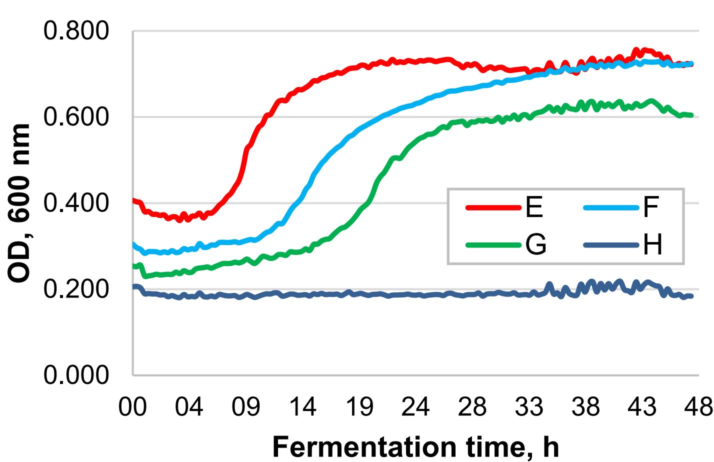


Figure 2. Growth of *L. helveticus* in skim milk permeate. Curve E: 8 log UFC mL^{-1}, curve F: 7 log CFU mL^{-1}, curve G: 6 log CFU mL^{-1}, and curve H: negative control.

Results obtained in this work differ from the results reported by Amrane (2005), who reported that lag phase took less than 1 hour when 11% of *L. helveticus* strain *milano* was inoculated in whey permeate supplemented with yeast extract; he also reported that growth remained exponential for 3 – 3.5 hours, deceleration growth took 5.5 hours, and stationary growth phase was reached after 9.5 hours of fermentation. Differences between results in this work comparing with that of Amrane (2005) in growth phases could be attributed to lack of essential nutrients (amino acids, vitamin and minerals) in skim milk permeate, as yeast extract was not added in this work. Hebert *et al*. (2000) found that the growth of *L. helveticus* CRL 1062 was stopped rapidly when $MgSO_4•7H_20$ or potassium phosphate was removed from medium, indicating an absolute requirement for these elements; omission of Tween 80 resulted in a reduction of growth of approximately 70%; and, calcium pantothenate, riboflavin, nicotinic acid, and pyridoxal were essential for the growth of

strains ATCC 15009 and CRL 1062. So the need to enrich SMP is a necessity in order to improve *L. helveticus* growth. Acidification curve (Figure 3) demonstrated how the pH value decreased when SMP was fermented by *L. helveticus*, this decrease of pH was associated to the production of lactic acid as result of lactose consumption. Lactic acid production seemed to be related to the growth phase as in lag and exponential phase there was a higher decrease of pH than the observed during stationary phase. After 14 hours of fermentation, pH of medium goes from 6.7 to 4.31; and after 24 hours, pH of medium was 3.67. These results are important because they demonstrated that *L. helveticus* can reduce the pH of medium to a level similar to those found in fermented dairy products. Results obtained also were similar to results reported by Morelli *et al*. (1986) who indicated that *L. helveticus* produces high levels of lactic acid in milk and can lower the pH of the medium in 24 h to values between 3.3 and 3.5.

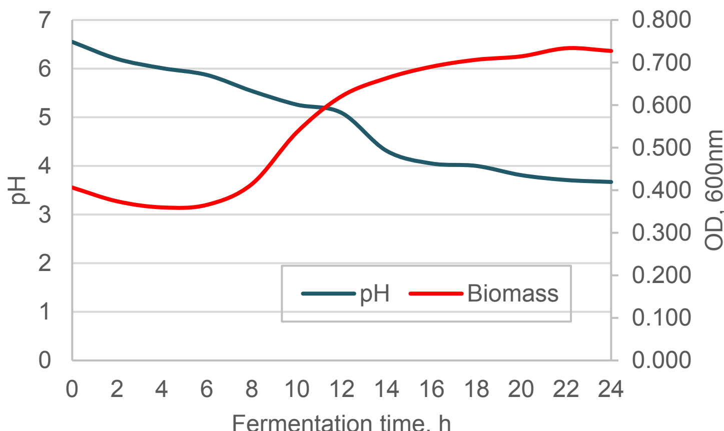


Figure 3. Relationship between growth of *L. helveticus* and pH in skim milk permeate.

Degradation of the proteins during fermentation was evaluated using an electrophoresis gel, and results are shown in Figure 4. These results show a very limited capability of *L. helveticus* to hydrolyze casein; this could be result of the lower growth rate previously observed when skim milk permeate was used as medium. Hebert *et al*. (2000) studied the ability of *L. helveticus* CRL 1062 to hydrolyze α-, β-, and κ-casein using MRS broth, SCDM and SCDM supplemented with different peptide sources as medium. They found that CRL 1062 proteinase hydrolyzes α- and β-casein predominantly and, these rates of hydrolysis by cell grown in peptide-rich mediums, such as MRS broth or SCDM supplemented with 1% Casitone, were significantly lower than those observed for cells grown in basal SCDM.

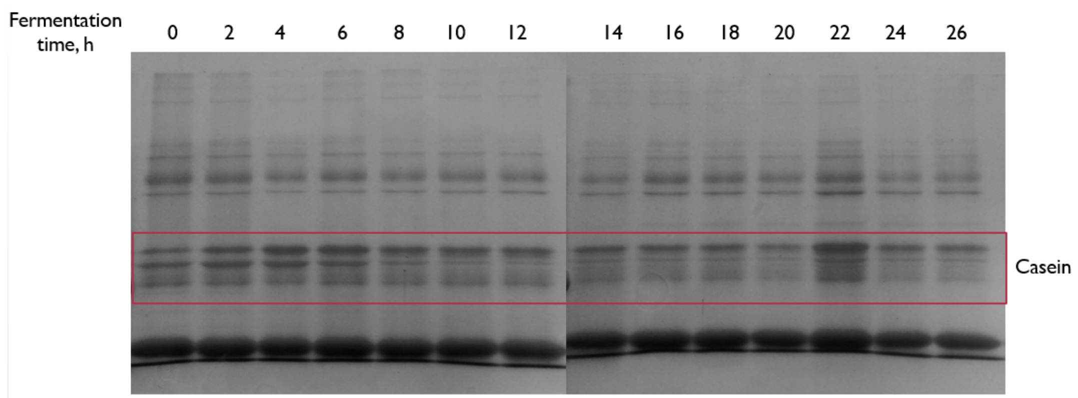


Figure 4. SDS-PAGE electrophoresis gel.

Conclusions: *L. helveticus* is capable to grow in skim milk permeate media, but growth rates are lower than growth rates obtained when MRS broth is used as medium. This difference in growth rates are related to the different nutrients found in each medium; MRS broth contain ingredients that supply nitrogen, carbon, minerals and other elements to support luxuriant growth of lactobacilli, and skim milk permeate only has lactose and some proteins as nutrients. One important result is that *L. helveticus* is capable to decrease pH of medium at levels similar to those found in some fermented products like yoghurt or fermented milks, and it means that this bacterium could be used for the production of fermented products using skim milk permeate as a main ingredient. Degradation of proteins was also observed during fermentation time, casein hydrolysis specifically; this casein hydrolysis occurs in lower quantities than expected. Those results could be improved by increasing the fermentation time, also increasing the growth rate by adding yeast extract or other ingredients that provide essential nutrients to the bacteria.

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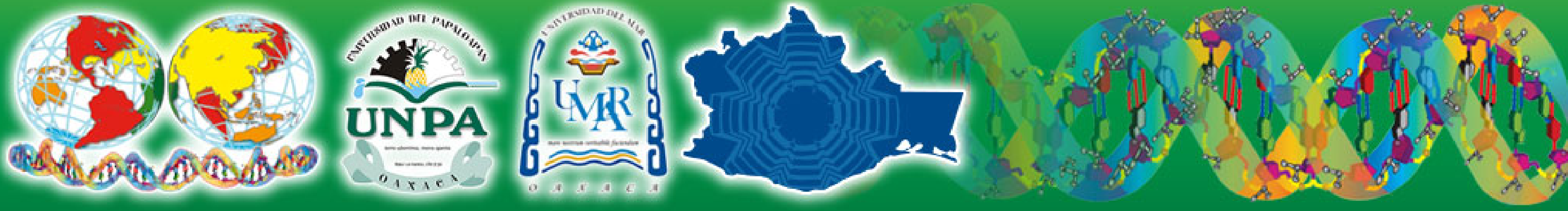

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1.3 BLUE AREA

Surface water microbiology characterization in Basaseachi National Park in Ocampo, Chihuahua, Mexico

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Abstract: In the past few years the Basaseachi National Park has presented seasonal gastroenteritis problems in local population, presumably by water contamination by human waste. The aim of this study was to examine water quality in order to respond an answer to this problem. Last year were collected 13 water samples of main rivers and the Basaseachi waterfall all long year (divided by the four station seasons), to examine fecal and total coliforms. The Mexican standard method (NOM-ecol-1996) for natural waters that we know are discharged with sewage pollutants is about 1,000 MPN 100 mL^{-1}. All samples that were above the standard where analyzed by microbiology standard techniques. The microorganism were isolated and characterized by biochemical methods; also were determined the antibiotic resistance profile of each isolate. The results showed a high prevalence of fecal and total coliforms in almost the four seasons' sampled and most of the samples. The antibiotic resistance profile showed multiresistance enteric microorganisms that may come from sanitary waste. Also we found microorganism with resistance to several antibiotics used in medical treatments, therefore it is presumed there is a horizontal contamination from enteric native bacteria.

Keywords: water pollution ● antibiotic multiresistance ● enteric microorganisms ● horizontal contamination

Introduction: Basaseachi National Park is located in the western side of the state of Chihuahua. This Park is named after Basaseachi Falls, the second tallest waterfall in Mexico with a height of 246 meters (853 ft). The park is famous for its natural beauty of pine-oak forest, spectacular rock formations, and breathtaking views off high cliffs. Enteric pathogens transmitted via drinking water are predominantly of fecal origin. These are common problems in rural areas in developing countries (Ashbolt, 2004). Seasonal water bodies like ponds, rivers, lakes and waterfalls are very productive ecosystems, where the biomass can conserve many kinds of plants and animals, and also contributed to the sustainable development. Water pollutants are generally attributable to the run-off from urban and agricultural areas, leakage from sewers and septic systems, and sewer overflows; these produce a serious public health problem in many countries (Girones *et al*., 2010). Every year there are nearly 1.7 billion cases of diarrheal in the world. It is the second leading cause of death in children under five years old; it kills around 760,000 children per year. It is both preventable and treatable, a significant proportion of diarrheal disease can be prevented through safe drinking water (WHO, 2013). Enteric bacteria are present in human guts and urinate tract, shed in the feces and may be present in environmental waters (Amdiouni and Maunula, 2012). Common bacteria involved in epidemic water outbreaks are *Campylobacter jejuni, Yersinia* spp.,

Vibrio cholerae, Salmonella enterica Serovar Typhi and Paratyphi*, Shigella and Escherichia coli* O157:H7. *Salmonella* and *Campylobacter* are the most frequent agents of bacterial gastroenteritis. Thermophylic *Campylobacter* species are widespread in the environment and are commonly found in surface water and sewage sludge. Even if *Salmonella* is not common in water isolation, its presence in water may pose a public health risk, due it need only a 15-100 CFU (colony-forming units) for cause a disease (Sidhu and Toze, 2009). *V. cholerae* caused the first pandemic in 1817 and it was not known until the 1850's whereby the English physician John Snow established that cholera was waterborne (Ashbolt, 2004). *Yersinia* is also linked to contaminated seafood and water outbreaks, whereas *Escherichia coli* have frequently being isolated from waters around the world. In fact, it has been found in 2% of raw sledges (Girones *et al*., 2010).

Materials and Methods:

The samples were collected in sterile 1.2 L propylene bottles (NOM-014-SSA1-1993) by the 2013 seasons. Upon location, 13 geographic referencing locations (Figure 1) were selected, including the waterfall, rivers and streams near to rural populations in Basaseachi National Park to analyze the type of human pollutants are discharched into the effluents. The samples were analyzed using the most probable number technique (MPN), accordingly to the Mexican standard procedure PROY-NMX-AA-042-SCFI-2005.

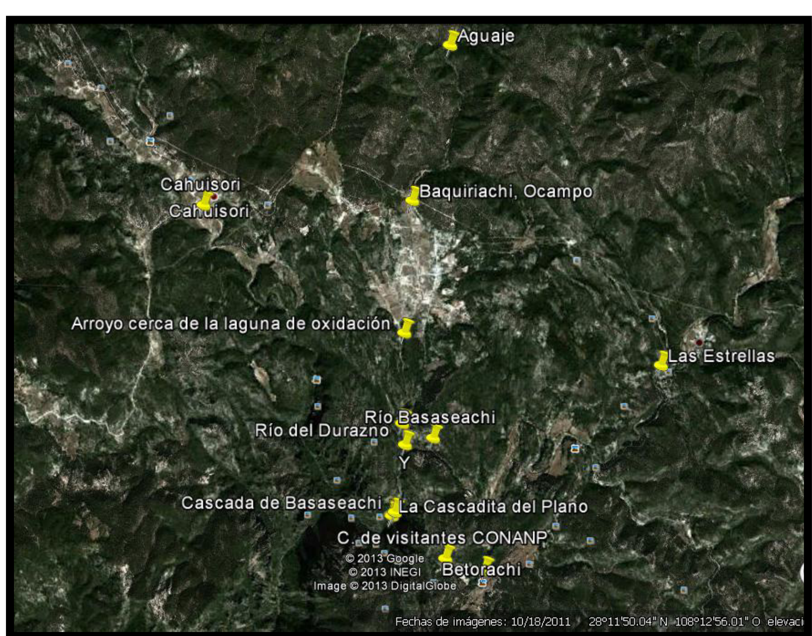


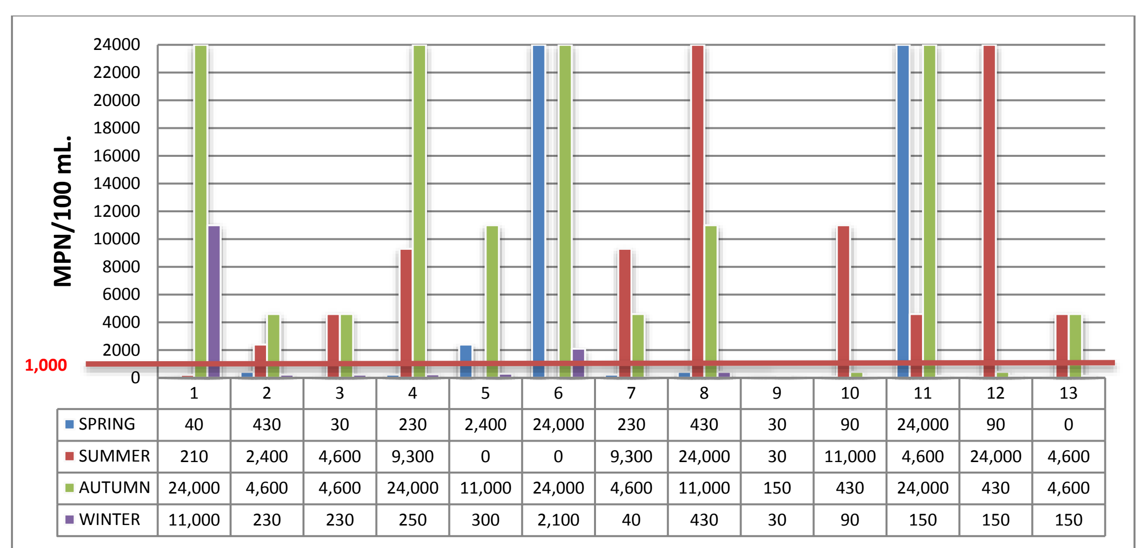
Figure 1. Geographic referencing points of Basaseachi National Park water sampling.

The Mexican standard methods (NOM-ecol-1996) for natural waters that are discharged with sewage pollutants allow up to 1,000 MPN 100 mL^{-1}. All samples that were above the given number by this standard were macroscopic and microscopic analyzed. For biochemical and antibiotic resistance profile probes, the AutoSCAN-4 system (MicroScan-Dade Behring) equipment was use. The rapid Combo panel NEG-44 is for gram-negative microorganism identification, based on

conventional and chromogenic test in a microplate with 96 wells and 27 dehydrated substrates. The normalized probability of acceptance identification is calculated from a list of microorganism, based on the probability of a positive reaction in each test. Five or less microorganism with the highest probability within a predetermine cutoff is evaluated to obtained the percentage probability. This result was based on the probabilities sum and the posterior division of each microorganism probability multiplied with the total sum. A percentage equal or above 85% means a high probability of acceptance identification, less of that means lows probability.

Results and Discussion:

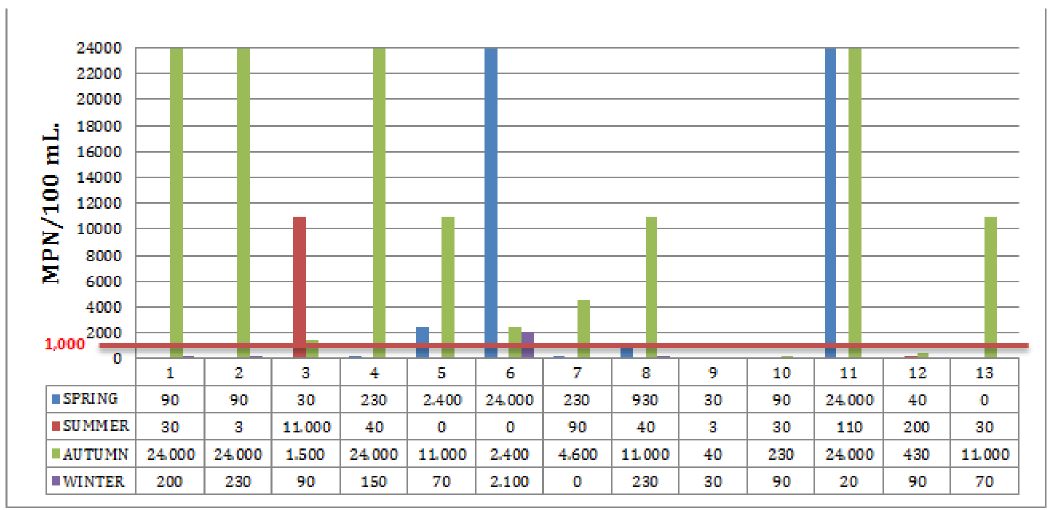
The results shown in the Figure 2 for total coliforms are above off the 1,000 MNP in most samples. The season with more probes is during autumn due the rainfall, followed by summer due the high temperatures and then spring. During winter the total coliforms were lower than the rest of the year, except for the sample 1 and 6.



	1	2	3	4	5	6	7	8	9	10	11	12	13
SPRING	40	430	30	230	2,400	24,000	230	430	30	90	24,000	90	0
SUMMER	210	2,400	4,600	9,300	0	0	9,300	24,000	30	11,000	4,600	24,000	4,600
AUTUMN	24,000	4,600	4,600	24,000	11,000	24,000	4,600	11,000	150	430	24,000	430	4,600
WINTER	11,000	230	230	250	300	2,100	40	430	30	90	150	150	150

Figure 2. Total coliforms counts from the Basaseachi National Park water within 2013.

Fecal coliforms count results for are shown in Figure 3. Results shown that most samples collected during the autumn season were above of the standard. Within the other seasons, counts were lower by the exception of few samples, collected mainly during the spring season. We isolated 51 enterobacteria from the samples that were above of the limit. Samples were analyzed by microbiology for isolation and bacterial characterization. Results showed nine different genera, as shown in Table 1. Bacteria are the most important pathogens in waterborne disease and the principal cause of outbreaks. Water is a very good way to transport enteropathogens like *Salmonella typhi, Salmonella paratyphi* also *Shigella spp., Vibrio cholerae, Clostridium spp. and Bacillus anthracis* (Gesche *et al.*, 2003). Presence of Escherichia coli is directly related to fecal contamination. Salmonella is considered a public health risk and should not be a common water inhabitant (Sidhu and Toze, 2009).



	1	2	3	4	5	6	7	8	9	10	11	12	13
SPRING	90	90	30	230	2.400	24.000	230	930	30	90	24.000	40	0
SUMMER	30	3	11.000	40	0	0	90	40	3	30	110	200	30
AUTUMN	24.000	24.000	1.500	24.000	11.000	2.400	4.600	11.000	40	230	24.000	430	11.000
WINTER	200	230	90	150	70	2.100	0	230	30	90	20	90	70

Figure 3. Fecal coliforms counts from the Basaseachi National Park water within 2013.

The antibiotic resistant profile is shown in Figure 4. *Salmonella paratyphi "A"* and *Vibrio parahemolyticus* were not resistant to any of the antibiotics tested; these were considered native bacteria. *Citrobacter freundii* and *Vibrio cholerae* showed resistance to one antibiotic; whereas *Enterobacter cloacae, Escherichia coli* and *Klebsiella oxytoca* showed a multi-resistant profile that indicated a human feces source. *Aeromonas hydrophila* are *Alcaligenes sp.* are saprophyte bacteria, not considered as a risk for human health. Nevertheless, since they showed an antibiotic resistant profile, might be considered they acquired this resistance by horizontal transmission, and may represent a risk for public health if this horizontal transmission goes further to other human pathogenic bacteria.

Table 1. Isolated microorganisms

Microorganisms	Identity percentage
Citrobacter freundii	99.99%
Aeromonas hydrophila	99.99%
Salmonella enterica Paratyphi A	99.99%
Escherichia coli	99.99%
Klebsiella oxytoca	99.99%
Enterobacter cloacae	99.99%
Vibrio cholereae	71.25%
Alcalígenes sp.	69%
Vibrio parahemolyticus	63%

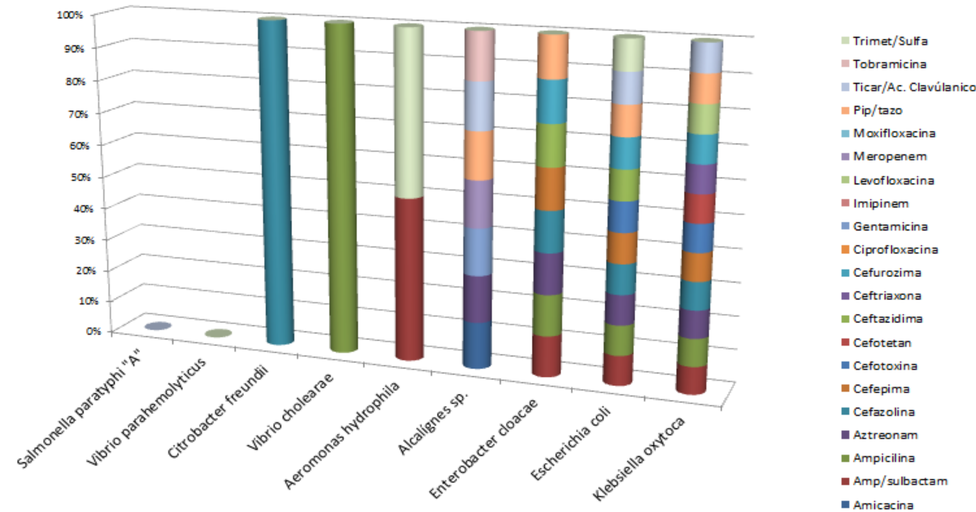


Figure 3. Antibiotic resistant profile found in the microorganisms isolated from water resources of the Basaseachi National Park.

Conclusions: Basaseachi National Park has very important contamination pollutants, these kind of problems are considered a problem in matter of public health. Bacteria found in analysis show a spread of sewage from rural locations to water bodies, where the people use water to drink. We also found environmental bacteria with resistant profile; this might cause future problems, and represents a health threat to the people who lives in this park.

Acknowledgments: This project was supported by CONACyT (scholarship to MCEDG, and project CB2010/155771 to PTG).

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1.4 GREEN AREA

Fermentation-assisted extraction of phenolics from desert plants

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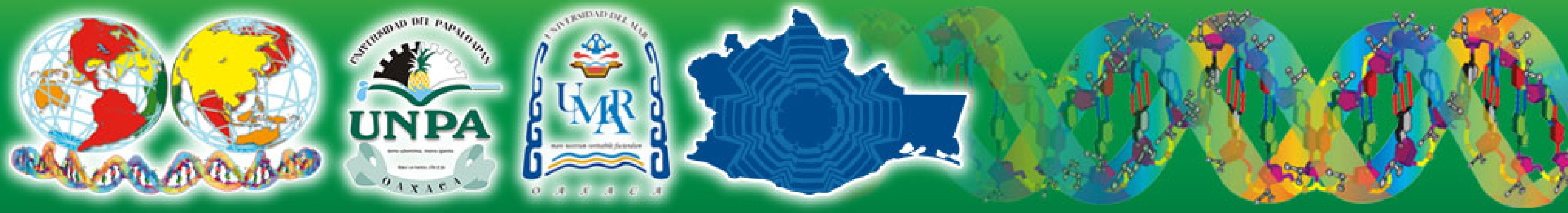
Abstract: Mexico has an important plant biodiversity and in its arid zone, several plants grow exhibiting an adaptation to extreme environmental conditions. Among these plants we can found "creosote bush" (*Larrea tridentata Cov.*), "tarbush" (*Flourensia cernua*), "damiana" (*Turnera diffusa*) and "leatherstem" (*Jatropha dioca*), which are used in phytotherapy and traditional medicine. Our research group has a great interest on the development of biotechnological processes that allow the liberation or extraction of bioactive compounds from these plants. In this work, fungal solid state fermentation was used as the pretreatment bioprocess to enhance the release of bioactive molecules. Some polyphenolic compounds, particularly condensed tannins, were identified in all the plants studied such as catechins and epicatechins. Hydrolysable tannins such as gallic acid were also identified at considerably high amounts. After fermentative process the biotransformed polyphenolic compounds were liberated to the culture medium which permitted a better recovery and quantification of these compounds. In this study, we have defined this process as Fermentation assisted extraction (FAE) as promissory emerging technology for recovery of important bioactive compounds.

Keywords: Fermentation-assisted extraction • biotransformation • polyphenols • deseart plants

Introduction: Creosote bush (*Larrea tridentata Cov.*), tarbush (*Flourensia cernua*), damiana (*Turnera diffusa*) and leatherstem (*Jatropha dioca*) are plants extensively distributed in arid regions. These natural resources have been deficiently studied and unfortunately badly profiteer, in spite constituting approximately one quarter part the national territory. Many of these plant species synthesise a great variety of primary and secondary metabolites, used empirically (like infusions or unguents) in the traditional medicine to treat some diseases by the natives and people of the rural zones in the south of United State of America and the North of Mexico (Meckes, 2004). Earlier, our group reported that some of such bio-active compounds isolated of these natural sources are important polyphenolic phytochemicals (Belmares *et al*., 2009), representing beneficial properties to human health, resulting attractive for different sectors, such as, modern medicine, pharmaceutical, food and cosmetology industries (Jasso *et al*., 2007). Also, we have evidenced the possibility that during the fermentative process of plant material, the polyphenols could be bio-transformed to simpler molecules with an important role in different industries (Robledo *et al*., 2008; Aguilera-Carbo *et al*., 2008; Ventura *et al*., 2008; Aguilar *et al*., 2008). Our studies have demonstrated that *Aspergillus niger* has the capacity to degrade hydrolysable tannins given as product, monomeric compounds (gallic and ellagic acid) of creosote bush and tar bush (Ventura *et al*., 2008). Also, Aguilera-Carbo *et al*. (2008) showed that biotransformation process of ellagitannins of creosote bush is directly released with enzymatic activity of a hidrolase ellagitannin showing that typical activity tannase is not sufficient to hydrolyze these compounds.



The objective of this work was to design a new method of extraction of bioactive phytochemicals, which was assisted by fungal fermentation using four species from semiarid Mexican zone of high ethnobotanical relevance.

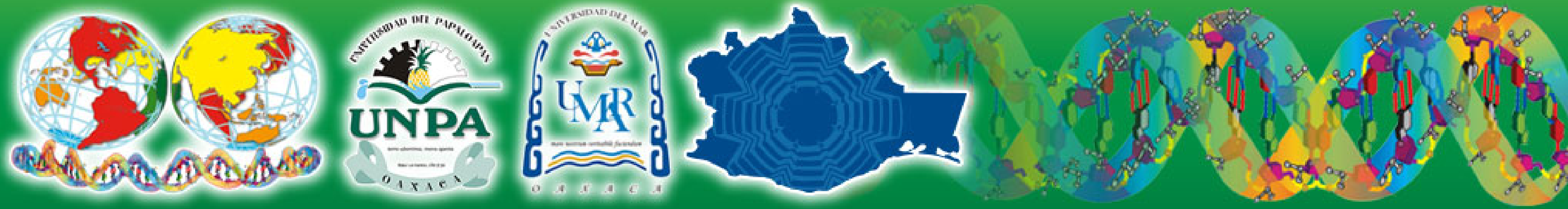
Materials and Methods:

Plants. The collection of creosote bush (*Larrea tridentata*), Damiana (*Turnera diffusa*) and letherstem (*Jatropha dioica*) and tar bush (*Flourensia cernua*) was realized in the state of Coahuila, México. Leaves of *L. tridentata*, *F. cernua* and *T. diffusa*, as well as root of *J. dioica* were dehydrated in at 60 °C and pulverized in a Torrey mill (LP 12). The pulverized plants were stored in black bottles until their use.

Fermentation assisted extraction (FAE). The biotransformation was evaluated for solid state fermentation with *Aspergillus niger* GH1 (Cruz-Hernandez *et al*., 2005). The fermentation conditions were: Carbon and energy source; Leaves of *L. tridentata, F. cernua* and *T. diffusa*, as well as, root of *J. dioica*. Humidity; 70% with Czapek-Dox. Temperature; 30 ºC. pH; 5.5. Inoculum; 2E7 spores of *A. niger* GH1/grame of dry plant. Time; 96 hours. Agitation; each 24 hours (Mercado *et al*., 2007). The vegetal plant fermented was dried in stove to 60 ºC for 24 hours. Fermented product was stored until its use later. The extraction was carried in a relation 1:4 (w/v) with methanol 70% (v/v). The sample was digested for system soxhlet, a temperature non-greater of 60 ºC with agitation (100 rpm) and under dark conditions for seven hours. After of extraction, the vegetal plant was filtrated with Whatman No 4, the residue was extracted for second time with acetone 70% (v/v) and the filtrates (methanolic and acetonic) were evaporates using a rotavapor apparatus (Yamato, RE540) in order to obtain aqueous extracts. This was store in amber bottler and cooled until its use.

Polyphenols compounds identification. It was used of method utilized for the polyphenols separation reported by Guyot *et al*. (1998), the thiolysis analysis has like objective dissolve procyanidines present in plants extracts of Mexican semiarid in solution anhydrous acid in presence of thioeter benzyl (mercaptan benzyl) giving like resulted terminals united of catechin and epicatechin, as well as, intermediaries united of polymers together with thioeter benzyl (epi-catechin-SR). The samples with and without thiolysis was analyzed by high performance liquid chromatography (HPLC) on column C18 with gradient of acetonitrile. The experimental conditions of analysis was: flow; 1 mL min^{-1}, movil phase, 50% of acetonitrile and 50% of water ultrapure with acetic acid (2.5%), Time; 10 minutes to operated the equipment, 50 minutes to conditioner the column and 60 minutes to analysis of sample.

Polyphenols compounds pre and post-fermentation evaluation. For quantification of hydrolyzed tannins (HT), the Folin-Ciocalteu method reported by Waterman and Mole (1994) was used. In this assay, 800 mL of the sample were put into a test tube and mixed with the same volume of Folin-Ciocalteu (Sigma-Aldrich) reagent, shaken and left for 5 min. Then this solution was diluted with 5 mL of distilled water and analyzed in a UV-Visible spectrophotometer at 750. The obtained absorbance values were analyzed against the standard curve of gallic acid (1000 ppm). For quantification of condensed tannins (CT), the catechin content was evaluated and analyzed by the HCl-butanol method reported by Swans and Hillis (1959). An aliquot of 0.5 mL of the sample was placed with 3 mL of HCl-butanol (10%, ratio 1:9), and then an aliquot of 0.1 mL of $(NH_4)Fe(SO_4)_2$ in 20% HCl was added. The mixture was sealed hermetically and heated for 1



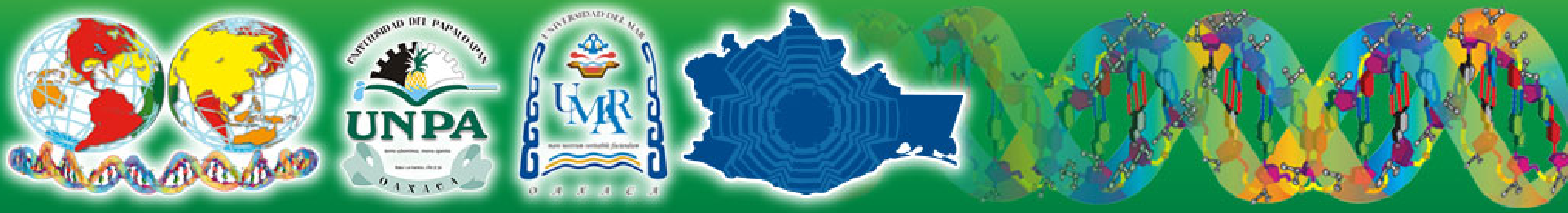
h at 100 °C in a hot water bath. After 1 h, it was cooled and the absorbance was read at 550 nm. The CT was calculated with reference to standard curve obtained using catechin (1000 ppm).

Evaluation of antioxidant activity. The evaluation of the antioxidant activity (AA) of polyphenolic extracts of plants of semi-arid Mexican zone (pre and post-fermentation) was following of method reported by Re *et al*. (1999), taking like reference commercial polyphenolic compounds with antioxidant action. The AA was measured by the method of bleaching of ABTS radical that consists of oxidizing a solution of ABTS (colorless) with a solution of persulfate (colorless) forming ABTS radicals (blue-green) that has its maximum of absorption to a wavelength of 734 nm; when these radicals are exhibited to antioxidant compounds they reduce forming neutral and colorless ions ABTS again. Polyphenolic standards commercialized by Sigma Aldrich were used: catechin (98%), galic acid (99%) and elagic acid (~95%) to a concentration of 200 ppm.

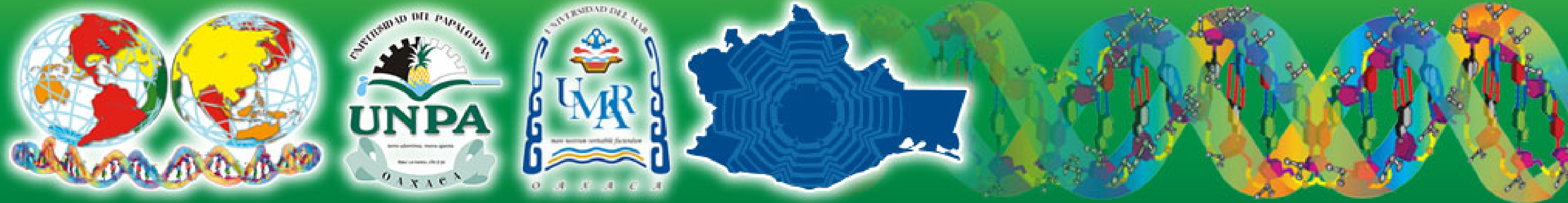
Statistical analysis. The data were analyzed with experimental designs SAS. The standard deviations were calculated from each group of experimental data obtained. The results presented a significant minimum difference with a probability of 0.05%.

Results and Discussions:

The chromatograms of polyphenols extracts without (A) and with (B) thiolysis at *L. tridentata* (Figure 1a), showed alteration over the composition of this specie after of the thiolysis presented a terminal catechin unit (T_EC) at retention time (RT) of 14.50 minutes and an extension epicatechin unit (T_ESR) at 35.34 minutes of RT. This behavior was similar in all the semiarid Mexican plants evaluated (Figure 1b, 1c and 1d). In this study the challenge was the complexity of its structures (catechin, epicatechin, procyanidins, etc.) that generated a complicated analysis when using natural extracts, providing an ample range of compounds that are not separated and quantified easily. Depolymerization of this compounds was carried in acid medium, where is donated a monomer originating of terminal procyanidin unit present in semiarid extracts plants and a carbocation of the extension unit; the mechanisms by which the polymerization to take place is unknown. A proposal speak of the capacity of received a hydrogen to generate a carbocation, this is immediately stabilized to a semiquinone. Finally is stabilized the aromatic property until a more stable quinone. For determine the monomers that participate in polymer formation, is used a hard nucleophile of type tyol forming a tyoether (Kenney *et al*., 2001). This procedure was used recently to obtain compounds new of extensions unit derivate of tyol group (Ramirez-Coronel *et al*., 2004), appropriate for recuperation of procianidins monomers of semiarid Mexican plants transforming them in a natural compounds source with new physicochemical and biological properties for industrial application. For other hand, the content of polyphenolics compounds was seen affected after of fermentation procedure with *A. niger* GH1 in agreement with the results in this study. In the Figure 2 is shower the quantification of HT and CT extracted with methanol and acetone from *L. tridentata* pre and post-fermentation, were the major polyphenols concentration was obtained with methanol like extraction solvent from HT and CT (469 and 570 mg g^{-1} of dry plant extracted, respectively), as well as, is appraised clearly that the fermentation procedure decrease the polyphenols amount in this plant. The changes happened received attention special because it is a plant distributed widely in the zone semiarid of Mexico and study realized by Luis *et al*. (2002) verifity the presence of compounds of polyphenolic nature, which later of fermentation procedure decreased due mainly to *A. niger*, that possess the capacity of using this plant like support from the enzyme degrading of tannins production, for example tannase (Treviño-Cueto *et*



al., 2007), futurmore can be using like carbon and energy source from antioxidant production of commercial interest. The results in this work coinciding with the reported by Aguilar *et al*. (2008) where later fermentation procedure the HT and CT decreased until 90 percent later of 96 hours and liberation simultaneous of gallic acid and catechin. Figure 3, present the concentration of HT and CT from *F. cernua* pre and post-fermentation, where the major concentration of polyphenols was obtained with acetone like extractor solvent in both compounds (150 and 180 mg g^{-1} of dry plant extracted, respectively), in this case the fermentation procedure decreased the polyphenols concentration in *F. cernua*. This specie is important by his polyphenols high levels (Martínez *et al*., 2002) which after being fermented with *A. niger* showed that fungus posed the capacity of degraded hydrolyzed and condensed tannins, results that agree with reported by Ventura-Sobrevilla *et al*. (2008) where later of procedure of degradation the polyphenols were transformed until monomers that can be consumed and accumulated. For other hand, the condensed tannins biodegradation in this specie and the accumulation of catechin monomers was proportional time of fermentation. Increase of gallic acid indicated the polymerization of gallotannins, results that agreed with the obtained in this investigation, this compounds can be using like substrate. The results obtained from *T. diffusa* pre and post-fermentation, is presented in the Figure 4, where the major polyphenols concentration was obtained with methanol like extractor solvent from HT and CT (213 and 250 mg g^{-1} of dry plant extracted, respectively) the fermentation decreased the polyphenols concentration and the biotransformation procedure is considered a source important of antioxidants (gallic acid and catechin) released by enzymatic hydrolysis of hydrolysed and condensed tannins. The biodegradation mechanisms of polyphenols from *T. diffusa* are actually unknown, but the low concentration of polyphenolic compounds later of the fermentation with *A. niger* shower that this microorganisms is able of transform or release compounds according with Mercado *et al*. (2007). In the Figure 5 is present the quantity of HT and CT extracted with methanol and acetone from *J. dioica* pre and post-fermentation, where the greater polyphenols concentration was with methanol; unlike the other species the fermentation procedure increased the HT and CT concentration (7 and 9 mg g^{-1} of dry plant extracted, respectively), is possible to mention that like *T. diffusa* this specie it has not been studied after a fungal biotransformation process, reason for study and know the way involved in the transformation process of polyphenols compounds. The tables above show that the results obtained of general form showed that the methanolic extracts pre-fermentation of *L. tridentata* presented a greater concentration of hydrolyzed tannins being 54.3% major that in *T. diffusa*, 82.4% major that in *F. cernua* and until 99.4% major that in *J. dioica*. The condensed tannins was major in *L. tridentata* until a 47.2% in comparison with *T. diffusa*, 53.5% major that *F. cernua* and 99.4% in *J. dioica*. The plants post-fermentation presented the same behaviour in each species, but in low levels. Realized studies by Druzynska *et al*. (2007), showed the influence of the time and solvent type used for extraction of polyphenols, and demonstrated that acetone is more efficient for the procedure of condensed tannins extraction in comparison with methanol, results that agree with two species used in this work (*L. tridentata* and *T. diffusa*). Similarly Sanoner *et al*. (1999) mentioned that used of solvent as methanol allows liberation of low molecular weight compounds and an extraction subsequent with acetone releases high molecular weight compounds. Therefore, is important to mention that results obtained in the present work demonstrate a greater amount of CT using methanol in *F. cernua* and *J. dioica*, possibly by the extraction time seven times major to used by Druzynska *et al*. (2007), furthermore was applied heat to accelerate the extraction of these compounds (Waterman and Mole, 1994). It is possible mentioned that differences in the concentration of polyphenols mainly must to genus and species used; in addition that the tannin content is affected by the climatic time of harvesting of the plants, phenological stage and plant part used (leaves,



stems, roots, etc.) (Hyder *et al*., 2005). Besides the chemical composition of the same and because some simple sugars, oligosaccharides and organic acid presents in these plants can be extracted with methanol, as well as, some phenols of low molecular weight (Guyot, 1998). In this investigation was decided to work with extracts obtained using methanol and acetone for being the appropriate dissolvent for obtaining the majority polyphenolic compounds (Guyot, 1999), yield for three of these vegetal species was of 16-17%, except for *J. dioica* (6%). The Figure 6 showed the capacity of vegetal extracts rich in polyphenolic compounds able to donate a hydrogen to stabilized and therefore cause the reduction of ABTS radical (blue to colorless); the smaller AA appeared in *J. dioica* (50%) that presented the smaller amount of polyphenolic compounds and *L. tridentata* was the extract with better antioxidant activity of 97% with the greater concentration of polyphenols; this species in comparison with the standards used was equally efficient that the galic acid and catechin, on the other hand, was much more effective that the elagic acid. The antioxidant activity of polyphenolic compounds is must mainly to its redox properties, which play an important role in absorption and neutralization of free radicals. The antioxidant potential of polyphenoloic compounds depends of number of hydroxyl groups and the conjugation degree of the structure; for example in flavonoids and phenolic acid compounds the activity improves as the number of hydroxyl groups increases (Sang *et al*., 2002), the results obtained in this stage agree with reported, is clearly visible the high correlation between the antioxidant activity and polyphenols concentration of the vegetal plants studied being the *L. tridentata* who presented a greater polyphenols concentration and therefore one better antioxidant activity.

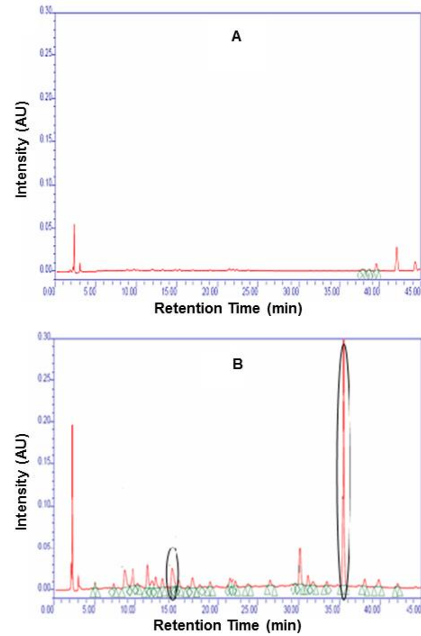


Figure 1a. Polyphenols identification for reverse phase HPLC from extracts of *L. tridentata* without thiolysis (A) and with thiolysis (B).

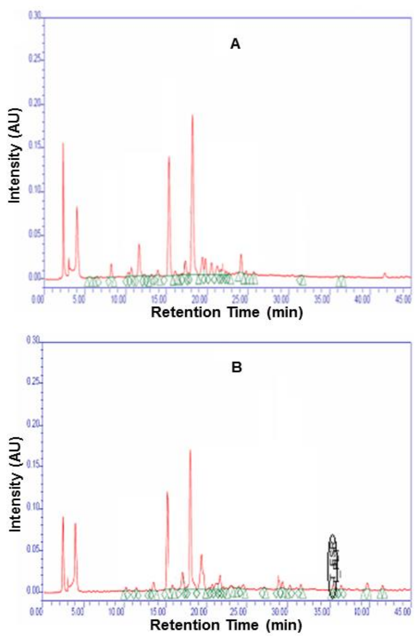


Figure 1b. Polyphenols identification for reverse phase HPLC from extracts of *F. cernua* without thiolysis (A) and with thiolysis (B).

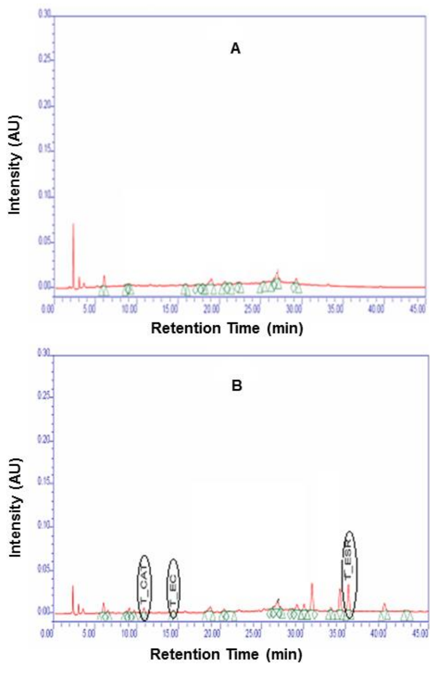


Figure 1c. Polyphenols identification for reverse phase HPLC from extracts of *T. diffusa* without thiolysis (A) and with thiolysis (B).

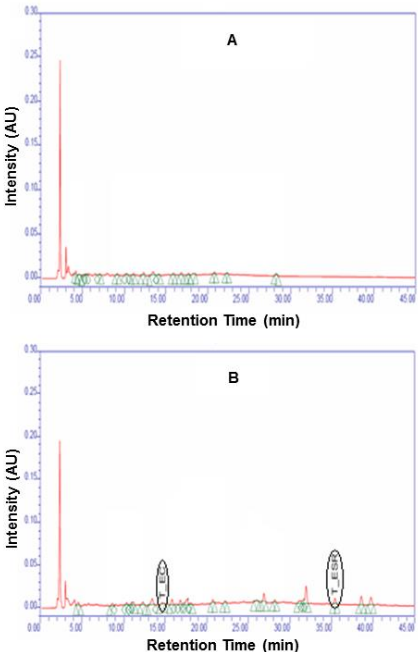


Figure 1d. Polyphenols identification for reverse phase HPLC from extracts of *J. dioica* without thiolysis (A) and with thiolysis (B).

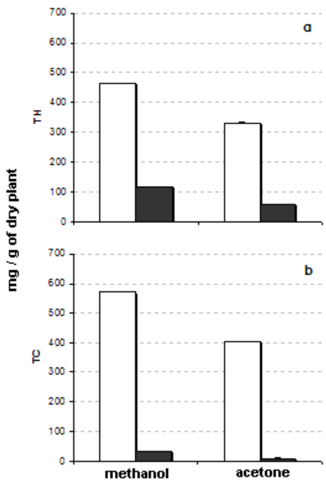


Figure 2. Effect of fermentation process on polyphenolic compounds concentration of *L. tridentata* extracted with methanol and acetone. a) Hydrolysed tannins (TH) equivalent to gallic acid and b) Condensed tannins (TC) equivalent to catechin. Pre-fermentation (□) and post-fermentation (■).

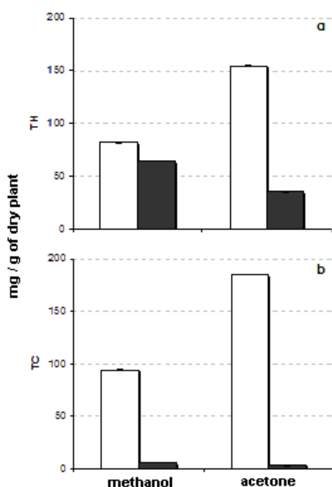


Figure 3. Effect of fermentation process on polyphenolic compounds concentration of *F. cernua* extracted with methanol and acetone. a) Hydrolysed tannins (TH) equivalent to gallic acid and b) Condensed tannins (TC) equivalent to catechin. Pre-fermentation (□) and post-fermentation (■).

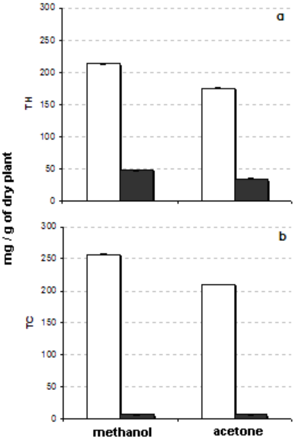


Figure 4. Effect of fermentation process on polyphenolic compounds concentration of *T. diffusa* extracted with methanol and acetone. a) Hydrolysed tannins (TH) equivalent to gallic acid and b) Condensed tannins (TC) equivalent to catechin. Pre-fermentation (□) and post-fermentation (■).

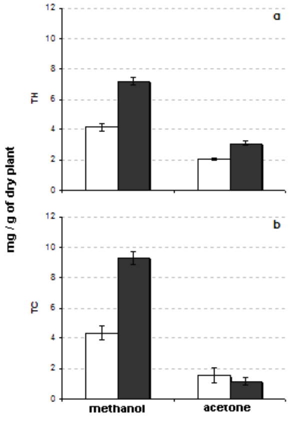


Figure 5. Effect of fermentation process on polyphenolic compounds concentration of *J. dioica* extracted with methanol and acetone. a) Hydrolysed tannins (TH) equivalent to gallic acid and b) Condensed tannins (TC) equivalent to catechin. Pre-fermentation (□) and post-fermentation (■).

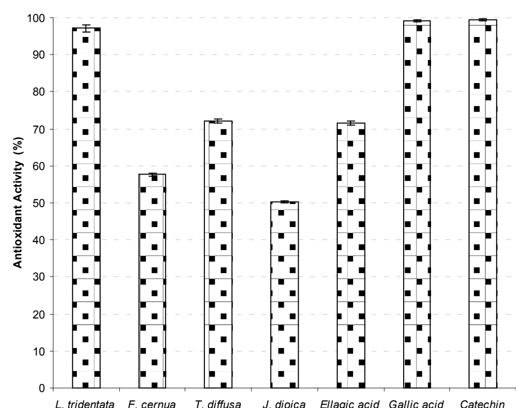
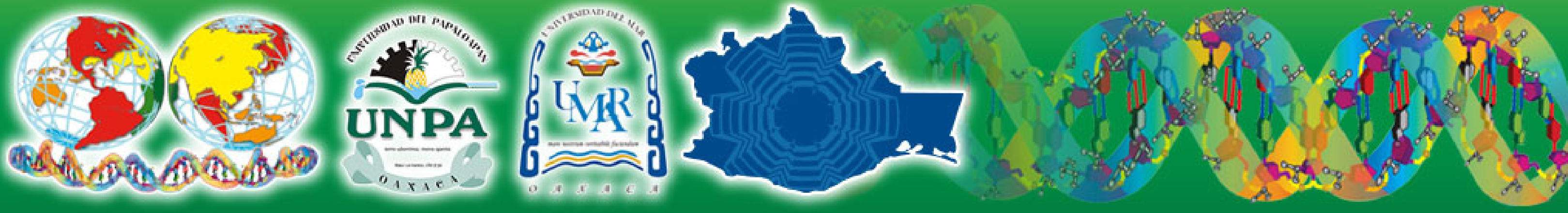


Figure 6. Antioxidant Activity of polyphenolic extracts of vegetal species from semi-arid Mexican zone and polyphenolic compounds (gallic acid, catechin and ellagic acid).

The antioxidant activity found in this plants of semi-arid Mexican zone, is greater to reported in *Allum sativum* L. (Eun-Jung, 2005), *Coriandum sativum* L. (de Almeida *et al*., 2005) and diverse varieties of raspberries rich in elagitaninos (de Ancos *et al*., 2000). Since the polyphenols can have unique papers like antioxidants and protectors of nutrients against oxidating damages, the investigations have been focused not only in foods and drinks like polyphenols source, but also in crude extracts and polyphenolic compounds structurally defined (Hagerman *et al*., 1998). For example, the good correlation between plum and apples trees polyphenols and their antioxidant capacity against the ABTS• radical (Kim *et al*., 2003). It is important to observe that the solid-state fermentation permits the release and recovery of potent phenolic antioxidants from plants from semi-arid Mexican zone, resulting in a high accumulation of gallic acid and catechin level and a biodegradation from polyphenols (Aguilar *et al*., 2008). Other phenolic antioxidants have been



produced in solid-state fermentation, mainly ferulic acid and *p*-coumaric acid from corn cobs enzymatically treated and fermented with *Sporotrichum thermophile* (Topakas *et al*., 2004).

Conclusions: Numerous studies have been conducted in the use of microorganisms under specific control processes (biodegradation) allows the achievement of new extracts and bioactive compounds with important applications on food and biopharmaceutical industries. These work is the first investigation that study the effect of a biotechnological process, such as solid-state fermentation, on the liberation or extraction of polyphenols from *L. tridentata Cov*., *F. cernua*, *T. diffusa* and *J. dioca* using *Aspergillus niger* GH1. The fermentative process is known about the pathways and the enzymes involved in this mechanism. This study is important, because the polyphenols could be biotransformed and liberated to simpler molecules with an important role in different industries, such as, agriculture, food and biopharmaceutical industries, easy to obtain. However, further research is needed on the purification of the bioactive compounds from the plants investigated in this study responsible for the beneficial effects observed. The use of microbial processes to obtain polyphenols compounds is also an interesting subject that should be studied in detail to ensure better applications of those compounds on the health field. The study of substances with neutralize properties of free radicals is an aspect of high medical importance, at present the interest has increased considerably to find antioxidant of natural origin for its use in food or medical material that allows replace synthetic antioxidants because the obtaining of the same is economic. In agreement with the results of this study the vegetal species evaluated here could be a new natural antioxidant source.

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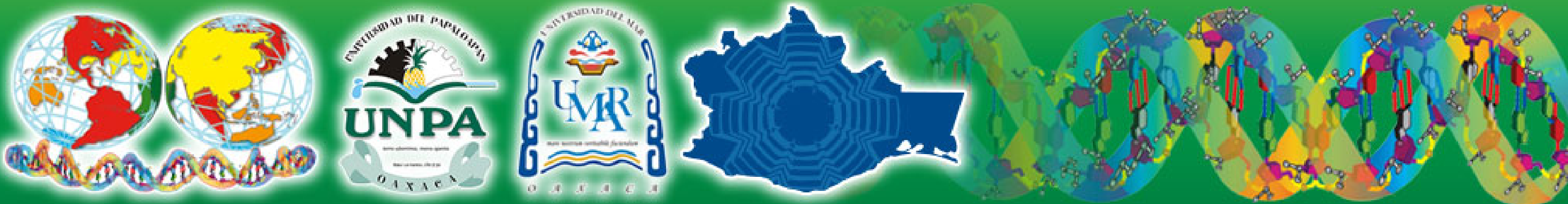

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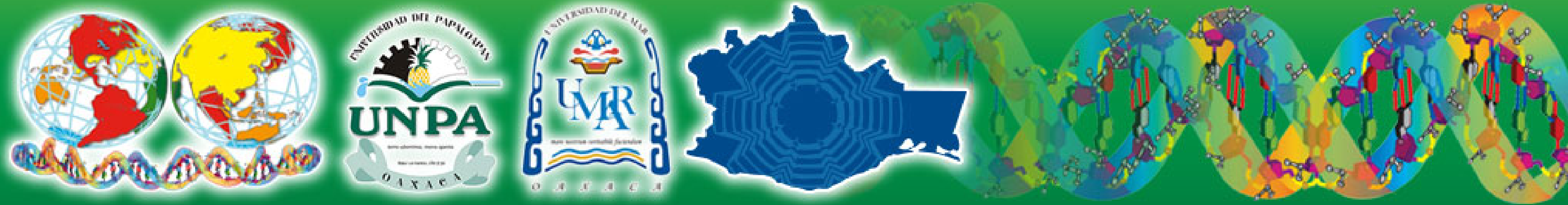

Lipid and protein productivity of *Scenedesmus* sp., *Chlorella* sp. and *Monoraphidium* sp. algae cultured in effluent from pisciculture

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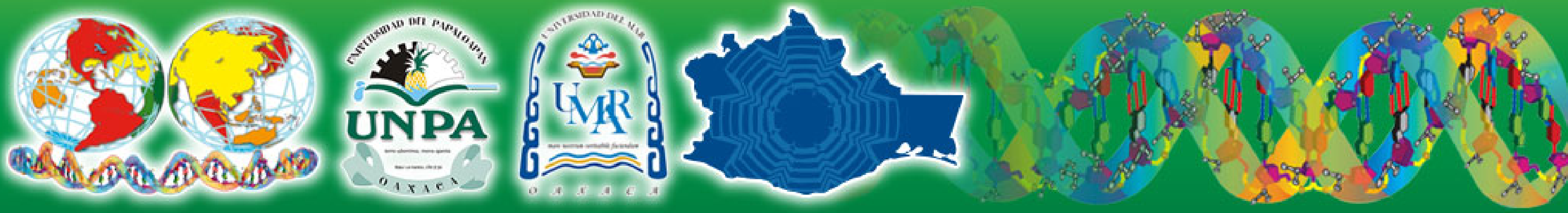
Abstract: *Scenedesmus* sp. *Monoraphidium* sp. and *Chlorella* sp. algae were cultured in two contrasting media: an effluent from tilapia pisciculture (TEM) and the synthetic basal Bold medium (BBM). Three volumes were used: 1.5, 4 and 9 L. Biomass productivity (Q_V), protein volumetric productivity (Q_{VP}) and lipid volumetric productivity (Q_{VL}) were estimated after 20 days in batch culture. Available phosphates and ammonium were measured before and after the cultivation period and then nutrient removal was estimated by subtraction. Data for each volume was analyzed as a 3 algae by 2 media factorial design with 2 replicates. Analyses of variance and Tukey tests for adjusted means were conducted at a 0.05 level. *Chlorella* sp. had the highest Q_V in BBM for the three volumes, followed in the 4 L volume by *Scenedesmus* sp. This last specie showed higher Q_{VL} than *Monoraphidium* sp. in BBM 1.5 L, as well as in the TEM 9 L photo-bioreactors (PBRs). *Scenedesmus* sp. showed higher Q_{VP} than *Chlorella* sp. and *Monoraphidium* sp. for the 1.5 L volume. *Scenedesmus* sp. and *Chlorella* sp. were able to remove more ammonium and phosphates from TEM, which is related with higher values of Q_V and Q_{VP}.

Keywords: Effluent for microalgae culture ● Freshwater microalgae in batch culture

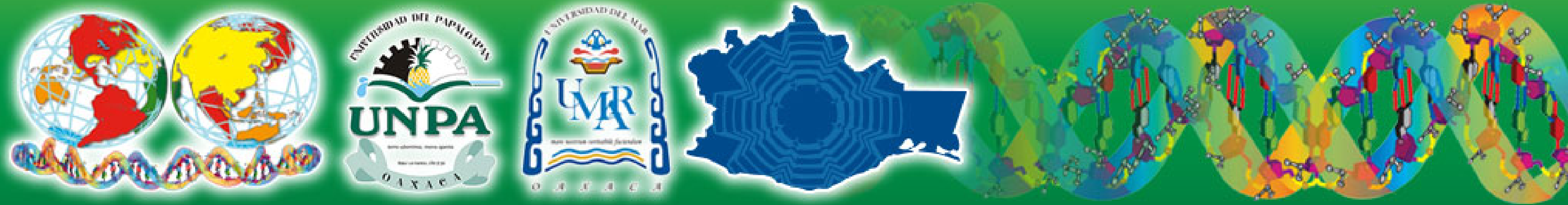
Introduction: Ability of freshwater algae to reduce organic compounds from effluents has long been studied in both tropical and temperate climates. Seasonal and long term changes in algae populations occur when they grow naturally in effluents under sanitization treatment. In those conditions, factors such as cell density, growth rate, biomass productivity and nutrient content can be used as the main indicators to choose, among many algae, the specie that produces efficiently in each medium (Barrera *et al.,* 2008). Effluents are comprised of many organic and inorganic compounds. Nutrient and pollutant content are present in a wide range depending on the industry from where they come from, for example: Oil, paper mills, livestock, wines, mining, leather, aquaculture, foods or services. Pisciculture effluent can commonly contain more than 7.3 mg L^{-1} of ammonium and more than 5.0 mg L^{-1} of phosphates.
Municipal wastewater may contain up to 20 mg L^{-1} of ammonium and up to 50 mg L^{-1} of phosphates, so this may be a suitable alternative for algae production. Besides, effluents do not contain toxic elements (Iturbide, 2008). Many algae species have been cultured for biomass production. According to Chisti (2008) algae biomass is one of the most promising sources of bioenergy, because of its high productivity. Bogen *et al.* (2013) found productivities from 0.03 to 0.36 g $L^{-1}d^{-1}$ with 31 and 15.9% lipid content for *Monoraphidium terrestre* and *Monoraphidium tortile*, respectively. In their data, among seven *Monoraphidium* species, the highest productivity occurred at the lowest lipid content and vice versa. Wu *et al.* (2013) studied the productivity of *Scenedesmus* LX1 algae in an open pond with domestic secondary effluent, finding a 20 g m^{-2} d^{-1} biomass production in 5 days long cultures, with 0.2 m depth, 16000 lx of light intensity and a biomass concentration of 500 g m^{-3}. They found that specific growth rate decreased sharply with depth. In another experiment with *Chlorella vulgaris* YSW-04 under batch culture, Ji *et al*. (2012) tried to optimize lipid content with piggery wastewater. In such conditions, biomass productivity



constantly increased as wastewater was partially replaced by a synthetic medium, so the pure synthetic medium produced the highest biomass. In the present experiment, productivity was compared among three sweet-water algae, in order to find the more suitable algae for biomass production under batch conditions, cultured in both Bold Basal Medium (BBM) and Tilapia Effluent Medium (TEM). Protein and lipid content were measured in the dry biomass produced. The main objective was to optimize biomass productivity by means of choosing the algae specie which better performs at each medium.

Materials and Methods:

The three algae species, *Chlorella* sp., *Monoraphidium* sp. and *Scenedesmus* sp., were isolated by sequential plate seeding method with Agar-Basal Bold Medium (BBM) from samples collected in natural freshwater ponds in Loma Bonita, Oax., Mexico. Morphological identification of the genera was completed with specialized taxonomical guides (Shubert, 2003). The strains were identified and named as Mon10UNPA-49, Sce10UNPA-44 and Chl10UNPA-45 from the Aquaculture Laboratory of the Universidad del Papaloapan. TEM was obtained from a 20 kg live weight stock of *Oreochromis niloticus,* line Gift, kept in freshwater glass aquariums in laboratory for 78 h under intensive feeding regime (3% of live weight in food per day). Then, the effluent was collected, filtered through a 55 µm zooplankton mesh and sterilized by boiling it for 10 min.
The effluent was cooled at room temperature and pH was adjusted to 7.5 (Hanna® Instruments pH meter) with 1M KOH and 1M HCl. Polyethylene terephthalate (PET) bottles with flat wall were used as photo-bioreactors (PBRs). Experimental dimensions were 1.5, 4 and 9 L. (diameter/height ratios were 9/21, 14.5/22, and 19.5/29 cm, respectively). PBRs were maintained in laboratory under steady light and temperature conditions. Two parallel lines of 60 W fluorescent Philips® T12 lamps (6000 lm) 20 cm apart were used, temperature was 25 °C. Air supply, without CO_2 enrichment, was adjusted from 1.5 to 4 L and from 4 to 9 L by a 1.7 factor according to preliminary assays, in order to optimize biomass productivity (data is not shown). Air flow was established at 1.6, 2.9 and 4.9 L min^{-1} for 1.5, 4 and 9 L, respectively. Cumulated biomass was harvested at the end of the growth period. Biomass dry productivity (Q) was measured in an electronic moisture analyzer (scale MOC-120H, Shimadsu®). Volumetric productivity obtained in g L^{-1} d^{-1} (Q_v) by means of the formula $Q_v = \mu Q$, where Q is biomass concentration in g L^{-1} and µ is the growth rate (Griffiths and Harrison, 2009; Li *et al.,* 2010). Dry matter samples were preserved to assess lipid concentration by the Bligh and Dyer method (1959) modified (Mandal and Mallick, 2009) through a 2:1 methanol/chloroform ratio by decantation.
Protein content was obtained by the Bradford method (Stepanchenko *et al*. 2011), Both lipid productivity (Q_L) and protein productivity (Q_P) were then expressed as volumetric productivities in accordance with Griffiths and Harrison (2009) (Q_{VL} and Q_{VP}). Available phosphates and ammonium were measured before the cultivation and after this period. Phosphates and ammonium removal was estimated by using contents measured before and after the culture period (Barrera *et al.,* 2008).
Statistical analysis was conducted by the MIXED procedure and LSMEANS were compared by Tukey test for multiple comparisons in SAS software. Analyses of variance for each volume were completed as a factorial design with 2 media by 3 algae with two replicates. The MIXED procedure gives a unique standard error for all the means in a media-algae group so all LSMEANS are compared, by the Tukey test, against any other mean in the 2×3 group. So the best combination media-algae can be found.



Results and Discussion:

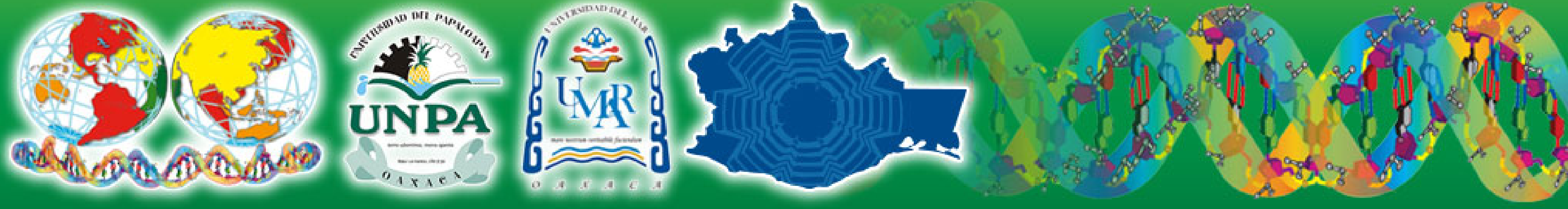
Volumetric productivity, lipid volumetric productivity and protein volumetric productivity (Q_V Q_{VL} and Q_{VP}) LSMEANS are shown by medium in Table 1 and for TEM+BBM results are showed in Table 2. Volumetric productivity, Q_V, was the highest for *Chlorella* sp. in BBM, followed by *Scenedesmus* sp. in the 4 L volume. *Monoraphidium* sp. showed the lowest Qv values for each of the three volumes. However, *Scenedesmus* sp. showed a higher Q_V when cultured in TEM for 1.5 or 4 L PBRs. Q_V was lower in *Chlorella* sp. when using TEM instead of BBM in 1.5 and 4 L volumes. This last finding agrees with Ji *et al.* (2012), who report that biomass production is reduced when an effluent substitutes a synthetic medium for *Chlorella* sp.

Table 1. Volumetric productivity (Q_V), lipid volumetric productivity (Q_{VL}) and protein volumetric productivity (Q_{VP}) of three sweet-water algae using Bold Basal Medium (BBM) or Tilapia Effluent Medium (TEM) in three volumes.

Volume/Specie	Q_V		Q_{VL}		Q_{VP}	
	BBM	TEM	BBM	TEM	BBM	TEM
1.5 L						
Chlorella sp.	0.152^{a}	0.07^{bc}	0.0094^{ab}	0.0037^{b}	0.0293	0.0165
Scenedesmus sp.	0.119^{ab}	0.153^{a}	0.0139^{a}	0.0129^{a}	0.045	0.0614
Monoraphidium sp.	0.034^{c}	$0.062b^{c}$	0.0035^{b}	0.0109^{a}	0.0088	0.0287
SE	0.017		0.0018		0.0089	
4 L						
Chlorella sp.	0.103^{a}	0.016^{d}	0.005	0.0015	0.0249	0.0102
Scenedesmus sp.	0.054^{b}	0.038^{bc}	0.0044	0.004	0.0205	0.0199
Monoraphidium sp.	0.021^{cd}	0.014^{d}	0.0024	0.0015	0.0096	0.0065
SE	0.005		0.0009		0.0037	
9 L						
Chlorella sp.	0.023	0.028	0.0014^{a}	0.0007^{ab}	0.0068	0.0105
Scenedesmus sp.	0.023	0.017	0.0007^{ab}	0.0014^{a}	0.0075	0.0037
Monoraphidium sp.	0.011	0.005	0.0015^{a}	0.0005^{b}	0.0044	0.0019
SE	0.002		0.0002		0.0014	

[a,b,c,d] :Means with different letter in the same column are different (P<0.05) for each volume.
[a,b,c,d] :Means with different letter in the same row are different (P<0.05) for each variable.
SE: Single standar error for the group of means above as reported by PROC MIXED.
*Any LSMEAN can be compared against any other in the six LSMEANS group (same SE).

Regarding Q_{VL}, only 1.5 L and 9 L volumes showed differences. *Chlorella* sp. had a lower Q_{VL} content than both *Scenedesmus* sp. and *Monoraphidium* sp. when cultured in TEM for 1.5 L volume. *Scenedesmus* sp. showed higher Q_{VL} than *Monorraphidium* sp. in BBM 1.5 L, as well as in the TEM 9 L PBRs. Volumetric productivity of proteins (Q_{VP}) was the highest for *Scenedesmus* sp. with respect to *Chlorella* sp. or *Monoraphidium* in the 1.5 L volume. This last alga had lowest Q_{VP} value in both BBM and TEM in the 4 and 9 L volumes in comparison with the other two algae species. Nutrient absorption (average for the three volumes) in TEM was: 82% of ammonium and



66% of phosphates by *Monoraphidium* sp., 92% of ammonium and 80% of phosphates by *Chlorella* sp. and 98% of ammonium and 90% of phosphates by *Scenedesmus* sp. This implies that algae adapt to produce in a mixotrophic environment (Sigee 2005; Barsanti and Gualtieri, 2006). *Scenedesmus* sp. had both higher nutrient removal and higher Q_V when cultured in TEM in comparison with those reached when cultured in BBM.

Table 2. Volumetric productivity (Q_V), lipid volumetric productivity (Q_{VL}) and protein volumetric productivity (Q_{VP}) in three sweet-water algae in three volumes (average for two culture media).

Volume - Specie	Q_V	Q_{VL}	Q_{VP}
1.5 L			
Chlorella sp.	0.111	0.0065	0.0229^b
Scenedesmus sp.	0.136	0.0134	0.0532^a
Monoraphidium sp.	0.048	0.0072	0.0187^b
SE	0.012	0.0012	0.0062
4 L			
Chlorella sp.	0.06	0.0032	0.0176^a
Scenedesmus sp.	0.046	0.0042	0.0202^a
Monoraphidium sp.	0.018	0.0019	0.0081^b
SE	0.003	0.0006	0.0026
9 L			
Chlorella sp.	0.026^a	0.0011	0.0087^a
Scenedesmus sp.	0.02^b	0.00108	0.0056^{ab}
Monoraphidium sp.	0.008^c	0.00101	0.0031^b
SE	0.001	0.00011	0.0009

^{a,b,c,d} :Means with different letter in the same column are different ($P<0.05$) for each volume.
SE: Standar error for the group of means above.

Conclusions: *Scenedesmus* sp. and *Monoraphidium* sp. are able to produce biomass in TEM or BBM equally, but *Monoraphidium* sp. had very low Q_V in both media. *Chlorella* sp. produces higher biomass than *Scenedesmus* sp. and *Monoraphidium* sp. when cultured in the BBM in 1.5 and 4 L PBRs. *Scenedesmus* sp. and *Chlorella* sp. are able to remove more ammonium and phosphates from TEM, they both give higher Q_V, higher Q_{VP} and greater Q_{VL} than *Monoraphidium* sp.

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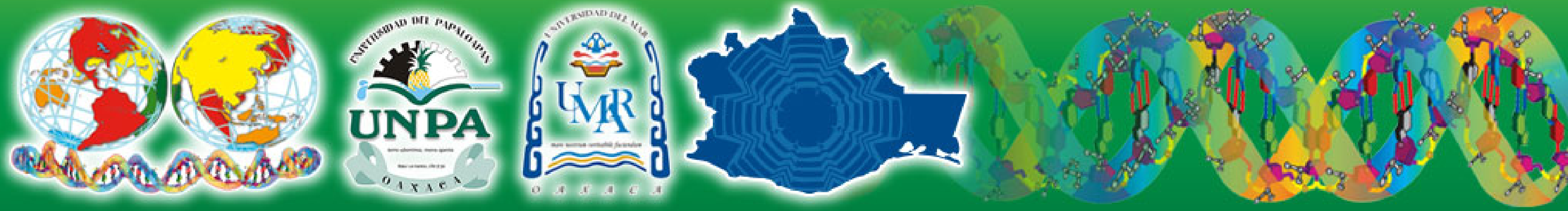

Antifungal activity of *Anagallis arvensis* extracts against phytopathogenic strains of *Rhizopus* spp., *Mucor* spp., and *Aspergillus flavus*

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Abstract: The biological activity of *Anagallis arvensis* against phytopathogenic fungi was investigated by evaluating the inhibitory effect of extracts of this plant on the *in vitro* growth of strains of *Rhizopus* spp., *Mucor* spp., and *Aspergillus flavus*. Ethanolic and petroleum ether extracts, prepared by the Soxhlet method, and aqueous extracts, prepared by decoction, were used. The antifungal activity was evaluated using the agar diffusion technique. For the concentrations used in this study, only the ethanolic extract was effective in inhibiting the growth of the plant pathogens tested. The zones of inhibition and the Minimum Inhibitory Concentrations (MICs) were determined and compared to those of Dithane®, which was used as a positive control chemical. The Activity Index (AI) was calculated. The MICs of *A. arvensis* ethanolic extract needed to inhibit mycelial growth were of 120 mg $well^{-1}$ for *Rhizopus* spp., of 120 mg/well for *Mucor* spp., and of 90 mg $well^{-1}$ for *Aspergillus flavus* ($p<0.05$). The corresponding activity indices were 0.93, 0.99, and 1.11, respectively. This research shows that the antifungal activity of ethanolic extracts of *A. arvensis* is comparable to that of Dithane® for all the fungi tested.

Keywords: Antifungal ● plant extracts ● phytopathogens ● *Anagallis arvensis*

Introduction: Fungal plant pathogens originate losses amounting to billions of dollars a year in Mexico. Fungi, such as *Aspergillus*, cause decay or deterioration of grains and legumes after harvest, during storage and transport, and fungi of the genera *Rhizopus* and *Mucor* sometimes affect fleshy fruits and vegetables (Agrios, 1996). In the search for new means to control plant diseases, natural products may become a source of environment-friendly fungistatic or fungicidal compounds. The efficiency of some saponin-rich crude plant extracts against plant pathogenic fungi has been already reported (Chapagain *et al*., 2007). In this regard, it may be useful to determine if the antifungal properties of *Anagallis arvensis*, which have been shown to exist against dermatophytes (Ali-Shtayeh *et al*., 1999), could be effective also against phytopathogenic fungi. *Anagallis arvensis* is an endemic plant in the State of Puebla and is widely distributed throughout almost all of the mexican territory and also throughout the temperate zones of the World (Hernández, 1987). The plant is widely used as a popular remedy for infected wounds and pimples, it is rich in saponins and has been shown to possess antioxidant properties (Lopez *et al*., 2008). Furthermore, triterpenes, sterols, flavonoids, and stigmasterol have been also isolated from the plant (Heitz, 1969). The objective of the present study was then to investigate the antifungal activity of extracts of *Anagallis arvensis* against phytopathogenic fungi.

Materials and Methods:

***Phytopathogens*.** Strains of *Rhizopus* spp., *Mucor* spp., and *Aspergillus flavus* were isolated and identified by the appearance of colonies through a macromorphological, micromorphological and physiological studies as stated in the litterature (Barnett *et al.,* 1998; Bonifaz, 2012), in the Mycology Laboratory of the BUAP. The isolated fungi were cultured in potato dextrose agar (PDA) tubes and incubated at 32 °C for 7 days to induce sporulation. For preservation purposes, was maintained in PDA culture tubes at 4 °C, and used as stock culture throughout the study.

***Preparation of extracts*.** Ethanolic, petroleoum ether, and aqueous extracts were prepared and used in the experiments according to a preliminary screening (Lopez *et al*., 2008), with some modifications. The powdered material (2 g) of all plants was extracted for 4 h at 4 °C with water (ratio 1:10) under stirring conditions. The homogenate was filtered and clarified by centrifugation at 5000 x g for 30 min at 4 °C. The supernatant was stored at -20 °C until further use.

***Evaluation of the antifungal activity*.** The minimum inhibitory concentration (MIC) of *A. arvensis* ethanolic extract needed to inhibit mycelial growth was determined for strains of three phytopathogenic fungi. Two different techniques were used to evaluate antifungal activity. For the aqueous and ethanolic extracts, the agar well-diffusion method was followed to determine the antimicrobial activity. Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with eight-hour-old broth cultures of fungi. Wells (10 mm diameter and about 2 cm apart) were made in each of these plates by using a sterile cork borer. About 20 µL of the concentration determined (MIC) for each extract were added with a sterile syringe into the wells and allowed to diffuse at room temperature for two hours. Control experiments comprising inocula without plant extract were set up. The plates were incubated at 32 °C for 7 days for fungal pathogens (Sen and Batra, 2012). The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. For the petroleum ether extract, the paper disc method to reveal any inhibitory effect of plant crude extracts. The discs were dried between each application. Negative control discs were prepared with 5×10 µL of the appropriate solvent, sterile water or ethanol. The agar well-diffusion method and the paper disc method were used positive control discs at concentration of 1 mg disc^{-1} were prepared with Dithane®. The Activity Index (AI) for a given extract was calculated as AI = zone of inhibition of the extract / zone of inhibition of the chemical control. Dithane® was the chemical control, an analysis of variance (ANOVA) and the mean comparison Tukey's test yield a significance level $p \leq 0.05$. All experiments for the antifungal activity were done in six replicates.

Results and Discussion:

The ethanolic extract of *A. arvensis* presented greater activity against *A. flavus* and had a lower MIC (90 mg well^{-1}) than against *Mucor* spp. and *Rhizopus* spp., for which it had the same MIC (120 mg well^{-1}) (Figure 1). The activity of the ethanolic extract is less than that of the chemical control in the case of *A. flavus* and *Mucor* spp. However, against *Rhizopus* spp, the extract, despite being at a lower concentration (120 mg well^{-1}), is more efficient than the standard (500 mg well^{-1}), as it may be seen in Table 1.
In the present study, the MIC value of the active plant extracts obtained in this study were lower than the Dithane values (Table 1) suggesting that the plant extracts were antimicrobial.

Table 1. Minimum inhibitory concentrations (MICs) of A. arvensis ethanolic extract and Dithane for the three phytopathogenic fungi tested.

Microorganism	*Ethanolic extract* MIC (mg well^{-1})	Dithane® MIC (mg well^{-1})
Mucor spp.	120	70
Rhizopus spp.	120	500
Aspergillus flavus	90	50

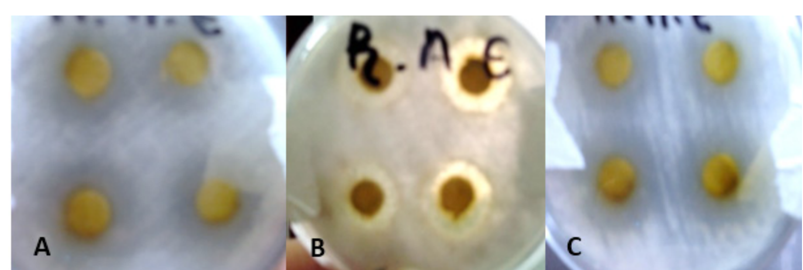


Figure 1. In vitro inhibition effect of ethanolic extracts of *A. arvensis* on PDA, A) *Mucor spp;* B) *Rhizopus spp* y C) *Aspergillus flavus.*

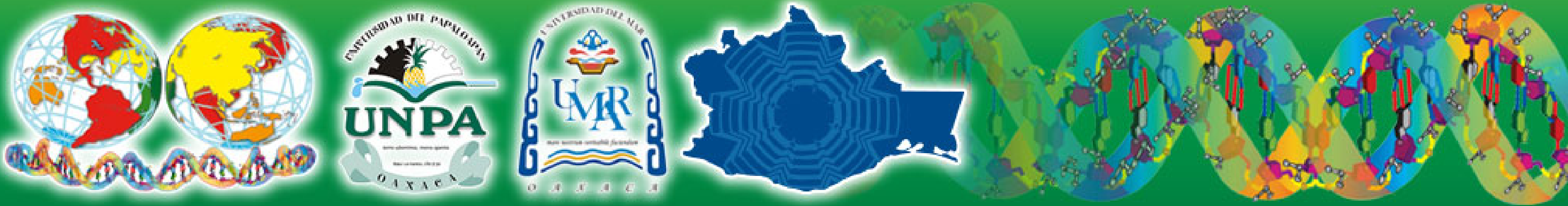
For the concentrations used in this study, neither aqueous nor petroleum extracts were effective in inhibiting the growth of the plant pathogens tested (data not shown). Other studies, however, have demonstrated the fungicidal activity of other polar extracts of *A. arvensis*, as reported by Lopez *et al*. (2011) for the case of methanolic extract, which has a high inhibition effect on the growth of *Candida albicans* (MIC = 0.31 mg mL^{-1}).

Table 2. Inhibition of the mycelial growth of three phytopathogenic fungi by *A. arvensis* ethanolic extract.

Microorganism	Zone of inhibition (mm)		Activity index (AI)
	Ethanolic extract	Dithane®	
Mucor spp.	13.13	14.08	0.93
Rhizopus spp.	12.80	11.50	1.11
Aspergillus flavus	15.04	15.13	0.99

Note. AI > 1: Activity of plant extract higher than activity of control chemical; AI <1: Activity of control chemical higher than activity of plant extract; AI = 1: No difference between activities of plant extract and control chemical.

Table 2 shows the antifungal AI of the ethanolic extract against all the fungi tested. These indices are 0.93 for *Mucor* spp., 1.11 for *Rhizopus* spp., and 0.99 for *Aspergillus flavus.* It may be seen that the highest antifungal effectiveness is against *Rhizopus* spp. Montes-Belmont (2009) has reported the antifungal activity of *A. arvensis* extracts against *Fusarium* and *Rhizopus,* as shown in this research for the latter. But there are no records in the literature of activity against the genus



Mucor. It is worth noticing the high antifungal activity that has been detected for the ethyl acetate extract of *A. arvensis*, with an inhibition of about 50% (Akarreta *et al*., 2008).

Conclusion: In this study, we evaluated the inhibitory activity of ethanolic, petroleum ether, and aqueous extracts of *A. arvensis* against the in vitro growth of *A. flavus*, *Mucor* spp, and *Rhizopus* spp. Only the ethanolic extract presented significant in vitro antifungal inhibitory activity, which was higher against *Rhizopus* spp.

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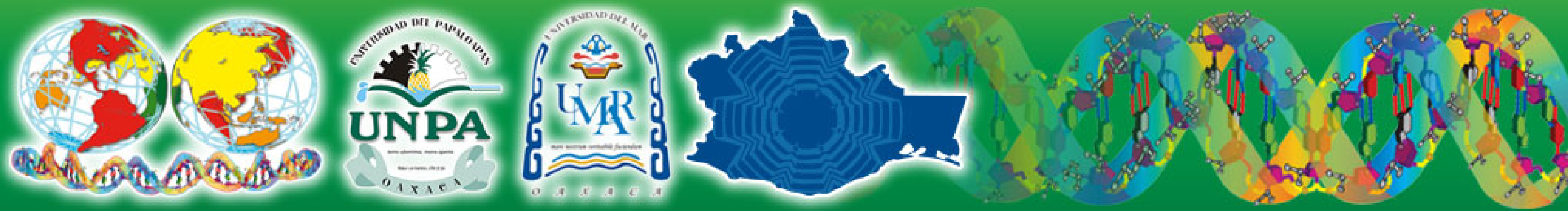

Biohythane production by two-stage anaerobic digestion process

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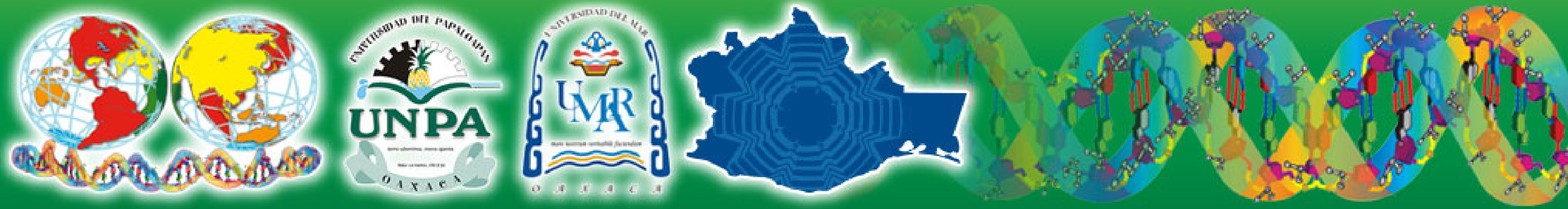
Abstract: Two-stage anaerobic digestion process has been previously studied in the form of an acidogenic step integrated with a methanogenic reactor to improve the methane production. Recently, the production of hydrogen and methane though two-stage anaerobic digestion process has attracted the attention of the researches. The mixture of hydrogen and methane derived from fossil fuels is called hythane and when it is obtained by biological means is called biohythane. When hydrogen is used as an additive to methane, some benefits are possible to obtain such as the increased flammability range of methane, and the decrease of the greenhouse gases emissions. This paper presents a mini review of biohythane production by two-stage anaerobic digestion process.

Keywords: Bio-hydrogen ● methane ● two-stage anaerobic digestion ● bio-hythane

Introduction: Energy is a key engine for the development of the society. Fossil fuels have favored the industrialization of the countries and improved the quality of life of the people. Around 85% of the energy demands worldwide are satisfied by the use of fossil fuels. However, the combustion of this non-renewable energy source produces many by-products that contribute to accelerate the climatic changes. Although the use of fossil fuels has satisfied the energy demands, this condition cannot be maintained in the future. Hence, the main environmental challenge that we have to face is to ensure the energy self-sufficiency and, at the same time, the reduction of the carbon dioxide emissions (Logan, 2008). It is necessary to develop a new energy platform to achieve this objective. An option can be found in the research and development of renewable energy carriers as hydrogen and methane produced by biological processes. The anaerobic digestion is a biological process that allows obtaining valuable products (e.g., bio-hydrogen, bio-methane, fatty acids) during the treatment of both liquid and solid wastes. Bio-methane is a versatile renewable energy source. It can be used to produce heat and electricity, and as fuel for combustion engines. Methane is frequently used not only as a chemical but also in transport industry in the form of compressed natural gas. On the other hand, hydrogen has been suggested as the energy vector of the future due to its advantages over the use of fossil fuels such as high energy content (120 kJ g^{-1}) and its combustion only produces water. Hydrogen has been pointed out as the cleanest energy vector while methane has been pointed out as a cleaner energy source in comparison to gasoline. (Hernández-Mendoza *et al*., 2013). The characteristics of bio-hydrogen and bio-methane make them an attractive option to obtain both energy carriers during the treatment of the wastes. This can be achieved by the use of two-stage anaerobic digestion process which couples the bio-hydrogen production with methane production by methanogenesis. The resulting mixture (biohythane) has been suggested as an adequate renewable energy source for the transition of a fossil fuel based economy to a hydrogen economy. The use of hydrogen as an additive for methane has many advantages. It can be used as a fuel in internal combustion engines without significant changes in its design (at low hydrogen content), improves the flammability range of methane, decreases the nitrogen oxides and greenhouse gases emissions, and reduces the



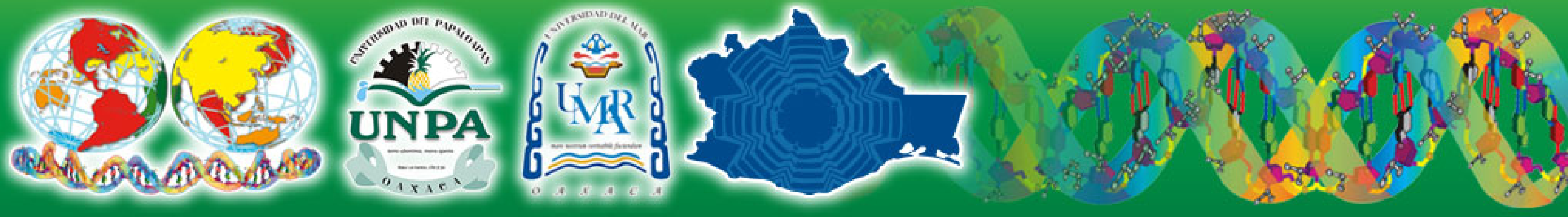
energy consumption (Villante and Genovese, 2012; Akansu *et al*., 2004, 2007). The main drawback to get the benefits of the use of biohythane is the necessity to have a hydrogen content in the gas mixture within 5% to 25%. The literature survey showed that when hydrogen-producing systems are properly integrated with methane-producing systems the hydrogen content in the biohythane is within the required range (Antonopoulou *et al*., 2008; Wang *et al*., 2009; Banks *et al*., 2010; Hafez *et al*., 2010; Cavinato *et al*., 2011; Hernández-Mendoza *et al*., 2013).

Material and Methods:

The papers for the analysis were obtained from the Scopus database. The search was done using the following keywords: two-stage anaerobic digestion, two-phase anaerobic digestion, bio-hydrogen, methane, and bio-hythane. After a refining search some papers were considered for this review.

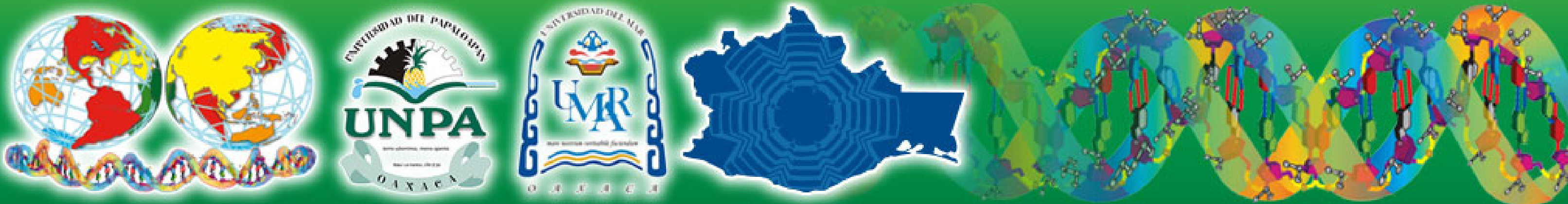
Results and Discussion:

Many researchers have evaluated the use of natural compressed gas (85-98% methane) mixed with hydrogen as an energy source (Das *et al*., 2000; Bauer and Forest, 2001; Akansu *et al*., 2004, 2007; Ortenzi *et al*., 2008; Villante and Genovese, 2012). Hythane has been commercially used as fuel in vehicles in USA and has attracted the attention of some companies as Volvo and Fiat. During the last two decades, the use of hythane has been extensively promoted for its use in public transport vehicles (Midha *et al*., 2011). However, the production of methane and hydrogen by an independent way from fossil-based materials is unsustainable and energy intensive. Currently hydrogen is produced by steam reforming of natural gas (40%), from oil and naphtha (30%), from carbon (18%), and from water electrolysis (4%) (Sinha and Pandey, 2011). Unfortunately, these processes release the same carbon dioxide quantity than the one produced by fossil fuels combustion (Lee *et al*., 2009). One of the challenges for the use of hythane as energy source lies on hydrogen production from an environmental friendly method. An option to overcome this challenge is by the use of two-stage anaerobic digestion process that is a modification of typical anaerobic digestion process. The anaerobic digestion is a complex biological process in which different microorganisms interacts to degrade the organic matter during four different and well defined steps (Gerardi, 2003). These include the hydrolysis of the proteins, cellulose, lipids and other complex organics. The acidogenesis in which is possible to obtain valuable by-products as hydrogen and volatile fatty acids (e.g., acetic acid, butyric acid, propionic acid). The conversion of hydrogen and carbon dioxide to acetic acid is carried out during the acetogenesis, and the methanogenic step in which the volatile fatty acids produced are converted into methane and carbon dioxide. In typical or single-stage anaerobic digestion the entire process is carried out in one reactor. Two-stage anaerobic digestion processes consist in separate, by a physical barrier (e.g., using 2 reactors), the acid forming steps from the methanogenic step. The hydrogen-to-methane pathway has to be blocked to obtain hydrogen from the overall process while avoiding its consumption by hydrogen-consuming microorganisms (e.g. methanogenic archeas, propionic-acid forming bacteria). In the first stage acidogenic bacteria hydrolysates the organic matter to transform it into volatile fatty acids, hydrogen, and carbon dioxide. In the second stage, the remaining organic matter is converted into acetic acid, by the acetogenic bacteria, that is further transformed into methane and carbon dioxide by methanogenic archeas. This configuration has improved the metanogenic step (Han and Shin, 2004; Ueno *et al*., 2007; Escamilla-Alvarado *et al*., 2010) increasing up to 21% the methane production in comparison with



single-stage anaerobic digestion systems (Liu *et al.*, 2006). Some researchers have reported that the total energy recovery (hydrogen and methane) was significantly higher when compared with a typical anaerobic digestion system (Escamilla-Alvarado *et al.*, 2010; Schievano *et al.*, 2014). The co-production of bio-hydrogen and methane reduces the fermentation time (Ke *et al.*, 2005; Ueno *et al.*, 2007), allows higher organic loading rate (Kongjan *et al.*, 2011; Luo *et al.*, 2011) and the solubilization and sacharification of biomass can be simultaneously done during the first stage (Ueno *et al.*, 2007). The anaerobic digestion of wastes is a mature technology in many aspects (Mata-Alvarez *et al.*, 2000). Bio-methane production and exploitation is an accepted and commercial technology (Reith *et al.*, 2003; das Neves *et al.*, 2009). Process parameters as reactor configuration, temperature, pH, nutrients and type of substrate have to be defined to increase the methane production and have a stable behavior of the system (Ke *et al.*, 2005). Methane obtained from anaerobic digestion is competitive in efficiencies and costs in comparison with other biomass energy forms as synthesis gases and ethanol (Chynoweth *et al.*, 2001). In contrast, bio-hydrogen production is yet a technology under development. Hence, the major drawback to produce bio-hythane at large scale is related with the bio-hydrogen production. To obtain a stable bio-hydrogen and methane production the integration of the bioreactors has to be done in an efficient way. Ke *et al*. (2005) indicated that the stability of both bio-hydrogen and methane producing systems can be affected in two-stage anaerobic digestion processes. However, other researchers have pointed out that the stability of the two-stage anaerobic digestion process was higher than the one-stage process (Kjongan *et al*., 2011; Luo *et al*., 2011) and instability of reactor is related with the physical separation of the acid-forming phases from the methanogenic phase which could negatively affect the hydrogen transfer and the syntrophic association of hydrogen and methane producing microorganisms. Supernatant recirculation, from the methane-producing bioreactor to the hydrogen-producing bioreactor is a common practice to mix and regulate the pH of the integrated system. Nevertheless, this could result in the contamination of the microbial community of the acidogenic process and the deviation of the metabolic pathway (Wang and Wan, 2009). Such pathway shift may reduce the hydrogen production due to hydrogen consumption by hydrogenogenic microorganisms and can favor the establishment of non-hydrogen-producing microbial community. The prevention of the proliferation of hydrogen-consuming microorganisms into hydrogen-producing systems is a major concern and challenge (Kraemer and Bagley, 2005; Zhu *et al.*, 2011; Hernández-Mendoza and Buitrón, 2014; Hernández-Mendoza *et al.*, 2014). To inhibit as much as possible the activity of those hydrogen-consuming microorganisms some pretreatment methods have been developed (Wang and Wan, 2009; Hernández-Mendoza and Buitrón, 2014). The operation of the hydrogen-producing reactor is complex and hydrogen-producing systems depend on different operational process parameters. Some of the parameters that influence hydrogen production are the inoculum source, bioreactor configuration, temperature, pH, organic loading rate, hydraulic retention time, and hydrogen partial pressure, among others (Wang and Wan, 2009). The interaction of such parameters can favor the hydrogen production pathway over other non-hydrogen-producing metabolisms. It is know that to achieve the maximal hydrogen production rate and yield is necessary to determine the optimal values of the operational parameters (Hernández-Mendoza and Buitrón, 2013). Unfortunately, those optimal values cannot be known previously and have to be determined during the reactor operation.

Conclusion: Biohythane is a renewable energy carrier that can be used as fuel for internal combustion engines and the main limitation to produce biohythane at commercial scale is related with bio-hydrogen production. The adequate integration of both hydrogen-producing and



methane-producing systems has many challenges. And major concern about biohythane production is related with the effects on the microbial evolution and stability of the hydrogen-producing system.

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MicroRNA expression and function in female reproductive tissue of *Arabidopsis thaliana*

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Abstract: MicroRNAs are noncoding RNAs of 21-22 nucleotides (nt) in length that act as repressors of genes, these genes are important in key developmental processes in many organisms. In plants target genes can be suppressed at translational level or degraded by action of Argonaute proteins, this molecular mechanism is called RNA silencing. More than half of the known Arabidopsis miRNA target genes encode transcription factors; thus, miRNAs regulate various developmental processes that include leaf morphogenesis, juvenile-adult transition, floral identity and fruit formation. In *Arabidopsis thaliana*, the AGO family protein have 10 members of which two have been unambiguously associated with different forms of RNA silencing. AGO mutants and other miRNA biogenesis genes, has been show the biological relevance of miRNAs in cell fate and identity of reproductive tissue, particularity the *ago9* mutant phenotype is reminiscent of apospory, a component of asexual reproduction through seeds (apomixis). Open questions about the biological function of miRNA interactors of AGO9 have not been explored. In this work we studied the pattern expression of 13 miRNAs that interact with AGO9 and with loss-of-function miRNA mutant, qRT-PCR and over-expression experiments we able to elucidate the partial function of two miRNAs in female reproductive tissue of *Arabidopsis thaliana.*

Keywords: microRNAs ● Arabidopsis ● ARGONAUTE

Introduction: MicroRNAs, are genome-encoded noncoding RNAs of 21 nucleotides (nt) in length that act as repressors of target genes, miRNAs use base pairing to guide RISCs to specific messages or partly complementary sequences. Repression of the target transcript by miRNAs may occur through translational inhibition, accelerated exonucleolytic mRNA decay, or slicing within miRNA-mRNA base pairing (Voinnet, 2009). MiRNAs act at transcriptional (TGS) and posttranscriptional (PTGS) level, where ARGONAUTA (AGO) proteins are involved; these silencing mechanisms are conserved across the organisms, to keep genome integrity. The AGO proteins bind miRNAs 21-22 nt in size, and together form a complex RISC that recognizes the target gene. In plants miRNAs are involved in various processes in development; mutants in the model plant *Arabidopsis thaliana* such as *ago1, dcl1, hen1, hyl1* and *hst*, have severe defects in the development of *Arabidopsis*, the null mutants of *ago1* and *dcl1* are embryonic lethal indicating the biological importance of miRNAs in the development of the seeds of flowering plants (Mallory and Vaucheret *et al*., 2006). In *Arabidopsis* the *ago9* mutant phenotype is reminiscent of apospory, a component of asexual reproduction through seeds (apomixis), the study of the molecular basis of miRNAs interactions of AGO9 opens the possibility to explore new possibilities to know more about miRNAs in female gametophyte (Olmedo-Monfil *et al*., 2010). In this work we studied the pattern expression of 13 miRNAs interactors with AGO9 and elucidated the function of two miRNAs in female reproductive tissue of *Arabidopsis thaliana.*

Materials and Methods:

Plant materials. All plants used in this work were of the Columbia (Col-0) ecotype. Seeds were surface sterilized with 100 % ethanol or with chlorine gas and germinated under stable long-day (16 h light/8 h dark) conditions in Murashige and Skoog (MS) medium at 22 °C. After one week seedlings were planted and grown under controlled greenhouse conditions (24 °C).

Constructs. Transcriptional fusions were generated by amplifying different segments of the regions corresponding to MIR genes. MiR1a was PCR amplified from wild-type genomic DNA, using the following primer combination (Figure 1):

MiR1a, 5'- AAGCTTGAGCTTTGTTACAGCCC -3' and TCTTACTTCAACTGCAACCTTG-3'

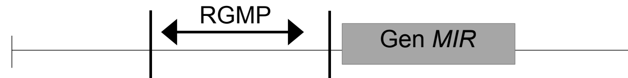


Figure 1. Promoter region of miRNAs (RGMP). Selection of the promoter of microRNAs was the region 5´uptream of MIR gene.

This promoter (RGMP) was cloned into pBlueScript KS (-) and digested with HindIII and XbaI, DNA fragments were subcloned into binary pBI101.3 to generate transcriptional fusions with the reporter gene uidA (GUS).

Generation of transformants. Resulting pRGMP::GUS plasmids were transformed into *Agrobacterium tumefaciens* strain C58C1 (Chen *et al*., 1994) and subsequently into *Arabidopsis thaliana* Columbia-0 by floral dipping as previously described (Zhang *et al*., 2006). Seeds obtained were germinated in MS medium containing kanamycin (50 mg mL^{-1}). Kanamycin-resistant individuals were confirmed as transformants by conducting PCR using the pBI101.3–Fw/GUS-Rv primer combination to amplify a RGMP fragment.

RT-PCR. Total RNA was isolated from flower, cauline leaf, rossete leaf and siliques by Trizol® reagent (Invitrogen). miRNA was amplified from cDNA with the following primers: miR1a, 5'-CGGGTGGTCACCGCATCTTTTGTT -3' and 5'-CGCGCCGGCGCTCAAGAAGA-3', ubiquitin was used as a loading control in RNA (Varkonyi-Gasic *et al*., 2007).

Histochemical Analisys. Inflorescences were fixed in FAA (10% formaldehyde, 5% acetic acid, and 50% ethanol) for 12 h and subsequently dehydrated in 70% ethanol. Gynoecia at different developing stages were dissected with insulin syringes, cleared in Herr's solution (phenol:chloral hydrate:85% lactic acid:xylene:clove oil in a 1:1:1:0.5:1 proportion), and observed with Nomarski optics using a DMR Leica® microscope. Histochemical localization of GUS activity was performed by incubating dissected gynoecia in GUS staining solution (10 mM EDTA, 0.1% Triton® X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid in 50 mM sodium phosphate buffer, pH 7.4) for 48 h at 37 °C.

Results and Discussion:

miR1a is expressed in female gametophyte. Our analyses show of promoter:GUS plants suggested that miR1a is expressed in multiple organs (data not show) but the expression in female gametophyte in more evident, specifically in the zone called micropylar pole (Figure 2C), where the synergid cells are located. This suggested that miR1a could regulate the process involved with the synergid cells and the pollen tube, when the double fertilization occurs. In most resistant tissues, staining patterns of the GUS fusions were very similar.

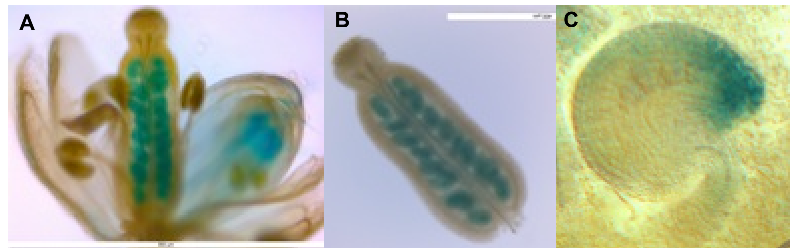


Figure 2. Expression patterns of miR1a promoter::GUS fusión. (A) Flower of *Arabidopsis thaliana*, promoter miR1a::GUS expression was observed only in ovules, (B) Carpel of *Arabidopsis thaliana*, promoter miR1a::GUS expression was observed in ovule also staining patterns was observed in petals and filament stament. (C) Ovule with staining patterns in the micropylar pole.

Target genes 1 and 2 are regulated by miR1a. To elucidate which are the specific expressions of miRNAs in diverse tissues of *Arabidopsis*, we made a RT-PCR for miR1a, the results show high expression in rosette leaf, cauline leaf, stem and silique, and however in flower the expression was low. Analyses for target genes of miR1a show low expression in all tissues except in flower, suggesting the regulation of target gene 1 and 2 by miR1a (Figure 3).

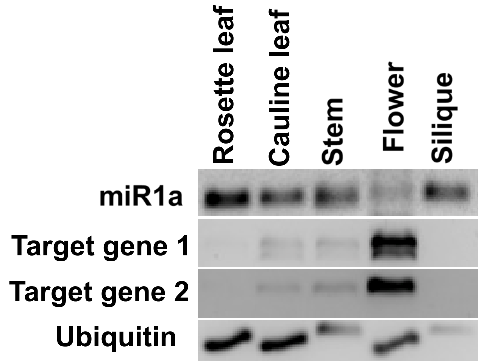
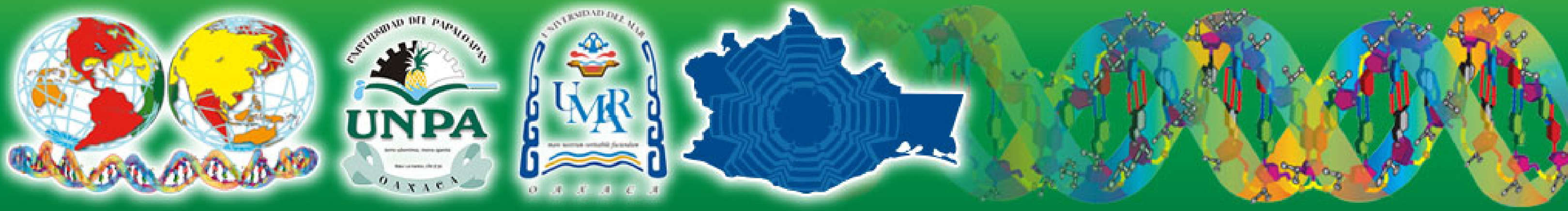


Figure 3. Semicuantitative RT-PCR of miRNA and target genes of miR1a. Analyses of miR1a in diverse tissues of Arabidopsis show the localization of this miR1a in all the tissues except in flower, however target gene 1 and 2 show low expression in all tissues except in flower, suggesting that target gene 1 and 2 is regulated by miR1a.



