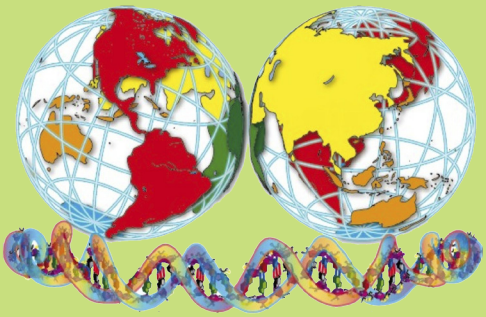

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Biotechnology Summit 2012

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Mexico

Editors

Fabián Fernández-Luqueño
Fernando López-Valdez
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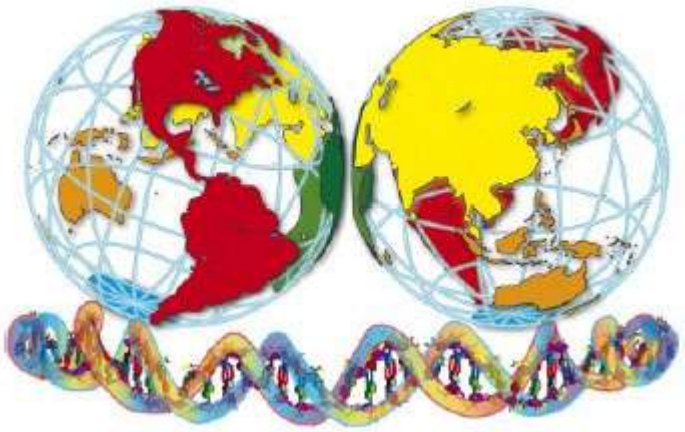

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Welcome to the Biotechnology Summit 2012

This year, the summit was organized by the non-profit organization International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society and will be held in the Hotel Maria del Carmen, in Merida Yucatan, in March 19 and 20, 2012.

It is gratifying to realize that the support of the ICGEB, CINVESTAV, AgroBIO MEXICO, ASM, SMBB delegation Merida, the Yucatan State Government and other local, national, and international institutions and companies, set the course and planning leading to the important field of biotechnology which will serve as a guideline to meet the country's priorities in this area and strengthens the emergence of biotechnology as a strategic element for the economic and social development of our country.
We hope that the biotechnology summit 2012 can contribute to the updating, distribution, information exchange, networking, and improvement of biotechnology and research activities in Mexico and other countries, and foster actions aimed at developing new processes, products and benefits for different needs and regions of our country. We have selected all critical areas of application of biotechnology and gathered them in one place in order to provide all participants, particularly young biotechnologists, a platform of interaction between academic researchers and industry, necessary to recognize the great diversity of scientific, technological and productive areas of biotechnology and sustainable development. The Scientific Sessions of Biotechnology Summit 2012 comprises the following areas:

1. ***BLUE biotechnology*** ***Aquaculture, Coasts and sea, Fish health and nutrition, Aquatic animals reproduction, Cloning and genetic modifications, Aquaculture and fisheries pest and Disease control.***
2. ***BROWN biotechnology*** ***Space and geomicrobiology, Arid Zone & Desert Biotechnology.***
3. ***DARK biotechnology Human and animal and pest control, Bioterrorism, Biowarfare, Biocrimes and Anticrop warfare.***
4. ***GREEN biotechnology*** ***Biotechnologies for the 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, Plant, Pets and Farm-animal disease, health, nutrition, reproduction, cloning and genetic modification, Plant micropropagation and plant tissue culture, Sustainable Design, Renewable energy generation: Resource-saving and energy-efficient, Bioremediation & Environmental Biotechnology, bio-fuel production and sustainable biotechnology development; biotechnologies for competitive production, New materials and new energy sources.***

5. *GREY biotechnology* ***Industrial biotechnologies: Classical Fermentation & Bioprocess / Bioengineering Technology. Engineering and technological re-equipment of bioproduction, output of science-intensive bioproducts.***
6. *PURPLE biotechnology* ***Strategy for the intellectual property protection, Patents, publications, Inventions.***
7. ***RED biotechnology*** ***Human Health & disease, Medical, Diagnostics and Tissue engineering.***
8. ***WHITE biotechnology*** ***Gene-Based Industrial biotechnologies.***
9. *YELLOW biotechnology* ***Food, Nutrition Science and Nutraceuticals.***
10. *PLATINUM biotechnology* ***Synthetic Biology.***
11. *SILVER biotechnology* ***Biobusiness, BioEntrepreneurship & Marketing; Development Economics, Biobusiness and Marketing.***
12. ***IRIS biotechnology*** ***Multidisciplinary Area: Biochemistry, Molecular Biology & Biotechnology and Applications based on omic's.***
13. *TRANSPARENT biotechnology* ***Bioethics, Biotechnology and Society: tools for asses the support to the scientific sector, including its biotechnological potential and human resources.***
14. ***GOLD biotechnology*** ***Bioinformatics, Nanobiotechnology, Microelectronic and Nicroelectromechanical systems (MEMS), Micro Systems Technology (MST), Nano Electro Mechanical Systems (NEMS) micromachines.***
15. *INDIGO biotechnology* ***Education & Early Childhood Stimulation In The Culture Of Health, Nutrition, Sport, Art, Science, BioTechnology & Society as Information and telecommunication technologies TIC´S: for integrating science, education and manufacturing.***

We will also hold the Symposium: Strategies to Monitor and Reduce Resistance to *Bacillus thuringiensis* among Targeted Insects.

Oral and poster session and workshops.

Thank you,

Dr. Susana Lozano

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IN EXTENSO PAPERS

RED BIOTECHNOLOGY AS AN EXCITING CAREER ALTERNATIVE FOR HEALTHCARE GRADUATES

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Abstract: The international healthcare (red) biotechnology area is an exciting intersection of science and business that has grown dramatically since its inception three decades ago. The present work summarizes the current state of bioeconomy and offers an exciting and rewarding career prospect for all healthcare graduates around the world.

Keywords: Red biotechnology • Biopharmaceuticals • pharmaceutical market • therapeutic classes.

Introduction: In 1953 Watson and Crick published a paper in Nature describing the double helix. In 1976 Swanson and Boyer founded Genentech, eventually were succeeding in launching the first biosynthetic insulin in 1982. Today there exist several commercial sectors, each given its own corresponding coding color, e.g. red, green, blue, and white. Healthcare biotech is color-coded red (from the red blood cells) and includes the biosynthetic production of medicines and vaccines, stem-cell research, DNA sequencing and more.

Materials and methods: This is a historical overview of red biotechnology scientific and business literature.

Results and discussion: In 2010, global red biotechnology had revenues of USD 84.6 billion, R&D expenses of 22.8, and net income of 4.7. It also employed 178,750 people, and had 622 public companies.

In 2010, the global pharmaceutical market reached USD 856.4 billion, of which U.S.A. accounted for 310.6, Japan for 96.5, EU5 (GER, FRA, UK, ITA, ESP) for 147.4, Canada for 21.5, South Korea for 11.1, and pharmerging countries (BRIC) for 150.5. In 2009, all three biosciences sectors (pharma – devices – labs) showed, on average, a positive net income.
Today red biotechnology is one of the most promising careers for bioscience graduates. Bioscience employment growth, led by research, testing and medical labs, greatly outpaced national employment growth