Conclusions: Promoter miR1a::GUS show specific expression in the ovule of Arabidopsis, especially in the micropylar zone. Our analyses of RT-PCR suggested that miR1a regulates both target genes 1 and 2 respectively, in all tissues except flowers. In order to understand the biological function of miR1a in reproductive tissue, actually we performed experiments for analysis of phenotype of loss-of-function mutant and over expression of miR1a. This results will be reflected in the symposium meeting

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Analysis of soil microbial community forest by PCR-DGGE and changes associated with the charcoal production in guanajuato

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Abstract: The temperate forest of oak (*Quercus* spp.) in Santa Rosa, Guanajuato, is considered by CONABIO as one of the priority regions for study and conservation of the country, due to its wealth of biodiversity. Its watershed also has a very important role in regulating the hydrological cycle of Bajio Guanajuato region. The main forestry activities are logging and process for charcoal in places called "kilns", both products are still used as fuel in rural and sub-urban areas in Mexico, Latin America, Middle East, and Asia. This activity has led to changes in the physicochemical properties of soil that affect the structure and function of soil forest microbial community. The high temperatures generated during the production of coal cause selective mortality of the microbial community and nitrogen loss by volatilization. Charcoal Production Area (CAR), Adjacent Charcoal Area (ADY) and Conserved (CON): In this study soil samples (0-10 cm) were taken from three sites. Soil DNA was obtained, the 16S genes and the genes coding for nitritoreductasa (*nirK*), nitrogenase (*nifH*) and amoniomonooxigenasa (*amoA*) were amplified by PCR to assess the functional diversity of microbial communities involved in the nitrogen cycle.

Keywords: Charcoal production ● Soil quality ● DGGE

Introduction: Electrophoresis DGGE denaturing gradient gels, is useful for directly determining the ecology and genetic diversity of complex microbial population as in the case of total DNA from soil samples tool. With the band patterns of each site was found: a decrease in the Shannon diversity index in the coal CON> ADY> CAR, the dendrograms generated showed differences between sites of the coal adjacent and conserved. The main objective of this work was to show how the production of charcoal affects the functionality and diversity of soil microbial community forest. Forest practices invariably change soil properties and processes in the (cycle N) are performed, with a generally negative effect. Understanding under what conditions affect the presence or absence of bands of microbial groups represented, as an indicator of the quality of the forest floor, they serve to adapt forest management practices. The overexploitation of forest resources of the Sierra de Santa Rosa was mainly the use of different tree species for selling firewood or charcoal. In the forestry you can see the disturbance by charcoal production in different microclimatic conditions to the rest of the forest, more solar radiation, exposure to wind and water erosion and extreme temperatures (Vázquez *et al.*, 2003). The bunkers are prepared by removing vegetation, litter and soil organic layer in a circular (7-10 m diameter). An oven with segment built by logs stacked in the center and branches, litter and logs on the top of them. This oven is set alight and smothered combustion which lasts 8 to 10 days is generated. The heat generated during the production of carbón is induced by chemical oxidation of organic matter altering the processing of carbon and nitrogen. High temperatures kill immediately part of the soil microbial community. Some groups of microorganisms are more sensitive to heat than others, especially those that are

highly specialized and nitrite oxidizers (*Nitrobacter* spp) have a 99% mortality at 80 °C and similarly the nitrifying bacteria (Hart *et al*., 2005).

Materials and Methods:

Soil samples (0-10 cm depth) of the forest of Santa Rosa, Guanajuato, 3 sites were taken from three sites: Charcoal Production Area (CAR), Adjacent to the Charcoal production Area (ADY) and Conserved (CON) at two sampling times (rainy and dry seasons). DNA extraction from soil was performed with the method Vázquez *et al*. (2002). The analysis of microbial communities from soil by PCR-DGGE was performed using the 16S gene and three gene regions for groups of N. Oligonucleotides for gene 16S Muyzer *et al.* (1993) amplify the región Variable V3 and 16S (Widmer *et al.,* 1998). For ammonium oxidizers were used oligonucleotides for the *amoA* gene (Avrahami *et al*., 2002), for denitrifying oligonucleotides for the *nirK* gene (Rösch *et al.,* 2002) and the fixing locations of N with oligonucleotides for the *nifH* gene (Rösch *et al.,* 2002). DGGE was performed in acrylamide / bisacrylamide 8% (Acrylamide / Bis 37.5:1). 16S gradients were 30-45%, *amoA* 15-40% and *nifH* and *nirK* 20-45% for 14 h at 60 °C and 45 V, the gels were silver stained. With the band patterns, the Shannon diversity index was calculated and dendrograms were generated by multivariable analysis with the program SYSTATMR ver. 11.

Results and Discussion:

It was possible to obtain total DNA samples from soil charcoal production area (CAR), adjacent to the charcoal production (ADY) and the conserved forest (CON) areas (Figure 1). Amplification products 1B obtained 16S and nitrogen groups are separated by DGGE gel (Figure 2). Multivariate analysis with banding patterns of each amplified region, generated a dendrogram clustering in Euclidean distances Figure 2A, B, C, D and E. The trend for the calculated Shannon diversity index for the gene 16S was CON> ADY > CAR; with amoA in the rainy season ADY> CON> CAR and dry season CON> ADY> CAR; nifH dry season CON> CAR> ADY and nirK dry season ADY> CON> CAR. The dendrograms showed differences between sites and sampling season.

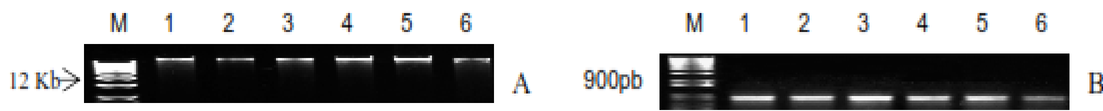

Figure 1. A) DNA from soil and B) Amplification of 16S. 1) Charcoal dry season CARS, 2) adjacent to the charcoal dry season ADYS, 3) Conserved dry season CONS, 4) Charcoal rainy season CARL 5) adjacent to the Charcoal rainy ADYL, 6) Conserved rainy season CONSL. M molecular weight marker 1 Kb plus ladder. Agarose gel 1% with ethidium bromide staining.

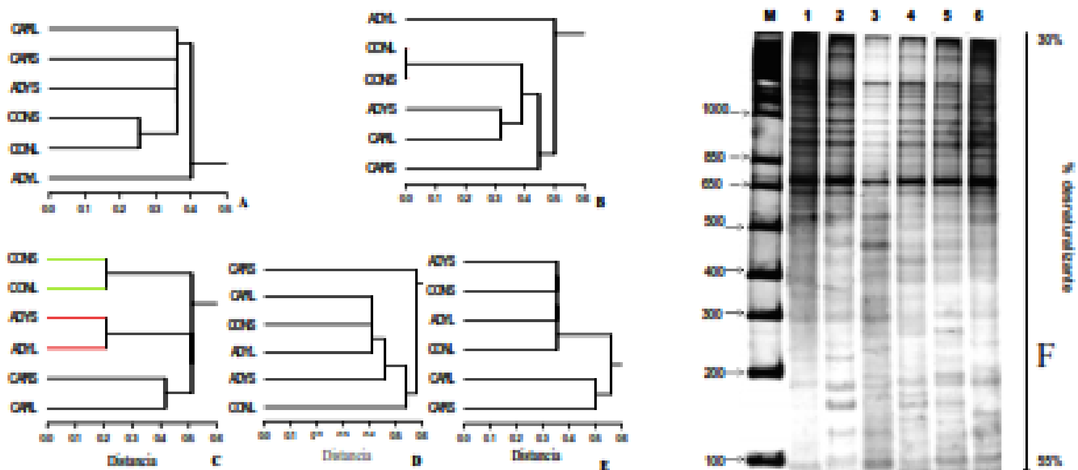

Figure 2. Dendrograms generated from multivariate analysis by A) 16S 900 bp, B) 16S 233 bp, C) *Amo*, D), *NifH* E) *nirK* and F) Image amplification by PCR-DGGE of 16S.

Conclusions: Changes in the forest soil of Santa Rosa, Guanajuato caused by charcoal production, generated a type of disturbance with particular characteristics, due to the high temperatures reached, the remains of coal, physicochemical changes of the soil (pH) and loss of organic matter, as well as microclimatic conditions resulting after using these sites. These generate changes changes in the structure and function of soil microbial community with a impact on short and long term. The analysis of the structure of specialized microbial communities such as the ammonium oxidizers, denitrifying and N fixers are excellent indicators of soil productivity and functionality, microorganisms are highly sensitive to changes, they are the first to respond, this feature makes them good indicators of quality, this characteristic allow us to assess the health of the forest ecosystem. To find some groups of microorganisms involved in of the N cycle are decreased or absent, could be assumed that storages result in shortages in the N availability will be observed in the sites of charcoal producction. The loss of key microbial groups alters natural soil processes (degradation of organic matter, recycling and nutrient availability) and cycles of C and N. The study of this type of disturbance should lead us consider improvements in forest management practices in order to become they sustainable.

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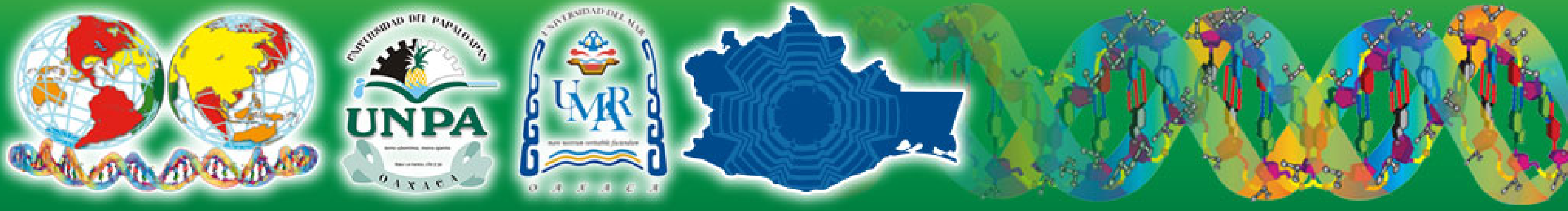

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Ultraviolet absorption of *Opuntia* mucilage

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Abstract: *Opuntia* is a plant widely known in Mexico and all around the world because of its nutritional benefits, resistance to stress factors and capacity to adapt to hard growing conditions. In recent years, several applications and curative properties of *Opuntia* have been proposed including its contribution to stabilize and regulate the glucose level in patients with diabetes type II, the antibiotic action of its crassulacean acid and the elaboration of cosmetics and paints with its chemical products. Here, the ultraviolet absorption of the *Opuntia* mucilage was analyzed in search of unconventional applications. Besides, we discuss about the feasibility of employing the *Opuntia* mucilage as active ingredient of sunscreens.

Keywords: Unconventional applications ● UV spectrum ● sunscreens ● active ingredient

Introduction: In the field of sun protection, dermatologists recommend the daily use of sunscreens and reapplication at regular time intervals to avoid injuries on the skin and diseases such as accelerated ageing, erythema, and cancer (Diffey, 2011; Latha *et al*., 2013; De Fabo *et al*., 2004; Thompson *et al*., 1993). However, the continuous and prolonged use of sunscreens elaborated with complex mixtures of chemical compounds also may induce non-desired collateral effects given that the skin may absorb chemicals producing a progressive intoxication of the human body until potentially dangerous levels (Scherschun and Lim, 2001; DeBuys *et al*., 2000). Currently, people are more conscious about the injuries that the chemical products may lead to the human health and the environment (Reena *et al*., 2012). Every day, more people prefer to consume organic items such as food and other products in order to minimize the risk of suffering harmful secondary effects of the chemicals used in the elaboration of a large number of common-use items, including sunscreens. For that reason, it was analyzed an organic material that may be employed in the elaboration of items such as sunscreens that are friendly with the human health and the environment. The organic material studied was the *Opuntia* spp mucilage due to its high availability, low cost, and relatively simple extraction. *Opuntia* is a cactaceae characterized by cladodes recovered by glochids (Figure 1). Usually, it has a high water content ranging from 90.0% to 92.5% and its main minerals are calcium and potassium, in addition to silica, sodium and marginal amounts iron, aluminum and magnesium. Recently, *Opuntia* was suggested as auxiliary remedy in the treatment of arteriosclerosis, constipation, gastric ulcers, diabetes type II, high levels of cholesterol and triglycerides (Saenz *et al*., 2006). Moreover, it is increasing the use of *Opuntia* in the development of cosmetics, especially in the manufacturing of products for hair and skin because its mucilage is a hydrocolloid able to retain moisture (Saenz *et al*., 2006).

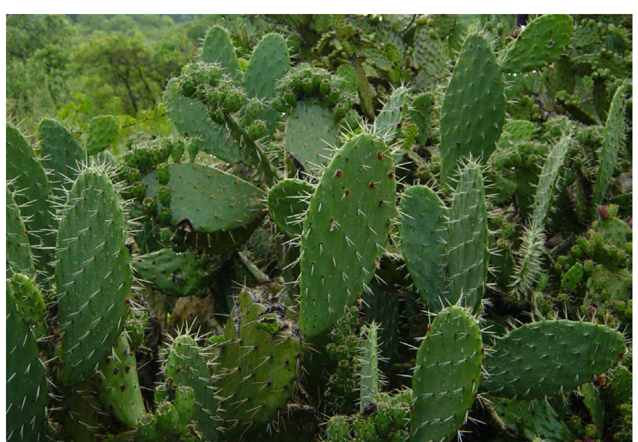

Figure 1. Cladodes of *Opuntia* spp.

In this work, the ultraviolet absorption of the *Opuntia* spp. mucilage is investigated in search of other unconventional applications. Also, the absorption of the *Opuntia* mucilage is compared with that of some commercial sunscreens. Finally, it is discussed the feasibility of using the *Opuntia* mucilage as active ingredient of sunscreens due to its absorption properties.

Materials and Methods:

The mucilage is a polymer extracted from the cladodes of *Opuntia*. To obtain a mucilage as concentrated as possible it is recommended to use 2-year-old cladodes harvested in the morning when the acidity of the vegetal tissues is higher. In order to extract the mucilage from the *Opuntia*, it was followed a procedure reported by Dominguez-Canales *et al*., 2011. After extracting the purified, dry and powdered *Opuntia* mucilage (Figure 2), its ultraviolet absorption characteristics were measured. The measurements were performed by means of a Perkin Elmer Lambda 900 spectrometer with PELA-1020 integrating sphere. This device has a sensibility from 200 to 2600 nm, measurement interval of 1 nm and minimal integration time of 0.08 seconds.

Figure 2. Purified, dry and powdered *Opuntia* mucilage.

For the measurements, it was acquired a reference spectrum without any sample in the spectrometer. Then, a small portion of the powdered *Opuntia* mucilage was put in a quartz cuvette to create a homogeneous film with thickness of 1 mm. After that, the mucilage sample was put on

the integrating sphere of the spectrometer to measure its absorption spectrum. Finally, the absorption of the analyzed sample was isolated by subtracting the reference spectrum.
Also, several samples of commercial sunscreens with different sun protection factors (SPF) were analyzed with the aim of comparing their absorption spectra with that of the *Opuntia* mucilage. In the same way as the mucilage measurements, it was put a small amount of each selected sunscreen in a cuvette to create a film with thickness of 1 mm. The absorption spectra of these sunscreens were measured with the same spectrometer setup. The obtained results and some discussions are presented in the next section.

Results and Discussions:

The absorption of the *Opuntia* mucilage and some commercially available sunscreens were measured. In Figure 3, the black continuous line corresponds to the spectrum of dry and powdered *Opuntia* mucilage, the red dashed line to a sunscreen with SPF 60, the green dotted line to a sunscreen with SPF 80, and the blue dashed-dotted line to a sunscreen with SPF 110.

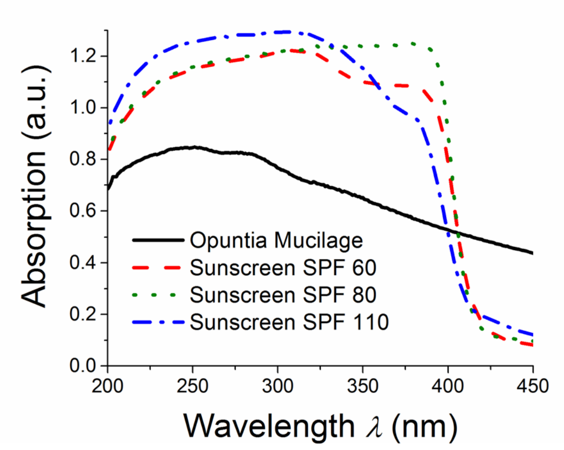

Figure 3. Absorption spectra of the *Opuntia* mucilage and of selected sunscreens.

In Figure 3, the *Opuntia* mucilage presents a strong absorption band with peak value at 250 nm. The full width at half maximum (FWHM) of this spectral band goes at least from 200 to 450 nm. This absorption range covers the entire UV region and a small part of the visible spectrum. For this reason, the *Opuntia* mucilage may be classified as a broad spectrum material in the field of sun protection. Figure 3 also shows the absorption spectra of the sunscreens selected for this research. One of the most important characteristics of these spectra is their notable similarity, even though these sunscreens were labeled and sold as products with different sun protection factors. Recently, the regulatory guidelines for Europe were revised and modified to indicate that the maximum SPF allowed on the labels of sunscreens commercialized in that region is 50+ (Federal Office of Public Health, Recommendations from the European Commission, 2014). In the United States of America, the applicable normativity specifies that if a sunscreen is labeled with a SPF higher than 50, then the label must include a warning text for the consumers about the absence of conclusive evidence to support that this kind of products provide higher protection against UV radiation than sunscreens with SPF ≤ 50 (FDA, Sunscreen Drug Products for Over-

the-Counter Human Use, Final Rules and Proposed Rules, 2011). Probably, this lack of conclusive evidence is closely related to an absence of enhanced absorption properties of the active ingredients in sunscreens with SPF > 50, just as the sunscreens analyzed here that have practically the same absorption spectrum.

Conclusions: The *Opuntia* mucilage has a strong absorption band with peak value at 250 nm that ranges at least from 200 to 450 nm covering the entire UV region. The ultraviolet absorption characteristics of this mucilage indicated that it is feasible to elaborate sunscreens with this organic material as single active ingredient due to its broad absorption spectrum in the UV. This advantage of the *Opuntia* mucilage would eliminate the need to combine several active ingredients to cover a wide spectral band minimizing the risk of suffering adverse effects associated to the organic absorption of every substance in a complex mixture. More research work is necessary to determine the efficacy of the *Opuntia* mucilage in sun protection.

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Construction of a hybrid system for wastewater treatment and phytotoxicological analysis of the influent

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Abstract: Aquatic pollution is one of the main causes of water shortage worldwide, impacting food security and public health. For wastewater treatment, emerging technologies such as phytoremediation and hybrid systems have shown overcome the insufficient performance of conventional technologies. One way to evaluate the effectiveness of these hybrid technologies is the use of toxicological bioassays which allows determining the concentration-response relationship of pollutant in the organisms. The aim of this study was to construct an hybrid system for wastewater treatment and to standardize acute toxicity bioassay for 96-h period for evaluating a As(V)-amended influent using lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*) and pea (*Pisum sativum*). The effective concentration ($EC_{50\text{-}96h}$) results were 3.70, 11.81 and 19.08% for *L. sativa, C. sativus* and *P. sativum*, respectively. *L. sativa* is less tolerant to influent toxicity while *P. sativum* is the most tolerant. It was also noted that the wastewaters unfortified, produced phytotoxicity ranging from 23.32 to 29.66% in the test organisms.

Keywords: Wastewater ● battery bioassay ● water pollution

Introduction: Water pollution due to agricultural activities is one of the main causes of water shortage worldwide impacting in food security and public health (Rajagopal *et al.*, 2013). This water shortage is promoting the use of wastewaters for agricultural irrigation (Iurciuc and Dima, 2013) promoting accumulation of metals, pathogens, changes in pH and salinity of irrigated soils (Boluda *et al.*, 2011; Iurciuc and Dima, 2013). For example, arsenic (As) is a water pollutant throughout trophic chain is biomagnified which can reaching humans by food from irrigated fields with wastewater polluted with this element. This is a fact found in Hungary, Mexico, Argentina, Australia, USA and Asia (Panda *et al.*, 2010). As a consequence, the use of emerging technologies for the remediation of soil and water, such as phytoremediation and hybrid systems for wastewater treatment which combine conventional technologies for wastewater treatment with phytoremediation modules offering the opportunity to use solid waste material of adverse environmental impact and high urban production such as automotive tires often associated to improper disposal (Pilon-Smits, 2005; Schwitzguébel, 2001; Schröder *et al.*, 2007). An approach to analyze the efficiency of these systems for wastewater treatment and the effluent toxicity, are bioassays by using plant species which have shown relation between dose and physiological response under field or laboratory conditions (Rizzo, 2011). This analysis allows predicting the ecological effects of pollutants at level of trophic chain. Its results can be used in programs of environmental monitoring and management of chemical product policies (Boluda *et al.*, 2011; Kusk *et al.*, 2011; Rizzo, 2011). In the present research evaluates a treatment system using waste

tires for substrate biofilm attachment process. The aim of this study was to perform a phyototoxicological analysis to the influent to be treated with a hybrid schem for following toxicological attenuation by the biological components of the system, as well as showing its construction and operation.

Materials and Methods:

For the phytotoxicological analysis were used lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*), and pea (*Pisum sativum)* seeds from Distribuidora Rancho Los Molinos S.A. de C.V. with the characteristics recommended by the Organization for Economic Co-operation and Development (OECD, 2006) which are shown in Table 1.

Table 1.Technical data for experimental seeds.

Common name	Species	Family	Germination (%)	Treatment
Lettuce	*L. sativa*	Astereaceae	>88	Without treatment
Cucumber	*C. sativus*	Cocurbitaceae	>96	Thiram
Pea	*P. sativum*	Fabaceae	>93	Thiram

Before wastewater analysis, Cr(VI) obtained from potassium dichromate ($K_2Cr_2O_7$)was standardized as a reference toxic following the calibration method (Díaz-Báez *et al.*, 2008; Silva *et al.*, 2007). A bioassay with concentrations of 500, 250, 125, 62.5, 31.125 mg L^{-1} of $K_2Cr_2O_7$ was realized and distilled water as control. Ten seeds of each plant species were placed on petri dishes using 5 repetitions per seed specie. After 96 h of germination the seeds were taken for measurement of radicle length for to calculate the percent phytotoxicity using the Equation 1 (Sahu *et al.,* 2008).

$$\text{Percent Phytotoxicity} = \left(\frac{\text{Radicle length of control} - \text{Radicle length of test}}{\text{Radicle length of control}}\right) * 100 \qquad (1)$$

Efective Concentration at 96 h ($EC_{50\text{-}96h}$) was calculated using the regression model of Morgan-Mercer-Flodin (MMF) in Curve Expert 1.4 environment. Equation 2 shows the MMF model.

$$y = \frac{ab + cx^d}{b + d^x} \qquad (2)$$

Where *y* is percent phytotoxicity; *x* is the concentration of $K_2Cr_2O_7$;*a* is *y* value when *x=0*; *d* is the parameter of the asymmetry of the curve; *c* is the value of *y* on the asymptote of the function, and $b = (x_{50})^d$, where x_{50} is the value of *x* at 50% of phytotoxicity. With the $EC_{50\text{-}96h}$, control charts were elaborated according to Díaz-Báez *et al.* (2008). Interlaboratory presition was expresed with the variation coefficient (VC), calculated from Equation 3. A value less than 30% is the minimun presition for each control chart (Silva *et al.*, 2007).

$$CV = [(\sigma/\text{mean})]*100 \qquad (3)$$

In order to characterize influent phytotoxicity, bioassays using plant species listed in Table 1 were conducted. Water samples were collected from a municipal wastewater treatment oxidation pond localized in Marin, Nuevo Leon, Mexico. The influent was amendment with As(V) from a stock solution prepared with 100 mg L^{-1} of heptahydrate disodium hydrogen arsenate ($HAsNa_2O_4•7H_2O$), this chemical presents 24.01% of As. From this stock solution, six fractions were prepared at 100, 80, 60, 40, 20 and 10% of stock solution for a As(V) concentration of 24.0, 19.21, 14.41, 9.6, 4.8 and 2.4 mg L^{-1}, respectively. A test with influent without As(V) was also conducted. Positive controls were a solution with EC_{50-96h} of $K_2Cr_2O_7$for each specie and negative controls were distilled water. Each experimental test was conducted by triplicated at 25 $\pm$ 2 °C during 96 h without artificial or natural light. Influent phytotoxicity was calculated by Equation 1 and the EC_{50-96} by Equation 2 but considering *x* as the fraction amended with As(V).

Construction of the hybrid system for wastewater treatment. Duckweed (*Lemna minor*) was collected from the oxidation pond in Marín, N.L., Mexico. The collected plants were propagated in the Environmental Remediation Laboratory at The Agronomy School Experimental Campus, Universidad Autónoma de Nuevo León, in Marín. The hybrid system for wastewater treatment consists of a 200 L tank, a peristaltic pump (Isco Sampler® 3700) connected to two anaerobic reactors, inoculated with sludge from the oxidation pond, with a volume of 60 L each, one of them with 3.97 kg of crushed tire. From the reactors, wastewater passes to six 20 L tanks, three for each reactor. One of them with 3.97 kg of crushed tire and *L. minor*, the second one with *L. minor* only and the third one with 3.97 kg of crushed tire. The Figure 1 shows the hybrid system.

Figure 1. System hybrid for wastewater constructed in this project.

Results and Discussion:

As shown in Figure 2, *L. sativa* was the less tolerant to $K_2Cr_2O_7$as reported by Aportela-Gilling and González-Pérez (2001), who observed a lethal concentration (LC_{50-96h}) at 18 mg kg^{-1} of soil .*P. sativum* was the most tolerant than the other species with a CE_{50-96h} of 266.34 mg L^{-1}. The control charts (Figure 2) obtained for the three species are considered acceptable because CV values were below 30% for all the experimental tests (Silva *et al*., 2007). For *C. sativus*, EC_{50-96h}has a value of 11.82% (2.84 mg L^{-1} of As(V)). This response is due to the fractions of the experimental influent at 99.33% (R^2 = 99.33, n = 21). For *P. sativum* the EC_{50-96h} was 19.08 % (4.58 mg L^{-1} of As(V)). In *C. sativus* and *P. sativum* their tolerance to As has been associated to genes present

in chromosomes 3 and 10 and to antioxidants enzymes. These physiological mechanisms could explains a EC_{50-96h} higher than *L. sativa* (Panda *et al.*, 2010)

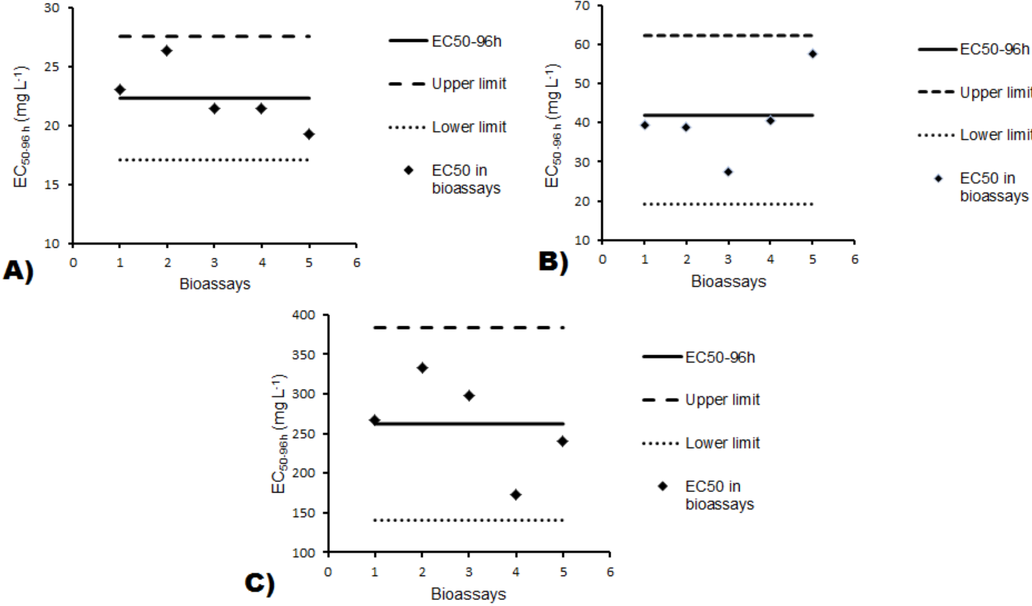

Figure 2. Control chart for $K_2Cr_2O_7$ in *L. sativa* (A), *C. sativus* (B) and *P. sativum* (C).

because CV values were below 30% for all the experimental tests (Silva *et al.*, 2007). For *C. sativus*, EC_{50-96h} has a value of 11.82% (2.84 mg L^{-1} of As(V)). This response is due to the fractions of the experimental influent at 99.33% (R^2 = 99.33, n = 21). For *P. sativum* the EC_{50-96h} was 19.08 % (4.58 mg L^{-1} of As(V)). In *C. sativus* and *P. sativum* their tolerance to As has been associated to genes present in chromosomes 3 and 10 and to antioxidants enzymes. These physiological mechanisms could explains a EC_{50-96h} higher than *L. sativa* (Panda *et al.,* 2010). Regarding wastewater without As enrichment assays, Figure 3 shows that this effluent produced 25.82, 29.66 and 23.32% phytotoxicity percent in *L. sativa, C. sativus* and *P. sativum,* respectively, because of an adverse effect of wastewater composition.

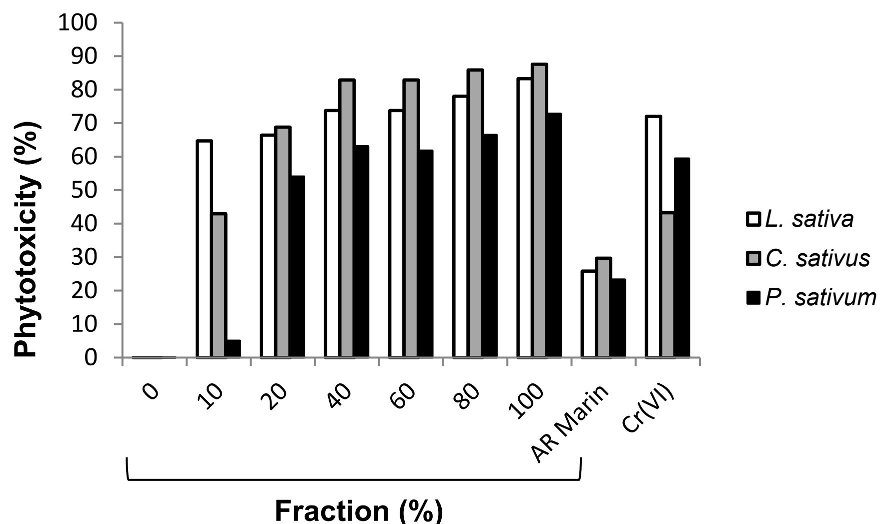

Figure 3. Percent phytototoxicity in the bioassay battery through the influent amendment with As, the influent without As and the reference toxic [Cr(VI)].

Conclusions: Toxicity levels of *L. sativa, C. sativus* and *P. sativum* for Cr(VI), which were used to develop control charts of Cr(VI) as a reference toxicant was established. Under the experimental conditions, *L. sativa, C. sativus* and *P. sativum* were sensitive to the tested influent fractions. Thus, these species can be considered sound pollution bioindicators to evaluate probable adverse effects of contaminated effluents on public health. In addition, the results from wastewater without As enrichment suggest that a phytotoxicity over 23% can be observed on plant species.

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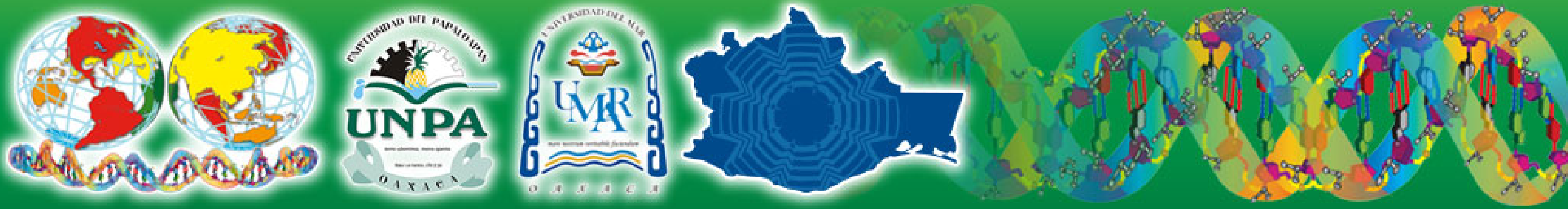

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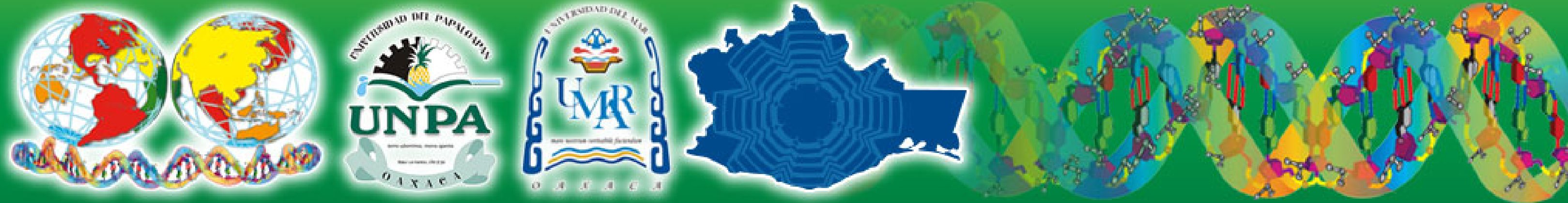

Feldspar filter prototypes for wastewater treatment

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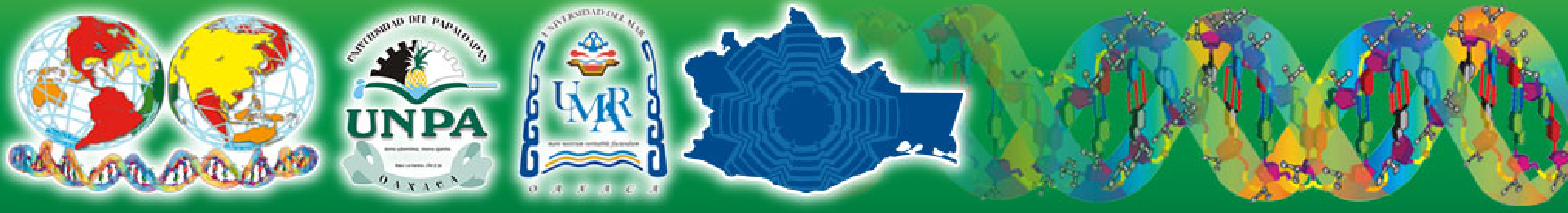
Abstract: The city of Taxco, Guerrero, does not have a wastewater treatment system. Added to this, the mining of silver and gold in the region have caused environmental damage to rivers and its tributaries by increasing the concentration of heavy metals such as Zn, Pb, Cu and Cd in a dissolved form. Feldspar is a mineral formed by aluminum silicates combined in its three forms: potassium, sodium, and calcium. The structural components of feldspar, such as TiO_2, can interact with wastewater, thereby producing a photocatalysis effect; in this way degrading part of the contaminants. Samples of wastewater were collected from the river Taxco within the area of the locality of Campuzano (UTM 14 Q East: 438397, 2039339.266245 North and 1160 m.a.s.l). 10 grams of feldspar per liter of sample were added, stirring constantly for 10 minutes. Each sample was exposed to sunlight for one hour. Subsequently, each water sample was poured in a column of zeolite and feldspar. The pH of the samples after treatment was 7.57. The wastewater color changed from greenish yellow to translucent, and the fetid odor was reduced. The feldspar has properties that significantly improve the quality of wastewater, which can then be used for agricultural purposes.

Keywords: Taxco ● feldspar ● wastewater ● zeolite

Introduction: The protection and conservation of natural resources is today one of the major social concerns. The water is considered a well valuable and scarce, that need a proper use and recycling. In addition, the need to preserve the environment has led to the search of new more efficient methods for the Elimination of chemical pollutants, mainly due to its toxicity or low biodegradable capacity for living organisms. To all this must be added the fact that the regulations impose more stringent criteria to achieve a greater and better water purification (Gómez and Fuentes, 2000). Currently the State of Guerrero have the problem of scarcity of water, due to the fact that around 80% of its rivers are contaminated (SEMARNAT, 2004). Such is the case of the river Taxco in the municipality of the same name, which is contaminated since 1543 when I start mining in the municipality (Castrejón, 2008). The contamination in its early days was caused by mercury which, they used the haciendas of benefit for the silver by method of amalgamation. Subsequently, in 1920 the mine started exploiting lead and zinc, which produced new pollutants (Romero-Martin, 2012). Mining pollution was previously increased the pollution produced by the population, according to reports of the Archbishopric of Mexico in the year 1685 the population was 2,156 inhabitants (Pérez-Rosales, 2012), for the year 1930 Taxco held 21 place on lists of municipalities with the largest number of inhabitants (Anon, 2010). Currently the growth of population in Taxco is also a factor that influences the increase in pollution, being the sixth municipality most populous in the State of Guerrero with 105,512 inhabitants (Anon, 2012).



Now the population of Taxco and theirs mining activities perform discharges the wastewater untreated to the Rio Taxco. The situation is exacerbated in the dry season since it has a higher concentration of metals such as: Zn, Pb, Cu and Cd in the form dissolved with values of 245.8, 0.21, 3.94 and 1.73 mg L^{-1}, respectively.

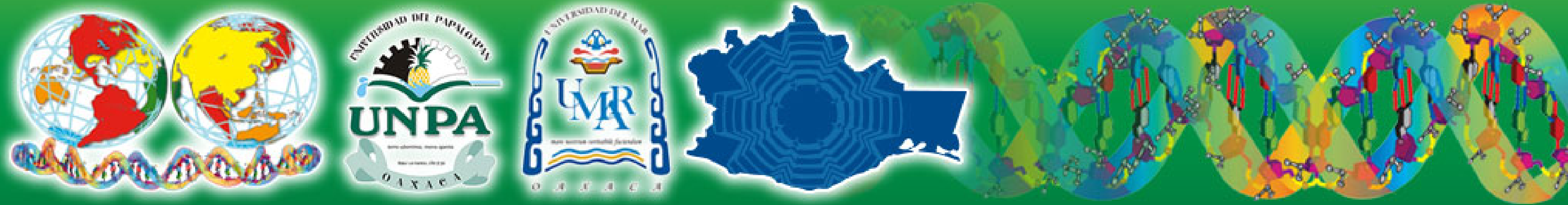
The more critical issue for the communities of the population for Taxco lies in the concentrations of heavy metals, which exceed the permissible maximum levels of Mexican regulations, therefore, this type of water should not be used for agriculture or domestic (Armienta, 2012). However, farmers in the area used the contaminated water from the River to irrigate their plots, which could have consequences on the health of the same by eating those foods. In addition, the use of water from the river not only is limited to planting, it is also occupied for cattle, and sometimes for domestic use. In the case of heterogeneous photocatalysis, there are references of applications with multiple semiconductors such as ZnO, $Fe2O_3$, Al_2O_3 and TiO_2 (Chen, 1997). However, the most widely used is titanium dioxide, since it presents a higher photocatalytic activity, is non-toxic, is stable in aqueous solutions, and is not too expensive, having evaluated different structures of the same (Cheng, 1995).

The mechanism of reaction that takes place in this case is as follows: a (n-type) semiconductor absorbs radiant energy of wavelength less than that corresponding to the "bandgap" (400 nm), generating the corresponding electron-hole pairs. These pairs are capable of catalyzing reactions of oxidation-reduction on the surface of the conductor, provided that the species present in solution have the correct potential. The distance from penetration of photons inside the particle of TiO_2 is shorter the smaller is the wave length, since they are absorbed by the molecules of the semiconductor with more power (Cheng, 1995).

This research intends the use of feldspar powder to wastewater treatment, for use in agricultural activities. Feldspar is a mineral formed by aluminum silicates combined in its three forms: potassium, sodium, and calcium. The chemical formula of feldspar is $XAlSiO_8$, where X can be sodium (Na), potassium (K), or calcium (Ca) (general coordination of mining, directorate general for development mining, 2011). Commercial varieties come mainly from granitic pegmatite dykes, in particular of the granite. Occur in areas of granitic and metamorphic rocks, and as it is common in pegmatites, are usually irregular, discontinuous and very unequal sized feldspar content. Another important source of feldspar is the exploitation of basic rocks called "syenites nefelinicas", which are gaining market at traditional feldspars. This Rock is free of quartz and its essential composition feldspar and nepheline {(na-k) $AlSiO_4$} and acts as a flux to lower temperature. The most important deposits of feldspar are located in the States of Puebla and Guanajuato (general coordination of mining, general directorate of mining development, 2011).

Materials and Methods:

Two samples of water were taken (1 litre) of Taxco River in the area of bridge Campuzano (geographic coordinates UTM 14 Q East: 438397, 2039339.266245 North and 1160 meters above sea level). The pH was measured of contaminated water. Later was added 10 grams of feldspar to each sample, stirring constantly for 1 minute. Once this process is finished, the samples were placed to receive the sunlight for an hour, so the photocatalysis process will take place. The zeolite-feldspar column filter was prepared; add 50 g of zeolite and 50 g of feldspar powder in a burette of 1.5 cm in diameter with a capacity of 100 mL. The water obtained from the photocatalysis process was placed to moved water through the filter (40 mL).



Results and Discussion:

The wastewater samples showed a greenish-yellow color and with the presence of sediments, was also perceived a foul odor. The pH value of the wastewater was 6.67. After added the 10 g of feldspar in the litre of contaminated water, the solid particles in suspension began to flocculate rapidly. The coloration of the wastewater changed from greenish-yellow to pale yellow. In addition to that diminished its foul smell. Ending the process of photocatalysis the pH was measured reaching a result of 7.57.

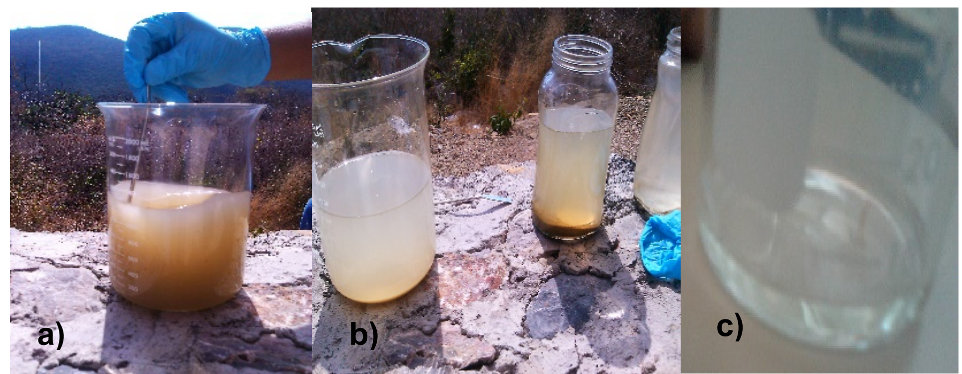


Figure 1. a) Addition of feldspar, b) flocculation and photocatalysis c) water treated with feldspar.

During the filtration process a 5 ml water sample was obtained in 2 h, the retrieved liquid is colourless and odourless, with a pH of 8.49. The pH of the water after this process was neutralized with 0.5 mL of HCl 0.1 M, generating an acceptable water quality.

Table 1. Chemical analysis of the wastewater treated with feldspar filter.

Sample	pH	Conductivity ($\mu S\ cm^{-1}$)	O_2 dissolved ($mg\ L^{-1}$)
Without treatment	7.64	1075	6.74
Phoyocatalysis	7.72	1176	6.99
Feldspar Filter	8.2	1187	6.60

The observed values of the partial chemical analysis can be to implement a secondary treatment to reduce salinity and increase oxygenation in the water.

Conclusion: In this study has been evaluated in a qualitative and quantitative way the use of feldspar for treatment of contaminated water, obtaining very encouraging results in a matter of minutes was observed to change colour, odour and turbidity, once the mineral aggregate began to react with the suspended solid particles. In the same way the results thrown by the filter, improved the quality of water obtained from the process of photocatalysis. In the next experiments

can be evaluated quantitatively both processes and determine the chemical contaminants in the samples. Improve containers for photocatalysis and design of the filter, improve the whole process for to do it in less time and with greater efficiency. On the other hand, the reason why we use this material is because industry of feldspar powder is considered waste and apparently has a useful application, but with the results, we can conclude that this mineral has properties that significantly improve the quality of water, which can be used for agricultural use.

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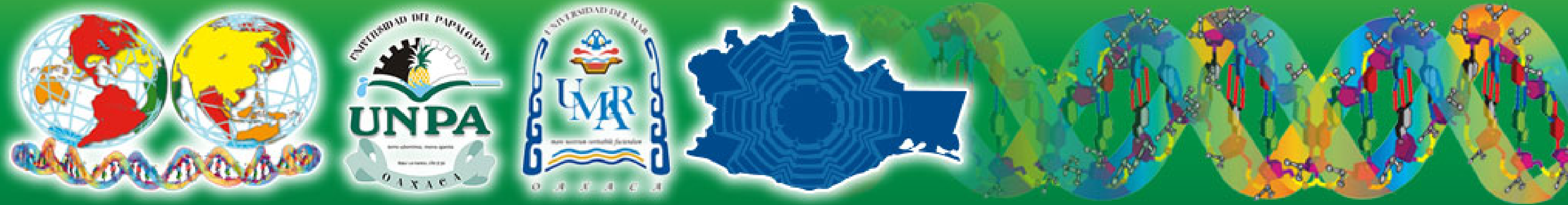

Preliminary design of filters based on chlorite for wastewater treatment

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Abstract: In the municipality of Taxco de Alarcón, Gro., there is the problem of availability of piped water and shortages in the supply of it. The importance of this research is based in the use of minerals and stony products that are able to do the process of filtration, this with materials that are relatively inexpensive, or even of natural acquisition. Were used minerals, which can be found on the soils, as a constituent part of rocks? Such is the case of aluminosilicates of igneous origin (feldspar and chlorite) which serves as a natural filter; this is due to the process of cationic exchange in the variety of minerals present in the soil structure. The flow of the filter was observed in 13 mL sec^{-1}, for to obtain water with pH 7.0 was made a correction of the pH by the addition of 8 mL of HCl to 0.1 M per liter of water. In this way, the results show promising data for work in the improvement of a filter that allows to give treatment to the wastewater that runs along the river and get a quality that could be used in agricultural activities and maybe for use domestic.

Keywords: Taxco ● chlorite ● feldspar ● wastewater

Introduction: Nowadays the emergence of new technologies have been implemented the use of new and different products for the improvement in the quality of water, products such as chemicals, processes and filter cloth; being the latter the greater widely used in households in rural areas where water sources used for primary needs are questionable. In the urban areas where water as a service becomes scarce, is a need of the population the development of plans for the reuse of water, derived from industrial processes or even wastewater, trying to reach the optimum level of purification for the use of the population needs (Arellano, 2002).
The importance of this research is based in the use of minerals and stony products that are able to do the process of filtration, this with materials that are relatively inexpensive, or even of natural acquisition. Using as active minerals, this can be found on the same soils, as a constituent part of rocks. Such is the case of aluminosilicates of igneous origin, which serves as a natural filter, this is due to the process of cationic exchange in the variety of minerals present in the soil structure.
Within processes that take part in the treatment of wastewater, have been described several phases. This taking into account aspects such as: a) the constituents in the water after its use in some industrial process b) if the water quality could cause some adverse effect. Already depending on these factors, the treatments that are performed can be the following: neutralization, removal of particles, of dissolved solids and of organic compounds.
Actually, the systems of wastewater treatment apply what is known as passive and active treatments. I) Liabilities, are those systems in which the intervention of man is minimal or nothing. These systems or biological environments wherein are included marshes, deltas, estuaries, mosses and mobs. This is to control acidity, metals content, reduction of solid suspended, etc. II)

Assets, on the other hand, they are implemented and constantly operated by man. Such is the case of the water treatment plants. In these plants, there are processes such as neutralization and precipitation, aeration, filtration, reverse osmosis, ion exchange and chemical softening.
At present are used feldspars, zeolites and clay materials for the treatment of wastewater. This is due to the high rate of feldspars cationic exchange, in the case of the zeolite by the form of crystallization of the elements and by the great capacity of absorption presenting clays. These mineral are able not only capture the heavy metals, also to do a process of elimination of microorganisms by means of natural disinfection. Based on this knowledge, was proposed a filtration system that can meets most of the requirements and quality controls to provide a quality of water for agricultural use and allows rural households take advantage of the use the water for domestic use.
For this work, was proposed the use of the mineral chlorite; a mineral of aluminosilicate compound of the group of the phyllosilicates of Fe, Mg; with metamorphic origin, it is a mineral of different origin to the before employees for the treatment of wastewater.

Materials and Methods:

Three samples of water were taken (1 litre) of Taxco River in the area of bridge Campuzano (geographic coordinates UTM 14 Q East: 438397, North: 2039339.26 and 1160 meters above sea level). The pH was measured of contaminated water. Later was added 2 grams of lime $Ca_2(OH)_2$ to each sample, stirring constantly for 1 minute. Once this process is finished, the samples were placed to flocculation process for ten minutes. The Chlorite-feldspar-activated carbon column filter was prepared; add 150 g of chlorite, 50 g of feldspar powder, and 50 g of activated carbon in a bottle of 10 cm in diameter with a capacity of 1 L.

Design for the filter. The first step was the removal of some suspended particles, for this we use a grid or fine mesh that could potentially retain and remove all these particles as organic and other sediments are that are included in these. In the second phase of filtration, was placed a layer of rock limestone, this ensures that the pH of the water was neutralized, obtaining values of 6.5 and 7.5 which are considered as optimal for human consumption. In a third phase we found an area of fine sand, this in order to filter some very small particles that might have escaped the mesh due to the size of the particle. The arena selected for this phase was of igneous origin, derived from granite, in what knows as moderate power zone. In this phase the application of the chlorite "aluminosilicates of Fe-Mg", promoted an area for the ion exchange, where occurs the sequestration of heavy metals, due to the properties of anions of the chlorite. At the same time we can see that there would be the release of Fe and Mg ions, which provide a reduced suspension for some aerobic organisms that die by lack of oxygen.

Results and Discussion:

In the first phase of filter the water flows through an area of fine sand, preventing particles of chlorite which could strain, in the same way to continue with the selection of arenas with origin of granites. In the final process of this first filter was placed a layer of cotton to pressure in a way such that there is no way that the sand can get to out of the filter. This cotton is considered as a prototype phase, because the accumulation of some bacteria and anaerobic organisms can be in this area forming a crop.

This is the first phase of the treatment of water where was can be appreciate that by natural processes and with simple materials was obtained water for irrigation in plots and fields of vegetables and other vegetables. This first filter can be apply in wastewater treatment with gray or black color water. Giving good results for irrigation purposes, because the coloration of the wastewater changed from greenish-yellow to pale yellow.
The second step is a single phase, the objective this phase is eliminate the anaerobic organisms with an oxygenation of the water. According with the literature it is knows that the chlorite create an environment reduced through the ions of Fe and Mg. For this reason the implementation of a mechanism of oxygenation is important to counter the effect of the exchange ionic of the chlorite. The third phase is disinfection by light UV, after having been filtered and aerated. According to Wright and Cairns (1990) the water passes to container, which is subjected to UV radiation, which kills any organism biological (bacteria, viruses, cells, etc.) by UV radiation. This filter was designed for help us in total and definitive removal of microorganisms that could be harmful to human beings, reason by which water could be used for domestic and agricultural use. The preliminary results for this moment only are visuals, where the water filtrated not presented color and odor in 30 days. In the next experiments can be evaluated quantitatively both processes and determine the chemical contaminants in the samples.

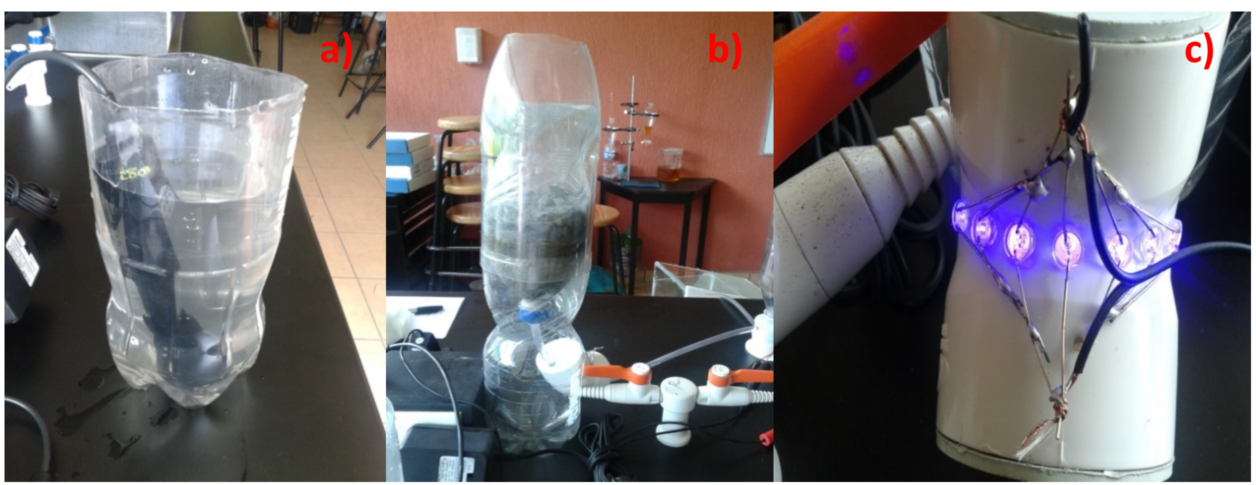

Figure 1. Process of treatment of the water of the river Taxco, where; a) Sample of water of the river Taxco, b) Filter of chlorite, and c) Light UV.

The flow of the filter was observed in 13 mL sec^{-1}, the pH values in the water filtered was increased because minerals are predominantly basic, likewise allows the cation exchange with the water and increase the electrical conductivity and the dissolved oxygen showed a decrement. For to obtain water of quality with pH 7.0 was made a correction of the pH by the addition of 8 mL of HCl to 0.1 M per liter of water.
To improve the results was proposed a fourth step. The fourth step is the distillation as last filter function; was based in the recommendation of boil the water before drinking it. This way can be separated the salts which are left to evaporate the water. The decision of this process is due an

accumulation of salts, the chlorite in conjunction with the limestone rock increased the salt in the water.

Figure 2. Process of treatment of the wastewater of the river Taxco. d) Oxygenation, and e) Container collector.

The data preliminary of water of the river and wastewater in this work are presented below.

Table 1. Parameter of the water after the first step in the filter.

Parameters	pH $-\text{Log}\ [H]^+$	Conductivity electrical ($\mu S\ cm^{-1}$)	Oxygen disolved ($mg\ L^{-1}$)
River Water	8.5 ± 0.05	667 ± 5.51	7.48 ± 0.08
Filtered Water	8.8 ± 0.03	862 ± 3.79	6.07 ± 0.02

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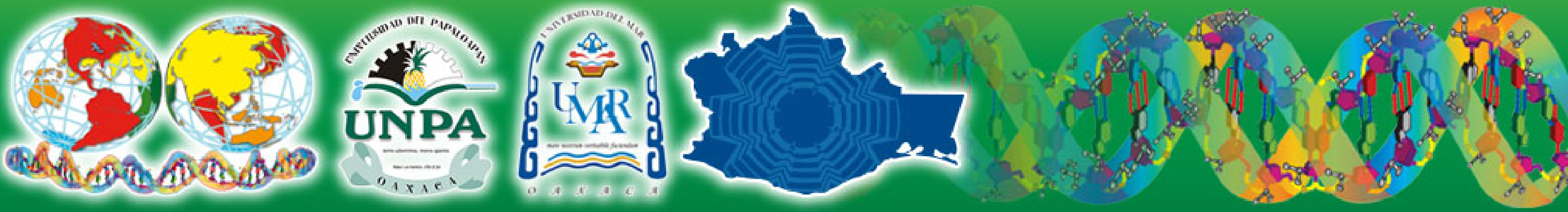

Conclusion: Materials recycled as containers of plastics and minerals which are found in rocks of the Taxco region were used for the design of the filter and was showed a significant improvement in color and odor of the water of the river. In this way, the preliminary results show promising data for work in the improvement of a filter that allows to give treatment to the wastewater that runs along the river and get a quality that could be used in agricultural activities and maybe as use domestic.

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Effect of ZnO, TiO_2 and Fe_2O_3 nanoparticles on the body mass change, reproduction and survival of *Eisenia fetida*

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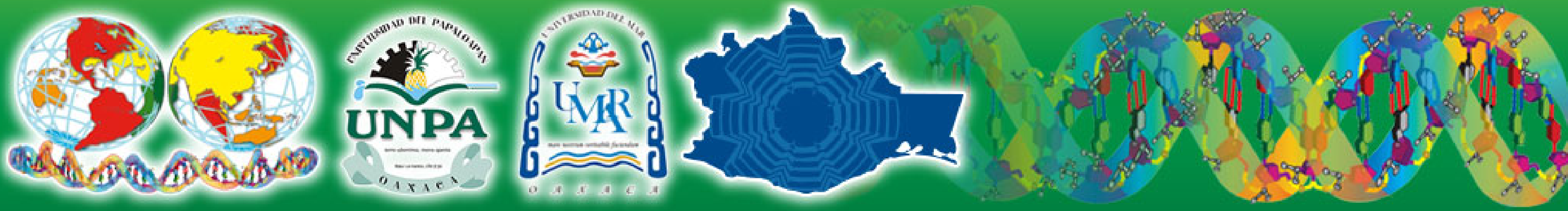
Abstract: The increasing production of nanoparticles and its byproducts bring as a priority the necessity of understand the real interaction between earthworms and nanoparticles (NPs) in an agricultural soil. The present study addresses the effects of zinc oxide, titanium dioxide and iron oxide NPs in the body mass change, reproduction and survival of *Eisenia fetida*. Earthworms were exposed to increasing concentration of NPs in a treated soil while total and bioavailable Zn, Ti and Fe were monitored in an aerobic incubation experiment of 60 days. Earthworms exposed to TiO_2NP at 2 g L^{-1} and ZnONP at 4 g L^{-1} did not lead to adults decease. In the case of Fe_2O_3 when the dose reached 2 g L^{-1} survival decreased compared with the control. In cocoons production there was not significant differences compared with the control, suggesting that NPs have no effect on earthworms' reproduction. ZnONP at 4 g L^{-1} enhanced juveniles on growing and survival. Bioaccumulation was not statically different in TiO_2NPs treatments, perhaps it has significant differences in ZnONPs and Fe_2O_3NPs, showing that bioaccumulation of Zn in earthworms increases on par with dose, meanwhile earthworms exposed to Fe_2O_3NPs bioaccumulated less iron than those that were in the control treatment.

Keywords: nanoparticles ● earthworms ● bioaccumulation ● reproduction

Introduction: The manufactured nanomaterials (MNMs), which are materials with at least one dimension between one and 100 nm, have found a wide scope in agriculture, energy generation, electronics, drug administration and medical diagnostic (Hu *et al*., 2010, Nel *et al*., 2006). The NPs are universally used in many products commonly employed by humans, such as food, clothing, medicines and cleaning products. In some cases, the NPs are made of materials having known toxicity; however, its properties may differ from their counterparts of higher mass, inducing additional biological activity in their smaller size, greater surface area and reactivity, which means that there is real potential in the NPs to exhibit toxic effects (Hooper *et al.,* 2011). Due to the previously mentioned, the estimated global investment in nanotechnology by 2014 is projected at $ 3 trillion (Hu *et al*., 2010). Nanotechnology will be the center of science, technology and business for the coming years, so due to the expected increase in MNMs production, government agencies and scientists have begun to investigate the environmental fate and behavior of these materials, in order to understand the potential risks to humans and other organisms that are exposed to NPs (Steward *et al.,* 2013). The widespread application of MNMs makes inevitable that NPs get discharged into the environment intentionally or accidentally. Most MNMs discharged into the wastewater stream are distributed to the activated sludge. Therefore, agricultural soils might serve as a sink for a significant fraction of the NMs released to the environment through the soil when activated sludge are poured into farm fields to improve soil fertility or during the atmospheric deposition of NPs (Keller *et al.,* 2013). However, despite the large amount of research conducted



about the potential applications of nanotechnology in recent years, relatively little has been done to assess their potential environmental risks, particularly in terrestrial ecosystems (Unrine *et al.,* 2011). *E. fetida* was selected as a model organism for this study due to it is an important species in toxicity testing of soils, standardized by the Organization for Economic Cooperation and Development (OECD) (OECD, 1984; Washington State Department of Ecology, 1996; Environmental Technology Centre of Canada, 2007). Besides being a species that plays an important role in terrestrial ecosystems, as it not only plays a critical role in biogeochemical cycles and the function of soils, it is also a common prey for a large number of consumers with high level in the food chain (Unrine *et al.,* 2011; Lee *et al.,* 2008; Sachová *et al.,* 2006). The present study aims to demonstrate the effects of three varieties of NPs, hematite (Fe_2O_3), zinc oxide (ZnO) and titanium dioxide (TiO_2) on the change in body mass, the survival and reproduction of the worm *E. fetida*.

Materials and Methods:

Nanoparticles of hematite (Fe_2O_3), zinc oxide (ZnO), and titanium dioxide (TiO_2) were purcheased from '*Investigación y Desarrollo de Nanomateriales S.A. de C.V.*' This study was carried out under plant growth chamber conditions by Sustainability of Natural Resources and Energy Program located in Saltillo, Coahuila, Mexico. The soil was taken to the laboratory and treated as follows. The soil from each plot was passed separately through a five mm sieve, adjusted to 40% water holding capacity (WHC) by adding distilled water (H_2O) and conditioned at 22±2 °C for 10 days in drums containing a beaker with 100 mL 1 M sodium hydroxide (NaOH) to trap CO_2 evolved, and a beaker with 100 mL distilled H_2O to avoid desiccation of the soil. Afterward this process the soil was tyndallized to remove any organisms that could be harmful to the earthworms. Vermicompost used to feed the earthworms were obtained from the worm culture maintained in our facilities, which is kept based on pre-composted organic material bedding.
All earthworms used in the present study came from a culture of *E. fetida* maintained in our facility, with a starter culture from the '*Universidad Autónoma Agraria Antonio Narro'* located in Coahuila, Mexico.
Earthworms were split into three treatments: a negative (control), a 2 g L^{-1} NP treatment and a 4 g L^{-1} NP treatment in which NPs of ZnO, TiO_2 and Fe_2O_3 were spiked to food. The experiment lasted 60 days, in which three destructive and random samplings were performed on days 20, 40 and 60. Food was added on day 30 and 50. For the purpose of the experiment, 900 mL glass jars were used, approximately 200 g aliquots of the mixed soil were distributed to each of the glass jars; subsequently ten adult *E. fetida* earthworms with fully developed clitella and average fresh mass of 0.42 g were added to each glass jar, which was covered with organza fabric, since this avoids excessive loss of moisture while allows the circulation of air within the glass jar. The experiment runs under plant growth chamber conditions, which means that the average temperature was between 22 ± 2 °C and the photoperiod was 12 hours light and 12 hours dark. During each sampling day adult earthworms, cocoons and juveniles were hand-sorted and counted. Adult earthworms were frozen at -20 °C until laboratory analysis.
To determine the total amount of ZnONP, TiO_2NP and Fe_2O_3NP present in the soil and earthworms of each experimental unit, the samples were subjected to wet digestion of dried material with a mixture of nitric and perchloric acids (White, 1988; Hoffman, 1996). The data were subjected to variance analysis and means compared with the Tukey test. Soil and earthworms characteristics were subjected to one-way analysis of variance using a general lineal models procedure (PROC GLM) to test for significant differences between treatments ($P < 0.05$). All

analyses were performed using Statistical Analysis System (SAS) software version 9.1 for Windows (SAS, 1989).

Results and Discussion:

After the experiment was mounted, it was observed that the treatments with Fe_2O_3NPs caused avoidance of earthworms at concentrations of 2 and 4 g L^{-1}, as these tried to leave the soil of the experimental unit. Similarly physical damage was detected in earthworms, such as inflammation and explosion in certain areas of the worm (Figs. 1A and 1B). However, in the treatment of ZnONPs-2 g L^{-1} it was observed that notwithstanding the earthworms had excellent mobility and color; they looked very thin and small (Fig. 1G). In the treatment of TiO_2NPs it was observed in the negative control (TiO-0 g L^{-1}) that all sampled individuals were in excellent conditions, however, in treatments TiO_2NPs-2 g L^{-1} and TiO_2NPs-4 g L^{-1} the earthworms looked very thin and lethargic (Figs. 1C and 1E). In sampling 3, day 60, in the treatment of Fe_2O_3NPs-0 g L^{-1}, earthworms had excellent mobility, while the few survivors of Fe_2O_3NPs-2 g L^{-1} and 4 g L^{-1} were too lethargic. In the case of ZnONPs-2 g L^{-1} and 4 g L^{-1} treatments, earthworms were observed very thin but with excellent mobility (Figs. 1H and 1I). Finally for TiO_2NPs-0 g L^{-1} treatment, earthworms looked very long and with no apparent physical damage, while earthworms in treatments 2 g L^{-1} and 4 g L^{-1} looked thin, slow and some of them stumpy (Figs. 1D and 1F).

Figure 1. NPs physical effects on *E. fetida*.

The results exhibited in Fig. 2A showed that both TiO_2NPs and ZnONPs did not lead to adults decease. When the doses reached 2 and 4 g L^{-1} respectively, the survival increased statically compared with control ($p<0.05$). In the case of the hematite, when the dose reached 2 g L^{-1} survival decreased compared with the control, suggesting that Fe_2O_3NPs are harmful for adult earthworms.

In cocoons production (Fig. 2C) there was not significant differences compared with the control, evincing that NPs do not favor nor disserve reproduction. Fig. 2B shows that ZnONPs enhances juveniles growing and survival at 4 g L^{-1} dose, since significant differences were observed when compared with control.

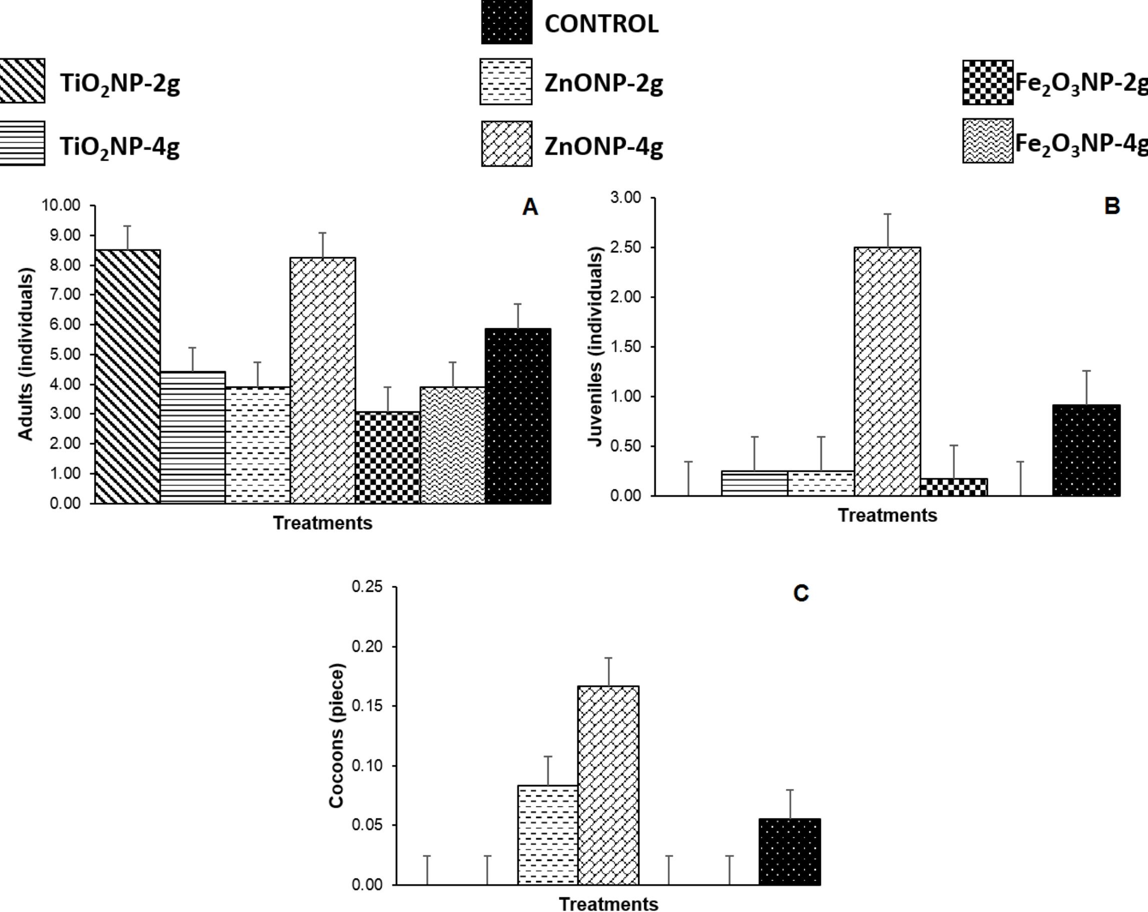


Figure 2. NPs biological effects on E. fetida.

Conclusions: The Fe_2O_3 NPs cause obvious physical damage to *E. fetida*. However, untreated worms and those exposed to TiO_2 and ZnO NPs grown without any change. The Fe_2O_3 NPs significantly decreased survival of earthworms, while TiO_2 and Fe_2O_3 NPs significantly decreased reproduction (number of young and number of cocoons) compared to ZnO NPs. The growth, development and survival of earthworms are altered when placed in contact with different doses or types of NPs, so it is necessary to develop further field and laboratory research for assessing ecological and environmental damage caused by the use and release of NPs.

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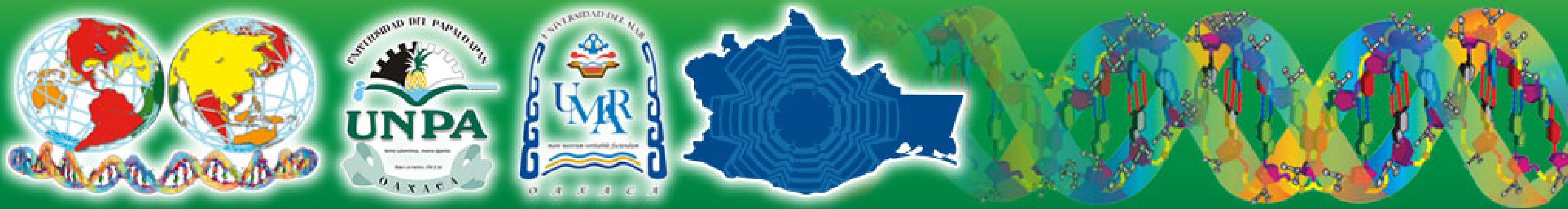

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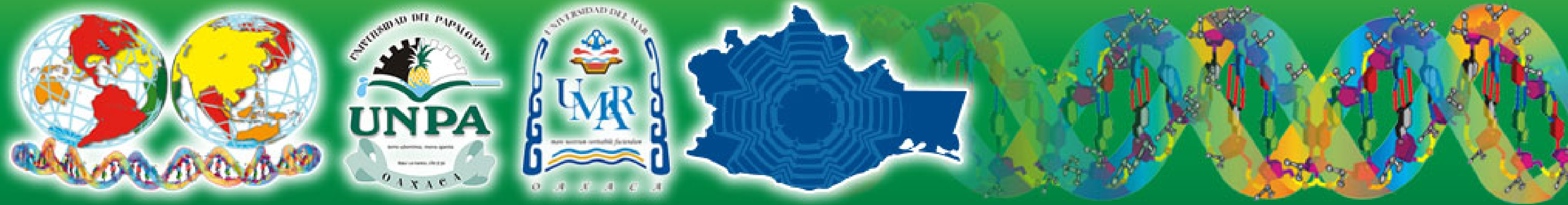

Enhancement of *Beauveria bassiana* virulence genes expression in selected culture media

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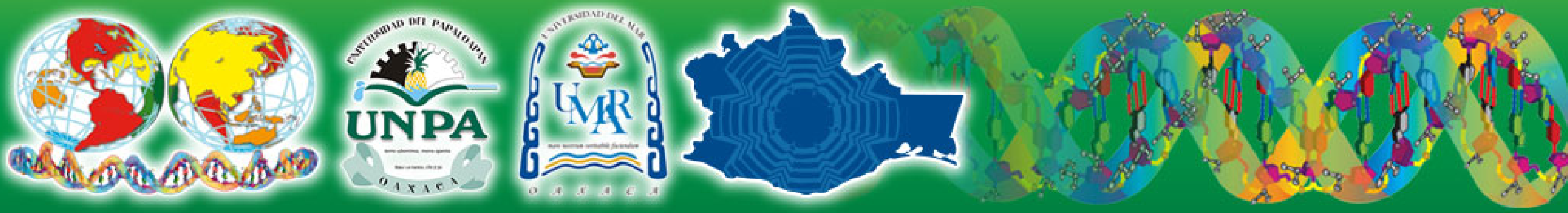
Abstract: The entomopathogenic fungus *Beauveria bassiana* is widely used as insect pest biocontrol agent and represents one of the most selected bioinsecticides worldwide. One advantage of the fungus (strange wording) are the fermentation production feasibility and wide virulence against several order insect pests. Its mode of action starts after achieving adherence to its host; propagule starts its vegetative growth and releases several insect cuticle (first defense barrier) degradation enzymes to invade/infect insect's body. Fungus micelia grow inside the body by taking the insect' nutrients, thanks to the combined action of enzymes and toxins. Fungus may keep growing until nutrients are consumed and host is dead. For fungi virulence improvement, during in vitro fungi propagule culture, selected ingredients as chitin may increase the enzymes and toxins production. In the present study, the expression of B. bassiana genes of proteases, and beauvericin and bassianolide toxins after cultured in a selected medium was evaluated. Gene expression was evaluated by real-time RT-PCR, using actin mRNA as internal positive control.

Keywords: *B. bassiana* virulence ● enzymes and toxins genes expression ● culture media

Introduction: It is known that up to 80% of the etiological agents involved in the insects' biocontrol are fungi, with around 90 genera and over 700 species. This is why a large investment by extending the investigation of these entomopathogens for application in biological control approaches has been reported (Destéfano *et al*., 2004). *Beauveria* (teleomorph: *Cordyceps*) *bassiana* (Hypocreales) is a broad host range facultative entomopathogen that plays an important role in the control of insect populations in nature. This fungus is being the most important mycoinsecticide currently used for controlling a variety of insect pests (Pathan *et al*., 2007). *Beauveria bassiana* (Bals.) Vuillemin occurs naturally in soils throughout the world, where many strains that exhibit considerable variation in virulence, pathogenicity and host range have been isolated. *B. bassiana* attacks larvae and adults upon contact and kills the host from the inside out. It produces spores (conidia, the asexual form), which after reaching the insect's skin proceeds to germinate and secretes enzymes (chitinases and proteases mainly) that dissolve the cuticle, to penetrate inside the body. Once inside, it produces beauvericin, a toxin that suppresses the host's immune system and in combination with other toxins improves the fungus virulence. The fungus grows by feeding on the host's internal organs and blood-like fluids, leading to the host death within three to seven days after contact, upon the size and host characteristics.
Research and development analysis to improve *B. bassiana* virulence against insects has indicated that selected culture media may lead to higher insect cuticle degradation enzymes and toxins production (Safavi *et al*., 2007). In this sense, after growing in a selected culture medium developed in our laboratory, one native *B. bassiana* strain produced significantly higher insect cuticle degradation enzymes, achieving 100% mortality by *Epilachna varivestis* Mulsant third instar larvae in half of the time (2 d *vs* 4 d) testing the same propagules dose (Tamez-Guerra and



Castrejon-Antonio, 2013). In order to determine if the increased speed of killing observed by propagules produced in the selected medium only relied on the enzymes or if this also improved the toxins production, the expression of the *B. bassiana* beauvericin and bassianolide toxins genes (*BBbeas* and *BBbass*, respectively) was evaluated after culturing in the selected medium by real-time RT-PCR technique using mRNA. For comparison purposes, the expression of proteases (*Pr1* and *Pr2*) and actin (as internal positive control) genes was evaluated.

Material and Methods:

***B. bassiana* strains and culture conditions.** Four native *B. bassiana* isolates coded PTG1, PtG2, PTG4 and PTG6 were evaluated (Tamez-Guerra *et al*., 2013). Same culture medium selected by Tamez-Guerra and Castrejón-Antonio (2013) was used for strains growth, but testing a longer fermentation time. Instead of up to 7 d, strains were kept at 26 °C in constant agitation for up to 13 d, taking samples after 3, 7, 9 and 13 d incubation and frozen at -20 °C until analysis.

***B. bassiana* proteases and toxins gene expression.** After the samples from different days and strains were collected, samples were thawed at room temperature. In each sample, 0.2 mL of chloroform was added and vigorously mixed for 15 s and incubated at room temperature for 2–3 min. Samples were then centrifuged at 12,000 g for 8 min at 2–8 °C. The upper layer (transparent phase) was isolated and transferred into a new tube, 500 µL of isopropanol was added, and the sample was mixed in a vortex, incubated at room temperature for 5–10 min, and centrifuged at 12,000g for 8 min. The supernatant was discarded, and the remaining pellet with RNA was washed with 1 mL of ethanol 75% in DEPC water (milli Q water mixed vigorously with 0.1% diethylpyrocarbonate for 2 h and autoclaved). The sample was centrifuged for 5 min at 7500 g. The supernatant was discarded and the pellet was air-dried for 5–10 min. The pellet was dissolved by pipetting in 50–200 µL of DEPC water and was incubated at 55–60 °C for 10 min. RT-PCR was used to synthesize complementary DNA (cDNA) from RNA. In each tube, 10 µL of 5x reaction buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, and 1.5 mM/$MgCl_2$), 1 µL of 50 mM dithiothreitol, 1 µL of 1 U of RNAase inhibitor, 2 µL of 800 µM of dNTPs, 2 µL of 2.5 µM of primer dT12-18, and 1 µL of 200 U of Maloney murine leukemia virus (MMLV) reverse transcriptase (PROMEGA) was added to 1 µg of RNA samples. This mixture was adjusted to 50 µL with DEPC water and was incubated at 37 °C for 2 h. The enzyme was inactivated by increasing the temperature to 60 °C for 10 min.
To identify transcripts of the constitutive ribosomal protein actin (as positive internal expression gene) and enzymes and toxins (proteases, beauvericin and bassianolide), specific internal gene primers were used in a polymerase chain reaction (PCR). In a final volume of 50 µL, 1X buffer (200 mM Tris–HCl at pH 8.4,500 mMKCl), 5 µL of template (cDNA), 3 µL 1.5 mMMgCl2, 1 µL of 100 mM dNTP's, and 10 pmol of each primer (Table 1) were mixed with 1 U of DNA taq polymerase (Bioline).

Results and Discussion:

Currect production of insecticides based on entomopathogenic fungi (myco-insecticides) is simple; however, their use as biological control agents critically depends on the standardization of production and stability as field biopesticide, wherein the formulation should allow the fungus to maintain their virulence (Deståfano *et al*., 2004).

Table 1. Selected primers for *Beauveria bassiana* enzymes and toxins expression genes.

Primer	Sequence	Expected size
Actin	*F* (5′-CCGAATTCCACCGATCCAGACAGAGTACTTTCGC-3′) *R* (5′-CCGAATTCGACATCAAGGAGAAGCTCTGCTACGTC	400 pb
Beauvericine	*F*: 5'-TCATGATTGAGCATCAAGCG-3' *R*: 5'-CTTGCCAATGAAACAGATGG-3'	401 pb
Bassianolide	*F*- 5´ AGCAGGATGCCATTGTTAGC 3´ *R*- 5´CTGAAGGAACGCCAGTAAGG 3´	506 pb
Proteases	EA1042*f*: 5-CTTCTGTTGTCAAGTTTGGCAAGG-3; EA1042*r*: 5-GCTTTCTATGAGTAGCTCGCCAAT-3	831 pb

In a previous study, propagules of one native *B. bassiana* strain produced in a selected culture medium produced significantly higher insect cuticle degradation enzymes, and the speed of achieving the insect pest mortality was reduced in 50% (Tamez-Guerra and Castrejon-Antonio, 2013). Nevertheless, the fungus toxins production was not evaluated. In this study, beauvericin (*Bbbea*) and bassianolide (*BBbass*) genes were amplified by RT-PCR. Analysis of collected samples from four *B. bassiana* strains grown in the selected culture medium at four different fermentation times (3, 7, 9 and 13 d), demonstrated the amplification of the four gene analyzed transcripts in the selected medium (Figure 1).
The expression of the *actin* gene transcript was constant and similar among strains and culture fermentation time. The expression of *Bbbea* was similar among strains in the first two collection times (3 and 7 d), was diminished by the PTG2 after 9 d, and only detected by the PTG6 strain after 13 d of fermentation. The expected band showing the expression of the *Bbbass* transcript was observed by all strains in the first collected sample (after 3 d). The PTG1 was the only strain that produced bassianolide to up to 9 d fermentation (Figure 1). Proteases transcript amplification was clearly observed in the first two collected samples (3 and 7 d) from PTG1, PTG2 and PTG6 strains, similar in the third sample (9 d) by the PTG1 and PTG2 but diminished by PTG6 strain, and slightly observed by the PTG1 strain after 13 d fermentation. The proteases transcript expression was not observed in any collected sample from PTG4 strain (Figure 1).
During its pathogenic phase, the developing *B. bassiana* hyphae penetrate the insect integument by producing extracellular enzymes (Fan *et al*., 2007). Virulence factors that modulate the insect immune system, and those that disable and finally kill the host, are also expected to contribute to the *B. bassiana mode of action*. In general, filamentous fungi with a pathogenic lifestyle often produce a large variety of peptides' based toxins that act as immunosuppressors or virulence factors (von Döhren, 2004). Toxins and enzymes are responsible for the virulence of each strain, where the speed of killing target insect pests may change upon nutrimental culture compounds. Out of four native *B. bassiana* tested in this study, all produced beauvericina especially in the early fermentation period, whereas bassianolide was only observed in samples collected after 3 d fermentation by the 4 strains and only one (PTG1) produce it for up to 9 d.

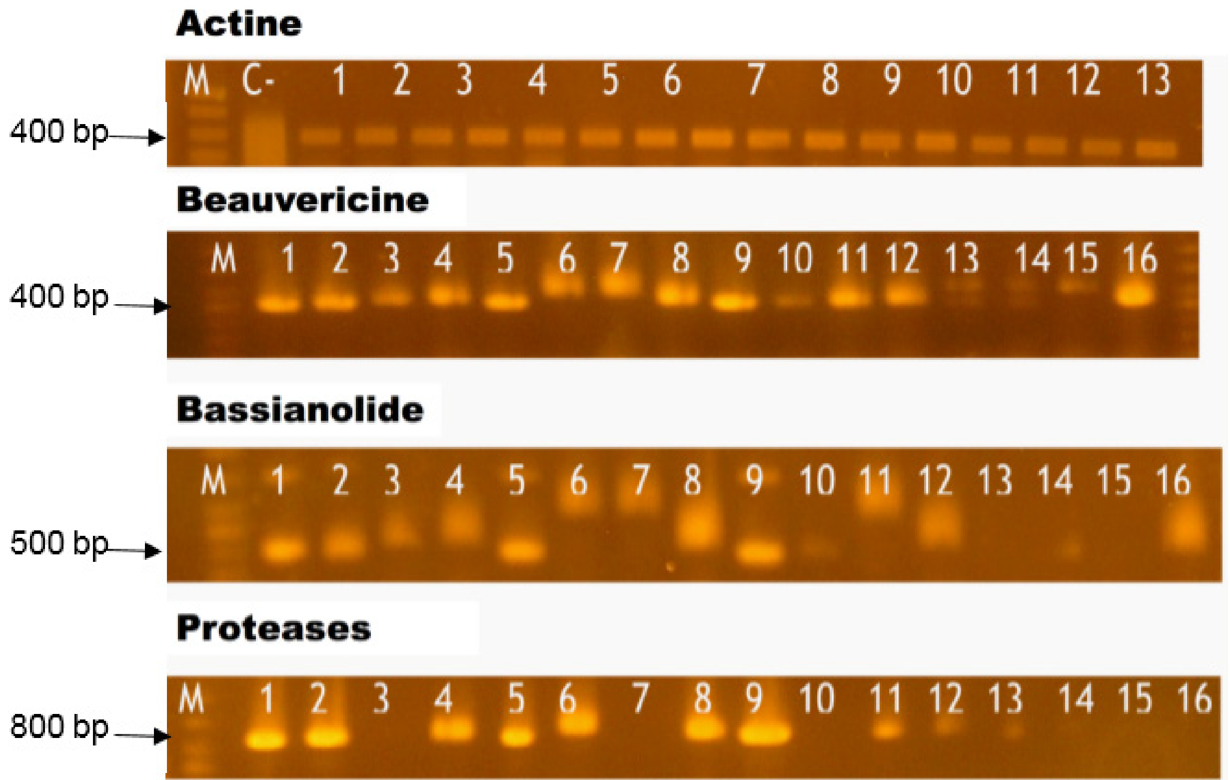


Figure 1. RT-PCR transcripts expression of genes: actine (*actine*), beauvericin (*Bbbea*), bassianolide (*BBbass*) and proteases (*Bbpro*) from samples of four *B. bassiana* strains culture medium at four different fermentation times (3, 7, 9 and 13 d). M= Molecular weight marker; lanes 1-4, samples collected at day 3; lanes 5-8 from day 7; lanes 9-12 from day 9; and lanes 13-16 from day 13 of fermentation of PGT1, PTG2, PTG4 or PTG6 strains, respectively.

Conclusion: The expression of three genes related to *B. bassiana* virulance; beauvericin, bassianolide, and proteases was observed by the strain PTG1 along the fermentation process, compared with the PTG2, PTG4 and PTG6 strains.

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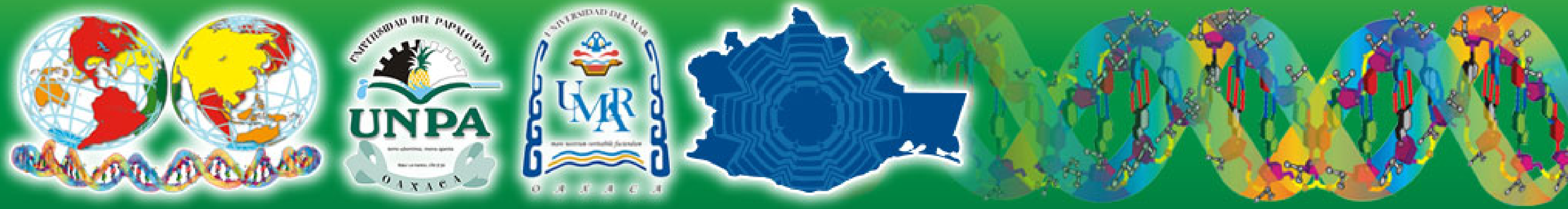

Gamma radiations (^{60}CO) on kiwi (*Actinidia deliciosa*) a Chev cv. Hayward) to induce tolerance to carbonates and alkaline pH

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Abstract: Mature kiwi embryos were given 4 doses of gamma radiation (Gy 0, 50, 100 and 150) and later cultured under *in vitro* conditions on Murashige and Skoog (1962) basal medium (MS) alkalinized by adding 4 different $CaCO_3$ concentrations (0, 0.4, 0.6 and 1.0%) with the pH adjusted to 5 levels (pH: 5.7, 7.2, 7.6, 8.0 and 8.4). The percentage of embryo germination (G) was evaluated 25, 30, and 40 days after culture establishment as was the percentage of seedlings. Showing tolerance to $CaCO_3$ and alkaline pH levels (T) 60, 120 and 180 days after germination. G was significantly affected by the Gy+Ca+pH interaction, with the combination 150 Gy, 1.0% $CaCO_3$ and pH 8.4 producing the lowest value (30%) while the value for the control was 70%. T was affected by Gy showing values of 3.3%, 6.0%, 7.3% and 2.7% for 0, 50, 100 and 150 Gy, respectively. From these observations we conclude that 100 Gy is the optimal irradiation dose (OD_{50}) for these crops.

Keywords: Kiwi ● *in vitro* ● cobalt 60 ● embryo germination ● alkalinity

Introduction: Kiwi, *Actinidia deliciosa* A Chev, is a fruit that has acquired great importance during the past two decades. However, its cultivation has been strongly limited by factors of soil, with calcium carbonates and bicarbonates $CaCO_3$, ($CaHCO_3$) and high pH levels as the main causes of physiological disorders in the plants. Ferric chlorosis, due to the lack of iron (Fe) availability, stands out among its serious problems. Depending upon the degree of deficiency, it can be the cause of low growth productivity and, in extreme cases, the premature death of vines (Tracme and Grass, 1987). Nevertheless, for many crops satisfactory answers have been found for conditions of salinity and alkalinity via the use of mutagenic agents and *in vitro* culturing. Handa *et al*. (1982), states that this is the most appropriate way to obtain genotypes tolerant to limiting soil conditions due to high concentrations of salts, the lack or overabundance of water, extreme temperatures, etc. However, within those limiting conditions, high salt concentration in water and soil have caused the most grief to farmers and perhaps have been subject to the most studies as well (Rengel, 1992). Ali *et al*. (1989) report having cultured citrus ovules *in vitro* in a MT medium supplemented with 0.1 mg L^{-1} of IAA and 1.0 mg L^{-1} of kinetin, and performed transferences over a period of two months to allow the formation of embryos, which were treated with 50-70 Gy of gamma radiation and transferred to the same medium (MT) but with the addition of a 0.8% concentration of NaCl. The tolerant progeny were selected through a gauze test and transferrals were performed 5 to 7 times in the same medium.

Material and Methods:

For the statistical analysis, a completely random experimental design with a 4 × 4 × 5 factorial array of treatments (radiation dose x $CaCO_3$ concentrations x pH levels) was used. The variables analyzed were germination, survival, degree of chlorosis, rate of stem growth, number of leaves, length and number of roots. The first variable was evaluated on the 25, 30, 35, and 40 days after being sown, and the next four variants were evaluated on the 60, 120 and 180 days after germination.

In order to determine the degree of chlorosis in the seedlings, the parameters were adopted according to the scale proposed by Amparano (1973). Data analysis was performed using the PROC GLM procedure for the SAS statistical program. Tukey test at a 5% probability range was used for media comparisons.

Results and Discussion:

Germination of mature embryos. The total germination of the control embryos was 75%, 35 days after culture began. Where only one factor was introduced into the system germination was only moderately affected: 65, 65 and 45% with $CaCO_3$ concentrations of 0.4, 0.6, and 1.0% respectively; and 75, 75, 70, 65 and 60% for pH levels of 5.7, 7.2, 7.6, 8.0, and 8.4 respectively. When there is an interaction between three factors, however, germination seems to be even more affected, to the extent that the interaction of the maximum treatment tested (150 Gy × 1.0% of $CaCO_3$ × 8.0 and pH 8.4) suppressed the germination rate by 70% (this percentage was the lowest of all the treatments and interactions). On the other hand, there was an exception regarding the interaction of the treatments at 50 Gy × 8.0 pH and 50 Gy × 5.7% pH x 0.4% $CaCO_3$, which showed the highest rate of germination (80%).
With these treatments, germination not only declined but was also delayed by 15 to 25 days compared to the control plants. Thus, we can infer that there was a negative interactional effect on kiwi embryos cultured *in vitro* in proportion to increased radiation doses, $CaCO_3$ concentrations, and pH levels.

Survival of Kiwi seedlings. The results show that the treatments have a growing negative interactional effect upon the survival of the seedlings. In interactions of 100 Gy radiation doses × 0.6% of $CaCO_3$ × pH levels of 7.2, 8.0 and 8.4, the percentages of survival were cancelled out 120 days after germination. These same calcium carbonate and pH concentrations exhibited greater suppressive effects on survival when the embryos were irradiated with 150 Gy doses, for at these doses, the survival of the embryos was nullified as of the 60th day of interaction with the 7.2, 7.6 and 8.0 pH levels.

Chlorosis. The results revealed that the seedlings grown from non irradiated embryos reached chlorosis levels of 2.5 (maximum) and 2.3 (minimum) by the 60th day. These had been established in cultures with a $CaCO_3$ level of 0.4% and 1.0%. Seedlings from embryos irradiated in doses of 50 and 100 Gy and inoculated under the same conditions showed lesser degrees of chlorosis (2.2 and 2.3 respectively). However, with radiations doses of 150 Gy the chlorosis levels were higher. In the second evaluation (120 days after germination), the chlorosis levels were higher for each treatment compared to those of the first evaluation. The increase was proportional to radiation doses and $CaCO_3$ levels, as expected. At this point, chlorosis levels reached maximums of 3.11 and 3.4 for radiation doses of 50 and 100 Gy interacting with the maximum tested percentage of $CaCO_3$ (1.0%). In addition, by this evaluation the dose of 150 Gy caused a high percentage of plantlets to be eliminated by the lethal effect of radiation and high levels of $CaCO_3$ and pH.
From these results we can conclude that radiation levels of up 100 and 150 Gy, 0.6 and 1.0% of $CaCO_3$ and a pH greater than 7.2 can cause high degrees of chlorosis in Kiwi seedlings cultured *in vitro*. Therefore, this deficiency resulted in diminished growth and seedlings survival. Upon determining the percentage of seedlings that presented normal characteristics in each and every one of the interactions of radiation doses, it was found that the radiation dose that produced the highest percentage of seedlings tolerant to high $CaCO_3$ and pH levels was that of 100 Gy (with a total of 73%

embryos germinating). This dosage produced 121% more tolerant seedlings than in the control. At a dosage of 50 Gy a rate of 6% was produced, an increase of 82%, while the control showed a rate of 3.3%, greater than the 2.7% produced by a dosage of 150 Gy.
These results demonstrated that radiation treatments of 50 and 100 Gy produced the least chlorosis, as well as the highest percentage of normal seedlings compared to those seedlings grown from non-irradiated embryos. It is suspected that during the radiation process the seedlings were forced to show their genetic and varietal differences (Warne and Hickok, 1986; Epstein, 1988) so they could adapt to the $CaCO_3$ concentrations and alkaline pH of the nurturing medium.

Stem growth. Variance analyses of the stem growth variations showed important differences on some evaluation dates for the combinations of radiation × $CaCO_3$, and radiation × pH level × $CaCO_3$. The combination of $CaCO_3$ and pH on the 120th day after germination showed a negative interactional effect. In other words, growth rates were inversely proportionate to $CaCO_3$ and pH. In fact, while the embryos at the time of germination reached a height of about 1 cm, with treatments with 0.6% of $CaCO_3$ × pH at levels of 7.6, 8.0, and 8.4, the growth rate was minimum, and in some cases non-existent. This behavior was also similar to the other treatments and their combinations.

Effects of radiation x $CaCO_3$. The highest number of leaves was produced (with the 0.4% $CaCO_3$ treatment) by those seedlings that came from embryos irradiated at 50 and 100 Gy. These results can be easily seen on the first and third evaluation, while the 1% $CaCO_3$ treatments produced the lowest number of leaves for each and every one of the dates that were evaluated. This suggests that this percentage of $CaCO_3$ is high for kiwi seedlings cultured *in vitro*.
It should be mentioned that the doses of 100 and 150 Gy produced a high percentage of seedlings that could not produce leaves, and in many cases the seedlings could only germinate and develop hypocotyl, but no leaves, and when leaves grew, they were deformed and small. Such abnormality is due mainly to the above mentioned radiation doses, since seedlings radiated at 150 Gy presented the most abnormalities. This caused the seedlings to have a low growth rate or none at all.

Number and length of roots. The variables of root length and number of roots showed highly significant differences in response to the three factors alone and in combination. The results showed, in general, that there was a linear negative effect on root growth from increases in radiation doses, $CaCO_3$ and pH. The longest average root length (3 cm) occurred with the embryos that were irradiated at 50 Gy and cultured in a medium with a pH concentration of 7.2, while the seedlings that received doses of 5 and 100 Gy and that were cultured in a medium with $CaCO_3$ concentrations of 0.4 and 0.6% achieved an average root length of 1.9 to 2.0 cm. Some $CaCO_3$ effects that could be clearly noticed at a glance were that on some seedlings, the roots were not capable of introducing themselves in the culture medium, forcing them to spread over the medium's surface. Other seedlings grew rather long, deformed roots without root hairs. In time, some of them thickened and became dark-brown colored, leading to the seedling's death.

Conclusions: The basic salts used on the Murashige and Skoog medium (1962) are adecuate for germinating and developing mature kiwi embryos *Actinidia deliciosa* A Chev. The Lethal Dose (LD_{50}) and the Optimum Dose to use on mature kiwi embryos is 100 Gy. 73% of the seedlings that grew from the embryos receiving the 100 Gy radiation dose and sown in a culture medium with 0.4% and 0.6% of $CaCO_3$ and pH levels of 7.2, 7.6, 8.0 and 8.4 were tolerant to such conditions. Only 33% of the seedlings that came from the non-irradiated embryos sown in the same alkaline conditions were resistant. The 50 Gy dose was the one that produced the highest germination rate (80%) with the

treatment of pH 8.0 and no $CaCO_3$, as well as in the treatment with a pH of 5.7 and 0.4% $CaCO_3$. The non-irradiated embryos showed a maximum germination rate of 75%. With this, we can deduce that the dose of 50 Gy is useful to enhance the germination of mature kiwi embryos cultured *in vitro*. The 180-day period allows the identification of seedlings tolerant to high $CaCO_3$ and pH levels. 0.6 and 1.0% concentration of $CaCO_3$, pH levels of 8.0 and 8.4, and radiation doses of 150 Gy strongly limit mature embryo germination, growth and survival of kiwi seedlings *in vitro*. The germination of mature kiwi embryos, and the survival and morphological appearance of kiwi seedlings *in vitro* are good indicators for tolerance and susceptibility to high $CaCO_3$ and pH levels. Considering the genetic variability of mature kiwi embryos, we can infer that the *in vitro* performance of those plants will indicate their *in situ* performance in calcareous soils.

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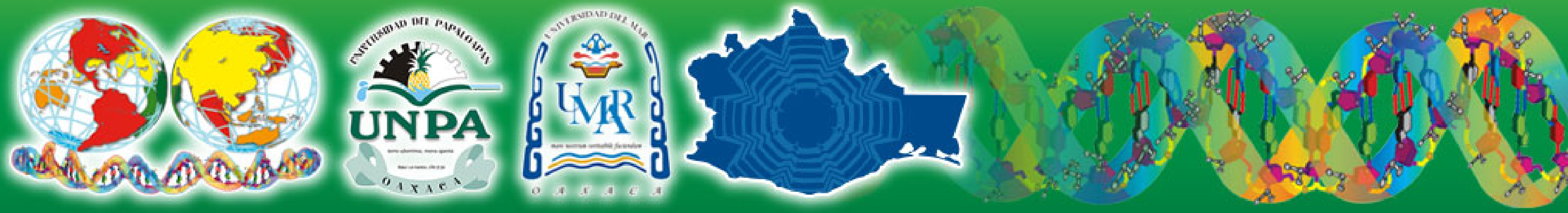

Effective use of recombinant *Brucella ovis* Omp31 antigen to detect cattle serum antibodies by the ELISA indirect test

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Abstract: Brucellosis is considered of great importance to livestock, since it significantly affects animal farming and market, causing a reduction in production. Serological diagnosis is the most common method used to confirm the disease. The practical use of diagnostic tests has not been entirely satisfactory. It is known that the immunogenic membrane protein Omp31 of *Brucella* can stimulates a humoral response in susceptible animals. In the present work, we obtained the recombinant Omp31 protein from *Brucella ovis* and used it to analyze 10 bovine sera positive to microbiological culture and fluorescence polarization assay (FPA), and 10 negative sera to Rose Bengal test (RBT, 8%), FPA and microbiological test, by indirect ELISA. The results of the serum samples from cattle not only showed antigen-antibody recognition, but also it was observed that the differences between the OD for the positive and negative sera were markedly different, showing the highest readings for sera positive cases. These results showed the effectiveness of recombinant Omp31 protein to detect antibodies against *Brucella* in cattle sera.

Keywords: *Brucella* ● recombinant Omp31 ● indirect ELISA ● diagnosis

Introduction: Brucellosis is a bacterial disease caused by microorganisms of the genus *Brucella*. Although different *Brucella* species are genetically closely related, there are differences in pathogenicity and preference of the host, which may be favored, at least in part, by the composition of the outer membrane (Martin-Martin *et al.*, 2009). Within the genus *Brucella* the following antigens have been identified: S-LPS lipopolysaccharide (smooth species), LPS-R (rough species) and outer membrane proteins (OMP) (Salas *et al*., 2001). The major *Brucella* OMPs were originally identified and classified according to their molecular weight (Moreno and Moriyón, 2006). Thus, the membrane proteins of group 1 have apparent molecular weight of 94-88 kDa, group 2 (omp2a and omp2b) having a molecular weight of between 36-38 kDa, and group 3 (omp25 and omp31) of 25-27 and 31-34 kDa, respectively (Cassataro *et al*., 2004). In *Brucella*, major OMPs are Omp25 and Omp31 (belonging to group 3).
Rough *Brucella* strains (*B. ovis, B. canis* and *B. abortus* RB51) lack of O-LPS chain and OMPs are more exposed on the surface and their role in the bacteria virulence has become very important in the search for antigens that can be used in the development of vaccines or diagnostic methods (Caro-Hernández *et al*., 2007).
In this work, omp31 *B. ovis* gene was cloned and expressed in *E. coli* system, using DH5α and TOP10 strains. Omp31 recombinant protein was obtained and analyzed by indirect enzyme

immunoassay (ELISAi). The results showed the effectiveness of recombinant Omp31 protein to detect antibodies to *Brucella* in bovine serum. Further investigation will be aimed at evaluating this protein in serological diagnostic tests like ELISA and FPA for diagnosis of brucellosis in domestic ruminants.

Material and Methods:

Cloning, expression and purification of Omp31 recombinant protein. DNA was extracted from *Brucella ovis* REO-198 strain kindly donated by Centro Nacional de Investigaciones Disciplinarias en Microbiología Animal (CENID-Microbiología) of Instituto Nacional de investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) and purified with PureLink Genomic DNA Minikit (Invitrogen®). Primers LCR04f 5'-TCCGTAATTTTGGCGTCCAT-3 and LCR04r 5'-TAGTTCAGACCGACGCGAACA-3', were designed from gen *omp31* sequence published by Vizcaíno *et al*. (1996, 2001b) in NCBI (using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). PCR test was performance using Touchgene Gradient (Techne®) thermocycler according with Tamez-Vielma, 2010. PCR product obtained (708 pb) was cloned in DH5α strain using the cloning vector pBAD/Thio-TOPO® (Invitrogen®). Plasmidic ADN was obtained and sequenced by Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental from Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, México and then subcloned in TOP10 strain. Cloning procedure was done in Laboratorio Central Regional de Monterrey A. C. (LCRM), from Comité de Fomento y Protección Pecuaria del estado de Nuevo León (CFPPN). To express *Brucella ovis* Omp31 recombinant protein, the TOPO/Omp31 recombinant strain obtained was grown on LB broth with ampiciline (50 μg mL^{-1}) and supplemented with arabinose at 37 °C for 4 h on a shaker. After that, the recombinant protein was purified using QIAexpress® Ni- NTA Fast Start (Qiagen®) and quantified in NanoDrop 2000c UV-Vis (Thermo Scientific®). We obtained 2 mg recombinant protein L^{-1} of purified culture.

ELISA indirect test. Ten negative serological and 10 RBT, FPA and microbiological positive cattle sera were tested by ELISAi in 96 flat-well microplate. In brief, a volume of 5 μg of recombinant protein diluted in 100 μL of carbonate buffer (pH 9.0) was placed in the plate well, incubated 2 h at room temperature and washed with 200 μL of wash solution PBS-Tween 0.05% (PBST) 10 min at room temperature on a shaker. After that, the plate was blocked with TBS-skim milk (2%) solution 2 h at room temperature and then washed. Sera and controls were diluted in PBST (ratio of 1:200) and 100 μL of these dilutions were added to each well in the plate, incubated at 37 °C in a humid chamber 1 h and washed with PBST. A volume of 100 μL of anti-ruminant IgG conjugate with HRP was added and incubated in a humid chamber 1 h at 37 °C and washed with PBST. One hundred microliters of 3,3′,5,5′-Tetramethylbenzidine (TMB) were added and incubated 15 min at room temperature in the dark. Then, 100 μL of stop solution were added. The absorbances were measured in a microplate reader at 405 nm. All experiments were performed in triplicate and its results analyzed by T-student test. Microbiological and serological positive and negative bovine sera were used as controls.

Results and Discussion:

ELISA indirect test. To determine whether the recombinant Omp31 protein is capable of acting as an antigen in the detection of antibodies to *Brucella* spp, a type indirect enzyme immunoassay (ELISAi) with 10 negative and 10 positive bovine sera was carried out. The analysis of the results of serum samples showed recognition of serum antibodies to the protein recombinant Omp31 and also differences between the OD of the positive and negative sera were markedly different, showing the highest readings corresponding to positive cases sera (Figure 1).

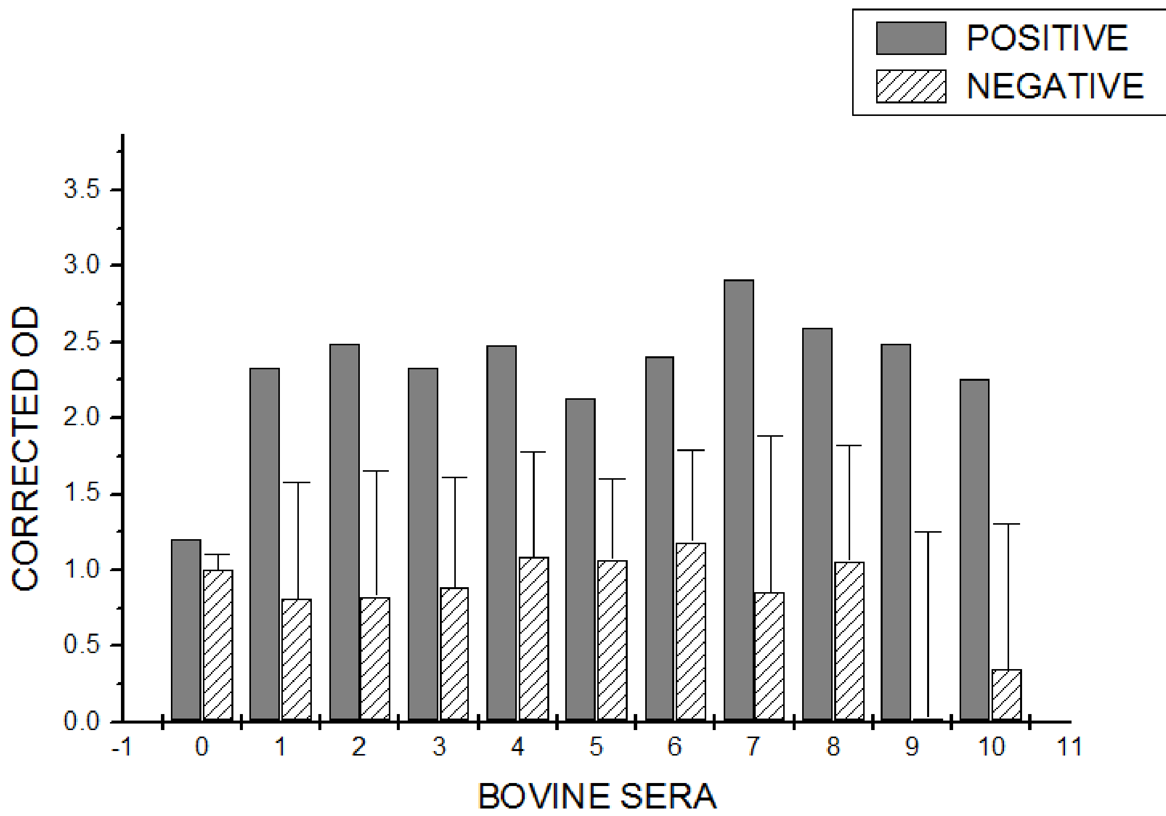

Figure 1. Graph showing corrected OD from bovine sera positive and negative to brucellosis. Column 0-9, (gray) samples of bovine positive sera; column 0-9 (pattern) samples of bovine negative sera; column 10 (gray) positive control; column 10 (pattern), negative control. (t=-8.822; *p-value*=2.49, α=0.05).

In genetic engineering, isolation of a specific gene that has information to encode a certain protein and its introduction into the DNA of an organism other than the initial (recombinant DNA) results in the production of a genetically modified organism to produce the protein of interest (recombinant protein). Among the many systems available for the production of recombinant proteins,

Escherichia coli is the most widely used in biotechnology because of its fast growth, high production density and lower cost substrates, also its genetics is widely characterized and coupled with this. There are a large number of strains and vectors available for use in this system (Baneyx, 1999).

In the genus *Brucella*, it has been discovered that the outer membrane proteins, especially those belonging to group 3 (Omp25 / Omp31), are sufficiently immunogenic to confer some protection against the disease and have been proposed as an option for the development of vaccines DNA as immune system induce both cellular and humoral response (Vizcaino *et al.*, 2001a).

In *Brucella,* major OMPs are Omp25 and Omp31 (belonging to the group 3), except for *B. abortus* which has been demonstrated by molecular techniques that lacks the gene coding for this protein and therefore the *omp31* may not be present in the outer membrane of this species (Cloeckaert *et al.,* 1996). However, in *B. abortus* has been described a membrane protein called Omp31b (~31 kDa) which has some similarity to Omp31 from the other strains of *Brucella*. Because of this, the nucleotide sequence of the recombinant Omp31 protein of *B. ovis* obtained was compared with the sequence reported in the BLAST Omp31b by Kim *et al.* (2011) to obtain a homology of 77%, a relatively high percentage that could explain the observed reaction in bovine sera.

Conclusions: The recombinant protein could be used to test antigen-antibody binding by ELISAi. The results obtained showed that the recombinant protein Omp31 produced reactivity with field sera from cattle. It is necessary to perform additional studies involving larger number of sera to determine the truly usefulness of recombinant *B. ovis* Omp31 protein in the diagnosis of brucellosis in different species of domestic ruminants.

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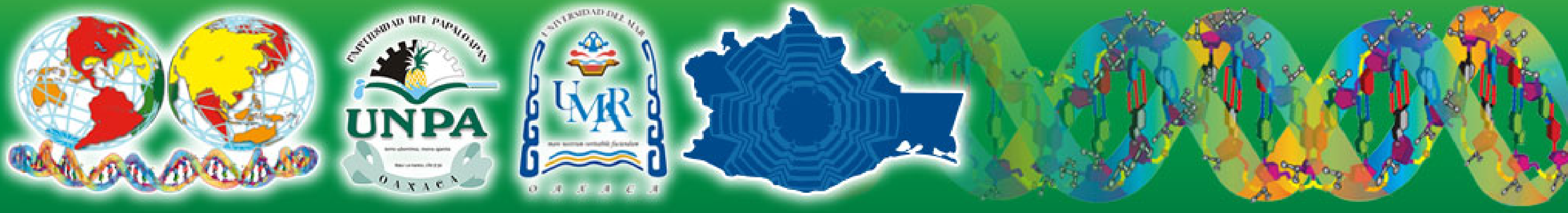

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Effect of nanoparticles on growth, development and yield of *Phaseolus vulgaris* L. and *Zea mays* L.

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Abstract: The increasing production of nanoparticles and its byproducts bring as a priority the necessity of understand the real interaction between crops and nanoparticles (NPs) in an agricultural soil. The present study addresses the effects of nanoparticles of ZnO, TiO_2, Fe_3O_4, Fe_2O_3 and FeOOH on growth, development and yield of *Phaseolus vulgaris* L. and *Zea mays* L. at greenhouse conditions, by detecting morphological and physiological changes that may also favoring nanoparticle bioaccumulation in the tissues. The experimental results shows that ferrihydrite and hematite nanoparticles at suitable concentrations (3 g L^{-1}) may actually benefit the development of some variables of the bean plants, whereas ZnO and TiO_2 nanoparticles at optimal concentrations benefited some plant characteristics. The phytotoxic effect of the nanoparticles was not hazardous to the yield of bean crop, just as for the maize crop. Nanoparticles had no effect on the generation of biomass, so that this trend indicate that there were not negative effects on crops.

Keywords: nanoparticles ● maize ● bean ● yield ● environmental pollution

Introduction: Nanoparticles are particles which dimensions are between one and 100 nanometers that occur naturally in case of forest fires, volcanic eruptions, etc., and can be manufactured in various industrial processes which has resulted in a major exhibition of these nanomaterials at the environmental level. Nanoparticles exhibit unique physical and chemical properties, which differ significantly from those for the same material at larger scale. (Nel *et al.*, 2006). Nel *et al.* (2006) mentions that there are nanoparticles, nanoemulsions and nanocapsules present in a wide range of products: chemicals, processed foods, food packaging materials and food contact materials, including food storage containers, cutlery and cutting boards. In Mexico some farming and ecological reserves with wild native flora are very close to the big cities. Therefore, the nanoparticles generated through the production process or during combustion processes in industry (thermoelectric, brickmaking, cement, etc.), diesel or gasoline engines, or the nanoparticles accumulated in the biosolids (sewage), have the potential to come into contact with crops and wild plants (Karl-Josef and Herth, 2011, Xingmao *et al.*, 2010). However, to our knowledge, in Mexico there is still no research evaluating the potential effect of nanoparticles on the growth and development of plants. It addition to the above, it is unknown the morphological and physiological responses of plants to high concentrations of nanoparticles and which storage sites in the tissues of these plants would be (Cañas *et al.*, 2008, Doshi *et al.*, 2008). Moreover maize and bean crops are important for almost all the countries and are part of the staple diet of many families (Cachorro *et al.*, 1993) The beans in Mexico ranks second in acreage and sixth in value of production. Its importance is ancestral and is the basis of food and nutrient source since prehispanic era. This work was conducted to determine the effect of nanoparticles of ZnO, TiO_2,

Fe_3O_4, Fe_2O_3 and FeOOH on growth, development and yield of *Phaseolus vulgaris* L. and *Zea mays* L. at greenhouse conditions, by detecting morphological and physiological changes that may also favoring nanoparticle bioaccumulation in the tissues (Asli *et al*., 2009; Jacob *et al.,* 2013; Presad *et al*., 2012; Seeger *et al.,* 2009; Zhu *et al.,* 2008; Jósko and Oleszczuk, 2013; Lin and Xing, 2008).

Materials and Methods:

This study was made in a greenhouse of the '*Grupo de Sustentabilidad de los Recursos Naturales y Energía del Cinvestav-Saltillo*' located in Saltillo, Coahuila, Mexico. The experimental setup was carried out from January to July 2014. This area is located in the southeastern state of Coahuila, centered at 25°31' N, 101°37 ' W, at an altitude of 1600 m above sea level with a mean annual temperature of 18 °C. The climate is generally dry and semiwarm to warm extreme to a large extent of Coahuila, with some variants through the regions of Coahuila. Temperatures average 12 °C in January, the coldest month, and 23 °C in June and July, the hottest months. Annual rainfall averages 369 millimeters, much of which falls during September and October. Based in the Köppen climate classification the semi-arid hot climate (BSh) is found in this area. According to FAO/UNESCO soil classification system, the soil is a Haplic Xerosol with pH 7.3 and electrical conductivity 4.8 dS m^{-1}, a water holding capacity (WHC) of 865 g kg^{-1}, an organic carbon content of 1.5 g C kg^{-1} soil, and a total N content of 0.7 g N kg^{-1} soil. Maize seeds were provided by 'Universidad Autónoma Agraria Antonio Narro', Coahuila, Mexico, while common bean seeds were donated by INIFAP-Celaya, Mexico. All seeds were kept in the dark at 4 °C until use. The experiment design was in complete randomized block with a bivariate array of treatments obtaining a total of 11 treatments, each with three replicates for a total of 33 experimental units per block. In addition, three blocks representing the three destructive sampling was used, giving a total of 99 experimental units. Then 300 mL of nanoparticles were applied to the soil contained in the bags, avoiding contact of the solution with the leaves of the plants. Destructive samplings per month were done, the plants fresh weight of shoot and root were mesured, after which it was subjected to drying for 7 days in an oven at 70 °C. Soil samples from the bags at two depths, 0-10 and 10-20 cm were also taken, the samples were allowed to dry for later analysis.

Results and Discussion:

For the variable height of the plant in the bean crop, significant differences were found, indicating that ferrihydrite nanoparticles at concentrations of 3 g L^{-1} were statistically better than the control, indicating that favored plant height. Zinc oxide nanoparticles with 3 g L^{-1} significantly affect plant height compared to the control, indicating that they had a negative effect on the plant. Lin and Xing (2008), found that zinc oxide nanoparticles administered to *Lolium perenne* L. (ryegrass) at concentrations of 8 to 1000 mg L^{-1} were translocated in the endoderm and cells of the plant, causing severe damage to the root and inhibiting plant growth. Ferrihydrite and zinc oxide nanoparticles at concentrations of 6 and 3 g L^{-1} respectively significantly affect stem diameter compared to control (Figure 1). A study performed by Lee *et al.* (2013) indicated that ZnO nanoparticles reduced the *Fagopyrum esculentum* biomass production by 7.7-26.4%, while the ZnO macroparticulates reduced biomass production by 11.4-23.5% under a hydroponic system subjected to concentrations from 10 to 2000 mg L^{-1} of this nanoparticle.
The appearance of the spike in the maize crop was affected by nanoparticles of titanium dioxide at high concentrations (6 g L^{-1}) because it was found to vary significantly compared to the other

treatments; it was also identified that the concentration of this nanoparticle affects the appearance of the spike because at concentrations of 3 g L^{-1} no significant difference was shown compared with the control (Figure 2). According Asli and Neumann (2009), colloidal suspensions of titanium dioxide nanometer sizes and a natural clay caused the pore size of cell walls of maize roots decreased from 66 nm to about 3 nm, promoting inhibition of the hydraulic conductivity, causing inhibition in the growth rate by approximately 10%, these changes may also delay or advance the apparition of maize spikes.

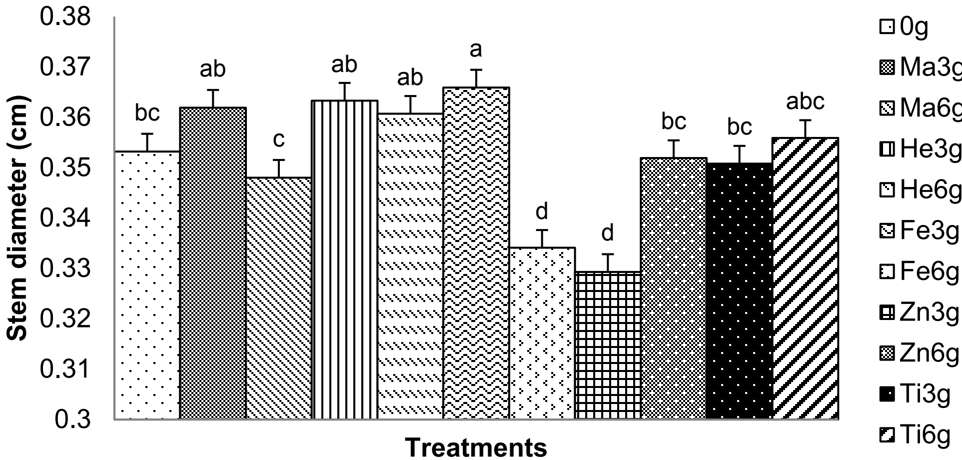

Figure 1. Effect of nanoparticles in the stem diameter of common bean.

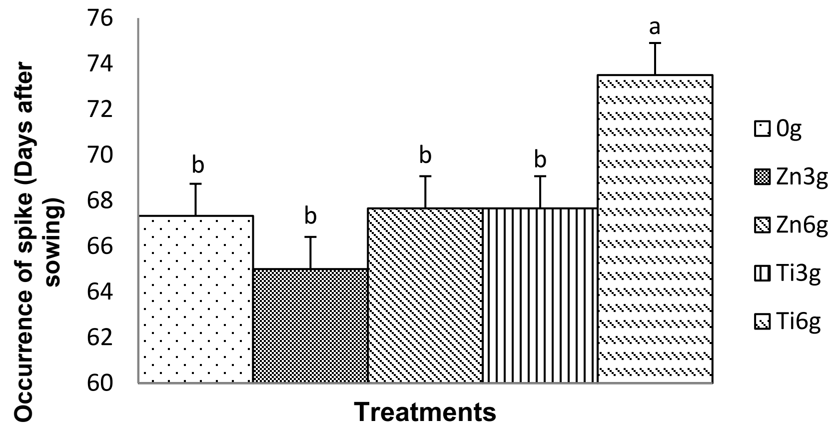

Figure 2. Effect of nanoparticles on the onset of flowering in maize.

In the bean crop, magnetite and titanium dioxide nanoparticles at concentrations of 3 g L^{-1} benefited significantly the pods formation compared within the control, as they advanced by a few days the appearance of these, other nanoparticles showed no significant differences over control. For the appearance of baby maize in maize all nanoparticles showed significant differences with respect to control, delaying the onset of baby maize. Demonstrating that the zinc oxide mainly affect this variable, followed by the titanium dioxide.

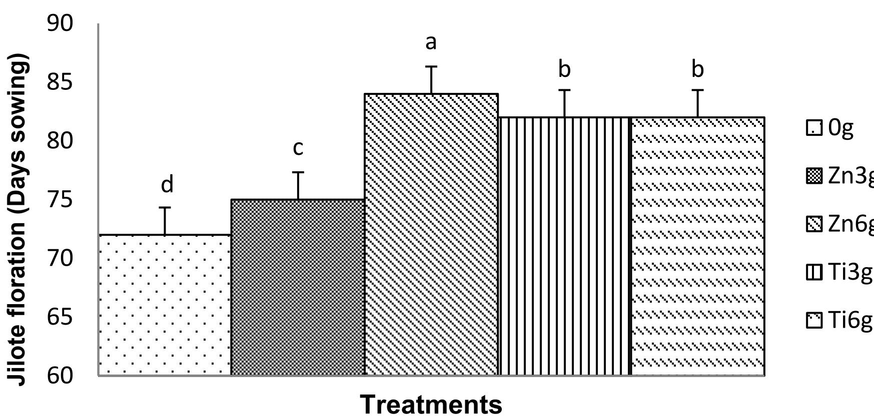

Figure 3. Effect of nanoparticles on the jilote formation in maize.

Conclusion: The ferrihydrite and hematite nanoparticles suitable as a 3 g L^{-1} concentrations may actually benefit the development of some variables of the bean plants, whereas ZnO and TiO_2 nanoparticles may affect some features of this crop, although the level toxicity of nanoparticles depends on many factors and one of them is the plant species with which they work.

Acknowledgment: This research was funded by '*Ciencia Básica SEP-CONACyT*' project 151881 and the Sustainability of Natural Resources and Energy Program (Cinvestav-Saltillo). M.F. V.-R., J.L. H.-L., and I. G.-O. received grant-aided support from '*Ciencia Básica SEP-CONACyT*'. F. F.-L. received grant-aided support from '*Sistema Nacional de Investigadores-CONACyT*'

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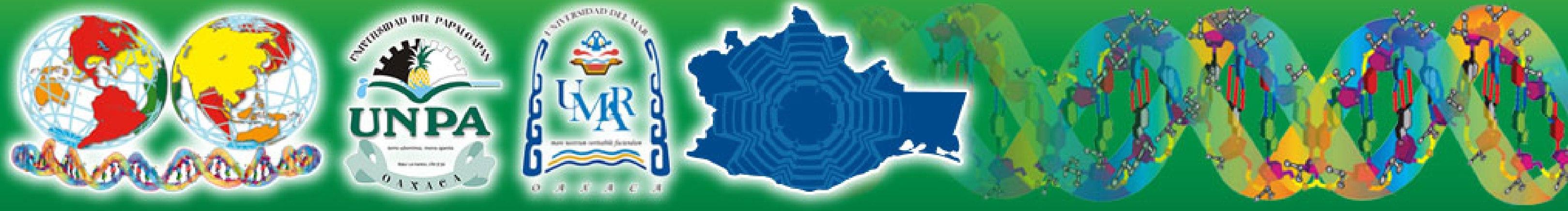

Agrobacterium mediated transformation of spanish red cedar (*Cedrela odorata* L.) and standardization of experimental conditions

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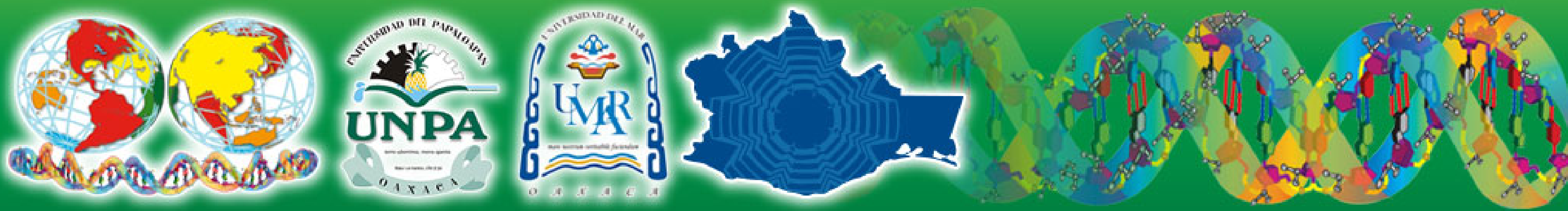
Abstract: A protocol was developed for *Agrobacterium* mediated genetic transformation of *Cedrela odorata* L., via organogenesis, by using hypocotyls as explants. More than 50% of cotyledon and 70% of hypocotyl explants, obtained from 30 day-old *C. odorata* seedlings, produced 2-4 shoots on MS medium supplemented with 2 mg L^{-1} 6-benzylaminopurine (BA), and 70% of shoots produced roots on MS medium with 0.1 mg L^{-1} Indole-3-butyricacid (IBA). These explants were co-cultured with *Agrobacterium tumefaciens,* strain C58C1, harbouring the pCAMBIA 1303 plasmid, carrying the hygromycin resistance gene (*hpt* II) as a selectable marker and β-glucuronidase (GUS) as a reporter gene. Factors affecting transformation efficiency were evaluated; such as age and type of explants, *Agrobacterium* concentration, infection and co-cultivation time period, presence of growth regulators, and the effects of cefotaxime in regeneration medium. A 50 mg L^{-1} concentration of both kanamycin and rifampicin was used to select pure *Agrobacterium* cultures, and 500 mg L^{-1} cefotaxime in addition to 10 mg L^{-1} hygromycin were used to select transformed cells. Four week-old *C. odorata* hypocotyl explants were co-cultured with *A. tumefaciens* at different times; our results showed a 44% transformation efficiency when a 10 minute period was employed. Putative transformed explants were immediately transferred to a regeneration medium in order to generate shoots.

Keywords: Tropical timber ● organogenesis ● transient GUS

Introduction: Tropical forests play an important role both ecologically and commercially. There are at least over 600 tropical timber species; in the form of plywood, logs, sawnwood and veneer; with commercial value in international trade markets (ITTO, 2011). The Meliaceae family is one of the best representatives because it includes the world's most valuable tropical forest species, as their wood is naturally termite and rot resistant. These ligneous species are included on the CITES Red List - Appendix III IUCN, since they are classified as vulnerable due to a population reduction of more than 20% in the last three generations, caused by deforestation and a decrease in their natural range. On the other hand, there is a lack of selection and domestication of species, as well as an inability to establish and maintain commercial plantations, so that international trade demands in tropical timber trees can be accomplished. After mahogany, spanish red cedar is the second tropical hardwood species with the largest economic value. In Mexico, there are *C. odorata* commercial plantations; however, there are limitations to their establishment due to the attack of *Hypsipyla grandella* (Lepidoptera: *Pyralidae*), an insect that limits the growth of meliaceae trees because their larvae feed on the apical meristem of young trees, resulting in loss of apical dominance and trunk deformation, which significantly reduces the economic value of timber



(Howard and Merida, 2005). Looking for natural resistant varieties through traditional breeding technologies would take a long time to be an effective strategy. On the other hand, recombinant DNA technology is a powerful tool for introducing foreign genes into long-lived perennial tree species (Pijut *et al*., 2012). Genetic transformation of tropical tree species is crucial for the development of elite germplasm with increased tolerance to biotic and abiotic factors (Pijut *et al*., 2012; Ho *et al*., 1998). Only a few papers on *Agrobacterium*-mediated transformation systems on tropical trees, such as *Azadirachta indica* (Widiyanto *et al*., 2009), and *Tectona grandis* (Naina *et al*., 1989), have been published, and there have been few reports on the reliable inheritance of their transformed genes. *Agrobacterium-mediated g*ene transfer has advantages over direct DNA delivery, like the preferential integration of foreign DNA into transcriptionally vigorous chromosomal regions (Ho *et al*., 1998). This study describes the transformation of spanish red cedar hypocotyls using a gene transfer system mediated by *A*. *tumefaciens*.

Materials and Methods:

Preparation of plant material*. C. odorata* seeds were obtained from 40 year-old elite trees, selected from natural populations present in the region of Sayula, Veracruz, Mexico (17°51'55.05" N, 94°57'37.09" W). The tree selection was based on anatomical characteristics (straight trunks at least 10 m long and 50 cm in diameter) as well as no signs of *H. grandella* attack. The seeds were disinfected by 20-min immersion in a sodium hypochlorite solution [NaOCl 10% (v/v)], which contained 50 µLL^{-1} of polyoxyethylene sorbitan monolaurate (Tween 20®). Immediately afterwards the seeds were immersed in 70% (v/v) ethanol for 5 min; after that, three rinses of 5 min each with sterile, distilled water were performed. Embryos were isolated aseptically from the seeds, and cultured on semisolid half-strength MSB5 medium [half-strength of MS salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), 30 g L^{-1} sucrose, and 0.7% (w/v) agar]. The pH was adjusted to 5.75 prior to autoclaving at 121 °C for 20 min. Cultures were kept at 25 °C under 140 µM m^{-2}s over a 16 h photoperiod. After 30 days, seedlings were obtained.

In vitro plantlet regeneration. The cotyledons and hypocotyls of 30 day-old seedlings, derived from *in vitro* germinated *C. odorata* seeds as mentioned above*,* were dissected in segments of about 5-10 mm in length. The explants were cultured on semisolid MSB5 medium supplemented with 0, 0.5, 1.0, 2.0, or 4.0 mg L^{-1} of N6–benzylaminopurine (BA), kinetin (KIN), or 6γ,γ-Dimethylallylaminopurine (2-iP) for 30 days for shoot induction. Adventitious shoots that were up to 1 cm in length were then excised from the explants, and placed for 30 days on semisolid rooting medium, which consisted of MSB5 medium plus 0, 0.1, 0.5, or 1.0 mg L^{-1} IBA.

Plant tolerance to cefotaxime and hygromycin*. C. odorata* hypocotyls and cotyledons were plated on semisolid MSB5 medium supplemented with different concentrations of filter-sterilized cefotaxime (0, 100, 250 and 500 mg L^{-1}) or hygromycin (0, 5, 10 and 20 mg L^{-1}). After 30 days of culture, the results obtained for each treatment were analyzed. Once the optimal cefotaxime and hygromycin concentrations were determined, these two were used simultaneously to eliminate *Agrobacterium*, and select transformants.

Agrobacterium strain*.* The *Agrobacterium* strain used was C58C1, a derivative of EHA101. This strain carried the pCAMBIA 1303 plasmid (http://www.cambia.org; Genbank access, AF234299). It was maintained on LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, 10 g L^{-1} NaCl, pH 7.0) supplemented with 50 mg L^{-1} kanamycin and 100 mg L^{-1} rifampicin.

Transformation procedure. Transformation experiments were performed in a growth chamber at 28 °C with a 16 h photoperiod (140 µ $Mm^{-2}s$). Hypocotyls and cotyledons were excised with a scalpel from 30-40 day-old seedlings of *C. odorata*, rinsed in liquid MSB5, dipped in 10 mL of an overnight grown culture of *A. tumefaciens* at different times (10, 15, 20 and 30 minutes), dried on filter paper to remove excess culture medium, and transferred to co-cultivation medium (semisolid MSB5 medium supplemented with 2.0 mg L^{-1} BA). Cultures were kept in darkness. After 3 days, explants were washed with sterile co-cultivation medium at 50 rpm, at 25 °C for 2 h. Dried explants were transferred to co-cultivation medium supplemented with 500 mg L^{-1} cefotaxime (selection medium). After 30 days, explants were placed on semisolid co-cultivation medium supplemented with 250 mg L^{-1} cefotaxime and 10 mg L^{-1} hygromycin in order to induce shoots.

GUS histochemical assay. Histochemical localization of *uid*A expression was performed on transformed tissue. During co-cultivation, control and putative shoots were subjected to β-glucuronidase (GUS) histochemical staining (Jefferson *et al*., 1987). Approximately 15-20 hypocotyls per experiment were used for the histological assay. After staining, explants were cleared in 70% (v/v) ethanol for 24 h prior to counting the number of GUS foci.

Results and Discussion:

Regenerated plants from cotyledons and hypocotyls of <u>C. odorata</u>. *C. odorata* mature embryos easily germinated when cultured on semisolid half-strength MSB5 medium without added plant regulators. After 30 days, explants from cotyledons and hypocotyls were excised, and cultured on semisolid MSB5 supplemented with three plant growth regulators (BA, KIN or 2-iP) at different concentrations, used separately. Under our experimental conditions, explants from both hypocotyls and cotyledons exhibited similar responses to BA, although hypocotyl explants produced more shoots. The finding that *C. odorata* hypocotyls are slightly better explants to form buds is consistent with observations done in *Perilla frutescens* (Kim *et al*., 2004). When a concentration of 2.0 mg L^{-1} of BA was used, a high responsive yield was obtained since 50% of the explants displayed shoot formation. Those responsive explants produced from 2 to 4 shoots (Figure 1 A, and B). As for the use of KIN or 2-iP on semisolid MSB5 medium, just 20% of the explants produced shoots when KIN was employed; while 2-iP produced calli. IAA, NAA and IBA auxins are frequently used to induce rooting in woody plant species (Azad *et al*., 2005), and this process is more difficult to achieve for woody species than for herbaceous ones. Interestingly, rooting in the woody *C. odorata* species was easily obtained by placing shoots on MSB5 supplemented with 0.1 mg L^{-1} IBA (Figure 1C), since other concentrations of the same regulator were not optimal for root induction.

Agrobacterium mediated transformation of <u>C. odorata</u>; effect of antibiotics. To evaluate the effect of cefotaxime and hygromycin on *C. odorata* explants, sensitivity assays were performed. Our results showed that doses up to 500 mg L^{-1} of cefotaxime did not affect the morphogenic capacity of explants; similar results were reported for *Eucalyptus camaldulensis* (Quisen *et al*., 2009). On the other hand, hygromycin had toxic effects on explants at high concentrations; however, a 10 mg L^{-1} concentration allowed us to select transformants. After *Agrobacterium* infection, explants were placed on selection medium with the appropriate antibiotic concentration as described above (500 mg L^{-1} cefotaxime and 10 mg L^{-1} hygromycin).

Type of explant. In this study, transformation efficiency for hypocotyl explants was notoriously successful since all of them (100%) were transformed as judged by GUS expression. On the other hand, cotyledons explants showed less transformation efficiency, just 50% of these explants subjected to *Agrobacterium* infection showed GUS expression. This last result agrees with that one reported for *Petunia hybrid* (Michalczuk and Wawrzy´nczak, 2004). Histochemical staining for GUS activity was not detected when *Agrobacterium* cells without the pCAMBIA13013 plasmid were tested (left tube; Figure 1D); conversely, GUS activity was observed in *Agrobacterium* cells transformed with the pCAMBIA1303 plasmid (right tube; Figure 1D).

Infection time. Another factor that was evaluated in this study was the infection time with *Agrobacterium*. We observed that *Agrobacterium* infection for 10 minutes produced the highest percentage of explants that exhibited GUS activity in their tissues (Figure 1 F, and Table 1). Under this infection time, resulting transformed explants were able to generate shoots on selection medium (Figure 1 I).

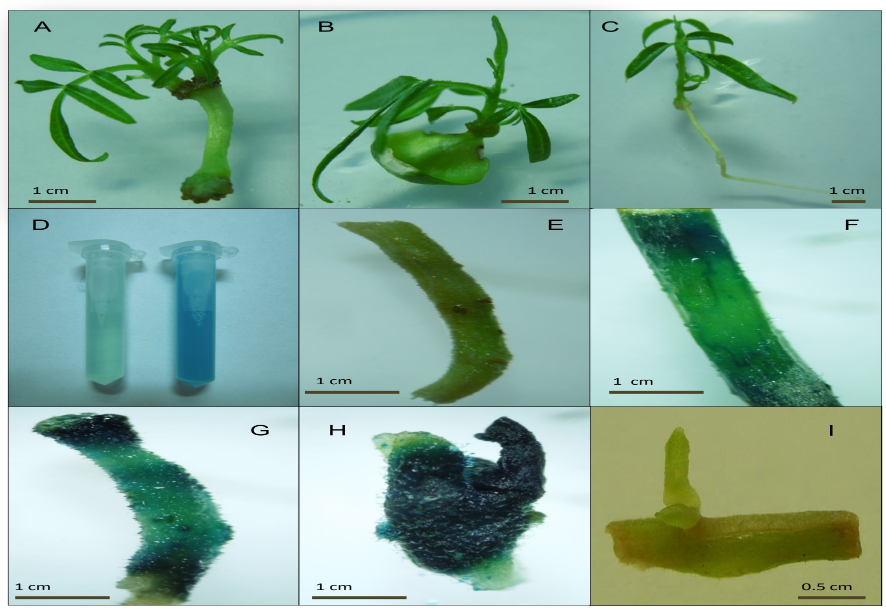


Figure 1. Regeneration and genetic transformation of *C. odorata*.

On the other hand, though GUS activity was detected on explants infected with *Agrobacterium* for 15 min (Figure 1G) and 20 min (Figure 1H); the percentage of explants that was positive for this assay diminished with longer infection times, as well as the shoot formation rate (Table 1). The longest period used (30 min) rendered neither shoots nor tissue positive to GUS reaction (Table 1). This study demonstrates for the first time the transfer and expression of elements present in the pCAMBIA 1303 vector to *C. odorata* tissue via *Agrobacterium*. In order to demonstrate this, experimental conditions were optimized for the detection of GUS activity in *Cedrela odorata*.

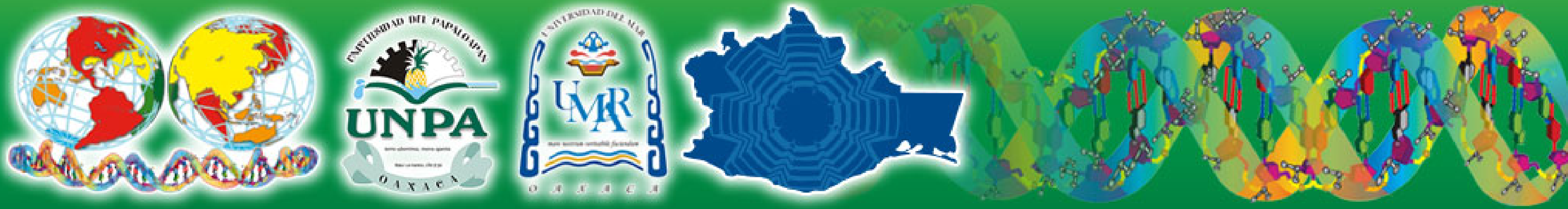


Table 1. Evaluation of *C. odorata* explants infected with *Agrobacterium* at different incubation times.

	10 min	15 min	20 min	30 min
Number of explants infected	42.0	42.0	42.0	42.0
Shoot formation rate	23.8	11.9	4.7	0
GUS positive (%)	67.0	29.4	15.3	0
Transformation efficiency (%)	43.5	15.2	8.10	0

Conclusions: A successful *A. tumefaciens*-mediated transformation, as well as a regeneration protocol of spanish red cedar are reported in this work. The transformation protocol required hypocotyl explants from 30–day *in vitro* seedlings. A 10-min incubation period with the *Agrobacterium* C58C1 strain, harboring the pCAMBIA 1303 plasmid, proved to be the best condition for tissue transformation and shoot generation. Selection and regeneration of transformed tissue was conducted on selection medium, which included 2 mg L^{-1} BAP. Rooting was carried out on rooting medium, which contained 0.1 mg L^{-1} IBA.

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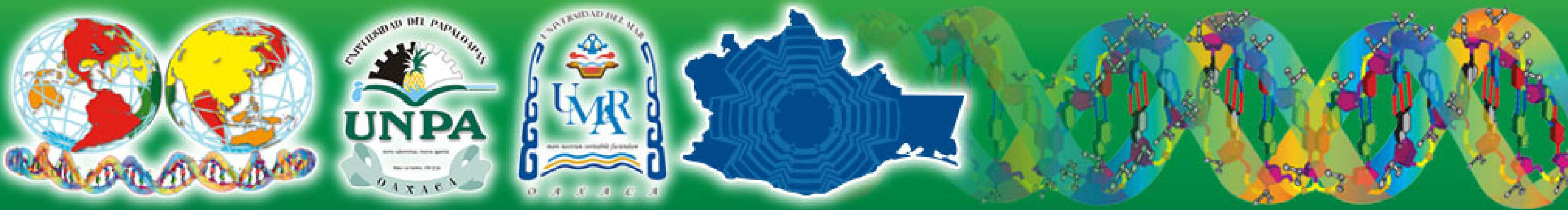

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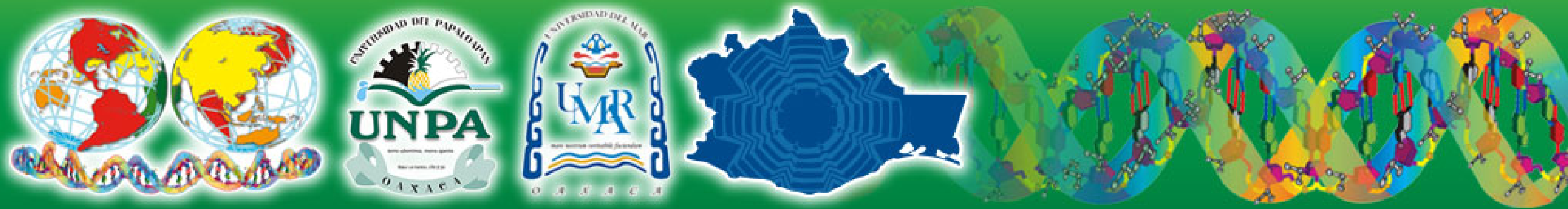

Influence of gut bacteria in the *Bacillus thuringiensis* susceptibility of *Plodia interpunctella*

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Abstract: In agriculture, the increased populations of insect pests have resulted in great crop (or crop products) yield losses. In the past decades, pest control was mostly relied on chemical insecticides, but these have caused considerable damage to the environment, in addition to animal and human health. Less hazard and control-effective alternatives includes the use of entomopathogens. The soil-bacterium *Bacillus thuringiensis* (Bt) is a biopesticide that has been commercialized since the 80s, mainly for Lepidoptera and Diptera pest control. Unfortunately, as observed among chemicals, insect may development resistance to Bt products if the resistant populations are selected. The first lepidoptera pest resistant to Bt was the grain/flour Indian meal moth *Plodia interpunctella.* In addition to the gut enzymatic activity, recent studies have suggested that intestinal microbiota is required for Bt insecticidal activity. Therefore, in this study we analyzed the effect of intestinal bacteria versus susceptibility to Bt in *P. interpunctella*. Our results demonstrated that in fact *P. interpunctella* intestinal bacteria increase the insect susceptibility to Bt, showing as low as 21% mortality after microbiota eradication, compared with 60% mortality in unaltered gut microbiota. Overall, it was concluded that Bt exposure without gut microbiota may lead to reduced *P. interpunctella* susceptibility to this bioinsecticide.

Keywords: Insect–microbe interactions ● insect pests ● *Bacillus thuringiensis*

Introduction: The production and excessive use of chemical insecticides to control insect pests have caused considerable damage to the environment and to animal and human health. Sustainable agriculture and new trends in food production strategies recommend the use of beneficial organisms (pathogens, predators and parasitoids) to reduce insect pest populations. Among the environmentally friendly biopesticides is listed the Gram-positive soil-bacterium *Bacillus thuringiensis* (Bt), which is characterized by its ability to produce crystalline parasporal inclusions during the sporulation process. Produced crystals can be toxic to certain insect orders among other organisms. These inclusions consist of proteins (Cry and Cyt protoxin) that have a specific insecticidal activity and are activated to the “toxic” form by enzymatic degradation in the insect gut (Höfte and Whiteley, 1989; Peyronnet *et al.,* 1997). However, the control of this pest by this bioinsecticide may be compromised due to the insect ability to develop resistance to Bt toxins if environmental selection pressure is high (McGaughey and Johnson, 1992; Yu-Cheng *et al.*, 2000; Candas *et al.*, 2003).

In some species of lepidopteran pests susceptible to Bt, have been noticed that removal of the gut microbiome with antibiotics supplied in diet, the susceptibility to this bioinsecticide is reduced (Broderick *et al.,* 2006). These results suggest that the toxicity of Bt is assisted through interaction with the native intestinal microorganisms. However, other authors report that these changes in susceptibility are due mainly to the use of antibiotics, no to the microbial load.

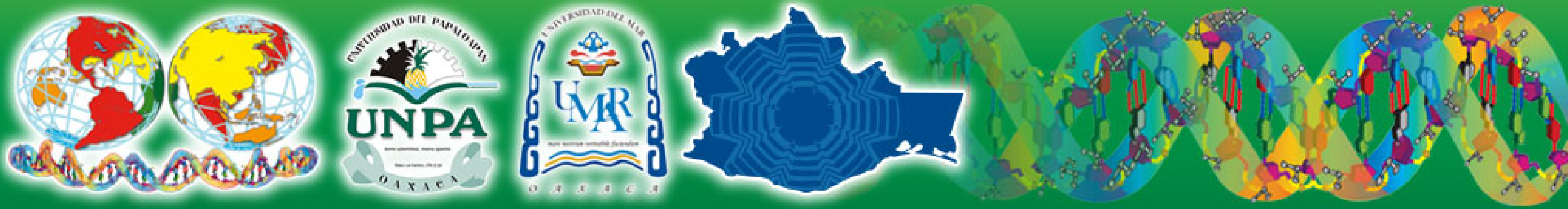
Microorganisms play an important role in the growth and development of many species of insects and contribute to their reproduction, digestion, nutrition and pheromone production (Allen *et al.,* 2009). Based on this, the elimination of the intestinal microbiota may help to understand their role in the insect physiology and the host-pathogen relationship. The first lepidoptera reported to show resistance to Bt toxins was the grain/flour pest Indian meal moth *Plodia interpunctella* (Hübner) which represents a major pest in grain and cereal (Oppert *et al.,* 1996). Survey of the *P. interpunctella* gut bacteria and the roles they can play to lead to entomopathogen resistance, could help to find new strategies for this pest control by Bt.

Materials and Methods:

Insect source. In this study the insect species tested was *Plodia interpunctella* (Hübner). Our Insect colony was maintained with artificial diet based on ground wheat, wheat bran, wheat germ, yeast, honey, glycerin and water (McGaughey and Beeman, 1988).

***Plodia interpunctella* LC_{50}.** Median lethal concentration (LC_{50}) was selected to evaluate the *P. interpunctella* susceptibility to Bt, using a commercial product based on Bt labeled as Bactospeine®DF (Valent Biosciences de México, S.A. de C.V.). Susceptibility of *P. interpunctella* larvae to Bt was assessed using a dose response bioassay using the commercial product Bactospeine® DF (Valent Biosciences de México, S.A. de C.V.), which is based on a *B. thuringiensis* var. kurstaki, containing bacterial cells, toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Ab Cry2A), and spores with an potency of 32,000 international units (IU) per milligram. To evaluate the median lethal concentration (LC_{50}), a dose-response bioassay was used. For this, assay was done on artificial diet (Tamez-Guerra *et al.,* 2006), where 6 ml of warm liquid diet was purred on Falcon® sterile Petri dishes, 50 × 9 mm, tight-fit lid (VWR, Monterrey N.L. Mexico). Assay was performed testing the selected doses of 0, 3, 30, 100, 500, 1000 and 3200 UI cm^{-2}. Fifty microliters of each dose was applied on dried artificial diet surface and dispersed with a sterile "L" glass, using an upper layer bioassay. After doses solutions were dried, 30 second-third larval instars for each concentration were transferred on diet and lid were tight closed. Larvae were incubated in dark at 25 °C ±2 °C until pupation. Mortality was recorded from live and dead larvae and LC_{50} was estimated by a probit (Bliss, 1934) analysis, using the Statistical Package for the Social Sciences version 17.0 (SPSS, 2008).

Microbiota gut eradication. For the *P. interpunctella* microbiota gut eradication, we use the methodology described in Broderick *et al.,* (2006, 2010) using artificial diet mixed with diferents antibiotic solution concentration 50, 100, 250, 500 and 1000 µg mL^{-1} of rifampicin, gentamicin, penicillin, and streptomycin per ml each. Antibiotic solution was mixed in the diet still warm and liquid (55 °C), trowed in each Petri dish per antibiotic dose and let to cold and harden. Then, 30 *P. interpunctella* second instar larvae were trasfered to each dish per concentration of the antibiotic mixture. After 48 h exposure, among the surviving larvae the presence / absence of aerobic/facultative bacteria present in the gut of third instar larvae was determined by colony forming units (CFU) technique on LB agar medium. In addition, gut content was analyzed using PCR DNA amplifying technique using *16 S rRNA* enterobacterial gene primers: 27F 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492R 5'-TAC GGC TAC CTT GTT ACG ACT T-3' refered by Frank *et al.* (2008), in addition to the survival or death of the exposed larvae.



Bioasssays. *B. thuringiensis* mortality bioassays were carried out by exposing *P. interpunctella* larvae to the calculate LC_{50} of the following treatments: Bactospeine® in artificial diet (Bt treatment), Bactospeine® treated with antibiotics (A/Bt treatment), Bactospeine® in artificial diet with antibiotics and maintained under sterile conditions (AE/Bt treatment), and their negative controls without the addition of Bactospeine® DF (artificial diet only, or adding A or AE treatments). Larvae were incubated in dark at 25 °C ±2 °C until pupation. The mortality percentage of surviving insects was then recorded.

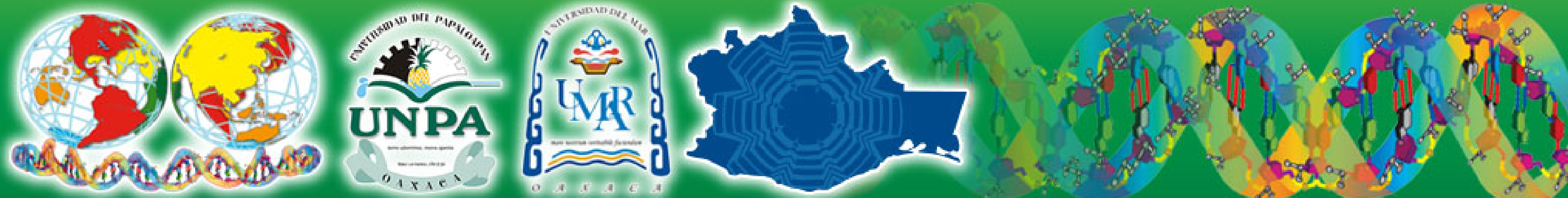
Results and Discussion:

Gut bacterial load after antibiotic exposure. Bactospeine dose response assays against second *P. interpunctella* larvae resulted in a calculated LC_{50} of 541.4 (95% confidence limits = 432.65 - 692.35) IU cm^{-2} artificial diet. Gut bacterial in treated larvae at concentrations of 250 mg mL^{-1} antibiotic or higher did not develop any colony on LB agar medium (Table 1).
Similarly, at a dose of 500 μgmL^{-1} antibiotic concentration or higher, the *16S rRNA* enterobacterial gene was not longer amplified by PCR among tested gut of exposed larvae.

Table 1. Gut bacteria in *Plodia interpunctella* treated with various concentrations of antibiotics on artificial diet.

Antibiotic Dose ($\mu g\ mL^{-1}$)	CFU/gut
1000	0
500	0
250	0
100	3.0×10^{2}
50	1.25×10^{4}
0	7.38×10^{7}

Bt susceptibility after antibiotic exposure. Bioassay testing second instar *P. interpunctella* larvae exposed to the calculated LC_{50} by Bactospeine (Bt) resulted in a mortality percentage of 60%, under the expected confidence limits. However, treatment with the same Bt dose but in diet mixed with antibiotics (A / Bt) resulted in a lower mortality (10%). This result is similar to that reported by Broderick *et al.* (2006, 2010). In this sense, recorded mortality is the effect of the antibiotics, which induce up to 21% mortality under sterile conditions AE/Bt treatment, still significantly lower than treatment with Bt, and higher compared to A/Bt treatment. This study results were consistent with those reported by Frankenhuyzen *et al.* (2010) who discussed that the changes in susceptibility could be due to the effect of the antibiotic in the insect, but no for the absence of the gut microbiome. However, this effect is partial and do not explain overall results. No significant differences (P <0.001) in larvae mortality were observed among no exposed Bt treatments (control, A and AE) (Figure 1).



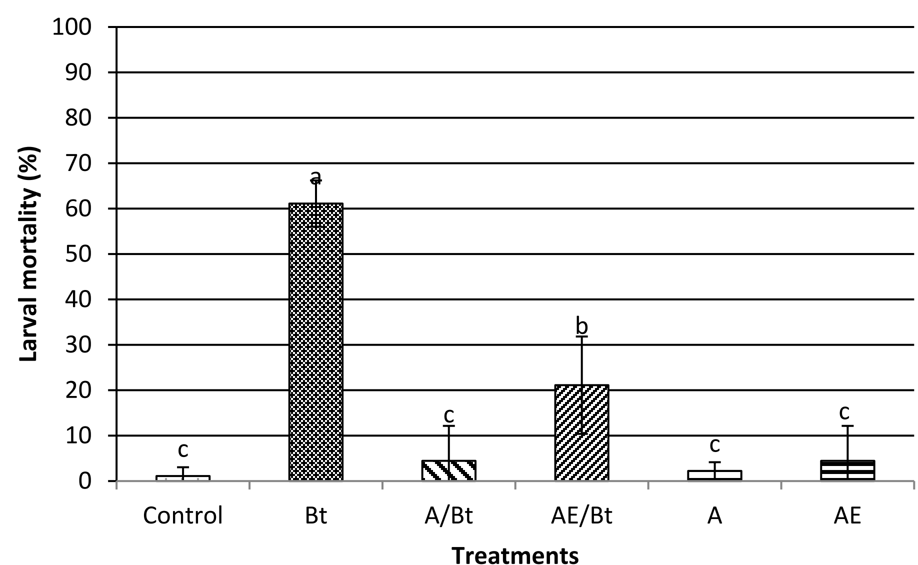


Figure 1. Second instar *Plodia interpunctella* larvae mortality percentage in artificial diet (control). Bt = exposed to Bactospeine® LC_{50}; A/Bt = Bactospeine® LC_{50} plus antibiotics mixture; AE/Bt = Bactospeine® LC_{50} plus antibiotics mixture maintained under sterile conditions, A= antibiotics mixture; AE = antibiotics mixture maintained under sterile conditions

Conclusion: The absence of gut microbiota of *P. interpunctella* reduces their susceptibility to Bt, however, antibiotics influence the susceptibility of the insect in bioassays. The intestinal microbiota is important factor in the effectiveness of Bt like biopesticide.

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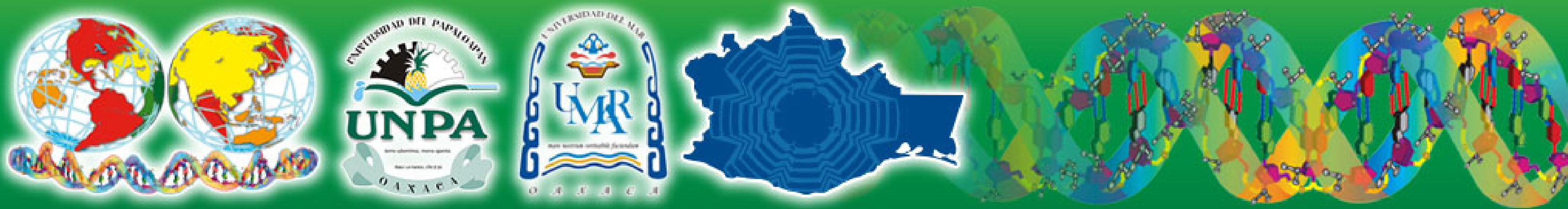

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Distribution analysis of the large rDNA subunit group I intron in *Beauveria bassiana*

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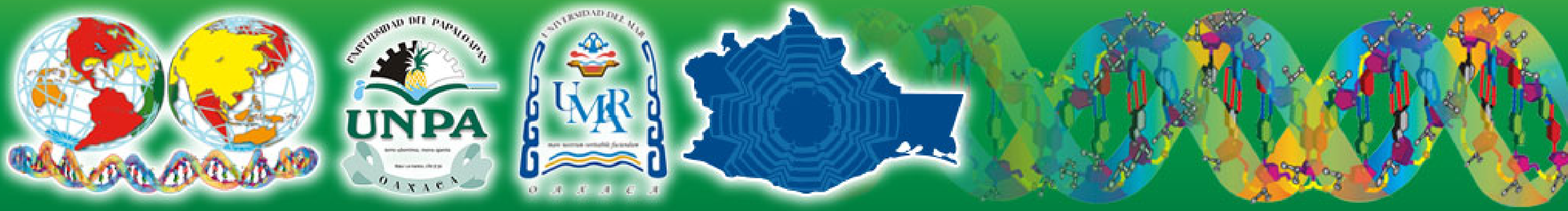
Abstract: *Beauveria bassiana* is a soil entomopathogenic fungus distributed around the world, used to control important insect pest species in agriculture and animal and human health. Therefore, it is very important to determine and to characterize the ecology of this biocontrol agent to achieve more precise control and to extend. In addition, *B. bassiana* knowledge will help to improve its efficacy in biocontrol and the biosafety while used in pest management programs. Classical methods for identifying fungi were mainly based on conidial morphology and biochemical characteristics. In the last two decades, the progress in molecular biology has given us a lot of useful tools, allowing a characterization at the genetic level and to be able to determine their biodiversity. In this study, the use of group 1 introns present in the genes of the large rDNA subunit was selected to differentiate between *Beauveria* genus isolates. Results may allow us to expand the knowledge of their distribution, prevalence and ecology of individual strains and their potential application in biocontrol.

Keywords: molecular study ● *Beauveria* populations' distribution ● group 1 introns

Introduction: *Beauveria bassiana* is a cosmopolitan entomopathogenic fungus widely distributed in soils around the world. Because of its efficiency to control insects, selected strains are being commercialize in control, being the most important biofungicide currently used for controlling a variety of insects that negatively affect crops, grains, and animal and human health (Pathan *et al.,* 2007). Classical methods for entomopathogenic fungi identification are mainly based on conidial morphology and biochemical and genetic characteristics. Molecular techniques are widely used as tools for the identification and phylogenetic analyzes of many fungal species. In general, molecular techniques involve the polymerase chain reaction (PCR) and DNA sequencing. These techniques are particularly valuable in cases in which the microorganism species are unable to grow in the laboratory (Bindslev *et al.*, 2002). The ribosomal DNA (rDNA) is a DNA sequence contained in the nuclear chromosomes, and encodes from the ribosomal RNA (rRNA). These sequences regulate the transcription and initiation of the gene amplification. While the final RNA product of a gene is being generated, any nucleotide sequence within a gene that is removed by RNA splicing is known as an intron (Berget *et al.,* 1977). Introns are found in the genes of most organisms and many viruses, but changes in introns are more currently found in bacteria and archea than that in eukaryotic organisms. When proteins are generated from intron-containing genes, RNA splicing takes place as part of the RNA processing pathway. This process is follow by transcription and precedes translation (Sharp, 1985). The low level of polymorphism in the rDNA transcription unit allows characterization of each species using only a few samples and



makes this DNA useful for specific inter comparison. In addition, replays of different rDNA coding regions show different rates of evolution. This DNA can provide information on almost any systemic level. As this study was conducted to analyzed the introns present in the large DNA ribosomal subunit (Wang *et al.,* 2003) to demonstrate the usage of this technique and its application as a molecular tool and to better understand how the fungal populations diversity of the genus *Beauveria* spread in soils. The aim of this study was to determine the genetic diversity among *B. bassiana* isolates by the absence / presence of their introns located in the LSU rDNA to identify the unique genetic patterns between isolates of this species.

Materials and Methods:

Genetic material. In this study, the genetic material of four monosporic *Beauveria bassiana* isolates, PTG1, PtG2, PTG4 and PTG6 (genebank accession: KC759728) and of the commercial strain GHA (used as a reference strain), were evaluated. The five fungi were grown in potato dextrose agar Petri dishes and incubated at 25 °C in dark for 10 d until the colony covered the 90% of the agar surface. On dishes with sporulated colony growth, an aliquot of 200 µL of sterile distilled water was added and mixed with the fungus to obtain an aliquot that was deposited in a test tube with 10 mL of sterile distilled water and stirred in vortex at high speed for 1 min. Tubes were then centrifuged at 10000 *g* for 10 min. Supernatant was discarded and pellet was collected in eppendorf tubes 1.5 mL and stored at – 80 °C until its use.

DNA extraction. For genomic DNA extraction, the extraction kit FastDNA™ SPIN KIT (MP Biomedicals) was selected and evaluation was conducted according to instructions and recommendations given by the manual.

PCR Amplification. The primers used for the specific amplification were previously reported by Neuveglise and Brygoo (1994) and Wang *et al*. (2003). The introns distribution determination among *B. bassiana* isolates were tested by primers combinations (Table 1). The PCR was conducted with GoTaq® Green Master Mix (Promega) and 1 µM for each oligonuceotide to observe the presence and absence of introns in the samples, amplification was carried out by the polymerase chain reaction (PCR) as follows. In a final volume of 20 mL, 1X buffer (200Tris-HCl pH 8.4, KCl 500 mM), 2 mL of fungus pellet (DNA), 3 µL of $MgCl_2$ 1.5 mM, 1 µL of 100 mM dNTPs, and 10 pmol of each primer (Table 1) was mixed with 1 U of Taq DNA polymerase (Bioline).
The cycling parameters were programmed into a Veriti Thermal Cycler (Applied Biosystems) as follows: 4 min of initial denaturation at 94 °C for 1 cycle, denaturation: 35 cycles, 45 s at 94 °C; annealing, 1 min at 59 °C; extension, 1 min at 72 °C followed by a final extension at 72 °C for 4 min.

Results and Discussion:

In the past, the introns of B. bassiana DNA using selected primers by the PCR technique has been reported as a tool for strains discrimination (Wang *et al.,* 2003). PCR products showed that the intron 2 of the large subunit rDNA group I intron was amplified, where the absence of intron 2 in the reference strain (GHA) and its presence among PTG1, PtG2, PTG4 and PTG6 isolates was observed (Figure 2).

Table 1. Selected primers for large rDNA group I subunit intron found in four *Beauveria bassiana* Mexican isolates.

Primer	Sequence	Expected size
Dominions D9-D11 LSU	I29 F (5´-CTGCCCAGTGCTCTGAATGTC-3´) M1 R (5´- GGTAAAACTAACCTGTCTCACG-3´)	2489 pb
Bb4 (In1)	I29 F (5´-CTGCCCAGTGCTCTGAATGTC-3´) I31 R (5´- CGCTGATTCTGCCAAGCCCAT-3´)	P= 620 pb A= 207 pb
Bb3 (In2)	I38 F (5´- ATGGGCTTGGCAGAATCAGCG-3´) I32 R (5´- CAGCCAAACTCCCCCCCTG-3´)	P=606 pb A=244 pb
Bb2 (In3)	I21 F (5´- CGATCCTTTAGTCCCTCGAC-3´) I22 R (5´- CGCTTACCGAATTCCTTCGG-3´)	P=656 pb A=157 pb
Bb1 (In4)	E23 F (5´- CCGAAGGAATTCGGTAAGCG-3´) M1 R (5´- GGTAAAACTAACCTGTCTCACG-3´)	P=501 pb A=84 pb

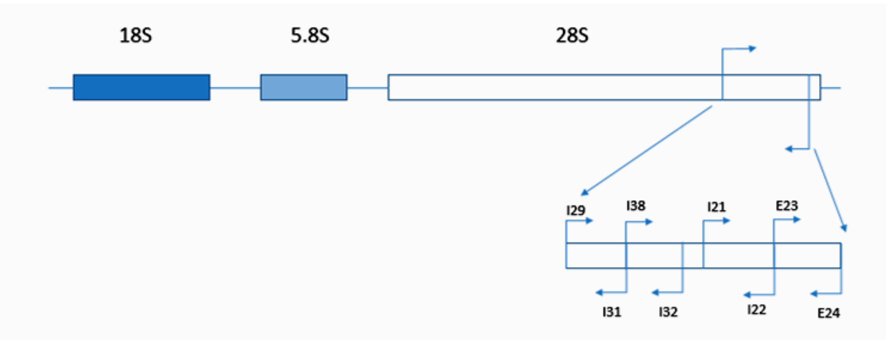


Figure 5. Schematic representation of the nuclear ribosomal repeat unit of *Beauveria bassiana*.

This study is the first approach to standardize the conditions to determine the introns presence among Mexican B. bassiana isolates. The aim is to use this technique to discriminate among strains, mainly after spraying in field trials. In our study, PCR products showed the presence of the intron 2 of the large subunit rDNA of group I introns, where the absence of intron 2 in the reference strain (GHA) and its presence in all native isolates PTG1, PtG2, PTG4, and PTG6 tested were observed (Figure 2). Although differences in introns between fungal isolates were previously reported in yeast (Langford and Gallwitz, 1983), our findings were accordingly to that changes in introns are more currently found in bacteria and archea than that in eukaryotic organisms (Sharp, 1985).

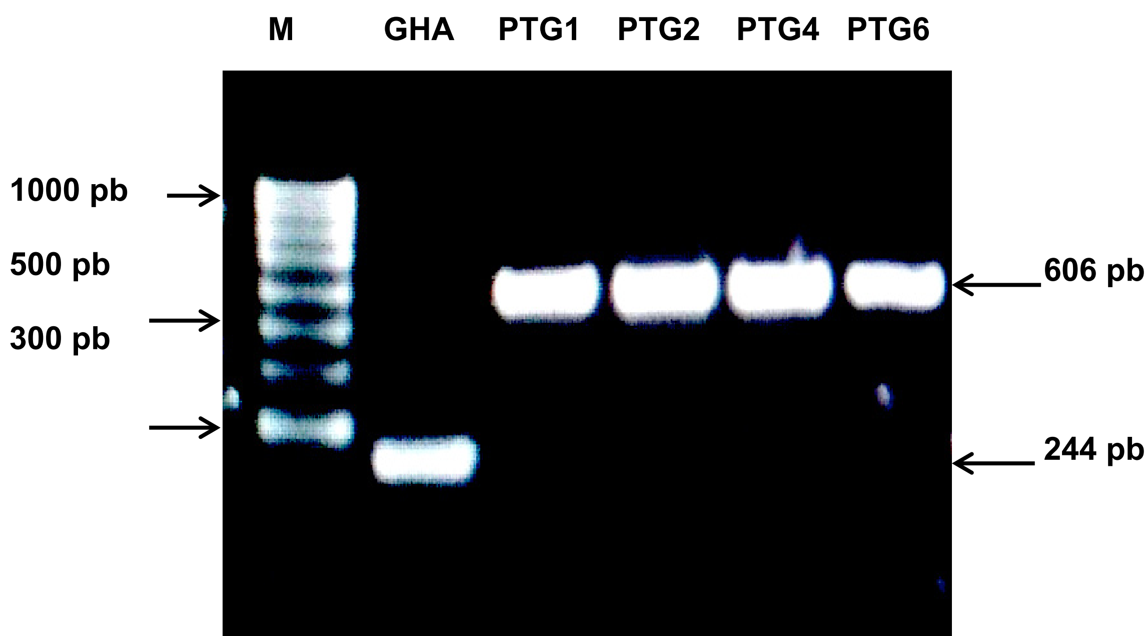


Figure 2. PCR products of Beauveria bassiana DNA showing the intron 2 of the large subunit rDNA group I intron. Lanes: M= weight marker; GHA = reference strain GHA showing intron 2 absence; PTG1, PtG2, PTG4 and PTG6, Mexican isolates showing intron 2 presence.

The relevance of this is that all the four strains were isolated from *Periplaneta americana* L., collected within the Biology Science School at the Autonomous University of Nuevo Leon facility (same insect, same location, different isolates) (Tamez-Guerra *et al.,* 2012). This tool allows us to associate these genetic variants with related strains tropism and virulence. As a perspective, this research could be used to complete studies of gene sequencing ITS regions association that lead us to recognize them as a molecular marker for dissemination and efficacy determination under field conditions.

Conclusion: In this work, the efficiency of this technique to differentiate between isolates of fungi of the genus Beauveria is demonstrated and proposed as a tool in understanding the population distribution of fungi is generated under natural conditions, in order to meet its spread and expand knowledge of the ecology of these microorganisms.

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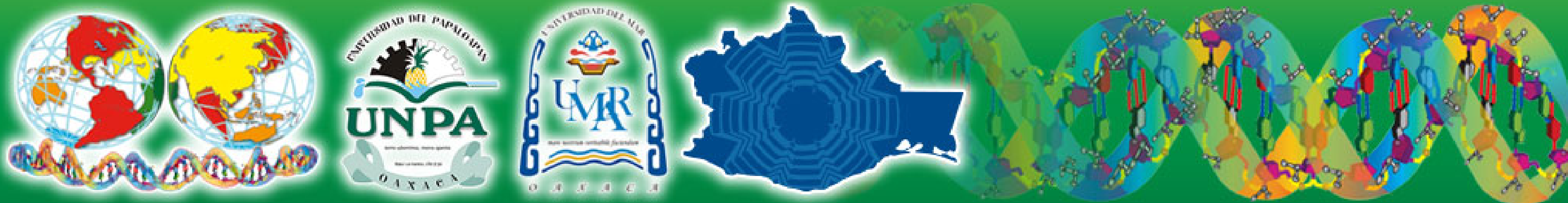

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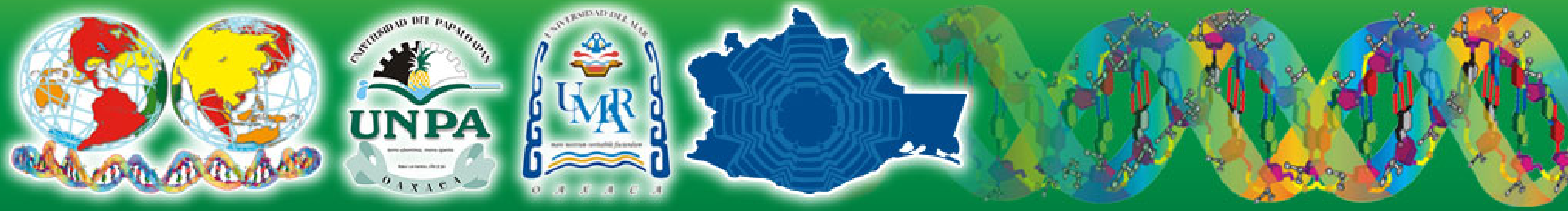

Native *Trichoderma* spp. isolates to control *Sclerotium cepivorum* Berk in garlic (*Allium sativum* L.) in the central region of Mexico

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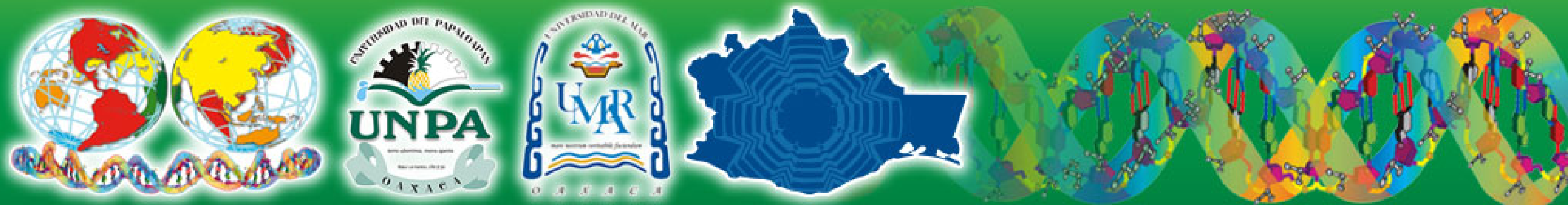
Abstract: Garlic is an important food seasoning used all over the word in cuisine. Unfortunately, the yield and quality of this crop could be reduced to 100% by the white rot *Sclerotium cepivorum* Berk., This high virulence is the result of high reproductive capacity and propagule densities and their longevity due to sclerotia production in soil. In recent years, species of the fungus *Trichoderma* have shown antagonistic activity against the white rot and other phytopathogen species important in agriculture. Since it is well know that microbial ecological antagonist-phytopathogen relationships are found in soils *Allium sativum* in crops, which are characterized morphologically, such as perspective relationship biocontrol. In this study we found *Trichoderma atroviride, T. aureoviride, T. harzianum, T. longibrachiatum, T. viride* and *Hipocrea gelatinosa*. These isolates will be evaluated for their antagonistic activity *in vitro* against sclerotia of *S. cepivorum* and to be considered as potential biocontrol agents.

Keywords: *Trichoderma* ● *Sclerotium cepivorum* ● *Allium sativum*

Introduction: Domestic production of garlic is strongly affected by white rot caused by *Sclerotium cepivorum* Berk. In the states of Zacatecas, Aguascalientes and Guanajuato have been reported incidents of diseased plants from 6 to 43%, where the severity of the attack is closely related to the level of soil inoculum (Delgadillo-Sánchez *et al*., 2004; Velásquez-Valle and Medina-Aguilar, 2004). *S. cepivorum* specializes in attacking species of the genus *Allium* L., and difficulty in control is due largely to its ability to develop resistance structures (sclerotia) that survive in the soil for more than two decades without losing its infective ability (Schwartz and Mohan, 2008). Various control methods have been evaluated from chemical fungicides, compost application, crop rotation, and solarization, among others. The lack of effective protection against this phytopathogen relies on its longevity, in addition to the sclerotia ability to germinate in soil by different time periods. It is also known that various species of the *Trichoderma* fungus have antagonistic activity against several phytopathogenic species of plant roots including white rot. *Trichoderma* mode of action relies on in-site competition for nutrients, antibiosis, and mycoparasitism by glucanolytic and chitinolytic enzymes action. The combined enzymes activity leads to the phytopathogenic fungi cell wall degradation (Dolatabadi *et al*., 2012). The aim present study to isolate and identify *Trichoderma* species from garlic cultivated soils within central Mexico stated (Guanajuato and Zacatecas) for further antagonistic determination against *S. cepivorum* sclerotia.



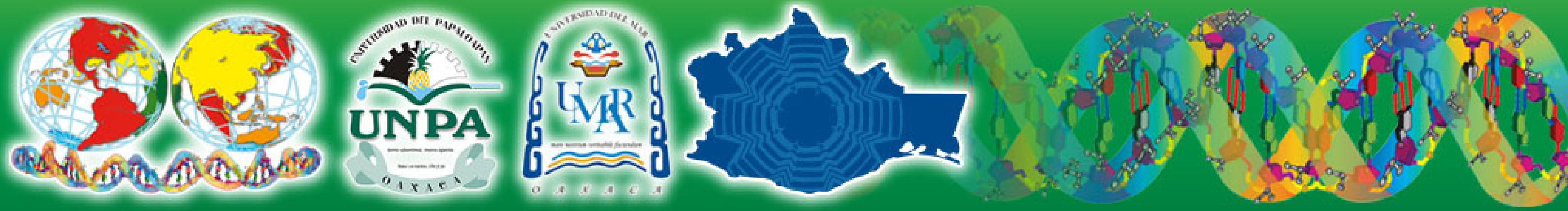
Material and Methods:

***S. cepivorum* field samples.** Field trips were conducted to collect samples from the main garlic production areas in the states of Zacatecas and Guanajuato with a history of white rot disease. Once there, garlic plants showing white rot disease symptoms were collected and placed in paper bags for storage and transportation to the Biological Formulation Unit laboratory located in the FCB-UANL. Each sample was labeled with the collection date, collection site, garlic variety, and geographic reference (this by using a Garmin GPS).

***Trichoderma* isolation.** To isolate the antagonistic fungus *Trichoderma*, soil samples from the same premises where garlic plants showing white rot symptoms were taken, five subsamples of each sample were taken at a depth to up to 30 cm of soil to achieve a homogeneous composite sample of 1 kg. Each sample was placed in a clear plastic bag and labeled in the same way that garlic plant samples and were taken to the Biological Formulation Unit laboratory.

***S. cepivorum* isolation.** Phytopathogen isolation was performed using the technique described by Clarkson *et al*. (2002) with some modifications. Briefly, garlic pieces from plants collected in the field showing mycelium or disease symptoms were disinfected using 15% sodium hypochlorite solution by 2 min and then rinsed three times with sterile distilled water, shaking vigorously to remove any disinfectant residue. Disinfected plant pieces were sown in Petri dishes containing potato dextrose agar (PDA) + chloramphenicol medium and were incubated in complete darkness at 20 °C and relative humidity > 80%. Subsequently, all dishes showing purified colonies with expected characteristics by *S. cepivorum* were selected and stored under refrigeration at 4 °C until use. From those, only the samples showing sclerotia production were selected for *in vitro* antagonist bioassays.

***Trichoderma* spp isolates preparation.** For detection of *Trichoderma* present in soil samples from garlic fields, serial dilution technique before drying and screening the soil samples (Tringiano *et al.,* 2008) was used. For this, 1.0 g of air dried soil was placed in a vial with 9 mL of sterile distilled water and stirred homogeneously by vortexing for 3 min. From that solution, 1.0 mL was transferred in another vial with 9 mL of sterile distilled water and stirred again. This process was repeated 4 times and the last dilution of an aliquot of 0.5 mL of the final soil dilution was inoculated and dispersed on PDA medium + chloramphenicol. For each soil sample, three replicates were prepared and incubated at 22 ± 2 °C in complete darkness for 24 h. Subsequently, colonial growth was observed under a stereoscope. Colonies were selected based on their distinctive characteristics such as fast growth, and initially submerged mycelium hyaline, woolly appearance, with colors ranging from colorless, dull red, yellow amber to yellowish green on the back of the culture medium trough the dish glass. Subsequently, monosporic isolates were obtained by the technique reported by Ho and Ko (1997). Selected dishes with a sporulated *Trichoderma* single colony growth, an aliquot of 200 µL of sterile distilled water was added and mixed with the fungus to obtain an aliquot that was deposited in a test tube with 10 mL of sterile distilled water and stirred in vortex at high speed for 1 min. Subsequently, the solution was poured into Petri dishes containing PDA and allowed to stand for 10 minutes at room temperature to remove excess moisture. Finally, they were allowed to stand under the laminar flow hood for 16 h where five germinated conidia were placed individually in Petri dishes with PDA medium and incubated at 22 ± 2 °C until growth and reached sporulation stage.



***Trichoderma* identification.** Identification of *Trichoderma* isolates from garlic fields samples collected from Zacatecas and Guanajuato soils was carried out by using fixed microcultures assemblies. Structural observations were done under compound microscope at 100X. Measurements for identification were performed using the classification keys descrived by Gams and Bisset (1988).

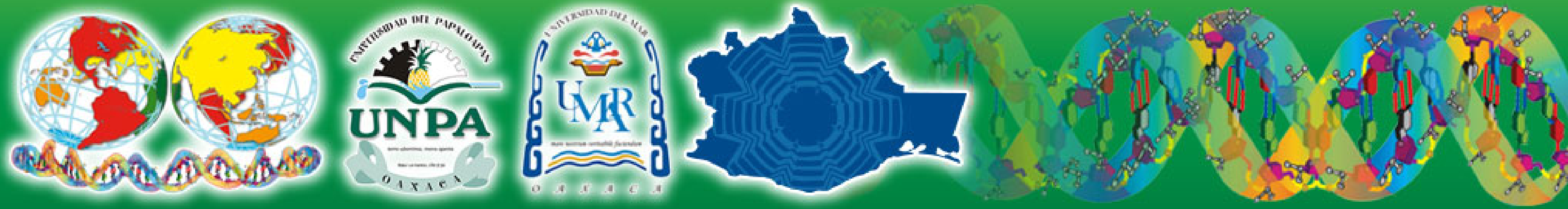
Results and Discussion:

Collected soil samples from garlic key producing areas from Zacatecas and Guanajuato Mexican states are shown in Table 1. After garlic samples showing the characteristic white rot symptoms were processed for fungi/microorganisms culture and isolation, the phytopathogen *S. cepivorum* was successfully isolated. This fungus genus was confirmed by the taxonomic classification reported by Bisset and Gams (1988).

Table 1. Origin of soil samples and plant for the production of *S. cepivorum* and *Trichoderma.*

Place	Municipality	State	Crop	Geographic location	
				N	W
Llano Blanco	Calera de V.R.	Zacatecas	Ajo	22.99222	-102.63789
San Jerónimo	Fresnillo	Zacatecas	Ajo	23.11992	-103.16635
La Purísima	General E. Estrada	Zacatecas	Ajo	23.00600	-102.70095
San Rafael	General E. Estrada	Zacatecas	Ajo	23.02287	-102.69888
Estancia de Ánimas	Villa González Ortega	Zacatecas	Ajo	22.75913	-102.59513
La Joya	Morelos	Zacatecas	Ajo	22.88591	-102.69799
La Laja	Salamanca	Guanajuato	Ajo	20.50384	-101.05962
Rancho Don Aarón	Juventino Rosas	Guanajuato	Ajo	20.62925	-101.00822
Rancho San Julián	Juventino Rosas	Guanajuato	Ajo	20.60447	-101.04692

Similarly, soil samples from garlic fields were processed for fungi/microorganisms culture and isolation, the antagonist *Trichoderma* was successfully isolated. Taxonomic classification indicated 2 genera and 6 different species (Table 2). In order to confirm the taxonomic classification, identification of these isolates will be corroborated by molecular techniques.
To date, *in vitro* confrontation bioassays o each antagonistic fungus against the phytopathogen isolates are currently underway. Results will help to select antagonist bioassays against *S. cepivorum* sclerotia and their enzymatic activity to better understand their mode of action. Several studies have reported diverse antagonistic activity by *Trichoderma* species to control the white rot of phytopathogen in garlic and onion fields (Ortega-Aguilar *et al.,* 2011; Rojas *et al.,* 2010; Coventry *et al.,* 2006; Granados-Motero, 2004). From species tested, *T. harzianum* and *T. viride* are being highlighted for their efficacy under natural and induced evaluations. This has reinforced the research looking for more isolates to apply selected antagonist and involve their use in



management programs. Preliminary results of tested isolates in the sampled soils give an indication of their antagonistic activity against *S. cepivorum*.

Table 2. Species of antagonistic fungi isolated from soils of garlic fields in Zacatecas and Guanajuato, Mexico.

Taxonomic identification	Isolates
Hipocrea gelatinosa (Tode) Fries.	1
Trichoderma atroviride (P. Karst)	1
Trichoderma aureoviride (Rifai)	2
Trichoderma harzianum (Rifai)	1
Trichoderma longibrachiatum (Rifai)	1
Trichoderma viride (Persoon)	3

Conclusion: In this study, six fungi isolates from Mexican garlic fields were identified as *Trichoderma* species. Preliminary results indicated they have potential as antagonist against the white rot phytopathogen *S. Cepivorum.*

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Wastewater sludge stimulates and accelerates removal of PAHs in polluted agricultural soils

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Abstract: Wastewater sludge is used throughout the world to dissipate hydrocarbon in PAHs-polluted soils. However, little is known about how wastewater sludge stimulates and accelerates the removal of PAHs from soils. Alkaline - saline soil from the former lake of Texcoco with pH 9 and electrolytic conductivity of 7 dS m^{-1} was contaminated with anthracene, and amended or not with wastewater sludge sterilized or not, and with or without polyacrylamide, meanwhile, the anthracene was monitored under aerobic incubation experiment for 112 days. An agricultural soil (from Acolman) and wastewater sludge were treated in the same way and were served as controls. After 112 days, the largest dissipation of anthracene was found in the Acolman soil amended or not with wastewater sludge, also with or without polyacrylamide. The largest dissipation of anthracene was found in both amended soils, with wastewater sludge and polyacrylamide. Meanwhile, the lowest degradation of anthracene was detected in PAHs-polluted sludge and also, in amended soils of Texcoco with sterilized wastewater sludge. It was found that polyacrylamide accelerated removal of PAHs from soils, while wastewater sludge increased the removal of PAHs from soils but the effect is controlled by the physical, chemical, and microbial soil properties, the contaminant and microorganisms in wastewater sludge.

Keywords: Microorganisms in wastewater sludge ● polyacrylamide ● PAHs

Introduction: Soil salinity is increasing at an alarming rate in the world through irrigation and excessive fertilization of crops while high salt concentrations limit crop production and reduce soil quality throughout the world (Tejada *et al*., 2006). Sludge is the residual product of wastewater treatment. The sludge contains nutrients and organic matter that is therefore widely used to improve soil fertility. However, it contains contaminants including metals, pathogens and organic pollutants. Current regulations therefore require pathogen reduction and periodic monitoring for some metals prior to land application (Harrison *et al*., 2006). Large amounts of polycyclic aromatic hydrocarbons (PAHs) are extracted, produced, refined and transported therefore contamination occurs frequently. PAHs are thus amongst the most widespread organic contaminants in soils, water and wastewater (Puglisi *et al*., 2007). The accidental release of PAHs causes serious damage to ecosystems when improperly managed and they may persist for a long time in soil. PAHs are ubiquitous, nonpolar, and highly hydrophobic due to their affinity for fatty tissues tend to accumulate in food. Although several hundred PAHs exist, most studies focus on a limited number of them, namely the 16 PAHs listed by the US Environmental Protection Agency and the European Community as pollutants (Puglisi *et al*., 2007). Seven of them including anthracene might be carcinogenic (Cai *et al*., 2007). In an earlier study, Fernández-Luqueño *et al.* (2009) found that after 112 days, polyacrylamide accelerated the removal of anthracene from an alkaline-saline soil and an agricultural soil *i.e.*, it was found that polyacrylamide accelerated removal of

anthracene from soil. However, it was not possible to determinate which wastewater characteristic increased the dissipation of PAHs. The objective of this research was to determinate how wastewater sludge stimulates and accelerates the removal of PAHs from polluted soils.

Materials and Methods:

This study was made in a greenhouse of the '*Grupo de Sustentabilidad de los Recursos Naturales y Energía del Cinvestav-Saltillo*' located in Saltillo, Coahuila, Mexico. The experimental setup was carried out from January to July 2014. This area is located in the southeastern state of Coahuila, centered at 25° 31' N, 101° 37' W, at an altitude of 1,600 m above sea level with a mean annual temperature of 18 °C. The climate is generally dry and semi warm to warm extreme largely of Coahuila, with some variants through the regions of Coahuila. Temperatures average 12 °C in January, the coldest month, and 23 °C in June and July, the hottest months. Annual rainfall averages 369 millimeters, much of which falls during September and October. Based in the Köppen climate classification the semi-arid hot climate (BSh) is found in this area. The first sampling site is located in the former lake of Texcoco in the valley of Mexico City (México) (19° 30' N, 98° 53' W) at 2,250 m above sea level with a mean annual temperature of 16 °C and mean annual precipitation of 600 mm (mainly from June through September). Briefly, the soil was characterized as loamy sand soil and alkaline-saline (NaCl and Na_2CO_3 as major minerals). The pH ranges between 8.5 and 10.5, electrolytic conductivity (EC) in saturated soil-paste extracts: between 4 and 150 dS m^{-1} and the soil has a large exchangeable sodium percentage (60-80%) (Table 1). Soil was sampled at random by augering the 0-15 cm top-layer of three plots of approximately 0.5 ha. The soil from each plot was pooled so that three soil samples were obtained. Second sampling site, is located near the ex-convent of Acolman in Acolman, State of Mexico (19° 38' N, 98° 55' W) and the former lake Texcoco at 2,250 m above sea level and with a mean annual temperature of 14.9 °C and average annual precipitation of 624 mm (mainly from June through August). Briefly, the soil was sandy loam with pH 6.0 and EC 2.4 dS m^{-1} is mainly cultivated with maize and that for >25 years, receiving a minimum amount of mineral fertilizer without being irrigated (Table 1). Soil was sampled at random by augering the 0-15 cm top-layer of three plots of approximately 0.5 ha. The soil from each plot was pooled so that three soil samples were obtained. As such, six soil plots were obtained, three from Acolman (served as control) and three from the former lake Texcoco. The soil was characterized and treated as follows. The soil from each plot at both sites was passed through a five mm sieve separately. It was adjusted to 40% of the soil water holding capacity (WHC) by adding distilled water (H_2O) and conditioned at 22±2 °C in drums containing a beaker with 100 mL of 1 M NaOH to trap the CO_2 evolved, and a beaker with 100 mL distilled H_2O to avoid desiccation of the soil sample, for ten days. Hydrocarbon was obtained from Sigma (USA) with purity >97% for anthracene. Acetone was purchased from J.T. Baker (USA) with purity 99.7%. Wastewater sludge was obtained from Reciclagua Ambiental (Sistema Ecológico de Regeneración de Aguas Residuales Ind., S.A. de C.V.) in Lerma, State of Mexico (México). Reciclagua Ambiental treats wastewater from several companies such as alimentary industries. Ninety percent of the wastewater is from alimentary industries origin, textile industries, and the rest from household. The sludge obtained after the addition of a flocculant is passed through a belt filter to reduce water content. Wastewater sludge was sampled aseptically in plastic bags. The pH of the sludge was 6.4, the water content 793 g kg^{-1}, the organic C content 509 g kg^{-1} dry sludge, total N 27.7 g kg^{-1} dry sludge, and P 1.65 g kg^{-1} dry sludge, while extractable P was 0.6 g kg^{-1} dry sludge and the concentration of NH_4^+ was 0.5 g N kg^{-1} dry sludge (Table 1).

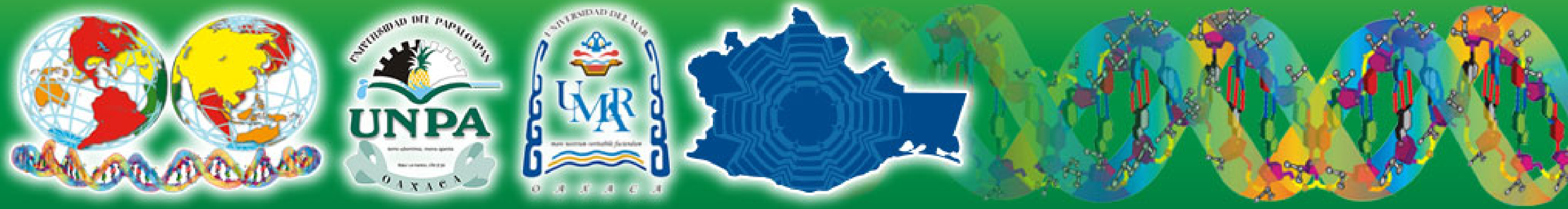

Table 1. Characteristics of the Texcoco and Acolman soils and the wastewater sludge.

	Acolman soil	Texcoco soil	Sludge
pH_{H_2O}	6.0	9.3	6.4
Water holding capacity (g kg^{-1}) [a]	674	659	ND [b]
Water content (g kg^{-1})	76	150	793
Organic carbon (g kg^{-1})	8.1	58.2	509
Inorganic carbon (g kg^{-1})	0.2	0.8	ND
Total Kjeldahl nitrogen (g kg^{-1})	0.7	1.2	27.7
N-NH_4^+ (mg kg^{-1})	3.4	3.7	500
N-NO_3^- (mg kg^{-1})	53	30	86
N-NO_2^- (mg kg^{-1})	0.6	0.3	7.9
Total phosphorus (g kg^{-1})	0.2	0.3	1.7
Extractable phosphorus (mg kg^{-1})	2.0	0.3	600
Electrolytic conductivity (dS m^{-1})	2.4	7.3	5.7
PAHs (mg kg^{-1})	NDT [c]	NDT	NDT
Clay (g kg^{-1})	38	58	ND
Silt (g kg^{-1})	267	80	ND
Sand (g kg^{-1})	695	862	ND
Textural classification	Sandy loam	Loamy sand	ND

[a] On a dry base; [b] Not determined; [c] Not detected.

One hundred and sixty-eight sub-samples (three plots × two soils × four treatments by soil × seven sampling days) of 20 g soil were added to 120 mL glass flasks. Additionally, twenty-one glass flasks were used to the treatment without soil, *i.e.* twenty-one flasks were used for each of the nine treatments (Table 2). The wastewater sludge used in the STERILE-SLUDGE treatment was sterilized three times with pressurized steam at 121 °C supplied by an autoclave for 30 min with an interval of one day.

Table 2. Treatments applied to the soils from Texcoco (TEX) and Acolman (ACOL).

Treatments	Characteristics
TEX-SLUDGE-POLY	Soil [a] + anthracene [b] + sludge-with polyacrylamide [c]
TEX-SLUDGE	Soil + anthracene + sludge [d]
TEX-STERILE-SLUDGE	Soil + anthracene + sterilized dry sludge [e]
TEX-PAH	Soil + anthracene
ACOL-SLUDGE-POLY	Soil + anthracene + sludge-with polyacrylamide
ACOL-SLUDGE	Soil + anthracene + sludge
ACOL-STERILE-SLUDGE	Soil + anthracene + sterilized dry sludge
ACOL-PAH	Soil + anthracene
SLUDGE-PAH	Sludge + anthracene

[a] 20 g dry soil; [b] 520 mg anthracene kg^{-1} dry soil; [c] 108 g dry sludge flocculated with polyacrylamide kg^{-1} dry soil; [d] 108 g dry sludge without polyacrylamide kg^{-1} dry soil; [e] 108 g sterilized dry sludge flocculated with polyacrylamide kg^{-1} dry soil.

Three flasks were chosen at random from each treatment of the six soil samples *i.e.*, 189 sub-samples. One-half gram of soil was extracted for PAHs with acetone and were analyzed by GC. The remaining 18.5 g soil was frozen. These provided zero-time samples. The remaining flasks were placed in 945 mL glass jars containing a vessel with 10 mL distilled H_2O and a vessel with

20 mL 1 M NaOH to trap CO_2 evolved. The jars were sealed and stored in the dark for 112 days at 22±2 °C. After 3, 7, 14, 28, 56, and 112 days, three jars were selected at random from each treatment and the soil was analyzed for PAHs as mentioned before. The remaining flasks were opened and aired for 10 min every five days in order to avoid anaerobic conditions, were sealed and further incubated. Chemical, PAHs and statistical analysis were determined according with Fernández-Luqueño *et al*. (2009).

Results and Discussion:

Our data suggest that the polyacrylamide accelerated the removal of the PAHs from the Acolman and Texcoco soils may be as an effect of the N release upon polyacrylamide decomposition further suggesting the polyacrylamide enhanced the dissipation of PAHs (Table 3). Sojka *et al.* (2007) stated that polyacrylamide affects the physical processes, such as adsorption of PAHs on the soil matrix thereby augmenting their bioavailability and degradation. Moreover, Wen *et al.* (2010) found microorganisms capable to degrade polyacrylamide from activated sludge and oil-contaminated soil. Additionally, Hu *et al.* (2012) state that polyacrylamide restores the soil structure and greatly increased soil aggregate stabilization, which the polyacrylamide and/or its decomposition could increase the supply of oxygen, regulate the water content and improve the nutrients bioavailability to remove PAHs.

Table 3. Dissipation of anthracene (mg kg^{-1} dry soil) in the Acolman and Texcoco soils amended with wastewater sludge with or without polyacrylamide, sterilized or left unamended, incubated aerobically at 22±2 °C for 112 days.

Treatments	Days after the onset of the experiment						LSD[a]
	3	7	14	28	56	112	
TEX-SLUDGE-POLY	168 ABa	166 Ba	143 Ca	206 Ca	194 Da	301 BCa	20.2
TEX-SLUDGE	107 BC a	112 BCD a	131 CD a	132 DE a	137 E a	211 DE a	123.9
TEX-STERILE-SLUDGE	44 C a	28 CD a	61 DE a	41 EF a	32 F a	69 DE a	164.9
TEX-PAH	164 AB a	243 AB a	265 AB a	253 BC a	259 CD a	265 BC a	122.2
ACOL-SLUDGE-POLY	196 AB b	243AB b	261 AB b	355 AB a	418 A a	443 A a	91.0
ACOL-SLUDGE	178 AB b	184 B b	188 BC b	354 AB a	354 AB a	389AB a	96.2
ACOL-STERILE-SLUDGE	212 AB c	231 AB bc	235 BC bc	319 ABC ab	322 BC ab	360 ABC a	96.72
ACOL-PAH	267 A c	344 A bc	368 A ab	420 A ab	432 A ab	437 A a	89.1
SLUDGE-PAH	4 C d	7 D d	8 E d	15 F c	31 F b	42 E a	5.5
LSD	119.9	140.5	116.6	115.4	90.4	155.2	

[a] LSD: Least significant difference ($P<0.05$). [b] Values with the same capital letter are not significantly different between the treatments, i.e. within columns ($P<0.05$). [c] Values with the same letter are not significantly different over time i.e. within the row ($P<0.05$).

Texcoco soil amended with sterilized wastewater sludge dissipated only 12% of anthracene, while Acolman soil amended with sterilized sludge dissipated 70% of anthracene at 112 days. Wastewater sludge has been used to remediate PAHs-polluted soils during many decades. The wastewater sludge contains nutrients, organic matter, polyacrylamide, and microorganisms, all of them are factors that improve the PAHs dissipation when it is mixed with soil, but these data suggest that the interrelationship between the wastewater sludge properties and the soil properties is very important to increase the dissipation of the PAHs in a polluted soil.
The dissipation of PAHs decreased significantly in soils amended with sterilized wastewater sludge. It implies that wastewater sludge contains microorganisms capable to degrade or reduce the PAHs and/or that some physical or chemical properties from wastewater sludge are affected during the sterilizing. However, Fernández-Luqueño *et al.* (2008) did not find changes in CO_2 emission, nor in NO_3^-, NO_2^- or NH_4^+ concentrations from sterilized wastewater sludge compared with unsterilized wastewater sludge. Additionally, it has been demonstrated that changes in pH as

effect of wastewater sludge addition or nutrients from the wastewater sludge had no significantly effect on the PAHs dissipation in soil spiked with phenanthrene and anthracene (Fernández-Luqueño *et al.,* 2008). It implies that wastewater sludge stimulate and accelerate the dissipation of anthracene from PAHs-polluted soil as effect of the polyacrylamide content and its microbial community but not by pH change nor by its nutrient concentrations.

Conclusion: It was found that polyacrylamide accelerated removal of PAHs from soils. Meanwhile, wastewater sludge increased the removal of PAHs from soils. The effects are controlled by the physical, chemical and microbial properties of soil; the contaminant; and microorganisms in wastewater sludge. Wastewater sludge polluted with PAHs must be treated with remediation technologies before its final disposal; otherwise, the PAHs contamination will be persistent.

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Physicomechanical properties of films based on orange and lemon peel: agroindustry residues

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Abstract: A series of agro-industry wastes, anionic starch and poly(vinyl alcohol) (PVA)-based single and layer by layer-layer films were prepared by casting method. Their tensile properties and water vapor permeability were investigated to examine the effect of lemon and orange peel wastes on the membrane performances. Starch is the most studied polymer in the biopolymers based membranes and it was used as reference. The films made in this study showed tensile strength values between 2.550 and 9.282 MPa, which match with those reported by several authors, who used materials of similar nature. Elongation valuespresented by the films ranged between 2.91 and 16.21%, showing a high significant difference between the formulations based on lemon peel and orange peel. The microstructure of films is related to the permeability to water vapor. Films with higher permeability showed more homogeneous surface than those with less permeability values.

Keywords: agro-industry ● waste ● packing industry ● biodegradable materials

Introduction: The benefits of naturally occurring polymers for material applications are important because their environmental compatibility. In addition, the use of renewable recourses provides an to the extended nonrenewable petrochemical supplies. The agriculture industry produces sufficient residues of some agricultural activities that could be used as renewable sources for polymer feed stocks. Biodegradability is an additional benefit of renewable polymers. Composites of polymers from renewable resources offer an answer to maintain a sustainable development of economically and ecologically attractive technology. A variety of naturally occurring biopolymers can be found. Some of these such as cellulose and starch are very actively used in several products today, while many other remain underutilized. The packing industry represents one of the most important areas of consumption and application of synthetic polymers, for the modern life. The biodegradable polymers have arisen as part of the new clean technologies, trying of minimizes the accumulation of solid wastes (Yiniang and Lina, 2009). The addition of agro-industrial byproducts with high content of lignin and cellulose, in a polymeric matrix, constitutes one of the innovations in the polymer development industry; in addition, the advantage of this type of agro-industrial byproducts represents an option for its suitable handling. The increase in demand of biodegradable materials for packing includes those with antimicrobial characteristics, which are defined as those active packing able to inhibit pathogenic or other microorganisms which could cause deterioration of food. The main objective of this study was to evaluate the physic- mechanical and barrier properties of films prepared from agro-industry residues. The main objective of this study was to evaluate the physico-mechanical and barrier properties of films prepared from agro-industry residues.

Material and Methods:

Lemon and orange peel wastes were obtained from Oranjugos, Co., Monterrey, N.L., Mexico, they were grounded by 72 h and passed through a mesh No. 120 until homogeneus particle size. Commercial grade glycerol (99.5%), Poly(vinyl alcohol) (PVA) and anionic starch were purchase from Analytika®, Arivol® 540 and Amifilm®, respectively. Anionic pectin solution with concentration of 2.35% (W/V) was prepared by dispersing it in the distilled water, the lemon peel or orange peel or starch solution were added respectively and stirring until they dissolved completely. The PVA (41.1% W/V) was added and after complete solubilization the glycerol as plasticizer (2% W/V) was added to the solution and stirred for 30 min. Different amounts of sodium benzoate as antimicrobial agent were added (0, 0.1 and 0.2 g L^{-1}). Films were prepared from aqueous dispersion and solutions with agro-industry wastes and poly(vinyl alcohol) (PVA), single and layer by layer membranes were prepared by casting method. The plates were placed for 24 h at room temperature for drying. Thickness of the films was measured with a precision digital micrometer (Digimatic Indicator Mod. 293, Mitutoyo Corporation, Japan). All films were conditioned prior to subjecting them to permeability and mechanical tests according to Standard method, D618-61 (ASTM, 1993a). Films used for testing water vapor permeability (WVP), tensile strength (TS) and elongation (E) were conditioned at 60% RH and 27±2 °C by placing them in a desiccators over a saturated solution of Mg $(NO_3)_2$ $.6H_2O$ for 72 h or more. For other tests, film samples were transferred to plastic bags after peeling and placed in desiccators. The mechanical properties were studied using an Electronic Tensile Tester Mod: QC II-XS, in accordance with ASTM D-882-91 (1996). The water vapor transmission rate was made using the standard method, E 97-87 (ASTM, 1989). Analyses by scanning electron microscopy (SEM) were done using a Jeol microscopy.

Results and Discussion:

Films made in this study, showed tensile strength values between 2.550 ± 0.161 and 9.282 ± 0.926 MPa, which match with those reported by several authors, who used materials of similar nature (Romero-Bastida *et al*., 2004; Fishman *et al*., 2006; Meneses *et al*., 2007). Also these results were higher than the reported by Cherian *et al*. (2005) who use lignin-starch formulations. The effect of the thickness in this parameter was evident when the results between the mono and bi-layer membranes for the three formulations (orange peel, lemon peel and anionic starch), were compare (Figure 1). Elongation values presented by the films under study ranged between 2.91 and 16.21%, showing a high significant difference between the formulations based on lemon peel and orange peel (Figure 2). There was no significant difference found in the percent elongation parameter in the films prepared from orange peel, the values obtained ranged between 2.9 and 4.6%. Otherwise, in the films based on lemon peel, the percent elongation was greater than the films prepared from orange peel. However, the values are lower than films produced with anionic starch and other films obtained with synthetic polymers like low density polyethylene (LDPE). Permeability values at time 0, after 24 h of test ranges between 6.150 E^{-5} g H_2O/mm^2 h in formulations based on orange peel whereas lowest values corresponded to 1.613 E^{-5} g H_2O/mm^2 to films elaborated with lemon peel (data not shown). The thickness of the films under study ranged from 0.717 ± 0.141 mm and 1.836 ± 0.129 mm (Figure 3). Thickness is a factor that directly depends on the chemical composition and concentration of the material, which is mainly due to the interaction of active organic groups capable of generating atomic interactions in the macromolecule when the components are mixed in solution, and this depends on the structural

behavior at the surface. Microscopy analyses showed films with smooth surfaces for the formulations prepared with both agro-industry residues (Figure 4). We can state that the microstructure of the two types of films made is related to the permeability to water vapor being able to appreciate films with more heterogeneous distribution for those who had higher permeability values.

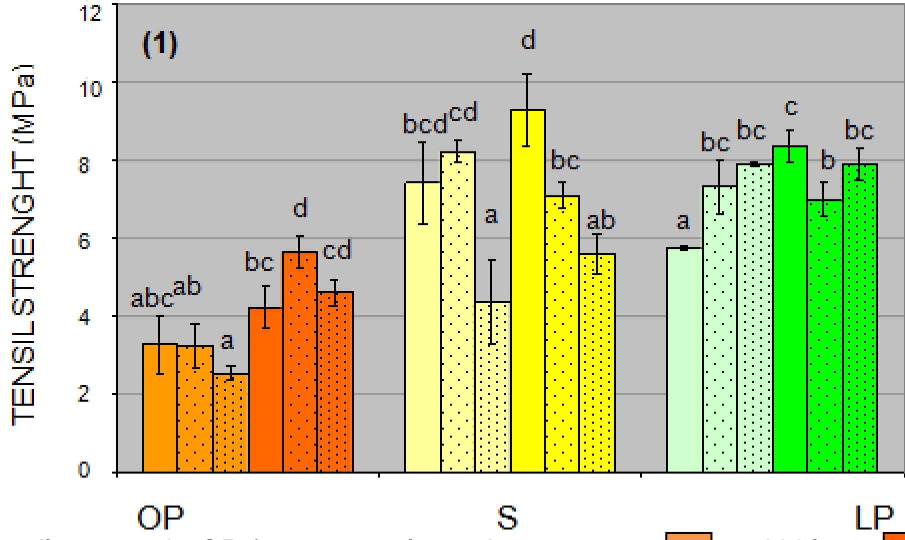


Figure 1. Tensile strength: OP (orange peel membranes mono and bi-layer); S (starch membranes mono and bi-layer); LP (lemon peel membranes mono and bi-layer); without sodium benzoate (SB) ; 0.1 g L^{-1} SB ; 0.2 g L^{-1} SB .

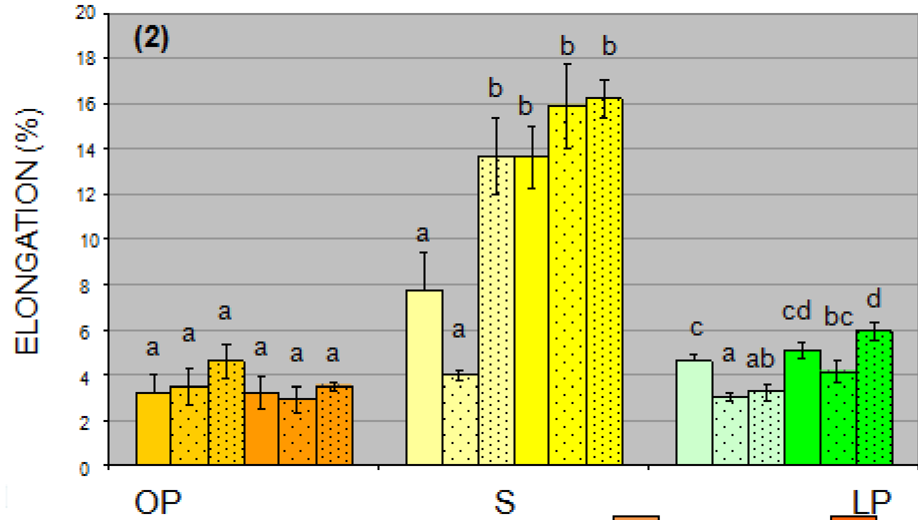


Figure 2. Elongation (%): OP (orange peel membranes mono and bi-layer); S (starch membranes mono and bi-layer); LP (lemon peel membranes mono and bi-layer); without sodium benzoate (SB) ; 0.1 g L^{-1} SB ; 0.2 g L^{-1} SB .

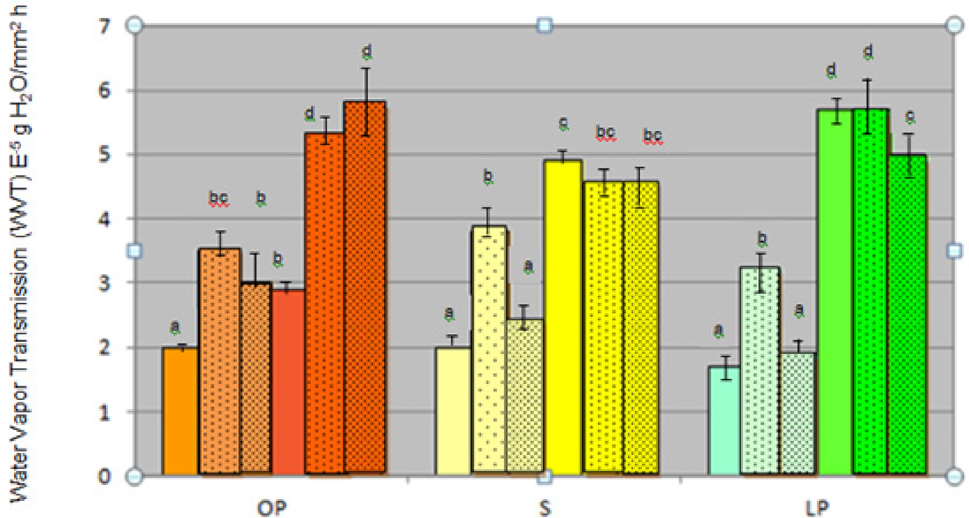


Figure 3. Water Vapor Transmission: OP (orange peel membranes mono and bi-layer); S (starch membranes mono and bi-layer); LP (lemon peel membranes mono and bi-layer); without sodium benzoate (SB) ; 0.1 g L^{-1} SB ; 0.2 g L^{-1} SB .

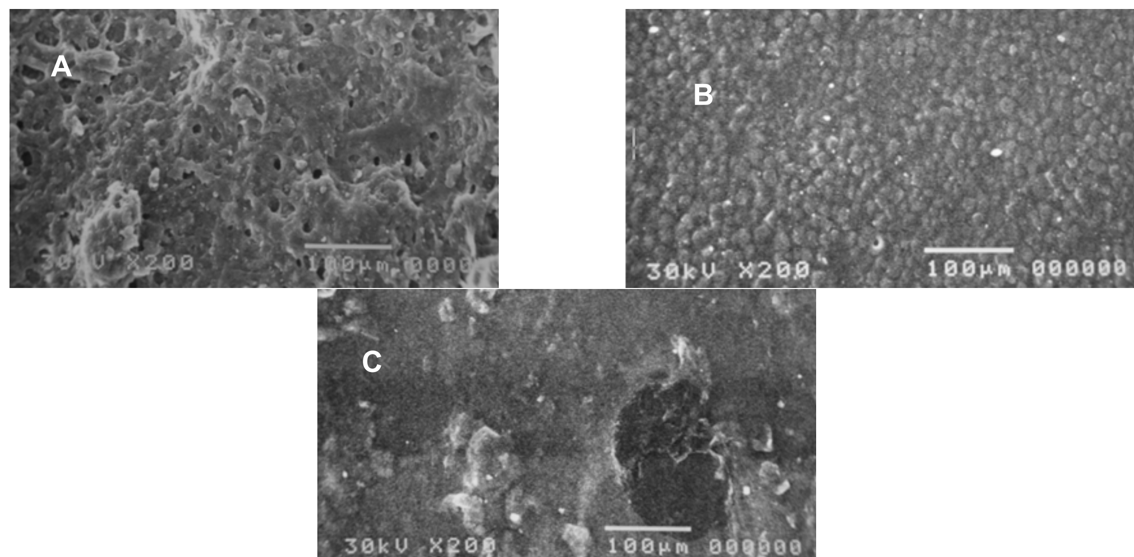
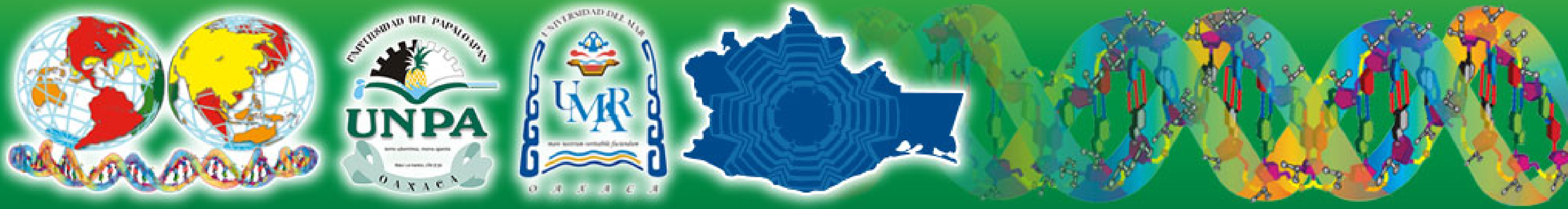


Figure 4. Monolayer membranes Microscopy Analyses, (SEM): A) Orange peel; B) Anionic Starch; C) Lemon Peel.

Conclusion: Because the abundant OH groups and good compatibilities, materials with high content of cellulose and its derivatives can be blended more easily with other natural polymers to obtain novel biodegradable materials with unique functions and good properties and these blends can be expected to substitute for a proportion for synthetic polymers. The physicomechanical properties of the films prepared from agro industry wastes in this work are comparable to studies with other biopolymers. Both agro-industry residues (orange and lemon peel) are plentiful in



Mexico, so it is important their achievement for preparation of biomaterials that can be used in different applications such as food packaging.

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Response Surface Methodology (RSM) for production of xylanases and laccases using spent coffee grains and spent grains as substrates in solid state fermentation

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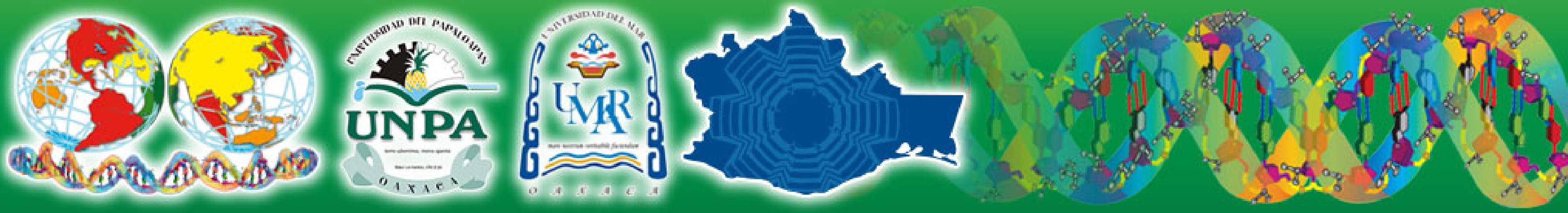
Abstract: Four different native strains identified as *Schizophyllum commune (Sc), RVAN2, CH5* and RVAN12 were grown in a basal media supplemented with spent coffee grains and spent grains from the brewing industry. Enzymatic activities of xilanase and laccase were measured. The RSM methodology used was a central composite design (CCD). The optimal point predictions for the strain RVAN 12, laccase activity using spent grains were 29.7 °C and 6.8 g with activities of 2254 U g^{-1}, Otherwise for *Sc,* the xylanase activity was 313 U g^{-1} at 31.1 °C and 6.73 g when the spent coffee grains were used. The two residues of beverage industry (spent grains and spent grain coffee), showing a great potential to be used as substrate for industrial interest enzyme production. Using Response Surface Methodology (RSM) it is possible to find the optimal values of specific variables such as temperature and substrate concentration for the design of production of lacasse and xylanase systems.

Keywords: solid fermentation ● RSM ● xylanase ● lacasse

Introduction: Population growth has generated the need for more efficient industries and a sustainable approach that allows the collection of large amounts of high consumption products in less time. This has motivated the application of enzyme technology, which in turn requires finding these enzymes producing organisms under different conditions, particularly fungi (Inglis *et al.*, 2000). The application of xylanases in the paper pulp industry is to confer the pulp brightness, its use decreasing the amount of organochlorine compounds traditionally used for the bleaching stage (Inglis *et al*., 2000). The laccase enzymes are widely distributed in white rot fungi and their function is the degradation of lignin, however not only are they used for the degradation of lignin in the paper bleaching process, but also promote the oxidation of a wide range of toxic organic compounds(Quiroz *et al*., 2011). Response Surface Methodology (RSM) uses contour plots to find the response surface and locate optimal parameters, which are useful to assess optimizing condition clearly (Suphamityotin, 2011). RMS has been successfully used for developing, improving, and optimizing biochemical processes including those related to enzyme systems (Boon-sawang and Wongsuvan, 2010). This method has been successfully utilized to optimize the improving solid state fermentation process (Gassara *et al*., 2011). Optimization of xylanase production using RSM have been reported for novel producers such as *Chaetomium thermophilum* (Katapodis *et al.,* 2007) and *Aspergillun niger* B03 (Dobrey *et al.,* 2007). Also there are reports on the optimization of xylanases production by recombinant strains as *Escherichia coli* DH4α (Farliahati *et al.,* 2010). Since any biotechnology process is likely to be based on crude enzymes, it is important to optimize simultaneous production of the constituent enzymes for



realizing their biotechnological potential. The aim of the present study was to evaluate the influence of fermentation parameters (temperature and substrate concentration) on the production of Xylanases and Laccases enzymes by native white rot fungi (basidiomycetes) from the Northeastern region of Mexico under solid state fermentation conditions, using Response Surface Methodology (RSM).

Material and Methods:

Four different native strains identified as *Schizophyllum commune (Sc), RVAN2, CH5* and RVAN12 belonging to the laboratory L1 of the Institute of Biotechnology were grown in a basal media supplemented with spent coffee beans and spent grains from the brewing industry (5 g, 30 °C) as the main carbon source. Enzymatic activities of xilanase and laccase were followed. To determine the xylanase activity, the method of Miller was used (Miller, 1959). The laccase activity was determined in the filtrate by the oxidation of 2,2 'azino-bis-3-etilbenz-thiazoline-6-sulfonic acid (ABTS). Two strains were selected and the quantitative effect of two independent variables, temperature (X1) and substrate concentration (X2), were evaluated to find the optimal concentrations of these two factors. Temperature ranges tested were 25, 30 and 35 °C and 3-7 g for substrate concentration. The RSM methodology used in this study was a central composite design (CCD), which is a first-order equation (2N). Data were analyzed in the Design Expert software V8.

Results and Discussion:

Activities for xylanase and laccase obtained by the four strains are showed in Table 1. Spent grains were the substrate where the highest lacasse activity was observed 1995 (±90) U g^{-1}, except for *Sc.* However, with spent coffee *Sc* reached xylanase activities of 237 (±13.9) U g^{-1}, whereas RVAN12 reached 97.5 (±31.3) U g^{-1}. These strains were selected for the optimization processes. The equation applied for two factors was: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2$.

Table 1. Xylanase and laccase produced by four native strains using spent coffee grains and spent grains.

	Spent coffee grains		**Spent grains**	
Strain	**xylanase U g^{-1}**	**lacasse U g^{-1}**	**xylanase U g^{-1}**	**lacasse U g^{-1}**
S. comune	237.8(DS±13.9)^{4b}	0	236.3(DS±12.3)^{4b}	0
RVAN 12	97.5(DS±31.3)^{8a}	0.58(DS±0.2)^{12a}	56.8(DS±7.0)^{4a}	1995.8(DS±90.0)^{16b}
RVAN 2	145.2(DS±6.6)^{4a}	1.25(DS±0.6)^{12b}	37.6(DS±2.0)^{4a}	1947.2(DS±40.1)^{16b}
CH5	80.6(DS±0.3)^{8a}	0	70.9(DS±23.1)^{20a}	164.3(DS±57.3)^{16a}

The optimal point predictions in agreement with the contour plots and response surfaces for both enzymes and with both selected fungi (*Sc* and RVAN12) are showed in Figures 1 and 2. For the strain RVAN 12, laccase activity using spent grains from the brewing industry the optimal conditions predicted were 29.7 °C and 6.8 g with activities of 2254 U g^{-1}, Figure1. Otherwise for *Sc,* the xylanase activity was 313 U g^{-1} at 31.1 °C and 6.73 g when the spent coffee beans were used (Figure 2).

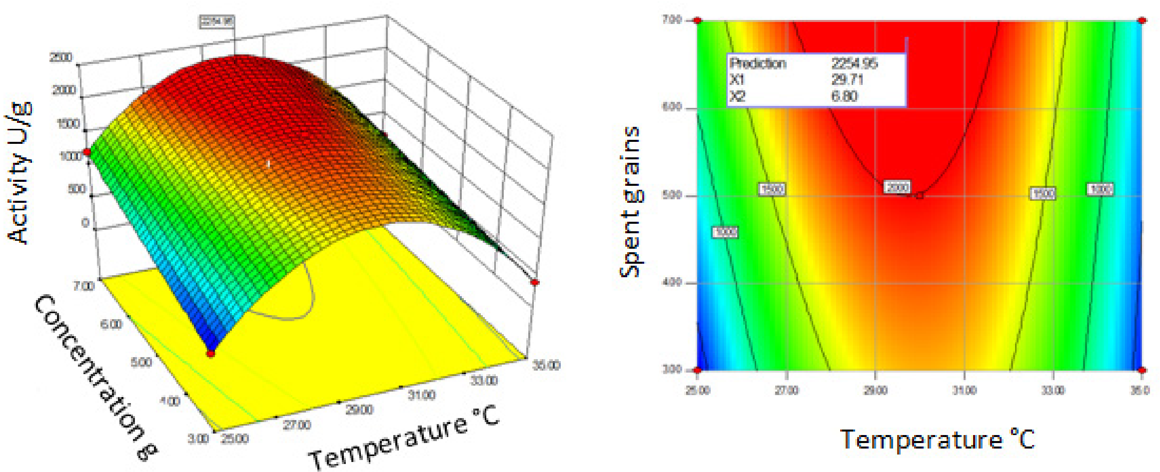


Figure 1. Response Surface Methodology (RSM), for lacasse activity, using spent grains, strain RVAN12.

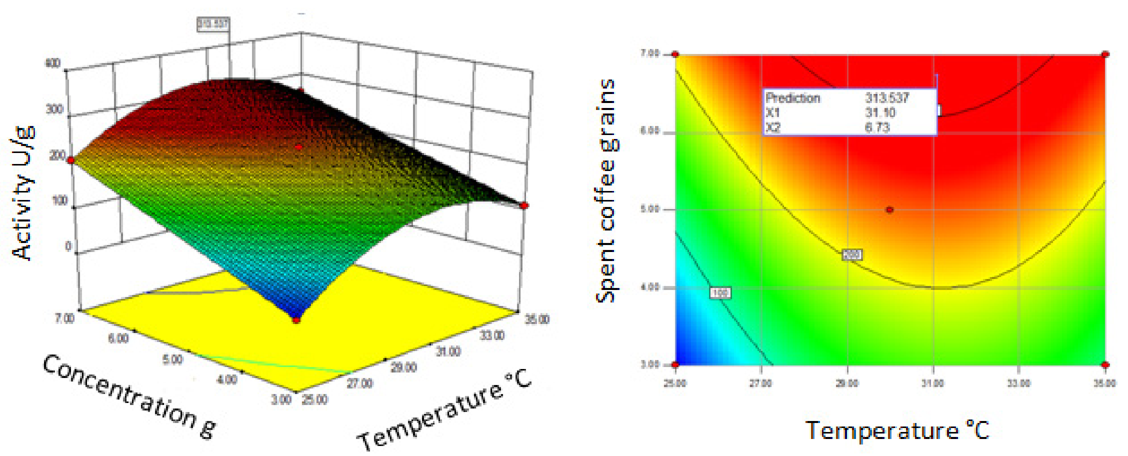


Figure 2. Response Surface Methodology (RSM), for xylanase activity using spent coffee beans, strain *Sc*.

Conclusions: Two residues of beverage industry (spent grains and spent coffee grain), showing a great potential to be used as substrate for industrial interest enzyme production, being that cost of raw material has direct impact on overall production of enzymes. Since many countries has an agro-based economy and generates huge quantities of agro waste, use of these agro or beverage residues should be promoted. Using Response Surface Methodology (RSM) it is possible to find the optimal values of specific variables such as temperature and substrate concentration for the design of production of lacasse and xylanase systems with a minimum number of experimental trials.

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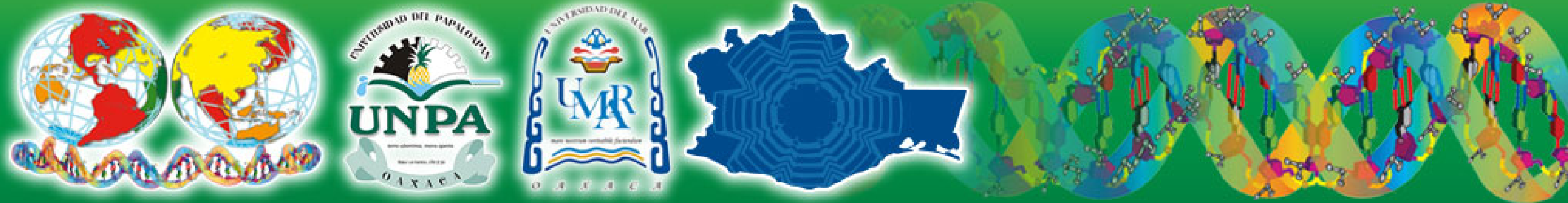

Sequential heterotrophic/autotrophic cultivation to improve xanthophylls production from *Scenedesmus incrassatulus*

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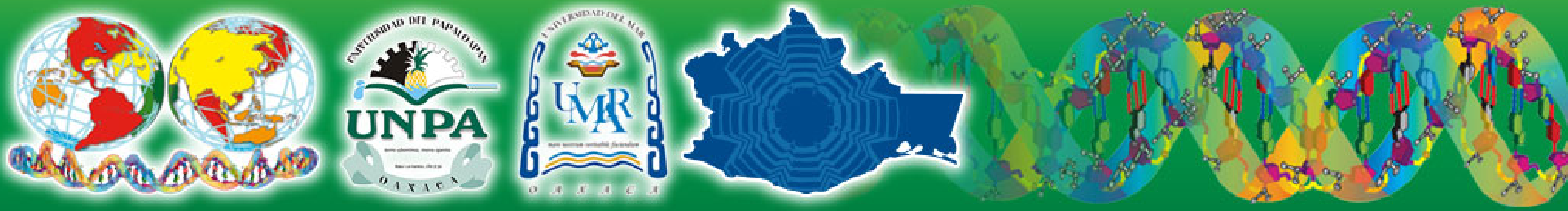
Abstract: The xanthophylls are naturals pigments widely used as food and feed additives. The chlorophycean *Scendesmus incrassatulus* can produce xanthophylls of industrial importance, mainly lutein, but the productivities in autotrophic cultures are low. In this work evaluated a sequential culture heterotrophic/autotrophic as an alternative to increase the productivity of xanthophylls in this microalga. At the end of heterotrophic cultivation the maximum biomass achieved was 6.86 ± 0.042 g L^{-1} with a xanthophylls content of 2.096 ± 0.02 mg g^{-1}. After transference of culture to the photobioreactor the content of xanthophylls was increased to 3.66 mg g^{-1}. The maximum productivity of xanthophylls was 3.33 mg L^{-1} d^{-1} with 17 h of autotrophic cultivation. The sequential heterotrophic/autotrophic cultivation achieved higher productivity of xanthophylls compared with mixotrophic, heterotrophic and autotrophic cultures.

Keywords: xanthophylls ● microalgae ● heterotrophic ● autotrophic

Introduction: Xanthopylls are oxygenated carotenoids. In the last years there has been an increasing interest in the application of xanthophylls on human food and feed. The xanthopylls of most commercial value are astaxanthin, lutein and canthaxanthin (Ahmed *et al.,* 2005; Breithaupt, 2007; Cardozo *et al.,* 2007). *Scenedesmus incrassatulus* is a chlorophycean microalga with several biotechnological applications from waste water treatment until carotenoid production. In previous studies from our work group it was established a lutein content of 2.59% dry weight of biomass from autotrophic cultivation of *S. incrassatulus* (García-Cañedo *et al.,* 2011). In heterotrophic cultivation from these microalgae, the biomass production was 41% and 24.2% higher than obtained in autotrophic and mixotrophic cultivation, respectively (Cañizares-Villanueva *et al*., 2011).

In heterotrophic culture the microalgae obtain the carbon and metabolic energy from organic substrates. The main advantages in heterotrophic processes are simple operation and easy maintenance obtaining with this a minor cost of production (Perez-García *et al.,* 2011). However, the heterotrophic cultivation limits the production of light metabolites induced like the pigments. The pigments are related to chlorophylls and they have a protective function against damage produced by solar radiation. Distinct chlorophytes can produce carotenoids in autotrophic cultivation under specific conditions of growing (Shi *et al*., 1999; Doucha y Lívanský, 2012; Perez *et al.,* 2011). In 1997, Ogbonna *et al.,* proposed a sequential cultivation whereby a microalgal culture grown in heterototrophy was transferred to photobioreactor to obtain accumulation of photosynthetic products. In this sequential process the protein and chlorophyll in the biomass were evaluated and it was shown that chlorophyll was increased around 50%. Thus, in this work, it is



proposed a sequential heterotrophic/autotrophic cultivation to improve the production of xhantophylls and biomass in *Scenedesmus incrassatulus*.

Materials and Methods:

Inoculum preparation. The microalgal strain of *Scenedesmus incrassatulus* used for inoculum was granted by the Laboratorio de Hidrobiología Experimental de la Escuela Nacional de Ciencias Biológicas del IPN (ENCB-IPN). It was cultivated in Erlenmeyer flasks of 250 and 500 mL with PCG medium (Perales, 2008) plus 1 g L^{-1} of glucose under controlled conditions of temperature (20-25 °C), illumination (120 µmol m^{-2} s^{-2}), photoperiod (12/12, light/dark) and air flow 0.5 vvm. Three sequential reseed were done at 120 h each one to ensure the culture synchronization.

Heterotrophic cultivation. Batch cultures were carried out in a stirrer tank fermentor Bioflo New Brunswick with 6 L total volume and 4.5 L operation volume. The pH was controlled at 7.5 by addition of NaOH 0.2 N, the temperature was controlled in 30 ± 1 °C. The culture medium used was designed from elementary composition of *Scenedesmus* sp. and contained glucose as carbon source. The samples were taken each 8 h for determination of biomass (dry weight) (Perales, 2008), glucose concentration (Miller, 1959) and xanthophylls content (AOAC 970.64).

Autotrophic cultivation. At the end of heterotrophic growth (with total glucose consumption) the biomass of *S. incrassatulus* was transferred to an air lift photobioreactor with 3 L total volume. In this photobioreactor the culture was incubated two days with 150 µmol m^{-2} s^{-1} superficial irradiation. Samples were taken each 6 h to evaluate the biomass (Perales, 2008) and xanthophylls content (AOAC 970.64).

Analysis of total xanthophylls content. In the pigment extraction from biomass of *S. incrassatulus,* a modification of method AOAC 970 64 was used. About 0.1 g of lyophilized biomass was placed in 10 mL volumetric flasks and 3 mL of HEAT solution (Hexane: Ethanol: Acetone: Toluene, 10:6:7:7, respectively) were added. Subsequently, the samples were disrupted with an ultrasonic processor and these were covered with aluminum for light protection; 200 µl of KOH 40% in methanol were added and the samples were heated at 56 °C during 20 min, after that, they were cooled and 3 mL of hexane were added with vigorous shaking. The flasks were gauged at 10 mL with 10% $NaSO_4$. The samples were left resting by around 1 h to get the phase separation. The superior phase was used to do the spectrophotometric measurement.

Results and Discussion:

At a first stage, *Scenedesmus incrassatulus* was grown in a batch culture in heterotrophic conditions. The maximum biomass concentration achieved was 6.86 ± 0.042 g L^{-1}, with a 0.05 ± 0.013 g L^{-1} h^{-1} productivity and the specific growth rate calculated by fit to an exponential model was 0.033 ± 0.003 h^{-1} (Figure 1). The experimental growth and glucose consumption data were fitted to a Monod model through simulation in Model Maker platform and its correlation coefficient was 0.95. The fit for $Y_{x/s}$ and μ_{max} values were 0.465 ± 0.092 g g^{-1} and 0.0601 ± 0.0007 h^{-1}, respectively.

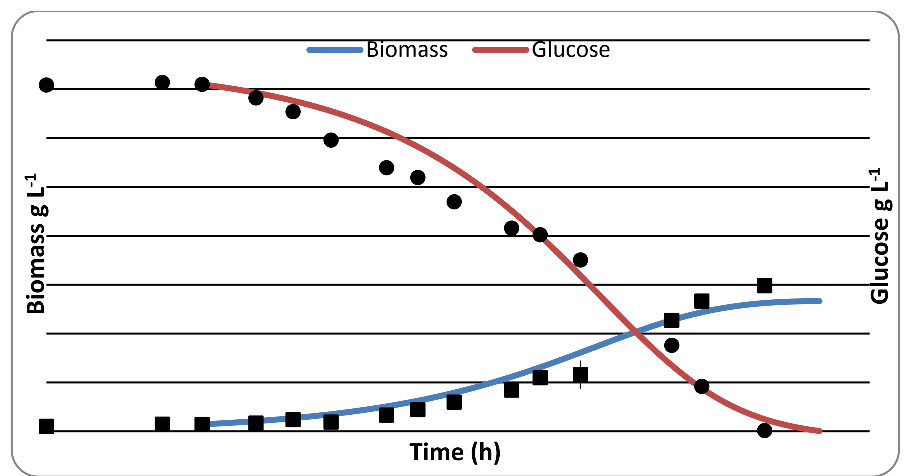


Figure 1. Kinetics growth and glucose consumption of *S. incrassatulus* in heterotrophic conditions. The markers are experimental data and lines the model prediction.

Regarding the specific content of total xanthophylls, a decrease of 53.45% was observed at the end of heterotrophic stage with respect to the start of culture (Figure 2). This result is consistent with the decrease of the specific content of total carotenoids reported by Canizares *et al.* (2011) for a culture of *S. incrassatulus* in heterotrophic conditions.

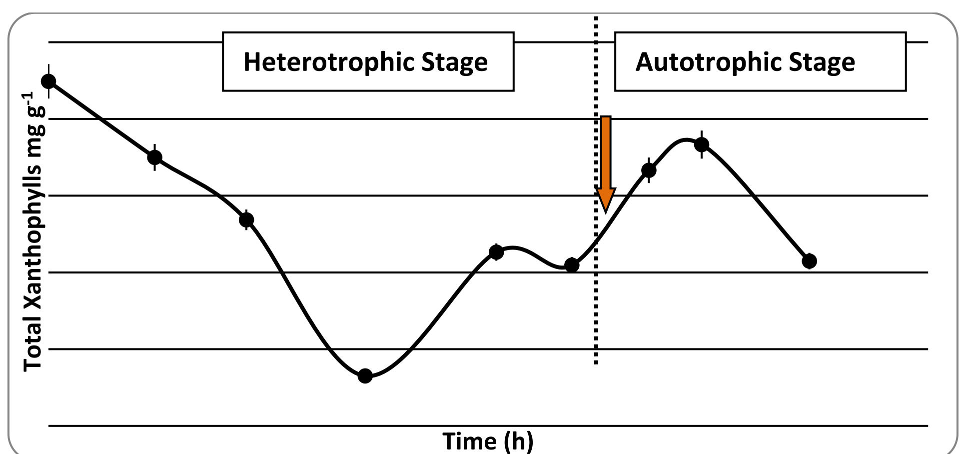


Figure 2. Production of total xanthophylls in sequential batch process of *S. incrassatulus* cultivation. The arrow indicate the begin of photoinduction.

After glucose was depleted into the batch culture of S. incrassatulus a subsequent stage for pigments production under autotrophic conditions was done in an air lift photobioreactor.The specific maximum content of xanthophylls in *S. incrassatulus* biomass was 3.66 mg g^{-1} after 26 h photoinduction (Figure 2). However, the maximum productivity of total xanthophylls was observed at 17 hours after autotrophic stage starting with 3.33 mg L^{-1} d^{-1} (Figure 3). The productivity and specific content of total xanthophylls maximum values were not reached simultaneously. This

behavior occurs because the biomass decrease during the light induction stage and thus affects productivity.

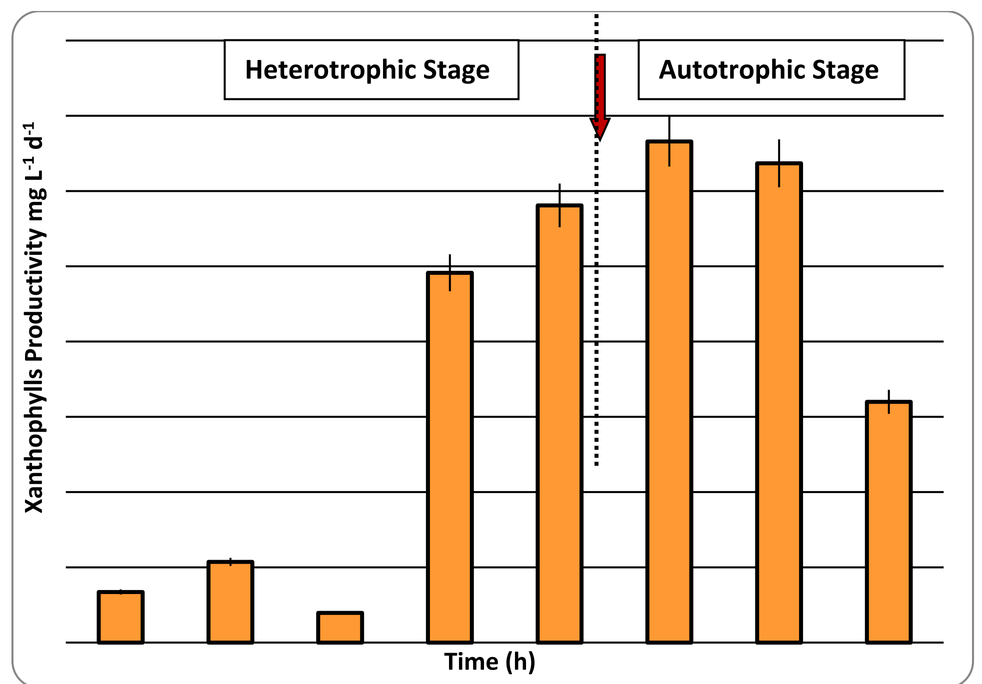


Figure 3. Productivity of total xanthophylls in sequental heterotrophic/autotrophic cultivation of *Scenedesmus incrassatulus.* The arrow indicate the begin of photoinduction stage.

Urbina (2010) referenced a xanthophylls productivity of 0.74, 0.28 and 0.34 mg L^{-1} d^{-1} in cultures of *S. incrassatulus* performed under mixotrophic, heterotrophic and autotrophic conditions respectively. The values obtained for the xanthophylls productivity in the present work are higher than the ones obtained by Urbina, 2010 using the same microlaga.

Conclusions: The implementation of sequential heterotrophic/autotrophic cultivation allowed to improve xanthophylls productivity from *Scenedesmus incrassatulus.*

Acknowledgements: Liliana Flórez Miranda thanks the Consejo Nacional de Ciencia y Tecnología, Government of México for the fellowship granted to perform this research at the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV).

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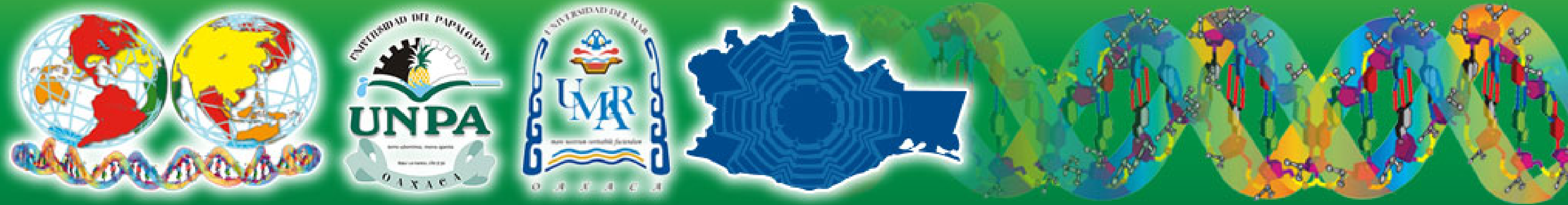

ScExlx1, an expansin-like protein from *Schizophyllum commune*

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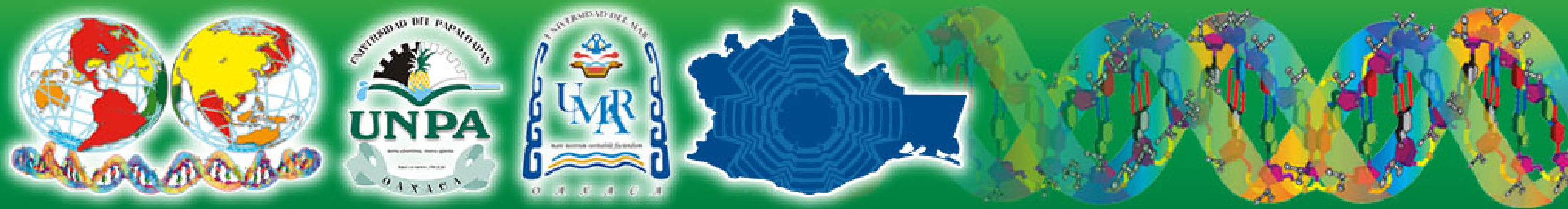
Abstract: The increasing need of renewable energy sources like bioethanol and efficient methods for its production creates an opportunity area of research looking for novel processes or molecules that can help to get yield increases. Expansins are proteins first discovered in plants where they promote cell growth through the breaking of hydrogen bonds that are formed between plant cell wall polysaccharides. These proteins had shown to act over on cellulose and some bacterial homologs have been reported to promote cellulose hydrolysis when combined with cellulase cocktail. In this study, we cloned an expressed a putative expansin-like protein from the basidiomycete *Schizophyllum commune*, providing information of a novel source for this kind of proteins.

Keywords: Basidiomycete ● expansin-like ● biofuel

Introduction: The high energy and portability of fuels from biological origin and its compatibility with the existing transportation infrastructure based on petroleum, helps explain its immense appeal as a fuel source. In addition to the increased use of biofuels such as biodiesel and ethanol derived from sugar or starch, the evidence suggests that transport fuels derived from lignocellulosic biomass represent the alternative fuel source with the greatest potential for scaling (Rubin, 2008).

The cellulose contained in the lignocellulosic biomass is the most valuable component if we are to take this biomass to produce biofuels. However, one of the main challenges to utilize cellulose as a carbon source is the difficulty to break its crystalline structure, which confers resistance to enzymatic possible attacks by microorganisms such as fungi and bacteria mainly, thus hindering their subsequent degradation (Martínez *et al*., 2008).

Nowadays, cellulolytic systems are not fully efficient for the production of second-generation bioethanol and hence can´t compete with the first generation bioethanol, it is necessary to continue with the search for proteins and cellulolytic systems that allow efficient hydrolysis and bioconversion of cellulose. For this reason, this study aimed to express heterologous protein from basidiomycete *Schizophyllum commune*, which by their degree of similarity to bacterial expansins could present amorphogenic activity on cellulose.



Materials and Methods:

A fungal strain of *Schizophyllum commune* named RVAN10 and isolated from the northeast region of Nuevo León state in México was used for this work. Mycelium was grown on YPD medium (Yeast extract 2%; Peptone 1%; Dextrose 1%; Agar 1.5%) for its propagation and storage. For isolation of ScExlx1 gene, *S. commune* was inoculated on mineral base media (7.8 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 18 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 500 mg L^{-1} $MgSO_4 \cdot 7H_2O$, 10 mg L^{-1} $ZnSO_4$, 50 mg L^{-1} KCl, 1 g L^{-1} K_2HPO_4 and 2 g L^{-1} NH_4NO_3, 1.5% Agar; pH 5) supplemented with 2% wheat straw as sole carbon source. The fungus was incubated in agar plates for 6 days at 28 °C.
Total RNA was isolated on 6-day-old culture of *S. commune* on wheat straw medium. First-strand cDNA synthesis was done using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer´s instructions. The full-length ScExlx1 cDNA was amplified using specific primers designed from *S. commune* H4-8 genome (http://genome.jgi.doe.gov; protein ID: 2642684). The 654 bp PCR fragment was purified and cloned in pJET (Thermo Scientific) vector resulting in pJET-ScExlx1. The ScExlx1 cDNA was sequenced using the pJET primers pJET 1. 2.
The ScExlx1 cDNA cloned into pJET vector was digested with *Kpn*I and *Xba*I and purified with GeneJET Gel extraction kit (Thermo Scientific). In parallel, pPICZαA was digested using the same restriction enzymes, and ScExlx1 was ligated at the corresponding sites into pPICZαA in frame with both the yeast α-secretion factor and C-terminal His_6 tag encoding sequences.
All methods for transforming and screening of *P. pastoris* are described in the Easy select Manual from Invitrogen. Culture supernatant was concentrated using Vivaspin centrifugal units (Sartorius) with a 10 kDa Cut-off at 7,000 rpm and 4 °C.
Total protein concentrations of crude supernatant or purified fractions were determined by Bradford assay (Bradford, 1976). Molecular mass estimation of recombinant ScExlx1 was done loading an appropriate amount of protein in 12% SDS-polyacrilamyde gel. Protein bands were visualized by Coomassie Blue R-250 (Sigma-Aldrich) staining and PageRuler Plus Pre-stained Protein Ladder (Thermo) was used for molecular mass estimation.
For western blot analysis, purified ScExlx1 was run on a 12% SDS-PAGE and blotted onto a nitrocellulose membrane (Bio-Rad) using a wet tank blotting system (Bio-Rad). After transference, the membrane was washed three times with phosphate buffer containing 0.1% Tween 20 (PBST) pH 8. Membrane was blocked with PBST plus skimmed milk (3%) for 20 min and washed with PBST. c-Myc (9E10) antibody (Santa Cruz Biotechnology) was used for immunodetection (dilution 1:5,000) and signal detection was visualized using an anti-mouse alkaline phosphatase conjugate (Sigma) (dilution 1:10,000) by incubating 30 min in PBST plus skimmed milk. The membrane was washed three times with PBST and 1 mL of Fast Red TR/Naphthol AS-MX (Sigma) was added for detection of alkaline phosphatase.

Results and Discussion:

After looking for expansin-like genes in *S. commune H4-8* genome, only one putative gene was found. We named this gene ScExlx1 accordingly to the nomenclature for expansin-like proteins reported recently (Kende *et al.*, 2004). ScExlx1 gene contained both Domain 1 (similar to GH45 endoglucanases) and Domain 2 (similar to Pollen allergens superfamily).
Once the ScExlx1 gene was found and named, total RNA isolation was made after growing *S. commune* in wheat straw for 6 days. Three bans were observed, corresponding to 28S, 18S and 5.8S ribosomal subunits (Figure 1) showing the integrity of isolated RNA.

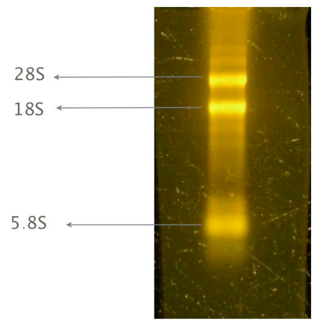


Figure 1. Total RNA extraction from *S. commune* growing on wheat straw.

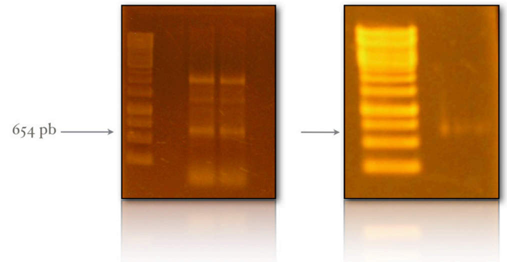


Figure 2. Amplification and purification of ScExlx1 cDNA.

Isolated RNA was used for full-length cDNA synthesis, resulting in 654 bp amplicon that was further purified for cloning into the pJET vector (Figure 2). The predicted aminoacids sequence obtained from ScExlx1-pJET and ScExlx1-pPicZαA constructions showed no significant changes between available expansins located in *S. commune* genomes deposited at Joint Genome Institute database. Alignment of ScExlx1 with EXPB1 and BsExlx1, showed a strictly conservation of aminoacids that form the shallow groove in D1 that potentially serves as a polysaccharide binding site (Figure 3; Kerff *et al*., 2008; Yennawar *et al*., 2006).

Screening of recombinant clones by SDS-PAGE, showed that three bands (~24, ~28 predicted size, and 30 kDa) were produced by transformed *P. pastoris* (Figure 4). These results suggest that two additional ScExlx1 isoforms were produced, indicating that one of them (24 kDa) can be suffering some kind of partial degradation. In the same way the bigger protein (30 kDa) can be recognized as the target for a glycosylation process.

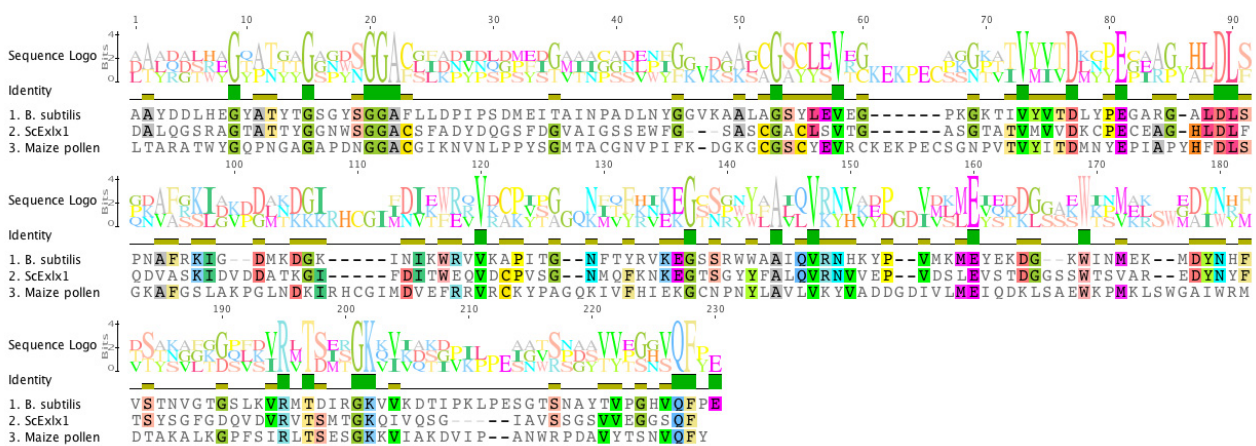


Figure 3. Alignment of expansin sequences from *B. subtilis* YoaJ, *Zea maiz* and *S. commune.*

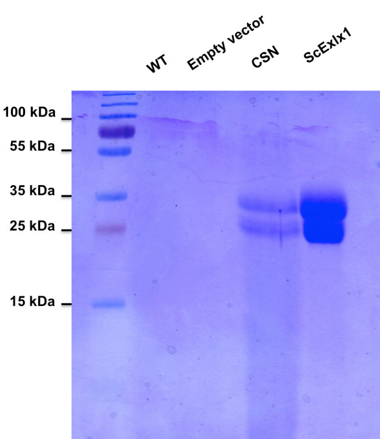
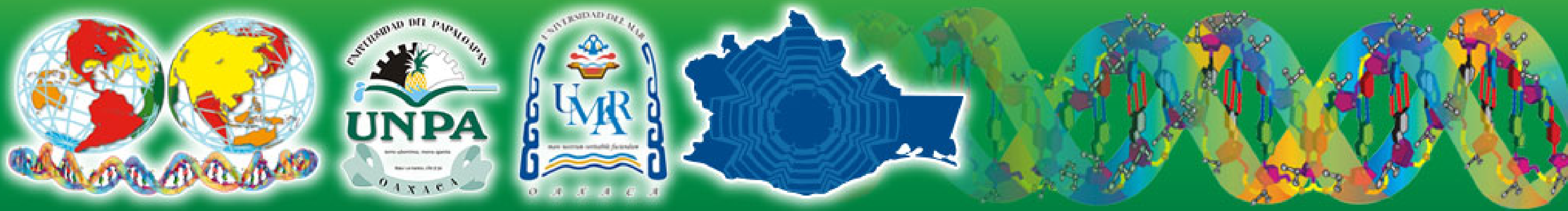


Figure 4. SDS-PAGE of recombinant *P. pastoris* clones. WT, *P. pastoris* X-33 wild type; Empty vector, *P. pastoris* transformed with pPicZαA empty vector; CSN, concentrated supernatant of *P. pastoris* transformed with pPicZαA:ScExlx1; ScExlx1, Purified ScExlx1.

Conclusion: The current analysis showed high sequence conservation at amino acids responsible for polysaccharide binding between previously crystallized expansins and ScExlx1, suggesting that ScExlx1 is a new member of the expansin superfamily. Also, we showed that *P. pastoris* is an efficient expression system for producing recombinant expansin proteins from microbial sources. As many studies of expansin and expansin-related proteins suggest that cell wall loosening proteins would be helpful additives to improve enzymatic saccharification of lignocellulosic biomass, we provide a new option to be evaluated as an opportunity to improve lignocellulose hydrolysis by pre-treating lignocellulosic biomass with this protein.



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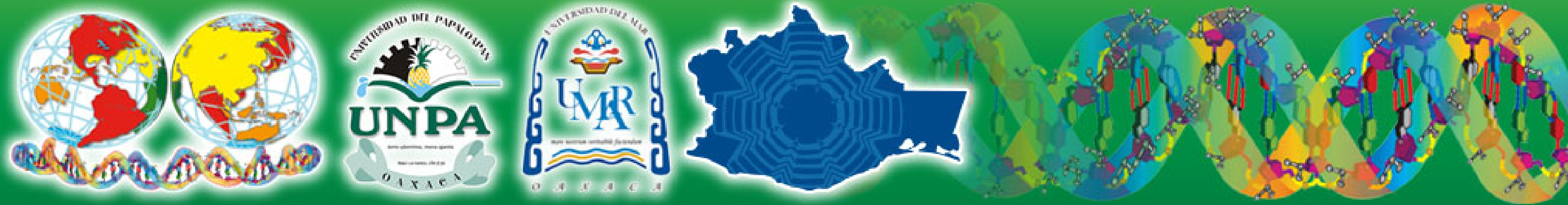

Effect of pH on the radial growth rate and pigment production of two strains of *Pycnoporus*

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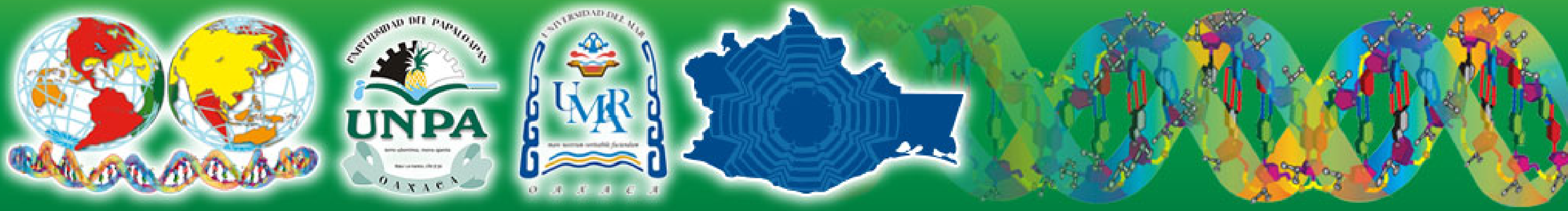
Abstract: The genus *Pycnoporus* includes saprophytes and basidiomycetes fungi common in tropical and subtropical areas of the world. These fungi have a great potential in the production of metabolites. In this study, two strains of *Pycnoporus spp* HEMIM-55 and 80 were investigated. The effect of pH culture media (4.5, 5.5, 6.5, 7.5 and 8.5) on radial growth rate and pigment production of two strains of *Pycnoporus* HEMIM-55 and 80 were evaluated. Optimal pH value for growth and pigmentation were 5.5 and 6.5. HEMIM-80 strain showed higher pigment coloration than HEMIM-55, but HEMIM-55 had a faster radial growth rate. In general, cells internal pH is neutral, but microorganisms have a mechanism for controlling inlet and outlet protons and cations flow, throughout the membrane, then they are capable to develop over different pH range. The culture media pH is an important factor for growth and pigment production in these fungi, however, the best pH conditions responsible for growth and pigment formation for *Pycnoporus* have been scarcely investigated.

Keywords: pH ● White-rot fungi● mycelium ●growth

Introduction: The genus *Pycnoporus* (Polyporaceae) is constituted by four species, *Pycnoporus sanguineus* (L. Fr.) Murr., *P. cinnabarinus* (Jacq. Fr.) Karst., *P. puniceus* (Fr.) Ryv., and *P. coccineus* (Fr.) Bondartsev and Singer (1980). *Pycnoporus* is a wild that grows on wood, both on fallen branches and stumps, preferring deciduous trees such as beech or oak. It can appear almost any time of year. Fungi of the genus *Pycnoporus* are white rot basidiomycetes, classified as microorganisms cosmetic and food grade. *Pycnoporus* is not edible in Europe, but belongs to the traditional pharmacopoeia of the countries of Africa and South America, which have been used to treat various diseases and skin lesions. It also produces cinnabarine that has shown antiviral activity and against bacteria undesirable in food (Smânia *et al*., 1995) and human pathogenic bacteria such as *Klebsiella pneumonia* and *Salmonella typhi* (Smânia *et al*., 2004) and others of clinical importance such as: *Bacillis subtillis* (Eherenberg) Cohn, *Escherichia coli* (Mingula) Castellani and Chalmers, *Listeria monocytogenes* Pirie. *Shigella flexneri* Castellani and Chalmers, *Salmonella typhi* (Schroeter) Warren and Scott, *Staphylococcus aeureus* Rosenbach, *Streptococcus agalactiae* Lehmann and Neumann (Sullivan and Henry, 1971). On the other hand, ergosterol with leishmanicidal activity was isolated from *Pycnoporus sanguineus* (Acosta-Urdapilleta *et al.*, 2010).

The genus is characterized by an orange-red color on the surface, pileus and pores. This color arises from the synthesis of various pigments such as cinnabarin, cinnabarinic acid and



tramesanguin (Correa *et al*., 2006). Pigments are chemical substances which impart color to another material by the optical effect of the refraction of the sunlight. It may also be defined as a powder which, when mixed with a liquid vehicle, imparts color (Wani *et al*., 2004). The importance of pigments is that they can be useful in industries like food, as additive or color enhancer and in the pharmaceutical (cosmetic). It has been investigated an alternative way to produce naturally occurring pigments where the sources may be plants and microorganisms (Duran *et al*., 2002). The pigments of filamentous fungi can be an alternative source instead of those from chemical or synthetic dyes. The objective of this study was to evaluate the effect of pH culture media (4.5, 5.5, 6.5, 7.5 and 8.5) on the radial growth rate and pigment production of two strains of *Pycnoporus* HEMIM-55 and 80.

Materials and Methods:

Organisms. Two strains of *Pycnoporus* (HEMIM-80 and HEMIM-55) were used. The inoculum was carried out on potato dextrose agar at a temperature of 25 °C for seven days and store at 4 °C. The strains were reactivated every month.

Medium and culture conditions. The composition of the culture medium was (gL^{-1}): glucose 10; yeast extract 5; K_2HPO_4 0.4; $MgSO_4$ 0.5; $CuSO_4$ 0.25; $MnSO_4$ 0.05 and bacteriological agar 15 (modified from Téllez-Téllez *et al*., 2008). The pH was adjusted at 4.5, 5.5, 6.5, 7.5 and 8.5. All the cultures were incubated at 25 °C for 5 days.

Morphological characteristics of the mycelium. The morphological characterization of the mycelium was made considering the color, texture, aerial mycelium and its density during the growth at different pH of the culture medium. The coloration of the mycelium obtained in all cases was determined using the Munsell catalog (Munsell, 1992).

Radial growth rate. Radial growth rate (Vr) was obtained as the slope of the mycelium radial growth (mm) *vs*. time (h).

Results and Discussion:

In general, HEMIM-55 strain showed greater Vr values than HEMIM-80 strain. HEMIM-55 strain presented the highest Vr value at pH 6.5, whereas HEMIM-80 strain had the highest Vr value at pH 5.5 (Table 1). In general, mycelium of HEMIM-80 strain was abundant and very little in HEMIM-50, in both cases there was not aerial mycelium.

Table 1. Radial growth rate (mm h^{-1}) of two *Pycnoporus* strains.

pH	HEMIM 80	HEMIM 55
4.5	0.102 (0.02)	0.158 (0.001)
5.5	0.115 (0.02)	0.158 (0.005)
6.5	0.089 (0.01)	0.161 (0.001)
7.5	0.113 (0.02)	0.147 (0.001)
8.5	0.113 (0.02)	0.146 (0.001)

The number in parentheses represents the standard deviation of three replicates.

Moreira *et al*. (2009), reported lower growth of *Lentinus crinitus* and *Psilocybe castanella* at pH 5.9 and 2.7, respectively, with the optimum at 4.5. In this work, HEMIM-80 growth best at pH 5.5, however it was able to grow a higher pH values such as 7.5 and 8.5. In the other hand HEMIN 55 has a faster growth radial rate at all pH values but it grows best at pH 6.5.
Pigmentation differences were observed between the strains (Figures 1 y 2). In general, HEMIM-80 strain showed higher coloration than HEMIM-55 strain. Both strains had higher pigmentation at pH 5.5 y 6.5. A positive correlation between the amount of mycelia and pigmentation was observed.

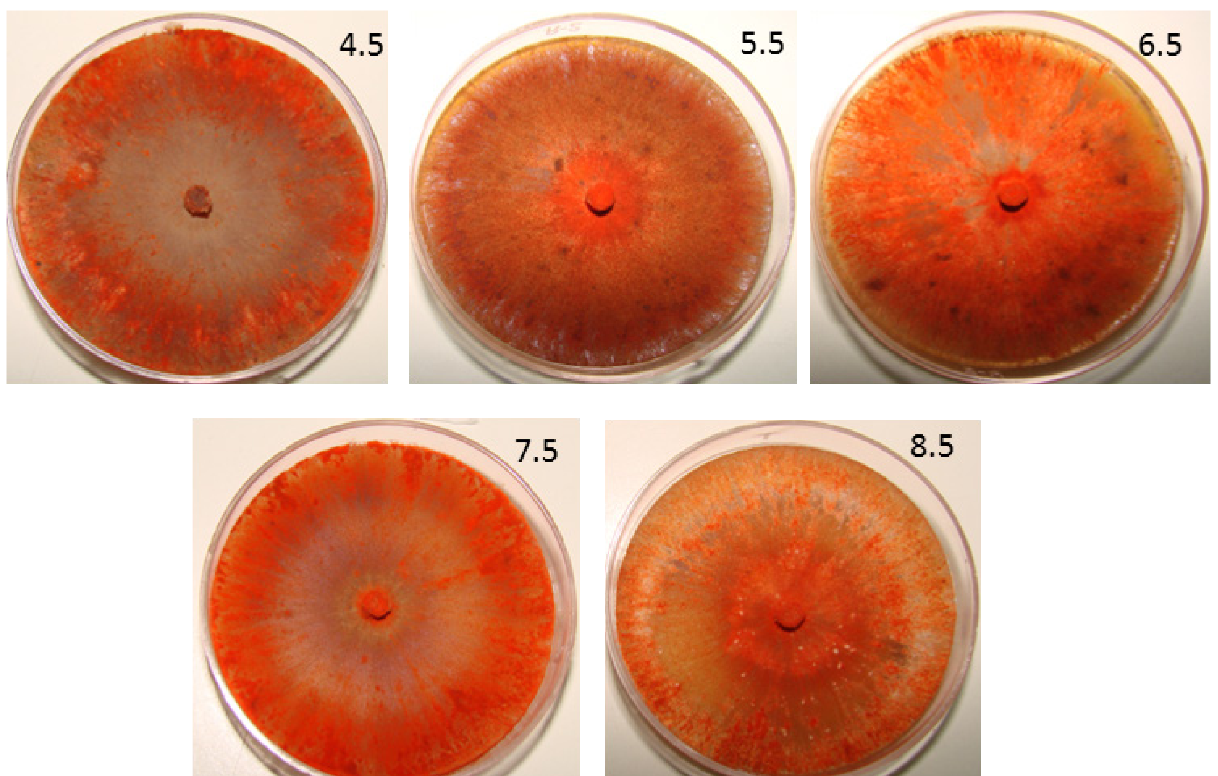


Figure 1. *Pycnoporus sanguineus* (HEMIM-80) grown at different pHs.

Chipeta *et al*. (2008), evaluated the effect of pH on xylanase production by *Aspergillus oryzae*, they found that the yield was higher at pH 7.5 than at pH 4.0, concluding that the pH is a factor that affects the production of fungal metabolites. In this work two *Pycnoporus* strains HEMIM 50 and 80 showed that growth and pigment production were function of culture media pH value, when media composition and temperature were fixed parameters during the experiments.

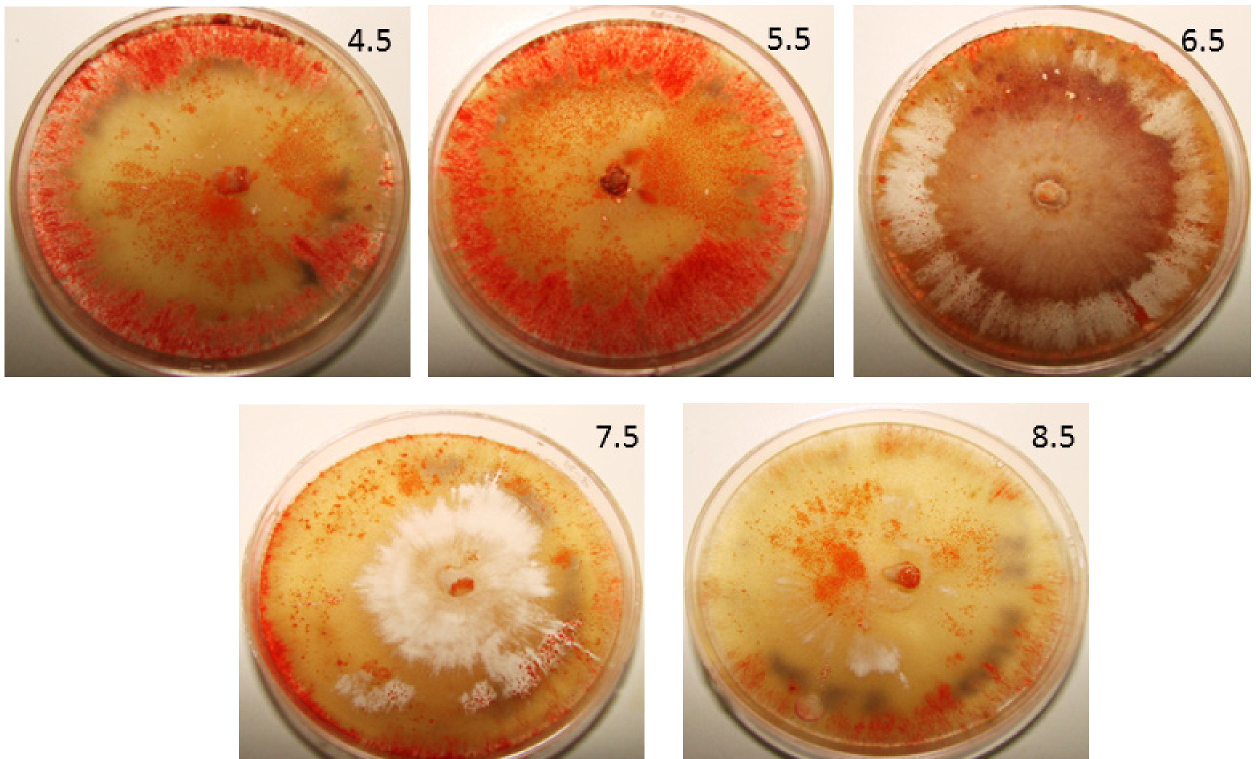


Figure 2. *Pycnoporus sanguineus* (HEMIM-55) grown at different pH´s.

Conclusions: The pH value in growth culture media is a very important factor for growth and development of basidiomycetes fungal mycelia and pigments. Culture media composition trigger specific fungal gene expressions according to the circumstances (pH, temperature and nutrient availability), that allows fungal enzymes to modify substrates according their needs. Most of basidiomycetes fungi tolerate extremes conditions of acidity or alkalinity (pH 4.5 and 8.5), however these conditions are characteristic of each strain even from the same genus. *Pycnoporus* HEMIM-80 showed no growth inhibition at any pH tested, contrary to this the strain HEMIM-55 that grew less at pH 6.5, where red pigment started to turn brown or darker than at pH 5.5. Furthermore at pH 7.5 and 8.5 HEMIM-55 reduces mycelia and pigment production. The best pH values to produce red pigment were 5.5 for HEMIM-80 and 6.5 for HEMIM-55. Furthermore, HEMIM-80 has the ability to grow at both acidic and basic pH values. *Pycnoporus* HEMIM-80 produced red pigment at all pH values but HEMIM-55 was not capable to produce pigments at basic pH values. Both strains HEMIM-55 and 80 are good candidates to be used to obtain pigments for biotechnology processes where pH values will play a determinant role in pigment metabolites production.

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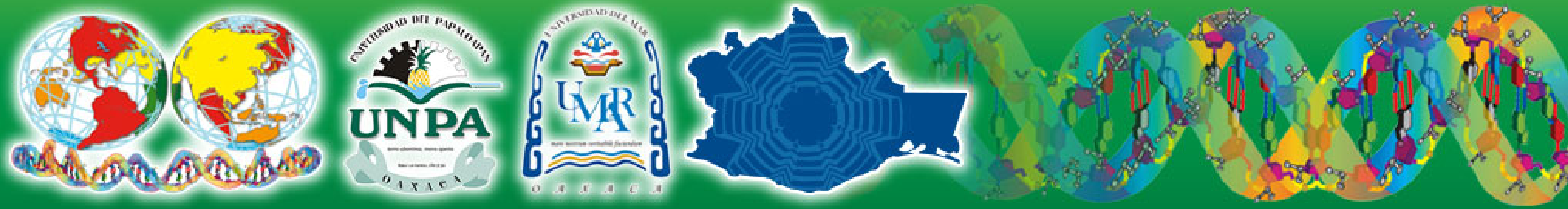

A *Pycnoporus sanguineus* laccase for denim bleaching and its comparison with an enzymatic commercial formulation

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Abstract: A laccase from the basidiomycete fungus *Pycnoporus sanguineus* strain RVAN-5 fungal crude extract (FCE) was evaluated for its ability to decolorize dyes. The dye decolorization was monitored at different dye/mediator concentrations and incubation time. Dye decoloration by the FCE ranged from 80 to 96% within 2-4 h at 25-65 °C. Similar results were obtained when violuric acid was added as mediator to the FCE; however, the number of decolorized dyes significantly increased. Of the five tested mediators, decolorization rates with violuric acid (VA) varied of initial and final optical density (595 nm) values of 2.5-3.0 to a final 0.2-0.02, respectively. Denim bleaching by alone *P. sanguineus* FCE had no effect at all; however, the mixture of FCE plus VA (1-10 mM) showed higher denim decolorization values than those obtained with a bleaching enzyme commercial formulation, as evidenced by the CIElab values obtained for FCE plus violuric acid mixture of $\Delta L = 6.4$, versus a ΔL 1.4 value obtained with the enzyme commercial formulation.

Keywords: laccases ● denim bleaching ● *Pycnoporus sanguineus.*

Introduction: It is estimated that approximately 10,000 different types of dyes and pigments are manufactured worldwide with a market of more than 7×10^5 tones per year. Approximately 30% of reactive dyestuffs are lost and discharged to the effluents and their persistence in the environment is deleterious. The synthetic dye molecules become recalcitrant, so chemical and physical methods must be used for the management of textile effluent with diverse biological treatments, such as enzymes and/or aerobic and anaerobic microorganisms for decolorizing the dyes (Chhabra *et al*., 2008). Several species of white rot fungi produce laccase enzymes (EC 1.10.3.2) that have oxidative decolorization properties of dyes. Laccases represent practical candidates for enzyme-mediated remediation processes because of their broad substrate specificity, easy production, and rapid action at milder pH and temperature values. Laccases cannot act on the nonphenolic of aromatic compounds because of their low redox potential (0.5–0.8 V). Moreover, the complex high molecular substrates cannot penetrate the active site of the enzyme. However, small organic compounds (mediators) having high redox potentials (>0.9 V) can be oxidized and activated by laccases, and these enable degradation of the substrate. A number of natural and synthetic mediators have been reported to be effective in lignin depolymerization and on synthetic dyes (Kunamneni *et al*., 2008). The use of laccases in the textile industry is constantly growing, especially in denim bleaching processes. All over the world, light washes on blue denim jeans are mainly bleached with sodium hypochlorite, which it has as main advantage of being cheap and the reaction takes place at room temperature; however, it has a number of disadvantages such as: chemically injurious and cause yellowness to the fabrics if not neutralized properly; it also attacks cotton and reduces its strength which is not desirable in light ounce denim. In addition, it cannot be used for lycra-containing garments, and most importantly, it is environmentally adverse

because its disposal cause diverse pollution problems, such as increase in biological oxygen demand (BOD) and in chemical oxygen demand (COD) level in effluent, with the subsequent increase of effluent processing cost (Rodriguez-Couto, 2012). There are at least 14 companies devoted for manufacturing more than 19 commercial products laccase based for denim bleaching located in Denmark, Hong Kong, China, Germany, Canada, USA and Colombia (Rodriguez-Couto, 2012).

Materials and Methods:

Fungus propagation and biomass extraction. *P. sanguineus* (L.) Murril strain RVAN-5 was cultured in Petri dishes containing YPD medium at 30 °C. Five tip mycelia growth portions 0.5 cm diameter were inoculated into 250 mL flasks containing 4.0 g of wheat bran and 12 mL of saline solution (g L^{-1} 2.0 g NH_4Cl, 0.5 g KH_2PO_4, $MgSO_4$, 0.5 g $CaCl_2$, 0.5 g KCl, pH 5.0) and incubated at 30 °C for 10 days; 70 ml of distilled water were added and the cultures were subjected at 200 rpm for 10 min in a shaker; the fungus biomass was then vacuum filtered through cheesecloth and the resulting fungal crude extract (FCE) was adjusted at 100 ml with distilled water and stored at -20 °C.

Dye decolorization. A dye color reduction assay was performed in 96 wells polyestirene microtiter plates. Microtiter plate wells were poured with 90 µL of 0.5 mM ABTS {2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid} in acetate buffer, pH 5.0, plus 10 µL of the FCE; microtiter plates were then incubated for 3 min at room temperature and read in a spectrophotometer plate reader at 405 nm. In separate microtiter plates, 95 µL per well of a 0.1% solution in acetate buffer of the following dyes (Remozal brilliant blue R (RBBR), Indigo carmine (IC), Bromophenol blue (BB), and Brillant green (BG); ten µL of FCE was added to each treatment, further incubated at 35, 45, 55 y 65 °C, for 1, 3 y 5 h and read at 595 nm.

Enzyme activity. The oxidative decolorization activity by the FCE was assayed by zymography. Ten µL of FCE were dispensed in 10 mL of sample buffer (glycerol, bromophenol blue 1m Tris, pH 6.8) and run under denaturing, non reduced SDS-PAGE conditions at 15 mA/4 °C; the gels were then subsequently washed for 5 min with phosphate buffer and 3 min with 40% methanol/10% acetic acid; afterwards, the gels were further incubated with a solution of 1 mM ABTS and 5 mM 2,6-DMP (Dimethoxyphenol) until color development. Individual zymograms were prepared and developed for RBBR, IC, BB, BG dyes at 0.25% in distilled water, until decolorization zone was visualized.

Denim bleaching. The effect in denim bleaching of FCE combined with individual synthetic mediators [violuric acid {2,4,5,6 (1*H*,3*H*)-Pyrimidinetetrone 5-oxime}; Vanillin (4-Hydroxy-3-methoxybenzaldehyde); 3-Hydroxyanthranilic acid; TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy) and 3,5-Dimethoxy-4-hydroxybenzoic acid], was evaluated. Square 7 x 7 cm (49 cm^2) portions of several denim brands (Old Navy®, Faded Glory®, Liz Claiborne®, Ralph Lauren® and a no Name bulk) were individually immersed in a 5 mM solution of the synthetic mediator plus 10% of the FCE in tap water. Each denim bleaching treatment was incubated at 55°C for 4 hr with occasional gentle agitation by hand and further rinsed with running tap water. After drying each denim square portions, bleaching treatment was evaluated for reflectance in a Perkin Elmer UV/VIS, Lambda 12 spectrophotometer and for CIE Lab values in a CR-300 Minolta® chromameter.

Results and Discussion:

Laccase enzymes produced by *Pycnoporus sanguineus* RVAN5 have an optimum pH of 3.0 and keep a 100% activity at temperature ranges between 25 and 45 °C; a 50% of their activity decreases at 55 °C in 30 h, at 65 °C in 6 h and at 75 °C in 1.5 h. (Fig. 1). At room temperature the activity is maintained for at least six months.

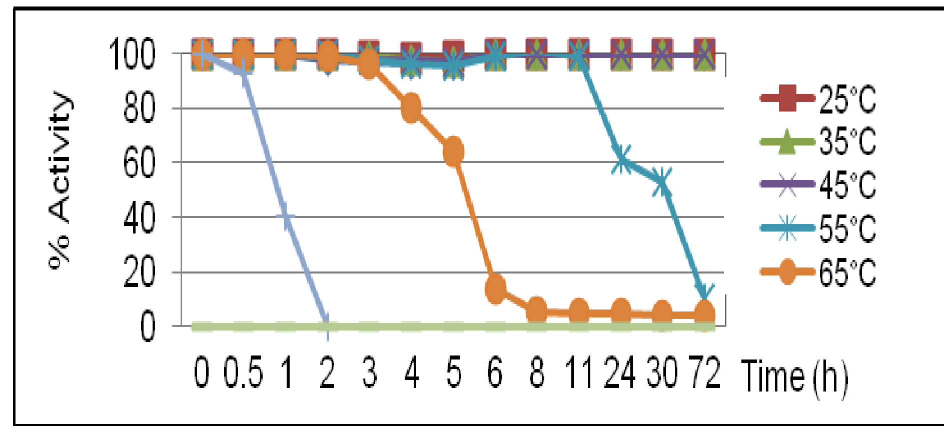

Figure 1. Stability of *Pycnoporus sanguineus* fungal crude extract (FCE) at different temperature ranges at pH 3.0 (190 µL acetate buffer, pH 5.0, containing 1 mM ABTS plus 10 µL FCE and read at 405 nm). Standard deviation (SD) < 0.09.

Pycnoporus sanguineus FCE decolorizated at least a 90% remozal brilliant blue R (RBBR) (0.15% solution), at pH 5 and 7, at 45-65 °C; however, only a 17-25% decoloration of RBBR was obtained at pH 3.0, at the same temperature values (Fig. 2).

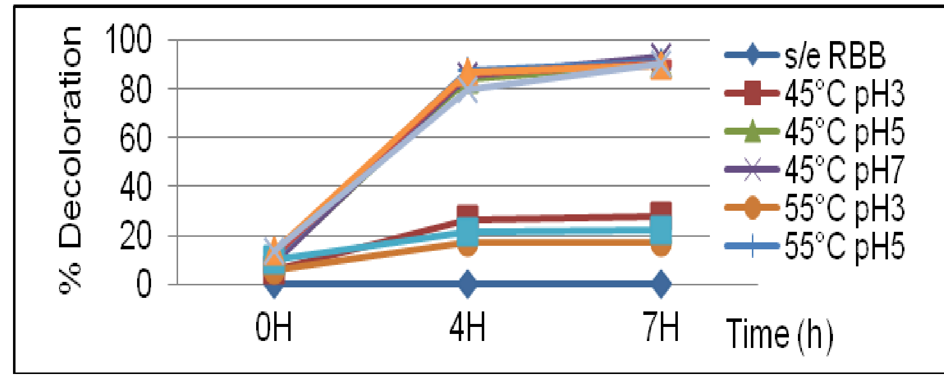

Figure 2. Effect of pH and temperature for dye decoloration by *Pycnoporus sanguineus* fungal crude extract. Standard deviation (SD) < 0.09.

Optimum pH values reported for fungal laccases on different substrates range between 4.0 and 7.0; likewise, laccasas are stable at 30 a 50 °C, but their activity rapidly decreases above 60 °C, with values of 1h at 70 °C and 10 min at 80 °C (Sadhasivam *et al*., 2008; Eggert *et al*., 1996).

Zymography conducted with our *P.sanguineus* FCE is shown in Figure 3. There was an enzyme with estimated molecular mass of Mrf 37 kDa which oxidated both ABTS (Fig. 3 lanes 1 and 2) and DMP (Fig. 3. lanes 3 and 4) within 1-2 min. An additional enzyme (Mrf 59 kDa) was also visualized in the zymograms, which showed its activity after an incubation time of 50-60 min (Fig. 3, lane 2 and 4). When using the RBBR, BB, and BG dyes as substrates, it was observed the decolorizing effect only for the Mrf 37 kDa enzyme (Fig. 3, lanes 5-8). It has been accounted that laccases have about 500-600 aa, with a molecular mass of 50-70 kDa (Lu *et al.,* 2007, Litthauer *et al*., 2007); there are reports that laccases produced by *P. sangiuneus* have a molecular mass of 68 to 80 kDa (Garcia *et al*., 2007).

Pycnoporus sanguineus FCE was evaluated along with some synthetic mediators for dye decoloration (Fig. 4). Control untreated (C) (dye alone) and violuric acid (VA) showed similar absorbance values (Fig. 4); whereas, the combination of FCE mixed with VA showed a significant decrease in absorbance values, which represented around a 96% decoloration. FCE alone without mediators was useful to decolorate only BB, GM, RBB dyes (Fig. 4).

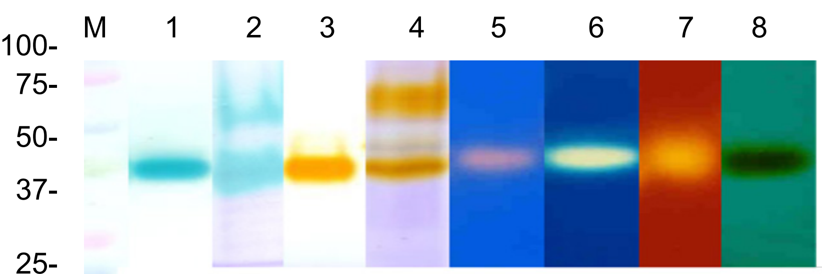

Figure 3. Zymogram of *Pycnoporus sanguineus* FCE with 12% SDS-PAGE. M (molecular weight markers), ABTS1mM (Lane 1, 2), DMP1mM (Lane 3, 4); dyes 1% concentration: IC (Lane 5), RBBR (Lane 6), BB (Lane 7), BG (Lane 8). Activity with 7.0 µL of FCE (0.02 1 U).

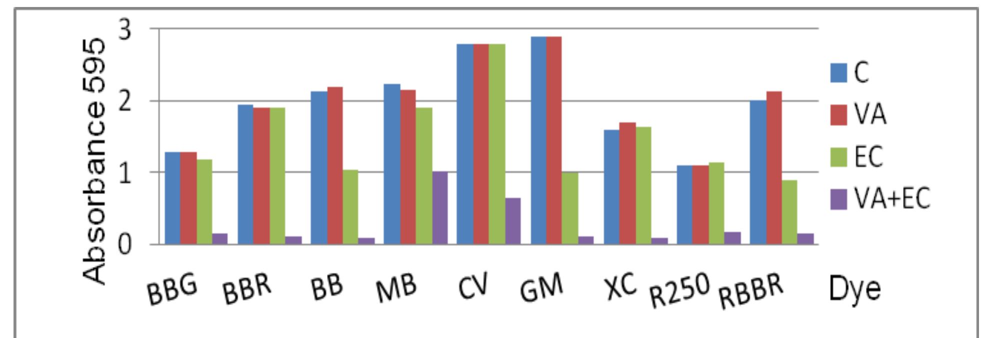

Figure 4. Evaluation of violuric acid as mediator for dye decoloration. BBG (Brillant Blue G), BBR (Brillant Blue R), BB (Bromophenol Blue), MB (Metilen Blue), CV (Crystal Violet), GM (Malaquite Green), XC (Xylen Cianol), R250 (Coomassie blue R250), RBBR (Remozal brilliant

blue R). C = Unteated control; VA =violuric acid; EC = fungal crude extract. Standard deviation (SD) < 0.09.

The effect of our FCE on denim bleaching was also evaluated by using synthetic mediators on several denim brands. Of all synthetic mediators evaluated only VA showed a remarkable denim color reduction, as quantified as reflectance values in an electromagnetic spectrophotometer (400 to 800 nm). Depending on the wave length range used, values of 5-20% higher reflectance were obtained with our FCE + VA than the untreated control (Fig. 5). From 500 to 600 nm, denim A and C presented a 10 % higher reflectance; from 780 a 800 nm, denim samples B and C, showed a maximum reflectance reading, representing a 16 a 28% difference in reflectance. Denim sample B showed a 12 % difference from 400 to 450 (Fig. 5). Denim sample D showed a 3% reflectance.

The relationship between the direct beam and the reflectant light by the denim will be higher as long as the fabric loss color. There are some reports on denim bleaching with an 8% and 17% for treated and non treated denim, respectively, with a 12% difference in reflectance (McAuliffe and Wang, 2008, Dascalu *et al*., 2008). The ideal redox mediator would be a small-size compound, able to generate stable radicals (in its oxidized form) that do not inactivate the enzyme, and which reactivity would allow its recycling without degeneration. In addition, laccase mediators should be environmental friendly and available at low cost. (Cañas and Camarero, 2010). Several studies on dye decolorization with laccase in the presence of naturally-occurring phenolic mediators of natural origin have been published recently. Acetosyringone and syringaldehyde, both di-methoxy substituted phenols derived from syringyl lignin units, where described as the fastest and most efficient laccase mediators, providing dye decolorization rates higher than those obtained with the powerful HBT mediator or other synthetic and natural mediator (Cañas and Camarero, 2010).

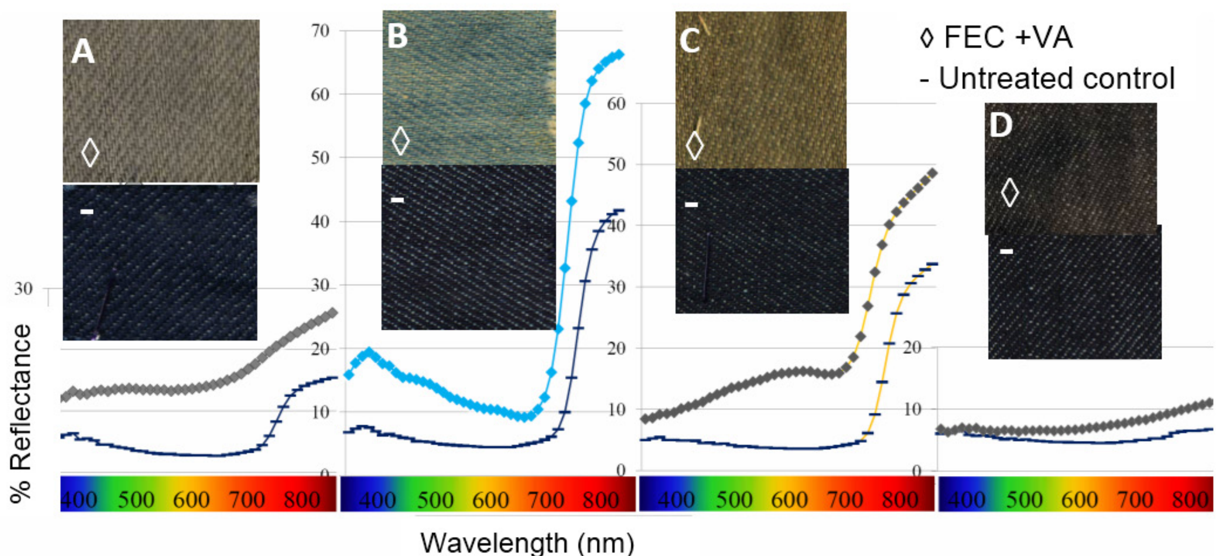

Figure 5. Spectrum curves of denim treated with *Pycnoporus sanguineus* (FCE) + violuric acid (VA).

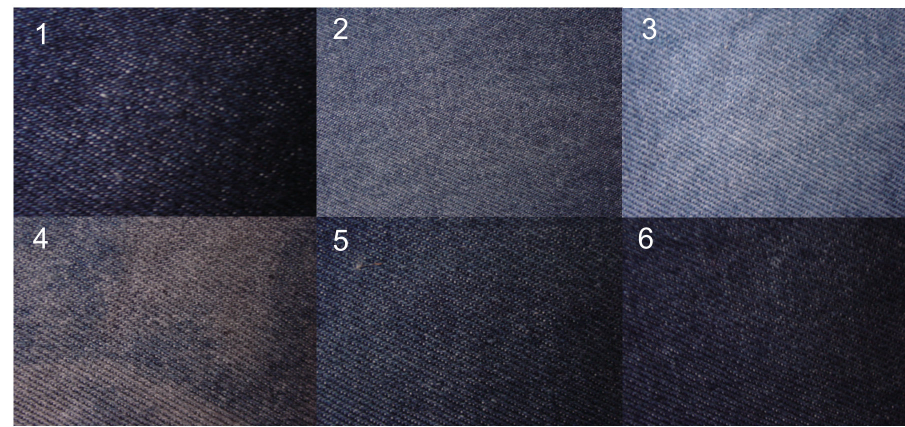

Figure 6. Denim bleaching comparison by *Pycnoporus sanguineus* fungal crude extract (FCE) with commercial treatment formulations. 1.- Untreated control; 2, 4, and 5.- FCE +violuric acid; 3 sodium hypochlorite; 6.- Laccase commercial product.

The differences in the CIELab scale (∆L values) on the Old Navy® denim bleaching by *P. sanguineus* fungal crude extract (FCE) and its comparison with a commercially available product (COM) is illustrated in Table 1; also a reported value for a patent enzymatic formulation is included (McAuliffe *et al*., 2008). Our FCE + violuric acid formulation gave a ∆L value of 6.4, contrasting with ∆L =1.46 of the commercially avalialble enzyme product (COM) included as control, and a ∆L = 2.4 reported for a patented enzyme product. Chlorine bleaching gave a ∆L value of 13.76 (Table 1).

Table 1. Differences in the CIELab scale on the Old Navy® denim bleaching by *Pycnoporus sanguineus* fungal crude extract and its comparison with a commercial available product.

		L*	a*	b*	∆L
ON	UT	21.94	2.04	-11.81	-
	FEC+VA	28.3	-0.67	-13.66	6.4
	COM	23.4	-0.29	-11.58	1.46
	Chlorine	35.7			13.76
REF	UT	22.9	1.6	-3.2	2.4
	ENZ	25.3	1.7	-3.7	

Conclusions:

A. Fungal laccase (FCE) produced by RVAN-5:

1. The fungal crude extract showed decolorizing properties up 60-100% within 4 h as measured with a 02% solution (reactive grade) of the following dyes RBBR, IC, BB, GB, without the use of synthetic mediators.

2. The fungal crude extract showed decolorizing properties on Denim only when used along a synthetic mediator.

B. Mix LacasaRNAN-5/VLA mediator (5% /10mM)

1. Denim color reduction occurred within 2 h at 25 - 55°C, reaching the major effect at 6 h.
2. VA was selected as the most appropriate mediator by its major denim color reduction effects, its cost ($5 dlls per gram reactive grade) and reported LD50 in rats (5g product/g weight).
3. Based on the ∆L values obtained, our laccase/mediator formulation is promising to use in denim bleaching.

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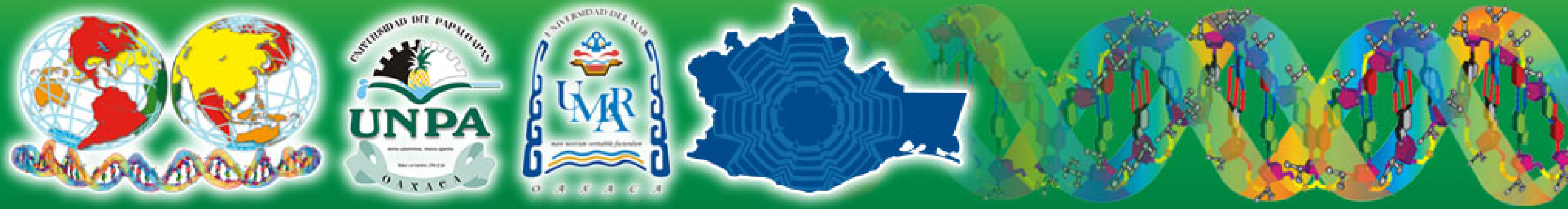

Impact of transgenic maize in Oaxaca, the maize origin land

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Abstract: The world needs producing maize sustainably. It can grow at all altitudes. In rural places it is produced mainly with traditional technologies, since the use of improved varieties is scarce. In Mexico the corn is the main food as: esquites & corn on the cob, masa for tortillas & antojitos (typical food) as: garnachas, molotes, tamales, atole, tejate, tlayudas, dobladas, quesadillas, memelas, etc. Oaxaca is known as "cradle of corn", proof of that is the breadth and variety of native corn color as white, yellow, red, and blue. It is this diversity of maize, which could risk to the possibility of interbreeding with genetically modified maize extensively studied in the world. Independently it is a tool to free hunger from other communities in the world, or to be used as an alternative source for biofuels. The impact of introducing transgenic maize in Oaxaca is a risk to the diversity of the center of origin of maize. Although it is a benefit to other communities that require more maize production for various needs such as feeding or biofuel production.

Keywords: corn ● diversity ● Oaxaca

Introduction: It was established that Mexico is the center of origin of maize, where the existence of at least 61 breeds of native maize (*Zea mays* subsp. *mays*) It is recorded; only in Oaxaca, have identified more than 30 maize landraces (Ramírez-Leyva, 2006). Thanks to the diverse and contrasting Oaxacan geography are developing organic farming origin of some of these varieties, ie, isolated cornfields where corn is planted in a specific race, to keep it pure (Ramírez-Leyva, 2006), hence the importance of trying to maintain the diversity of these varieties in this area of Mexico.

Materials and Methods:

We use a bibliographic method for search information about the maize culture in the world and Mexico. For this methodology we made an extensive review of statistics data about maize in FAO, INEGI, NCBI, PNAS, and other web sites available and we made graphically analyze the data in Excel Microsoft Office: Mac 2011.

Results and Discussion:

According the Figure 1, the global population in growing in the last time, but the rural population not. This is preoccupying because in some years (actually is beginning) the food will be insufficient for the population.

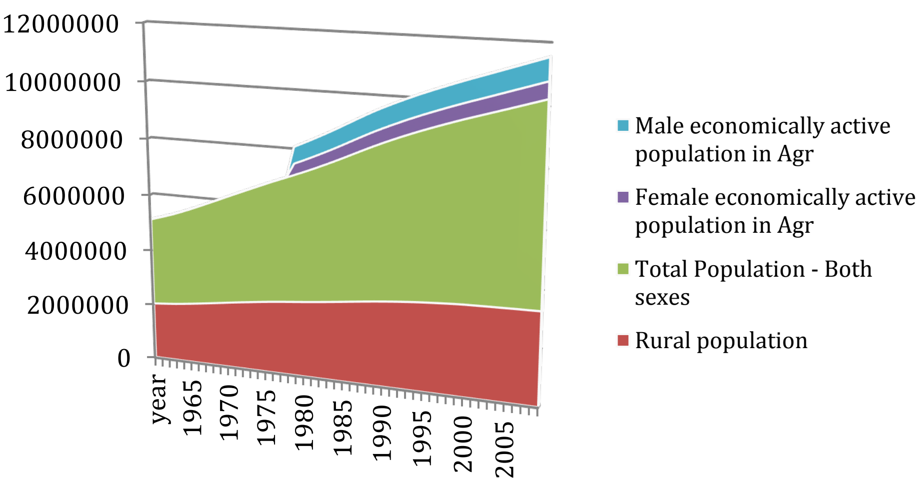

Figure 1. Global population dynamics from 1961 to 2010. (http://faostat3.fao.org/faostat-gateway/go/to/browse/O/*/E)

In the Figure 2 is demonstrated the importance of the Maize, because is the second commodity in the world, and as we can see in Figure 3, Mexico is the fifth producer in the world, but many foods in this country are based in corn, for example typical food and beverage. But, these foods are elaborated with some Maize varieties for example blue tortillas and yellow tortillas.

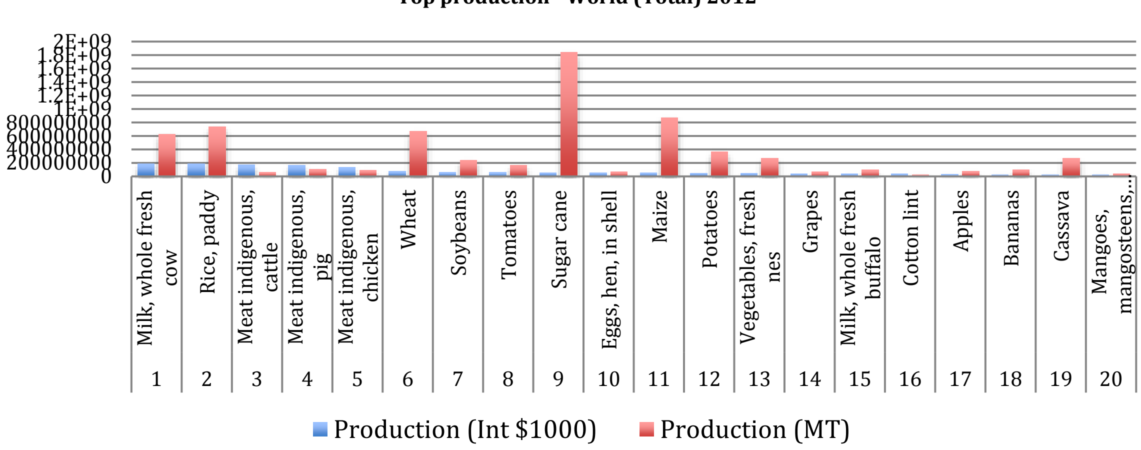

Figure 2. Ten top commodities in the world. (http://faostat.fao.org/site/339/default.aspx)

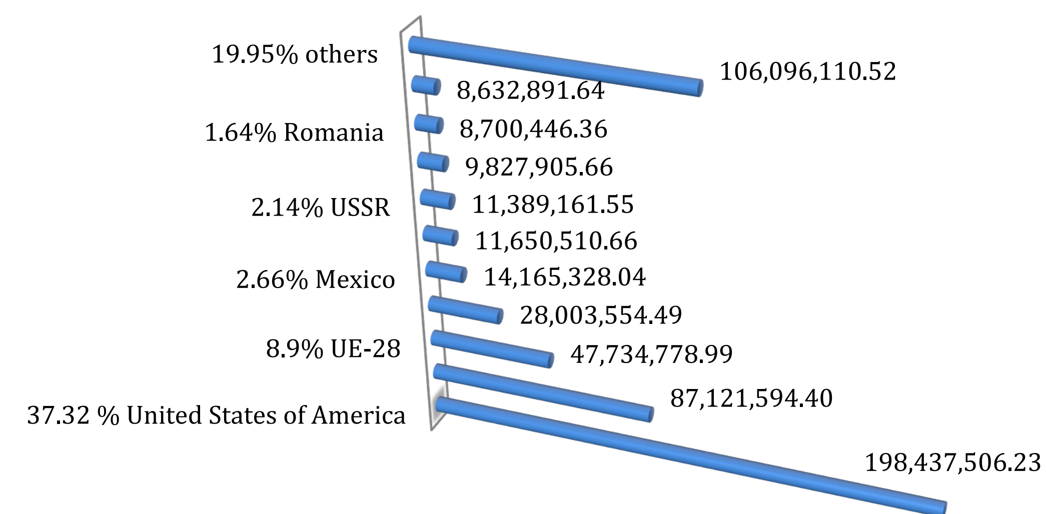

Figure 3. Corn production in world in tonnes in 2013.
(http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QC/E)

Another importance for the Maize is the nutrient content (Figure 4), which is some different in the varieties, as we can observe in the Figure 5.

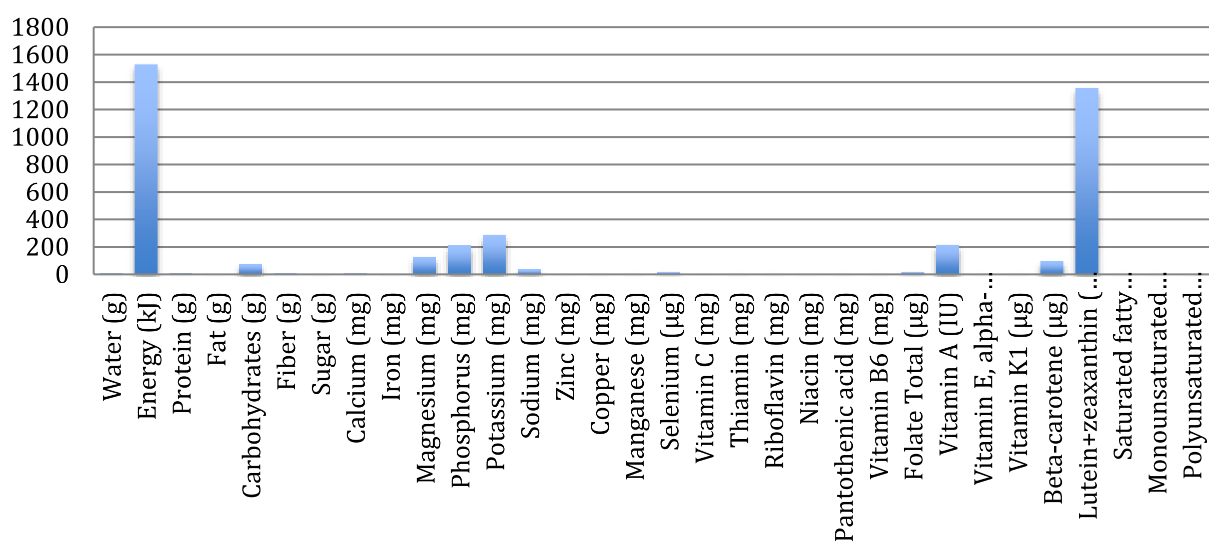

Figure 4. Maize nutrient content.
(http://en.wikipedia.org/wiki/Maize#endnote_reference_name_AA)

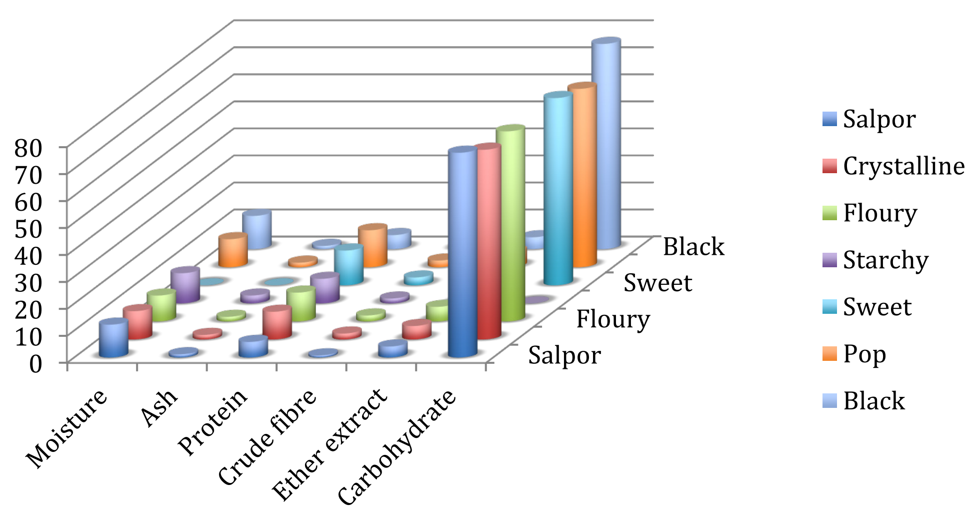

Figure 5. Nutrient content in different kind of corn. (http://www.fao.org/docrep/t0395e/t0395e03.htm)

In the figure 6 we present information about the production in different states of Mexico, we can observe that Oaxaca is the fifth producer, in spite of be the origin of this vegetable. And in the figure 7 we illustrated the principal areas where the Maize is produced in this place.

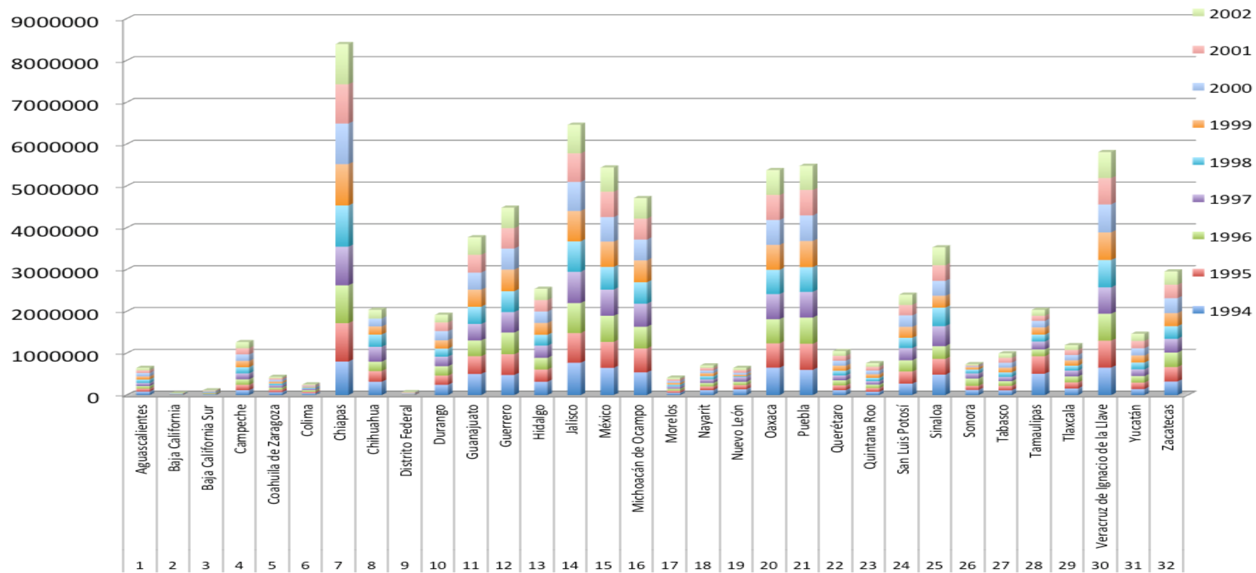

Figure 6. Production of maize in the states of Mexico since 1994 to 2002 in hectares. (http://www3.inegi.org.mx/sistemas/biinegi/default.aspx)

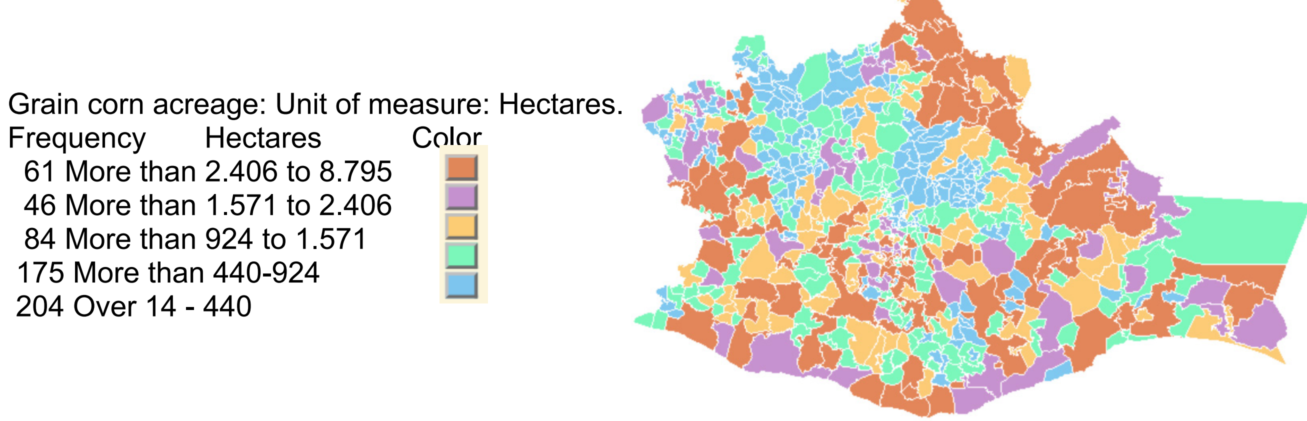

Figure 7. Production of maize in the state of Oaxaca, Mexico. (http://www3.inegi.org.mx/sistemas/mapatematico/default.aspx)

Another use for Maize in the world is oil maize as an energy source. In the Figure 8 we can observe that the oil maize production in the USA is growing with the time, and in the Figure 9 is possible see, that this country is the first producer in the world, but Mexico produce just a little.

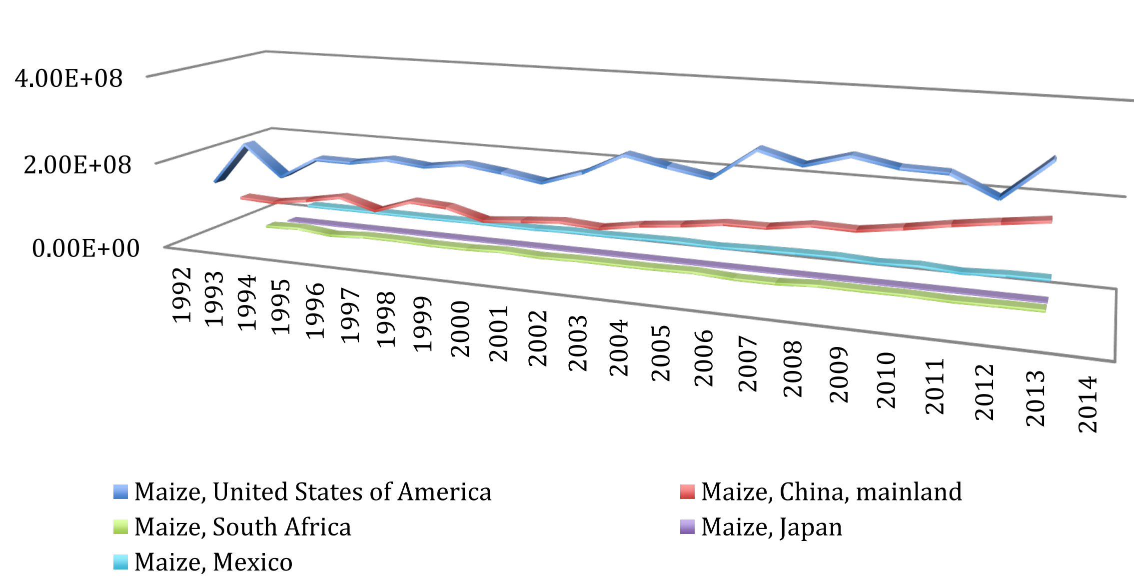

Figure 8. Production of oil maize from USA. (http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QD/E)

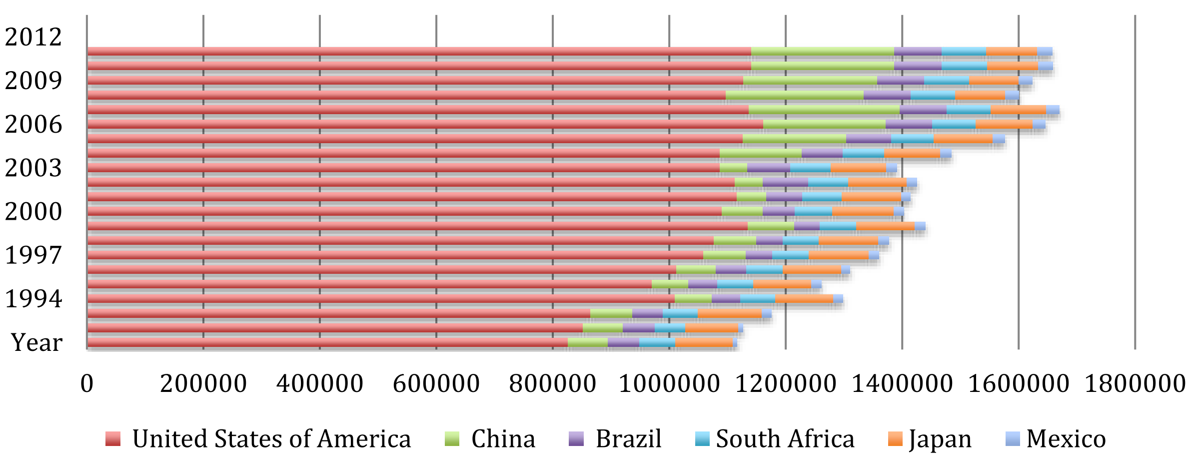

Figure 9. Top countries that produce oil maize.
(http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QD/E)

Aragón *et al.* (2006), made a study where they find different color of maize in Oaxaca, Mexico as yellow, blue, white, orange, red & black. They collected maize in Oaxaca and they found 35 landraces: Ancho, Arrocillo, Bolita, Celaya, Chalqueño, Chiquito, Comiteco, Conejo, Cónico, Cónico Norteño, Elotes Cónicos, Elotes, Occidentales Mixeño, Mixteco, Mushito, Nal-Tel, Nal-Tel de altura, Negro de Tierra Fría, Negro Mixteco, Olotillo, Olotón, Olotón imbricado, Palomero Toluqueño, Pepitilla, Serrano, Serrano de Oaxaca, Serrano Mixe, Tabloncillo, Tehua, Tepecintle, Tuxpeño, Vandeño, Zamorano, Zapalote Chico, Zapalote Grande. In the last information we can see the importance for the maize, for improve agricultural policies to drive for a best careful of the diversity.

Conclusion: There is a biological diversity in Oaxaca, which includes corn; this diversity can be supported in various studies, such as the ancestors have searched. Find varieties with good quality in various aspects such as nutrition, resistance to moisture or drought or pests. The impact of introducing transgenic maize in Oaxaca is a risk to the diversity of the center of origin of maize. Although it is a benefit to other communities that require more maize production for various needs such as feeding or biofuel production.

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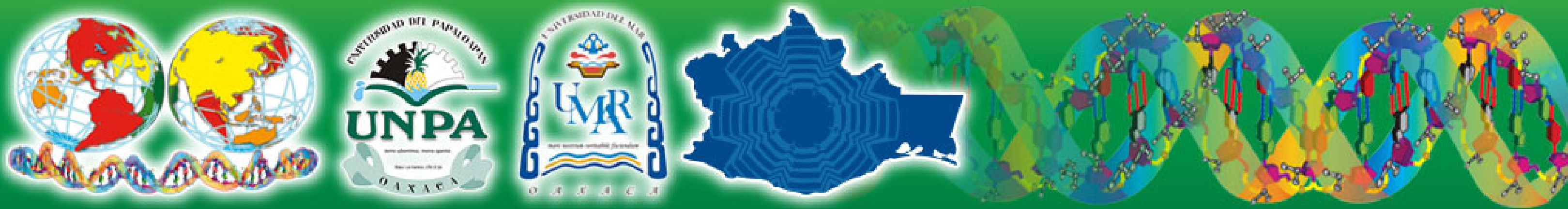

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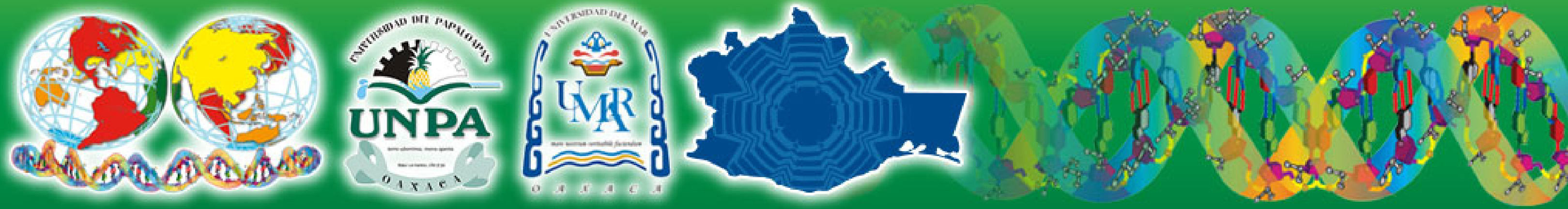

Expression and characterization of lignocellulolytic enzyme in tobacco chloroplast

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Abstract: The plastid genome has been an attractive targeting genome for plants transformation, several advantages exist in the plastid protein expression comparable with nuclear transformation, as high-protein expression levels, the opportunity to express operons in a natural containment; because of this; plastid genome is an attractive target for biotechnological applications. Therefore, in this work was performed the chloroplasts genetic transformation of tobacco plants to express and characterize biochemically the lignocellulolytic enzyme genes such as Pectin lyase (*Pel-A*) and manganese peroxidase H3 (*MnP-2*). Synthetic genes were obtained and cloned into pPRV111 vector to constructed pES4 and pES5 vectors which contain the *Pel A* and *MnP-2* genes respectively, with *rrn16S* and *3'rps12* genes as homologous recombination site and *aadA* gene as selection marker. Two vectors were used to transformed chloroplast of tobacco by biolistic. The transplastomic plants were obtained after three rounds of selection on RMOP/spectinomycin and plants were characterized by PCR and Southern blot verifying the efficient gene integration. The mRNA production was established by Northern blot analysis. Enzymatic activity of each enzymes were obtained by specific enzymatic analyses, which confirmed that the chloroplast plants is a good option to express this type of enzyme and used like bioreactor for biotechnological applications.

Keywords: Chloroplast transformation ● *Nicotiana tabacum* ● lignocellulolytic enzyme

Introduction: Chloroplast genetic engineering has led to significant advances in plant biotechnology and continue playing a crucial role in the genetic improvement of plants in the areas like agriculture, food, medicine and environment (Quesada-Vargas *et al.*, 2005). This technology involves the transgenes insertion into the chloroplast genome, which provides several advantages over nuclear transformation like the recombinant protein expression, no positional effects, absence of epigenetic effects and uniparental transgene inheritance and possibility of expressing multiple transgenes in operons (Daniell *et al.*, 2005); chloroplast transformation has been successfully tested in tobacco '*Nicotiana tabacum*' , potato '*Solanum tuberosum*' (Sidorov *et al.*, 1999), tomato '*Solanum lycopersicum*' (Ruf *et al.*, 2001), soy , cauliflower '*Brassica oleracea*' and lettuce '*Lactuca sativa*' (Kanamoto *et al.*, 2006). Currently, more than 100 transgenes has been integrated (Verma and Daniell, 2007) including genes that encoding enzymes with industrial value, biomaterials, biopharmaceutical, and of agronomic importance (Bock, 2007) with expression levels up to 70% TSP (Maliga and Bock, 2011). Due to the impact of the chloroplast transformation, have been conducted research for the expression of proteins with biotechnological

interest and emphasis on the environmental impact (Maliga and Bock, 2011) including lignocellulolytic enzymes (Papini-Terzi *et al.*, 2009); although expression of these enzymes has been reported for bacterial systems, there are few studies that focus on the production of hydrolytic enzymes and overexpression in chloroplast compartments (Petersen and Bock, 2011). Because of this, the plastid biotechnology was used to overexpress cell wall hydrolytic enzymes to study their expression, folding, stability and activity in chloroplast compartments and the effect it would have the overexpression on metabolism and normal growth of plants thus generate a model of kinetics photosynthetic processes and the impact of overexpression of such enzymes in chloroplastidic compartments.

Materials and Methods:

Cloning of genes of interest in chloroplast expression vector. Two genes of cellulolytic enzymes were used: pectin lyase gene (*Pel A)* from *Streptomyces thermocarboxydus* (Tonouchi *et al.*, 2010) and manganese peroxidase H3 gene (*MnP*-2) from *Phanarochaete chrysosporium* (Orth *et al.*, 1994). The sequences of each gene were designed *in silico* using the Serial Cloner 2.6.1 software and subjected to elimination of unwanted restriction sites; full CDs were designed according to the codon usage of the chloroplast genome (Sinagawa-Garcia *et al.*, 2009); The sequences of *Pel A* (GenBank: AB513441) and *MnP-2* (GenBank: U10306) were synthesized by GeneScript (*New Jersey, USA*). *Pel A* gene (804 bp) was synthesized flanked by the *rrn16S* promoter (P) and *rbcL* terminator (T); including the Shine-Dalgarno (SD) sequence and the leader sequence (LS) of *rbcL* gene in the 5'end of the coding region of the gene (P*rrn16S*, SD, LS *rbcl Pel A*, T*rbcL*). The full sequence was synthesized flanked with *EcoRI/HindIII* sites to be cloned into the same sites in the vector pPRV111; addition, the coding region of the *Pel A* gene was flanked with recognition sites to enzymes *5'Nhe I/3'Xba I* to replace it within the vector by *MnP-2* gene.

Plant material and chloroplast transformation vectors. Tobacco plants (*Nicotina tabacum* var. Petitte havana) were obtained by germinating seeds under sterile conditions on RM culture medium. For the maintenance and propagation of plasmids *Escherichia coli* strains DH5α (Invitrogen ®, Carlsbad CA, USA) were used. The genes of interest were cloned into the vector pPRV111 for expression of heterologous genes into the chloroplast genome (Sinagawa-Garcia *et al.*, 2009; Tungsuchat-Huang *et al.*, 2010). The plasmid DNA was obtained using QIAGEN Plasmid Maxi Kit columns (QIAGEN Inc., Valencia, CA). Gold particles of 0.6 µm (Bio-Rad ®) were used to transformation. Tobacco leaf bombardment was performed according to Svab and Maliga (1993) and Lutz *et al.* (2006). High pressure gun S1000He Bio-Rad with a Hepta adapter (Bio-RAD, Germany, Munich) was used at 1100 psi and 11 cm shooting distance. Bombarded leaves were incubated for 37 h in dark, cut into 3 × 3 mm and placed on RMOP medium supplemented with 500 mg L^{-1} of spectinomycin/streptomycin (Sigma-Aldrich, Japan).

Molecular analysis. Total DNA was extracted of tobacco plants by Doyle (1991) protocol. PCR was performed using specific primers: *Pel A* gene Fw-5'ATGACATCCGCGACACGA3' Rv-5'TGATGTCGGACGAGCTGTACT3' and *Mnp*-2 gene Fw-5'ATGGCCTTTGCATCCTCA3' Rv-5'TTATGCAGGGCCGTTGAAC3', under the following condition: 2 min of denaturing at 94 °C, followed by 25 cycles of amplification (45 s at 94 °C, 45 s at 60 °C, 1 min at 72 °C). To Southern blot analysis the samples was digested with *HindIII*, a probe *rRNA16S* was used with the primers Fw-5'TGAGAATGGATAAGAGGCTC3' Rv-5'TGTTGTTCCCCTCCCAAGGG3'; to RNA analysis a

Northern blot was performed using a specific primes of each genes afore mentioned, the probes were labeled with digoxigenin-11-dUTP (Roche, Mannheim, Germany).

Results and Dicussion:

Chloroplast transformation. From pPRV111 vector were constructed two vectors (pES4 and pES5) containing the genes *Pel A* and *MnP*-2 respectively, these vectors have at *rrn16S/3'rps'12* like homologous recombination sites in inverted repeat (IRA/IRB); also include the *aadA* gene as a selectable marker (Figure 1A, 1B). With that vector, tobacco leaves were bombarded obtained explants putatively transformed at the fifth week with a transformation efficiency of 3.14% (Figure 2A); these shoots were subjected to a second and third round of selection (Figure 2B) to remove mutant clones. Shoots of the third round of selection were placed in RM medium without antibiotic to normal development and then placed in pots in the greenhouse until flowering (Figure 2E).

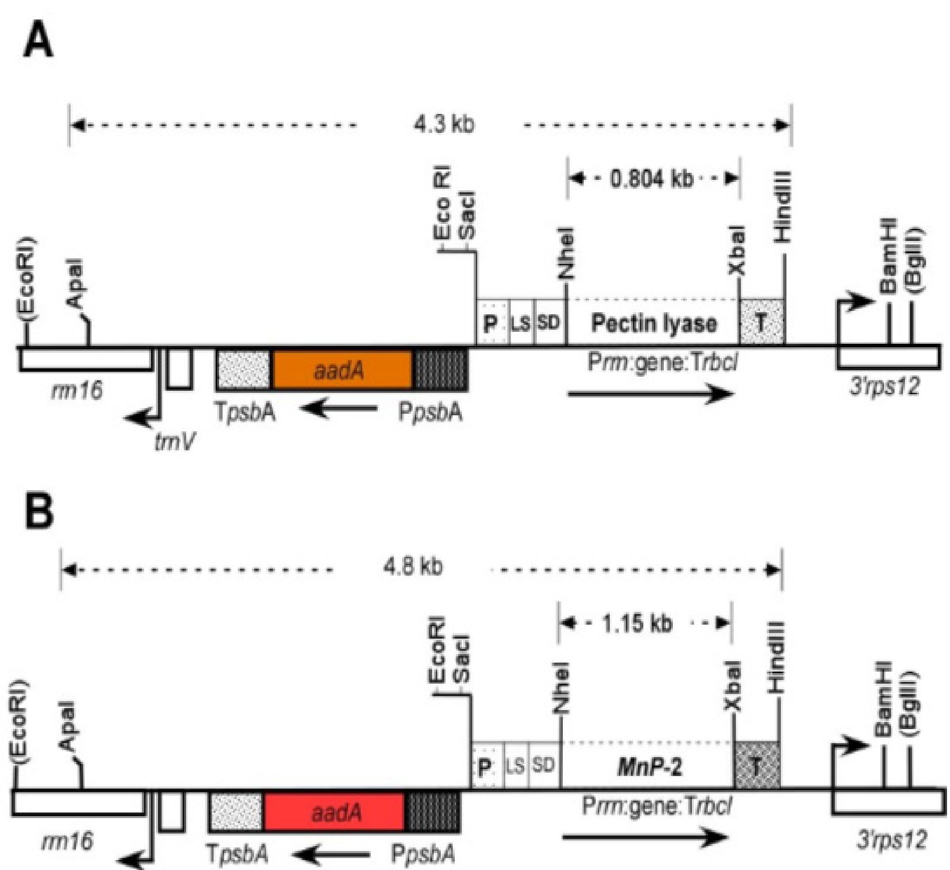

Figure 1. Restriction maps of the plastid transformation vectors *A*) pES4 (Pectin lyase gene) and *B*) pES5 (manganese peroxidase H3 gene) for cloning into IR of the chloroplast genome in *rrn16* and *3'rps12* homologous recombination site, these vectors contain *aadA* like selection marker gene; also, the genes have an *rrn* promoter and *rbcl* terminator sequence.

Integration of DNA analysis in tobacco plants. To confirm the integration of transgene, putative plants were screened by PCR using specific primers of each gene. The samples showed PCR-positive results, the amplicon of *Pel A* gene was of 0.804 kb, whereas to *MnP-2* gene was of 1.115 kb confirming the integration of the transgene into the chloroplast genome (Figure 2 C).

To investigate if the transplastomic lines achieve homoplasmy a Southern-blot was performed. The probe was made by digestion of total DNA with *BamHI.* The probe hybridized with a single 3.0 kb fragment in untransformed line. In transplastomic lines, the probe hybridized with a single fragment of 4.3 kb to *Pel A* lines and 4.8 kb to *MnP-2* lines. The absent the 3.0 kb in both lines suggest that all the chloroplast genome are transformed (Figure 3A). Total RNA from untransformed and transformed *PelA/MnP-2* lines was extracted and used to perform a Northern-blot analysis with specific probes. Both transplastomic lines (*PelA/MnP-2*) showed monocistron transcripts (804 nt and 1115 nt) of stable form (Figure 3B, 3C). Seed from the two lines of transformed plants were placed in selection medium supplemented with spectinomycin obtaining germination and development at the six days and confirming the gene segregation (Figure 2d).

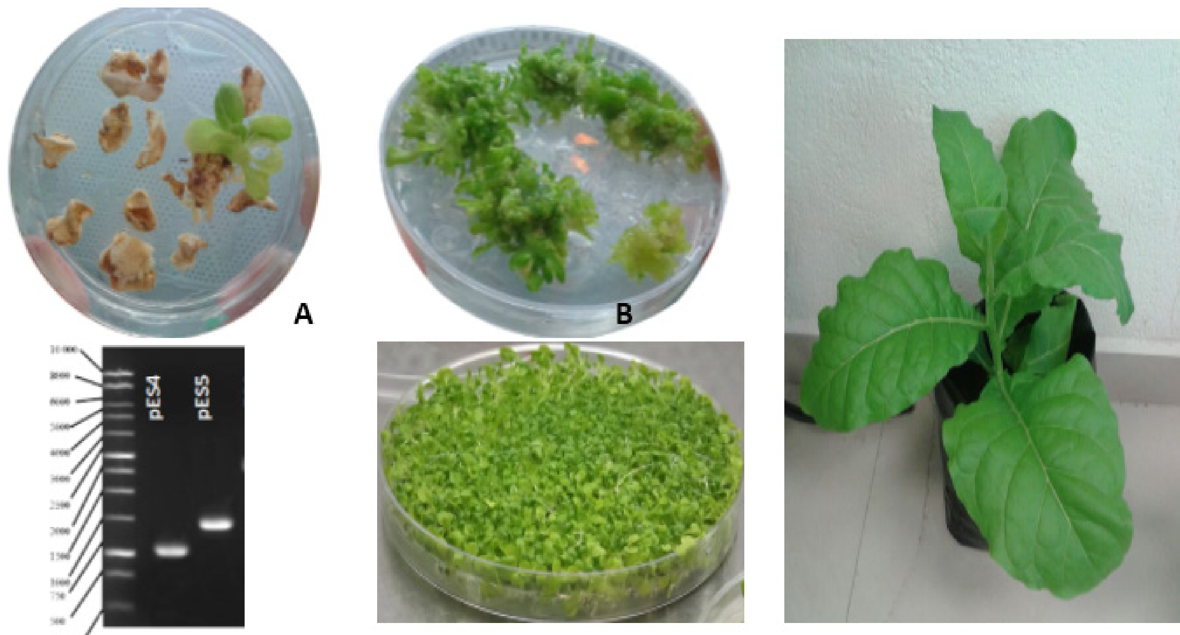

Figure 2. *A*) Regenerating shoots of tobacco after bombardment (5 weeks). *B*) Third selection round in RMOP medium supplemented with Spectinomycin 500 mg L^{-1}. *C*) PCR analysis of pES4 and pES5 vectors amplified 804 pb (*Pel A*) and 1115 pb (*MnP-2*), *D*) Shoot from seed of transplastomic plants. *E*) Transplastomic plant of two months in greenhouse.

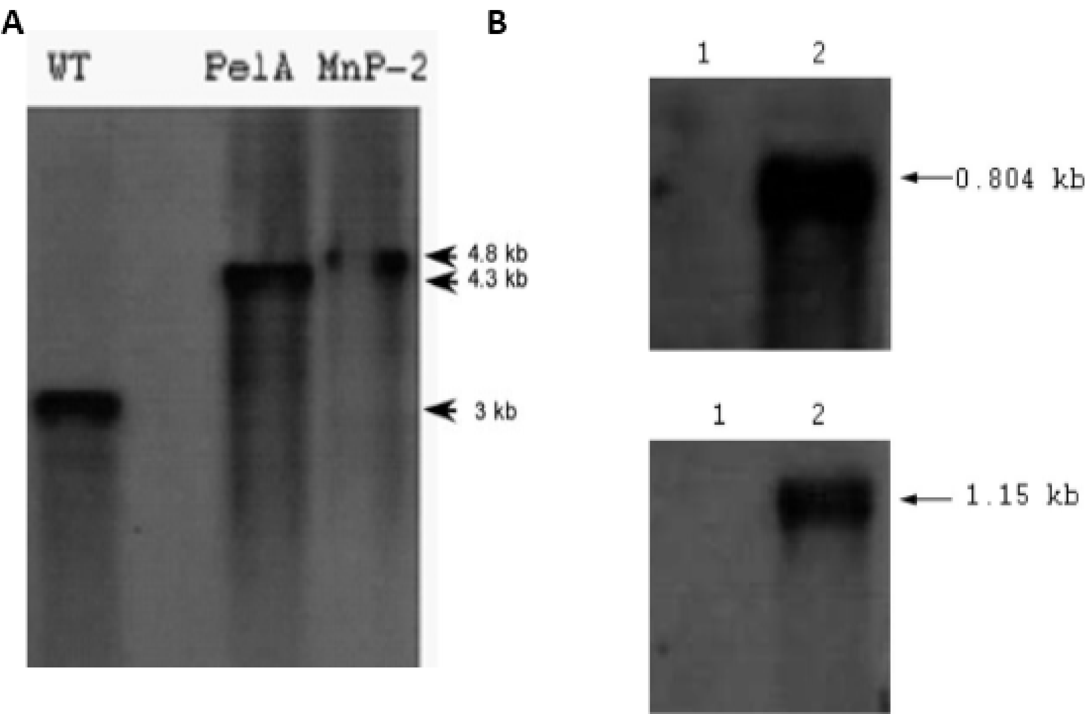

Figure 3. *A*) Southern blot analysis of pES4 and pES5 show efficient chloroplast genome transformation; to *Pel A* gene the probe showed a 4.3 kb fragment while to *MnP-2* gene the probe showed 4.8 kb. *B, C*) Northern blot analysis shown transcription of mRNA in both constructions '*Pel A* gene: 0.804 kb and *MnP-2* 1.15 kb' (*1: wild type, 2: transplastomic plan*t) according at the interest genes weight.

Analysis of protein expression. To date has confirmed stable integration of heterologous DNA in chloroplast genome, is currently working on the analysis of total soluble protein; also is pending enzymatic analysis to confirm the activity levels of the proteins produced from the transgenes and a analysis to verify segregation of heterologous genes in seeds.

Conclusion: The chloroplast genetic transformation can be used of efficient form to over expression of hydrolytic enzymes of cell-wall expressing functional proteins with high levels of expression. This evidence contribute with new information to realize the genetic improvement in others expression system with these enzymes that impacting on the agricultural sector and biofuel.

Acknowledgments: We would like to thank Dr. Pal Maliga, Waksman Institute, Rutgers University, USA for providing pPRV111 plasmid. Also a special thanks to CONACYT for funding for the development of this project.

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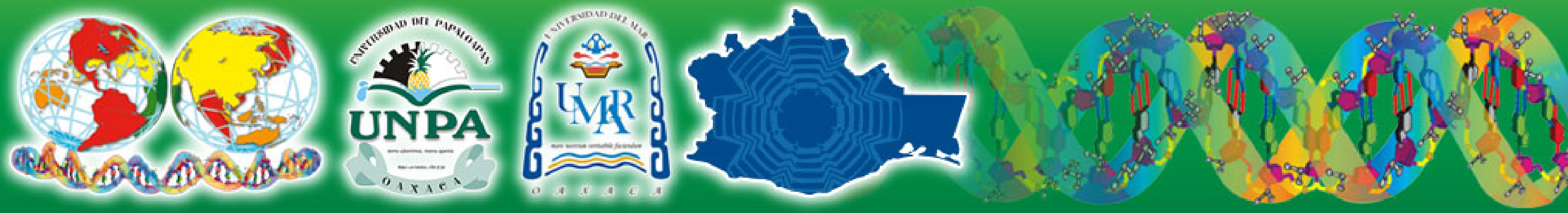

Design of short alpha-helical peptides and their activity against pathogenic bacteria *Mycobacterium tuberculosis*

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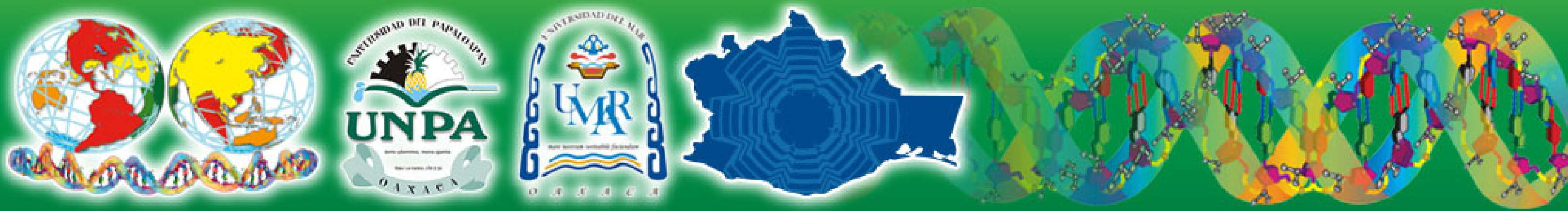
Abstract: The contention of multidrug-resistant bacteria requires the use of new antibiotics. Pandinin 2 (Pin2) is a highly hemolytic antimicrobial peptide that has a central proline residue. Proline forms a structural ‘‘kink’’ linked to its pore-forming activity towards human erythrocytes. In this work, the residue Pro14 of Pin2 was both substituted and flanked by Glycine residues according to the low hemolytic activities of antimicrobial peptides as Magainins and Ponericins, respectively. Both Pin2 variants showed antimicrobial activity against *E. coli*, *S. aureus*, and *M. tuberculosis*. However, Pin2 [GPG] was 30% less hemolytic. To avoid the drawback associated to the cost of synthesis of large peptides, two short peptides were designed and synthesized based on Pin2 [G] and Pin2 [GPG]. Both Pin2 [14] and Pin2 [17] short variants, showed antibiotic activities against *E. coli* and *M. tuberculosis.* Besides, Pin2 [14] presented only 25% of hemolysis at 100 µM, while the peptide Pin2 [17] did not show any hemolytic effect at the same concentration. Furthermore, these short antimicrobial peptides showed better activity at molar concentrations against multidrug resistant *M. tuberculosis* respect to conventional antibiotics. Pin2 [14] and Pin2 [17] have the potential to be used as alternative antibiotics with reduced hemolytic effects.

Keywords: antimicrobial peptides ● *Mycobacterium tuberculosis* ● hemolytic activities

Introduction: Cationic Antimicrobial Peptides (CAMPs) are components of the biological defense system of microorganisms, plants, animals and humans (Bulet *et al*., 2004). CAMPs with alpha-helical conformation share some common characteristics such as broad-spectrum antimicrobial activities at low micromolar concentrations and alpha-helix conformation in hydrophobic environments (Nguyen *et al*., 2011). They have potent antibacterial activities that made them promissory candidates to develop novel antibiotics towards multidrug resistant pathogenic bacteria, as well as towards clinically important yeasts such as *Candida albicans* (Park *et al*., 2011; Yeung *et al*., 2011). Pin2 is a 24-residue alpha-helical antimicrobial peptide characterized from the venom of the African scorpion *Pandinus imperator*, this peptide has broad spectrum antimicrobial activities in the micromolar range; however, it shows hemolytic activity at similar concentrations (Corzo *et al*., 2001).

Pin2 shows an alpha-helical with a central proline *“kink”* structure. The proline *‘‘kink’’* is a structural characteristic of some CAMPs, such as Alamethicin, Melittin and Pardaxin, that confer them high pore-forming abilities but also a high hemolytic activities (Dathe *et al*., 1998; Dempsey *et al*., 1991). On the other hand, CAMPs such as Magainin 2, Oxypinin 2b and Ponericin G1, showed antimicrobial activities with low cytotoxic effects towards erythrocytes (Corzo *et al*., 2002; Orivel



et al., 2001; Zasloff, 1987). These peptides have different amino acid motifs in the central region of their primary structures. For example, Magainin 2 has a single glycine in the middle of its structure, Oxypinin 2b (Oxki2b) has a GlyValGly motif, and Ponericin G has glycine residues flanking the central proline, a GlyProGly motif. Likewise, the substitution of the proline residue (P14) in Pin2 for the residues Val, GlyVal, ValGly or GlyValGly reduced the hemolytic activity of Pin2 without any significant changes in its antimicrobial activity (Rodriguez *et al*., 2011). In this work, based on the low hemolytic activities shown by the antimicrobial peptides Magainin 2 and Ponericin G1, two synthetic variants of Pin2, Pin2 [G] and Pin2 [GPG] were chemically synthesized with the aim to reduce the hemolytic activity and preserve the antibiotic activities of Pin2. In addition, two short variants of Pin2, with 14 and 17 residues, respectively, were designed and chemically synthesized with the aim to continue reducing their hemolytic but keeping their antimicrobial activities as well as to reduce the number of residues to have a low cost CAMP. Here these antimicrobial peptide variants are proposed as potential antibiotics for the clinical treatment of pathogenic bacteria including *Mycobacterium tuberculosis*.

Materials and Methods:

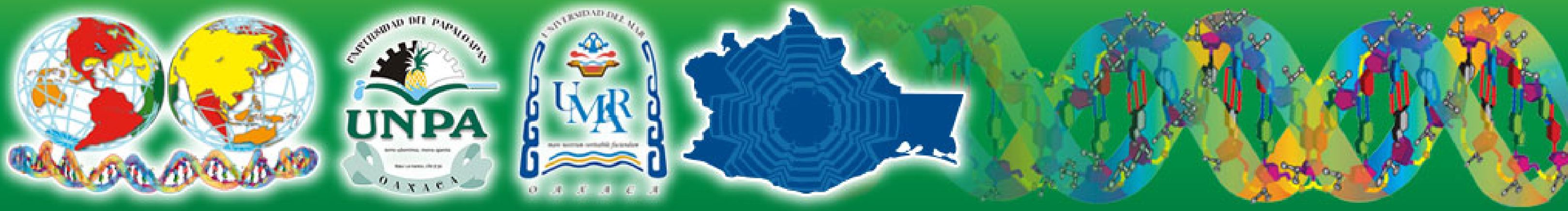
Bacterial strains and peptides. The bacterial strains used in this report were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and *M. tuberculosis* multidrug resistant strain (MDR). Pin2 and its variants were chemically synthesized by solid phase method using the Fmoc methodology. Each crude synthetic peptide was then purified by reverse phase HPLC (RP-HPLC) on an analytical C18 column. The final purity of the peptides was higher than 95%.

Antimicrobial assays. Minimal inhibitory concentrations (MIC) and bacterial growth inhibition curves were obtained using pure peptides, at different concentrations, in the presence of bacteria using two different methods, agar diffusion susceptibility assays and broth microdilution assays in accordance to the procedures from the Clinical and Laboratory Standards Institute (CLSI, http://www.clsi.org).

Mycobacterium tuberculosis assays. The Resazurin microtitre assay plate (REMA) method was conducted to determine the *M. tuberculosis* susceptibility to the action of Pin2 and the variants studied in this work. Resazurin is an oxidation–reduction indicator and has been used to assess viability, bacterial contamination and to test for antimicrobial activity. The *M. tuberculosis* H37Rv (ATCC 27294) and a clinically isolated multidrug resistant (MDR) strain were used in these experiments.

Hemolytic assays. Hemolytic activity was determined by incubating suspensions of human red blood cells with serial dilutions of each selected peptides. Red blood cells were counted by a hemocytometer and adjusted to $7.7x10^6$ cells mL^{-1}. Red blood cells were then incubated at room temperature for 1 h in 10% Triton X-100 (positive control), in PBS (blank), or with amphipathic peptides at concentrations of 0.4, 0.8, 1.6, 3.1, 6.2, 12.5 and 25 µM, only for Pin2 [14] and Pin2 [17] the 50 and 100 µM concentrations were evaluated.

Circular Dichroism (CD) measurements. The CD experiments were recorded on a Jasco model J-720 spectropolarimeter (Tokyo, Japan). The different spectra were measured from 260 to 190 nm on samples in water, 20, 40 and 60% trifluoroethanol (TFE), at room temperature, with a 1-



mm pathlength cell. Data were collected at 1 nm with a scan rate of 100 nm min^{-1} and a time constant of 0.5 s. The concentration of each peptide was 150 µg mL^{-1} Data were the average of five separate recordings and were analyzed on line by the software K2d.

Results and Discussion:

Solid phase antimicrobial activities. The antimicrobial activity of Pin2 and its variants were tested in solid phase against *S. aureus* and *E. coli*. Pin2 [G] variant displayed the highest antimicrobial activity with a MIC value of 12.5 µM, towards both *S. aureus* and *E. coli*. Both parental Pin2 and Pin2 [GPG] showed MIC values of 37.5 and 25 µM for *S. aureus*, and 18.8 and 25 µM for *E. coli*, respectively. However, the short variants Pin2 [14] and Pin2 [17] had MIC values >300 and 80 µM towards *S. aureus*, respectively, but they both Pin2 [14] and Pin2 [17] were more active towards *E. coli* with MIC values of 25 µM.

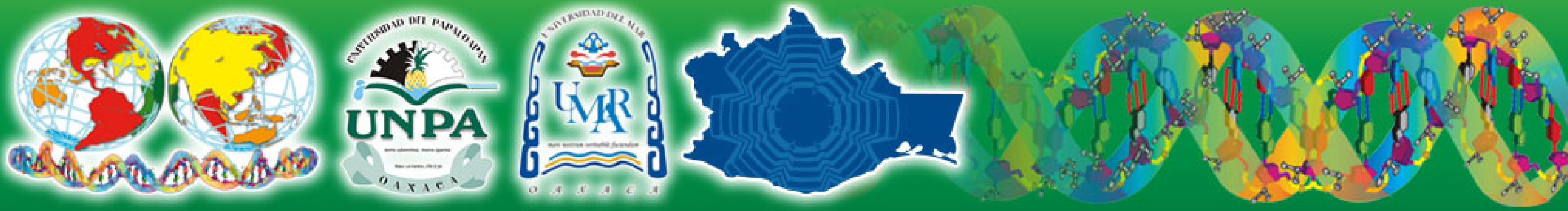
Broth culture antimicrobial activities. The bacterial growth of both *S. aureus* and *E. coli* was observed in the presence of serial concentrations of all Pin2 variants from 25 to 0.4 µM. Most of the synthetic peptides showed bactericidal or bacteriostatic antimicrobial activity against the two strains with MIC values of 12.5 and 25 µM. Pin2 [G] and Pin2 [GPG] showed MIC values of 12.5 µM for both *E. coli* and *S. aureus*. The short variant Pin2 [14] had a MIC value of 25 µM against *E. coli* but a bacteriostatic effect at 25 µM against *S. aureus*. Finally, the short variant Pin2 [17] only showed bacteriostatic effect on *E. coli* at 25 µM, and no antimicrobial activity towards *S. aureus* was observed (Table 1).

Table 1. Antimicrobial and hemolytic activities of Pin2 and the Pin2 variants.

		Minimum Inhibitory Concentrations (µM)		
Peptide name	**Assay**	***S. aureus***	***E. coli***	**Hemolysis (IC_{50})‡**
Pin2	MHA	37.5	18.8	3.3 [1.9-5.7]
	MHB	12.5	12.5	
Pin2 [G]	MHA	12.5	12.5	1.4 [0.4-4.3]
	MHB	6.25	12.5	
Pin2 [GPG]	MHA	25	25	46.6 [34-64]*
	MHB	25	25	
Pin2 [14]	MHA	>300	25	418.4 [291-602]*
	MHB	50	25	
Pin2 [17]	MHA	80	25	ND§
	MHB	NA	>25†	

MHA, Mueller-Hinton Agar; MHB: Mueller Hinton Broth; ‡ Mean and 95% confidence intervals, values expressed in µM; *The numeric IC_{50} value was obtained from the Boltzmann sigmoid equation fit; † Bacteriostatic effect; NA Non Activity observed; § Any hemolytic effect at 100 µM was observed because of that the value of IC50 did not calculate.

***M. tuberculosis* activity.** The antibiotic capacities of Pin2 variants were tested in two strains of *M. tuberculosis* using the REMA methodology. All synthetic peptide variants showed antimicrobial activities over both *M. tuberculosis* strains (Table 2). The MIC values observed were from 11 to 30 µM for the *M. tuberculosis* H37Rv and from 6 to 33 µM for *M. tuberculosis* MDR. It was interesting to observe that the two short variants Pin2 [14] and Pin2 [17] had MIC values of 11.9



and 11.6 µM towards *M. tuberculosis* H37Rv and MIC values of 6 and 14.8 µM against *M. tuberculosis* MDR, respectively.

Table 2. Antimicrobial activity of Pin2 and the Pin2 variants on two *M. tuberculosis* strains.

		***Mycobacterium tuberculosis* H37Rv**		***Mycobacterium tuberculosis* MDR***	
Peptide name	**MW (Da)**	**MIC (µg mL^{-1})**	**MIC (µM)**	**MIC (µg mL^{-1})**	**MIC (µM)**
Pin2	2612.1	57.7±22.3	22.1±8.6	86.5	33.1
Pin2 [G]	2572.0	48.1	18.7	48.1	18.7
Pin2 [GPG]	2762.2	80.1±24.8	29±9	48.1	17.4
Pin2 [14]	1680.1	20 ± 6.2	11.92±3.7	10± 3.1	6±1.8
Pin2 [17]	1891.3	22 ± 4.9	11.65±2.59	28.04 ± 9.814	14.8±5.2
Ethambutol§	204.3	0.5	2.5	20	97
Isoniazid	137.1	24 ±8.8	175.1±63.9	6 ±2.2	43.8±16
Rifampicin§	823.0	0.4	0.5	32	38.9

The MIC values were calculated using the Resazurin dye reduction method, 500,000 bacteria per well were evaluated; §, MIC values reported by Rastogi, et al., (1996); *, Clinically isolated strain characterized with resistance to rifampicin and isoniazid in UIMZ-IMSS, Zacatecas, Mexico.

***Hemolytic activity of Pin2 variants*.** The hemolytic assays of Pin2 and the Pin2 variants on human erythrocytes (Figure 1) showed that the parental peptides Pin2 as the Pin2 [G] variant at 25 µM had the highest hemolytic activities of all five peptides evaluated, with a 91% and a 100% of hemolysis, respectively. However at the same concentration the Pin2 [GPG] variant showed only a 30% of hemolysis, indicating that the insertion of the GPG motif is relevant for hemolytic activity reduction. The two short variants had not hemolytic effects at 25 µM. That is, they were assayed up to 100 µM observing that the peptide Pin2 [14] showed only 25% of hemolysis, while Pin2 [17] did not show any hemolytic effect at such concentration.

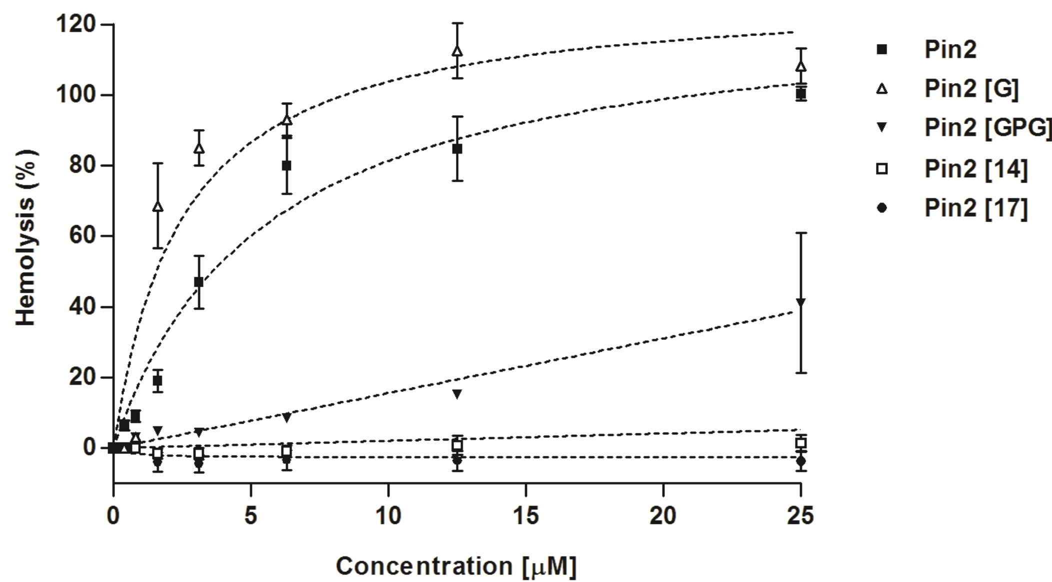


Figure 1. Hemolytic activity in human red blood cells. Data are the average of at least four independent experiments. Error bars represent the standard deviations.

Circular dichroism secondary structure analysis. The CD spectrum of Pin2 was compared to the CD spectra of the Pin2 [G] and Pin2 [GPG] variants. The secondary structure analysis was performed using CD spectral data from 190 to 260 nm, in the presence of 60% aqueous TFE, the CD spectra of Pin2 [G] and Pin2 [GPG] showed a clear ordered structure with two minimum ellipticity values at 208 and 222 nm (Figure 2A), indicating an alpha-helix conformation. In order to obtain more information concerning the propensity of these peptides to adopt alpha-helical structures, their CD spectra at different TFE proportions (0, 20, 40 and 60%) was acquired. The parental peptide Pin2 (Figure 2B) and the Pin2 [GPG] (Figure 2D) variant showed a clear ‘‘random coil’’ CD profile in the absence of TFE, but interestingly, the Pin2 [G] variant showed a 20% of alpha-helical structure in the absence of TFE (Figure 3C), suggesting a more structured Pin2 [G]. Pin2 [G] and Pin2 [GPG] adopted a maximum of 60% of alpha-helical structure at 40% TFE. Similarly, the CD spectra of the short variants Pin2 [14] and Pin2 [17] were conducted at 0, 20, 40 and 60% TFE (Figures 2E and 2F). In the absence of TFE both Pin2 [14] and Pin2 [17] had clear positive ellipticities around 220 nm, and the increment in the TFE proportion induced a reduction in their ellipticity values. Furthermore, in the absence of TFE Pin2 [14] showed a strong negative ellipticity at 205 nm, and the increase of TFE enhanced its CD ellipticity value. Similar behavior was observed for Pin2 [17]. The CD deconvolution for Pin2 [14] and Pin2 [17] resulted in unstructured peptides even at the highest TFE concentration of 60%.

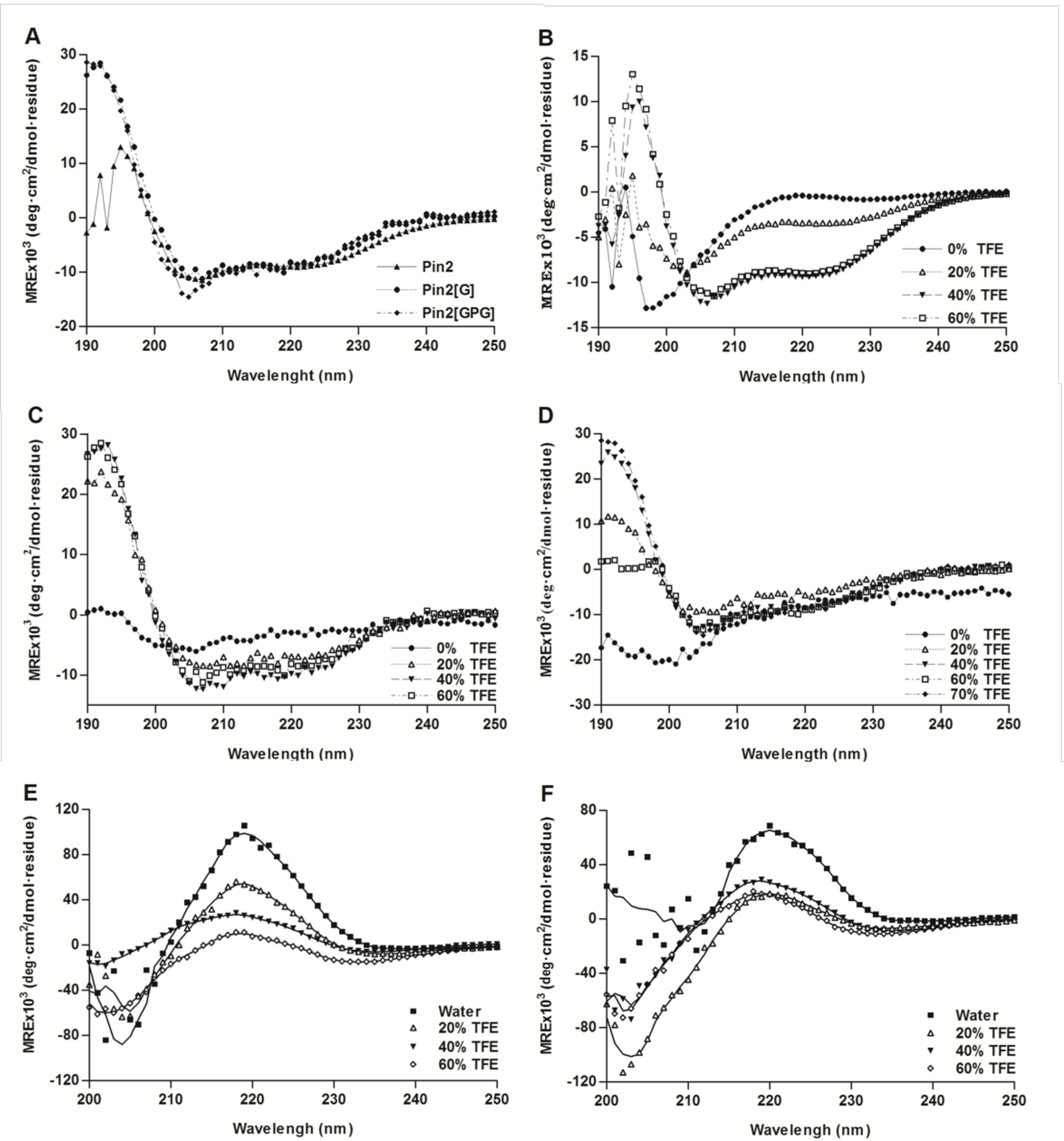


Figure 2. Circular dichroism Pin2 and its variants. A. Circular dichroism spectra of Pin2 and its variants in 60% TFE, B. Pin2 TFE alpha-helix induction kinetics, C. Pin2 [G] TFE alpha-helix induction kinetics, D. Pin2 [GPG] TFE alpha-helix induction kinetics, Pin2 [14] TFE alpha-helix induction kinetics and Pin2 [17] TFE alpha-helix induction kinetics.

The differences among the antimicrobial activities of the different peptides reported here are first related to the length of to peptides, as was observed by Deslouches *et al*. (2005), in which peptides with short sequences display lower antimicrobial activities, observing that for their study a 24 amino acid length is optimal for antimicrobial activity. Respect to the selectivity, in the same study

short peptides showed better activity towards Gram negative bacteria, indicating an effect of the thickness of the cell wall of Gram positive bacteria. As was observed here, short peptides displayed lower antimicrobial activities towards Gram positive *S. aureus*. In other study, Liu *et al*. (2007) observed that the antimicrobial activity of a peptide series was increasing according to length of the peptides observing the lower MIC values with peptides with larger peptides. Here, the same profile is observed in both solid phase and broth culture assays. The lower MIC values observed in the solid phase respect to the broth culture assay could be related to a limited diffusion through the agar media primarily because of the hydrophobic nature of the peptides. Similar results were observed for Nisin an antimicrobial peptide produced by *Lactococcus lactis* (Chandrapati and O'Sullivan, 1998).
Respect to the antimicrobial activity towards *M. tuberculosis*, better molar antimicrobial activities were observed with the short variants, respect to the other CAMPs and conventional antibiotics evaluated here, especially with the MDR strain. These short peptides could be evaluated in combination with commercial antibiotics as Rifampicin to increase the affectivity of these antibiotics over clinically important Mycobacterium strains, in especial those with antibiotic resistance (Khara *et al*., 2014). The antimicrobial potency and hemolytic profile of CAMPs are closely related to the alpha-helical structure (Jiang *et al*., 2014; Ramón-García *et al*., 2013). So that, the peptides with higher helical profiles have lower MIC values but also they become more hemolytic. The hemolysis reduction observed in the Pin2 [GPG], Pin2 [14] and Pin2 [17], could be related to the DC profiles observed here. Pin 2 [G] has a higher helical profile respect to the other peptides and it is more hemolytic. While the short peptides, they do not show an alpha-helical profile, displaying low or none hemolytic effects.

Conclusions: Here we based the design of low hemolytic and short antimicrobial peptides on observed patterns in nature and on theoretical calculations. We found a strong correlation in hydrophobicity and alpha-structured molecules with high hemolytic activity; however, the antimicrobial capacity could be sustained with low eukaryotic lytic activities in short hydrophilic antimicrobial peptides such that Pin2 [14] and Pin2 [17] with a plus of maintaining a wider antimicrobial spectrum; that is affecting Gram-positive and Gram-negative bacteria as well as multi-drug resistant *Mycobacterium tuberculosis*.

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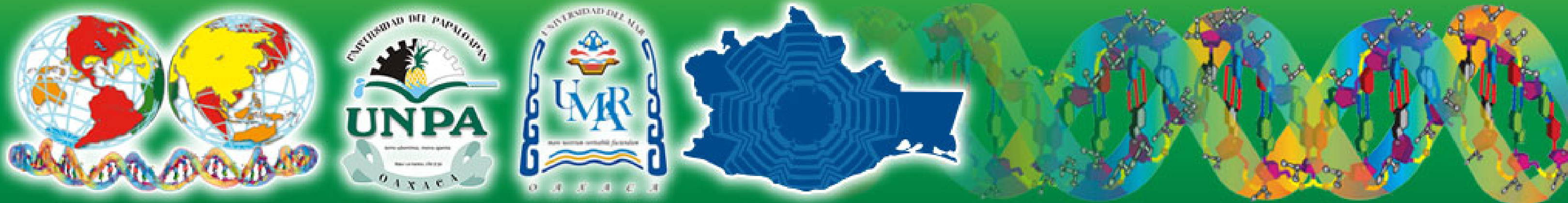

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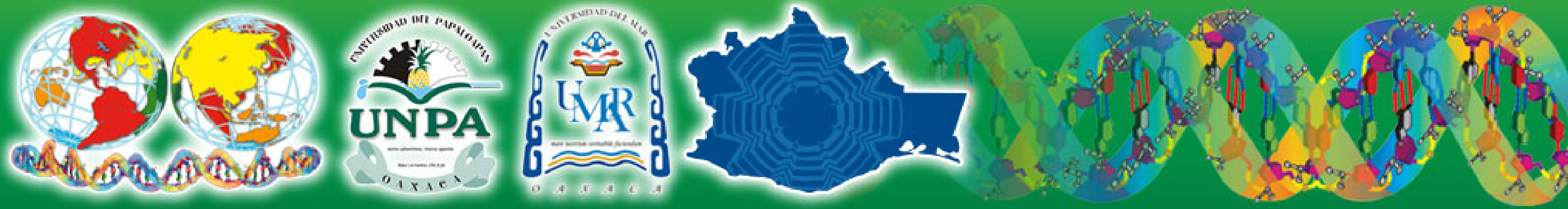

Use of biotechnology in controlling preharvest aflatoxin contamination, a major agricultural problem

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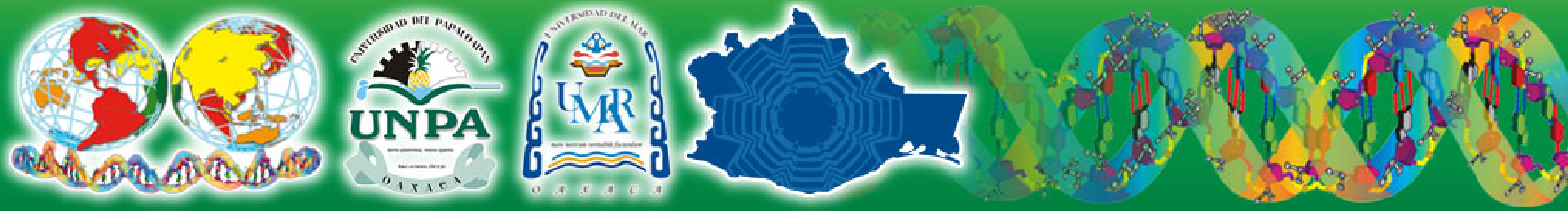
Abstract: Aflatoxin is a human carcinogen and acutely toxic to humans, pets, livestock, and wildlife. It is produced by the fungus *Aspergillus flavus* prior to harvest when it invades crops such as maize, peanuts, treenuts, and cottonseed, thereby greatly reducing their value and marketability. This contamination is a worldwide problem, especially in the warmer parts of the world. Researchers from around the world have attempted to control this contamination using traditional methods such as crop management and breeding resistant crops; these approaches have found limited success. The use of biotechnology has enabled researchers worldwide to decipher and find solutions much more rapidly too many health and agriculture problems. These technologies are now being applied to understand the aflatoxin contamination problem, and to develop strategies to control this significant agricultural issue. The methods used are the study of the genomics of the fungus, proteomics of the crops, metabolomics and transcriptomics to decipher the host-plant fungal interactions.

Keywords: Plant-*Aspergillus flavus* interaction ● biological control ● genetically engineering host

Introduction: Fungally produced mycotoxins such as the aflatoxins, trichothecenes, and fumonisins account annually for hundreds of millions of dollar equivalents (internationally) of crop losses, as well as loss of health and human life across the world, thus affecting world health, trade and food security on a global basis (Ehrlich *et al*., 2011a; Bhatnagar *et al*., 2002). Also of concern is the connection between a lack of food security and social unrest across the world. Thirty countries experienced food-related riots in 2008, half in Africa. The severe loss of life (over 130 deaths reported) in Kenya as recently as 2004, due to acute toxicity from consumption of aflatoxin contaminated maize, highlights the health implications of mycotoxin contamination in developing countries, where regulations are non-existent or not applied, and where clean, uncontaminated corn is at a premium. Elimination of mycotoxin concerns through application of innovative research solutions using biotechnology (genomics, proteomics, transcriptomics and metabolomics) could prevent food losses, unsafe food grains, and losses in human and animal life as threats to world food security (Bhatnagar *et al*., 2008).

Aflatoxins are natural poisons produced predominantly by two common fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. *A. flavus* is most commonly found in agricultural fields, and when it invades crops like maize, peanuts, cotton, and tree nuts, it produces aflatoxins whenever the environmental conditions permit. Chronic, as well as sporadic, aflatoxin contamination in a variety of field crops and agricultural commodities worldwide has had a serious impact on the economics and food safety of these products. The number of deaths in Indonesia due to aflatoxin-induced liver cancer is estimated at 20,000 year^{-1} (reviewed in Bhatnagar *et al*., 1994). The short-term toxicity of aflatoxins and the chronic exposure of humans to these compounds in foods leading to liver cancer or death have been well established. Aflatoxins, furanocoumarin compounds, are the most widely studied of all mycotoxins because of their potent toxicity and carcinogenicity. And, the most significant research progress towards controlling mycotoxins has been made with aflatoxins.



Over one hundred countries are known to have regulations limiting mycotoxin levels with most having some specific regulatory levels for total aflatoxins in foodstuffs, and a few dozen having regulations for aflatoxins in feedstuffs. The Food and Drug Administration in the United States has set limits of 20 ng g^{-1} total aflatoxins for interstate commerce of food and feed and 0.5 ng g^{-1} of aflatoxin M_1 in milk.
The limits on aflatoxin contamination can result in severe economic losses, *i.e.*, greater than $250,000,000 in direct losses to farmers (Richard and Payne, 2003). FDA economists estimated the annual cost of aflatoxin contamination in the United States at ~ $500 million through two categories of loss, market rejection and animal health impacts. It has been suggested by Wu (2004) that the total economic impact of aflatoxins should also include many other factors, *e.g.*, export market losses, sampling and testing costs, costs to food processors, grocery markets and consumers, and human health effects. Therefore, it is imperative for researchers worldwide to develop strategies using modern technologies for effective control of aflatoxin contamination of crops. Strategies to minimize or control aflatoxin contamination will economically benefit the agricultural industry in developed countries, where regulatory agencies follow strict guidelines against the sale of contaminated commodities, but also will increase food safety for populations in developing countries where such regulations, if they exist, may not be strictly enforced.

Three lines of defense against toxin contamination. Researchers trying to eliminate preharvest aflatoxin contamination are basically engaged in developing three lines of defense that will work together in concert. Sincet there is not "magic bullet" for solving the aflatoxin contamination problem, so several strategies must be utilized simultaneously to ensure a healthy crop, free of aflatoxins. The first line of defense attempts to keep the toxigenic fungus from reaching the crop. This goal is being achieved by biological control (preferably with native, atoxigenic strains of *A. flavus*), and effective, targeted cultural management practices. The second line of defense occurs if the toxigenic fungus does reach the crop. Attempts are being made to prevent the fungus specifically from growing in the seed. Strategies to achieve this goal include enhancing host resistance either by breeding for resistance or by genetically engineering host/non-host resistance traits into the crops affected by preharvest toxin contamination. The third line of defense is activated if the fungus grows into the seed. Once the fungus has colonized the seed, it is undesirable for the fungus to make toxins in the seed, *e.g.*, in the embryo in the case of maize. Once again, preventing the fungus from making the toxin can be achieved by enhancing host resistance and targeting factors that inhibit toxin synthesis.

Results and Discussion:

A more complete understanding of how secondary metabolism in the aflatoxin-producing fungus is affected during plant contamination is very important if effective and permanent strategies for control of aflatoxin contamination are to be developed. *Aspergillus flavus* invades crops as diverse as maize, cottonseed, peanuts and tree nuts, and has been isolated from soils across the globe. The common feature in aflatoxin contamination is the fungus. Therefore, to understand the preharvest aflatoxin contamination process, it is important to understand the genetic make-up and the gene expression profile of the fungus under various environmental conditions, during host-fungal interaction as well as during aflatoxin production. Identifying plant factors that prevent or enhance toxin production when the fungus invades the crop is also essential for finding solutions to the aflatoxin contamination problem.

***Aspergillus flavus* genomics.** After the discovery of all the genes involved in aflatoxin biosynthesis and the elaboration of the gene cluster (Yu *et al*., 2004; Bhatnagar *et al*., 2006), *A. flavus* genomics was carried out and aimed at understanding the genetic control and regulation of toxin production in response to environmental influences on the fungus, such as temperature, water stress, pH, and soil micronutrients and microflora, as well as the nutrition status of the susceptible crop (Payne *et al*., 2011; Yu *et al*., 2011a; Ehrlich *et al*., 2011b). Genomics can help in understanding the ability of *A. flavus* to adapt to inhibitory plant defenses by examining changes in gene expression profiles that affect signal transduction within the fungus upon contact and invasion of the plant. Results from comparing the whole genome sequences show that the genome of *A. flavus* (36.3 Mb) is larger than that of *A. nidulans* (30.1 Mb) or *A. fumigatus* (29.4 Mb) and thus capable of producing a more complex pattern of secondary metabolites. The expanded genome of *A. flavus* over other *Aspergillus* species is support for the hypothesis that adaption to growing in complex environments requires increased availability of secondary metabolite biosynthesis genes. Functional genomics has helped to increase understanding of fungal adaptations to the host plant that are required for invasion and secondary metabolite biosynthesis. Metabolic profiling along with structural and functional genomics is providing significant information on the fungal responses to various alterations in its ecology; as well as information on the numerous secondary metabolic pathways that have been discovered from genomic studies (Ehrlich *et al*., 2011b,c).

There are strong connections between environmental conditions and occurrence of aflatoxins in world crops. Apparent global climate changes, particularly warming, are implicated in promoting toxin contamination increases in certain regions of the world (Wu *et al*., 2011). Not enough is known about the effects of environmental stresses on toxin contamination in world crops or in predicting environmental conditions conducive to outbreaks of mycotoxin contamination of crops. The most convincing body of research suggests that environmental stress such as high night time heat and drought promote elevated levels of aflatoxin in corn. On a fungal genomic level, data has indicated an oxidative stress pathway existing in the aflatoxin producing fungus, *Aspergillus flavus*, which may be triggering aflatoxin biosynthesis in crop tissues subjected to heat stress. Gene expression in the fungus is also up-regulated with incremental increase in temperature (Yu *et al*., 2011b).

Enhancing Host-Plant Resistance. Recent research through proteomics demonstrating the correlation of elevated levels of gene activities encoding proteins in corn kernels implicated in heat and oxidative stress tolerance and lower levels of aflatoxin accumulation implies the intriguing possibility of breeding stress tolerant crops to reduce mycotoxin accumulation, as well as further suggesting the need to address climate change truly as a negative factor in crop production, quality and safety, particularly targeting mycotoxins as a safety threat to human populations on a global basis (Chen *et al*., 2014).

Further corroborating evidence for the role of plant stress in aflatoxin outbreaks is the finding that corn lines bred in collaboration with ARS by the Institute for International Tropical Agriculture, Ibadan, Nigeria, for resistance to ear rot and aflatoxin accumulation, expressed much elevated levels of stress protective proteins in kernels of resistant inbreds. These inbred lines, bred from crosses between resistant U.S. and resistant African parents, to address the aflatoxin problem in both the U.S. and Africa, have been released this year (Brown *et al*., 2013).

Conclusion: Understanding the complex inter-relationships of plant and fungal gene products during the host plant -*A. flavus* interaction is the key to developing strategies to interrupt the aflatoxin contamination process through enhancing host-plant resistance. *A. flavus* genomics and proteomics of seed-based resistance have provided the best investigative tools for simultaneous discovery and analysis of the biochemical function and genetic regulation of the critical genes governing fungal development, plant fungal interaction and aflatoxin biosynthesis.

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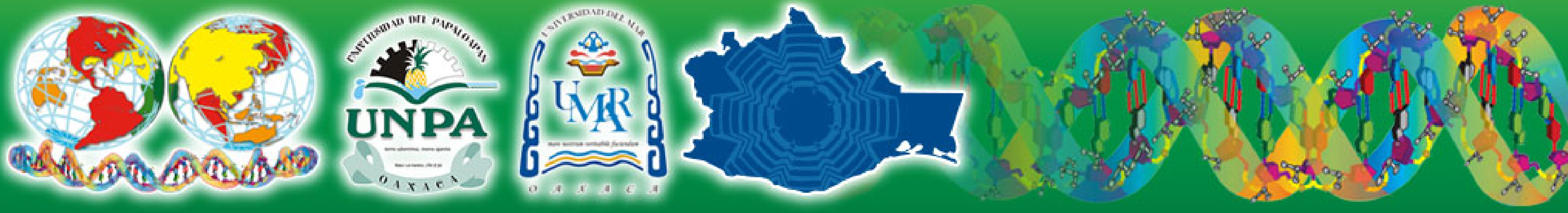

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Effect of alkaline treatment for inactivation of *Salmonella* sp. and coliphages in wastewater sludge

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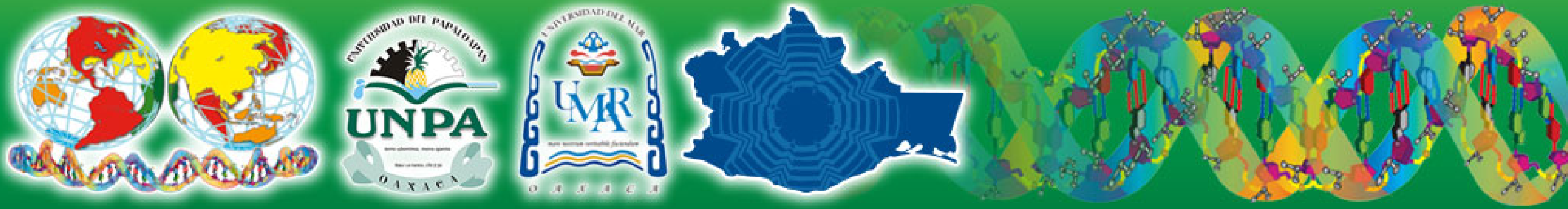
Abstract: The wastewater treatment plants can produce organic matter from its primary treatment. This organic matter is derived from organic household and industrial waste such as food industry. The organic matter is separated by flocculation, in order to avoid the saturation of biological treatments (secondary treatment). This organic material is known as sewage or wastewater sludge. Generally, there is not application or use for the sludge, they are disposed to landfills or incineration. The wastewater sludge is a good source of nutrients, their high content of carbon, nitrogen, phosphorus, sulphur and diverse materials or trace elements that can be beneficial for the plants. The organic matter applied to degraded or depleted soils can help to recover or restore the fertility, structure and physicochemical properties of the soils. Therefore, the wastewater sludge can be a soil conditioner and a nutrient source for plants. However, one disadvantage of these is the high counts of the pathogens and heavy metals, depending on the origin of wastes, for a reason the application of wastewater sludge to soil is limited. The objective of this research is take advantage of the organic matter derived from wastewater sludge in a sustainable way. They can be useful as nutrient source for the plants or improving soil properties through of the microbiological stabilization in an economical way. In addition, we promote culture to reuse or recycle of the wastes making them a valuable by-product for the organic and conservation agriculture, in sustainable way.

Keywords: Sewage sludge ● wastewater sludge ● alkaline treatment ● enteropathogens

Introduction: The wastewater sludge is a common wastes derived from industrial and municipal wastewater treatment, which separated by flocculation of the organic matter from wastewater by chemical agents (Dumontet *et al.,* 2011). The amount of sewage sludge and its composition depends on the characteristics of the influent and treatments used to treat wastewater (Enrica *et al.,* 2012). The sewage sludge has higher organic matter content and essential nutrients for plants that can be recycled as fertilizers or soil ameliorants. However, some disadvantages are the heavy metals content and pathogenic microorganisms that can be a limiting factor for their exploitation on the urban and agricultural applications (NOM-004 2002). There are several treatments that perform the stabilization of the wastewater sludge *i.e.* where they are subject to a treatment that gives place to a suitable by-product for its subsequent use, reducing fermentation capacity of the organic matter and pathogenic organism's content (Haubry *et al*., 1992). The alkaline treatment can reduce bacterial pathogenic organisms and viral up to 99% or more and have effect on the resistant forms like helminth ova (USEPA, 2003). The objective of this research was stabilize



microbiologically the wastewater sludge from a wastewaters plant treatment ('Reciclagua Ambiental S.A. de C.V.') in order to take advantage of use.

Materials and Methods:

The count of virus (F+ coliphages) was determined by the use of *Escherichia coli* strain (enterohemorrhagic serotype) as host in soft agar medium. The soft agar is over a first layer of nutrient agar at 37 °C for 12-16 hours (double layer technique). This method quantifies the number of lytic plate by the dilution factor that corresponds to the total coliphages in the wastewater sludge sample. The results were recorded as plaque forming units (PFU g^{-1} ST). The experiment was carried out two times per season (warm and cold).
Salmonella (NMP g^{-1} ST) quantification was done using Enrichment Broth of tetrationate (MCD Lab.). Identification was done using selective mediums and the presence of colonies was confirmed by biochemical techniques (APHA, AWWA, WPCF, 1999). A variance analysis was performed, where response variables were in function of the number of pathogen microorganisms. Data were analysed by comparison of means using a LSD test with three observations per day per treatment, with $\alpha = 0.05$. The analysis was programmed using the SAS software, version 9.0 (SAS Institute, Inc., 1989).

Results and Discussion:

The coliphages count was zero PFU g^{-1} ST on alkaline treatment, but in non-alkaline treatment was not, at the beginning of the experiment under both conditions (warm and cold seasons).The coliphages count was zero PFU g^{-1} TS on alkaline treatment, but no on non-alkaline treatment, at beginning of the experiment in both seasons (Figures 1a and 1b). The alkaline treatment was significantly different compared with non-alkaline treatment (P<0.05). However, the coliphage counts increased in alkaline treatment on cold season at fifth day (Figure 1a). On fifth day, it was observed that viral count increase due to factors as temperature and humidity in a favorable way for viruses. The pH was one of the main factor involved on coliphage counts reduction at the beginning of the experiment. The limed sewage sludge was stable during to 45 days. The environmental temperature could be related with the coliphages decrease due to 50 and 55 °C, at warm season and 33 °C, at cold season. A positive effect has been demonstrated when the temperature increase from 15 to 40 °C on the inactivation of the Type I polivirus (Straub *et al*., 1992).

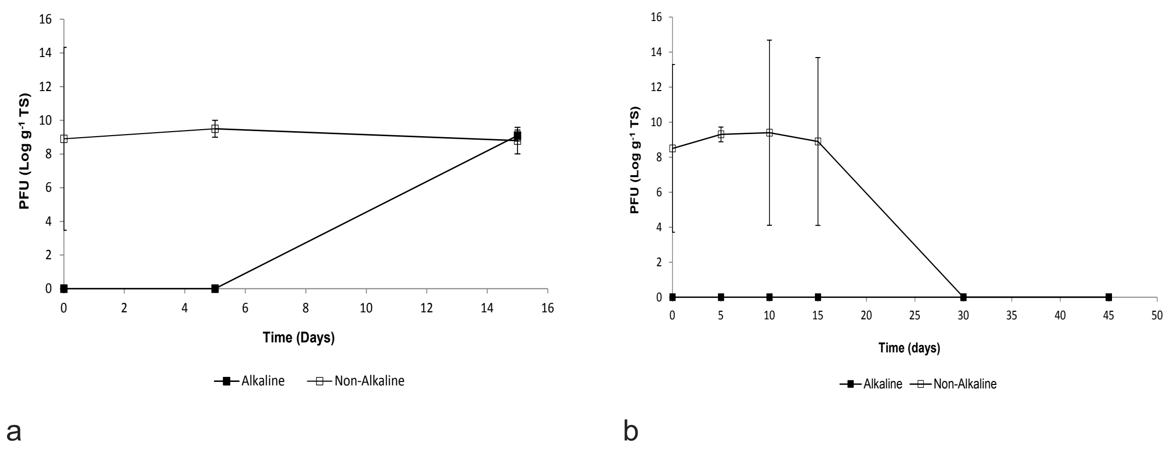


Figure 1. Inactivation of coliphages a) at cold season and b) at warm season.

The count of *Salmonella* sp. fell below the permissible limits established by the USEPA (2003), from zero time, and remained so for the first 5 days of contact. The control treatment showed no significant differences in the content of pathogenic bacteria during the period of the experiment (Figure 2a). However, this effect only persisted until the 5th day. A significant increase in the bacteria population was observed, at 15th day. Mignotte-Cadiergues *et al.* (2001) reported the elimination of *Salmonella* sp. in sewage sludge at 24 h, at doses of 15, 30 and 45% CaO, showing pH 10, 11.4 and 12 units, respectively. Inactivation of *Salmonella* sp. was achieved without presenting regrowth during the 60 days of monitoring. Where the experiment was carry out at temperatures near to 0 °C, *i.e.*, the conditions were not favorable for the bacteria growth, keeping them on dormant state, so it was not observed regrowth. Rising pathogenic account in the alkali treatment during cold weather bacteria could be due to the conditions of temperature and water content of 33 °C and 14.6%, respectively; conditions were favorable for the repopulation of bacteria. Yeager and Ward (1981) stated that re-contamination can occur when the water content of the material can reach a value of about 15%. Moreover, the use of lime at 10% also considerably affected populations of pathogenic organisms, making obtaining a sludge class "A" from day 0 and unlike the cold season, remained in the same class within 45 days of the experiment. The main factors involved on the pathogenic microorganism reduction were high pH, environmental temperature and water content of sludge.

Control treatment was observed that from day 0 to 5 the population was increasing, reaching a maximum at 10 days and from day 15 it was decreasing. Control treatment, despite being exposed to temperatures above 50 °C and a moisture content lower than 5%, no significant differences were observed on the counts of *Salmonella* sp. by day.

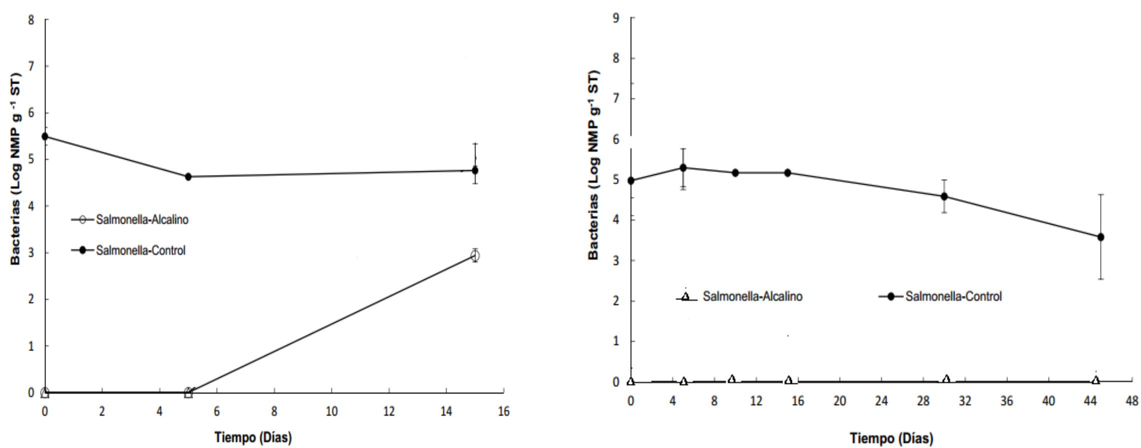


Figure 2. Counts of *Salmonella* sp. on alkaline treatment a) at cold season and b) at warm season.

The microorganisms can be protected from heat and other adverse factors because they are embedded in the matrix of the sludge (Mocé-Llivina *et al.,* 2003). Parmar *et al.* (2001) reported that raising the temperature to 50 °C decreased the populations of fecal coliforms and *Salmonella* sp. However, they were eliminated not at all, finding values of 1×10^3. The height at pH 10 by itself failed to eliminate pathogenic bacteria. They found that the joint effect between the elevation of the pH to 10 and the temperature at 50 °C on the elimination of pathogenic microorganisms. Meckes and Rhodes (2004) reported that the lime treatment plus heat (water bath at 55 °C) significantly reduced the number of fecal coliforms, *E. coli* and *Salmonella* sp. The joint action of pH, environmental temperature and water content were a key factor in reducing pathogens and the sludge stability can be classified as type "A".

Conclusions: Alkaline treatment by CaO application significantly reduces the count of coliphages and *Salmonella* sp. Factors such as environmental temperature and the water content involved in the stabilization process of sludge, allow a joint action of the stabilization. The alkali treated sludge can potentially be used as ameliorant of soil but additional studies are necessary.

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1.5 WHITE AREA

Stress resistance in *Azospirillum* strains overexpressing genes involved in poly-β-hydroxybutyrate biosynthesis

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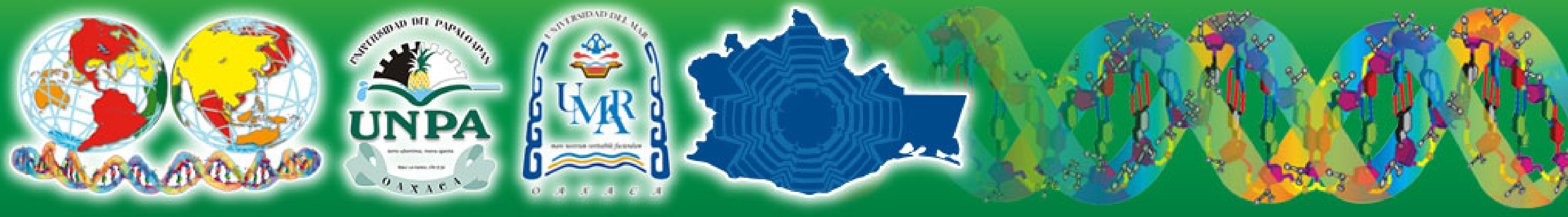
Abstract: Poly-β-hydroxybutyrate (PHB) is a biopolymer produced by bacteria when they are subjected to nutritional stress. The use of PHB under stress is a mechanism that favors their establishment, proliferation, survival, and competition. Biopolymer synthesis involves three chemical reactions by enzymes: β-ketothiolase, acetoacetyl CoA reductase and PHB synthase, encoded by *phb*A, *phb*B and *phb*C genes, respectively. The aims of this research were to quantify PHB production in *A. brasilense* Sp7 strains when *phb*A and *phb*C genes were overexpressed. Also, recent reports suggest a relationship between antioxidant activity and PHB production; therefore, antioxidant enzyme activity (SOD, CAT and APX) was quantified in cultures subjected to osmotic stress induced by NaCl. PHB quantification showed that *phb*A gene overexpression increased around 60% respect WT strain. Instead *phb*C gene overexpression did not alter PHB production compared to the WT. On the other hand, the data obtained by enzymatic quantification infer that *A. brasilense* uses another mechanism to contend with environmental stress. Such a mechanism could be related to the PHB biosynthesis, however studies are necessary to demonstrate our hypothesis.

Keywords: Poly-β-hydroxybutyrate genes ● antioxidant enzyme activity ● *Azospirillum*

Introduction: Many bacteria accumulate granules of poly-β-hydroxybutyrate (PHB). The Gram negative, α-proteobacteria *Azospirillum brasilense* Sp7 accumulates (PHB) up to 75% of the cell dry weight (Tal *et al*., 1990). PHB is synthetized under unbalanced growth conditions: an excess of carbon and a limitation on the nitrogen sources. In bacteria, PHB functions are: carbon and energy reserve, reducing power, encystment, sporulation, stress resistance and desiccation. There are three genes considered to be essential in PHB biosynthetic pathway: *phbA*, *phbB* and *phbC* that encode necessary enzymes to synthetize PHB (β-ketothiolase, acetoacetyl-CoA reductase y PHB synthase, respectively). Biopolymer synthesis begins with the condensation of two acetyl-CoA molecules by β-ketothiolase forming acetoacetyl-CoA, an acetoacetyl-CoA reductase catalyzes the conversion of acetoacetyl-CoA to β-hydroxybutyryl-CoA. Finally, the β-hydroxybutyryl-CoA is polymerized by a PHB synthase (Kadouri *et al*., 2002).

It has been suggested for diverse ecological systems that the accumulation, degradation, and utilization of polyhydroxyalcanoate (PHA) like PHB by several bacteria under stress is a mechanism that favors their establishment, proliferation, survival, and competition, especially in competitive environments where carbon and energy sources are limiting, such as those encountered in the soil and rhizosphere (Okon and Itzigsohn, 1992).



Recently studies suggest an association between PHB accumulation and high stress resistance in bacteria adapted to extreme environments (Ayub *et al*., 2004). Due to this we decided analyze stress tolerance to high NaCl concentrations (150 and 300 mM) in *A. brasilense* strains that overexpressing genes involved in poly-β-hydroxybutyrate biosynthesis. For this, antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase) were quantified.

Materials and Methods:

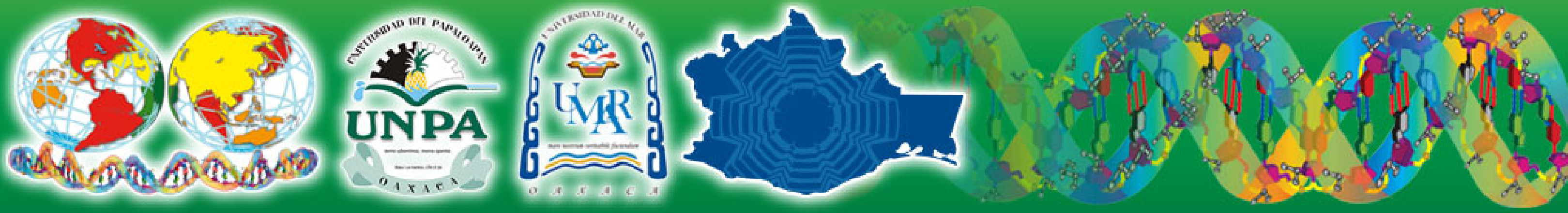
Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids are listed in Table 1. For *E. coli* growth Luria Bertani (LB) media was used. *Azospirillum* transconjugants were selected on minimal medium (MMAB).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype	Reference
A. brasilense Sp7	Wild-type strain	Nur *et al*. 1982
Ab7A	*A. brasilense* Sp7 overexpressing *phb*A gene	This work
Ab7B	*A. brasilense* Sp7 overexpressing *phb*B gene	This work
Ab7C	*A. brasilense* Sp7 overexpressing *phb*C gene	This work
Ab7-206	*A. brasilense* Sp7 harbouring pMMB206	This work
E. coli DH5α	ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-I relA1	Gibco-BRL
E. coli S17-1λpyr	Sm^r recA thi pro $hsdR^-$ λpir	Biomedal Lifescience
pMMB206	Expression vector Cm^r	Addgene
p206A	pMMB206 harbouring *phb*A gene	This work
p206B	pMMB206 harbouring *phb*B gene	This work

Cloning. PCR-amplified *phb*A and *phb*C genes were cloned into the broad-host-range expression vector pMMB206. The *phb*A gene was PCR-amplified by using the primers phbA-FEco (5′-AGGAATTCAATGACCTCGGCGCCTTTGACC-3′), with an *Eco* RI site (underlined) located upstream of the start codon, and the primer phbA-RBam (5′-CGGGATCCTCAGACCGCTTCCAGGACC-3′), with a *Bam* HI site (underlined) located downstream of the stop codon. Similarly, the *phb*C gene was amplified by using the primers phbC-FPst (5′-CTGCAGATGGTCGGAGATCTGGGTGTC-3′) and phbC-RPst (5′-CTGCAGTCAGACGATGCGCACCTTGGC-3′) with *Pst* I sites (underlined) located upstream and downstream of the gene. The amplicons were digest with *Eco* RI and *Bam* HI (*phb*A) and *Pst* I (*phb*C) and ligated with the similarly digested expression vector pMMB206. *E. coli* DH5α was transformed with the ligation mix and transformants were selected by blue/white selection on LB plates containing 1 mM IPTG, 80 µg mL^{-1} X-Gal and 35 µg mL^{-1} chloramphenicol. Obtained constructions were named p206A and p206C respectively. Lately constructions were transferred into *E. coli* S17-1 λpyr, and then conjugatively mobilized into *A. brasilense* Sp7. Obtained strains were named Ab7A and Ab7C, respectively. Likewise pMMB206 expression vector was transferred into *E. coli* S17-1 λpyr and conjugatively mobilized into *A. brasilense* Sp7, obtaining Ab7-206 strain. Transconjugants clones were selected in LB agar and minimum medium (MM) with 100 µg mL^{-1} ampicillin and 35 µg mL^{-1} chloramphenicol.

Poly-β-hydroxybutyrate quantification. Estimation of PHB content was determinate as Law and Slepecky (1961) and the amount of PHB produced was calculated from the standard curve



prepared by using PHB (Invitrogen). *A. brasilense* Sp7 strains were growth in MMAB containing malate and NH_4Cl as sole source of carbon and nitrogen, respectively. Cultures were incubated with shaking at 32 °C until OD_{600nm} reached 0.3. At this stage, IPTG was added to 1 mM final concentration to induce the expression of the cloned genes. Bacterial culture was centrifuged at 8,000 rpm al 4 °C for 6 min and the pellet was washed with $MgSO_4$ 10 mM. It was mixed thoroughly in NaClO by vortexing. Mixture was incubated at 37 °C for 90 min and again centrifuged at 8,000 rpm for 10 min to sediment the lipid granules. Supernatant was decanted, and the pellet was washed successively with distilled water, acetone and ethanol and centrifuged at 8,000 rpm for 10 min each wash. Pellet for granules was dissolved in chloroform at 60 °C until all the chloroform gets evaporated. H_2SO_4 was added and the tubes were heated for 20 min in boiling water bath, cooled and mixed thoroughly. Absorbance was read at 235 nm against a H_2SO_4 blank on UV-Vis spectrophotometer. PHB assays were performed for triplicate.

Enzymatic assay. *A. brasilense* Sp7, Ab7A, Ab7C and Ab7-206 strains were cultivated in MM supplemented with NaCl, 0, 150 and 300 mM in order to induce osmotic stress. Crude extracts were prepared as Clara and Knowles (1984). Cell suspensions were disrupted by sonication for a 30-s burst for a total of 10 min. Cellular debris was removed by centrifugation at 8,000 rpm for 20 min. The supernatant was retained for the enzymatic assays. Superoxide dismutase activity (SOD, EC 1.15.1.1) was measured on the basis of SOD´s ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated photochemically (Beyer and Fridovich, 1987). Data are presented as U SOD/mg protein. Catalase activity (CAT, EC 1.11.1.6) was measured as described (Aebi, 1984). Consumption of H_2O_2 (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min was monitored. Data are shown in mKatal/mg protein) Ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured accord Amako *et al*. (1994). Specific activity is presented as mMol min^{-1} mg^{-1} protein.

Total protein (TP). TP was determined using the Bradford method and BSA as a standard (Bradford, 1976).

Statistics. The results are given as mean $\pm$ standard deviation of three independent determinations. Data were analyzed using ANOVA test with a significance level of $P < 0.05$.

Results and Discussion:

Azospirillum, like other microorganisms, synthetizes PHB under unbalanced growth conditions: an excess of carbon and a limitation on the nitrogen sources, in our study *A. brasilense* strains were cultivated in MMAB containing malate and NH_4Cl as sole source of carbon and nitrogen, respectively. And IPTG was added to induce the expression of genes cloned into broad-host-range expression vector. So PHB contend is showed in Table 2.
It has been reported that *A. brasilense* is able to accumulate PHB up to 70% of their cell dry weight in presence of adverse factors like UV radiation, desiccation and osmotic stress, and the polymer-rich cells have better survival (Okon and Itzigsohn, 1992). However our data cannot been compared due to methodology employed in both cases. Nevertheless our results show that in Ab7A strain, *phb*A gen overexpression leads to increased PHB production. This may be due to an increase in β-ketothiolase enzyme activity by raising Acetyl-CoA efflux from TCA cycle to PHB pathway, altering the production of PHB. On the other hand, when *phb*C gen is overexpressed a minor production of PHB in Ab7C can be observed in comparison with *A. brasilense* Sp7. This

can be explained due to a mayor amount of PHB synthase bound to PHB granules. However, is necessary corroborates it, by granule dye and TEM.

Table 2. PHB/mg protein data obtained in *A. brasilense* strains.

Strain	PHB/mg protein
A. brasilense Sp7	617.10 ± 79.91
Ab7A	1007.27 ± 23.04
Ab7B	*
Ab7C	443.96 ± 35.46
Ab7-206	747.76 ± 52.72

* Ab7B status: in process.

In order to evaluate an association between PHB accumulation and stress resistance in bacteria, we decided analyze stress tolerance to high NaCl concentrations (150 and 300 mM) in *A. brasilense* strains used in this study. So that, SOD, CAT and APX enzyme activity were quantified. Figure 1 shown SOD, CAT and APX contents in *A. brasilense* strains.

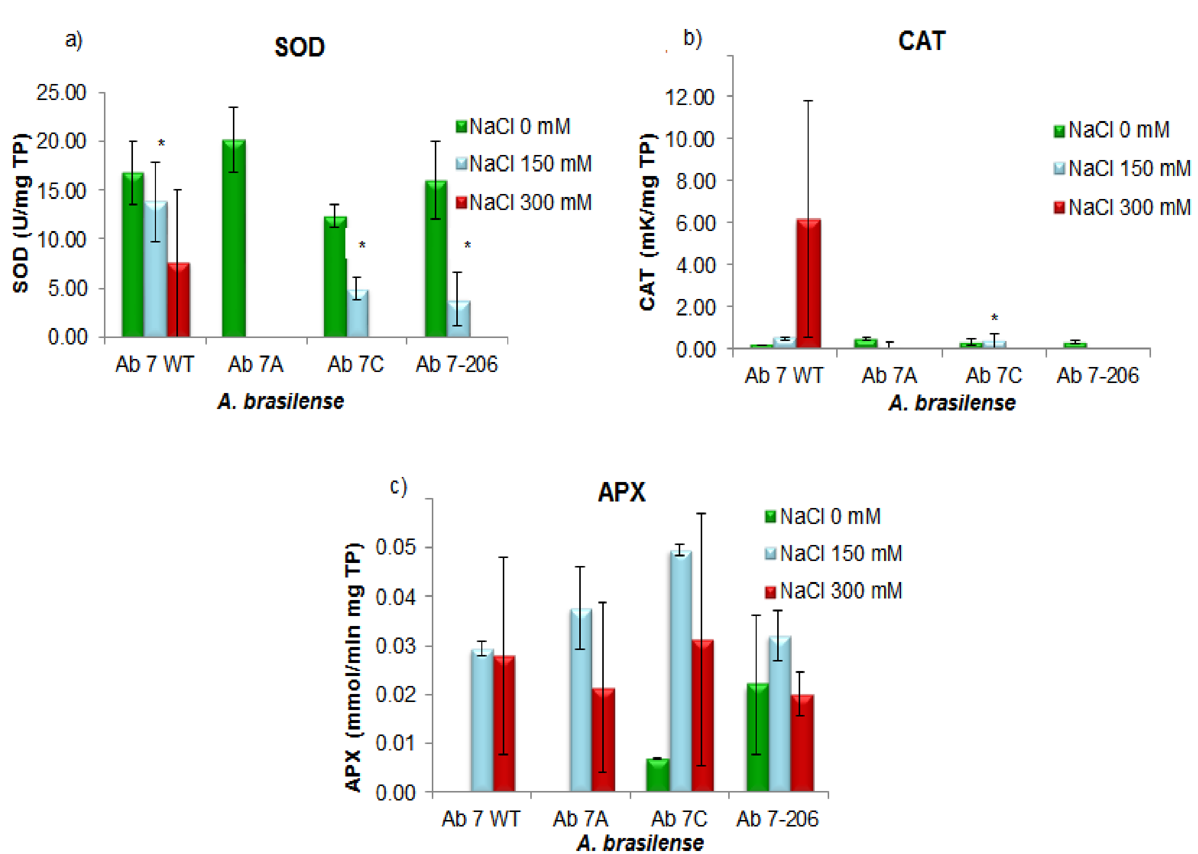


Figure 1. SOD (a), CAT (b) and APX (c) contents in *A. brasilense* strains (*P<0.05; asterisks represents significant differences between *A. brasilense* Sp7 and genetically modified strains).

SOD and APX production in *A. brasilense* Sp7 was similar that Clara and Knowles (1984) reported. In our study CAT activity was no detected in *A. brasilense* cultures without stress. It was founded that Ab7A produce major SOD levels respect Ab7C in non-stressed cultures. However, when strains are cultivated in NaCl 150 mM, SOD production is null or weakly in all strains except in *A. brasilense* Sp7. In NaCl 300 mM SOD levels were non-detected. Furthermore, CAT was present in all strains from 0 to 1 mK mg^{-1} protein. Nevertheless, in NaCl 300 mM, CAT was only detected in strain Ab7 WT. Regarding the activity APX in non-stressed conditions reported only for Ab7C and Ab7-206 strains. While at NaCl 150 mM an increased APX production was observed, highlighting it in Ab7C and Ab7-206 strains. Finally in NaCl 300 mM, APX production was decreased in all strains. Has been documented that osmotic stress can serve as a direct inducer of the oxidative stress (Aldsworth *et al*., 1999) and *Azospirillum sp.* has high rate of respiratory activity (Bergersen and Turner, 1980) and also, carries enzymes that detoxify O^{2-}, and H_2O_2 (Nur *et al*., 1982). Stouthamer *et al.* (1979) suggested that activity of enzymes of decomposition of O, and H_2O_2 is insufficient to prevent damage to the cell. This may apply also to *A. brasilense*. According to the above, we hypothesize that *A. brasilense* strains employed another defense mechanism to counteract free radicals generated during exposure to NaCl. This can be explained because non-stressed cultures have higher levels of enzyme activity when overexpressed genes involved in PHB biosynthesis (in SOD and CAT). Currently there are reports indicating that PHB production is increased when cultures are subject to osmotic stress induced by NaCl (310 mM). Likewise, there is evidence suggesting the involvement of PHB as an antioxidant response in bacteria (Ayub *et al*. 2004), given that, PHB are carbon and energy reservoirs. So, once obtained PHB production in *A. brasilense* strains overexpressing *phb*A and *phb*C genes, would be useful quantify the PHB production in cultures subjected to osmotic stress.

Conclusions: PHB production was raised upon 60% in Ab7A strain. Nevertheless *phb*C gene overexpression in Ab7C resulted in PHB levels similar to reported by *A. brasilense* Sp7. Considering previous reports that suggest a relationship between antioxidant activity and PHB production, antioxidant enzymatic activity was quantified in the all strains subject to osmotic stress by adding NaCl. The data obtained from enzyme quantification in working strains allow us to inference that other mechanism was used to counteract the effects caused by osmotic stress. That mechanism could be PHB production. However, the metabolism of antioxidant enzyme activities in *A. brasilense* is not completely understood in relation to the ability of these microorganisms to produce PHB. So, once obtained PHB production in *A. brasilense* strains would be useful quantify the PHB production in cultures subjected to osmotic stress.

Acknowledgments: This work was partly supported by CONACYT, grant 273381, and by VIEP-Benemérita Universidad Autónoma de Puebla.

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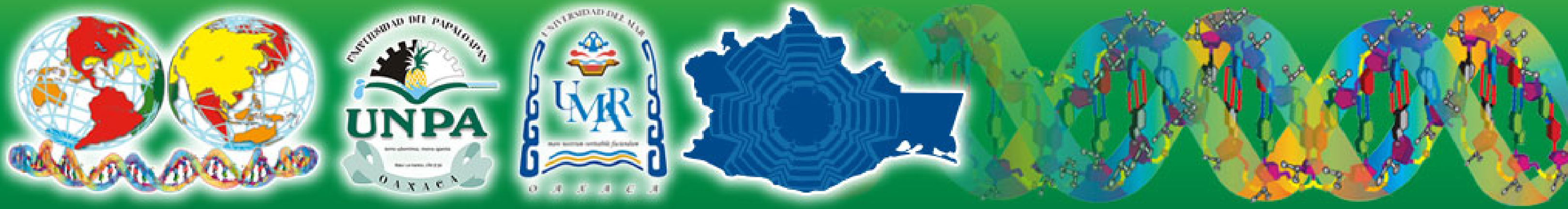

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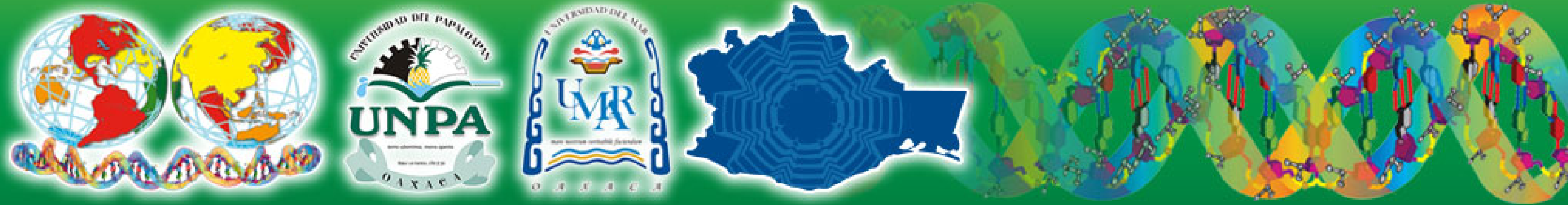
1.6 GREY AREA



Influence of thiamine and C/N ratio on production of pDNA of *E. coli* DH5α in a chemically defined culture medium

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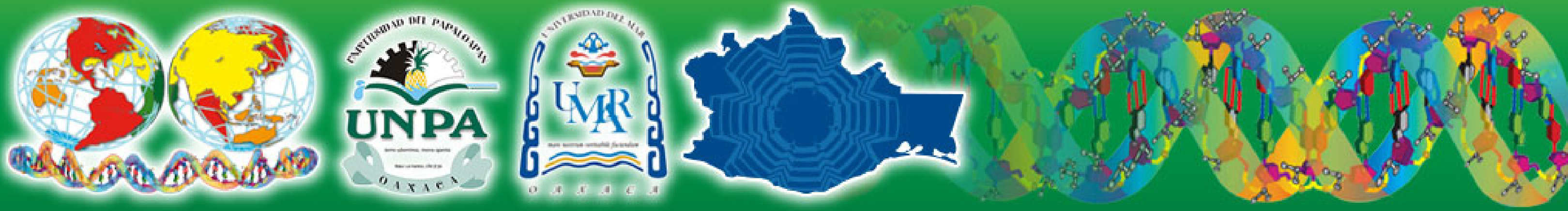
Abstract: DNA vaccines are an alternative for immunization against bacterial and parasitic diseases. However, purification and immunization protocols test required high plasmid yield for the initial steps in the laboratory. In this work, the individual effect of thiamine (0 to 10 mg L^{-1}) and C/N ratio (by varying glycerol and ammonium sulfate concentration) on plasmid DNA production (pDNA) of *E.coli* DH5α (pVAX-NH36) was evaluated in flask culture at 30 and 37 °C using a chemically defined medium. The pDNA concentration increased by increasing thiamine from 0 to 2.5 µg L^{-1} in both temperatures, but maximum pDNA concentration (40.71 mg L^{-1}) and cell density (5.76 g L^{-1}) were obtained at 37 and 30 °C respectively. The C/N ratio had not significant effect on pDNA concentration at 37 °C when C/N ratio was increased from 9.34 to 21.79 (by varying ammonium sulfate at glycerol concentration of 25 g L^{-1}) and increased from 5.6 to 13.08 (by varying glycerol at ammonium sulfate concentration of 4.97 g L^{-1}).

Keywords: plasmid ● DNA ● vaccine ● flask

Introduction: Plasmid DNA vaccines are an alternative to treat various diseases. These vaccines are based in the direct injection into the host of plasmid DNA, which encodes for one or several pathogen antigens, instead of protein antigen or attenuated/dead pathogen (Ellis, 2001). The production of such vaccines has been extensively studied in recent years, however production conditions vary in changing the construction of the plasmid and the selection of the host organism (Carnes, 2005). Currently many researches are focused on increasing the production of pDNA to begin with preclinical testing, which required higher yields of plasmid (Schalk *et al*., 2006). However, fed-batch cultures to obtain this, required a complex infrastructure that is not available in several cases and is not necessary for the first steps of the investigation. Several researches have been done to increase productivity of plasmid DNA using different approaches, such as, using different strains and plasmids, optimization of the media, varying culturing systems and conditions, as well as genetic engineering (O'Kennedy *et al*., 2003). The effect of nutrients on the production of pDNA can be masked in a complex medium (Sanchez-Casco *et al.,* 2013). For this reason, a chemically defined medium is suggested in order to elucidate the nature of nutritional effects as far as possible. In this work, influence of thiamine and C/N ratio on production of pDNA was evaluated in chemically defined medium designed previously.



Materials and Methods:

Bacterial strain and plasmid. *Escherichia coli* DH5α transformed with the 3936 bp plasmid pVAX-NH36 (which containing a 936-bp DNA fragment coding the antigen NH36 of *Leishmania donovani* and a kanamycin resistance gene) was propagated in LB medium and mixed with 20% v/v glycerol at 80% w/v and stored in cryovials at -80 °C.

Inoculum. A stored cryovial was inoculated in a 250 mL baffled flask with 50 mL of chemically defined medium (CDMJES) containing (g L^{-1}) 25 glycerol; 7.1 $(NH_4)_2$ SO_4; 2.3 $MgSO_4$ $7H_2O$; 2 NaCl; 0.00285 $FeCl_3$ $6H_2O$; 0.5 mL of trace metal solution containing (g L^{-1}): 2 $ZnCl_2 \cdot 4H_2O$; 2 $CoCl_2 \cdot 6H_2O$; 2 $Na_2MoO_4 \cdot 2H_2O$; 1.9 $CuCl_2 \cdot 2H_2O$, 1.6 H_3BO_3; 1.6 $MnSO_4 \cdot H_2O$; 0.6 Citric acid and 1 $CaCl_2 \cdot 2H_2O$ and 5 mL of phosphate buffer 1.22 M plus kanamycin (50 µg mL^{-1}). The flask was incubated in an orbital shaker at 200 rpm and 30 °C. When the optical density (at 600 nm) of culture reached 1.5 mg mL^{-1}, the culture was used as inoculum (10% v/v). All experiments were performed in a 250 mL baffled flask with 50 mL of CDMJES medium plus kanamycin (50 µg mL^{-1}) admixed with 5 mL of inoculums. Deionized water was used to prepare all the culture media.

Analytical methods. Bacterial growth was measured turbidimetrically at 600 nm in diluted aliquots and converted to dry cell weight by means of an appropriated calibration curve. Plasmid DNA was extracted and purified using a commercial kit Invisorb Spin Plasmid Mini two. DNA concentration was determined by measuring absorbance at 260 nm.

Condition 1. Effect of thiamine (sterilized for filtration) on pDNA production of *E. coli* DH5α (pVAX-NH36) in flask culture using CDMJES medium: The medium was supplemented with different amounts of thiamine (0, 1.0, 2.5, 5.0, 7.6 and 10 µg L^{-1}) and incubated at 30 and 37 °C until the stationary phase was reached.

Condition 2. Effect of C/N ratio by varying glycerol and ammonium sulfate concentration on pDNA production at 37 °C: According to a factorial design 3^2 varying the concentration of glycerol (15, 25 and 35 g L^{-1}) and ammonium sulfate (2.13, 3.55 and 4.97 g L^{-1}) in the mineral CDMJES medium with kanamycin and thiamine according the results of Condition 1.

Results and Discussion:

Condition 1. Biomass and pDNA production from *E.coli* in flask culture at different amounts of thiamine and temperature are shown in Figure 1. The pDNA concentration increased by increasing thiamine from 0 to 2.5 mg L^{-1} in both temperatures, but the maximum pDNA concentration (40.71 mg L^{-1}) and cell density (5.76 g L^{-1}) were obtained at 37 and 30 °C, respectively.

Condition 2. The pDNA production when the C/N ratio was varied by varying ammonium sulfate and glycerol concentration is shown in Figure 2. The C/N ratio had not significant effect on pDNA concentration at 37 °C when C/N ratio was increased by increasing glycerol from 15 to 35 g L^{-1} keeping the ammonium sulfate concentration at 4.97 g L^{-1} and when it was increased by decreasing ammonium sulfate from 4.97 to 2.13 g L^{-1} keeping the glycerol at 25 g L^{-1}. However, the pDNA decreased when ammonium sulfate was decreased in both glycerol concentration of 15 and 35 g L^{-1}.

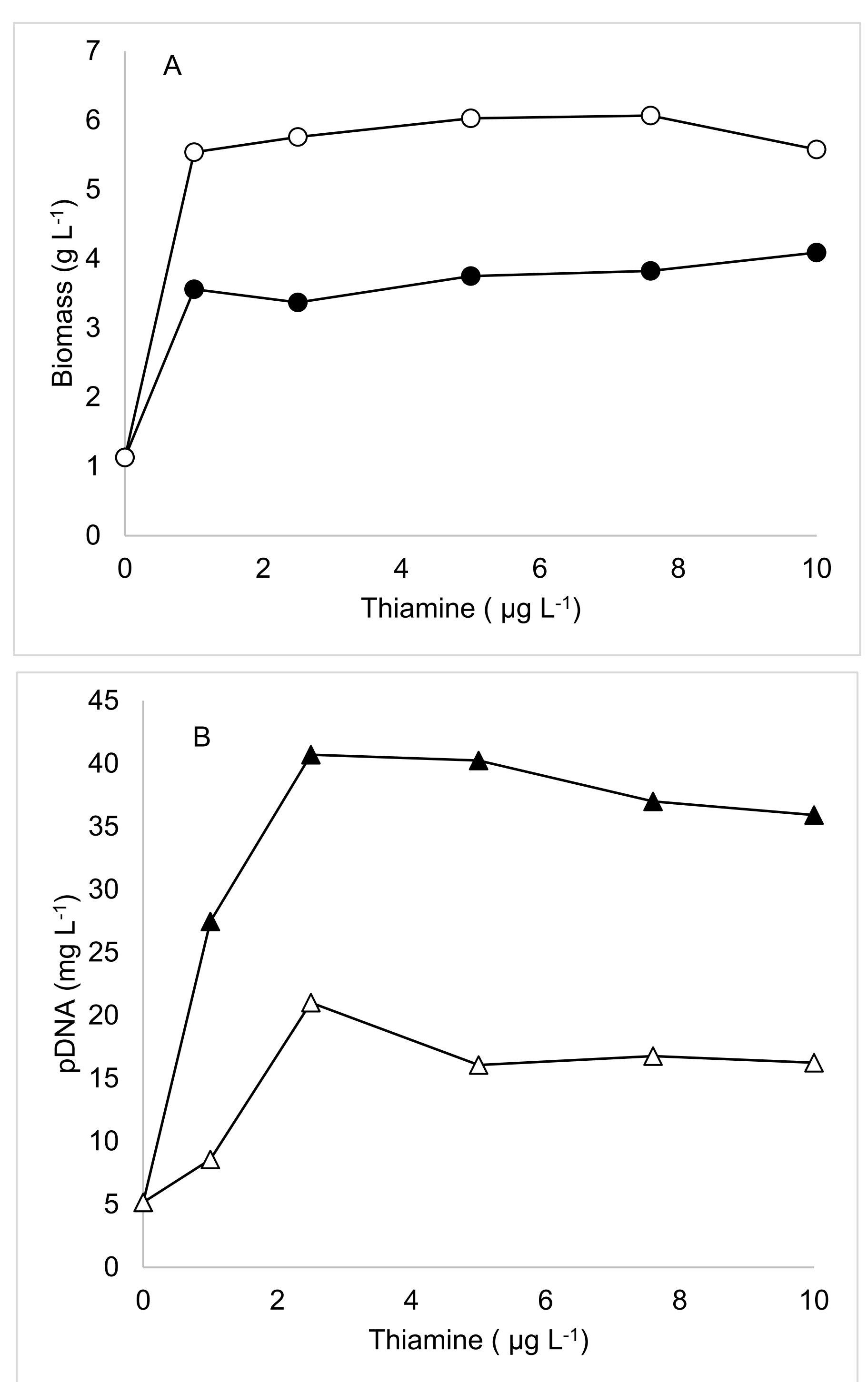


Figure 1. Effect of thiamine on biomass and pDNA of *E.coli* DH5α (pVAX-NH36) in flask culture at 30 °C (empty) and 37 °C (filled) using chemically defined medium.

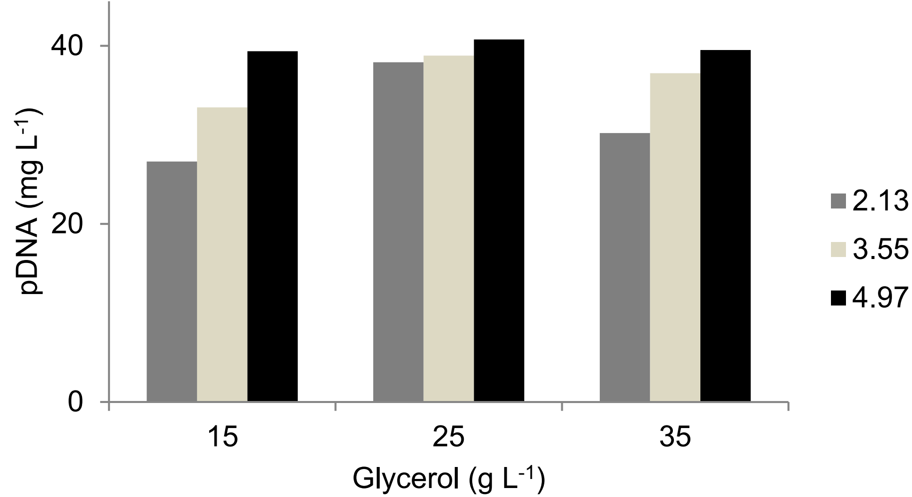


Figure 2. Effect of glycerol and ammonium sulfate on pDNA of *E.coli* DH5α (pVAX-NH36) in flask culture at 37 °C using chemically defined medium.

Conclusion: In the mineral CDMJES medium with an initial concentration of glycerol at 15 g L^{-1}, ammonium sulfate at 4.97 g L^{-1} and thiamine at 2.5 μg L^{-1} the maximum production of plasmid is obtained (40.71 mg L^{-1}) at 37 °C. This medium represents the first step to evaluate the effect of nutrients on the pDNA production by avoiding the use of complex media.

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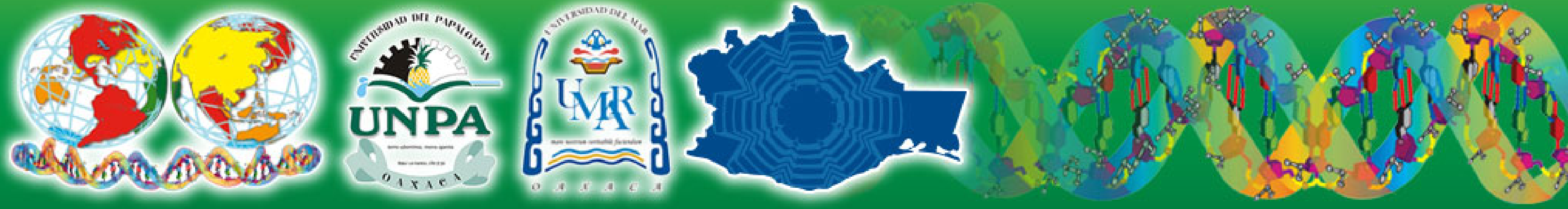

Chlorella sp. and *Scenedesmus sp.* microalgae cytotoxic activity against L5178Y-R *Murine lymphoma* cells

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Abstract: Cancer nowadays is responsible for 13% of deaths worldwide. Treatments have the drawback of serious secondary effects to the patients, leading to constant research for new therapeutic alternatives. Bioactive compounds produced by microalgae from around the world have shown cytotoxicity against several cancer cell lines. The aim of this study was to evaluate cytotoxic activity of a number of microalgae isolated from the state of Nuevo Leon, Mexico, against the murine lymphoma cell line L5178Y-R. Aqueous extracts from such isolates were tested using the MTT colorimetric reduction microassay. Results showed that *Chlorella* sp. and *Scenedesmus* sp. aqueous extract were the most toxic against L5178Y-R cells, showing 84.99% and 77.48 % cell toxicity, respectively.

Keywords: Microalgae ● Cytotoxicty ● Lymphoma ● L5178Y-R ● Cancer

Introduction: Microalgae are the simplest and most primitive organisms on the planet, located at the food chain end. They are prokaryotic or eukaryotic unicellular and photosynthetic organisms distributed worldwide among aquatic and terrestrial niches, since they may easily adapt to environmental stress conditions, such as extreme temperatures, high osmotic pressure and UV radiation exposure (Guedes *et al*., 2011). These organisms are extremely diverse and have demonstrated several biotechnological advantages in addition to photosynthesis, such as the use of simple nutrients, rapid growth, and ability to accumulate and secrete diverse secondary metabolites. In this sense, microalgae may synthesize bioactive compounds such as pigments, sterols, polyphenols, fatty acids, proteins, vitamins, alkaloids, and sulfated polysaccharides. Selective compounds have shown biological properties including antiviral, antimicrobial, immunomodulatory and anticancer activities (Lordan *et al*., 2011; Teas and Irhimeh, 2012). Since cancer or malignant tumor cells show with progressive and invasive growth behavior (Qian *et al*., 2001), treatments include several drugs. Nevertheless, cancer cells might resist drugs, leading to unhealthy side effects.
The aim of the present study was to evaluate cytotoxic activity of aqueous extracts from cultured native microalgae isolated from Nuevo Leon state, in Mexico, against the murine lymphoma L5178Y-R cell line.

Materials and Methods:

Several water bodies from Nuevo León state were randomly sampled by gathering water in plastic bottles, subsequently 5 mL of these samples were taken and transferred to 250 mL Erlenmeyer flasks with 100 mL of the culture medium reported by Chuken-Lopez *et al*. (2010). All samples were incubated in flasks at room temperature (25 ± 3 °C) and 120 rpm orbital speed, with

continuous artificial light (100 lux light source), for three weeks. Four samples, out of 19 collected, showing no evidence of growth after incubation were discarded.

Table 1. Identification of microalgae isolated from Nuevo Leon.

Microalgae isolate	Sampling location	Identified as:	Photo (100x)
1	Pesqueria River, Gral. Escobedo N.L. 25°48'04.8"N 100°16'43.5"W	*Chlorella pyrenoidosa*	
2	Topo Chico Creek Monterrey, N.L. 25°44'16.1"N 100°20'38.1"W	*Chlorella* sp.	
3	Recreational Park “La Turbina” Sabinas Hidalgo, N.L. 26°29'13.7"N 100°12'53.7"W	*Chlorella* sp.	
4	Donated by Facultad de Ciencias Químicas, UANL	*Chlorella* sp.	
5	Dam “Los Guerra” Mina N.L. 26°00'12.2"N 100°32'56.7"W	*Chlorella* sp.	
6	Salinas River Mina N.L. 26°00'11.8"N 100°32'41.7"W	*Chlorella* sp.	
7	Las Encinas Creek Gral. Escobedo N.L. 25°47'48.2"N 100°18'40.0"W	*Chlorella* sp.	
8	La Silla River Guadalupe N.L. 25°40'30.5"N 100°14'13.7"W	*Scenedesmus* sp.	
9	La Silla River Guadalupe N.L. 25°40'39.9"N 100°13'04.0"W	*Scenedesmus* sp.	
10	Topo Chico Creek San Nicolás, N.L. 25°43'47.1"N 100°19'13.4"W	*Scenedesmus* sp.	

Table 1 (continued).

Microalgae isolate	Sampling location	Identified as:	Photo (100x)
11	El Indio Creek Juárez N.L. 25°37'53.6"N 100°09'20.3"W	*Navicula* sp.	
12	La Estanzuela, Natural Park Monterrey N.L. (El cielo) 25°32'16.9"N 100°16'31.6"W	*Nitzchia* sp.	
13	Niños Héroes Park Monterrey N.L. 25°42'56.0"N 100°18'49.3"W	*Cymbella* sp.	
14	La Silla River Guadalupe. N.L. 25°40'35.0"N 100°13'37.2"W	*Oocystis* sp.	
15	La Estanzuela, Natural Park Monterrey N.L. 25°34'16.6"N 100°15'31.1"W	*Oocystis* sp.	
16	La Turbina, Recreational Park Sabinas Hidalgo, N.L. 26°29'06.0"N 100°12'54.8"W	*Oocystis* sp.	
17	Donated by Facultad de Ciencias Químicas, UANL	*Tetraspora* sp.	
18	La Estanzuela, Natural Park Monterrey, N.L. (El cielo) 25°34'03.0"N 100°15'49.1"W	*Kirchneriella* sp.	
19	Cola de Caballo, Natural Park Santiago, N.L. 25°22'03.5"N 100°09'38.4"W	*Kirchneriella* sp.	

In order to obtain microalgae cultures from a single genus, samples that showed growth were inoculated on Petri dishes containing the culture medium solidified with 1.5% agar, and the plates were incubated at 30 °C with a 100-Watt spotlight as continuous source, until microalgae colony growth was observed. Colonies were then collected with a bacteriological loop, transferred to a 250 mL Erlenmeyer flask with 50 mL of the culture medium (liquid), and incubated under the conditions described above.

In order to assess axenic cultures from each cultured sample, they were treated for 48 h with an antibiotic solution consisting of penicillin (500 IU mL^{-1}), streptomycin (500 mg mL^{-1}), gentamicin (50 µg mL^{-1}), and fungizone (1.25 µg mL^{-1}). After this, cultures were transferred to an algae culture medium without antibiotic and incubated under the same conditions until growth was observed. Microalgae axenic culture samples were confirmed by microscopy. Algae genus identification was performed by guides identification based on morphological characteristics (Prescott *et al.*, 2002; Graham *et al.*, 2008; Bellinger and Sigee, 2010). After 19 isolated microalgae were identified, the cytotoxic activity of the aqueous extracts of 15 microalgae was evaluated. To achieve this, 200 mL of culture medium for algae were incubated under the same conditions until an abundant growth was observed (approximately 3 weeks). Biomass was collected by centrifugation and filtration, and then samples were dried by freeze-drying. Aqueous extracts were prepared using a 250 mL beaker with 20 mL of boiling distilled water. The lyophilized microalgae biomass was added and mixed for 10 min, centrifuged and filtered to eliminate the remaining biomass. Collected supernatant was lyophilized and then dissolved in RPMI culture medium at 1 mg mL^{-1} concentration, and used as stock solution. The cytotoxic effect of aqueous extracts against L5178Y-R cell line was performed by microplate technique using MTT as an indirect cell activity indicator (Gomez-Flores *et al.*, 2009). Treated cells were incubated for 48 h with one out of seven aqueous extracts used as treatments. Boiled water was used as control. Cells mortality was compared against the control.

Results and Discussion:

After identification of isolated microalgae, mainly *Chlorella* sp., *Scenedesmus* sp. and *Oocystis* sp. were identified (Table 1). *In vitro* cytotoxicity assays of 15 aqueous extracts of microalgae isolates against the L5178Y-R cell line demonstrated the potential of seven isolates identified as *Chlorella* sp., and *Scenedesmus* sp. (Figure 1).

As observed in Figure 1, several strains showed cytotoxic effect, and the best results were obtained with strains of *Chlorella* sp. (isolate 6) and *Scenedesmus* sp. (isolate 10), reaching a cytotoxicity of 84.99% (±1.79) and 77.48% (±3.95) against mouse lymphoma cell line at a concentration of 500 µg mL^{-1}, respectively. Although the concentrations used in the present study can be considered high, these results indicated that there are at least trace amounts of compounds which can inhibit the growth of tumor cells in the aqueous extracts of these strains. However, results obtained in the present research are comparable to authors from different geographic regions such as Yusof, who reported cytotoxic activity of an aqueous extract of *Chlorella vulgaris* against the HepG2 cell line with an IC50 of 1.6 mg mL^{-1} (Yusof *et al.*, 2010), and the results shown by Oftedal, who used concentrations as high as 4 mg/mL against cell lines of lymphona and leukemia (Oftedal *et al.*, 2011), in both studies, they used and reported concentrations higher than those in our report. These results showed that some genus of microalgae from Nuevo Leon, produced compounds with antitumor activity potential which warrants further research, using extracts from different polarity solvents.

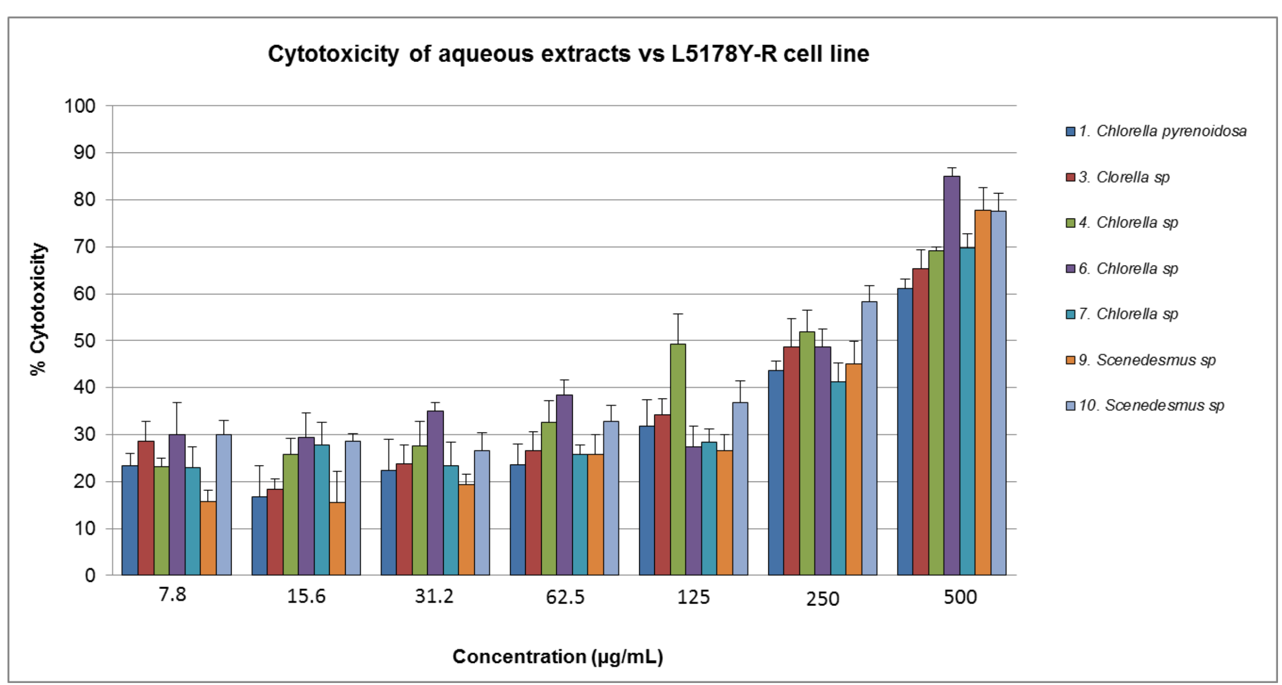

Figure 1. Cytotoxic effect of seven concentrations of the aqueous extracts of seven selected microalgae isolates, based on their efficacy to kill the L5178Y-R cell line.

Conclusions: The results obtained from the present research showed that aqueous extracts of microalgae from Nuevo Leon, Mexico, produced compounds with cytotoxic activity against L5178Y-R cell line growth, *in vitro*. Further research in this area will focus on isolating strains from the genus *Chlorella* and *Scenedesmus,* as well as in the extraction, purification (using different polarity solvents), and evaluation of cytotoxic compounds produced by these strains.

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Determination of parameters to set up a methanol feeding strategy to express TvLEGU-1 in *Pichia pastoris*

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Abstract: *Trichomonas vaginalis*, a sexually transmitted parasite responsible of human trichomonosis, has many cysteine proteinases (CPs) and some are involved in trichomonal virulence. Antibodies against TvLEGU-1 have been detected in patient sera with trichomonosis. TvLEGU-1 is a legumain-like CP that plays a major role in trichomonal cytoadherence and it is a potential biomarker for serodiagnosis of trichomonosis. However, its characterization has been limited by the lack of a reliable recombinant expression platform to obtain this protein in its correctly folded conformation. Therefore, the precursor of TvLEGU-1 was expressed using *Pichia pastoris.* To improve the expression of recombinant protein at 5L bioreactor scale, in this work we determined the adaptation time to the methanol, the substrate maximum rate specific uptake during and after the adaptation phase of *Pichia pastoris* X-33 strain transformed with the pPICZαB-*tvlegu1* in order to set up a methanol feeding strategy to improve the recombinant protein expression.

Keywords: *Pichia pastoris* ● protein expression ● fed batch culture ● cysteine proteinase

Introduction: *Trichomonas vaginalis*, a sexually transmitted parasite responsible of human trichomonosis, has many cysteine proteinases (CPs) and some are involved in trichomonal virulence (Petrin *et al*., 1988; Carlton *et al.,* 2007; Arroyo and Alderete, 1989, 1995; Mendoza-López *et al*., 2000; Hernández *et al*., 2004; Ramón-Luing *et al*., 2011; Lehker *et al.,* 1990; Provenzano and Alderete, 1995). Antibodies against TvLEGU-1 have been detected in patient sera with trichomonosis. TvLEGU-1 is a legumain-like CP that plays a major role in trichomonal cytoadherence and it is a potential biomarker for serodiagnosis of trichomonosis (Rendón-Gandarilla *et al*., 2013; Ramón-Luing *et al*., 2010). However, its characterization has been limited by the lack of a reliable recombinant expression platform to produce this protein in its correctly folded conformation. TvLEGU-1 has been cloned and expressed in *Escherichia coli* (Rodríguez-Cabrera, 2007; Reséndiz-Cardiel *et al*., 2011), nevertheless all the efforts to obtain this protein in native conformations by using different vectors and *E. coli* strains, have failed. Therefore, the methylotrophic yeast *Pichia pastoris* expression platform was selected and DNA sequence of the precursor of TVLEGU-1 was cloned into the pPICZαB vector. Several clones of the *P. pastoris X-33* strain transformed with the pPICZαB-*tvlegu1* were grown in flasks. A clone was chosen to express the recombinant TvLEGU-1 in Fed-Batch fermentation in a 5 L bioreactor. However, one of the critical parameters during the Fed-Batch fermentation is the methanol adaptations phase, as well as, the methanol feeding strategy (Trinh *et al*., 2003; Khatri and Hoffmann, 2006).
To induce the expression of recombinant protein in *P. pastoris* with methanol many of the fermentation strategies reported were defined by using constant feeding profiles during Fed-Batch

cultivations (Invitrogen protocol; http:// tools.invitrogen.com). Nevertheless, this approach does not allow the improvement of protein expression easily. A reliable alternative is to determine strain characteristic parameters to set-up a methanol feeding strategy during the Fed-Batch production processes. Recently, Dietzsch *et al.* (2011) reported a fast and easy-to-do characterization of recombinant *P. pastoris* based on batch cultivations with pulses of methanol. Using this protocol methanol adaptation time ($\Delta time_{adapt}$), specific substrate uptake rate during the adaptation phase ($q_{s\ adapt}$) and maximum specific substrate uptake rate ($q_{s\ max}$), are estimated to set-up a feeding strategy of methanol based on q_s strain-specific values(Dietzsch *et al*., 2002). In the present work, we report the q_s values of a recombinant *P. pastoris* X-33-pPICZαB-*tvlegu1* clone with phenotype Mut$^+$ expressing a legumain-like TvLEGU-1 from *T. vaginalis* using the method described above.

Materials and Methods:

Microorganisms. *P. pastoris* X-33 strain (Invitrogen) whit a phenotype Mut$^+$ and pPICZαB vector (Invitrogen) were used in this study. Chemically competent *P. pastoris* X-33 cells were transformed with the pPICZαB-*tvlegu1* that contains the DNA sequence (*tvlegu1*) encoding the precursor of the cysteine proteinase legumain-like TvLEGU-1 from *T. vaginalis*.

Culture media. A preculture was done in a buffered glycerol complex media (BMGY; Invitrogen, 2002), batch culture and repeated pulses methanol were done in a fermentation basal salts media (BSM) as previously described by Dietzsch *et al.* (2011).

Preculture. Frozen stock (-80 ºC) was precultivated in 100 mL of BMGY in a 1000 mL flask at 28 °C and 250 rpm for 16 hours.

Batch cultivation and specific rate calculations. Batch cultivation was carried out in a 5 L working volume BioFLo 115 bioreactor (New Brunswick) at 28 ºC and fixed agitation speed of 700 rpm. The culture was aerated with at least 1 vvm dried air to keep dissolved oxygen levels > 30% and pH was adjusted and controlled to pH 6.0. Gravimetric method and a paramagnetic cell (New Brunswick) were used to measure the CO_2 off gas and O_2, respectively. Process parameters were recorded and logged using the BioCommand software (New Brunswick). After the consumption of glycerol (increase of dissolve oxygen and drop in off-gas activity), a first methanol pulse (adaptation pulse) was fed to the bioreactor to a final concentration of 0.5% (v/v) and following pulses were done to a 1% (v/v). Several samples were taken at time points during the methanol pulses (*i.e.* beginning, maximum, and end off gas pulse) to determine the methanol concentration by gas chromatography and dry cell weight. The values estimated at beginning and end of each pulse were used to calculate the average rate of specific substrate uptake (q_s).

Western blot assay. Proteins secreted to supernatant after 48 h induction were TCA-precipitated and analyzed by SDS-PAGE using 12% polyacrylamide gel. TvLEGU-1 was detected by Western blot assay using commercial anti-poly His-tag antibodies (1:5000 dilution, Roche) and chemiluminescence SuperSignal Pico Maximum Sensitivity Substrate kit (Pierce).

Results and Discussion:

A batch culture of *P. pastoris* X-33 transformed with the pPICZαB-*tvlegu1* using glycerol as substrate was done. After 24 hours of culture, an increase of dissolved oxygen and drop in off gas activity were detected that indicated the depletion of glycerol. Then, first methanol pulse was applied (adaptation pulse; black arrow, Figure 1). After 4.28 hours of the first methanol pulse, the maximum in off gas activity was observed, indicating that it is the time required by this *P. pastoris* clone to adapt and use methanol as the sole source carbon (adaptation time -$\Delta time_{adapt}$). The biomass and methanol concentrations determined during the methanol pulse allowed us to estimate a specific rate for methanol uptake ($q_{s\ adaptation}$) of 0.7031 mmol g^{-1} h^{-1}. After the adaptation methanol pulse, two pulses with 1% (v/v) where conducted and a maximum specific methanol uptake rate ($q_{s\ max}$) of 1.306 mmol g^{-1} h^{-1} and 1.198 mmol g^{-1} h^{-1} were determined for the first and second pulse, respectively. The cell culture adapted to methanol was maintained with a methanol feeding rate of 1 mmol g^{-1} h^{-1} (lower than $q_{s\ max}$) and recombinant TvLEGU-1 was immunodetected in the supernatant after 48 h induction (55 kDa band, Figure 2B). The $qs_{\ adaptation}$ and $qs_{\ max}$ estimated for this *P. pastoris* clone can be used to establish an optimal feeding strategy to prevent the methanol accumulation during the adaptation phase, as well as, during the Fed-Batch cultivations (Dietzsch, 2002).

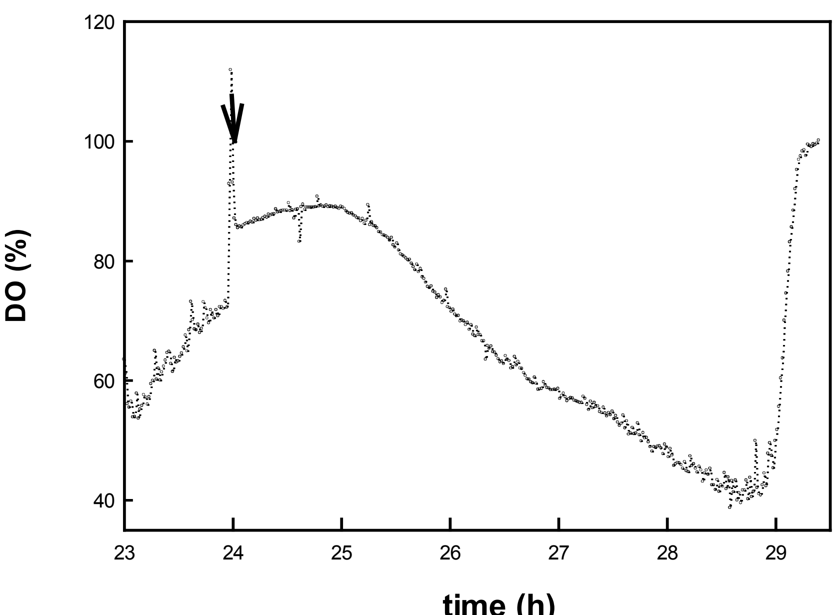

Figure 1. Increase of dissolved oxygen. **%** dissolved oxygen (OD %), 0.5% (v/v) methanol adaptation pulse (↓).

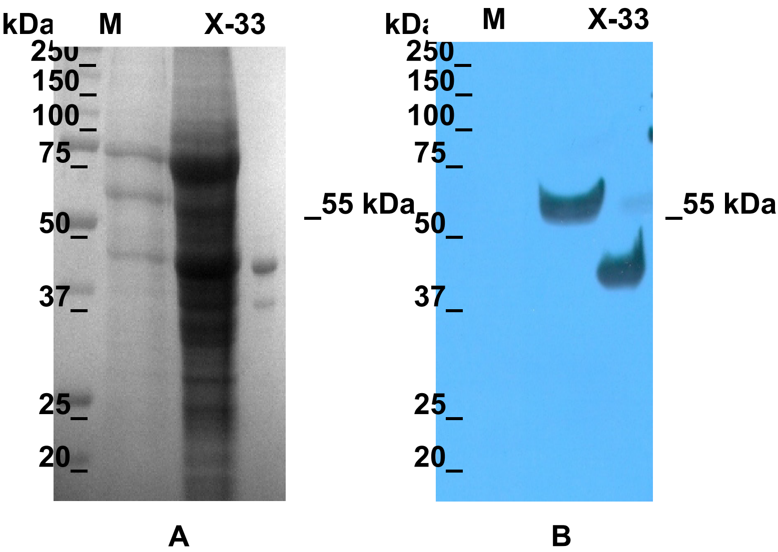

Figure 2. Recombinant TvLEGU-1 expression in *P. pastoris* after 48 h methanol induction. A) 12% SDS-PAGE, B) Western blot assay against poly His-tag. M: molecular weight marker, X-33) *Pichia pastoris* X-33 supernatant, R) *P. pastoris X-33*-pPICZαB-*tvlegu1* supernatant , C)TvLEGU-1-His tag control expressed in *Escherichia coli* as inclusion bodies.

Conclusion: The $\Delta time_{adapt}$, $q_{s\ adaptation}$ and $q_{s\ max}$ specific of a clone of *P. pastoris* X-33 were determined to set up a fed batch methanol feeding strategy and improve the expression of the recombinant TvLEGU-1; a potential biomarker for the diagnostic of trichomonosis.

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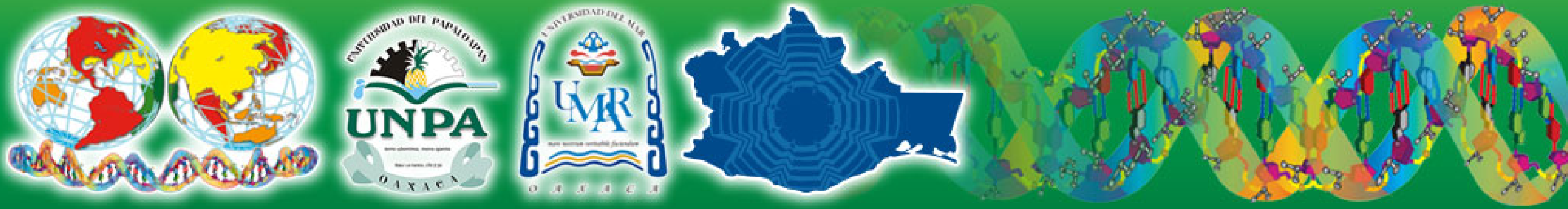

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Use of solid-state fermentation as a treatment to reduce anti-nutritional factors in mango seed kernel

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Abstract: Mango seed kernel (MSK) is rich in nutritional compounds as mineral and protein with a good profile of essential amino acids but contains antinutritional factors (AFNs) which avoid the use for human consumption. The aim of this work was to investigate the influence on reduction of antinutritional factors of MSK flour through solid state fermentation (SSF). MSK flour was fermented by *A. niger* N402 in a SSF method until 72 h, at 30 and 40 °C, the reduction of AFN's was monitored each 24 h. Also a proximate composition was realized. The effect on the antinutritional content showed that there was an effective decrease at 30 °C in 72 hours over phytates (0.98 to 0.40 mol kg^{-1}), oxalates (0.97 to 0.65 mg 100 g^{-1}) and tannins (6.23 to 5.45 g 100 g^{-1}), this last one was better reduced at 40 °C/72 h (3.11 g 100 g^{-1}). Whereas HCN content increased, being constant at 40 °C. The use of SSF as a treatment to reduce this compounds is an effective technique, also has the advantage of not lose nutritional compounds or improve them. The flour produced could be an ingredient for baked products even use it as an optimum protein supplementation in cereal mix products.

Keywords: Mango seed kernel ● antinutritional factors ● solid state fermentation

Introduction: One of the most common problems in food processing is the disposal of the sub-products generated, this waste material produces severe ecological problems, so that strategies for the profitable use of these material are needed. Mango seed which represents between 20-60% of the fruit, has limited food or industrial use in most producing countries and is wasted, the kernel contained in the seed (mango seed kernel MSK) is a good source of carbohydrates, specially starch, fat; which have unique physical and chemical characteristics similar of cocoa butter, not only contain proteins but also has a good essential amino acid profile better than maize. However, despite these attributes mango seed kernel even contains antinutritional factors (AFNs) which limits its utilization as a food ingredient (Diarra, 2014; Fowomola, 2010). The importance of AFNs is because of these substances affect nutritional value of food, especially seeds, due to assimilation of nutrients (proteins and minerals) is inhibited, causing non desirable physiological effects like stomach distension, flatulence, etc. Antinutritional factors are natural substances non fibers, created by secondary metabolism of plants like defense mechanism to stressful situations (D' Mello, 2005). Solid state fermentation (SSF) has emerged as a potential technology for the production of enzymes, fuel, industrial chemical and pharmaceutical products even to detoxify vegetables products. This process involves the growth and/or cultivation of microorganisms under controlled condition in the absence or near absence of free water for the production of desired products of interest, several agro crops such as cassava, sugarcane bagasse, seeds, and tea waste are the most often and commonly used as substrates for SFF reducing pollution concern.

SSF processes are simple, use low volume equipment (lower cost) and are effective by providing concentrated products (Ojokoh *et al*., 2012). The aim of this study is to evaluate the effect of solid state fermentation over mango seed kernel to reduce the presence of antinutritional factors and the flour produced be employed as a source of nutrients in the human consumption.

Materials and Methods:

Preparation of material. Matured fresh mango cv. Oro sample were obtained from a local market of Chiapas, México. The peels and pulp were removed by washing in clean water, while the seeds were separated manually cracking endocarp. The kernels were cut into 1 cm^3 and dried at 40 °C for 28 hours in forced air oven, followed was ground by using a food processor, then through sieve 50 until a fine powder. Stored at ambient temperature in polyethylene bags (Medina *et al.,* 2010).

Inoculum preparation. A strain of *A. niger* N402 was evaluated, the propagation was realized on Erlenmeyer flask containing potato dextrose agar (PDA) sterile (50 mL) and incubated at 30 °C for 7 days. Later conidia were collected by adding 25 mL Tween 80 0.1% (v/v) and gently homogenized with magnetic stirrer. Conidial concentration was determined by using a Neubauer hemacytometer taking 1 mL of suspension (Martinez, 2005).

Culture media and growth conditions. Pontecorvo medium sterile was used to provide 50% of humidity, adjusting pH to 5.5. 130 g of mango seed kernel flour sterile as solid support was homogenized with a volume of Pontecorvo medium (128 mL) and a volume of conidia suspension (2 mL) to obtain a concentration of 2.7×10^7 conidia g^{-1} of mango seed kernel flour, all was placed into a polycarbonate tray 2 ¼ in. Inoculated trays were grown until 72 hours at 30 and 40 °C, in each temperature ferment samples (32 g) were taken every 24 hours and stored at -20 °C until treatment (Martinez, 2005). Samples obtained were dried at 55 °C for 24 hours, after were milled to a fine powder and stored at 4 °C prior to analysis (Fadahuns, 2009).

Proximate composition and antinutritional factors analysis. Dry matter, ash, crude fat, crude fibre and protein (Nx6.25) were carried out according to AOAC methods (2000) and carbohydrates were calculated by difference. Total soluble tannin was determined using copper acetate gravimetric method by Joslyn (1970). Phytates were measurement according to the method described by Wheeler and Ferrel (1971) with modifications of Kayode *et al.* (2013) described briefly, 4 g of milled mango seed kernel were soaked in 100 mL of 2% HCl for 3 hours and then filtered through Whatman No. 1 filter paper. A volume (25 mL) of the filtrate was placed in a 100 mL conical flask and 5 mL of 0.3% ammonium thiocyanate solution was added as indicator. Then, 53.5 mL of distilled water was added to the mixture to give the proper acidity. A titration was carried on with an Iron III chloride solution, which contains about 0.00195 g of iron mL^{-1}, until a brownish-yellow color appeared which persist for 5 minutes. Oxalate content was determined using the titrimetric method by Ukpabi and Ejidoh (1989) while hydrocyanic acid by alkaline titration method (AOAC, 2000).

Statistical analysis. Experiment was a completely randomized design. The analysis of variance and the mean comparison (Tukey, $p\leq0.05$) were run in JMP 8.0 software (SAS Institute, 2008).

Results and Discussion:

The proximate composition of mango seed kernel flour is shown in Table 1. The fat (7.57%), crude protein (8.36%) and carbohydrates (74.37%) content are the major components in this variety, however the value of crude protein is significantly lower that the value reported by Kayode & Sani (2008), 15.56% on cv. Oori. Meanwhile fat and carbohydrates content are higher than values reported by the same author, 6.98% and 64.84%, respectively. This differences are attributed on variety and conditions of the harvest. The amount of these nutrients present in mango seed kernel flour showed that have high potential as energy source to allow it use in solid state fermentation. The anti-nutrients content of mango seed kernel is represented in Table 2. Kayode *et al.* (2013) found a content of tannins higher (15.73 g 100 g^{-1}) that the value present in this study (6.17 g 100 g^{-1}), it is well established that tannins can bind and precipitate proteins, by inhibiting the activities of proteolytic enzymes or by increasing losses of endogenous protein (Cannas, 2014; Ravindran and Sivakanesan, 1996). On the other hand, the phytates have been shown to be involved in the complexing of dietary essential minerals in legumes and cereals which diminishes their intestinal absorption, besides a complex phytate-protein can be formed. MSK flour evaluated has a content 0.97 mol kg^{-1}. The value of oxalates is significantly lower (1.03 mg 100 g^{-1}) than reported by Dakare *et al*., (2012),1192.5 mg 100 g^{-1}; oxalate can have a harmful effect on human nutrition and health, especially by reducing calcium absorption and aiding the formation of kidney stones (Fekadu *et al*., 2013). Hydrocyanic acid (HCN) quantifies the presence of cyanogenic glycosides, which are a potent specific inhibitor of several enzyme-catalyzed processes (Montgomery, 1980). The present study showed that HCN content is 12.47 mg 100 g^{-1}.

Table 1. Proximate composition of mango seed kernel flour.

Chemical composition	Content
Dry matter (%)	95.18±0.01
Fat (%)	7.57±0.04
Ash (%)	2.47±0.02
Crude Protein (%)	8.36±0.75
Crude fibre (%)	2.23 ± 0.06
Carbohydrates (%)* (by difference)	74.37

Values are mean ± standard error of triplicate determinations, expressed on wet basis.

Table 2. Mango seed kernel flour content of some anti nutrient factors.

Antinutritional factor	Content
Tannins (g 100 g^{-1})	6.17±0.11
Phytates (mol kg^{-1})	0.97±0.06
Oxalates (mg 100 g^{-1})	1.03±0.01
Hydrocyanic acid (mg 100 g^{-1})	12.47±0.06

Values are mean ± standard error of triplicate determinations

The effect produced by SSF process over these compounds is shown in Figure 1. In the case of HCN, the kinetic shown a behavior contrary to a reduction, at 30 °C a higher content of HCN is found, being at 72 hours, 14 mg 100 g^{-1}, more than the initial value. The increase is gradual, however at 40 °C this increment is produce at 24 hours (15.6 mg 100 g^{-1}) and is maintain constantly until 72 hours. The HCN is released from the interaction between a (non-toxic) cyanogenic glucoside and a hydrolytic enzyme (β-glucosidase), in a process known as

cyanogenesis. Nonetheless, Kayode and Sani (2008) reported the increment of glucose due to fungal enzymes such as endo carbohydrates and β-glucosidase in the fermented mango kernel cake, this indicate the production of interest enzyme capable to produce HCN. Baraldo *et al*. (2014) demonstrated that higher β-glucosidase activity was found in the range of 40-55 °C, at pH 4.5-5.5, produced by *A. niger* using wheat bran in SSF, this could explain the increment of HCN in this study due to the experimental condition established. It has been reported that only plants that accumulate more than 50 to 200 mg are considered to be dangerous (Kingsbury, 1964). The values of tannins in MSK flour were increased, at both temperatures, with a significantly reduction at 40 °C 72 h^{-1} (3.11 g 100 g^{-1}). Perez *et al*. (1997) proposed that the formation of resistant starch together with condensed tannin-protein content may be responsible for the increase in tannins. While phytates content is reduced more than 50% at 30 °C 72 h^{-1}, a wide range of micro flora has been known to possess phytase activity, which may be partly responsible for reduction in phytic acid content in the fermented samples (Ojohok, 2005). In the case of oxalate content, a significantly reduction is presented at 30 °C 72 h^{-1} (0.65 mg 100 g^{-1}), the decrease in the levels could be due to the production of various enzymes during the vegetative and reproductive phases of the fungi and these could have contributed to the detoxification of the flour (Belewu and Sam, 2010).

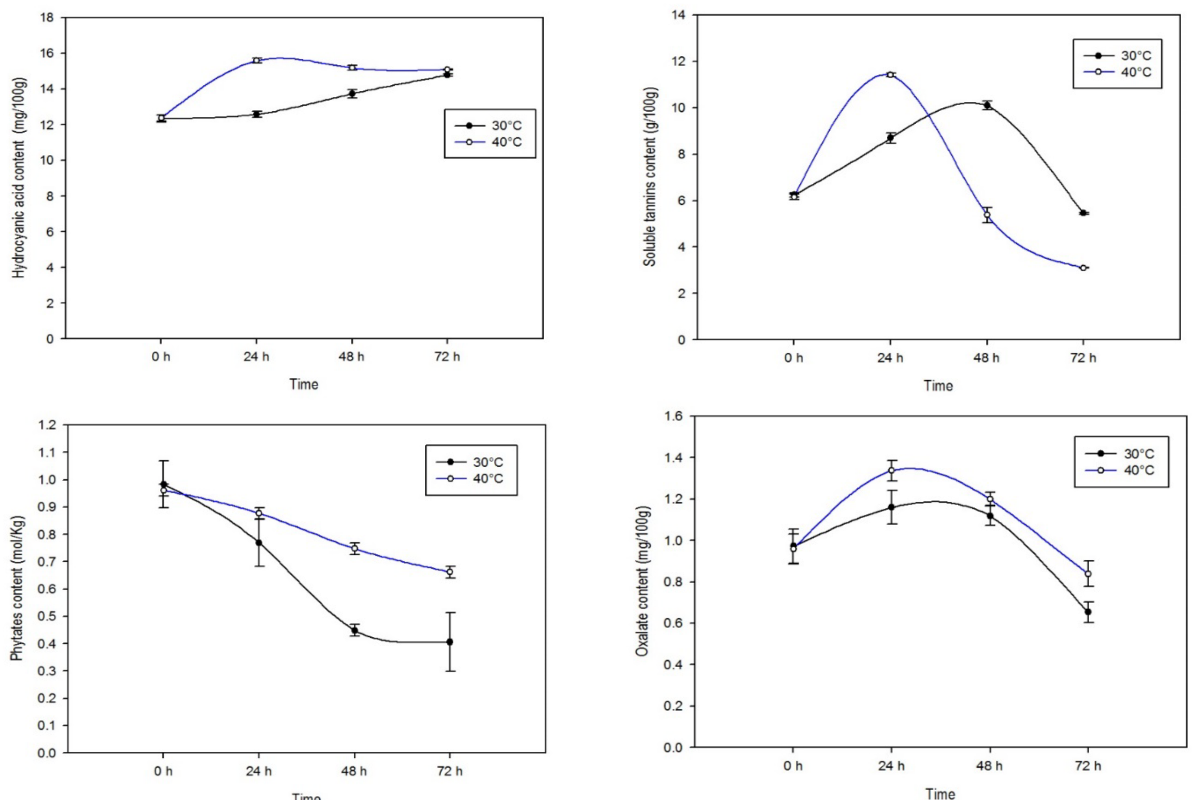
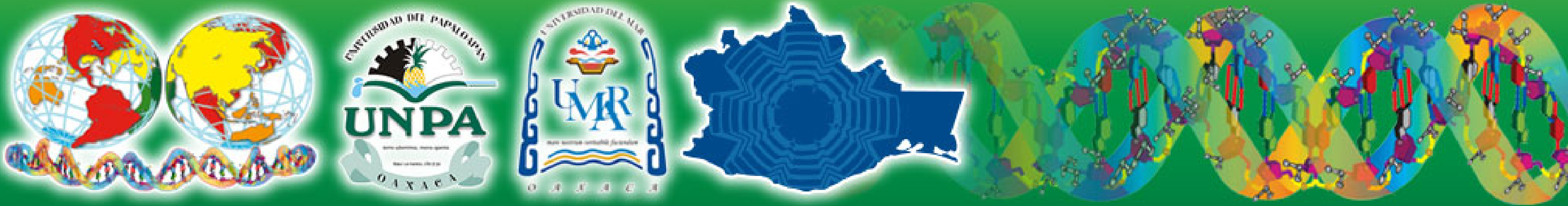


Figure 1. Kinetics of antinutritional factors on mango seed kernel flour by solid state fermentation with *A. niger* N402 30 and 40 °C. Values are mean of duplicate determinations from two independent experiments ± standard error.



Conclusion: The use of solid state fermentation process employing *A. niger* is a good method to reduce antinutritional factors present in mango seed kernel flour, lowering the contents about 40%, the best condition found in this study was at 30 °C until 72 hours. This treatment had a great impact in reducing of the AFNs preserving the nutritional compounds. So fermented mango seed kernel flour could be a principal ingredient for making products such as biscuits, pastry, also bread and pasta, even utilize it as an optimum protein supplementation in cereal mix products. The use of flour produced can benefit human nutrition in areas where this agro waste are abundant. It is then recommended that mango seed kernel be fermented before consumption.

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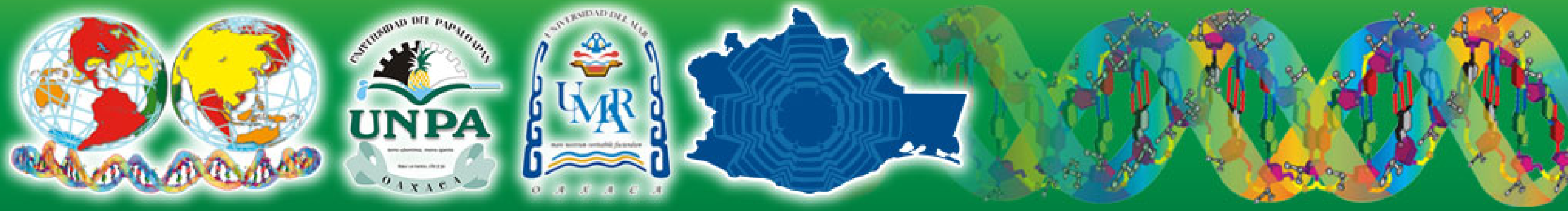
1.7 TRANSPARENT AREA



Artificial Intelligence applied to biotechnology

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Abstract: Sciences such as artificial intelligence and biotechnology do not seem to have much in common, but they indeed have crossed their paths and put their efforts together to come a long way in helping each other by means of artificial neural networks, genetic algorithms and multi agent systems applied to food technology, agricultural and livestock production and renewable energy generation.

Keywords: Artificial intelligence ● Biotechnology ● Artificial Neural Networks ● Genetic Algorithms.

Introduction: Life has become a subject of study for many disciplines, which while they may be very different and even opposite in its structure and mechanisms, they have the human well-being as central objective of their daily work. This talk presents together two of these apparently very different disciplines, whose achievements have impact on human welfare and they affect many aspects of everyday life, almost without us realizing their presence: artificial intelligence and biotechnology, with their different branches or specialties have jointly reached unthinkable goals until recently. Let us briefly consider each of these disciplines.

Background, aim, scope, and discussion:

Artificial intelligence. Artificial intelligence, which deals with the *study of computations that allow to perceive, reason and act* (Winston, 1992), seeks to understand the functioning of the brain and to mimic it in order to make machines more useful to humankind. Artificial intelligence focuses on developing and applying new techniques (paradigms) to classic problems that are often easy for a person but that its mechanization is no simple matter to teach to a machine, computer, algorithm or robot. You can also define artificial intelligence as a branch of computing which deals with the automation of intelligent behavior (Luger and Stubblefield, 1993). Artificial intelligence has had great progress since its origin in the Conference of Dartmouth College (Hanover, New Hampshire, USA) in 1956, that is, more than 50 years ago. It is there where the name artificial intelligence was first used and it opened a new field of formal study with the possibility of simulating human intelligence in a machine. Although it is a young science, advances in technology are taking artificial intelligence by the hand and thus allowing it to evolve very quickly.

Biotechnology. On the other hand we have biotechnology, which can be defined as any technological application that uses biological systems, living organisms, or by-products to make or modify products or processes for specific uses, according to the FAO (1992) on the Biological Diversity Convention which is an international treaty with three main goals: the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of benefits arising from the utilization of genetic resources. Its general objective is to promote measures that will lead to a sustainable future. In addition, this same international agency defines

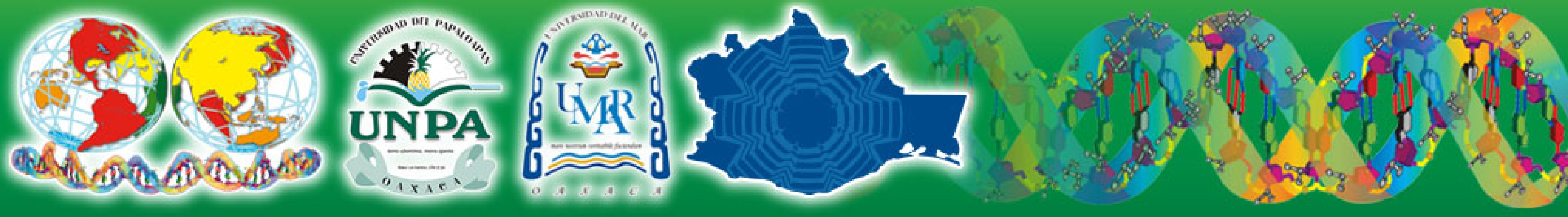
biotechnology as a "set of molecular technologies such as manipulation and gene transfer, DNA typing and cloning of plants and animals."

Artificial intelligence and biotechnology together. It is not unreasonable to say that both disciplines have very different objectives and functions: machines opposed to living organisms, algorithms opposed to production processes, field experiments opposed to simulators, reality (the real world) opposed to virtual reality. Diametrically opposed and yet... is there some point of convergence? Apparently, there is. In principle, they both seek human well-being and they both are based on the process of life and try to imitate and improve it; finally, they both require the power of technology: computers, sensors, circuits, etc. There is no technobiologist without a computer! So you can see that these disciplines can, in fact, merge but also complement each other and fit together in such a way that by joining their objectives they can come up with projects like an artificial nose, imitation of skin with bioengineering and artificial cloning, a farming multi agent system or renewable energies with artificial neural networks and genetic algorithms.

Artificial nose. Food is subject to quality control processes in order to authorize its trade and marketing (Pagani *et al*., 2014). Either by taste, touch or coloring, sensory analysis is considered to be the most subjective of all standards but it offers the least certainty. Sensory analysis is performed by experts in the tasting of food, but as every human being, experts in certain foods can be scarce (or not exist at all) or be wrong or get ill. Therefore, automating this delicate process is quite desirable. Emerging techniques and tools, which are closely correlated with sensory data, have been developed as alternatives to the human sensory assessment. Among these techniques, there is an electronic nose, a relatively new tool that can be used to monitor the safety and quality of a product, generating instant answers. The electronic nose is a device comprising a chemical sensors and an electronic system associated with artificial intelligence algorithms. Chemical identification is performed by comparison of the pattern of response of the sensor (electronic nose) of vapor (from the tested food) with previously established patterns of the corresponding known vapor (Nagle *et al*., 1998).

Artificial skin with bioengineering and artificial cloning. This work was developed jointly by the Universidad Autónoma de Bucaramanga and the University of Pamplona, Colombia (Muñoz *et al*., 2012). Their research project allowed to build a prototype of an intelligent lower limb prosthesis. Stem cells obtained with bioengineering and artificial cloning were used. The prosthesis is equipped with a system for data acquisition that takes signals from the healthy leg while in motion and are then replicated to be learned with training through artificial intelligence techniques; the signals are then imitated by artificial cloning in the prosthesis, where the behavior from the stem cells is copied and supported in the mechatronic systems (imitation patterns of movement - intelligent controller - articulation - sensors).

Multi agent system for farms. The role of agriculture has become an increasingly important process in food supply, environmental management and energy supply. Then, caring for the Earth is a fundamental factor which makes it crucial to maintain control over the use of the soils to prevent its deterioration and to ensure the economic sustainability of people, regions and countries.
This multi agent system (MAS) developed in Colombia (Wanumen, 2013), allows to simulate a farm divided into lots with livestock and farmland subsystems. This software implements separate and disjoint activities such as "cultivate", "grazing" and "land rest and recovery". The question is:



what is the strategy to achieve the greatest economic benefit while maintaining quality and productive capacity of the soil and optimally using the resources of the ranch? The system is designed to carry out actions taking into account variables such as the number of cattle taken down by illnesses even when they eat and move to different lots. In addition, you can choose to cooperate (or not) with the productivity system to meet goals like A. comply with higher productivity, B. do not allow cattle to die and C. move cattle to lots through the best route.
After a number simulations and parameter adjusting, some interesting conclusions arose such as: death of crops occurs when the initial payment is less than a certain parameter, but when it is higher than the same figure, the crop may die depending on other factors (life time or the attack of pests). It was also observed that increasing the initial fertilizer increases the total gain but there comes a time when more than the initial amount of fertilizer will not increase the gain of the system significantly. Furthermore, productivity increases when cattle are augmented but when there is too much cattle, total gain turns to zero: livestock dies so much because they are too many eating all the available grass on the farm…. dead cattle are very expensive!

Hybrid renewable energy with artificial neural networks and genetic algorithms. The Applied Science and Technological Development Center of the Autonomous National University of Mexico presents the development of a computational web tool, which optimizes the use of renewable energy in residential buildings, to meet a fraction of the energy needs in a hybrid way (electric and thermal) through the application of artificial intelligence techniques (Ochoa *et al.*, 2013).
From a web portal, users access the system to design a hybrid system of renewable energy, based on energy consumption needs estimated for a residential building for both electric and thermal energy; it also allows to estimate solar radiation with a large degree of certainty by using an intelligent model based on an artificial neural network architecture which takes meteorological measurements and solar radiation data obtained from the national weather database. The intelligent system implements an optimization model based on genetic algorithms whose fitness function is based on the economic savings generated by the use of a solar heater and a photovoltaic panel, as well as economic-environmental savings induced by non released greenhouse gas emissions; thus satisfying fully or partially the specified energy demand.
This tool has been transferred to the National Housing Commission, given that this project was funded by the Housing Conavi-Conacyt Fund Section. It was developed in ASP.NET platform and it uses a Microsoft SQL Server Data Base Manager.

Conclusions: Artificial intelligence extends very long arms (and not exactly of the robotic kind) to all the sciences and today I am very pleased to chat with you all, specialists and scholars of biotechnology, to thank you all for the great opportunity offered to us engineers in computer systems, both of this burgeoning field of artificial intelligence and many other computer specialties such as the BD and computer networks to name a few. I would also like to invite you to talk to your closer peers and ask us, dare us to support you on the wonders that biotechnology works. Let us give the opportunity to future professionals in these areas to work together and design promising projects that carry aloft the well-being of us all.

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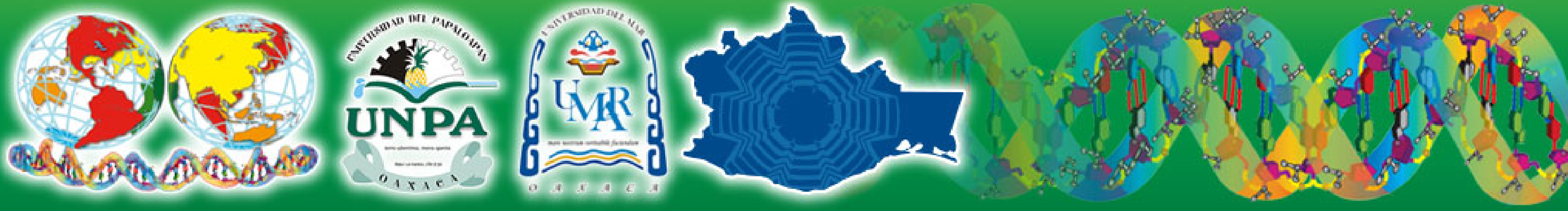

Attitudes toward animal research among Mexican undergraduate science students

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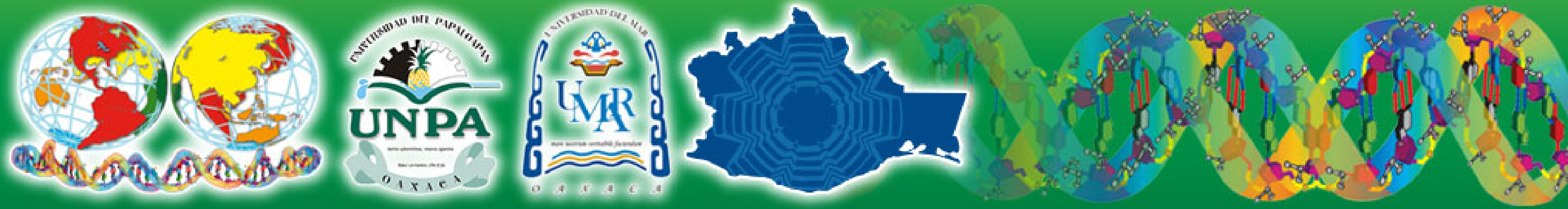
Teaching of bioethics has become an important part of scientific careers curricula offered by universities. One of the many topics covered during such courses is the use of animals in research, an issue that for the general public is very controversial given the growing concerns for animal welfare ultimately leading to the decreasing support for the use of animals in research, testing and entertainment. Attitudes regarding this topic from students of scientific careers are of particular interest, since they will likely be confronted with related situations during their training or as professionists. We have applied the Gallup & Beckstead questionnarie to a group of undergraduate mexican students to explore their attitudes toward animal experimentation. The survey sample consisted of 127 students currently enrolled in two Mexican universities; Universidad Autónoma de Nuevo León and Universidad del Papaloapan, majoring in diverse disciplines including, biology, chemistry, food science, nursing and biotechnology. Student participation was anonymous and voluntary, to participate they were provided a link to the online questionnarie to be completed on a web browser. The questionnarie also requested basic personal information from the participants; group age, gender, academic year, and career. According to the results, the students showed a strong concern for animal well-being and a strong agreement with the need for more regulation of animal research; at the same time, the surveyed students recognized the importance of animal research for the advancement of knowledge. This is a small-scale study, results are preliminar, a full-scale study is needed in order to reach solid conclusions.

1.8 IRIS AREA



Bioinformatics approach for microRNAs targets prediction and analysis in papillary thyroid cancer

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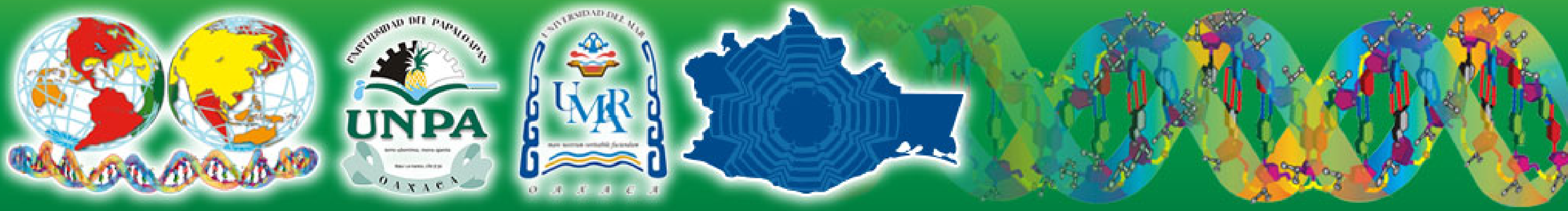
Abstract: Thyroid carcinoma is the most frequent endocrine cancer accounting for 1-2% of thyroid nodules. Papillary thyroid carcinoma (PTC) is the most prevalent form representing about 80% of all thyroid carcinoma. In this study, we applied a bioinformatics approach to analyze 38 microRNAs (miRNAs) related with PTC cases. We identified 8 down-regulated and 30 up-regulated miRNAs that exhibit a fold change ≥ 2. We then conducted target prediction and pathway enrichment analysis with these miRNAs to investigate the potential associated gene and pathway targets. Pathway analysis identified that some biological processes are regulated by altered microRNA expression, including cell proliferation, cell death, apoptosis and KEGG cancer pathways, cell cycle, focal adhesion, MAPK, mTOR, Wnt, ErbB, p53, ECM receptor interaction, *cytokine-cytokine receptor interaction,* Jak-STAT, TGF-beta, VEGF and –PI3K-Akt signaling pathway. We concluded that this set of deregulated miRNAs might have an important impact on cancer related cellular pathways whose role in papillary thyroid cancer has not been previously studied. Additional studies should be conducted to confirm our bioinformatics results.

Keywords: Papillary thyroid cancer ● MicroRNA ● Target prediction ● Pathway analysis

Introduction: Thyroid cancer is the most common malignancy of the endocrine system; it accounts for approximately 1-2% of all newly diagnosed cancer cases, and its incidence is increasing worldwide (Colamaio *et al*., 2011). Papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) are the most frequent thyroid tumors, corresponding to about 80% and 15% of all thyroid cancers, respectively. Both tumors are well differentiated, are originated in thyroid follicular cells, and may progress to completely non-differentiated anaplastic thyroid carcinoma (ATC), a very rare type of thyroid cancers (2–5% of all cases). PTC generally has a benign course, with a 10-year survival rate of more than 95% of patients (Marini *et al*., 2011; Hung and Sarlis, 2002). Overall, long-term outcomes for children and adolescents with PTC are excellent, with 2% cause-specific mortality at 40 years (Hay *et al.,* 2010).
Micro-RNAs (miRNAs) are small single stranded non-coding RNAs of about 18-25 nucleotides that are negative gene expression regulators. They are known to play important roles in various cellular processes including reproduction, proliferation, differentiation, cell survival and carcinogenesis (Braun and Hüttelmaier *et al.,* 2011). The deregulation of miRNAs expression is thought to be an important regulator of tumor development and progression in several human tissues. Several miRNAs are selectively increased in cancer cells, but more often, miRNAs show a decreased expression in cancer cells in comparison with normal cells. Considering their influence on cancer cell phenotype, some miRNAs are considered to be oncogenic (oncomiRs), while others are considered to be tumor-suppressive (TS-miRNAs; Samimi *et al.,* 2013). The overexpression of specific miRNAs leads to the repression of tumor suppressor gene expression,



and conversely the downregulation of specific miRNAs results in an increase of oncogene expression. These situations induce subsequent malignant effects on cell proliferation, differentiation, and apoptosis leading to tumor growth and progress (Grammatikakis *et al.,* 2013). Notably, it has been demonstrated that numerous miRNAs are transcriptionally deregulated in PTC when compared with unaffected thyroid tissues (Marini *et al*., 2011). To elucidate the potential role of deregulated miRNAs and miRNAs target in the carcinogenesis of PTC, we performed a bioinformatics approach.

Materials and Methods:

We made an extensive review of independent reports that collectively analyzed more than 387 thyroid tumors. We selected a set of differentially expressed miRNAs (DEMs) that were either upregulated or downregulated among distinct samples and/or experimental conditions, with a fold change ≥ 2. miRNAs targeted genes were searched using three gene target prediction algorithms in Diana-mirPath, namely: TargetScan v5, PicTar 4-way, and DIANA MicroT v4 (Papadopoulos *et al*. 2009). Subsequently, these target genes were validated through the search in databases of experimentally validated targets, such as miRTarBase and miRWalk (Hsu *et al.,* 2010; Dweep *et al*., 2011). The expression of DEM targets in cells and tissues examined was verified using UniGene database. Finally, molecular networks and pathways related to DEM targets were identified by using pathway analysis tool DAVID, version 6.7 (Huang *et al*., 2009).

Results and Discussion:

We identified 30 upregulated miRNAs species in PTC: hsa-miR-146b, 187, 222, 221, 32, 551b, 21,155,122a, 31, 205, 224,181b,125b, 222a, 96, 220, 34a, 30d, 375, 146a, 181a-2-3p, 15a-3p, 26a, 30a-5p, 181a, let-7c, 181c, 181a-3p, 223, and 135b. While the downregulated miRNAs included hsa-miR-345, 300, 374b, 218, 7, 486-5p, 451 and 335. In PTC tissue, we identified 26 targets for downregulated miRNAs and 194 targets for upregulated miRNAs. The characterization of the molecular network of these 220 potential targets, theoretically deregulated in PTC tissues, evidenced the relevance of cancer-related pathways (p=3,4E-34), that are strongly enriched in the annotation categories as shown in Figure1. The upregulated target genes are indicated in yellow, while downregulated target genes of miRNAs in red (Figure 1). Gene ontology enrichment analysis of miRNA-targeted genes using the Functional Annotation tool DAVID revealed that several pathways and molecular functions are modulated in PTC tissues, including cell cycle, focal adhesion, MAPK, mTOR, Wnt, ErbB, p53, ECM receptor interaction, *cytokine-cytokine receptor interaction,* Jak-STAT, TGF-beta, VEGF and the, PI3K-Akt signaling pathway. Some important genes reported in other cancers were also found in our analysis, for example; CCND1, IGF1, PTEN, RASA1, AKT3, APC, CASP3, SOS1, and WNT1, among others.

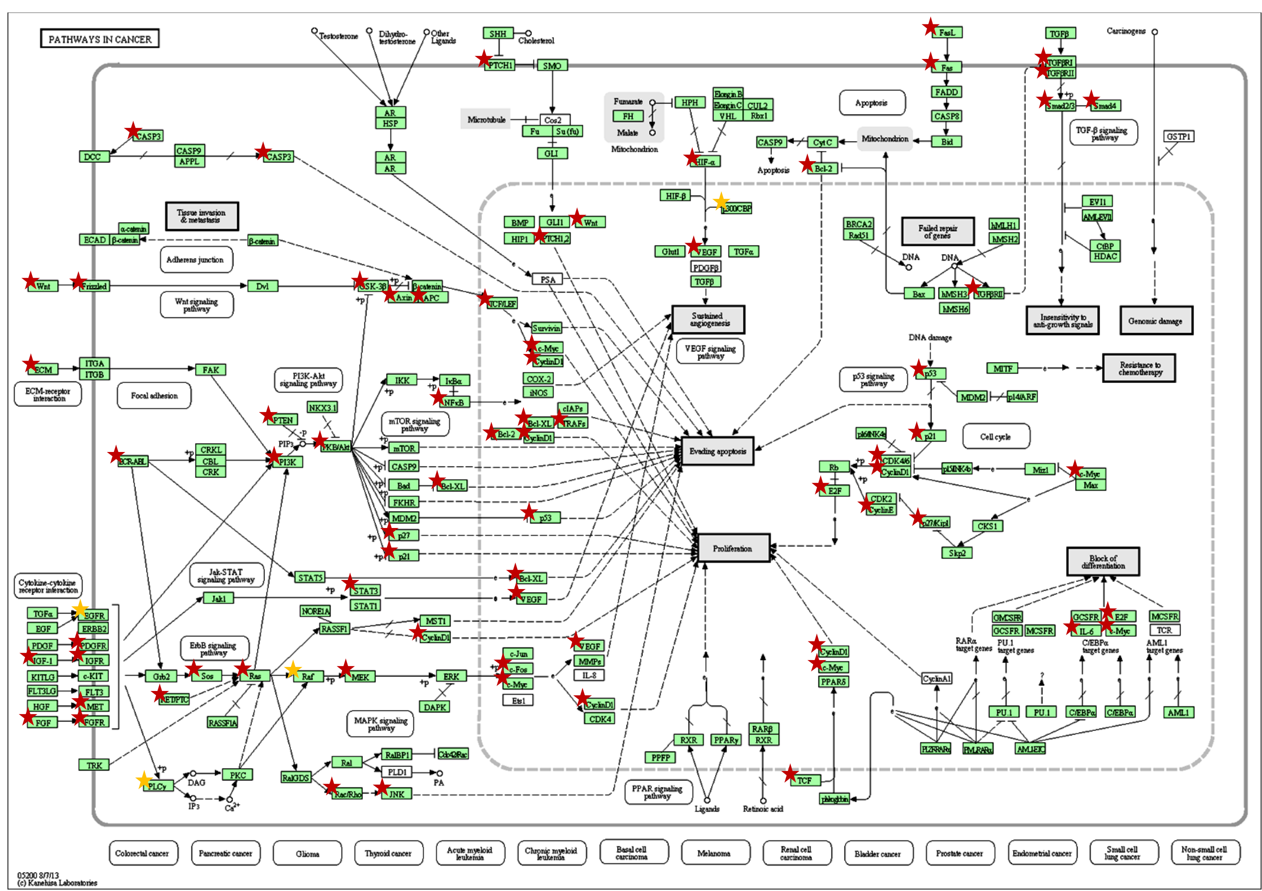


Figure 1. Gene network of the differentially expressed genes in papillary thyroid cancer involved in cancer-related pathways.

Conclusions: Our results indicate that the identification of deregulated *miRNA and their target-genes, together with* their effects on signaling pathways and regulatory processes of cell proliferation, cell death, apoptosis and angiogenesis, might contribute to a better understanding of the molecular mechanisms involved in PTC. Experimental studies are required to confirm our bioinformatics findings and further contribute to the development of miRNA-based therapeutics.

Acknowledgments: This work was supported by SIP-IPN, COFAA-IPN.

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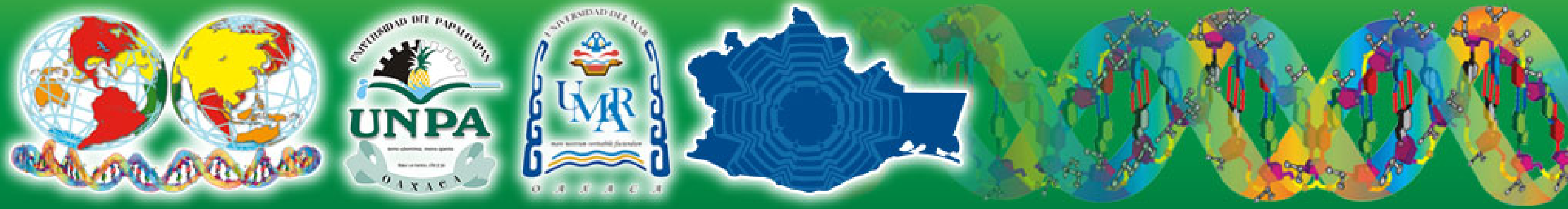

In silico design of multiple input genetic circuits using logic gates

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Abstract: Many synthetic genetic circuits have been proposed aiming to obtain processing and control devices. Here we depict an *in silico* design of a set of logic gates based on a previously exposed three-terminal architecture named the transcriptor that uses bacteriophage serine integrases to control the flow of RNA polymerase along DNA. The main objective of this work is to design biological logic circuits by a computer simulation approach simplifying the logic circuits engineering. It was established a mathematical model representing the dynamics of the logic gate's components using both deterministic and stochastic approximations. Two biologic virtual circuits were constructed: a full adder and a 2x4 decoder. Results suggest that it is possible to acquire predictive information from models previously to circuit construction phase. This allows the preview of issues related to biologic systems such as synchronic genetic expression, undesirable transitory expression and incompatibility between gates in consequence of the stability of involved molecules.

Keywords: Logic gates ● Deterministic ● Stochastic

Introduction: Boolean logic gates integrate multiple digital inputs into a digital output. In cells, regulatory networks encode logic operations that integrate environmental and cellular signals (Morris *et al*., 2010). Researchers have independently constructed artificial logic gates that have been used in pharmaceutical and biotechnological applications (Callura *et al*., 2012). However, it remains difficult to predict how a combination of gates will behave based the function of each individual gate (Cheng and Lu, 2012; Guet *et al*., 2002).
From the synthetic biology's point of view, most studies involving biological design are carried out empirically with a relatively small number of repurposed parts without predictive modeling. Thus, significant efforts to develop an integrated and extensible biological design cycle are necessary. This design cycle would enable practitioners to develop high-level conceptual designs, translate these designs into potential circuit implementations, model and verify their behavior *in silico*, construct the designs and modulate and probe the resulting constructs for proper operation (Cheng and Lu, 2012).
Following the idea of this integrated design cycle, and as part of it, we realized the study of dynamic behavior and the use of stochastic modeling in order to reproduce the experimental data and to predict the operation of a set of logic elements encoding Boolean AND, NAND, OR, NOR, XOR and XNOR functions proposed by Bonnet *et al*. (2013). We also used these models to construct two electronic circuits *in silico*.

Materials and Methods:

The architecture of each logic gate consisted of a three-terminal device where input and output signals were composed of transcription rates at positions on DNA marking logic element boundaries.

Table 1. ODE's system described a single logic gate's dynamic. The constants k represent synthesis or degradation rates if they are in positive or negative terms, respectively.

Molecule	Expression	Molecule	Expression
Integrase A mRNA	$\frac{dIntAm}{dt} = k_{tra} \cdot P_{BAD} - k_{dam} \cdot IntAm$	Inverted state of integrase A target	$X_{LRa} = \frac{IntA^n}{IntA^n + a^n}$
Integrase A	$\frac{dIntA}{dt} = k_{tna} \cdot IntAm - k_{dap} \cdot IntA$	Inverted state of integrase B target	$X_{LRb} = \frac{IntB^n}{IntB^n + b^n}$
Integrase B mRNA	$\frac{dIntBm}{dt} = k_{trb} \cdot P_{Tet} - k_{dbm} \cdot IntBm$	Gfp mRNA	$\frac{dGFPm}{dt} = k_{trg} \cdot f(X_{LRa} \cdot X_{LRb}) - k_{dgm} \cdot GFPm$
Integrase B	$\frac{dIntB}{dt} = k_{tnb} \cdot IntBm - k_{dbp} \cdot IntB$	Gfp	$\frac{dGFP}{dt} = k_{tng} \cdot GFPm - k_{dgp} \cdot GFP$

Logic elements used asymmetric transcription terminators as reversible check valves that disrupted RNA polymerase flow. For every gate, the terminators position, and consequently, the transcription rates were regulated by the inducible expression of a pair of integrases. This goal was achieved using two inducible promoters sensitive to arabinose (ara) and anhydrotetracycline (aTc) controlling the expression of TP901-1 and Bxb1 integrases, respectively. The integrases allowed the inversion of targeted genetic elements, in this case, asymmetric transcription terminators and a promoter. These integrases were used as a 2-input signal and, depending on a specific gate's construction, their presence-absence combinations controlled the 1-output GFP expression signal.

In order to represent the behavior of every single logic gate, we set a system of six ordinary differential equations. Each ODE describes the dynamic of a single molecule in the network: integrase A, integrase B, their respective mRNA, GFP and its mRNA. Table 1 shows the ODE's system. The production term of the output signal (Gfp) mRNA contains a specific gate expression that relates each logic function with the integrase's control signals. The functions were obtained from enzymatic kinetics and are showed in Table 2. We translated the ODE system to a stochastic approach given by Gillespie's algorithm, which describe the system through the probabilities for it state defined by the number of molecules of each type at a given time, replacing the differentiable concentrations (Thattai and Van Oudeaarden, 2001).

Two electronic based circuits were assembled using both deterministic and stochastic methods: a full adder and a 2×4 decoder. For this purpose, multiple gates were layered using integrases expression as wires. Simulations were carried out using Matlab software package.

Results and Discussion:

Simulations were carried out using all the possibilities within a 2-input truth table for all gates and expression levels obtained were normalized. Figure 1 shows the results obtained.

Table 2 (Left). Logic functions controlling output signal. **Figure 1** (Right). Predicted logic.gate performance. Light green and dark green bars stand for deterministic and stochastic normalized results, respectively.

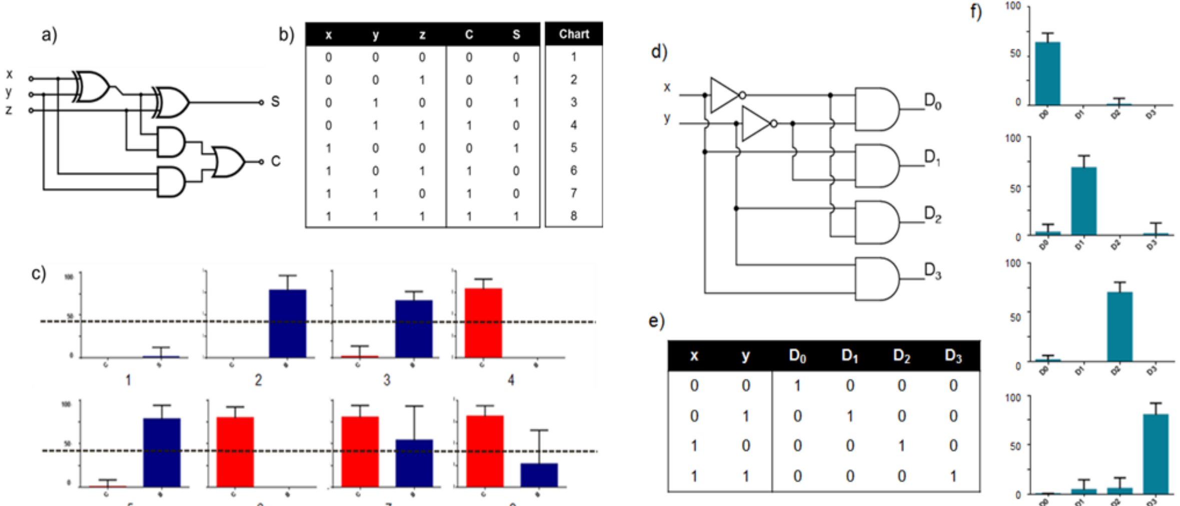
Operation	Expression
AND	$f_{(ON)} = \frac{XlrA \cdot XlrB}{1 + XlrA + XlrB + XlrA \cdot XlrB}$
NAND	$f_{(ON)} = \frac{1 + XlrA + XlrB}{1 + XlrA + XlrB + XlrA \cdot XlrB}$
OR	$f_{(ON)} = \frac{XlrA + XlrB + XlrA \cdot XlrB}{1 + XlrA + XlrB + XlrA \cdot XlrB}$
NOR	$f_{(ON)} = \frac{1}{1 + XlrA + XlrB + XlrA \cdot XlrB}$
XOR	$f_{(ON)} = \frac{XlrA + XlrB}{1 + XlrA + XlrB + XlrA \cdot XlrB}$
XNOR	$f_{(ON)} = \frac{1 + XlrA \cdot XlrB}{1 + XlrA + XlrB + XlrA \cdot XlrB}$

Gate	Truth Table		
AND	0	0	0
	1	0	0
	0	1	0
	1	1	1
NAND	0	0	1
	1	0	1
	0	1	1
	1	1	0
OR	0	0	0
	1	0	1
	0	1	1
	1	1	1
NOR	0	0	1
	1	0	0
	0	1	0
	1	1	0
XOR	0	0	0
	1	0	1
	0	1	1
	1	1	0
XNOR	0	0	1
	1	0	0
	0	1	0
	1	1	1

Deterministic system was able to correctly reproduce each logic operation gate. However, it was complicated to establish a threshold value in order to accurately define ON/OFF output states. This is due to the restrained dynamic range of the AND and OR logic gates. This effect was not an issue with the stochastic model. Stochastic model was described by the probabilities reflected in the propensities from each reaction, and logic functions are implicit in them. This probabilistic scenario enhances the dynamic range for AND and OR gates, and consequently decreases the probability of getting false ON/OFF output states (Guet *et al*., 2002). Furthermore, stochastic model considers intrinsic noise, and consequently allows a better representation of the random nature inherent to any genetic expression system. Nevertheless, a greater expression level variation was also observed from stochastic model in comparison to experimental results, which complicates output digitalization.

The output variation issue was overcome considering integrases encoded genes copy number. Experimentally, Bxb1 and TP901-1 genes were cloned in plasmids with pSC101 origin of replication. pSC101 origin leads to low plasmid copy numbers, ranging from 5 to 10 copies (Peterson and Phillips, 2008). It was observed, as expected (Gonze *et al*., 2002), that coefficient

of variation decreases as the copy number increases. According to these probes and to information previously reported, a copy number of 7 plasmids was settled for subsequent simulations.
Previous efforts have been made in order to create genetic logic gate collections (Tamsir *et al*., 2011; Moon *et al*., 2012); however they were constructed through the assembly of a reduced group of simple gates. This increases the size and complexity of circuits made with multiple gates and hampers their physical construction. Also, it has been found that in biological networks, the noise is transmitted from upstream genes and became more significant in longer cascades (Pedraza and Oudenaarden, 2005). In this context, the set of logic gates studied here is advantageous given that each of them is constructed independently.

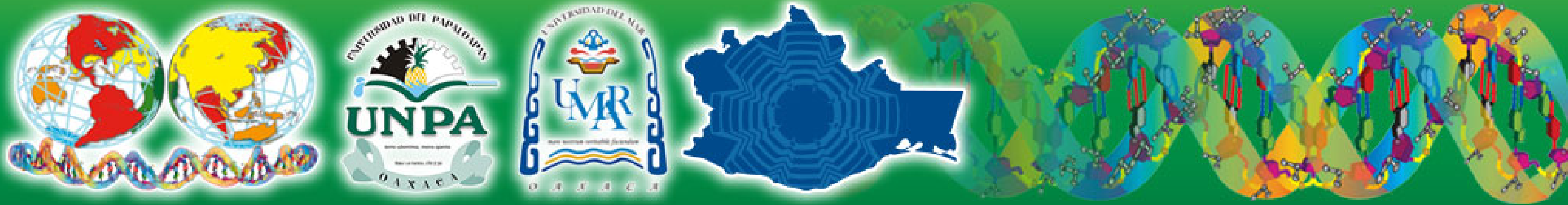


x	y	z	C	S	Chart
0	0	0	0	0	1
0	0	1	0	1	2
0	1	0	0	1	3
0	1	1	1	0	4
1	0	0	0	1	5
1	0	1	1	0	6
1	1	0	1	0	7
1	1	1	1	1	8

x	y	D_0	D_1	D_2	D_3
0	0	1	0	0	0
0	1	0	1	0	0
1	0	0	0	1	0
1	1	0	0	0	1

Figure 3. Full adder and 2×4 decoder circuit assembles. Left-sided schemes represent the full adder circuit, whereas at right is exhibited the 2×4 decored. a) and b) symbolize the building diagrams, b) and e) depict the true tables, c) and f) show the stochastic prediction for each input combination. In c) blue and red bars stand for sum (s) and carry (c) outputs, respectively.

As stated before, two virtual circuits were assembled by means of proposed models layering logic gates: a full adder and a 2×4 decoder. These constructions are inspired in integrated circuits, with the idea to allow the introduction of electronic engineering and design concepts in biologic systems. The complete adder is a combinational circuit that permits three-bit input addition. The other circuit, a decoder is a digital function that translates binary information from one coded form to another. Figure 2 shows the circuit's structure, true tables and expression levels predicted by stochastic models.
The full adder can accept eight different input combinations. Its dynamic range easily differentiates ON/OFF output states for six cases, except for the last two combinations (110) and (111). The digitalization cannot be achieved as consequence of integrases transitory expression that should be in OFF state. It is suggested that transitory expression generates sufficient enzyme molecules to perform the inversion process of its targets leading to erroneous logic operations. The 2×4 decoder presents similar issues, however, the transitory effect was eliminated raising the probability of involved integrases degradation (output NOT gates signals, see scheme). Analogously, half-life of integrases can be experimentally reduced with degradation tags.



Conclusions: Predictive computational models that are validated by experimentation represent a well-suited tool for acquiring predictive information of specific circuit's designs. The analysis proposed here expedites the construction of logic circuits of higher complexity using layered gates.

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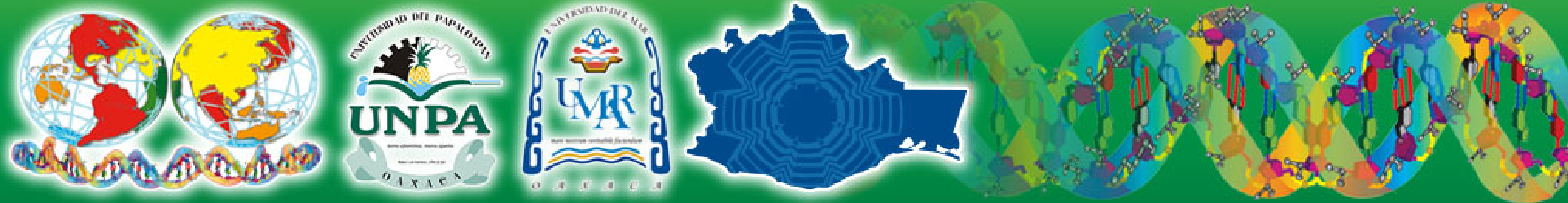

Identification of calcium-activated chloride channels in sea urchin sperm

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Abstract: Searching for membrane receptor proteins from sea urchin spermatozoa we discovered a member of the TMEM16 family protein, also named anoctamins (ANOs). This novel ion channel family is responsible of calcium activated chloride currents, whose members share considerable homology at the eight transmembrane segments (TM1-TM8) and particularly around the pore forming region including TM5 and TM6. We also found other two members (predictions) in the *Strongylocentrotus purpuratus* genome. Subsequently, by molecular biology methods, the three transcript sequences were obtained. This work compares their primary structures of the pore region with the ten members of the mammalian TMEM16 family and presents the first evidence for the presence of calcium dependent chloride channels, in sea urchin spermatozoa.

Keywords: Calcium-dependent chloride channels ● sea urchin sperm

Introduction: Sea urchin spermatozoa are model cells for studying signal transduction events underlying flagellar motility. According to the current model of the signaling pathway triggered by speract, the sperm activating peptide in *S. purpuratus*, a Calcium-activated Chloride Channel (CaCC) could be modulating the calcium influx (Darszon *et al.*, 2008; Aguilera *et al.*, 2012), consequently, the flagellum motility.
Calcium-activated chloride currents are present in most cell types and control the most diverse functions such as photo transduction or neuronal and cardiac excitability, epithelial Cl^- secretion and fertilization (Kunzelmann *et al.,* 2009; Duran *et al.*, 2010). TMEM16 protein family or anoctamin family is a novel ion channel family responsible of calcium activated chloride currents, whose members (10 in mammals) have eight transmembrane domains (Hartzell *et al.,* 2009). They are found in all eukaryotes, however, the functions of someTMEM16 proteins remain unknown. While isolating plasma membrane receptor proteins from sea urchin spermatozoa, we discovered a 122 kDa sperm membrane protein. Peptide sequences were obtained, and the cDNA sequence was cloned from sea urchin testis. Secondary structure predictions show eight putative transmembrane segments. We found the cDNA sequence corresponds to a member of the TMEM16 family. The full-length cDNA sequence encoding this new channel, and two more TMEM16 members were obtained by standard molecular biology methods.
The present report describes for the first time the presence of three members of the TMEM16 family in sea urchin testes. Phylogenetic analysis, comparisons of the primary structures, especially from the putative ion pore region, of the three SpTMEM16s with the 10 members of the mammalian TMEM16 family, and analyses of expression were conducted for the three SpTMEM16s.

Materials and Methods:

Sperm and isolation of membranes. Sperm of the sea urchin *S. purpuratus* were spawned by injection of 0.5 M KCl. Undiluted sperm were stored on ice for <12 h before use. Sperm were homogenized to break the flagellum from the sperm head and these two parts of the cell were separated by differential sedimentation (Vacquier and Hirohashi, 2004).

Cloning. Flagellar membranes were passed through a WGA column to isolate potential glycosilated membrane proteins. One of the bands obtained, of 122 kDa, was subjected to tandem mass spectroscopy. A BLASTp search at NCBI (*National Center of Biotechnology Institute*) was performed using the "RefSeq Protein" database from the sea urchin genome (Sodergren *et al.* 2006). A predicted partial gene sequence for this and two more predicted TMEM16 gene sequences were used to design exact primers to get the whole SpTMEM16 sequences. The full-length cDNA sequences were then obtained by PCR amplification using a testis cDNA library as template. Testis total RNA was also isolated and cDNA was synthesized by standard procedures. 5' RACE was performed to obtain the 5' end, and the 3' ends. PCR amplifications were performed under the condition of pre-denaturation at 94 °C for 4 min, 32 cycles of 94 °C for 1 min, 55–65 °C for 30 s and 72 °C for 1.5 min. The expected PCR fragments were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA) and sequenced.

Sequence analysis. The secondary structure prediction shows eight putative transmembrane segments. Sites and motifs were found using ProfileScan (http://hits.isb-sib.ch/cgi-bin/PFSCAN) and transmembrane segments and domains were predicted with SMART (http://smart.embl-heidel berg.de/) (Schultz *et al.*, 1998). BioEdit and MEGA6 programs (Hall, 1999; Tamura *et al.,* 2013) were used for alignments. The GenBank accession numbers for SpANO4, SpANO7 and SpANO10 are: KM017612, NP_001232902 and NP_001232900, respectively.

Phylogenetic analysis. Complete sequences were used to generate a neighbor-joining phylogenetic tree of ANO proteins using MEGA6 (Tamura *et al.,* 2013) and 5000 replications. (Figure 3).

Results and Discussion:

The three complete sequences were analyzed and they share a predicted topology of eight transmembrane segments, denoted as S1–S6 in Figure 1, and the putative ion pore region, boxed in yellow.
An antibody raised against a region between TM5 and Tm6 of SpTMEM16D (labeled as Ab, in Figure 1) shows a preferent distribution at flagellum and mitochondrion´s region, the power supply of the flagellum (data not shown).
The putative pore forming region for ANO channels has been suggested by mutating conserved positively charged amino acids between TM5 and TM6, which changed the ion selectivity of the channel (Yang *et al*., 2008). ANO1 (TMEM16A) and ANO2 (TMEM16B) confers receptor-activated calcium-dependent chloride conductance, (Yang *et al*., 2008). Also, three cysteines have been identified in the pore forming loop by binding to cysteine-reagent MTSET which indicates those cysteines are exposed (Yang *et al*., 2008). We also identified those cysteines in SpTMEM16 sequences, labeled with blue arrows in Figure 2. There is also a small hydrophobic region (LSIIM) between TM5 and TM6 (the putative p-loop) well conserved in all TMEM16 members, this region

is found in the canonical ion channel pore (Kunzelmann *et al.,* 2009). In all three SpTMEM16 proteins we found this small hydrophobic region, for *Sp*TMEM16D (IAITM), *Sp*TMEM16G (LFIIM), and for *Sp*TMEM16K (LSTLLI) (See Figure 2).

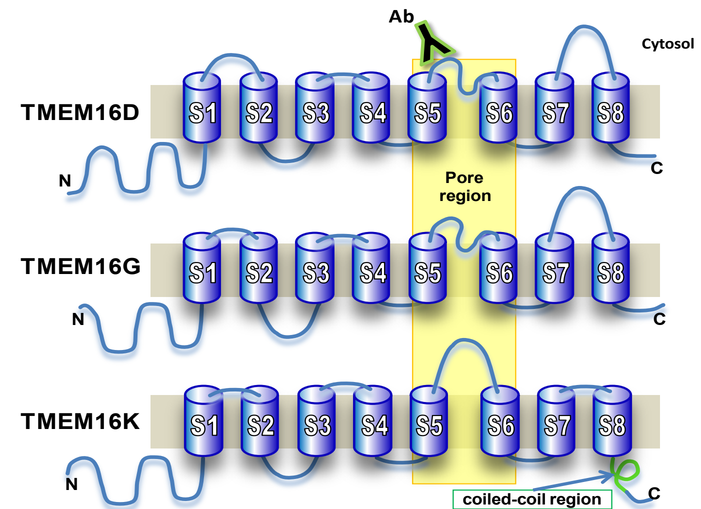

Figure 1. Hypothetical two-dimensional model of SpTMEM16s.

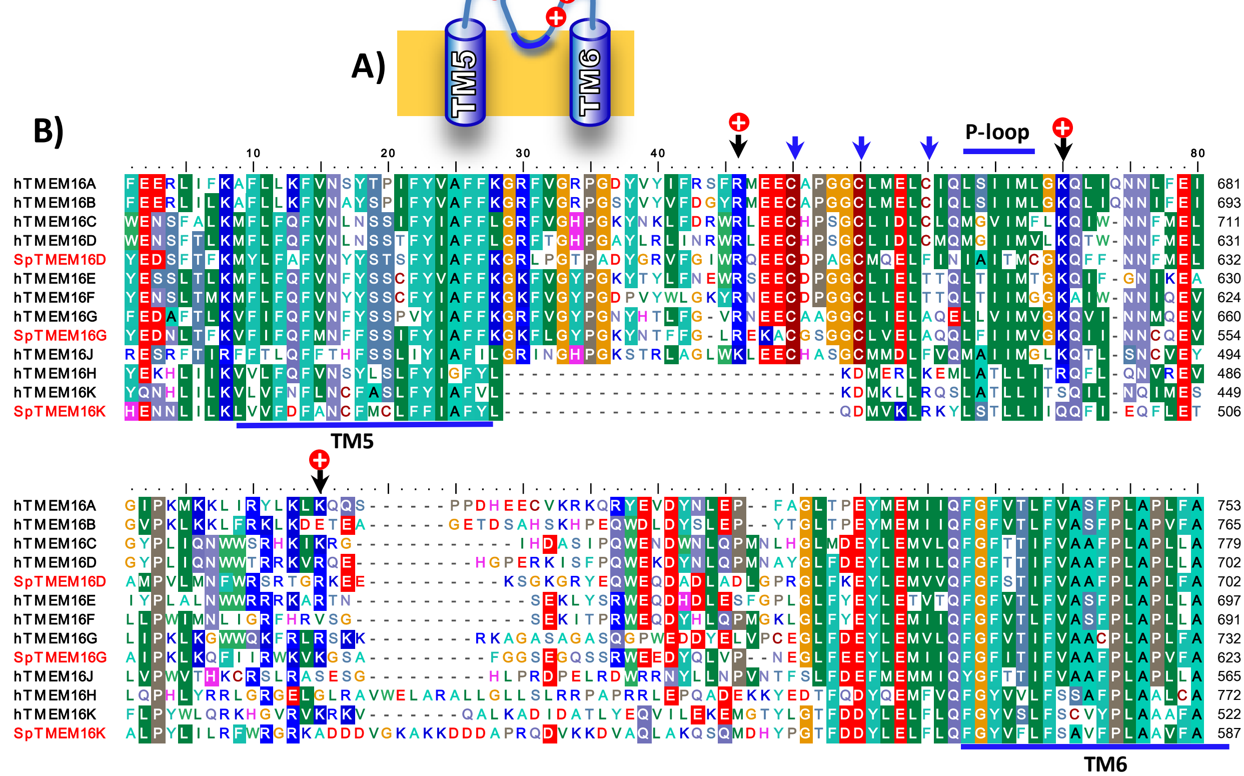

Figure 2. Pore region comparison of SpTMEM16s (red) with the human TMEM16 family. A) Pore region model. B) Alignment of the putative pore region of TMEM16s.

The region between TM5 and TM6 in TMEM16H and TMEM16K are very dissimilar to the other TMEM16 members, they do not have the three cysteines mentioned above. Moreover, the three positively charged amino acids are not completely preserved. Then, *Sp*TMEM16K does not have any of the cysteines and the region between TM5 and TM6 is very short. While structure predictions suggest chloride channel function for TMEM16A-J, there is no clear prediction for TMEM16H and TMEM16K. A radiation and neighbor-joining tree was constructed with 36 selected full-length TMEM16 sequences present in GenBank and SpTMEM16 members found in sea urchin, highlighted in yellow in Figure 3. The ten human homologues are in blue. A human voltage-dependent chloride channel was used as outgroup. Units in Figure 3 indicate the number of amino acid substitutions per site and bootstrap support values are shown at nodes. The homologues parse into distinct families and plants cluster together. The tree shows that the differentiation of these genes is ancient.

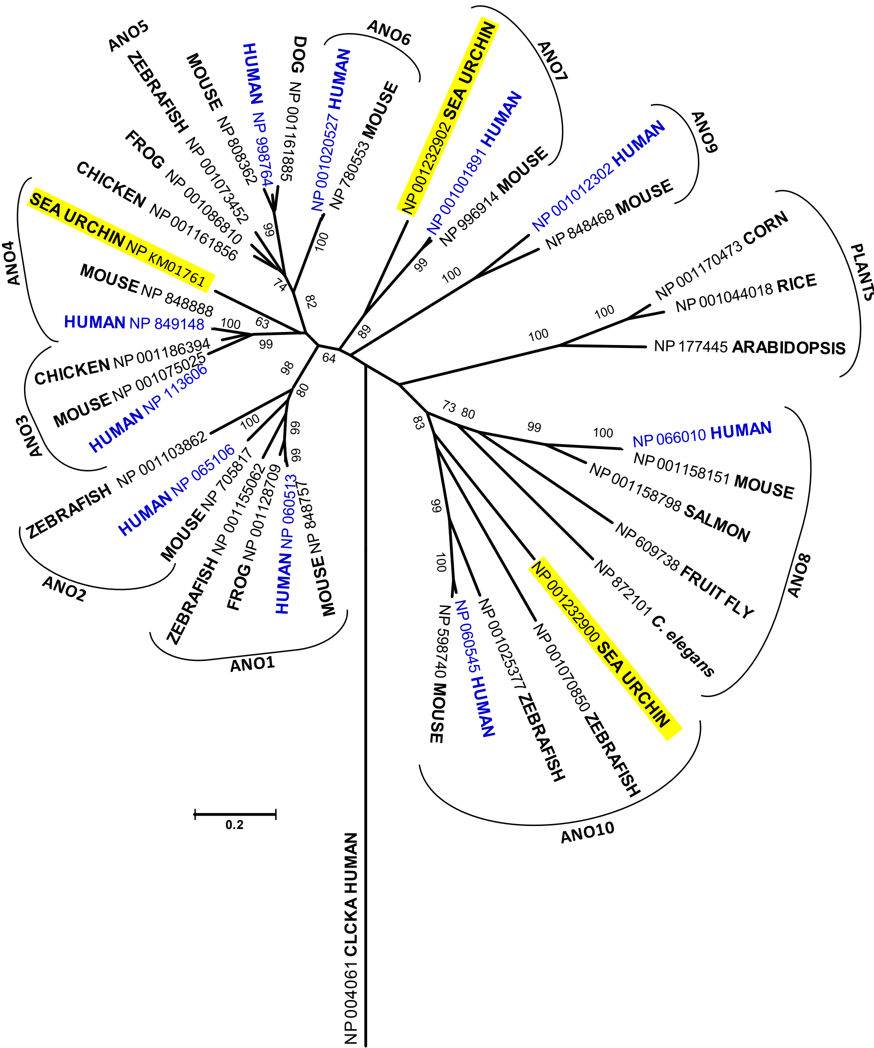

Figure 3. Phylogenetic relations of the TMEM16 (ANO) family.

Conclusion: Three members of the TMEM16 family were discovered in sea urchin sperm. These proteins are evolutionarily basal to other TMEM16 members. Although Sp TMEM16D and Sp TMEM16G share the secondary structure characteristics of the mammalian ANO members 1 to 4, so do Sp TMEM16K with its corresponding mammalian homolog, it will be important to study their electrical properties, ion selectivity, and calcium dependence in order to help to elucidate their function as CaCCs and their participation in the signaling pathway triggered by speract and their possible role in flagellum motility modulation.

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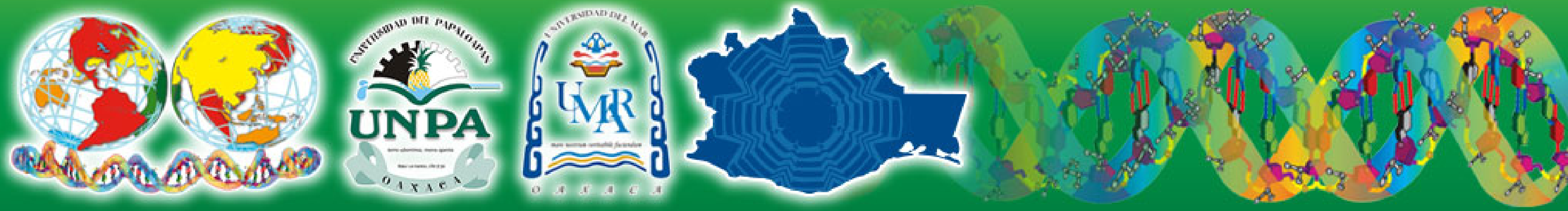

Pharmacological evidence supports CFTR participation in the sea urchin sperm motility

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Abstract: Sperm motility is a prerequisite for the fertilization process. In this work, we explore the involvement of Cl^- in the motility of *Strongylocentrotus purpuratus* sperm by analyzing sperm swimming in a confined space in the presence of different inhibitors of Cl^- transport. An algorithm was developed based on the particle tracking to obtain the swimming trajectories. Then, an analysis of instant speed and mean radius of curvature of sperm motion was performed. It was found that the trajectory, instant speed and changes in mean radius of curvature are significantly altered in the presence of six inhibitors of channels or anion transporters: DIDS, DPC, NPPB, Bicuculline, R-(+)-DIOA, and CFTRinh−172. Five of them are inhibitors of a Cl^- channel (the CFTR), which is known produce the cystic fibrosis disease in humans. Our results suggest that the Cl^- transport has an important role in sperm motility. And because the inhibition with CFTRinh−172 (a specific inhibitor for CFTR) we believe that this channel is responsible for at least part of the Cl^- transport across membrane during sea urchin sperm motility.

Keywords: Sperm motility ● chloride transport ● CFTR

Introduction: Chloride (Cl^-) is the most common anion present under physiological conditions and it has an essential role in many physiological processes. Like the K^+, Cl^- helps to keep potential cell membrane and it is involved in the processes of hyperpolarization and repolarization of cell membrane potential. The Cl^- can be internalized into the cell through various transport mechanisms such as ion channels and transporters. The intracellular Cl^- levels are determined by the contribution of all Cl^- transports present in the plasmatic membrane of each cell type. Despite the importance of Cl^-, there is not a description of the mechanism that regulates the transport of Cl^- in sea urchin sperm.

Sperm motility is a prerequisite for the fertilization process. It is known that sea urchin sperm, while being in the gonads, are immotile and their motility is subsequently activated when they are released into the external environment (Darszon *et al*., 1999). Sperm flagellar beating is regulated by the ionic composition of the medium. It has been shown that different cations such as Ca^{2+}, Na^+, and K^+ have a direct or indirect effect in motility (Darszon *et al*., 2008). However, nothing is known about the involvement of Cl^- in such an important function as is sperm motility for the fertilization process. The present work focuses on studying if the Cl^- permeability has an effect on sperm motility of sea urchin *S. purpuratus* by analysing sperm swimming in a confined space in the presence or absence of Cl^- transport inhibitors.

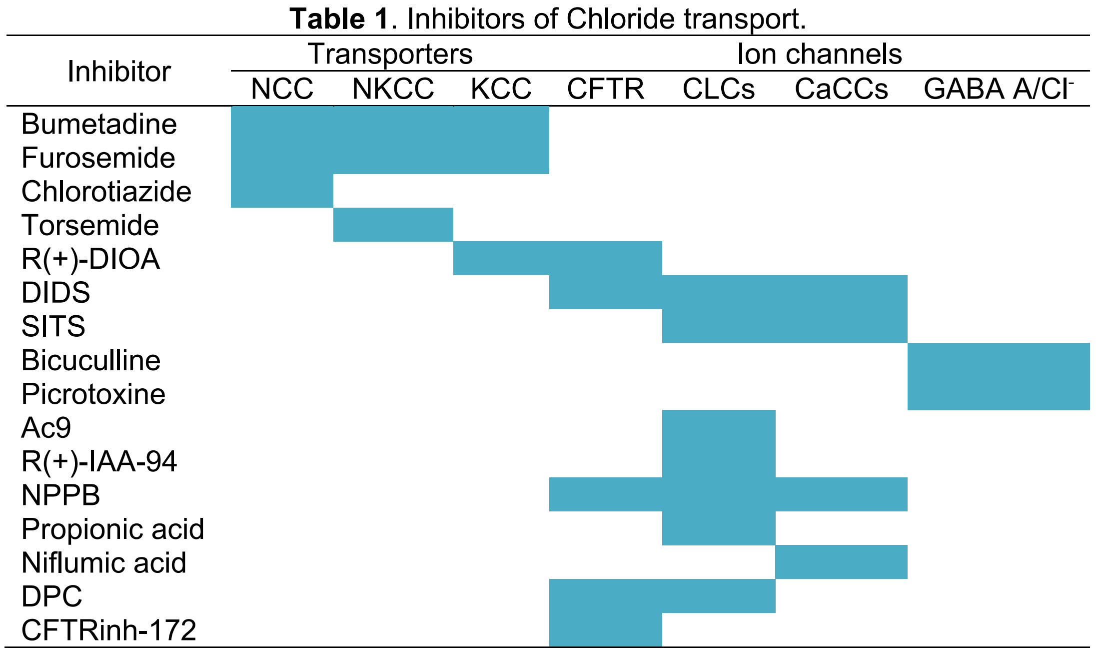
CFTR is a Cl^- channel whose mutation results in human cystic fibrosis. It is known, by immunolocalization, and pharmacological evidence that CFTR is present in mammalian sperm (Figueiras *et al*., 2013), and that it is essential for fertilization but nothing is known about its role in sperm motility (Chu *et al.,* 2010). According to our results, it is likely that a channel of this type

is present in sea urchin sperm and that it may be actively involved in the regulation of ion permeability required for motility.

Materials and Methods:

Sperm of the sea urchin *Strongylocentrotus purpuratus* were spawned by injection of 0.5 M KCl, and diluted 1:1000 in artificial seawater (ASW) at 4 °C and pH 7.0, to keep still until the time of use. For each assay, a second dilution, 1:100 in pH 8.0 ASW (ASW8), was made in the presence or absence of different inhibitors of Cl^- transport (see Table 1). Forty seconds later, 30 s videos of swimming sperm were acquired.

Table 1. Inhibitors of Chloride transport.



Inhibitor	Transporters			Ion channels			
	NCC	NKCC	KCC	CFTR	CLCs	CaCCs	GABA A/Cl^-
Bumetadine	■	■	■				
Furosemide	■	■	■				
Chlorotiazide	■						
Torsemide		■					
R(+)-DIOA			■	■			
DIDS				■	■	■	
SITS					■	■	
Bicuculline							■
Picrotoxine							■
Ac9					■		
R(+)-IAA-94					■		
NPPB				■	■	■	
Propionic acid					■		
Niflumic acid						■	
DPC				■	■		
CFTRinh-172				■			

Chambers for video recording. Disposable chambers of Polydimethylsiloxane (PDMS) with the "SYLGARD ® 184 SILICONE ELASTOMER KIT" (Dow Corning Corporation) were made as follows: 4 mL of PDMS were poured in a glass of 5 cm by 5 cm and left to polymerize at 120 °C for 30 minutes, so that a square of PDMS ~ 1 mm high and 5 cm per side was formed. The square was cut into four equal parts and two joined circles of 8 and 11 mm in diameter each, were drilled at each quarter. Chambers were placed on coverslips treated with 0.05% w/v of polyvinylpyrrolidone (PVP40) (Sigma-Aldrich, St. Louis, MO, USA), to prevent sperm from sticking to glass.

Video acquisition was made by a MyoCam-S digital camera (IonOptix LLC, USA) in an Eclipse Ti inverted microscope (Nikon Instruments Inc.) with a 40x/0.75 objective and an optical magnification of 1.5x. Video recording was made at 90 frames per second (fps) and was defragmented in images for further analysis. To obtain the swimming trajectories, an algorithm was developed based on particle tracking. Once obtained, the trajectories were divided into blocks of 1 s. Then, they were subjected to an analysis of the instant speed and mean radius of curvature.

Results and Discussion:

To explore the involvement of Cl^- permeability in the motility of sea urchin sperm, we used 16 different inhibitors for Cl^- transport (see Table 1). We observed that R (+)-DIOA, Bicuculline, DIDS, NPPB, DPC and CFTRinh - 172 altered the form of the trajectory described by the swimming sperm (see Figure 1).

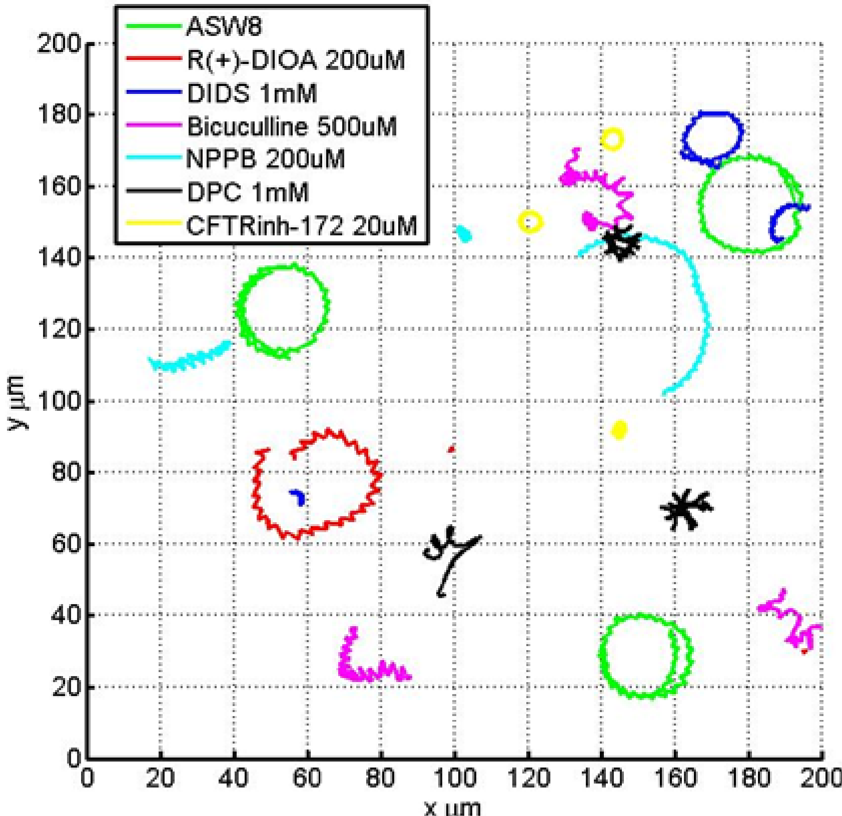


Figure 1. Characteristic trajectories.

Sperm trajectories were analyzed to obtain the speed of sperm each time instant, by the following equation:

$$\text{Speed(t)} = \sqrt{V_x(t)^2 + V_y(t)^2}$$

where $V_x(t)$ and $V_y(t)$ is the velocity in x and y, respectively, at a given time t.

The mean instantaneous speed for the control group (ASW8) was 158.38 ± 41.15 µm s^{-1}, consistent with the values found in the literature (Riedel *et al*., 2005). Figure 2 shows the speed averages obtained for each condition, normalized to their respective control. There is a statistically significant inhibition (*p <0.001) of the mean instantaneous speed in the presence of R(+)DIOA, Bicuculline, DIDS, NPPB, DPC and CFTRinh-172. Interestingly, in the presence of R(+)-IAA94 a

significant increase was observed in the mean value of the speed even though the shape of the trajectory is seemingly unaltered.

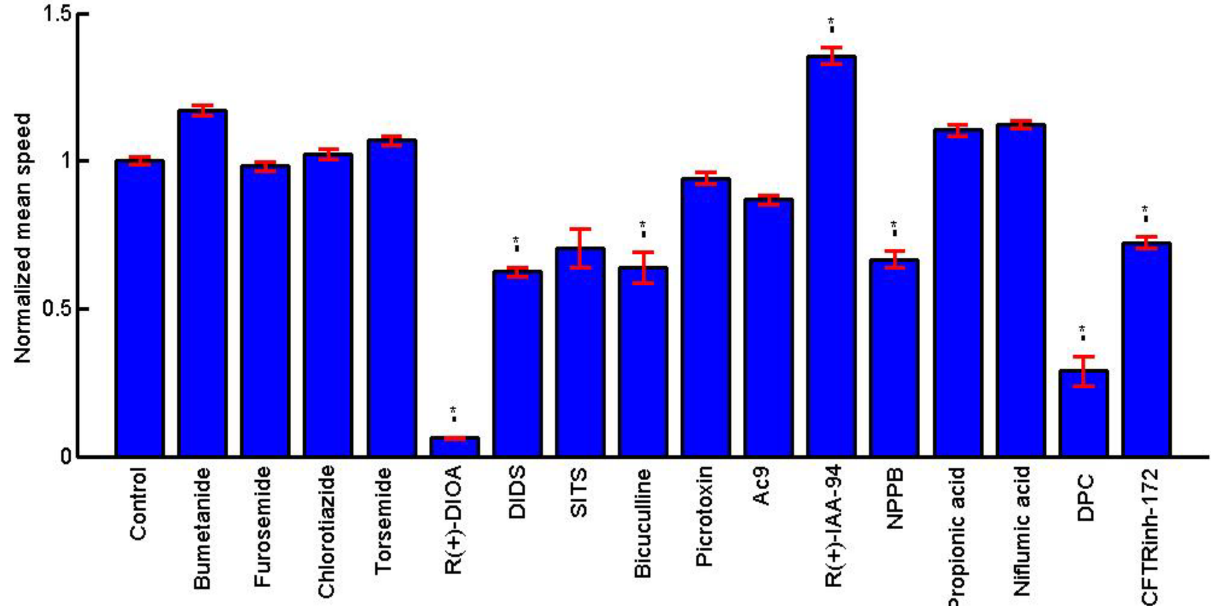


Figure 2. Normalized mean speed.

A time series was generated taking as a reference the position of the sperm head in x. From that, the spectra amplitude was obtained by the discrete Fourier transform (DFT). The maximum value in the spectrum amplitude corresponds to the curvature radius of the sperm trajectory, that is, the radius of the circle described by the trajectory. Figure 3 shows the box plot for the maximum value in the spectrum amplitude for all conditions, where the central mark in each condition corresponds to its median, the edges of the box are the 25th and 75th percentiles, the whiskers extensions are the most extreme data points, and "+" markers correspond to the outliers. In the presence of the inhibitors: R(+)-DIOA, Bicuculline, DIDS, NPPB, DPC and CFTRinh-172, the shape of the trajectory and the instantaneous speed, were dramatically affected, with a statistically significant ($p < 0.001$) as compared to the control .

We propose that the Cl^- channel, CFTR, plays an important role in Cl^- permeability regulation since: 1) It is reported that DPC inhibits CFTR (Zhang *et al.,* 2000); 2) DIDS and SITS are general Cl^- channels blockers, however, one of the few channels that do not block in common is the CFTR, only DIDS does, (Lu and Ding, 2012); as in our case. 3) Of the four drugs used to suppress CCls (Ac9, R (+)-IAA-94, propionic acid and NPPB) NPPB did not affect the sperm motility, and this in contrast to the other three, also inhibits the CFTR (Zhang *et al.,* 2000); 4) Of the five blockers of cotransporters (Bumetanide, Furosemide, Clorotiazide, Torsemide and R (+)-DIOA) only R (+)-DIOA had a significant effect and it is known that this inhibitor also blocks CFTR (Ito *et al*., 2001); finally, 5) CFTRinh – 172 is a specific inhibitor for CFTR and effectively inhibits motility (Verkman *et al*.; 2006).

Finally, to know the effective concentration of those inhibitors altering sperm motility, we made dose response curves for the six inhibitors affecting all parameters discussed above (curves not

shown). The IC_{50} reported for these inhibitors (DIDS, NPPB, DPC, CFTRinh-172 and R (+)-DIOA), for CFTR, falls within the window of the effect in our assays.

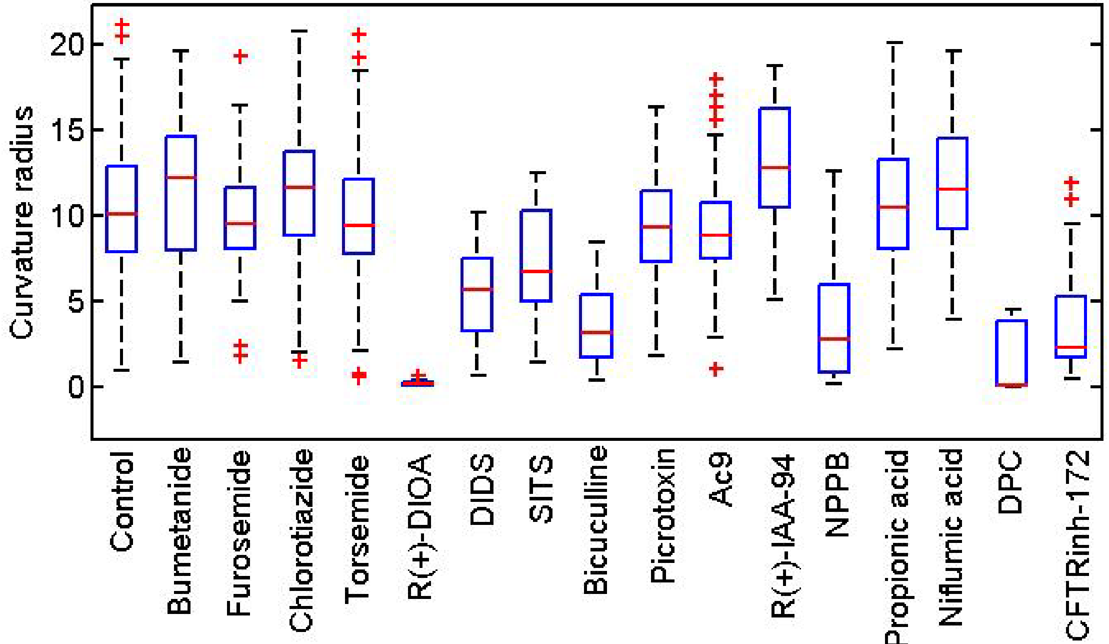


Figure 3. Curvature radius.

Conclusions: The Cl^- permeability has an important role in regulating sperm motility of sea urchin *Strongylocentrotus purpuratus*, since it appears that, when the Cl^- permeability is affected by the presence of these inhibitors of Cl^- transport: R (+)-DIOA, Bicuculline, DIDS, NPPB, DPC and CFTRinh-172, changes occur in the trajectories described by the sperm. By parameters such as instantaneous speed and mean radius of curvature, it was possible to quantify the changes in the trajectories confirming that there is a statistically significant change in sperm motility in the presence of these six inhibitors. Since DIDS, NPPB, DPC, CFTRinh-172 and R (+)-DIOA share the common characteristic of being CFTR inhibitors, we propose that this Cl^- channel is required for sperm motility, however, this does not discard the participation of other proteins that could be also involved in the regulation of Cl^- permeability during sperm motility.

Acknowledgements: This work was supported by a Conacyt grant 82831 and CINVESTAV to BEG.

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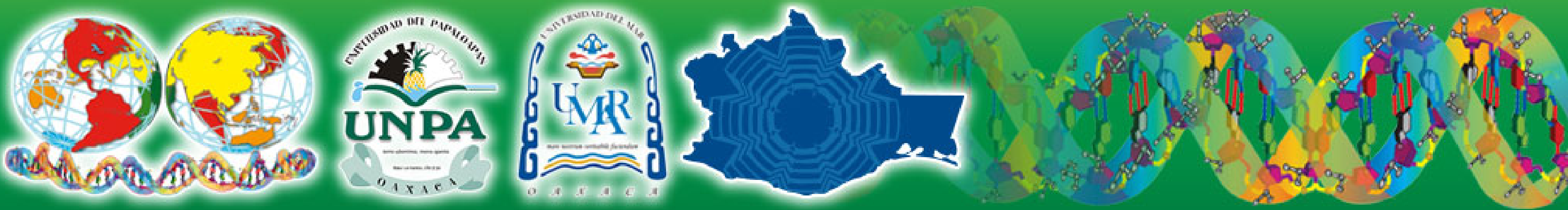

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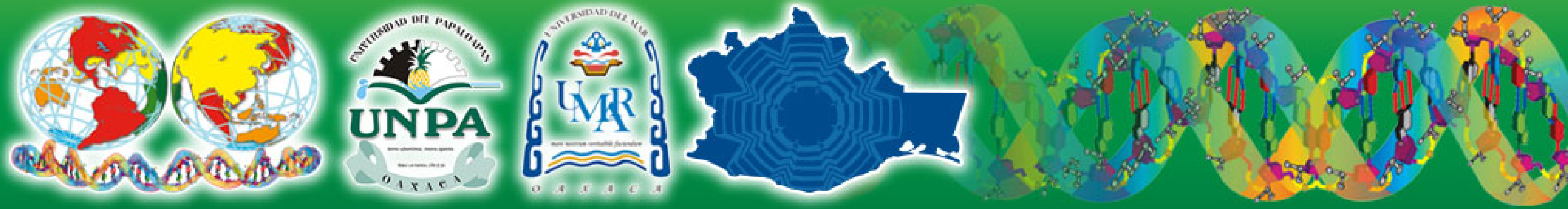

Calcium-activated potassium channel identification in *Strongylocentrotus purpuratus* sperm

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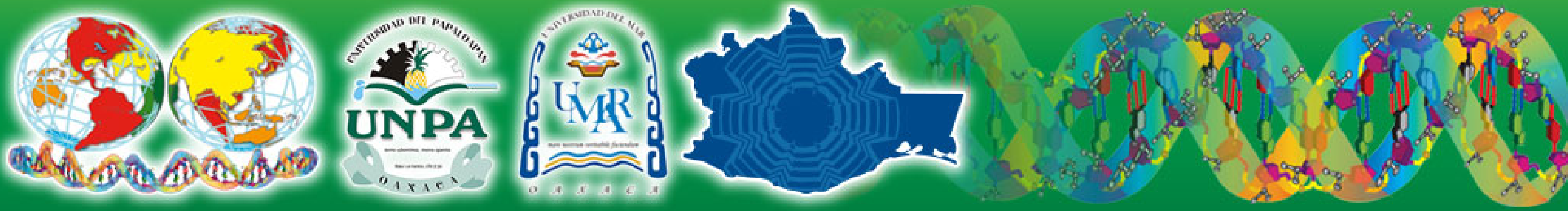
Abstract: The interaction of egg and spermatozoa has been extensively studied in sea urchin because of the communication between both gametes trough the sperm-activating peptides (SAPs), that diffuse from the outer layer of eggs and influence sperm swimming behavior. In the sea urchin *Strongylocentrotus purpuratus* is speract, a decapeptide that induces physiological changes like increased flagellar motility, oxygen consumption and calcium oscillations. It is believed that these calcium oscillations are influenced by the activity of calcium-activated potassium channels (CaKCs). Studies with inhibitors and mathematical models support this idea, but the existence of these channels in the sea urchin spermatozoa remains unproved. In this paper we report the presence of CaKCs in sea urchin sperm. We have identified transcripts for three CaKCs in sea urchin testis similar in sequence to Kcnma1 and Kcnt1. In addition, the proteins KCNMA1 and a KCNN3 were detected by Western-Blot in membrane protein extracts of sea urchin sperm. These results demonstrate for the first time the presence of CaKC family members in *S. purpuratus* sperm.

Keywords: Speract ● Calcium-activated Potassium Channel ● Sea urchin sperm

Introduction: Sea urchin sperm are model cells for studying signal transduction events underlying flagellar motility. According to the current model of signaling pathway triggered by speract, the sperm activating peptide in *S. purpuratus*, a CaKC could be modulating the calcium influx, consequently, the flagellum motility.

Speract, a decapeptide isolated from eggs of *S. purpuratus*, activates a signaling pathway in the flagellar membrane, which induces intracellular calcium oscillations, changing the flagellar movement pattern by reducing the turns and promoting more episodes of straight swim. The actual models propose that Speract binds to a receptor and this binding activates a membrane guanylyl cyclase, the increase of cyclic GMP levels activates a cyclic nucleotide-activated potassium channel (KCNG) that hyperpolarizes the cell. This hyperpolarization removes inactivation of voltage-gated calcium channels (Ca_v) and activates a series of responses that end up with depolarization of the cell and the opening of the Ca_v channels, which in turn depolarize even more the membrane through the influx of calcium ions. After the calcium is cleared and the Ca_v channels are closed, there must be a new hyperpolarization to start again and eventually open the Ca_vs. It has been proposed that the calcium oscillations reported in sea urchin sperm are caused by this sequence of hyperpolarizations-depolarizations (Darszon *et al*., 2008). It has been suggested that this action could be done by calcium-activated potassium channels (CaKCs) (Wood *et al*., 2003, 2007; Greenwood *et al*., 2007) since they are active when the intracellular calcium concentration is high. This suggestion aroused of the observation that the niflumic acid, a non-specific inhibitor of CaKC channels, increases the amplitude and the period of the intracellular calcium oscillations (Wood *et al*., 2003, 2007). Later, two mathematical models predicted that a CaKCs absence would



affect both amplitude and period, as seen in the niflumic acid experiment. Furthermore, when Iberiotoxin, a specific antagonist of the CaKC channels, was added to sperm in the presence of Speract, the same alterations were observed (Espinal *et al*., 2011). This is a strong experimental evidence of the participation of the CaKC channels in the Speract signaling pathway.
There are three identified subfamilies of this protein family, the BK, the SK, and the recently discovered IK channels. Besides their participation in multiple functions, BK channels perform the fast after-hyperpolarization that follows the action potential in neurons (Lee *et al*., 2010) whereas the SK channels perform the slow after-hyperpolarization (Tuteja *et al*., 2005). These two functions are quite similar to the role that CaKCs would play in the sea urchin speract-signaling pathway.
Although there is evidence of the possible participation of CaKCs in the Speract pathway of sea urchin sperm, their existence in this species and its localization are unknown. The present work seeks to identify the presence of CaKCs members in sea urchin sperm. This evidence could give support to the hypothesis of the CaKCs participation in the Speract pathway, although functional analysis must be conducted.

Materials and Methods:

Biological material. The *S. purpuratus* sea urchins were provided by Panames (Ensenada, Baja California). They were maintained in an aquarium at 13 °C with artificial sea water (Kent marine) with pH 8 and were spawned by injection of 0.5 M KCl.

RNA extraction. The gonads of the sea urchins were removed and homogenized in *Tri-Reagent* (Sigma) to extract the total RNA in the tissue. Later, the messenger RNA was purified with the commercial kit *Poly(A)Purist* (BioRad) .

RT-PCR. The cDNA synthesis was made using the kit *Smartscribe* (Clontech) according to the manufacturer's protocol. The PCR reactions were carried out with the following conditions: 95 °C, 1 min; 10 cycles at 95 °C, 15 s, 65 °C, 20 s., 68 °C, 1 min; 10 cycles at 95 °C, 15 s; 60 °C, 20 s; 68 °C, 1 min; 10 cycles at 95 °C, 15 s; 57 °C, 20 s; 68 °C, 1 min; and a final extension at 68 °C, 7 min. Specific primers were designed to amplify the cDNA segments for the predicted Kcnma1, Kcnt1 and Kcnn3 genes found in the sea urchin genome. The primers and expected sizes are shown in Table 1.

Sequence analysis. Sites and motifs were found using ProfileScan (http://hits.isb-sib.ch/cgi-bin/PFSCAN) and transmembrane segments and domains were predicted with SMART (http://smart.embl-heidel berg.de/) (Schultz *et al.*, 1998). BioEdit program (Hall, 1999) was used for alignments.

Western Blot. A 10,000 g supernatant of a 1% NP40 non-ionic detergent extracts of whole sperm was dissolved in Laemmli sample buffer and separated by SDS–PAGE. Proteins were transferred to PVDF membranes, blocked with non-fat dry milk in 150 mM NaCl/10 mM Hepes, pH 7.5. Blots were probed with the primary antibodies SK1, and Max-K (Santa Cruz Biotechnology), diluted 1:400 and 1:250, respectively; and incubated for 1 h at RT. Washes were in NaCl/Hepes containing 0.1% Tween 20. Signals were detected with the ECL system *SuperSignal West Dura Extended Duration* (Pierce).

Results and Discussion:

CaKC homologous in sea urchin. With the purpose of identifying the homologous genes for each of the eight members of the CaKC family in sea urchin, a BLAST (Basic Local Alignment Search Tool) at the NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) site was carried out. Only three predicted gene sequences, in the sea urchin genome, aligned with significant score to members of the human CaKC channels: SpKcnma1 (XP_783726), which aligned to hKcnma1 (AAI44497) with 53% of similarity; SpKcnt1 (XP_785197) to hKcnt1 (AAI36619), with 65% similarity and SpKcnn3 (XP_780648) to hKcnn1 (NP_002239), with 66% similarity.

Transcripts of SpKcnma1, SpKcnt1 and SpKcnn3 mRNA. The RT-PCR reactions that result in product amplification are shown in Figure 1.

Table 1. Primers and expected sizes for the amplification of Kcnma1, Kcnt1 and Kcnn3.

Gen	Reaction No.	Forward Primer	Reverse Primer	Expected size
Kcnma1	1	gagtctatcacctctacatacg	Ttcataccacctccctcagc	321 bp
	2	ttacacaatgtggagagg	Catacacatcaccgtagccg	828 bp
Kcnt1	3	tccatagcgatcataagc	Tggtagaatgacgagagc	495 bp
Kcnn3	4	cttgttgtagcagtcttgg	tgtagctggtctagtcttcc	392 bp
	5	cagtactccttgtcgtaacc	actgtgcttgcttcgtcagc	496 bp
	6	cagtactccttgtcgtaacc	tgtagctggtctagtcttcc	613 bp
	7	cttgttgtagcagtcttgg	actgtgcttgcttcgtcagc	275 bp

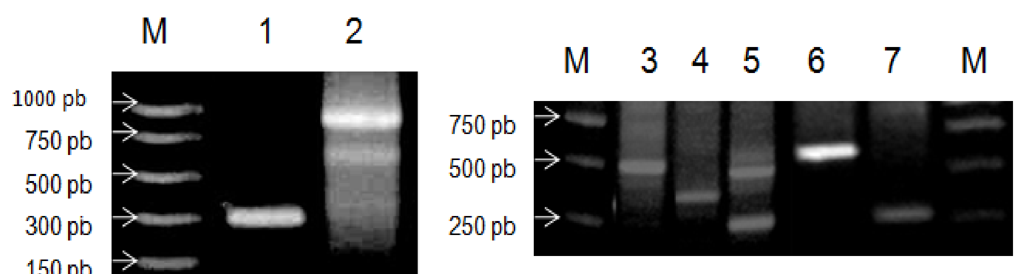


Figure 1. cDNA fragments of SpKcnma1 and SpKcnn3, amplified by RT-PCR. The lane number is the same as the reaction No. in Table 1.

Here we show the RT-PCR reactions showing the cDNA fragments of the expected size (Table 1) for transcripts from Kcnma1, Kcnt1 and Kcnn3. The cDNA bands were cut, gel purified and sequenced. Not all the sequencing reactions were successful; however, we did obtain sequences for SpKcnma1 and SpKcnn3, RT-PCR reactions 1 and 4-7, respectively, see Table 1 and Figure 1. We obtained a transcript sequence of 163 bp that corresponds to SpKcnma1 (position 2941-3102, accession number XP_783726) and a 395 bp transcript sequence for SpKcnn3 (position 1680-2074, accession number XP_780648). These results confirm the presence of RNA transcripts for SpKcnma1 and SpKcnn3 in sea urchin gonads. The fragment 3 couldn't be

sequenced, therefore the Kcnt1 expression still unclear. More RT-PCR reactions with specific primers should be done in order to confirm or discard the presence of Kcnt1 transcript.

Sequence analysis. The secondary structure prediction shows that the predicted SpKcnma1, of 1249 aminoacids, has the characteristic regions of the Kcnma1 channels: seven transmembrane domains, the BK subfamily conserved domain and four intracellular hydrophobic domains. The SpKcnn3 also has the characteristic domains of the SK subfamily: 6 transmembrane domains and the SK subfamily conserved domain. Both SpKcnma1 and SpKcnn3 show a pore region with the selectivity filter "*GYGD"* that is the signature motif of the K^+ channels superfamily.

Presence of the KCNMA1 and KCNN3 proteins in sea urchin sperm. The antibodies employed were raised against mouse CaKC channels. We performed a search in available commercial antibodies in order to use only those antibodies whose epitopes are shared with the predicted sea urchin CaKCs.
Two antibodies were used for protein detection by Western blot: The mouse anti-SK1, an antibody that recognizes a shared region between the 3 members of SK family: KCNN1, KCNN2 and KCNN3 and, the mouse anti-Maxi-K, specific for KCNMA1. No appropriate commercial antibodies could be found for SpKCNT1.
Anti-SK1 recognized a band of ~150 kDa (Figure 2), when the calculated MW of SpKCNN3 is 92 kDa, although this protein has two predicted glycosylation sites, we do not know yet if it is glycosylated. Anti-Maxi-K detected a ~91 kDa. More studies must be carried out to address the differences in molecular weight that can be due to post-traductional modifications, alternative splicing or protease cleavage.
Finally, immunofluorescence was detected in sea urchin sperm membrane and the base of the head where the mitochondrion is located with anti- SK1 antibody (data not shown).

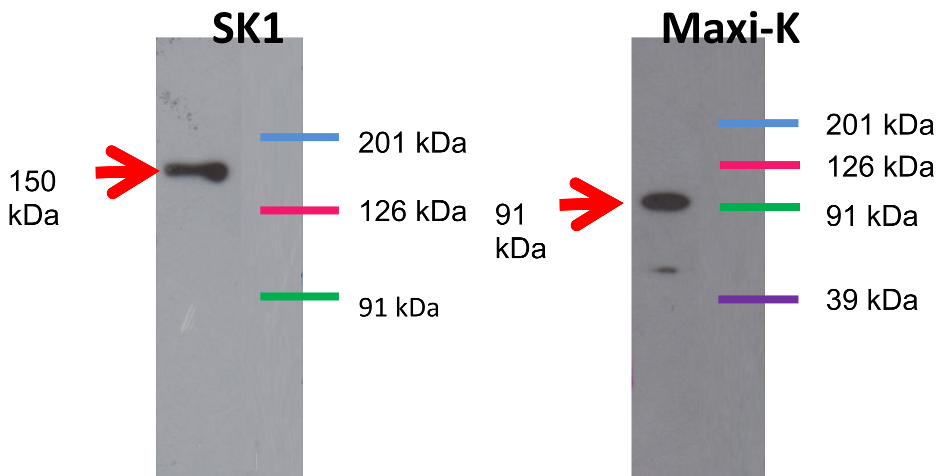


Figure 2. Western-Blot analysis with anti-SK1 and anti-Maxi-K.

Conclusions: Consistent with the findings above described, by using RT-PCR, sequencing and, *in silico* tools, we found in *S. purpuratus* testis two partial cDNA sequences for Kcnma1 and Kcnn3 that share homology with the human CaKC family members hKcnma1 and hKcnt1. The corresponding predicted genes in the *S. purpuratus* genome are SpKcnma1 and SpKcnn3. Furthermore, using commercially available antibodies to mammalian CaKCs, we detected by Western blot, as well as by immunocytochemistry, signals in sperm with two of those antibodies.

Functional studies using CaKCs blockers in sea urchin sperm could indicate their participation in motility and/or the acrosome reaction. To our knowledge, this is the first time the presence of members of the CaKC family in sea urchin sperm is reported. Our work may provide a basis for investigating the role of CaKC in sea urchin sperm motility.

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Genome annotation and reconstruction of the metabolic model of the fungus *Leucoagaricus gongylophorus*

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Abstract: The fungus *Leucoagaricus gongylophorus* is a fungus living in symbiosis with the ant *Atta mexicana* and it has been isolated from the nest and cultivated at laboratory conditions. This fungus has the capability to degrade different polysaccharides contained in plant biomass to produce more simple sugars; where the major fraction of these sugars is storage as glycogen that serves to feed the ant. Due to the observed metabolic capability, the fungus is an attractive biologic system to be explored for Biotechnological applications, specifically for biomass utilization and biofuel production. Moreover, as the fungus degrades the biomass to simple sugars, these ones can be used as raw material for other processes. To achieve a systems view and explore the metabolic capabilities, in this project, the objective is to obtain the full genome sequence of the fungus *Leucoagaricus gongylophorus* and perform functional annotation of the protein-coding genes. In specific, we are focused in the identification of the enzymes with lignocellulosic activity and the metabolic enzymes to reconstruct the genome scale metabolic model to optimize the production of sugars using the natural capability of the fungus. The genome scale metabolic models are mathematical models extendedly used in Systems Biology for Metabolic Engineering applications.

Keywords: metabolic-model ● biomass ● fungus ● DNA-sequencing

Introduction: For the production of the second generation of biofuels one of the central objectives is the use of biomass as the main source of fermentable sugars. These sugars are useful for the cultivation of microorganisms used as cell factories. As an example, microorganisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis* are attractive for industry as they produce ethanol; reaching almost 91% of theoretical yield of ethanol based on glucose. Nevertheless, these microorganisms are not capable to degrade plant biomass. Therefore, different efforts have been directed to optimize microorganism that naturally degraded biomass and they can be manipulated to overproduce metabolites. As an example, *Clostridium cellulolyticum* has been a model to extract different family of enzymes with lignocellulosic activity (Desvaux *et al*., 2001a; 2001b), and it has been genetically engineering to overproduced biofuels, such butanol, through the ABE pathways (Formanek *et al.,* 1997; Jones *et al.,* 2008; Papoutsakis, 2008). Even when there are useful applications of *Clostridia* species, the processes are not reaching economic feasibility and there is room for improvement. To overcome these situations other strategies are based on design bioprocess to degrade plant biomass.
The identification and characterization of microorganism that ease biomass hydrolysis and metabolic production is a researching line still open (Li *et al.,* 2010). Under this panorama, the fungus *Leucoagaricus gongylophorus*, a symbiont of the Mexican ant from *Atta* genus, has been isolated and cultivated in laboratory conditions to its manipulation and characterization, as it has

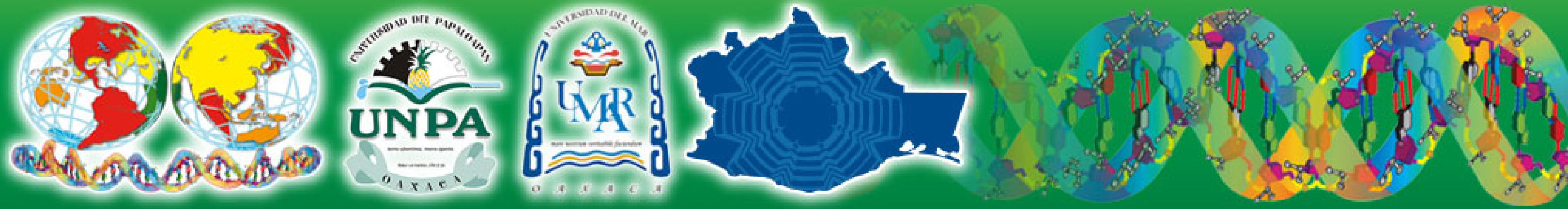
the function of degrading the biomass plant carried by the ants and storage carbon sources in form of glycogen (Manuscript in Preparation). The same fungus has been isolated from other ant species (Gomes *et al.,* 1998; Silva *et al.,* 2003). For instance, Siqueira *et al.* (1998) reported the findings of *L. gongylophorus* in the nest of *Atta sexdens,* express enzymes with different biomass hydrolysis, such as, xilanase, cellulase, pectinase and maltase. Furtheremore, Aylward *et al*. (2013a, 2013b) reported the first draft of the genome sequence of the fungus, but this one is a symbiont from the *A. cephalotes* and *Acromyrmex echinatior* ants, originally from Panama. Using bioinformatics tools and metaproteomics the authors found 145 enzymes with lignocelulase activity, including celulases, laccases, pectinases, xilanases and amylases.
To explore the metabolic capabilities and acquire systems understanding, we obtained the genomic sequence of the fungus *Leucoagaricus gongylophorus* to annotate and reconstruct the genome scale metabolic model (GSMM) and identify the different genes with enzyme activity for hydrolysis of biomass. The GSMM models are mathematical tools useful to generate rational strategies for Metabolic Engineering (Otero and Nielsen, 2009). The GSMM have been improved by the omics tools developed in Systems Biology (Tyo *et al*., 2007). Finally, the genomic data and the functional annotation is not limited to model reconstruction. This cumulus of information will allow the development of other types of omics tools, and picture the systems perspective of the microorganism.

Material and Methods:

Samples of the fungus *Leucoagaricus gongylophorus* were collected from the nest and ADN extracted using toolkits. The semi-solid ants solid state-cultures were made on potato dextrose agar (PDA) and malt extract (MEA-LP) as it was reported by Miyashira *et al*. (2010). The genome sequencing was made using two NGS platforms. Pyrosequencing with GS XL+ from 154-Roche with the capacity of generating 550 Mb and 800,000 reads with average size of ±550 bases. The other platform used was MiSeq from Illumina with the capacity of generating 5.4 Gb and 11 millions of reads with average size of 250 bases using paired-end. The hybrid *de novo* genome assembly was performed using the workflow suggested in Wang *et al*. (2012) and it based on the assemblers, Newbler (454 ROCHE software), Velvet (Zerbino and Birney, 2008) and Phrap1.090518. Based on the assembled genome sequence we used the programs GeneMark (Borodovsky and Lomsadze, 2011) and Augustus (Stanke *et al.*, 2006a; 2006b) the gene predictions. The predicted proteins were identified with BLASTP. The databases used for functional identification are Pfam (Jones *et al.,* 2008), NR (Li *et al*., 2010) and COG (Aylward *et al.*, 2013). For the biochemical reactions the stoichiometry is verified in KEGG database (Tanabe and Kanehisa, 2012), BRENDA (Schomburg *et al*., 2004) and publications. For the identification of carbohydrate-active enzymes (so called CAzymes) we used the method described in Suen *et al*. (2010).

Results and Discussions:

In this project the genome sequencing of *L. gongylophorus* has been performed. Based on the genome sequence assembly the aim of the project is the functional annotation of the enzymes with lignocellulosic activity and the metabolic enzymes to build the GSMM to explore the metabolic capabilities the fungus has to produce glycogen. The size of the genome sequence is about 101 Mb and the number of contigs resulted from the *de novo* assembly is 40,578. The number of



predicted proteins was 4,921, from this set, 156 were identified with activity for biomass degradation (CAZymes).
It is important to mention that at the moment the proposal of this project was submitted to get funding, the first draft of the genome sequence of the *L. gongylophorus* was published, nevertheless, this sample of fungus comes from the nest of the ant *Atta cephalotes* that lives in Panama (Aylward et al., 2013) . As a consequence from this publication we were able to perform a comparative analysis of the quality and annotation of both draft genomes. Therefore, from the sequence obtained in this project we obtained about 50% less contigs compared with published draft. Regarding the predicted proteins there are 354 possible proteins and 11 CAZYmes more. As part of the GSMM reconstruction, from the set of predicted proteins, it was possible to identify the enzymes from the glycolytic pathways, as well as TCA and pentose phosphate.

Conclusions: The genome sequencing and *de novo* genome assembly from the fungus *Leucoagaricus gongylophorus* that is in symbiosis with the ant *Atta mexicana* has been obtained. The functional annotation is in process and the first results show more predicted proteins compared with an already published draft of the genome sequence from other sampled fungus obtained in the nest of the ant *Atta cephalotes*. Whether these observations are the result of the differences in the quality of the draft genome sequence, or these are the results of biological differences, it is a pending issue that needs to be analyzed by performing a more extended comparative analysis of the sequences. Meanwhile, for the reconstruction of the GSMM, it was possible to identify the pathways of central carbon metabolism. Besides the process of functional annotation and reconstruction of GSMM, some experiments are taken place to obtain consumption rate of different model substrates (like alpha-celullose and sugar cane bagasse) and production rates of glycogen, cellular biomass and CO_2. The data will be used to validate predictions with the GSMM.

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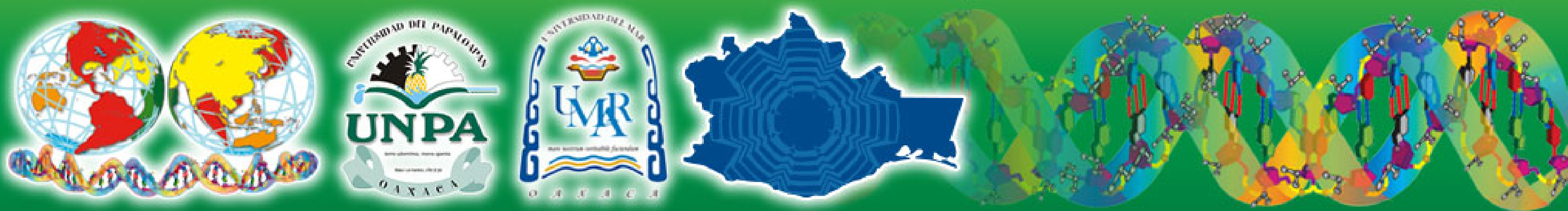
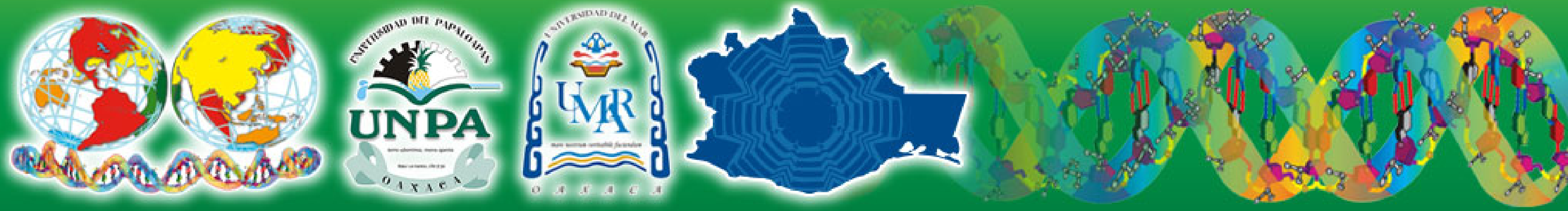


1.9 INDIGO AREA



Chemistry Student's interesting for Biotechnology

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Abstract: The training of professionals dedicated to biotechnology in Mexico has failed to have the expected rebound in the diversity of areas of impact. To investigate what happens to students, a questionnaire and an interview with two careers students' from chemistry applied area. The results allow us to see that the information on biotechnology in general, and how that impacts their careers in a particular way is scarce and in some cases appears to be distorted. These results indicated the importance of bringing children to accurate and timely scientific information.

Keywords: Biotechnology ● Learning Biotechnology ● Professional formation

Introduction: According to Trejo (2010), biotechnology is one of the areas of scientific knowledge that has achieved a more rapid evolution in recent decades and one that has had more impact on the development of various economic sectors, particularly those aimed at improvement in health, agricultural production, livestock production, preventing deterioration and improving the environment and oriented to the production of various goods, pharmaceuticals and food processing industry. Biotechnology applications have growing importance in the national and international economies; sectors such as pharmaceuticals, agriculture, food processing and stabilization, remediation of soil and water and the generation of specialty chemicals for the production of polymers, flavors and fragrances, as representative examples (Bolívar-Zapata, 2002; Trejo, 2010). Despite this context, Herrera-Estrella (2013) mentions that there is a shortage of scientists in all areas and specifically in biotechnology in Mexico, for example compared to Korea, which has 25,000 researchers in biotechnology and Mexico has 27 thousand for all areas, and if that is the fact that there are 26,000 species of plants in the country, of which 14,000 are endemic, ie, there is a universe that should serve 30 researchers working in biotechnology. That is why the aim of this study was to determine some of the causes of the lack of students majoring in Chemistry for Biotechnology. As De Melo and Melo (2009) says "Chemistry is a central science in technological applications that have great impact on development of areas such as biotechnology".

Materials and Methods:

This work is a qualitative, descriptive and exploratory study about the interest or disinterest on chemistry students for the biotechnology. This study was made in a public University in Mexico City. In order to investigate the interest of students of both careers Chemistry (Food Chemical and Biological and Pharmaceutical Chemistry) for their career and its relationship to biotechnology, a questionnaire (Figure 1) was applied to 120 students majoring mentioned enrolled in a microbiology course because it is subject to prior biotechnology curriculum careers. The results were analyzed by determining the frequencies of the answers given in the questionnaire

administered and conducted brief interview. It is noteworthy that data collection instruments were validated by three judges, they were modified and a pilot was conducted with students of social service prior to their implementation with students.

This questionnaire is to know you better and to meet the expectations you have about the microbiology course. Therefore, we ask that you answer as honestly as possible to the following questions.

Name: ______________________________

1. In what career are you registered?______________________________
2. Why did you choose this career? ______________________________
3. What are the areas or subjects that you like or appeals to you? ____________

   ____
4. Why? ______________________________
5. At the end of your career, what you want to work? ____________________

   ____
6. Why? ______________________________

Figure 1. Questionnaire interest in selected career.

Subsequently the responses provided by the students and in connection therewith underwent a brief interview during class in order to contribute their ideas spontaneously and truthfully about their knowledge and interests in biotechnology were analyzed. The interview was semi-structured and included the interview guide shown in Figure 2.

Option 1: When the student said something about biotechnology

Hi, in the questionnaire administered at the beginning of the course, I was struck by your interest in biotechnology.

Why the interest? ______________________________

Have you read about some topics of biotechnology as they relate to your career and what you think about it?______________________________

____

Option 2: If the student does not mention biotechnology

Hi, in the questionnaire administered at the beginning of the course, I noticed that not mention anything in relation to biotechnology.

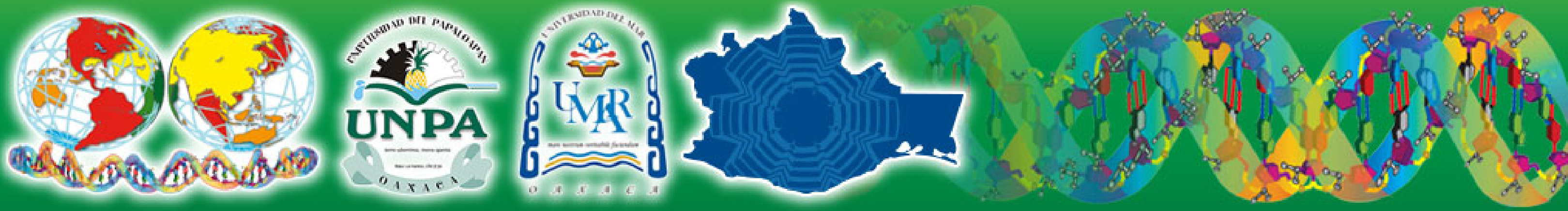
Have you read or heard about some topics of biotechnology as they relate to your career? When the answer is yes, continue

What do you think about this?

____

If the answer is no certain career-related student and was asked if he would consider further in any of the above topics or work on it examples were provided.

Figure 2. Interview Guide investigates the knowledge and interest in biotechnology.



Results and Discussion:

In the Table 1, we show the results obtained with the application of the questionnaire. It's important to say that in most cases were made categories to group the responses by students.

Table 1. Results of the questionnaire application.

Question	Results
In what career are you registered?	65% Biological Pharmaceutical Chemistry 35% Food Chemistry
Why did you choose this career?	30% Because I like chemistry and other area 25% Because I like 20% Because with it I can make money by working 15% Because I consider interesting and useful 10% Because I care research
What are the areas or subjects that you like or appeals to you?	45% Chemistry related 25% Area-related pharmacy or food 10% Biology related (cell biology, microbiology, biotechnology) 10% Development of new products (pharmaceuticals or food) 5% Administration 5% Humanistic partner
Why?	30% No Response 20% Because I like the chemistry 20% Because they help you get a good position in the industry 15% Because It help to progress 15% Because since I was a child I like the chemist
At the conclusion of your career, what you want to work?	67% In industry (pharmaceutical or food) 20% In the health sector 5% In a postgraduate 5% Teaching or research 3% Do not know or did not answer.
Why?	45% Because I would like to have a good job and salary 25% Because I can apply what I learned 15% Because it helps me progress 10% Because its satisfies me 5% Because I can share what I had learned and help others

Then, the results obtained by applying the interview students are presented, it will be recalled, and the interest here is to know your opinion and interest in biotechnology. Of the students who mentioned biotechnology as an area of interest, 100% were knowledgeable about different aspects in which biotechnology affects their careers. Some of them mentioned their interest in continuing his graduate studies in any area of biotechnology either food or health sector because since children have heard related to biotechnology or have seen TV news which touches the subject and the have retained this concern. However, none of them mentioned his interest in the environmental aspect. Among students who mentioned that like subjects related to the

development of new products, none of them sees biotechnology as a tool for this, but lean towards the chemical synthesis products. Of the students who did not mention biotechnology (90% of students who answered the questionnaire), responses are variable and range from the lack of impact of biotechnology in his career, to those who have a negative opinion of it. Following their answers grouped by categories are broken down:

52% of students do not know what biotechnology can serve in their careers, but to present some examples, some (25%) were interested and said they would like to know more about it perhaps be losing any chance of a better employment or personal growth. The rest showed disinterest in the comments of teachers (Figure 1). 22% of students indicate that they disagree with biotechnology, which has a negative impact on the maintenance of biological diversity both animal and vegetable. Almost all of them consider genetic engineering as a synonym for biotechnology and even some mention being against the use of GMOs (Figure 3). This result is similar as reported for Pedrancini *et al*. (2007) who's detected through qualitative analysis that the student's opinion about GMOs is erroneous, because is influenced by the communication media, but not for a scientific knowledge. Cabo *et al*., (2006) observed this same behavior in teachers who have as their main source of information on television, followed by newspapers and magazines finally.

18% recall at some point have read or heard from their teachers theory some aspects of biotechnology, but do not consider it of interest because they would like more professional capacity, chemical industry that apply the knowledge acquired (Figure 1).

The remaining 8% believe that the information provided by the teacher is interesting and leave open the opportunity to learn about the impact of biotechnology in their careers; they do not even have very well defined what they want to devote their professional lives (Figure 3).

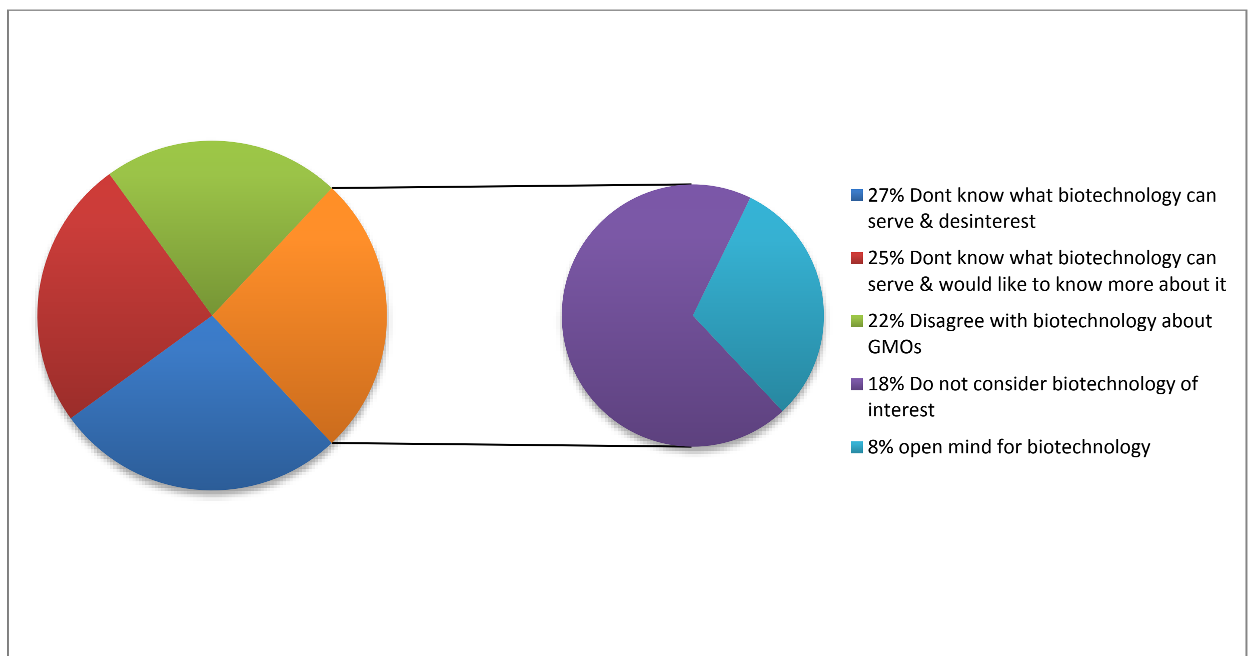
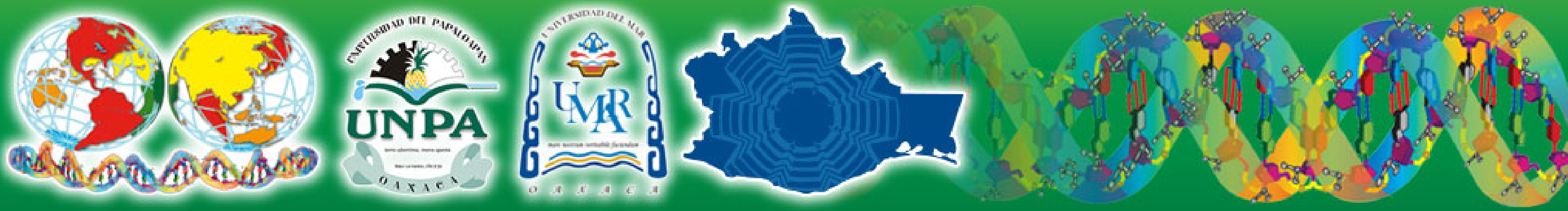


Figure 3. Their answers grouped by categories.



It is important to mention that according to Cabo *et al*. (2006) results on degree of interest and information to the Biotechnology teachers show similar results to the general public; the balance between interest and information is negative it would be interesting to know what the image transmitted on biotechnology teachers.

Conclusions: From the above results, we can conclude that there is a serious lack of information about what is involved in biotechnology careers mentioned. However, it is unknown whether in high school biology teachers taught the subject and the students do not remember or if in fact it was not considered at this stage of training. Clearly the information or tastes students developed in childhood positively influence the interest in certain aspects of the career, so it is considered desirable that there is a wider dissemination of truthful information about what is biotechnology and its potential uses for the progress of humanity. At this point, there is the suggestion of the development of workshops or books that bring to children the different areas of knowledge. It is also important that information about biotechnology is viewed from a historical point of view where there was no genetic manipulation so that students know exactly what the impact of biotechnology and genetic engineering differences.

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Process of forming of biotechnology PhD program on network: Case study

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Abstract: The main goal of this work is to illustrate the challenges faced by doctoral training program in Biotechnology, which must attend three challenges: 1) to consolidate as recognized postgraduate program that performs basic and applied science in a recent and necessary field. 2) Take advantage of and capitalize on its innovative networking experience, and 3) strengthen internal academic life and capitalize on previous experiences of graduate programs, in whole context of the demands of science and technology policies from Mexico. The new requirements of these policies and the multidisciplinary characteristics of Biotechnology pose new challenges to institutionalize, organize, and develop doctoral studies. The Biotechnology PhD program in network could be characterized by two outstanding features: multidisciplinary training and inter-institutional interaction. As part of the National Program for Postgraduate Quality ('Programa Nacional de Posgrados de Calidad', PNPC) from CONACYT in order to share the limited infrastructure available, maximizing resources for the benefit of the students, and encourages collaboration and leverages the expertise of researchers belonging to the Network of Biotechnology, National Polytechnic Institute.

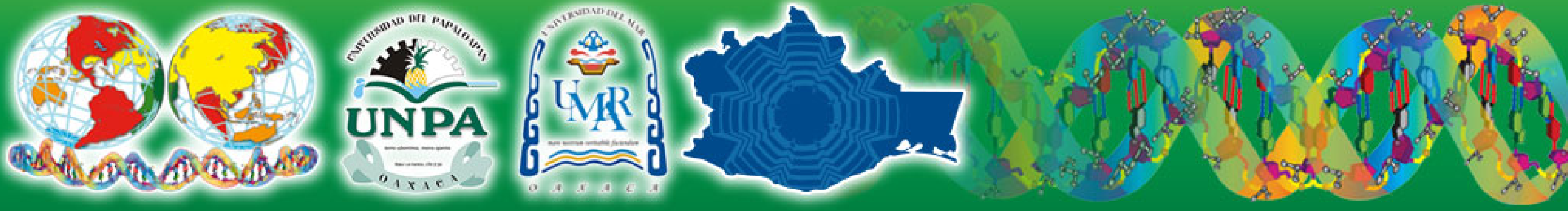
Keywords: PhD in Biotechnology ● institutionalization ● PhD program in network ● multidisciplinary training.

Introduction: The Doctorate in Biotechnology is an innovative program, organized by Biotechnology Network with six nodes that compose this Network, National Polytechnic Institute. This way of working is a specific feature that distinguishes this program from other programs of scientific doctorates in Mexico. The PhD is currently recognized by the PNPC - CONACYT and just six years after its creation is known for its international relevance.

It is interesting to study the creation and playback of innovative institutional forms that organize and validate the scientific and technological activities as integral part of the institutionalization processes of the science (Kent, 2011; Vazquez, 2014).

The Biotechnology is one of the main areas of science education has been significantly developed, combines knowledge of biology, genetics, engineering, computer science, and biochemistry, among others. That is its multi-disciplinary and innovative nature resides. Secondly, the Biotechnology has shown got numerous applications in industry, society, and in the study of complex processes such as climate change.

This article explores the learning potential and organizational research challenges of a multi-site, multi-disciplinary program that promotes the interaction of students, faculty and institutional administrative support. Which, converges in an academic doctoral program in order to benefit students, academic experiences, human resources, and infrastructure of the Institute academic



units in five different entities that outstanding in the area of Biotechnology, Medicine and Environment. (http://www.dcb.rsip.ipn.mx/Paginas/Inicio.aspx).
According to Whitley and Gläser (2007) and Scott (2005) understand the process of institutionalization of the scientific and technological system as a set of changes in the governance of science driven by elites and scientific leaders, and the establishment of new and diverse models formalizing organizational structures and administrative procedures designed to promote and regulate the scientific and technological activity. Three crucial aspects of the institutionalization of science are 1) the design of a scientific career, 2) building of disciplinary identities and 3) legitimacy spaces into the society. In Mexico, the guideline of scientific careers is in charge of the National System of Researchers ('Sistema Nacional de Investigadores', SNI), which recognizes and legitimizes the achievements of researchers to the scientific community and society. Different disciplines develop different ways of working, organizing and formulating research agendas.
In the current context of furthering science, technology and innovation, the processes of scientific institutionalization acquire a new state of complexity to be incorporated as important players to the employers and/or clients of goods and technological services (Jacob, 2001; Gibbons *et al*., 2007). This represents changes and tensions in the identities of the researchers. On the one hand, seek to advance their careers through the National System of Researchers (SNI), but otherwise must interact with customers and business partners. It also involves challenges for the organization and management of Applied Research Centres.
Therefore, institutionalizing a multi-disciplinary PhD, multi-venue with a combined agenda of basic and applied science poses special organizational challenges. The CIBA is a successful example of this.

Materials and Methods:

The study of organizations is complex. To do this, the case study is a useful resource (Yin, 1994). It was conducted an exploratory study through interviews with CIBA community and with its founders. It was relied on written documents that inform their creation and development, and policy documents. The information from the interviews and documentary data were processed as qualitative data, coding and identifying patterns and trends, in order to explain the institutional process.

Results and Discussion:

The institutional network doctorate in Applied Biotechnology taught at CIBA entered to date 21 students, which the first three graduates in the first half of 2014.
The master's and doctoral programs originally offered the Centre were oriented to the Basic Science, while recent programs in Productive Biotechnology are focused on applied science and innovation. A finding of this case study is the existence of a debate on the desirable orientation programs, challenges to meet the demands of the national science and technology that is expressed in the difficulties that this transition between basic and applied science is for managers and researchers.
The institutionalization of this innovative way of PhD in network represents a major coordination effort, not without its problems and tensions, involving all levels of organization of the National Polytechnic Institute (IPN). Since in the vertical plane, from the researchers of each node to the Office of Research and Graduate Studies (Secretaría de Investigación y Posgrado). In the horizontal plane, meets the participation of researchers, students and directors of six academic

centres in several regions of the Republic. The transition between the organizational form of CICATA -focused on Applied Science and Technology development- the organizational form of Centro de Investigación en Biotecnología Aplicada (CIBA) master's and a doctorate in network has been an experience of great complexity.

On one hand, it is an expression of adaptation to a complex institutional environment: IPN system as a multidisciplinary educative organization that sets targets and regulates research and training on PhD. On the other hand, involves internal adaptive challenges and complexities in the Centre, where researchers and managers must decide on the research agenda, the forms of engagement with companies and training of young scientists. At this level, the collegial form of organization was fundamental to process different positions and objectives, although it was not without its tensions.

To close this paper it is worth remembering that doctorates, where new scientific form, implicit models of being a scientist and making science learning standards adopted by the institution (usually implicit) of the discipline and colleagues who collaborate from other countries and institutions with local researchers. These rules translate into expectations for the young man who started a career PhD; requirements posed by public policies for doctoral programs, program internal structures, its management, syllabus, tutorials, requirements for a doctorate, the practices, discourses and expectations of the researchers.

In the current context, this whole scheme to train young scientists is on the process of change: the transit from science discipline to the multi-disciplinary, from basic to applied research and the innovation, and the network organization. It is necessary to recognize the difficulties and challenges that this new context to represent for the organization, the management and the work of the Research Centres and doctoral programs. The institutionalization of innovative ways of organizing science, technology and innovation is not a simple process, and the practical challenges of requiring greater attention by policy makers.

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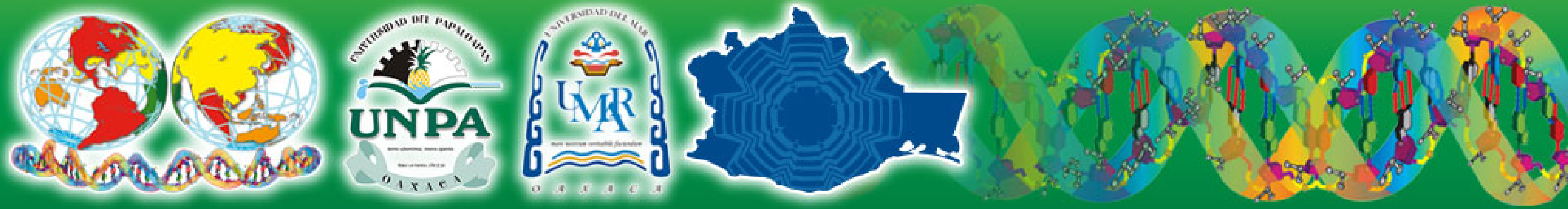
1.10 SILVER AREA



Strategic overview of the use of Malanga in the production of tostadas

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Abstract: The objective of this research is to design a strategic overview for a legally constituted company engaged in the production and marketing of food products. According to the latest trends in the food industry Innova Market Insight predicts that health and nutrition require a holistic approach to provide nutritious foods to consumers; that is why an investigation needs to be carried out with a strategic overview for producing and marketing baked tostadas, made from Malanga (also known as Taro, Dashen or yam). It is considered one of the species of roots and tubers with great potential in the tropics. The conclusion reached is that the product is an innovative solution with high nutritional value, catering to consumers with a healthy lifestyle that will benefit the company financially.

Keywords: Strategic planning ● nutritious food ● environment

Introduction: Due to the constant changes in the environment, and in the food industry, the latter has felt the need to innovate their products. This makes Latin America and Asia sources of new flavors that increase the taste and nutritional value of their products. Innova Market Insight is dedicated to the production and marketing of toast and corn chips, made with quality and 100% natural ingredients, without preservatives and with typical Mexican flavor. Today it wants to innovate its main product, the toast fries, and aims to produce and commercialize baked toast with malanga, and that requires strategic flavor overview research. Malanga is considered one of the species of roots and tubers with great potential in the tropics. Is part of the exotic or non-traditional products whose consumption has had a major surge in Mexico. The only state producing of malanga in the last three years has been Veracruz; only in the years 2001 and 2005 has Malanga production gone unreported (Bancomext, 2011).

Materials and Methods:

The company subject to this investigation is located in the Port of Veracruz, México. The methodology used in this deductive research is descriptive and explanatory. The techniques that were used in the research are surveys, interviews, and questionnaires, which help reveal the progress and results of the investigation. The research instruments designed will be used in the compilation of information from the field research.
The study was conducted using the strategic management methodology of Balanced ScoreCard which allows us to clarify, deploy, and give follow-up to the strategy through its four perspectives: finance, customers, processes, and human capital (Kaplan and Norton, 1996).

Results and Discussion:

Carrying out a preliminary study on the malanga and its nutritional value, we investigated the chemical composition of the corms of the taro root, which is high in nutrients, carbohydrates and proteins. In addition it is highly digestive, so it is considered an excellent food for consumers (Photo 1).

Photo 1. Malanga plant.

The corporate vision, was determined through the participation of the members of the company; "Be a leader in the national and international food market".
Business values obtained were as follows: Honesty, responsibility, commitment, initiative and teamwork.

The analysis of the environment, according to the Theory of Affinity, was (Daft, 2008):

- Unknown potential markets
- Need to improve the quality standards according to the competition.
- Required knowledge and mastery of the production process for baking tostadas made with malanga.
- Diversify the product according to the trends of the food market.

The definition of strategic objectives provide direction, in assessment and coordination; they are essential the activities of control, motivation, organization and planning effective in a company.

To achieve the business vision of the company, 7 strategic plans were defined. (Diagram 1). Designing the business strategy to conceptualize the future alternatives is also useful as a way to verify the previous decisions and analyze the company's presence in futures markets.

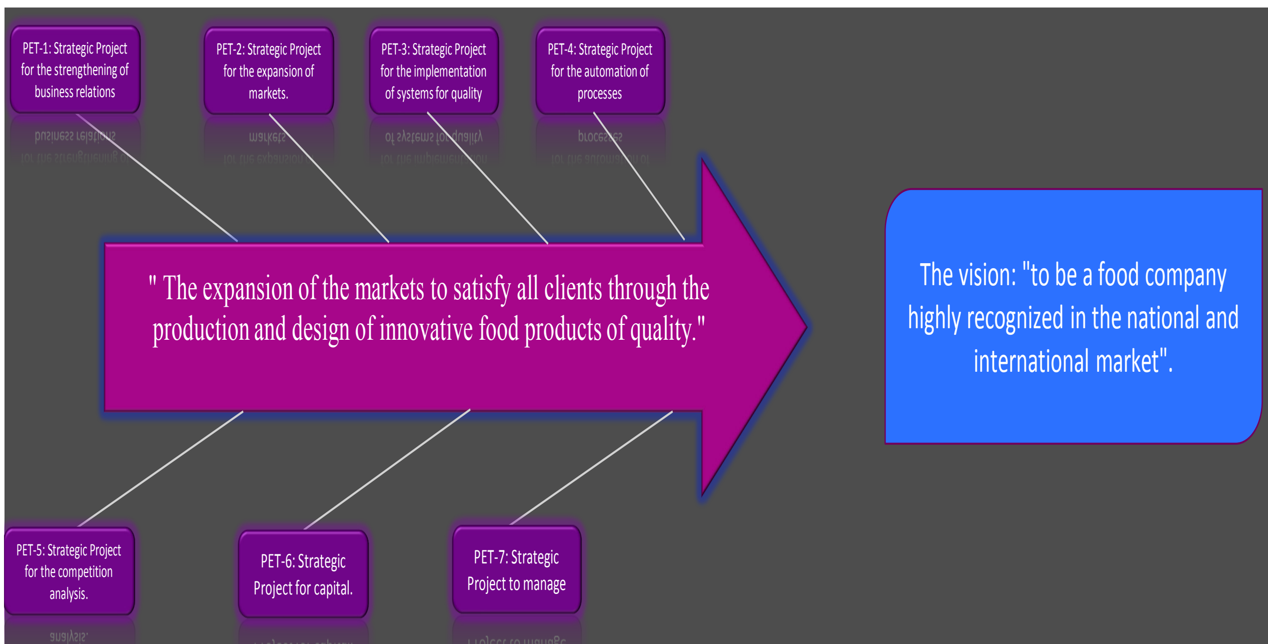


Diagram 1. Design of Strategic Plans.

As the organization conceptualizes its future, it must identify the specific means to measure the progress towards that future, by identifying the critical indicators of success (Norton and Kaplan, 2011). The strategic map for the business, according to their strategic plans, is as follows (Diagram 2):

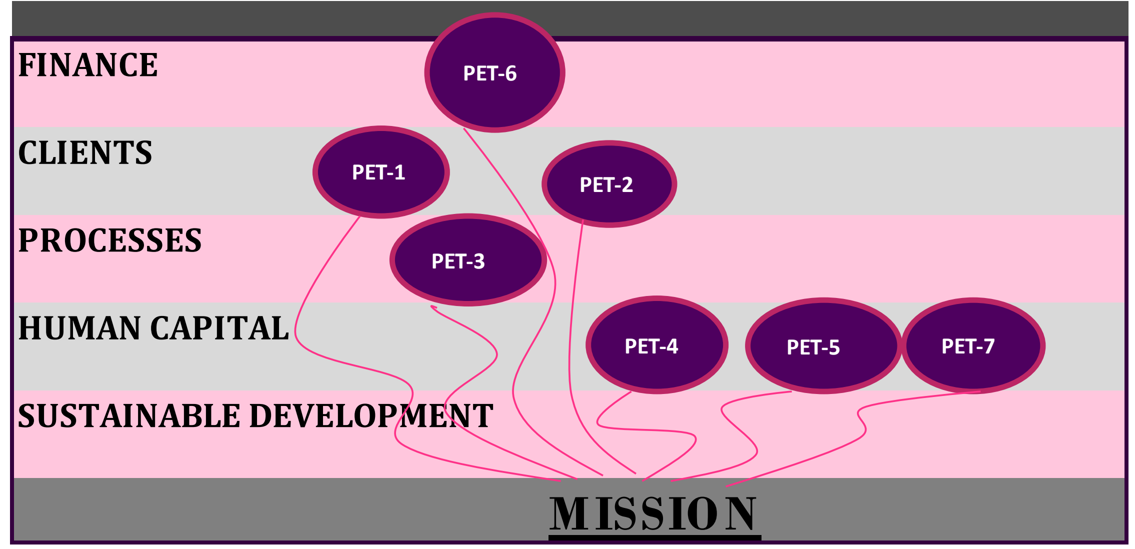
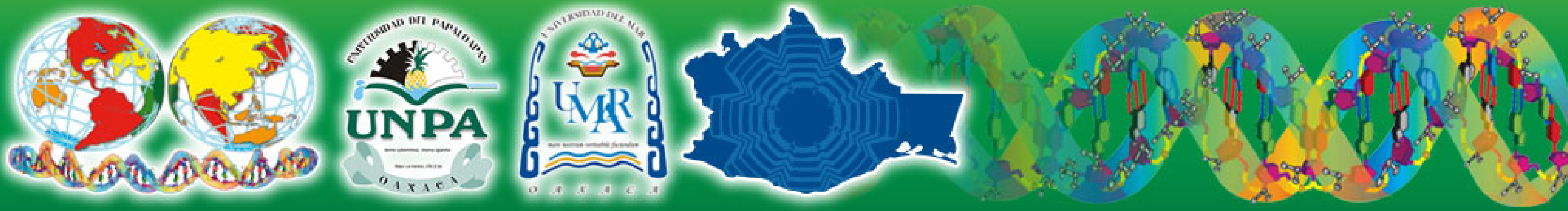


Diagram 2. Strategic Map.



Conclusions: The strategic actions constitute tasks, processes, and goals that are considered necessary steps to create the organization's strategic plan and achieve the mission. The Balanced ScoreCard is a tool to mobilize people toward the full implementation of the mission through channeling energies, skills and specific knowledge of persons in the organization towards the achievement of long-term strategic goals.

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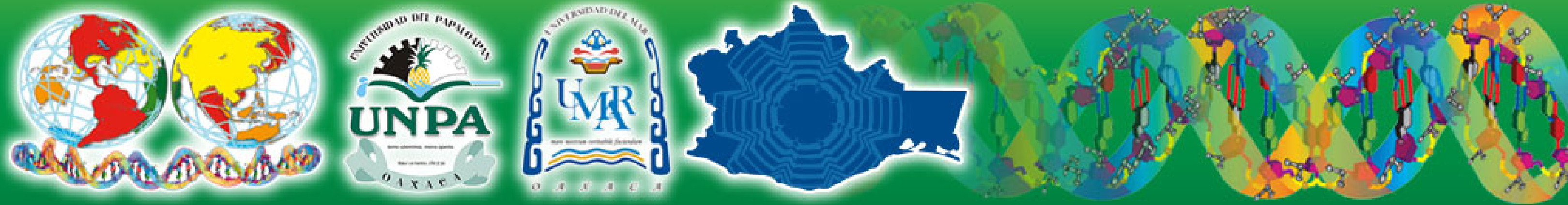

Market and economic-financial feasibility of a power-saving system

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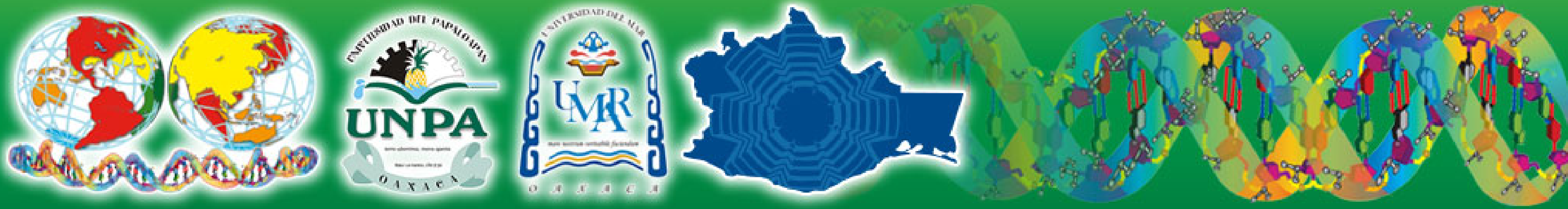
Abstract: The objective of this research is to analyze and evaluate, through a study of market and financial feasibility, a power-saving system project in the city of Coatzacoalcos, Veracruz, México. Electrical energy is essential for the development and productivity of nations and its use has become indispensable. Therefore it is necessary to optimize electrical usage, in order to avoid wasting energy resources and causing air pollution. The findings of this research are: the project is viable from the marketing and financial perspective, and contributes to the strategic objectives of the Secretary of Energy of the Mexican Federal Government.

Keywords: Ecology ● electrical energy ● market study ● financial study

Introduction: Mexico has a wide variety of energy sources, but most electricity is generated from coal, natural gas and oil. All of them are non-renewable natural resources and their use generates a large quantity of gases which affect the atmosphere, causing global warming and devastation to the environment. The Energy Saving Trust (Fideicomiso para el Ahorro de Energía Eléctrica) promotes, through its programs, efficient energy consumption, as well as energy-saving and clean technologies, and seeks to change the culture of energy consumption and to support research projects focused on energy saving. The State of Veracruz, México, makes a significant contribution to the development of the country. In the case of electric energy, both Petróleos Mexicanos and Comisión Federal de Electricidad extract petroleum and produce energy in the state, which makes it possible to efficiently supply electricity throughout Mexico.
The search for alternative energy and efficient energy consumption has led to new technologies in home automation, which are applied in the automation and control of energy use in the home. These technologies provide security, comfort and efficient use of electrical energy, reducing consumption. These energy saving technologies were used in this investigation (SENER, 2014).

Materials and Methods:

This research was conducted in the city of Coatzacoalcos, Veracruz, México, which supplies electricity to 85,933 households. The methodology used in this research was descriptive, explanatory, and deductive. The techniques that were used in the research were surveys, interviews and questionnaires, which were applied to a representative sample in the city, with a significance level in the research of 0.05. The pilot project involved 100 homes in the center of the city, where the home-automation technology was installed. The performance level of this home automation technology was measured using a criterion-referenced test.



Research instruments were designed and used in the compilation of the field research to generate the market study. A financial study to determine the economic viability of the project was also carried out. Both studies demonstrated the viability of the technology.

Results and Discussion:

The purpose of the project was to analyse the viability and feasibility of the financial market and an energy system based on the use of electronic components (sensors) and electrical components (relays), whose main function is to reduce energy consumption in rooms by means of motion detectors.

The aim is to maximize energy savings in the use of electrical and electronic equipment automatically, inside a room in a house and to assess economic and financial viability.

The system's operation begins with a motion sensor, goes through a control system, which sends a signal to the relay and optimizes the electric energy consumption of the lightbulb (Figure 1).

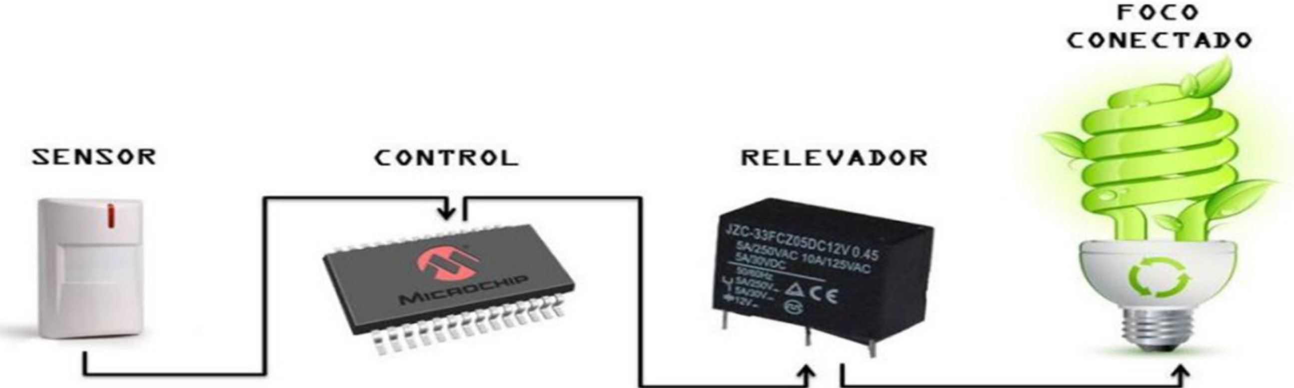


Figure 1. Process Sensor System.

In the research a SWOT analysis (Strengths, Weaknesses, Opportunities and Threats) was carried out, from which the following strategies were obtained (FIDE, 2014):

Strategy 1: Perform an advertising campaign focused on energy-saving and ecological awareness.
Purpose*:* To spread the savings culture by means of the advertising campaign, focused on ecological awareness (Veracruz, 2014).

Strategy 2: Promote plans for funding the product.
Purpose*:* To make the product available to the population.

Installation costs of this home automation technology are compatible with a family budget, making the project financially viable.

Conclusions: This research is feasible from the perspective of environmental care and consumer cost, because: i) The installation price is $ 843 Mexican pesos ($65 USD) per household, ii) The breakeven point is the sale of ten systems per month. Cash flow is positive starting the first year. Internal rate of return for the project is 23.8%, leading to a reasonable prediction that the project will be financially viable, and iii) on average, the 100 households in the pilot project reduced their energy consumption by 104 Kwh. At a price of $ 0.798 Mexican pesos per Kwh this resulted in a savings of $82.99 Mexican pesos per household, validating the research.

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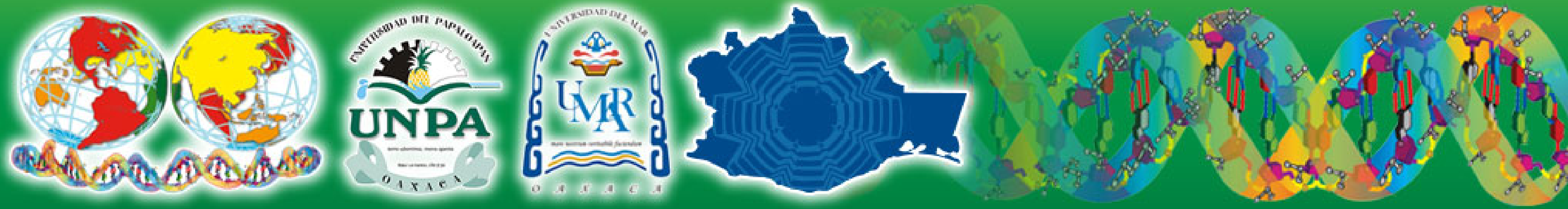

Bio-businesses and their economic value added

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Abstract: Bio-businesses are organizations that are more focused on research and development than the primary and secondary activities in their value chain. Herein lies the importance of analyzing whether science and technology are functioning properly in these companies. Sometimes bio-businesses are confused with traditional businesses which sell their products to industries that use biotechnology. The difference between a bio-company and a traditional company is that the bio-company is mainly dedicated to research, and its essential goal is to provide innovative market solutions. If a company is a bio-company, its products will have value in accordance with market trends. However, greater market risk is a downside of this type of organization. Normally these bio-enterprise are consolidated, profitable, competitive and economic value added industries, with a growth future linked to innovation. The importance of this research lies in a financial analysis of the paper industry and determination of its economic value added, as their contribution to environmental protection is undeniable. Currently two thirds of the input for the paper production comes from recycled paper, and the remainder comes from wood. There is a financial advantage of using recycled paper, as there is a 60% saving in electricity; and for every ton of recycled paper, 17 fewer trees are felled, 25,000 liters of water are saved, and the use of chemicals needed to convert cellulose to paper is reduced.

Keywords: Bio-businesses ● financial benefit ● environment

Introduction: This research carries out a comparative financial analysis between a traditional company and a paper industry bio-company, to assess financial impact through the generation of economic value added of both businesses (Ibañez, 2004). The bio-company connected to this research generates an EBIT (Earnings before interest and taxes) of twenty million pesos, its net operating profit after tax is about nineteen million pesos, and its economic value added is positive. In contrast, the traditional paper industry generates a positive EBIT, but its economic value added is negative. This bio-company aims to make inroads into the emerging green market composed of those sub markets which, in the medium and long term – given the increasing demand for green products and increased environmental education and awareness – will have an impact on its growth and development.

Material and Methods:

This paper company emerged in the forests of Mexico, with a modest sawmill and a dream: to create value chains from comprehensive and regenerative forestry in harmony with the environment. To foster a low-carbon economy, protect forests and become part of the solution to the problem of global warming, the company designed a sustainable business model that allowed it to move away from the conventional paper industry mode, characterized by a high social cost of paper manufacture from intensive tree felling. The methodology used in this research is

descriptive-explanatory. The method is deductive. The techniques used are surveys, interviews and questionnaires which aid in presenting the progress and results of the research.
For the financial analysis and obtaining economic value added, financial engineering was used, as it allows for the evaluation of the maximization of company resources and their application in productive and profitable projects. In this way, the company can meet its social and economic commitments, reducing risk and increasing value for its shareholders.

Results and Discussion:

It is essential for corporations and shareholders to assess whether managers are creating or destroying value, and it must be understood that book value is usually a very inaccurate measure of value perceived by shareholder.
Financial objectives are typically related to profitability and value drivers such as (Diez, 1994):

- Economic Value Added.
- Sales Growth.
- Profit margin earnings before interest and taxes (EBITDA); profits after tax.
- Working capital.
- Cost of capital.

Some measures of financial performance that were used to conduct this research into the paper industry are listed below:
A measure of profitability is Net Operating Profit after Taxes (NOPAT), which highlights the return on capital investment. Another measure is the residual income (RI), sometimes called economic benefit, which can be defined as: RI = NOPAT - Capital x Cost of Capital (COC).
If we define the return on capital (ROC) as the ratio of NOPAT to capital, the formula would be: ROC = NOPAT / Capital). The residual profit is what is left for shareholders after the suppliers have been compensated, and in this way we can qualify the company as profitable or not. The residual profit is generated when the return on invested capital is greater than the cost of capital (Marshall, 2002).

If RI> 0 the company creates shareholder value.
If RI <0 the company destroys shareholder value.

The concept of residual benefit is not new. It was created in the twentieth century by General Motors, and later Stewart called it economic value added (EVA) and defined it as:

$$EVA = NOPAT - Capital \times COC$$

A company creates value when the EVA is positive and destroys value when it is negative.
The research results obtained regarding the conventional paper industry and the recycled paper industry are listed in Table 1.

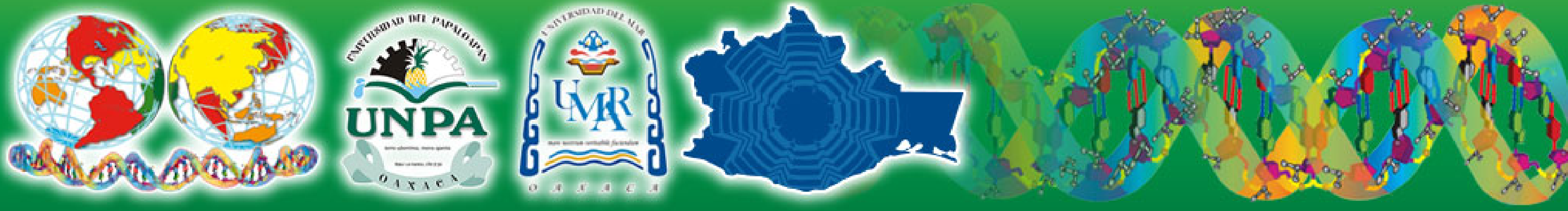

Table 1. Measures of financial performance.

CONCEPT	PAPER INDUSTRY (Mexican pesos)	RECYCLED PAPER INDUSTRY (Mexican pesos)
EBIT	917,097	24,308
NOPAT	- 2,803,670	19,755
CAPITAL	26,235,042	40,000
COC	12%	12%
ROC=NOPAT /CAPITAL	-11%	49%
RI=CAPITAL x (ROC _ COC)	- 5,951,875	14,955
EVA=NOPAT-(CAPITAL x WACC)	- 5,951,875	14,955
ION=NOF + FIXED ASSETS	19,031,973	59,903
ROCE=EBIT/CAPITAL	3%	61%
FACTOR Z	- 110.71	1.13
FREE CASH FLOW	- 5,593,820	18,857
RION = EBIT/ION	5%	41%
RIONDI= RION X (1-RATE)	4%	29%

Conclusion: It can be seen that the industry which uses recycled paper as raw material has a Net Operating Profit after Taxes (NOPAT) of $ 19.755, which highlights the return on invested capital. Additionally, its Economic Value Added is positive with $ 14.955, which means it is creating value for shareholders.

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Characterization of micro, small & medium enterprises (MSMEs) at Tuxtepec, Oaxaca: Classification of commercial business

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Abstract: The Papaloapan basin region is characterized by extensive economic activity within the city of San Juan Bautista Tuxtepec; which is a point of convergence for economic activities for the states of Oaxaca, Veracruz and Puebla, because it has important economic activity in the primary, secondary and tertiary sectors. This paper presents the preliminary results of the classification of the twists of Micro, Small, and Medium Enterprises (MSMEs) in the first quadrant of the city of Tuxtepec, Oaxaca.

Keywords: MSMEs ● commercial business ● economic sectors

Introduction: At present the role played by companies in Mexico is visible in job creation, growth and development, because there are approximately 4 million 15 thousand business units, of which 99.8% are MSMEs which generate 52% of Gross Domestic Product (GDP) and 72% of employment in the country (INEGI, 2009). The Red-Cumex MSMEs (2010) describes the complex problems with them noting aspects such as lack of experience and training of entrepreneurs, lack of proper public policies and their problems and present reality. However for (Kauffman, 2001) the most important problems they present are: inadequate formation of the economic system that favors, and gives priority to large and very large companies and corporate; inadequate government policies; corruption of public officials; high cost of financing; inadequate technical and production infrastructure; lack of technological resources; almost no application of appropriate tools of business planning; unfair competition from informal trade; globalization and unfair practices internationally; the lack of a corporate culture of Mexican businessmen. According to the Observatory MSMEs Mexico (2003), based on (Jiménez, 2010), another problem that these companies face in Mexico is the lack of available statistical information on the economic role and performance. On the other hand to identify and understand the degree of development of an economy is necessary to analyze its economic sectors (primary, secondary, tertiary) integrating them and the ability of each of them to meet the demands of supplies, materials, machinery and skilled labor within the sector and the national or regional economy work. Where the primary sector includes all activities related to natural resources such as agriculture, fishing, mining and extraction of oil, coal, nickel, emeralds and gas, among others; the secondary sector meets all industrial activities, whereby raw materials and goods are transformed; there are additional characteristics for different degrees of development. In this sector industry is at its manufacturing industries such as the manufacture of processed food, beverages, furniture, glass or clothing. This sector also includes the construction and metalworking, steel and textile industries; and finally the tertiary sector includes transportation, education, entertainment, health, justice, communications, tourism and services water, electricity and banking (Pachon, 2005).

Therefore this paper presents the classification of types of business in each industry used to make a diagnosis of MSMEs in the first quadrant of the city of Tuxtepec, Oaxaca, population 101,810, the second most populous city in the state of Oaxaca (INEGI, 2010) city. The service sector is the largest and fastest growing activity due to its location in the center of the Papaloapan basin, the city has become the main meeting point for buying and selling activities of the surrounding areas (H. Ayto. Municipal of San Juan Bautista Tuxtepec, 2014).

Materials and Methods:

The Weisbord Model is an instrument of organizational diagnosis that indicates where and what to look for to diagnose the problems of an organization; this model consists of six critical areas: purpose, structure, rewards, helpful mechanisms, relationships and leadership, which must function properly if you want the organization to succeed (French, 1996). According to the revision made to the diagnostic model Marvin Weisbord noted that the variables are not sufficient to analyze situations and important in business today, primarily to the characteristics of the city MSMEs Tuxtepec. So in addition to the six variables listed by Marvin Weisbord, the variables considered for obtaining information that shaped sections in the questionnaire are: identification of the business; profile of the respondent; information technology; marketing and sales; administrative management; social responsibility and change; finances. A questionnaire with 95 items, including Likert scales, dichotomous and open questions was used for data collection.
Types of business were classified by identifying the business sector and where the specific type of different MSMEs surveyed, based on the North American Industry Classification System (NAICS) version 2013 established as the classification of economic activities of the National Institute of Statistics and Geography allowing to interpret all categories, which are grouped into sections according to their specific type and commercial activity (NAICS, 2013).
Also an object of this work is to identify the characteristics that meet MSMEs classification as a biobusiness, such as performing a set of activities of collection, production, processing and marketing of goods and services derived from native biodiversity under the criteria of environmental sustainability, social and economic, this will be achieved using the variable of social responsibility that are handled in the questionnaire in order to analyze various actions such as saving electricity, saving and caring for water, collecting plastic, paper, cardboard for reuse, prevent polluting the air with smoke, fumes, noise and toxic waste channeled to the right places; what MSMEs do to benefit society taking into account their impact on the natural environment.

Results and Discussion:

Table 1 shows the preliminary results of the classification of the specific types of MSMEs Tuxtepec City, Oaxaca. The classifications of the types of the businesses surveyed are five sections that make up the first quadrant of the city of Tuxtepec, which include the colonies: The Piragua, Lazaro Cardenas, Maria Luisa, Ex-normal, Heladio Ramirez Stage 1 and 2. As shown in the table, the sector that has the smallest economic impact is the industrial sector, because the area and objects of study correspond to the first quadrant of the city and only to MSMEs; however the commercial and service sectors are the two most predominant sectors in the classification of the specific business types as in the study of the city of Tuxtepec is most focused on these commercial business activities.

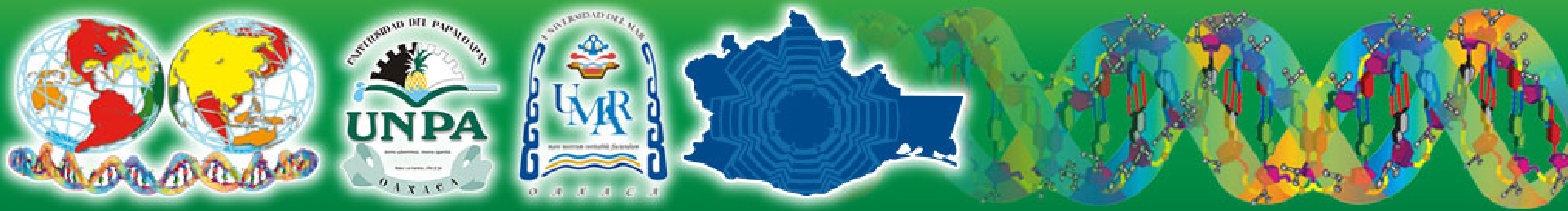


Table 1. Classification of the specific orders of the MSMEs Tuxtepec, Oaxaca.

	Industrial
	Food and Drink
1	Ice Maker
2	Popsicles, ice cream and prepared waters
3	Bakery
4	Purifying and bottling water
5	Selling tortillas
	Durable Goods
6	Blacksmithing and metal structures
7	Sheet shop
8	Metalworking (around)
9	Carpentry
10	Print Workshop, bound
11	Printing and advertising
	Personal items
12	Tailoring and sewing garments
13	Manufacture of uniforms
14	Jewellery (Costume)

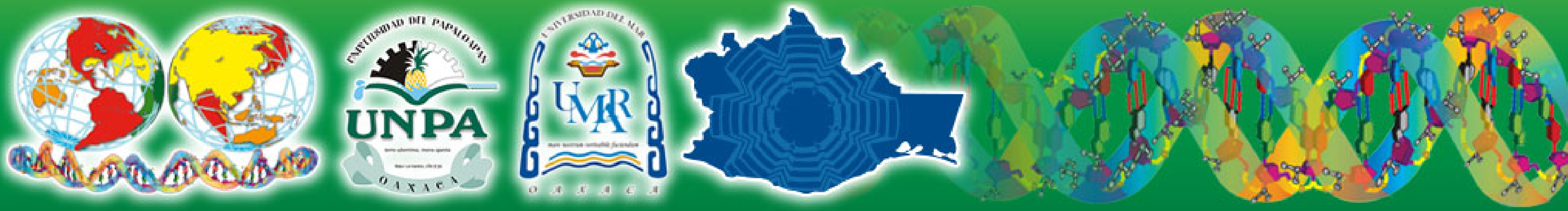
	Commercial
	Food and Drink
1	General groceries
2	Meat (beef and / or pork)
3	Dried chiles and seeds
4	Beer tank
5	Sweets
6	Soda fountain
7	Dairy and meats
8	Fish and Seafood
9	Poultry
10	Wholesale egg Sale
11	Of tamales Sale
12	Fruit and vegetable shop
13	Wines
14	Raw materials for confectionery
15	Palettes and Cream
	Durable Goods
16	Accessories and / or auto parts
17	And various import items
18	Glassware, and utensils slab
19	Hardware and tlapalería
20	Agricultural and gardening tools
21	Cordage (cleaning supplies)
22	Tires and tubes
23	Knitting and hosiery
24	Furniture and fixtures
25	Bicycle Parts
26	Electronic Parts and appliances
27	Motorcycle Parts
28	Fabric and white
29	Motor vehicles
30	Glass, mirrors and aluminum
	Personal items
31	Orthopedic footwear
32	Clothing of Use
33	New clothes
34	Clothing and accessories for babies
35	Hats
36	Shoe Sale
	Electrical and electronic products
37	Electronics
38	Furniture, computer equipment and periphera
39	Power supplies
40	Cell Phones

	Commercial
	Real luxury amenities
41	Handicrafts and decor
42	Sporting Goods
43	Jewellery
44	Audio and video discs
45	Florist
46	Studio picture
47	Musical Instruments and audiovisual
48	Jewelry and Watches
49	Toy store
50	Bookstore
51	Perfumery
52	Newspapers and magazines
53	Flooring and ceramic coatings
54	Pet stores
55	Gift shop
56	Dresses for parties and special events
57	Vivarium
	Chemicals
58	Automotive oils and lubricants
59	Agrochemicals
60	Fuels
61	Paints, sealants and solvents
62	Cleaning chemicals
	Hygiene and Health
63	Braces
64	Pharmacy
65	Imagery
66	Nutritional supplements
67	Optics
	Multiple products
68	Department store
69	Supermarket stores
70	Convenience stores
	Other:
71	Religious articles
72	Forage
73	Stationery
74	Disposable products

	Services
	Food and Drink
1	Bar, canteen and similarly
2	Cafeteria and soda fountain
3	Fonda and / or soup kitchen
4	Pizzeria
5	Restaurant
6	Rotisserie and grilled
7	Selling tacos
	Entertainment and fun
8	Musical instrument rental
9	Holiday party rooms
10	Banquets and social events
11	Library
12	Billiards
13	Singers and bands
14	Nightclub
15	Film
16	Discotheque
17	Fashion Design
18	Lottery outlets and tips
19	Gimnacio
20	Groups of Performing Arts
21	Video and photography
22	Video Games

	Services
	Hygiene and Health
23	Fitness centers
24	Office for health care
25	Gynecologist
26	Health and personal care
27	Hospital
28	Clinical laboratory
29	Laboratory of X-ray and ultrasound
30	Medical
	Maintenance and Repair
31	Car washing
32	Washing, polishing and waxing automotive
33	Renewal of footwear
34	Repair of computer equipment
35	Watch repair
36	Automotive mechanical and electrical workshop
37	Visual arts workshop
38	Bicycle workshop
39	Auto electronics and appliances
40	Rolling mill and painting
	Tourism and related activities
41	Travel agency
42	Lodging houses
43	Internet café
44	Parking and pension
45	Hotel
46	Motel
	Telecommunications and transportation
47	Phone booth
48	Radiodifusora y/o televisora
49	Servicio postal, mensajería y paquetería
50	Telephony and communications
51	Freight
52	Passenger transport
	Crafts and other specialized activities
53	Locksmithing
54	Construction company
55	Professional design
56	Graphic design
57	Pest exterminanción
58	Funeraria
59	Private nursery
60	Public nursery
61	Laundry and / or dry cleaning
62	Home cleaning
63	Lettering
64	Private security
65	Photocopies
66	Upholstery
67	Veterinary
	Administrative, financial and legal
68	Lawyer
69	Employment agency
70	Consulting and advisory
71	Pawn shops
72	Accountants
73	Accounting firm
74	Audit office
75	Legal office
76	Financial and insurance institutions
77	Public desktop
78	Real estate
79	Notary

	Services
	Public and private education
80	Public preschool
81	Private Preschool
82	Private primary education
83	Public Elementary Education
84	Public secondary education
85	Private Secondary Education
86	Average public higher education
87	Private Higher Secondary Education
88	Public higher education
89	Private higher education
90	Computer schools
91	Language Schools
	Other:
92	Asociaciones y organizaciones



Conclusions: Upon completing this work we can conclude that the classification and identification of the specific types of business that was used led to a better analysis of the different study variables (size of business, type of business, age, mode of operation, and other sectors) related to the particular characteristics which are the object of study and allows MSMEs to develop a way to identifying the problems and characteristics the operation of these businesses.In addition, the classification of business types was achieved following the model of the six critical areas of Marvin Weisbord (aims, structure, rewards, helpful mechanisms, relationships and leadership), however as we want to know the prospects and current status of MSMEs in first quadrant of the city of Tuxtepec, Oaxaca, we included in the questionnaire paragraphs identifying the business; a profile of the respondent; information technology; marketing and sales; administrative management; social responsibility and change; finance, this is in order to later determine what percentage of MSMEs belong to each sector and to specific type of business.

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Health and marketing by PREVENIMSS

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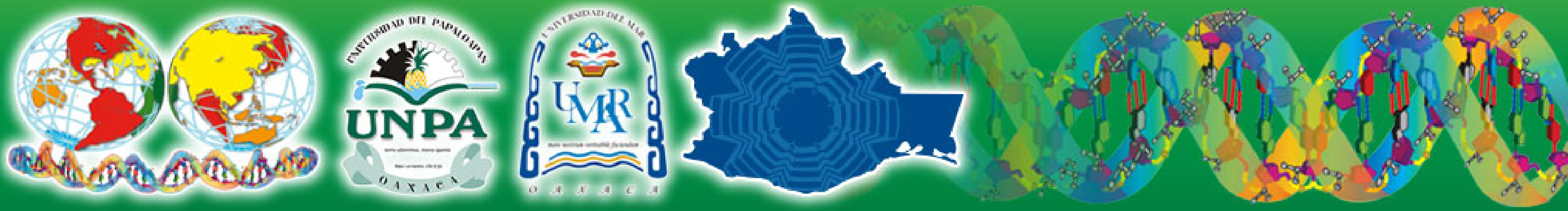
Abstract: It show the link between the health and Marketing as it provides customer satisfaction making profits in return, as a useful tool to address many of the challenges generated by the field of health in our country with the creation PREVENIMSS for its acronym in Spanish prevention and Mexican Institute of Social Security, program in which nurses wearing it just in the field of Public health.

Keywords: Health marketing ● marketing ● PREVENIMSS ● social marketing

Introduction: Any health institution, even the smallest, put into practice the principles of Marketing, from the nurse-patient relationship event if does not have a specialized department in a community, what makes for common sense, which is reflected in the success of the community, orienting to different groups in order to change attitudes, beliefs and interests individually and collectively through health education. The Health Marketing is a discipline that has great potential for application in the field of Public Health. The American Marketing Association defines marketing as the process of planning and executing the conception, pricing, promotion and distribution of ideas, goods and services to create exchanges that satisfy individual and organizational objectives. For the British Institute of Marketing is: responsible for identifying, anticipating and satisfying the needs and requirements of the consumer process.

Materials and Methods:

This was prepared based on the size, significance, impact and vulnerability of damage or prevent risk factors. Of disease prevention and specific danger to the health protection: With the foregoing the traditional approach to health programs is changed. Through its strategic design of Health Marketing to promote and strengthen habits and healthy lifestyles with a proactive, innovative and different approach. In combination with these other disciplines, allow to implement strategies in different subjects of health promotion and disease prevention.
It is the process of planning and execution of the concept, establishment of promotion and distribution of ideas and services to influence the target audience to acpetar, reject, modify or abandon some behavior for the benefit of the individual, group or society. Health Promotion: Support strategies for health education for risk management and developing personal skills, capabilities and competencies in health. Influence public opinion and promotes advocacy to strengthen community action and creating healthy public policy. Causes creations of social support networks (communities) to develop environments conducive to health.
With this we can define the importance of linking health and Marketing as it provides customer satisfaction making profits in return. A linking strategy is the PREVENIMSS program that provides delivery service that has the general purpose systemic and orderly provision of health-related actions ranked into five age groups: 1 Child (under 10 years), 2 Teen (10-19 years), 3 Women



(20-59 years), 4 Male (20-59 years) and 5 Senior (60 and over) and five components: i) promotion health, ii) nutrition, iii) disease control and prevention, iv) early detection of diseases, and v) reproductive health.
With this there is exchange between the client and the nurse in public health programs, through a specific methodology for its implementation and development, but flexible to suit the needs and strategic and cultural realities of each State or locality.

Results and Discussion:

PREVENIMSS is one of integrated health programs, is a model of health care of the Mexican Social Security Institute, which links the health with Marketing. With the actions taken by the Marketing as a process of planning and executing the conception of the product, price, promotion and distribution of creativity to create exchanges that satisfy individual and organizational goals of the organizations.
The strategy is PREVENIMSS fundamental commitment to improve the quality of life of users, through raising their culture and information in health care, for active and responsible participation among eligible and nurse.

Conclusions: The purpose is linking health and Marketing is understood as administrative guidance that concerns a process of exchange between the client and the practitioner formally in the various approaches to the handles and characteristics of health services service, price-time, distribution-location, promotion and communication PREVENIMSS creation program in which nurses wearing it just in the field of Public health to achieve quality standards - Four product areas.

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2. Abstracts

2.1 Plenary lectures

Molecular modeling of the pathogenic Gly753Arg mutation in the HECT domain of Smurf1

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Smurf1 is a very important gene because it encodes an ubiquitin ligase that is specific for receptor-regulated SMAD proteins in the bone morphogenetic protein (BMP) pathway (Zhu *et al*., 1999). Smurf1 acts as a negative regulator of the BMP signaling pathway. The Smurf1 gene is highly expressed in embryonic and adult cells and in tissues. Enriched expression is observed particularly in developmental stages (Rotin and Staub, 2011). Thus, defective Smurf1 can cause birth defects and genetic diseases. Smurf1 is an acronym for Smad Ubiquitination Regulatory Factor-1. The gene codes for an E3 ubiquitin-ligase protein. This protein has four subdomains: C2, WW1, WW2 and HECT. The Smurf1 E3 ubiquitin-ligase is localized to the plasma membrane, in close proximity to RhoA (Rotin and Staub, 2011). The C2 domain is the first domain of Smurf1 and has an affinity for phospholipids (Tian *et al*., 2011). The C2 domain is required for the Smurf1 protein to target RhoA and interacts directly with it (Wang *et al*., 2006). The two WW domains (WW1, WW2) interact with substrates that contain PY patterns, like Smad1/5/6 (Sangadala *et al*., 2007). The HECT domain (homologous E6 associated protein C-terminus) is involved in the ubiquitination reaction, a post-translational modification. The secondary structure of the HECT domain consists of six alpha helices and six beta strands. Poly or mono ubiquitination allows target proteins to follow the cellular pathways to which they are intended. The poly-ubiquitination of lysine at position 48 of the target substrates will be recognized by the proteasome and will allow degradation of the protein having at least four added ubiquitin chains. There are two major families of E3 ubiquitin ligases, one with a RING subdomain and one with an HECT subdomain. E3 ubiquitin ligases having a RING subdomain, unlike E3s containing an HECT subdomain, catalyze directly the isopeptide bond between the C-terminus of ubiquitin conjugated to E2 and the lysine on a given specific substrate (Ozkan *et al*., 2005). Smurf1 contains an HECT subdomain. In this region, there is an active cysteine in position 725 that will make a covalent glycyl thioester intermediate linkage with the C-terminus carboxyl group of ubiquitin. Once transferred to the HECT subdomain, a pseudo-peptide bond is catalyzed between the C-terminus of ubiquitin and the specific lysine on the substrate (Kornitzer and Ciechanover, 2000). The different types of ubiquitination will allow target substrates to be recognized by other proteins and follow different metabolic pathways (degradation; Sadowski and Sarcevic, 2010). This process is reversible through the action of deubiquitinases (DUBs) that remove ubiquitin chains linked to the target protein (Komander *et al*., 2009). Many proteins interact with Smurf1 but are not ubiquitinated. They facilitate the interactions between Smurf1 and the target substrates. Smurf1 mediates ubiquitination and degradation of SMAD1 and SMAD5, two receptor-regulated SMADs specific for the BMP pathway. It also promotes ubiquitination and subsequent proteasomal degradation of TRAF family members and RHOA (Zhu *et al*., 1999; Bryan *et al*., 2005; Li *et al*., 2010; Lu *et al*., 2011). Smurf1 induces also TGF-beta type I receptor degradation (Ebisawa *et al*., 2001; Tajima *et al*., 2003; Asano *et al*., 2004).

Three polymorphisms have been reported for human Smurf1, none of which led to disease: one in the region between the second WW subdomain and the HECT subdomain, and two in the HECT subdomain (Domain Mapping of Disease Mutations database, bioinf.umbc.edu/dmdm).
In this work, we will study a glycine to arginine mutation at position 753 in the C-terminus of the HECT domain of the Smurf1 gene product by means of molecular modeling. The goal of protein modeling is to predict a 3D structure from its sequence with accuracy comparable to the best results achieved experimentally. This germ-line mutation was reported by Qatar medical doctors and geneticists (Shafallah Medical Genetics Center, Doha, Qatar) on people with a form of autism. We will try to understand the molecular and cellular effects of this mutation and how it can possibly lead to the disease.

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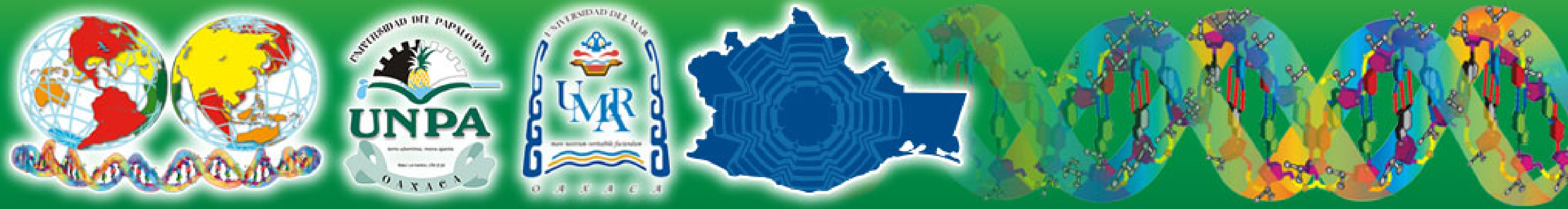

Theranostic nanoplatforms in cancer: an overview

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Nanobiotechnology in medicine is an area of emerging interest and opens up a whole universe of new possibilities for the early stage diagnosis and treatment in cancer. Developing countries are also taking part in the development of this technological revolution in medicine. In Latin America, the leading nations in this area are Brazil, Mexico and Argentina. The National Nanotechnology Initiative (Arlington, VA, USA) defines nanotechnology as 'the understanding and control of matter at dimensions of roughly 1-100 nm, where unique phenomena enable novel applications'. In fact, nanomaterials are designed to have at least one dimension (length, width, height) at the nanoscale of 1-100 nm. Nanoscience is defined as the study of phenomena and the manipulation of materials at the atomic, molecular and macromolecular scales, where the properties differ from those at a larger scale. Nanomaterials that have a nanoscale length, width and height are known as nanoparticles. As the size of the particles gets reduced to nanoscale range, there is an immense increase in the surface to volume ratio which increases reactivity and change the mechanical, electrical, and optical properties of the particles. The nanoparticles, having much larger specific surface area than their coarse analogs, exhibit enhanced biological activity and present undeniable interest as carriers or in drug delivery. Many types of nanoparticles exist with respect to their size, shape, material, and coatings. The specific properties of the core materials provide distinct monitoring and therapeutic applications. For example, nanoliposomes and nanocasules have been evaluated over the years, and a significant amount of evidence has been obtained showing that these carrier materials are able to improve the balance between the efficacy and the toxicity of therapeutic interventions. Besides for therapeutic purposes, nanomedicine formulations have in recent years also been increasingly employed for imaging applications. Theranostics is a concept which refers to the integration of imaging and therapy.

Starter cultures in the meat industry

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Many years ago, the agro-food industry has used microorganisms and their enzymes to improve and transform the properties of some raw materials. Different cultures of microorganisms called “starter cultures” are used in the meat industry, to increase up some processes of maturation. Among the microorganisms that have been investigated for such use are: bacteria, yeasts and fungi, from which takes advantage of the ability to metabolize proteins and lipids, as well as synthesize compounds that give it aroma and flavor to the product.

Microbial cultures in food production.

Starter cultures are the ones that try to induce desirable sensory changes on the product. They are microorganisms that occur in pure or mixed state, selected according to their specific properties and which are added to certain foods, especially meat and dairy products, to improve its appearance, aroma and flavor, as well as the conservation of the same. The most commonly used are lactic acid bacteria (LAB). They are used mainly in the dairy industry and growing amount also in the meat industry. With their use they present mainly 4 functions, in varying proportions:

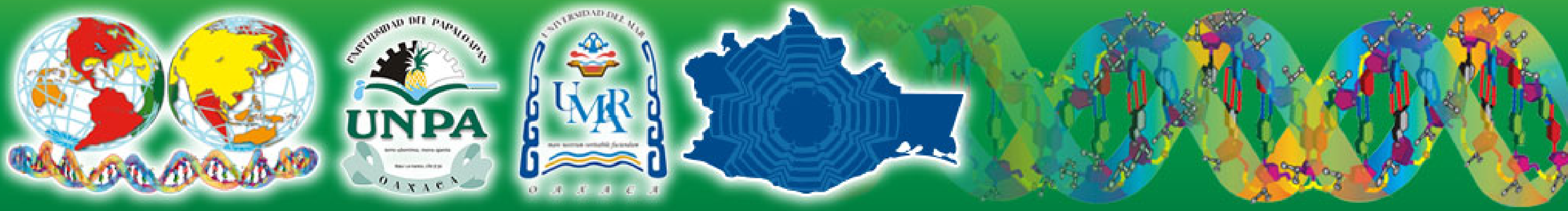
- Decrease the value of the pH at given speed within certain limits.
- Action alternative native germs and germs that infect food by microbial contamination.
- Generator effect of aroma as a consequence of the microbial metabolism in feed (only in certain species), to obtain certain qualities.
- Reduction of enzymes to induce hydrolytic and/or catalytic processes in the food.

The LAB form enzymes that participate in a decisive manner on the transformations that occur in foods, mainly lipases and proteases, which influence or determine the phenomena of maturation in a series of products.
The mostly used genera are of the genus *Lactobacillus, Leuconostoc, Pediococcus and Streptococcus*.

The main purpose the starter cultures are added to matured sausages can be summarized in the following points:

- Control of the maturation process.
- Inhibition of undesirable microorganisms.
- Reduction of health risks.
- Increase of quality and standardization.
- Control of specific taste and aroma.

In several European countries, including Russia, Germany, Bulgaria, Yugoslavia, Finland, and France and in the United States they are being used to prepare cured raw meats. They are sometimes used in combination with micrococci or other species of LAB.



Increased use of starters cultures are: *Lactobacillus plantarum, L. brevis, L. farciminis, L. alimentarius and L. curvatus.* These species are added as initiators to ensure a flavor and aroma uniform and good conservation. The effectiveness of a starter culture for the meat industry is defined by its metabolic ability in the flesh under the conditions present in the sausage, concentration of salt, moisture, water activity, redox potential, pH, temperature, availability of nutrients, etc. Starter cultures are marketed typically frozen or freeze-dried. Also being use fungal starter cultures (from molds), preferably: *Penicillium candidum, P. camemberti and P. roqueforti.* Used species of mould most not have toxic properties, so it can be used without any risk in the meat industry.

A fungal Starter culture must have the following characteristics:

- Don't have to produce toxins or antibiotics,
- be able to develop in the conditions of maturation of a sausage,
- Do not produce flavors and aromas of anomalous but do produce proteases and lipases that can contribute to improve the sensorial characteristics of the product.
- They must grow up fast and smooth to prevent the growth of other undesirable moulds and develop a mycelium whose color confers to the product a nice appearance.

If the Starter culture used features previously mentioned, its use would provide the following advantages:

- The sausage will be dry, uniform layer, an attractive appearance, aroma and flavor,
- Its fast growth will prevent the development of other fungi or yeasts that could devalue the product, commercially,
- Lower the risk of crusting due to inadequate drying conditions,
- It will contribute to the delay of rancidity to decompose the peroxide,
- It will prevent other molds, potentially toxigenic, to colonize and develop in the sausage.

Due to the importance that has been acquiring the use of starter cultures in the meat industry, and it has been used in Europe for many years and in the United States. Its use is increasing gradually in Mexico. The growing presence of cured raw meat products (mainly of foreign origin) has been found in big supermarkets. Therefore, our Institute is initiating research projects related to the production of this type of food, in order to reduce costs since industrialization could perform at the local level and eliminate the costs of importation.

Preparedness and response highlighting red sky

Wilton C. Menchion

Centers for Disease Control and Prevention

This year's Biotechnology Summit brings together a host of academic and luminary though leaders that may be instrumental in changing and advancing the face of Biotechnology and health security for generations to come. At the Centers for Disease Control and Prevention, we have be working in collaboration with a cross-section of stakeholders and partners both internal and external to the United States Government (USG) to speed the delivery of actionable data and associated information to decision makers at all level of the Health Security spectrum. These and other efforts have led to the development of a mass data gathering and information sharing tool called Red Sky. Toward this end, this presentation will focus on the activities of CDC's Health Security actions and high the capabilities of Red Sky. What is Red Sky? CDC Red Sky is a secure, easy-to-use, web-based dashboard and knowledge management system that allows stakeholders and leadership to access critical public health emergency information anywhere and anytime. Red Sky improves CDC's capacity to collect, share, and improve comprehension and understanding of critical public health events to enable faster and more effective decisions to save lives and reduce morbidity.

What does CDC Red Sky do?

- Provides a single integrated information sharing and data visualization capability to notify CDC Director, leadership, and staff of significant or developing public health security events and enables programs to report, collaborate, and apply their collective knowledge in one location.
- Provides partners a web-enabled capability to monitor, publish, and share verified and validated information concerning public health security events; enabling mutual support and faster response.
- Improves timely and accurate information sharing with internal & external stakeholders; reduces cost, improves collective action, and reduces duplication of effort.

Entrepreneurship: A choice for life?

Ricardo Gómez Flores

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The word "entrepreneur" comes from the French word *entreprendre*, 19th century, meaning "one who undertakes". According to the Oxford dictionary, an entrepreneur is someone "who establishes a business, taking on financial risks in the hope of profit." Entrepreneurship is much more than the process of starting a business. Entrepreneurship is a habit and an attitude; a set of skills applied to the search for innovation in the business, personal and professional contexts, and an approach to life around innovative thinking, calculated boldness, and proactive behavior.

Why is entrepreneurship important in our lives? There are several important reasons. First, entrepreneurial skills protect us in a weak labor market, giving us the tools to create our own businesses and jobs. Second, learning about entrepreneurship broad our perspectives on ways to further our career with an entrepreneurial spirit, instead of the traditional ways in particular fields. Third, today, more than ever, companies seek employees with some kind of business experience. The leading companies of all sizes understand that they must innovate to survive and thrive. Students who have an entrepreneurial mindset have developed strong communication skills, recognize how to take the initiative, and know how to execute it. By hiring entrepreneurs, these companies are bringing innovative perspectives to the workplace.

From a business perspective, entrepreneurial spirit is often associated with business creation, which can be taught in the classroom, and is commonly associated with the process of defining the business idea and identifying business opportunities, the development of a business plan, and finding financing needed to establish and grow our business. In the Autonomous University of Nuevo León (UANL), entrepreneurship is taught, and business initiatives are stimulated. UANL nurtures an entrepreneurial ecosystem in growth, ranging from the operation of the entrepreneur program at the middle and higher education, to prompt the foundation and business incubation, acceleration of business, and partnering with companies to support their technology demands. In this ecosystem, the key message is "entrepreneurship for life".

From a personal perspective, entrepreneurship motivates us to get out of our sphere of comfort, facing challenges and risks to build our own future, being tolerant to stress and failure to transform the obstacles encountered along the way, into opportunities. The entrepreneurial philosophy seeks to provoke a change in the form of being, doing and thinking of individuals by developing skills, abilities, values, motivations, and attitudes that enable one to develop the entrepreneurial spirit that dwells in every human being, making it increasingly competitive in their field, increasing the feeling of professional accomplishment, and feeling a useful person to society.

Undoubtedly, education is critical to every entrepreneur, it means, such people are made, not born, but being born into a family of entrepreneurs can be very motivating and influential, although this does not guarantee a future as an entrepreneur. The financial support to entrepreneurs in Mexico today is very exciting and should be taken; incubators of higher education institutions or governments, facilitate the process of entrepreneurship. It is important to understand that SMEs generate most of the country's GDP, therefore Mexico's economic future depends on them.

Although entrepreneurship is essentially the creation of a company, by finding and taking advantage of an opportunity, and by gathering resources to make that opportunity to become a business, it is actually also a way of life. Once you have been bitten by the excitement of

discovering an opportunity and the creation and design of a new business, it is hard not to seek opportunities everywhere one looks. But it is recognized that this lifestyle is not for everyone. And the reason is not for everyone? Because it comes with serious challenges-after all, the best things in life are never easy. Some of the most common challenges that all entrepreneurs face, are listed below:

- Creating the right opportunity.
- Working long hours, often without pay.
- Dealing with uncertainty and high risk.
- Needing to make important decisions that often affect others.
- Relying on the expertise and resources of others.
- No having previous experience in this type of business.
- Failing at some point (which is inevitable).
- Finding the right people for your team.
- Increasing capital and other resources.
- Dealing with a sense of isolation and disillusionment.

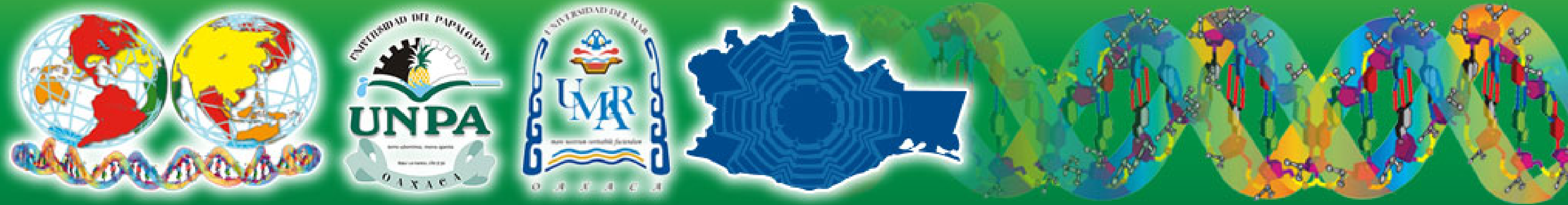
It is known that most entrepreneurs have no idea how hard it is to create a successful business. They have no idea of the amount of self-discipline and perseverance it takes to survive the first year. At some point, every business is overwhelmed with everything you have to think, especially in the early stages, when it all falls on the shoulders of the employer. If the challenges of entrepreneurship seem daunting, consider the rewards. Entrepreneurs become independent and able to take charge of their careers. Entrepreneurship is still the best way to create real wealth and potentially help others, and provides an exciting job, and a way to make a difference. The entrepreneur is for life, but it is important to distinguish the “one-day” entrepreneur, which has passion and energy, but is not interested in undertaking business, he is only interested in the opportunity; in addition, this type of entrepreneurs think all the time on how much they can sell the idea or the company, in many cases in the early years, regardless of whether the new owner destroys it or merges with a larger brand. They are also good in increasing sales, but their focus is not long-term sustained profitability. Meanwhile, entrepreneurs known as "lifetime", are entrepreneurs of a task, they do one business or more all their lives, but in the same activity and sector. Although over time some have diversified their businesses, they do them as secondary activities without ever neglecting their "Origin." These entrepreneurs believe in sustained long-term profitability, because they are 100% committed to the cause of their company for life.
Creativity, critical thinking, resourcing, searching and seizing opportunities, time management, persuasion, negotiation, oral and written communication, leadership, and decision making are essential skills that every entrepreneur should develop. Leadership reflects the personality of the entrepreneur who must be someone who knows the market to take advantage of opportunities that arise to add value to his business, which fulfills his ambitions. The entrepreneur must be tolerant to risk, stress and failure, because the environment in which he operates is characterized by financial or personal potential risk; in addition, he must be tenacious, determined, disciplined and persistent, and have a great willingness to work to make a commitment. He must also have confidence in himself and have a strong passion to achieve goals, as well as having the ability to create and innovate to provide a fast and effective solution to the problems of invention and business development that are encountered.

The critical stages of entrepreneurship include a) Having a business idea, b) Having a business plan, c) Forming the company, d) growing as a company, and e) expand and diversify. Companies begin with the selection of a business idea, for which the entrepreneur should find something he likes to do and he is good at; he should also assess whether his idea can satisfy a need in the market. It is very important to make a list of our interests and skills, list the types of businesses that relate to our interests, identify future needed products still not for sale, evaluate goods and services and ways we can improve existing ones, choose a business that offers profit potential, conduct market research to determine the potential profitability, and learn all we can about the industry of interest. Consider that many ideas remain just ideas, as there is no technical and / or economic feasibility.

It is advisable to have a business plan because it helps the entrepreneur to prepare enough resources and stay focused on the key objectives. This includes executive summary, industry analysis, company description, products and services, market, operations and staff, marketing strategy, financial projections, and capital needs. Another key aspect for new businesses is to have initial funding for the development and operations as demand grows. This can be achieved through family or friends, from the sale of assets (cars, laptops, cell phones, real estate, appliances, etc.), angel investors, or state or federal support as provided by the National Institute of the Entrepreneur in Mexico, and it is essential to formalize the company through formal and legal constitution of the company and registration in the Internal Revenue Service (in Mexico this is the Servicio de Administración Tributaria), and legally protect the brand, logo or distinctive sign.

It is important to recognize 3 types of business personalities. The typical personality of a joint venture or risk company is to be 70% technical (technical knowledge of the product or service), 20% administrative, and 10% entrepreneurial (attitude). The typical personality of a successful business is to be 33% of each technical, administrative and entrepreneurial. Finally, the typical personality of a successful and sustainable business is to be 25% technical, 25% administrative, 25% entrepreneurial, and 25% innovative.

Some of the reasons why people become entrepreneurial include the desire to be their own boss, have financial success, and have job security and a better quality of life. In addition, entrepreneurs today have resources available to support them and expand their opportunities. One of these resources is globalization, where the entrepreneur has a global market of their products and services, aided by information technology, also globalized; they also have the evaluation and use of demographic and economic trends as a permanent market, and finally they have education that allows the entrepreneur to be prepared and updated in all aspects related to their business function.



Bio-businesses and their economic value added

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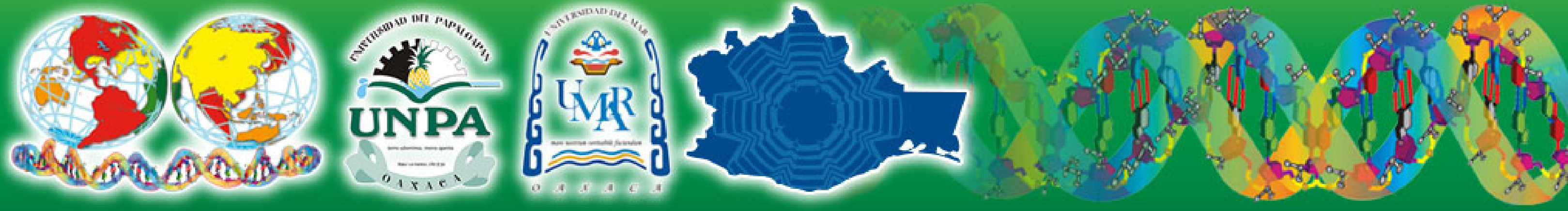
Bio-businesses are organizations that are more focused on research and development, which are the primary and secondary activities in a value chain; herein lies the importance of analyzing how technology and science, properly work in these companies. Sometimes confounds the bio-enterprise when really we are talking about traditional businesses who sell their products to the industries that use biotechnology. The difference between a bio-company and a traditional company, is that the bio-company is mainly dedicated to the research and its essential goal is to provide innovative solutions to the market. If is a bio-company, according to the trends of the market will value your product, however, there is a downside to this type of organizations, the risk is in the market; normally these bio-enterprise are consolidated industries, profitable, competitive and economic value added, with a future of growth, linked to innovation. This research carries out a comparative financial analysis between a company and a traditional bio-business devoted to the paper industry, to assess the financial impact through the generation of economic value added of both businesses. The bio-business object of this research generates an EBIT (Earnings before interest and taxes) of twenty million pesos, It is net operating profit after tax amounts is about nineteen millions and its economic value added is positive; in contrast, the traditional paper industry generates a positive EBIT, but its economic value added is negative. This bio-business pretend to pertain to the green market composed of those emerging markets in the medium and long-term due to the trends in demand for organic products and to the extent that education and environmental culture increase, will have an impact on its growth and development.



Towards resource mobilization from global wheat gene bank to the farmer's field

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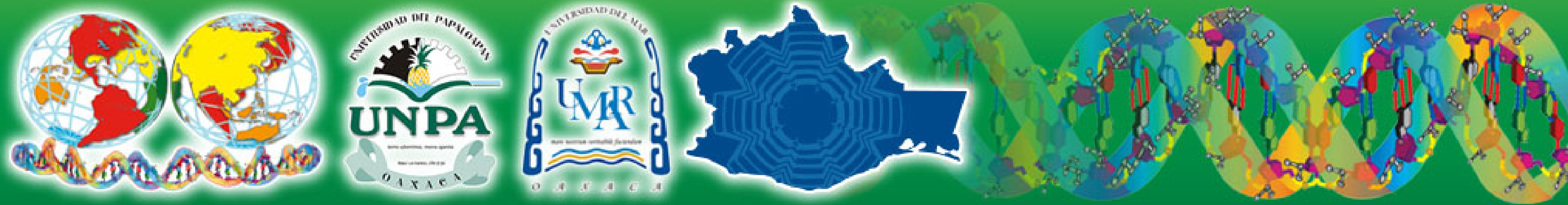
Wheat, world's most important calorie source is a staple food crop and in coming decades, its demand is projected to increase by 60%. Increasing population pressure, climate change, shrinking farm resources and reduced genetic diversity are the major challenges for ensuring global food security. Broadening of the genetic base is very important step in accelerating progress in breeding programs. One of the ambitious initiatives of CIMMYT, *Seeds of Discovery* (SeeD) has made efforts to characterize and mobilize novel, useful genetic variation from landraces, wild relatives and other underutilized sources into adapted elite genotypes. (1) Genome-profiling of more than 50,000 wheat gene bank accessions were carried out using a genotype by sequencing (GBS) platform. (2) In way to identify trait donors approximately 15,000 wheat landraces were characterized for heat and drought, ~20,000 gene bank accessions for grain quality and 6000 for diseases. (3) Wheat landrace core sets developed using GBS and phenotype information represent a unique resource. (4) Bridging germplasm was also developed for mobilization of useful phenotypic variations from exotics to elite germplasm. The linked top cross population panels (LTP) were developed in which exotic alleles were mobilized from 200 diverse accessions (landraces and primary synthetics) to farmer adapted elite cultivars. Within panels populations were linked through common elite parents. These resources will be used for Genomic selection (GS) and nested association mapping (NAM). The SeeD-wheat project has a unique balance between conventional and advanced breeding methods as well as between germplasm characterization and product delivery. Wheat researchers world-wide can utilize these resources for targeted wheat genetic improvement.



Evaluation of antimicrobial and insecticidal proteins in arachnid venoms

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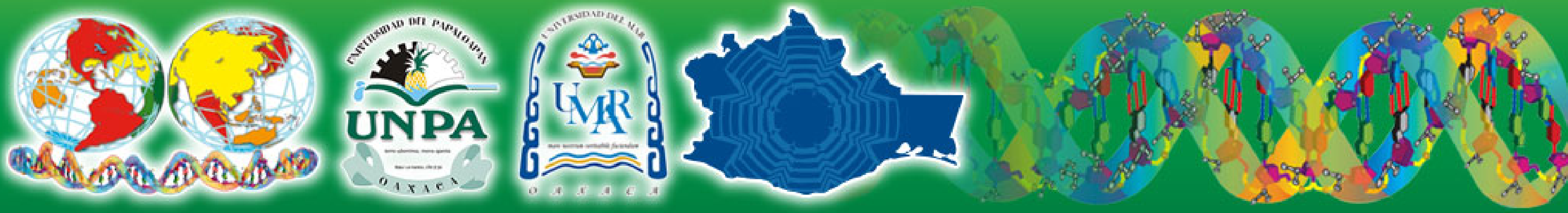
Abstract: Spider venoms are complex mixtures of biologically active compounds such as, proteins, antimicrobial peptides, enzymes, nucleotides, aminoacids, lipids, biogenic amines among other compounds. Antimicrobial peptides (AMPs) and insecticidal specific toxins (IST), once purified and characterized can be chemically synthesized for further research. L-Pin2 is an antimicrobial peptide isolated from the venom of scorpion and interacts with the outer and cytoplasmic membranes of bacteria. PaluIT1 is an insecticidal peptide isolated from the venom of spider. It binds the *para*-type voltage-gated sodium channel of insects altering its inactivation properties. In this work, we present the potential use of an enantiomer of L-Pin2, D-Pin2, as therapeutic agent and the insecticidal properties of PaluIT1 towards Lepidoptera species. D-Pin2 was more stable to human serum and to *Pseudomonas sp.* proteases than that of L-Pin2. Furthermore, D-Pin2 was less toxic towards human erythrocytes than L-Pin2. On the other hand, PaluIT1 median lethal dose (LD_{50}) was determined towards five different Lepidoptera larvae, interestingly PaluIT1 is very effective towards *Spodoptera exigua* but it was not to *Heliothis viriscens*.

Keywords: Antimicrobial peptides ● antibiotic ● insecticidal activity

Introduction: Antimicrobial peptides (AMPs) have potent antibacterial activities that make them good candidates to develop novel antibiotics because of their broad-spectrum of action towards multi-resistant pathogenic Gram-positive and Gram-negative bacteria, as well as towards clinically important yeasts such as *Candida albicans* (Yanmei *et al*., 2012). However, some AMPs are susceptible to protease degradation and they are toxic to eukaryotic cells (Moncla *et al*., 2011). There are different approaches for protecting antimicrobial peptides from the proteolytic action, among them the incorporation of D- amino acids, which have been studied and proved to be effective. Since enantiomeric D-peptides are considered to be resistant to protease degradation, the replacement of all L-amino acids by their corresponding D-analogs often help to keep its antimicrobial activity and bring in protection against several pathogenic bacteria and their proteolytic enzymes. Some examples of D-AMPS protease resistant to are OmpT from *Escherichia coli* (McCarter *et al*., 2004); autolysin V8 protease, staphopain A and staphopain B the last two papain-like cysteine proteases, all *Streptococcus aureus* and *Streptococcus agalactiae* (Potempa *et al*., 1988); elastase A, elastase B, alkaline protease and protease IV from *Pseudomonas aeruginosa* (Caballero *et al*., 2001). Among D-AMPs that had been synthesized are D-cecropinA (Wade *et al*., 1990), D-melittin and D-mastoparan M (Yanmei *et al*., 2012), which have shown higher resistance to proteases degradation under systemic usage, and at the same time. Have shown lower toxicities towards red blood cells. L-Pin2 is an AMP of 24 residues long (FWGALAKGALKLIPSLFSSFSKKD), which belongs to the group of short magainin-type helical peptides. It was isolated from the venom of the African scorpion *Pandinus imperator* (CL Kock,



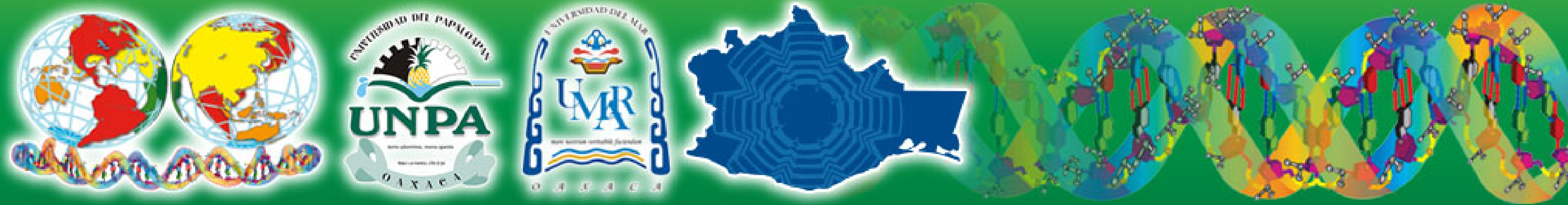
1841) (family: Scorpionidae Latreille, 1802) distributed in Nigeria, Togo, Sierra Leone, Ghana and the Cong. It possesses high antimicrobial activity against a broad range of Gram-positive and negative bacteria (Corzo *et al*., 2001). On the other hand, the voltage-gated sodium channel (Na_v) is a target of insecticides, neurotoxins and several drugs. These compounds are able to bind to at seven identified neurotoxin binding sites in tNa_v and either block conductance or modulate sodium channel gating. From spiders venoms different insect toxins have been determined to interact with some of these binding sites. PaluIT1 was purified from the venom of the spider *Paracoelotes luctuosus*, and it is homologous to the sequences of mu-agatoxins from the spider *Agelenopsis aperta*, which alter the inactivation properties of Na_v in a similar way to alpha-scorpion toxins, but they bind on site4 of the Na_v in a way similar to beta-scorpion toxins. In this work, the antibiotic capacity of D-Pin2 toward pathogenic bacteria, and the insecticidal properties of PaluIT1 towards Lepidoptera larvae were investigated.

Materials and Methods:

Antimicrobial and hemolytic activities. Antimicrobial activity was determined by agar and broth microdilution susceptibility assays (CLSI). Pathogenic bacteria, such as, *P. aeruginosa*, and *S. aureus* were cultured overnight to half of bacterial log phase in Mueller-Hinton broth (MHB) at 37 °C. Stock solutions of each AMP at 50 µM were diluted serially up to 25.0, 12.5, 6.25, 3.1 and 1.5 µM to a final volume of 200 µL, placed in polypropylene microtubes and vacuum dried. Ampicillin 30 µM was used as negative control. Next a volume of 200 µL aliquots of the bacterial suspension ($1x10^8$ CFU mL^{-1} in MHB with A_{625nm}=0.5) was diluted 1:10 poured into each of the AMPs stock solutions and the control microtubes. Then each was poured into three well of a 96-well microtiter plate in triplicate. Growth of each sample was evaluated by measuring absorbance every 2 h until 10 h of incubation time at 37 °C in a Spectramax plus 382 plate reader from Molecular Devices (Sunnyvale, CA, USA). Reading results were obtained comparing the growth by OD (A_{625nm}) in samples containing L- or D-Pin2 (MHb + Peptide + bacteria inoculum), with growth in the positive control sample (MHb + bacteria inoculum). The resulting MICs were defined as the lowest peptide concentration that showed zero visible growth or absence of growth, that is growth inhibition (100%). The minimal inhibitory concentrations (MIC) values were the mean result of all three independent experiments.

Hemolytic activity. Hemolytic activity was determined by incubating a 10% (v/v) suspension of erythrocytes with both L- and D-Pin2. As positive controls, red blood cells were incubated at room temperature for 1 h in deionized water and 10% Triton X100 and an appropriate amount of antimicrobial peptides in 1xPBS as a blank Human erythrocytes were centrifuged at 4,000 g for 5 min, supernatant was separated from the pellet, and its absorbance measured at 575 nm. This relative OD value was compared to that of the suspension treated with 10% Triton X-100 and defined as the percentage of hemolysis. Mainly the mechanical handling of the samples produced the hemolysis detected on PBS buffer, so it was subtracted from the hemolysis caused by the action of the peptides and the positive controls.

Stability to proteases and to human serum. For isolated proteases, twenty micrograms of each D- or L-Pin2 and either 1 µg of bovine pancreatic trypsin or of human elastase from leukocytes were added (ratio 20:1) to each vial and incubated for 4 h at 37 °C. For bacterial proteases, Twenty micrograms per microliter from a cryopreserved supernatant of *Pseudomona* DFU3 was incubated with 20 µg of either L- or D-Pin2 for 0, 4, 8, 12 and 24 h at 37 °C, a negative control with either of



L- or D-Pin2 was used (no protease activity). For human serum, serial dilution concentrations from 1.5 to 50 µM of D or L-Pin2 were placed in polypropylene microtubes and vacuum dried. Then, 200 µL of diluted human serum were added to each sample and incubated for 4 h at 37 °C. Aliquots of each experiment were withdrawn each hour, enzyme activity was inhibited by heating at 70 °C for 5 min and precipitated using a solvent mixture of acetonitrile (50%) with trichloroacetic acid (0.1%). Finally, the supernatant was recovered, vacuum dried, resuspended and analyzed by HPLC.

***LD_{50}* Determination.** A completely random design test was stablished, including seven treatments and negative control, with three replicates of 15 larvaes each one. Palult1 was injected in third instar larvae pronotum with a micro-injector. Toxin effect was observed for 24 h and the data recorded. Statistical analysis using Probit (POLO 1.0 program) were performed to analyze the results. Paralytic and lethal effects were registered at different time intervals up to 48 h.

Bioinformatics. BLAST searches of *para* insect Na_v protein were done using Gene Bank, (www.ncbi.nlm.nih.gov/genbank/) multiple alignments of the whole *para*-type alfa subunit. Local alignments of site4 and site3 of the same alfa subunit channel were performed using clustalW2 program (http://www.ebi.ac.uk/Tools/ msa/clustalw2/), to estimate a global similarity among the *para*-type Na_v of several lepidoptea (i.e. *Plutella xylostella*, *Px* (BAF37095.2), *Bombyx mori, Bmo* (NP_001136084.1), *Bombyx mandarina, Bm* (ACD80425.1), *Helicoverpa zea*, *Hz* (ADF80418.1), *Helicoverpa armigera*, *Ha* (ABE60888.1) and *Heliothis virescens, Hv* (AAC26517.1)) and of Blattodea (i.e. *Periplaneta americana*, *Pa* (ACX44801.1) and *Blattella germanica, Bg* (O01306)). For comparison to human Navs, the following Nav isoforms were used; $Na_{v1.1,}$ (P35498**)**, $Na_{v1.2}$ (AAG53413.1), $Na_{v1.3}$ (Q9NY46**)**, $Na_{v1.4}$ (P35499), $Na_{v1.5}$ (Q9JJV9**)**, $Na_{v1.6}$ (Q01118**)**, $Na_{v1.7}$ (Q01118.2), $Na_{v1.8}$ (Q9UQD0) and $Na_{v1.9}$ (Q15858**)**.

Results and Discussion:

The antimicrobial assays showed equivalent antimicrobial behavior for both L- and D-Pin2 with a MIC value range from 3.1 to 50 µM in culture broth. *Pseudomona aeruginosa* was more resistant than *S. aureus* to this antimicrobial peptide (Table 1). Although the resistance mechanisms of mobile genetic elements is always a concern, the most difficult challenge we have faced with *P. aeruginosa*, a bacteria responsable for hospital-acquired infections, a challenge to patient because of its ability to rapidly develop resistance during the course of treating an infection. The chromosomally encoded AmpC cephalosporinase, the outer membrane porin OprD, and the multidrug efflux pumps are particularly relevant to this therapeutic challenge.

Table 1. Minimum inhibitory concentrations of D- and L-Pin2 against bacteria.

	MIC (µM)			
Microorganism	L-Pin2	D-Pin2	Ampicillin	Pexiganam
	MHB		MHA	
P. aeruginosa	50.0	50.0	30 R	32
S. aureus	3.1	3.1	30 R	8

The concentration of bacteria inoculated into MHA or MHB was 108 CFU mL^{-1}. L- or D-Pin2 was added into each culture at concentrations from 1.6 to 50 IM. Na: no activity, n: not determined, n = 3 experiments, R = Resistance to 30 IM ampicillin (29 mm); Pexiganan was used as AMP of reference.

Concerning eukaryotic toxicity, L-Pin2 showed higher hemolytic activity than that of D-Pin2. That is, at 10 µM, the hemolysis cause for L-Pin2 was 80% and for D-Pin2 only 50%. That means D-Pin2 is 30% less hemolytic than L-Pin2, it is for the moment an important improve caused for the substitution of L- for D- amino acids (Figure 1).

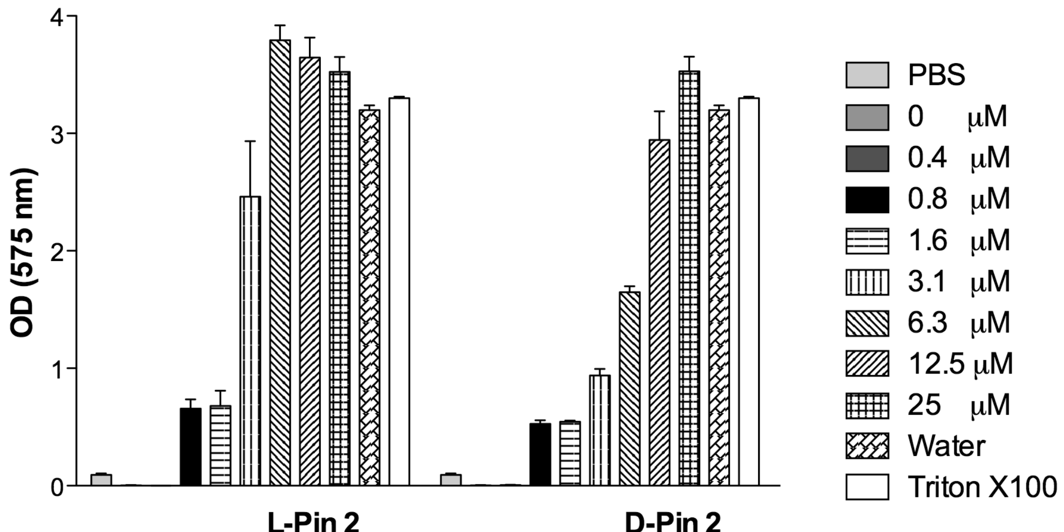
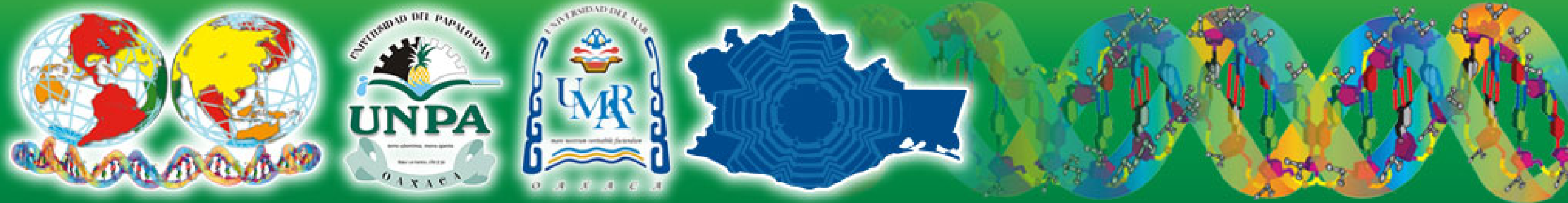


Figure 1. L- and D- haemolytic activity towards red blood cells.

D-Pin2 was not cleaved by either bovine pancreatic trypsin or human elastase up to 4 h; however, L-Pin2 was rapidly enzymatically digested. In the presence of trypsin L-Pin2 was digested in two shorter peptides, which corresponded to molecular masses of 1,225.4 and 791.9 Da and to the amino acid sequences LIPSLFSSFSK and FWGALAK, respectively. L-Pin2 was cleaved also by human elastase into several proteolytic fragments. Three of those proteolytic fractions have molecular masses of 919.7, 663.4 and 1,571.8 Da, and they agreed with the calculated molecular masses of the amino acid sequences FWGALAKGA, FWGALA and FWGALAKGALKLIPS, respectively. *Pseudomonas aeruginosa* DFU3 proteases cleaved L-Pin2 in the first hour of incubation contrary to this D-Pin2 was not cleaved up to 4 h, but then after 8 h a reduction of 20% of D-Pin2 was registered, and after 24 h only 5% of D-Pin2 was left. Whole human serum was diluted (1:4) and incubated with L- and D-Pin2 for 24 h. The results demonstrated that L-Pin2 was degraded more rapidly than D-Pin2 using the same incubation conditions.
On the other hand, the neurotoxic effects of PaluIT1 were clearly observed in larvae after half an hour post-injection. Toxic symptons such as paralysis, dehydratation, tissue necrosis and death were observed in *S. littura, S. frugiperda, S. exigua, D. magnifactella* and *G. mellonella* but no effect was observed in *Heliotis virescens.* The LD_{50} values obtained for *S. exigua* were LD_{50} 1.96 $\pm$ 0.3 µg g^{-1}, *S. frugiperda* LD_{50} 6.3 $\pm$ 0.2 µg g^{-1}, *S. litura* LD_{50} 9.5 $\pm$ 3.7 and for *D. magnifactella* LD_{50} 8.5 $\pm$ 0.4 µg g^{-1}, and *G. mellonella* 9.36 $\pm$ 0.2 µg g^{-1}, respectively. Therefore, PaluT1 was



insecticidal towards four of five Lepidoptera larvae used. Binding site 4 sequences local alignment of Lepidoptera Na_v obtained from the Genebank revealed that there is not similarity with that of the loop 1 of *Heliothis virescens*. Furthermore, the percentage of amino acid sequence similarity of the Lepidoptera alfa-subinit *para*-type Na_v with that of the human Nav1-9 are shown in Table 2. It is observe that the Lepidoptera alfa-subunit *para*-type Nav and the human Nav alfa-subunits present more sequence identities than that of the Nav from *Heliothis virescens* (Table 2). In Table 3 and 4 it is observed that *Heliothis virescens* had the lowest score in % of similarity among Na_v alfa subunits of several insect species including *D. melanogaster* from where the *para*-type Na_v was isolated.

Table 2. Percentage of similarity alignment analysis of Lepidoptera *para*-type Na_v alfa-subinit and 1-9 Human Nav.

Lepidoptera	**Lepidoptera Na_v *para* type**						**Human sodium Na_v1-9**								
Especies	Bm	Bmo	Hz	Ha	Px	Hv	$Na_{v1.1}$	$Na_{v1.2}$	$Na_{v1.3}$	$Na_{v1.4}$	$Na_{v1.5}$	$Na_{v1.6}$	$Na_{v1.7}$	$Na_{v1.8}$	$Na_{v1.9}$
B.mandarina	-	88.6	90.6	20.5	86.7	85.4	41.8	41.6	41.6	39.9	40.3	31.0	31.0	41.5	39.9
B. mori	88.6	-	86.6	18.1	84.6	78.8	44.3	44.4	44.1	42.9	43.2	32.8	32.8	45.0	43.0
H. zea	90.6	18.1	-	20.7	84.3	23.1	41.0	40.6	40.7	39.2	39.5	30.4	30.4	41.1	39.6
H. armigera	20.5	18.1	20.7	-	19.3	23.1	10.9	11.0	11.1	11.3	10.3	9.4	9.4	10.7	10.0
P. xylostella	86.7	84.6	84.3	19.3	-	83.2	42.6	43.2	42.7	41.6	41.4	31.8	31.8	43.2	41.6
H. virescens	85.4	78.8	84.8	23.1	83.2	-	41.9	42.3	42.1	44.7	40.4	34.2	34.2	42.6	40.9

Ha= *H. armigera*, Bm= *B. mandarina*, Bmo= *B. mori*, Hz= *H. zea*, Px= *P. xylostella*, Hv= *H. virescens*, $Na_{v1.1}$ - $Na_{v1.9}$=Human Nav. Nr = not reported

Table 3. Percentage of similarity alignment analysis of loop 1 site 4 os alfa subunit of *para*-type Na_v channels of Lepidoptera, and Diptera orders Blattodea.

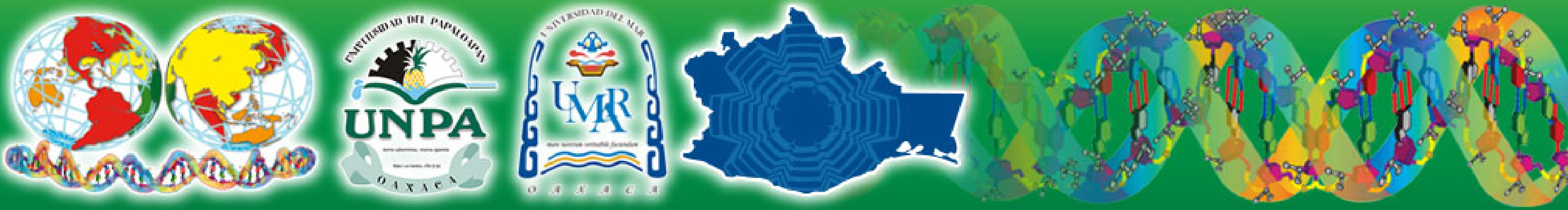
			Canales de Na_v *para* de insectos										
	Especie	Secuencia del loop 1	Ag	Bg	Bm	Bmo	Cq	Dm	Hz	Hv	Md	Pa	Px
Canales de Na_v *para* de insectos	*A.gambiae*	IVVNTLFMALDHHDMDPDMEKALKSGNYFFT	-	72.7	72.7	72.7	81.8	63.6	81.8	9.1	63.6	72.7	90.9
	B.germanica	IVVNMLFMALDHYDMNKDMEKALKSGNYFFT	72.7	-	72.7	72.7	81.8	81.8	81.8	9.1	72.7	72.7	90.9
	B.mandarina	IVVNTLFMALDHHNMDKDMDKALKSGNYFFT	72.7	63.6	-	100	54.5	54.5	72.7	0	45.5	81.8	72.7
	B.mori	IVVNTLFMALDHHNMDKDMDKALKSGNYFFT	72.7	63.6	100	-	54.5	54.5	72.7	0	54.5	81.8	72.7
	C.quinquefasciatus	IVVNTLFMALDHHDMNPDMERALKSGNYFFT	81.8	72.7	54.5	54.5	-	81.8	81.8	9.1	72.7	72.7	72.7
	D.melanogaster	IVVNTMFMAMDHHDMNKEMERVLKSGNYFFT	63.6	72.7	54.5	54.5	81.8	-	81.8	0	72.7	72.7	63.6
	H.zea	IVVNTLFMALDHHDMDKDMERALKSGNYFFT	81.8	72.7	72.7	72.7	81.8	81.8	-	0	54.5	72.7	81.8
	H.virescens	IESMWDCMLVGDVSCIPFFLATVVIGNLVVL	9.1	0	0	0	9.1	0	0	-	9.1	0	0
	M.domestica	IVVNTMFMAMDHHDMNPELEKVLKSGNYFFT	72.7	63.6	45.5	45.5	72.7	72.7	54.5	9.1	-	63.6	63.6
	P.americana	IVVNTLFMALDHHDMNKDMDKALKSGNYFFT	72.7	81.8	81.8	81.8	72.7	72.7	72.7	0	63.6	-	72.7
	P.xylostella	IVVNTLFMALDHHDMDRDMEKALKSGNYFFT	90.9	72.7	72.7	72.7	72.7	63.6	81.8	0	63.6	72.7	-

Ag= *A .gambiae*, Bg= *B. germanica*, Bm= *B. mandarina*, Bmo= *B. mori*, Cq= *C. quinquefasciatus*, Dm= *D. melanogaster*, Ha= *H. armigera*, Hv= *H. virescens*, Hz= *H. zea*, Md = *M. domestica*, Pa= *P. americana*, Px= *P. xylostella*.

Table 4. Similarity alignment analysis of loop 2 site 4 os alfa subunit of *para*-Type Na_v channels of Lepidoptera, and Diptera orders Blattodea.

Insect similarity percentage of loop 2 site 4												
Specie	Sequencia	Ag	Bg	Bm	Bmo	Cq	Dm	Hz	Hv	Md	Pa	Px
A.gambiae	VALSLLELGLEGVQGLSVLRSFRL	-	100	66.7	100	100	100	100	0	100	100	100
B.germanica	VALSLLELGLEGVQGLSVLRSFRL	100	-	66.7	100	100	100	100	0	100	100	100
B.mandarina	VALSLLELGLEGAQGLSVLRSFRL	66.7	66.7	-	66.7	66.7	66.7	66.7	0	66.7	100	100
B.mori	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	-	100	100	100	0	100	100	100
C.quinquefasciatus	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	100	-	100	100	0	100	100	100
D.melanogaster	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	100	100	-	100	0	100	100	100
H.zea	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	100	100	100	-	0	100	100	100
H.virescens	FNRISRFIDWVKRNVADVMKLLKN	0	0	66.7	0	0	0	0	-	0	100	100
M.domestica	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	100	100	100	100	100	-	100	100
P.americana	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	100	100	100	100	100	100	-	100
P.xylostella	VALSLLELGLEGVQGLSVLRSFRL	100	100	66.7	100	100	100	100	100	100	100	-

Ag= *A .gambiae*, Bg= *B. germanica*, Bm= *B. mandarina*, Bmo= *B. mori*, Cq= *C. quinquefasciatus*, Dm= *D. melanogaster*, Ha= *H. armigera*, Hv= *H. virescens*, Hz= *H. zea*, Md = *M. domestica*, Pa= *P. americana*, Px= *P. xylostella*.



Conclusions: Arachnid venoms are an important sources of antimicrobial and insecticidal peptides among other compounds. The specific way of interaction of these peptide allow us to control them for specific applications. For antibiotic use, the proteolytic activity of bacteria such *S. aureus* and *P. aeruginosa* has to be taking in account for the design of antimicrobial peptide derivatives such inclusion of D-amino acids. For insecticidal activity, the cell receptor Na_v is just a target place but for other insect pest is not always the best target (*i.e. H. virescens*), other insecticidal peptides with different mechanism of action have to be searched.

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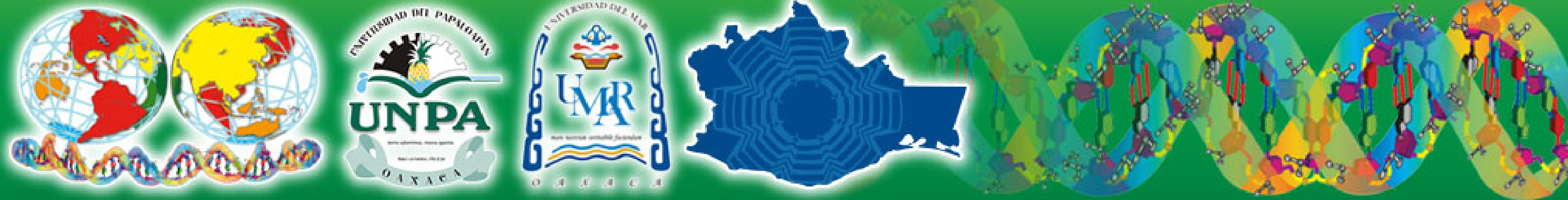
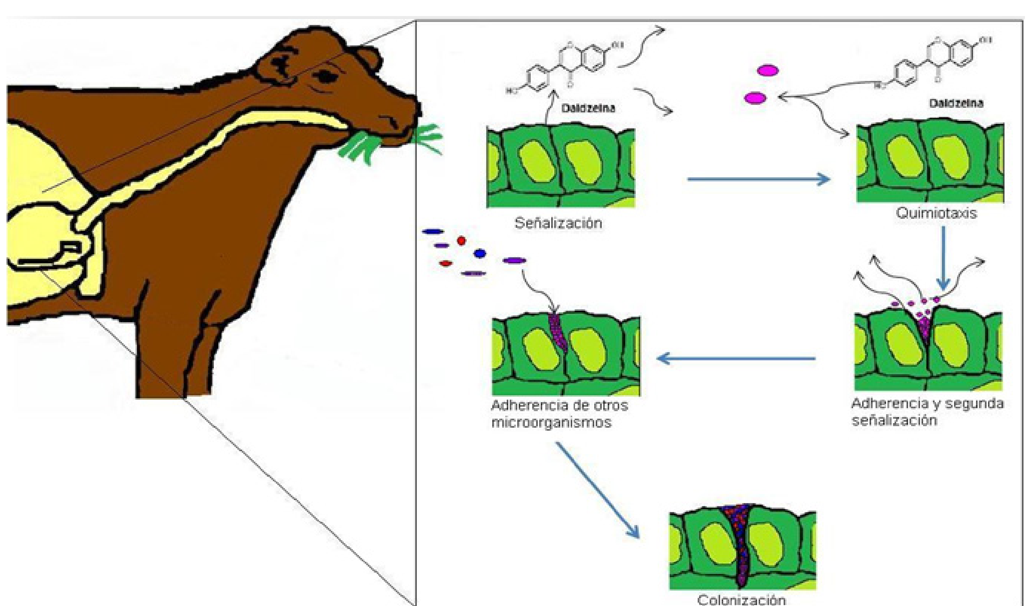


Interaction ruminal bacteria and food particles: usefulness as symbiotic

Mónica Marcela Galicia-Jiménez

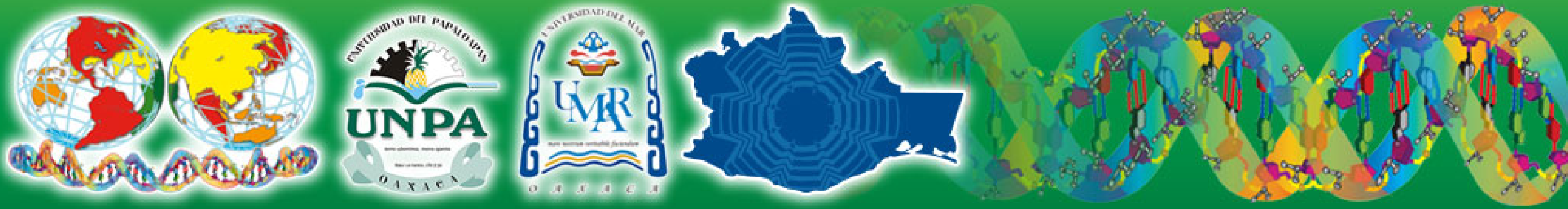
Institute of Genetics. Universidad del Mar, Campus Puerto Escondido

The application of different diets has focused on finding higher production in ruminants varying the diet of the animal, affecting rumen microbial population. However, the production efficiency has been reported ruminant inconsistently. One option could be daidzein, which have a role in plant-microbe interaction. Our objective is integrate knowledge of this interaction, understanding the signaling pathways used by bacteria in the rumen for the colonization of food particles and its degradation, thus ask the manipulation of rumen fermentation, thus creating the symbiotic cultures for cattle. By performing *in silico* experiments allowed us to discover putative genes in *Ruminococcus albus* chemotactic, also, daidzein was found in 4 plants forage potential use and chemotaxis assay for rumen bacteria was standardized by modifying known aerobic capillary method by combining with the technology used for the measurement of gas production *in vitro* allowing demonstrate the attractive effect of daidzein.



2.2 Symposium 1

Massive Sequencing of DNA & Current and Future Strategies for the Analysis of the Results and Success Stories Using these Methodologies



Exploring gut microbiota alterations associated with HIV infection using 16S rRNA sequencing and the Ion Torrent PGM

Sandra María Pinto-Cardoso

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HIV-1 infection induces a rapid and massive depletion of gut TCD4+ cells. Loss of these cells in the gut-associated lymphoid tissues (GALT) allows for the translocation of bacterial products to the systemic circulation. Microbial translocation is a contributing factor to systemic immune activation and disease progression. As the immune system in the GALT plays such an important role in shaping the gut microbiota composition, and considering the prevalence of gut-linked diseases in HIV infection, it is important to understanding how alterations in the gut microbiota composition may influence the progression of the infection. I will share our experience at the CIENI, the center for research in Infectious Diseases, on analyzing and comparing diversity and composition of microbial communities in fecal samples from HIV-positive samples (people living with HIV) with or without combined antiretroviral treatment (cART) and HIV negative samples using both an “in house” research method for amplifying and sequencing the V3 region of the 16S rRNA gene, as well as the Ion Torrent Metagenomics 16S kit (as part of the early access program the CIENI participated in).

SAGA – A high-throughput genotyping platform for Mexican agriculture

Petroli César D[1], Sansaloni Carolina P[1], Galvez Hector J, Martinez Manuel H, Sierra Aleyda A, Campos Jaime A, Hearn Sarah[1], Soca Giovanny[1], Kilian Andrzej[2], Wenzl Peter[1]

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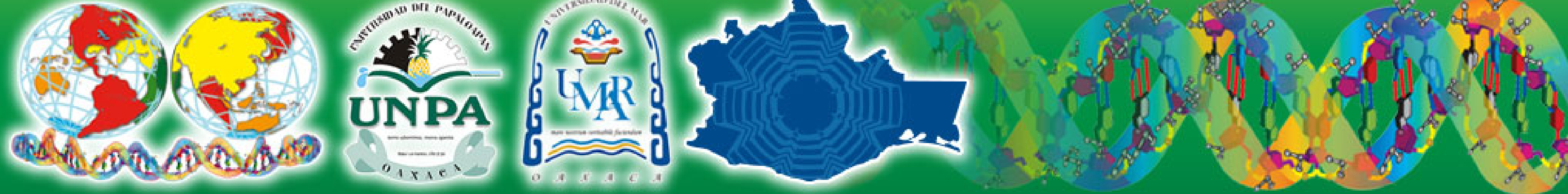
Human population is growing fast, natural resources are decreasing and food security of coming generations is at risk. Crop genetic resources are one of few options left to continue raising food production to feed humanity by 2050. The MasAgro-Biodiversidad project, the most upstream component of the Mexican Government-funded MasAgro program, systematically characterizes and mobilizes novel genetic variation from maize and wheat genebanks into breeding programs. A strategic partnership was established with DArT PL (www.DiversityArrays.com) establish, in Mexico, a "Genetic Analysis Service for Agriculture" (SAGA – *Servicio de Análisis Genético para la Agricultura*). SAGA is on its way to generate tens of thousands of high-density genome profiles from DNA samples using the "DArTseq" Genotyping by Sequencing (GBS) method developed by DArT PL. Each genome profile typically generates several tens of thousands of both Single Nucleotide Polymorphism (SNP) and Presence/Absence Variation (PAV) molecular markers. SAGA uses a HiSeq2500 next-generation sequencer (Illumina) and DNA-barcode-enabled multiplexing of individual samples. The principal goal of SAGA is to genotype more than 100,000 maize and wheat samples from genebanks at CIMMYT, INIFAP and others organizations. SAGA also offers advice and support in the interpretation and application of GBS data for a variety of applications. All data generated will be made available to the public via an internet portal. This data will contribute to diversifying the genetic base of maize and wheat breeding programs in order to accelerate the development of high-yielding and climate-read cultivars of two of the three most important crops of humanity. As the number of samples analyzed for MasAgro-Biodiversidad decreases, and subject to sufficient demand, SAGA will seek to offer its GBS-based genome-profiling services to the Mexican research community at large.

2.3 Symposium 2

Monitoring Resistance to *Bacillus thuringiensis* and New Approaches to Control Targeted Insects



Symposium welcome

Carlos A. Blanco[1], Patricia Tamez-Guerra[2]

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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 is important to approach this potential issue proactively utilizing the best management programs. As a results, scientists from academic, research institutions and seed companies have organized symposia in Mexico since 2003 (Fig. 1). Now in Huatulco 2014, presentations in the sixth symposium will include results not just on Bt topics, but new insect pest control strategies as well. You are welcome to attend.

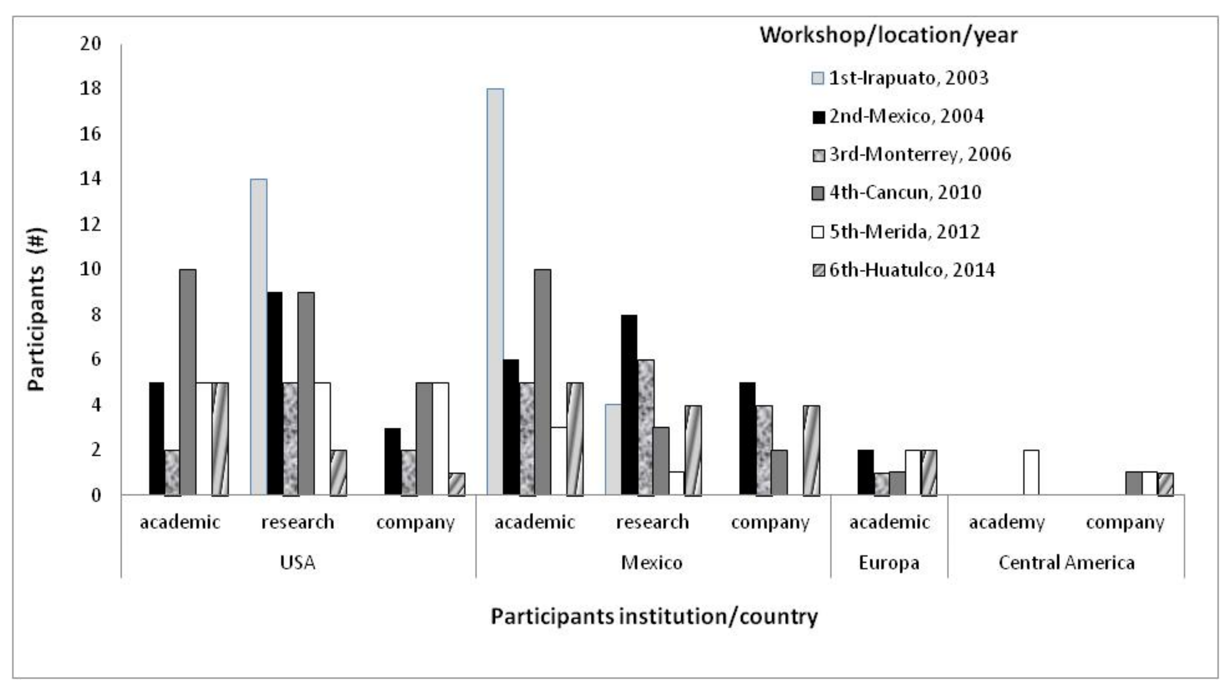
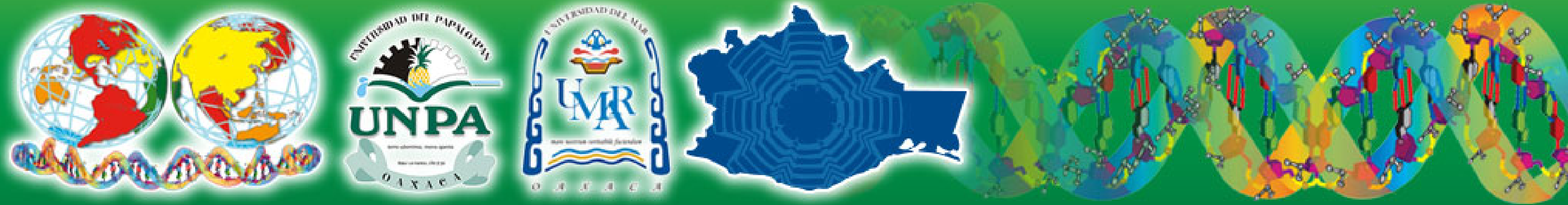


Figure 1. *Bacillus thuringiensis* resistance monitoring workshops in Mexico.



Introduction - status and global networks to address this issue

William Moar

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Maintaining the durability of Bt crops against targeted pests globally presents challenges due to differences in pest biology, product performance, and grower needs and capabilities. Recent initiatives to maintain or increase durability of Bt crops include gene pyramiding, enhanced refuge options such as refuge-in-a-bag (RIB), and industry alignment through ETS (Excellence through Stewardship) that will improve the effectiveness and consistency of IRM programs. This presentation will expand on these topics and discuss how industry is moving forward to provide more tools for insect control globally.

Alternative splicing and highly variable cadherin transcripts are associated with field-evolved resistance of pink bollworm to Bt cotton in India

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Evolution of resistance by insect pests can reduce the benefits of insecticidal proteins from *Bacillus thuringiensis* (Bt) that are used extensively in sprays and transgenic crops. Despite considerable knowledge of the genes conferring insect resistance to Bt toxins in laboratory-selected strains and in field populations exposed to Bt sprays, understanding of the genetic basis of field-evolved resistance to Bt crops remains limited. In particular, previous work has not identified the genes conferring resistance in any cases where field-evolved resistance has reduced the efficacy of a Bt crop. Here we report that mutations in a gene encoding a cadherin protein that binds Bt toxin Cry1Ac are associated with field-evolved resistance of pink bollworm (*Pectinophora gossypiella*) in India to Cry1Ac produced by transgenic cotton. In laboratory diet bioassays we confirmed previously reported resistance to Cry1Ac in pink bollworm from the state of Gujarat, where Bt cotton producing Cry1Ac has been grown extensively. Analysis of DNA from 436 pink bollworm from seven populations in India detected none of the four cadherin resistance alleles previously reported to be linked with resistance to Cry1Ac in laboratory-selected strains of pink bollworm from Arizona. However, DNA sequencing of pink bollworm derived from resistant and susceptible field populations in India revealed eight novel, severely disrupted cadherin alleles associated with resistance to Cry1Ac. For these eight alleles, analysis of complementary DNA (cDNA) revealed a total of 19 transcript isoforms, each containing a premature stop codon, a deletion of at least 99 base pairs, or both. Seven of the eight disrupted alleles each produced two or more different transcript isoforms, which implicates alternative splicing of messenger RNA (mRNA). This represents the first example of alternative splicing associated with field-evolved resistance that reduced the efficacy of a Bt crop.

Insect nutritional ecology and environmentally-mediated variation in Bt susceptibility

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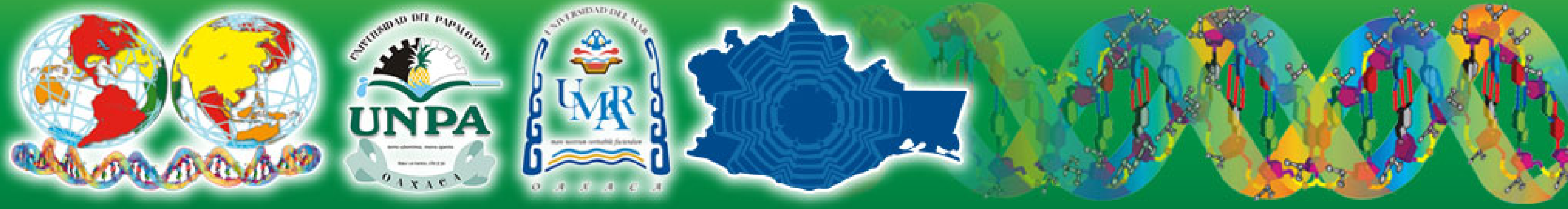
Insecticide resistance in agricultural pest species represents a costly and ever-increasing problem that poses significant challenges for meeting the nutritional demands of our growing global population. The evolution and spread of genetic mutations conferring resistance is the primary explanation for observations of low pesticide efficacy and resulting poor pest population control in agricultural systems. Unfortunately, in many cases it takes years to identify the mutations and alleles responsible for these resistant phenotypes, with most incidents assumed to be the result of mutations without any substantiation. As a result, the potential for gene-by-environment interactions to play a dominant role in mediating pesticide resistance is often overlooked. One environmental factor that has been shown to be highly variable is plant macronutrient content. Macronutrients such as protein (P) and carbohydrates (C) have also been shown to strongly affect insect behavior, physiology, and performance, including detoxification potential. Studies have shown that dietary protein-to-carbohydrate ratios (P:C) can influence feeding behavior and detoxification ability in locusts, as well as immune function in caterpillars, indicating that the impact of nutritional variability on insecticide resistance is probative. In this study we explored the potential for plant macronutrient content to impact susceptibility to Cry1Ac endotoxin, found in transgenic cotton, in the generalist herbivore *Helicoverpa zea*. Using artificial diets, we mimicked the macronutrient content of different cotton tissues and reared larvae on diets either without Cry1Ac, with a low concentration of Cry1Ac, or with a high concentration of Cry1Ac. We then measured the main and interactive effects of Cry1Ac and diet macronutrient content on larval survival, pupal mass, and eclosion success. Our results clearly demonstrate an effect of specific diet macronutrient content on Cry1Ac susceptibility and suggest that variation in insect performance in both the field and laboratory assays may be at least partially attributed to variation in diet nutritional quality.



Analysis of the immune response in wild *Helicoverpa zea* (Lepidoptera: Noctuidae) populations and host relationship

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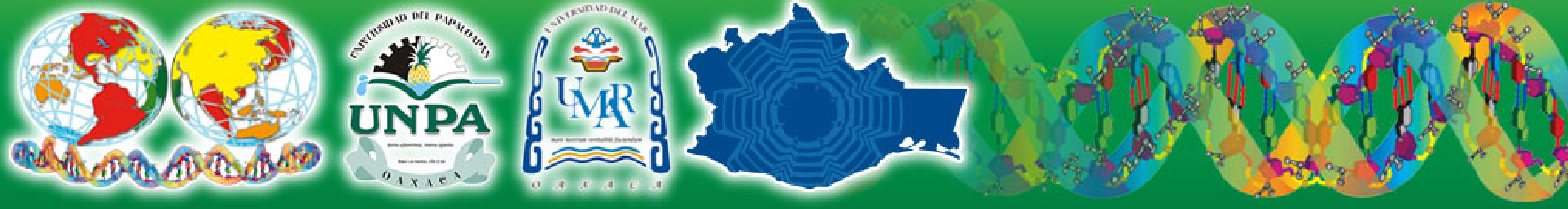
The expression of genes encoding to lysozyme (*lis*), three antimicrobial peptides (AMPs) (galiomicin (*gal*), cecropin (*cec*) and gloverina (*glo*) and peptidoglycan receptor protein (*pgrp c*), were evaluated by real-time RT-PCR, using mRNA sequences reported in *H. armigera* to assess differences related to larvae growth instar, exposure to host plant, and *Bt* as a commercial product or as Cry toxins in transgenic crops. Transcripts amplification was analyzed in 2nd, 4th and 5th instars: i) collected from conventional (C) or transgenic crops (Ct) in field conditions; ii) collected among different geographical areas from Mexico and USA; iii) exposed under three laboratory conditions (LB): a) after exposure to conventional cotton plants (LBA), b) exposed to *Bt*-cotton (LBAt), or c) to *Bt* commercial product Bactospeine® (LB*Bt*) on artificial diet. Identified sequences were reported in the GenBank. Results found in larvae reared on artificial diet with *Bt*-cotton (LBAt), *cec* expression was repressed in 2nd instar, whereas Bactospeine® (LB*Bt*) repressed *gal* in 4th instars. By comparing the expression in larvae fed on C and LBA, *pgrp c*, *gal* and *lis* were suppressed in the 2nd instar fed on C, whereas *glo* was suppressed in the 4th instar, and *gal* was suppressed in the 5th instar. By increasing exposure time, in LBA and LBAt expression of *LIS* in the 5th instar was stimulated. Larvae collected form field crops resulted in AMPs expression upon the variety and crop conditions. In fact, the highest expression of AMPs and *lis* was among larvae collected from crops subjected to drought stress and *Bt*-transgenic crops in Sinaloa state.

Acknowledgements: This project was supported by CONACyT 418967 (scholarship to MMV), CONACyT-TAMU 2011-049 (to PTG and PP) and PAICYT-UANL CA760-11 to PTG.



Cry1Ac protoxin from *Bacillus thuringiensis* affects the fitness of *Helicoverpa zea* B. (Lepidoptera, Noctuidae)

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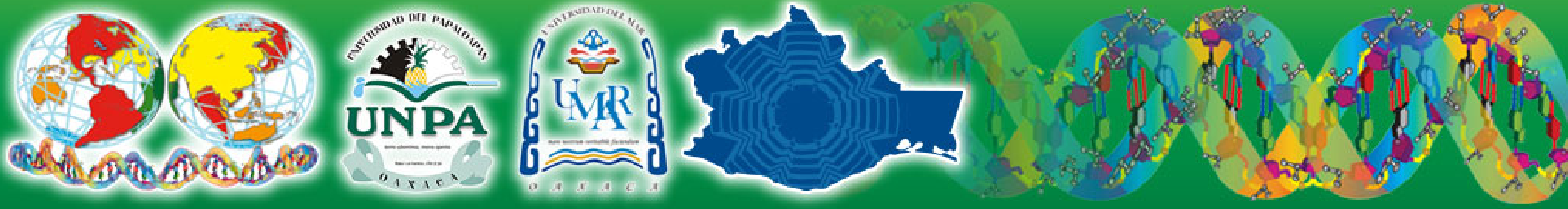
Biotechnological cotton known as Bollgard II™ express Cry1Ac and Cry2Ab *B. thuringiensis* toxins, which control two of main lepidopteran cotton pests: *Pectinophora gossypiella* (pink worm) and *Helicoverpa zea* (corn earworm). However, the constant pressure of selection could to drive the apparition and evolution of resistance. At laboratory level there is evidence of resistance to Cry1Ac for more than 500 times. Our goal was to obtain colonies of *H. zea* with resistance to the protoxin of Cry1Ac and to know the how this trait is transmitted to offspring. Wild type insects (eggs, pupae and adults) were collected and established in our laboratory. Laboratory diet bioassays demonstrated a baseline (LC50) of 1.3 µg g^{-1} diet for Cry1Ac protoxin. From this susceptible colony, we obtained resistant colonies to 10, 20 and 50 µg g^{-1} diet. Resistant colonies showed a lower fitness, high mortality at pupae phase, a progressive decay of amount of progeny, and an unusual male:female rate (2:1). Last result is controversy, because currently female amount is higher in male comparison. Another important result was the observation of very lower egg fertility (< 5%) and after 4th matting generation, fully eggs were no fertile. Interesting was to note that when resistant female was matting with wildtype males, the eggs was no fertile. Nowadays, we are involved to demonstrate changes in genetic expression of several important genes involved in resistance such as cadherin, aminopeptidades and alkaline phosphatase, putative receptor(s) of Cry1Ac in *H. zea*.

Acknowledgements: To CIBIOGEM for the grant 164429.



What could be the benefits of planting genetically-engineered maize in Mexico?

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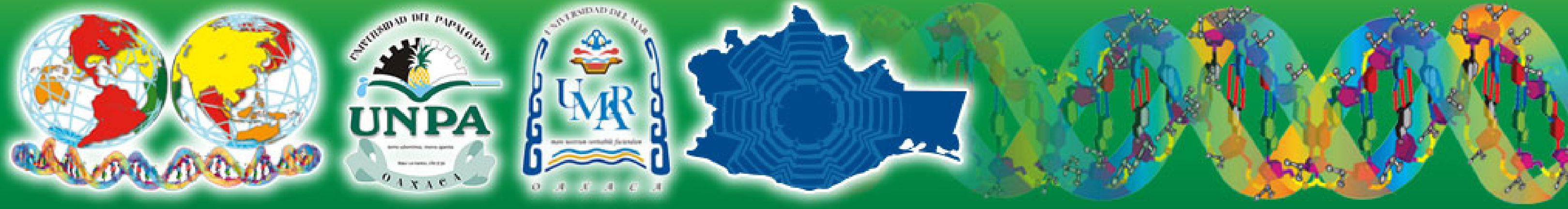
Mexico is the fifth largest maize producer of the world, representing close to a third of its internal agricultural production. Paradoxically, Mexico imports a third of the maize that it consumes, and its internal production yields 40% below of the world's average. Arthropod pests are partially responsible for lowering Mexican pest production. A group of Mexican scientists and crop advisors conducted a census of the impact of arthropod pests throughout the country. Results indicate that *Spodoptera frugiperda,* the most problematic pest, requires up to 3 sprays per crop season, representing 3,000 tons of insecticidal active ingredients (a.i.) for its control, followed by other destructive Lepidoptera such as *Agrotis ipsilon* and *Helicoverpa zea*, requiring also 1-3 insecticide applications per crop cycle, amounting to 2.8 and 0.8 tons of insecticidal a.i. for its control, respectively. Coleopteran pests require less insecticide than the previous pests and have narrower special distribution. The white grub complex (*Phyllophaga* spp.) is controlled in only a few Mexican regions with 1-2 insecticide applications, representing up to 1.6 tons of a.i., while *Diabrotica* spp. only requires an average of 1.5 applications but in a larger area of the country, amounting to 0.4 tons of a.i. Since genetically engineered (GE) maize hybrids have proven to be effective against these pests, and because these arthropods occur at different times of the maize development, in some regions of Mexico this crop is sprayed multiple times to control Lepidoptera and Coleoptera that otherwise could be effectively managed with certain GE maize hybrids, with the potential of reducing thousands of insecticidal active ingredient.



Susceptibility of the fall armyworm, *Spodoptera frugiperda* to Bt toxins and conventional pesticides among different maize production systems in the US and Mexico

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The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) is one of the main pests of corn in many areas of the American continent. If uncontrolled this insect causes severe damage to the crop. Genetically modified (GM) corn with the ability to produce Bt proteins is regarded as a major tool for pest control in modern and high-tech agriculture, but recent isolated cases of resistance of targeted pests threatens the useful life span of this technology. While this technology is a major method of controlling corn insect pests in the U.S., in Mexico pest control strategies are based almost entirely in the use of conventional pesticides. For instance, in the past few years in some areas of Mexico it has been necessary to spray up to six times during the season to control FAW. The overuse of pesticides for FAW control may lead to development of insect resistance more quickly than with use of GM Bt corn. Despite the economic important of FAW in the Americas there are few studies that provide information about the adaptation of this insect to insecticides whether applied as a spray or deployed through genetic modification of the plant. Our hypothesis is that FAW from Mexico would exhibit less susceptibility to conventional insecticides whereas FAW from the US would exhibit less susceptibility to Bt proteins. We are conducting bioassays to determine the susceptibility of FAW to Bt proteins and five conventional pesticides (chlorpyriphos, spinetoram, permethrin, flubendiamide and methomyl) in FAW populations from Mexico and the US.

Susceptibilities of geographic populations of *Helicoverpa zea* (Boddie) in Mexico to Bt ∂-endotoxins Cry1Ac and Cry2Ab; a 13 year study

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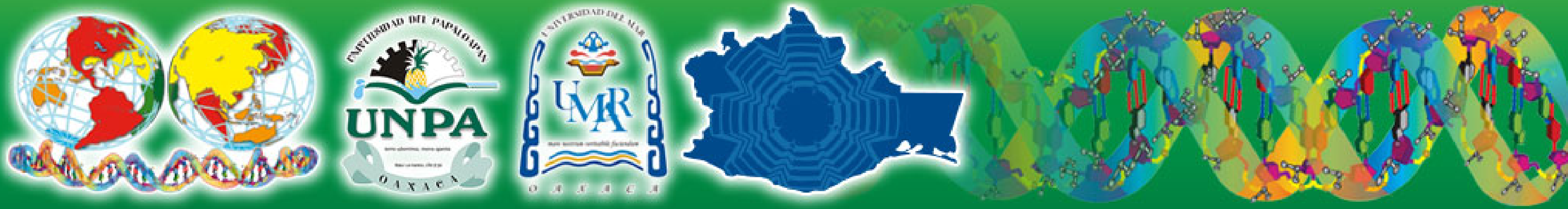
An insect resistance monitoring program was developed for Mexico to accommodate the commercial introduction and stewardship of Bt cotton. In 1998 and 2003 field-collected geographic populations of the cotton bollworm *Helicoverpa zea* (Boddie) were evaluated against *Bacillus thuringiensis* Berliner crystalline δ-endotoxins Cry1Ac, and Cry2Ab, respectively, to establish baseline susceptibility data in preparation for the commercial introductions of Bollgard® (expressing Cry1Ac) and Bollgard® II (expressing Cry1Ac and Cry2Ab) cottons, respectively. The sub-lethal response of growth inhibition was evaluated as measured by larval weight reduction (Inhibition of Weight: IW) and inhibition of normal progression through developmental stadia (Inhibition of Development: ID). Informed by the baseline susceptibility data, an annual monitoring program was subsequently established under which a single diagnostic concentration of Cry1Ac and Cry2Ab was used in diet bioassays to test for continued "normal" susceptibility to these insecticidal proteins among geographic populations. The diagnostic concentration selected was suitable since it aligned with previous work and represented the concentration under which larvae evaluated in baseline studies were reduced in weight by 98% or more (362pprox. IW_{98}) relative to untreated controls, or under which 97% or more failed to molt to 3rd instar (362pprox. ID_{97}), after 5 days of exposure. In the monitoring study, populations were tested against Cry1Ac from 1998 through 2010, and against Cry2Ab from 2002 through 2004, and again from 2007 through 2010. None of the Cry1Ac-exposed larvae tested during the 13 year period reached the third larval instar by five days and mass reduction relative to untreated control larvae was uniform at 98 to 99%. For the seven years of Cry2Ab monitoring, no treated larvae reached third instar, and mass reduction was uniform at over 98% relative to controls in five-day assays. These results illustrate the value of a single diagnostic concentration and sub-lethal response criteria to monitor susceptibility of target pests to Bt proteins expressed in crops over time and suggest that susceptibilities to the Cry proteins expressed in Bollgard (Cry1Ac) and Bollgard II (Cry1Ac; Cry2Ab) cottons have not changed during the period these technologies have been grown in Mexico. This report is also unique since it is one of relatively few internally consistent long-term studies on resistance monitoring for this or any other geography.



What has happened in Mexico after nineteen years of releasing pest resistant GM cotton? An efficacy analysis related to Bt crops based on environmental release reports

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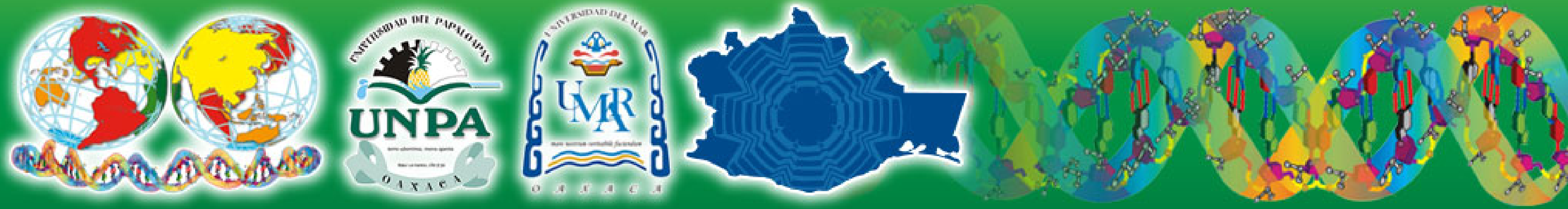
The first GM resistant cotton was probably released in Mexico during 1995 in the State of Tamaulipas, in a permitted area comprising 35 ha in the municipios of Altamira and Aldama. From this moment on, eight GM cotton events containing at least one *Bacillus thuringiensis* (Bt) gene have been given release permits in different regions of the North of the country. Out of these eight GM cotton events, six have been developed by Monsanto, one by Bayer and one by Dow AgroScience. Three GM cotton events accumulate the highest number of release permits granted by the Mexican authorities, these are: *Bollgard* cotton (MON-ØØ531-6), requested to be released in the States of Baja California, Sonora, Chihuahua, Coahuila, Durango, Colima, Sinaloa, San Luis Potosí, Tamaulipas and Veracruz between 1995 and 2010; *Roundup Ready™ Bollgard™* cotton (MON-ØØ531-6 x MON-Ø1445-2), which accounts for release solicitations in the States previously mentioned as well as Baja California Sur, and released between 1997 and 2010; and *Roundup ready™ Flex™ Bollgard II™* cotton (MON-88913-8 x MON-15985-7) which started being released in the year of 2003 and has been solicited for different localities in the states of Baja California, Sonora, Chihuahua, Coahuila, Durango, Sinaloa, San Luis Potosí, Tamaulipas, Veracruz and Zacatecas. This last GM cotton event is still being released nowadays. Only the stacked GM events *Roundup Ready™ Bollgard™* and *Roundup Ready™ Flex™ Bollgard II* include experimental, pilot and commercial phase releases as well as reports of their performance during several of these releases. The most important cotton pests in Mexico are the pink bollworm (*Pectinophora gossypiella*) and the boll weevil (*Anthonomus grandis*) while others are of a secondary nature including the tabacco budworm and corn earworm complex (*Heliothis virescens* y *H. zea*). The eight Bt cotton events that have been released in the country have at least one or several of the following Cry genes: Cry1Ab, Cry1Ac, Cry2Ae y Cry1F. A recent permit was also given to a stacked VIP3(a) containing GM event. Cry1Ab, Cry1Ac, Cry1F and Cry2Ae have shown efficacy, among other insects, to *Heliothis virescens*, *H. zea,* a lepidopteran complex identified as a secondary plague in the different cotton producing States of the country. The Cry1Ac gene is part of seven GM cotton events released in Mexico; the Cry1Ab and Cry1F genes are present each in one GM cotton event released in Mexico, while Cry2Ae is only present in *Roundup ready™ Flex™ Bollgard II* (MON-88913-8 x MON-15985) together with Cry1Ab. Given this context, it is of our interest to contrast, by revising and analyzing the data included in the environmental release reports generated in Mexico, how these GM cotton events have shown to be effective in the control of cotton plagues, in the States in which the permits have been given by the competent authority. It is because of this reason that in this presentation we will address the following question: Do GM cotton events show efficacy in relation to the present cotton plagues in the areas of release? We show a preliminary analysis based on report data generated during releases in the Mexican territory.



Studies of Cry3Aa-intoxication identify strategies to increase potency

Brenda Oppert[1], Elena N. Elpidina[2]

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The yellow mealworm, *Tenebrio molitor*, is sensitive to the Cry3Aa coleopteran-specific toxin from *Bacillus thuringiensis* (Bt) but is insensitive to the lepidopteran-specific toxin Cry1Ac. These two toxins were used to evaluate differences in gene expression in *T. molitor* larvae exposed to Cry toxin for 12 hours, a timepoint that was previously determined to provide the greatest difference in gene expression. Approximately 237 million paired-end sequence reads (250 bp insert size) were obtained from control (no toxin), Cry1Ac-fed (negative control), or Cry3Aa-fed larvae, with triplicate biological replicates for each treatment group (nine samples total). Statistically significant changes in gene expression were found in Cry-treated compared to control larvae (Student t-test, ≥ 90 C.I. and >8-fold change). However, there were only 23 genes that were differentially-expressed in Cry1Ac-challenged larvae, whereas there were 438 genes (428 specific to Cry3Aa) differentially expressed genes in Cry3Aa-challenged larvae, reflecting the relative sensitivity of *T. molitor* larvae to these toxins. Using Blast2GO, we determined that most of the differentially expressed genes are involved in metabolic and cellular processes, binding, and catalytic activity. These changes are likely related to the cessation in feeding that occurs in intoxicated larvae and induction of immune-like defenses that we have observed in previous studies. The most severely repressed transcripts (<0.02-fold) in Cry3Aa-intoxicated larvae included lipase, serine proteases (8), dipeptidyl peptidase, and hexamerin (2); the most highly increased transcripts (>50-fold) were mostly unidentified or hypothetical proteins (9), serine and cysteine proteases, lysosomal proteins (2), lipase, and transport-related proteins (2). Bioassays indicate that protease inhibitors can reduce the time to kill and increase mortality in *T. molitor* larvae exposed to sublethal doses of Cry3Aa. These data demonstrate that understanding insect responses to Bt toxins is valuable, and can be used to increase the potency of coleopteran specific Bt toxins.

Effect of *Bacillus thuringiensis* Cry3Aa toxin on the expression of gut peptidases in *Tenebrio molitor* larvae

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We studied *Bacillus thuringiensis* Cry3Aa intoxication and the expression of insect gut peptidases in *Tenebrio molitor* larvae. Larvae digest protein by cysteine (papain family) peptidases in the anterior midgut and serine peptidases (chymotrypsin family) in the posterior midgut. The data demonstrate that after 12 h of intoxication, 11/26 cysteine peptidase transcripts were significantly increased in expression (2-4-fold), including the main digestive cathepsin B, but after 24 h these transcripts mostly were decreased. Serine peptidase transcripts included 88 presumably active peptidases and 104 inactive homologs. Thirty two transcripts of 88 significantly changed expression after 12 h intoxication, and changes ranged from approximately 6-fold increase to 20-fold decrease. Expression of the main digestive trypsin and chymotrypsin mRNAs decreased. Among different groups of serine peptidases with altered expression levels, only chymotrypsin-like peptidases were significantly increased after 12 h intoxication. By 24 h, the changes in expression were similar to the 12 h profile. The majority of serine peptidase homolog mRNAs were decreased in expression. The severe change in expression of serine peptidases induced by intoxication may be associated with the close proximity of secreting cells to the lesion focus in the posterior midgut. At the same time, the insect maintained the production of critical digestive cysteine peptidases synthesized and located in the anterior midgut. The data on suppression of insect digestive system after 24 h intoxication correlate to observations of intoxicated larvae and cessation of feeding.

Acknowledgments: The research was performed with the support of the Russian Foundation for Basic Research (grants #14-04-91167-NSFC_a and #12-04-01562-a).

Countering multiple resistance with modified Bt toxins

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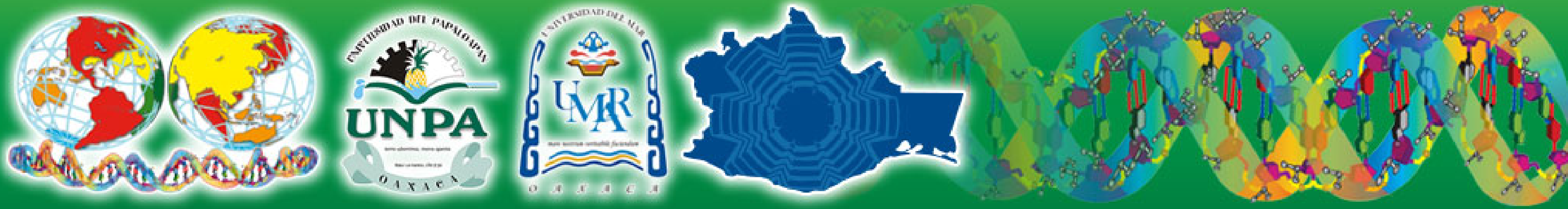
Evolution of resistance in pests threatens the long-term efficacy of insecticidal proteins from *Bacillus thuringiensis* (Bt) used in sprays and transgenic crops. For example, field-evolved resistance to Bt cotton producing the single Bt toxin Cry1Ac has been documented for pink bollworm (*Pectinophora gossypiella*) in western India. In laboratory diet bioassays, we found that the genetically modified Bt toxins Cry1AbMod and Cry1AcMod effectively countered pink bollworm resistance to the native Bt toxins Cry2Ab, Cry1Ab and Cry1Ac. Resistance ratios based on the concentration of toxin killing 50% of larvae for a lab-selected resistant strain relative to a susceptible strain were 210 for Cry2Ab, 270 for Cry1Ab, and 310 for Cry1Ac, but only 1.6 for Cry1AbMod and 2.1 for Cry1AcMod. For both the resistant and susceptible strains, the results show slight but significant synergism between Cry1AbMod and Cry2Ab, whereas the other combinations of toxins tested did not show consistent synergism or antagonism. The results suggest that the modified toxins may be useful for managing populations of pink bollworm resistant to Cry1Ac, Cry2Ab, or both. Competing interests: AB, MS and BET are coauthors of a patent "Suppression of Resistance in Insects to *Bacillus thuringiensis* Cry Toxins, Using Toxins that do not Require the Cadherin Receptor" (patent numbers: CA2690188A1, CN101730712A, EP2184293A2,EP2184293A4, EP2184293B1, WO2008150150A2, WO2008150150A3). JAF is coauthor of a patent ''Cadherin Receptor Peptide for Potentiating Bt Biopesticides'' (patent numbers: US20090175974A1, US8354371, WO2009067487A2, WO2009067487A3). Pioneer, Dow AgroSciences, Monsanto and Bayer. CropScience did not provide funding to support this work, but may be affected financially by publication of this paper and have funded other work by AB, JAF, MS and BET.



Resistance management for Bt crops: successes and failures

Bruce Tabashnik

Department of Entomology, University of Arizona.

Transgenic crops producing insecticidal proteins from *Bacillus thuringiensis* (Bt) decrease reliance on insecticide sprays, but evolution of resistance by pests can reduce the efficacy of these crops. A review of data from 77 studies conducted in eight countries reveals that reduced efficacy of Bt crops with practical consequences for pest control was associated with field-evolved resistance in some populations of 5 of 13 species of major pests by 2010, compared with only one such species in 2005. Factors contributing to this surge in documented cases of resistance include more extensive monitoring as well as increases in the area planted to Bt crops, the number of pest populations exposed to Bt crops, and the cumulative duration of exposure. Whereas most previous assessments characterized pest populations only as resistant or not, the new analysis introduces a series of five color-coded levels ranging from strong evidence of sustained susceptibility to the most serious cases of resistance. Field outcomes support theoretical predictions that factors delaying resistance include recessive inheritance of resistance, low initial frequency of resistance alleles, and abundant refuges of non-Bt host plants. The results imply that proactive evaluation of the inheritance and initial frequency of resistance are useful for predicting the risk of resistance and improving strategies to sustain the effectiveness of Bt crops.

Vip3A proteins for the control of caterpillars

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Vip3 toxins are *Bacillus thuringiensis* (Bt) proteins which are secreted to the medium during the vegetative growth and, for this reason they do not contribute to the insecticidal activity of bioinsectices prepared from Bt spore/crystal mixtures. Nevertheless, some of the Vip3 proteins are highly active against Lepidoptera and show a relatively broad insecticidal spectrum. Because Vip3 proteins share no homology with the Bt crystal (Cry) proteins and because they have different modes of action, Vip3A proteins have been combined with Cry1 proteins in transgenic crops (Bt crops). The mode of action of Vip3 proteins is not well understood. It is known that there are some insect species susceptible to Cry1 proteins which are practically completely tolerant to Vip3 proteins and *vice versa*. Although Vip3 proteins and Cry1 proteins share general features in their modes of action, they differ notably when the different steps are studied in detail. For example, we have found that, contrarily to the Cry1 proteins, Vip3 proteins do not have a protease-resistant core, or at least not so resistant as in the case of Cry1 proteins. This implies that the activation step within the insect's midgut is more critical in the case of Vip3 proteins because there is a dynamic equilibrium between protoxin activation and toxin degradation. A second differential feature between Cry1 and Vip3 proteins is that they bind to different membrane binding sites. This has been shown with different insect species using either labeled Cry1 proteins or labeled Vip3 proteins, and performing competition studies with unlabeled heterologous competitors. This feature means that, for resistant management purposes, it is a good strategy to combine *cry1* and *vip3* genes in the same plant because mutations in insect populations conferring resistance to one toxin (due to alteration of its receptors) would not confer cross-resistance to the other toxin.

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Effects of endophytic fungi on fall armyworm, *Spodoptera frugiperda* Smith, and host plant damage

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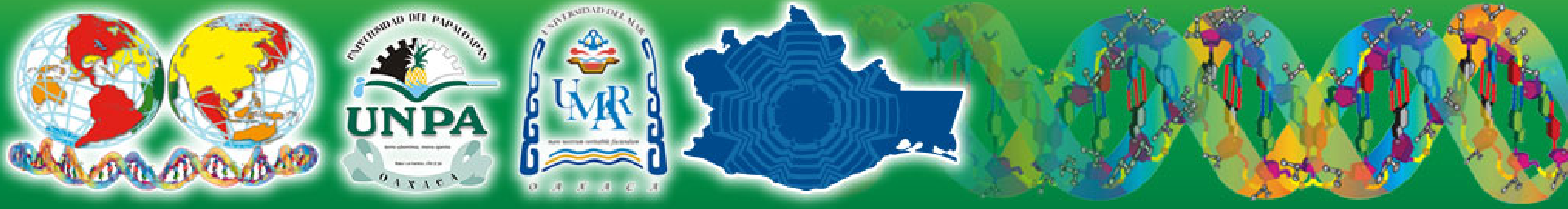
Fungal endophytes are microorganisms that can live within plant tissues without causing apparent damage. Among the different kinds of endophytes isolated to date, there are several that have potential beneficial uses as biocontrol agents, including endophytic entomopathogenic fungi. A recently survey isolated a range of putative beneficial fungal endophytes in different tissues of cotton cultivated in Texas. We examined the endophytic effects of one of these isolates (*Paecilomyces sp.*) along with a commercially available fungal entomopathogen (*Beauveria bassiana*), identified as strain GHA, on host plant use by fall armyworm (*Spodoptera frugiperda*). First, we measured preference and performance using no-choice tests in laboratory feeding assays. *S. frugiperda* larvae were assayed for feeding on foliage obtained from control and inoculated cotton plants. Results indicated that 4th instar larvae fed less on leaves obtained from *B. bassiana* inoculated plants and inoculation with *Paecilomyces sp.* does not seem to have any effect on fall armyworm feeding preference. As *Spodoptera frugiperda* is an economically important pest in both México and US, we tested the effect of both endophytes on cotton field trials during the years 2012 and 2013 in Texas. To monitor for the presence of fall armyworm, we did regular scouting along the cultivation period. We did not find evidences of fall armyworm on control, *Paecilomyce sp.*-inoculated nor *Beauveria bassiana*-inoculated plants. However early in the season we observed some moths in the periphery of our cotton field trials. In addition, in 2014 we conducted two field trials using corn and sorghum to test for the effect of *Beauveria bassiana* strain GHA and another *Beauveria bassiana* strain, isolated in México and identified as BB42. Results early in the season indicated that both *Beauveria bassiana* strains have positive effect on the germination and plant-stand up in both corn and sorghum field trials.



Manipulating secondary plant compounds for enhance crop protection

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Traditional plant breeding methods to improve a crop's ability to tolerate or reduce insect damage are well established; however, relatively little research has been done on major commodity crops exploring cisgenic or transgenic approaches for improving host plant resistance by manipulating secondary plant compounds. The purpose of this talk is to review current research in this area, discuss potential hurdles, and explore future opportunities for crop protection and resistance management.

2.4 Roundtable

Analysis of the complexity in the evaluation of adverse effects caused by GMOs in Mexico

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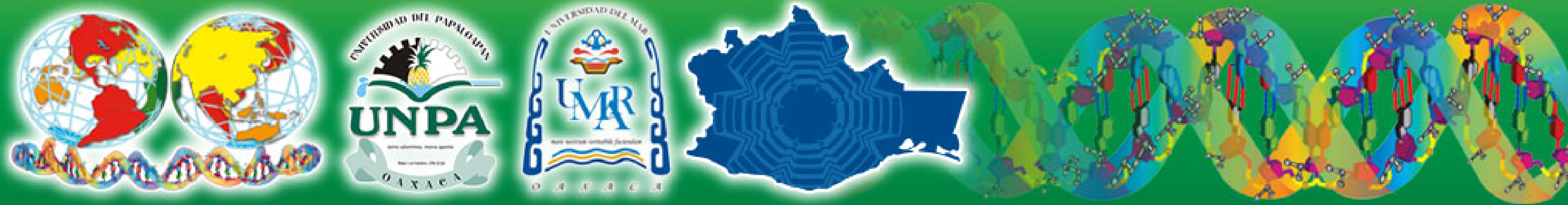
The environmental damage caused by genetically modified organisms (GMOs) is mentioned from international treaties to local laws, however, it is still necessary to discuss the theoretical and practical framework for its evaluation in organisms that are not the object of the genetic transformation (non-target organisms, NTO). Currently, Mexico is an ideal location for this kind of assessment, since it is the center of origin and diversity of cotton *Gossypium hirsutum* and, therefore, the area where this species has developed interactions with other organisms from thousands of years to more recent ones. At least 50% of wild cotton metapopulations in the country express a recombinant protein, product of transgene flow events with genetically modified crops (GM). These proteins have two objectives: herbicide resistance and tolerance to Lepidoptera. This particular scenario is set in a mega-diverse country that lacks baseline information to perform research about potential environmental impacts of GMOs on the habitats where wild relatives of cotton live (e.g. lowland and coastal dunes). This situation calls for decision making: 1) monitor the possible loss of diversity of NTO and wild cotton plants; 2) Remove the plants with transgenes sacrificing the environment and the diversity of cotton populations, but prevent effects on NTO; or 3) just observe the possible long-term consequences on population, community and ecosystem levels. However, in any of the options above, we need to have information about: how can we make an assessment of the potential adverse effects caused by insecticidal proteins in arthropofauna community? In this study, different experimental designs to assess the effects of GMOs on non-target organisms and observe the potential in real scenarios were developed from different ecological approaches: the first is general and studies the composition of communities and their functionality; the second focuses on a group selected for its representation in the community: Lepidoptera; and the third refers to the analysis of a single functional group: floral visitors. Each of the methods was accompanied by its particular collecting strategy. Samples were cataloged, identified by taxonomic specialists and DNA (COI), photographed and deposited in collections. For each of the approaches, conditions that reflected different types of adverse effects were simulated over time. Different statistical analyzes were programmed in R (www.r-project.org) to share the statistical design. In the simulated results from real data, the parameters that are significantly affected differ between ecological levels. In general, species diversity (diversity index Shannon-Wiener), equity and species composition (X2) are more sensitive than abundance and richness. The communities where wild cotton lives are subjected to a set of anthropogenic pressures on the habitat, therefore these plants and their associated diversity could be affected by several reasons, mainly the change in land use for tourism development and growth of urban centers, however, the effects caused by transgenes are a new pressure that we do not know how quickly it can act and it can not be removed without destroying diversity itself, which should be taken into account in the risk analyses and biosecurity measures for the release of GMOs. This research was conducted only for the analysis of environmental damage, however, we can see that, for now, in the legal crossroads where the absolute effect of the presence of a new gene in

an ecosystem must be proved, in settings outside the laboratory, is technically impossible. On the other hand, again only in environmental terms, performing good risk analyses, with sufficient baseline information and adequate monitoring, had been less expensive than remediation itself. We recommend monitoring the diversity associated with wild relatives of GMOs, generating baseline information and effective approaches to risk analysis, monitoring programs and strategies to mitigate damage before releases are approved.



Construction process of a non target organisms research network in Latin America

M. Andrea Orjuela-R., Francisca Acevedo-Gasman

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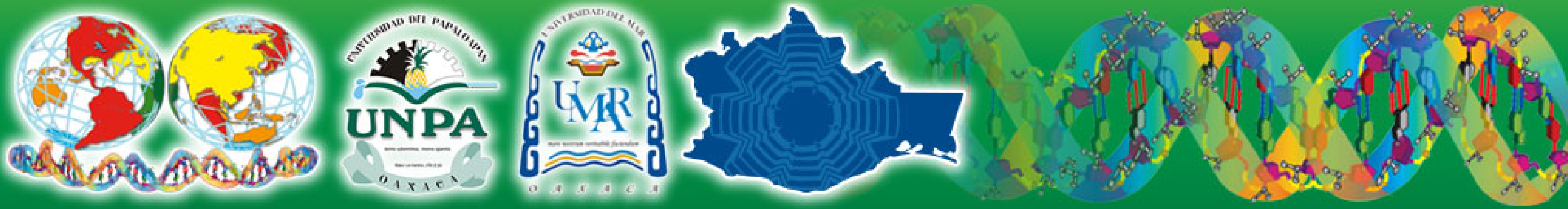
Biotech crops have expanded their commercialized crop area to 175.2 million hectares distributed among 27 countries since 1996. Applications for the environmental release of 21 genetically modified (GM) plant species have been registered in Mexico since 1988. Some transformation events of GM cotton and soybean have reached commercial status, while GM maize has gone through experimental and pilot releases. Risk assessments consider the occurrence of possible adverse effects on the conservation and sustainable use of biodiversity, one of these being the damage that could be eventually generated on non target organisms (NTO). These kind of effects are important not only because they could translate into biodiversity loses but also because losing certain species might affect ecological functions and services provided, i.e. pollination, natural plague control or soil fertility. In order to address this subject, CONABIO promoted a workshop entitled “Advances in development of methodologies for assessment and monitoring of potential effects of genetic modified crops on non target organisms” which took place in July 2013; this work reports on this meeting. The event had an assistance of 51 participants (2 from Colombia, 2 from Brazil, 1 from USA and 46 from Mexico). The main goal was to bring together a group of researchers as well as people involved in risk assessment to learn about the subject, discuss and generate strategies to collaborate in identifying a common way forward for working together. During four days we discussed some strategies and tried to put in practice a methodology already developed in Brazil using information obtained from different Mexican sources and taking advantage of the expertise of the different researchers participating in the activity. Information gaps were identified and a directory of experts and research groups was obtained. One of the principal conclusions of the Workshop was the need to build an interactive network which could be a powerful tool for knowledge exchange between people working and living in different countries. We are now working in building this network, we have a Facebook profile named “Red Temática de Organismos No Blanco” and a twitter account @redtemonb that are the first steps in this process.



Environmental interaction studies in support of environmental risk assessment of biotech crops

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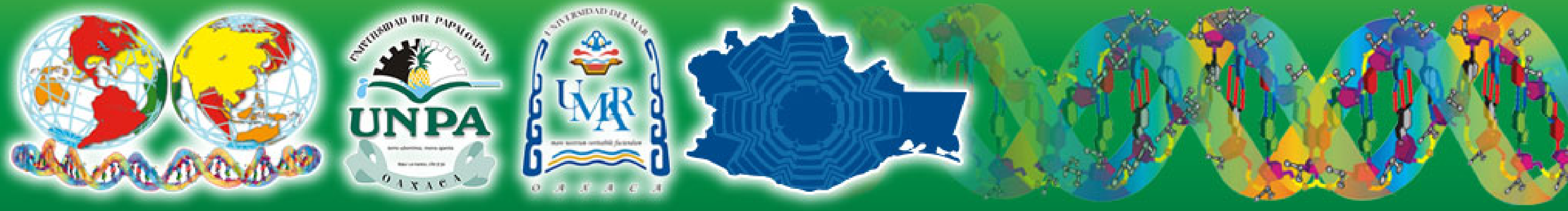
Environmental interaction evaluations are conducted as part of the plant characterization of a biotech crop and are considered in an environmental risk assessment (ERA). The purpose of environmental interaction evaluations is to assess interactions of the biotech plant with the receiving environment, including non-target arthropods (NTA), abiotic stressors, and diseases relative to a conventional control. Data generated by these evaluations are used outside of the tiered system where they are useful during problem formulation and aid in the environmental risk assessment to reduce uncertainty of unintended effects through collection of en planta data. The approach taken can be utilized across crops, product concepts, and world areas, and can be developed on a case by case basis for different regulatory requirements. An overview of the study design and key recommendations are discussed here. The design of field evaluations for the biotech plant includes replicated test, control, and reference plots in randomized blocks. The references are commercially available varieties or hybrids. The inclusion of references and the conduct of experiments at different locations provide a context for interpreting measured differences due to natural variability and local differences in agro-climatic conditions such as soil, weather, nutrients, and other abiotic and biotic stressors. This study design collects meaningful arthropod abundance data while maintaining a reasonably sized study to minimize land requirements and to allow for multiple sites in the interest of more robust study. This study focuses on arthropods closely associated with the plant, exhibiting low mobility, and a clear path of exposure (e.g., non-target herbivores) for detecting potential effects. Statistical analyses are performed on taxa present in sufficient numbers. This approach is to have a minimum level of abundance for each taxa sampled as a criterion to allow for a more robust analysis of potential treatment effects. An inclusion criterion is established where a given arthropod must have an average count per plot per collection time (across all materials) of ≥ 1. The environmental interaction data is compared between the biotech crop and the conventional control. Potential significant differences are first assessed in the context of the reference range generated from the conventional references that are also included in the design to aid in the interpretability of the data. Data from multiple sites and years allow for an evaluation of the consistency of potential differences. Local NTA field evaluations are commonly required for cultivation approvals of *Bt* crops often without consideration for the existing tiered approach data or other lab and field data from other geographies. The uniqueness of the agricultural setting or the diverse nature of a country are sometimes referenced as justification for local NTA field trials. As such, an assessment of the similarity of arthropod taxa across regions can help clarify how commercial maize fields compare across regions. The occurrence of similar taxa in commercial maize growing areas across regions can be used to justify the transportability of NTA data. Furthermore, any differences noted in the assessment would aid in identifying taxa that require additional consideration.



Implementation of normalized procedures (biosafety measures, standards & technical guides) for the risk assessment of NTOs in Mexico

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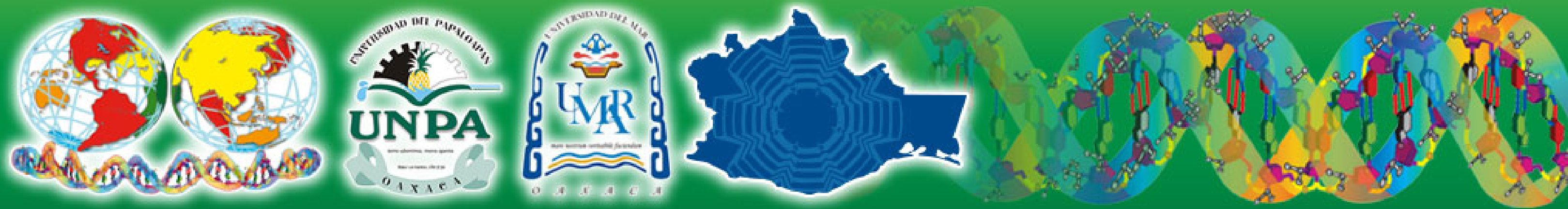
Environmental Risk Assessment (ERA) is a previous requirement to the environmental release of GM crops in many countries such a as Mexico. Here, there is an extensive legislation covering agbiotech applications including the Biosafety Law on GMO (124 articles), its By-law (73 arts.) with most of regulatory procedures, which contains the 'Special Regime for Maize Protection' and additionally, national standards related to biosafety. Procedures for the ERA of GM agricultural or forest varieties are being elaborated through one of these NOM (Norma Oficial Mexicana), which should, among other requirements, document most biosafety measures planned/ imposed in release applications/ permits. Furthermore, a multi-sectorial group also gathered to discuss and elaborate a technical guide for addressing potential risks to Non-Target Organisms (NTOs) from *Bt* (Resistant to Insect Pests) GM varieties in field conditions. The guidelines include criteria for: a) the inventory of entomofauna present in different eco-regions (level IV according to the North American Commission for Environmental Cooperation)[1]; b) the selection of representative or surrogate NTO species within functional groups; c) a tiered evaluation of potential adverse effects and d) some monitoring during advanced phases of evaluation. In Mexico, GM cotton is already at the commercial phase in extensive northern areas, but maize is still stuck at the pre-commercial phase in reduced areas, so different schemes have been adopted for each crop under the principles of Annex III of the Cartagena Protocol, the 'problem formulation' approach, and the experience on NTO work of selected authors and institutions around the globe. Advances on this implementation process will be briefly presented.

[1] http://www.cec.org/Page.asp?PageID=122&ContentID=1329&SiteNodeID=498&AA_SiteLanguageID=3



2.5 Pre-congress Workshops

Micropropagation ornamental plants

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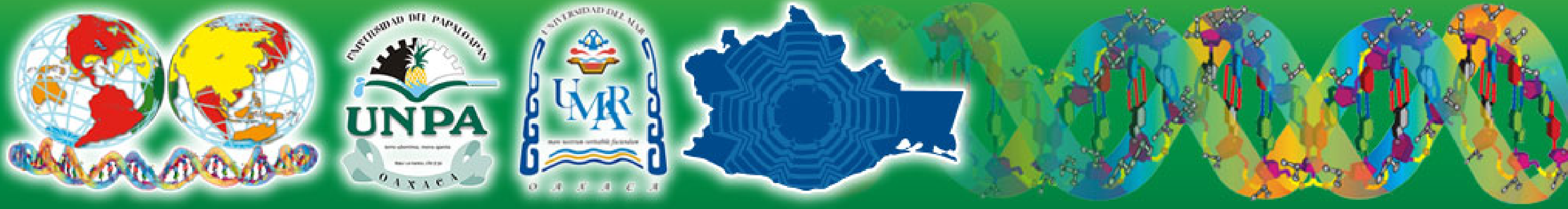
Floriculture in our country is going through great obstacles. One is the payment of royalties for the propagation or multiplication of the plant material. The geographical possession of our country is excellent, being neighboring United States, the biggest buyer of flower, however, is located between the fourth and fifth leading flower producer in Latin America. External dependence, low production, the quality of the plant produced in Mexico is sold in the domestic market (70% of production), and very little to export, no effective disease control and technology in general, we are limited and low competitiveness. We are not up to the major exporting countries and leaders that dominate the world, such as Holland and Colombia. The quality of plants partly determines the productivity of plantations. The availability of healthy and vigorous plants originating from genetically tested plants. Plant biotechnology and especially micropropagation is one of the useful tools for mass commercial production. A plant multiplication done under controlled conditions and in miniaturized form (*in vitro* culture) represents the best option to keep production level than the national and international market demands. This course aims to share lessons learned on the *in vitro* reproduction of ornamental species. It is aimed at students of plant biotechnology, agronomy, biology and related areas. It has been designed from the basic knowledge to the management of micropropagated plants for commercial purposes. It is expected that this information is input to interest in relation to the factors affecting the *in vitro* propagation of ornamental plants and to establish protocols to resolve handling yet productive cultivars make decisions with different plants that potentially are of academic interest and economical. The objective of this course is to share lessons learned in the micropropagation of ornamental species.



Tuning optimal-robust linear MIMO controllers of bioreactors by using Pareto optimality

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Pareto optimality was introduced in order to find the better equilibrium between performance and robustness of linear controllers by the simultaneous minimization of the quadratic-error and quadratic-control functions integrals. The Pareto optimization problem was solved setting the characteristic matrix eigenvalues in the region of left complex semi plane where |Im/Re|<1 as constraint. 2D Pareto fronts were built with the quadratic-error function integral vs. quadratic-control function integral. The proposed method was applied for tuning linear controllers of two bioreactors one of them unstable SISO and the other one stable MIMO.

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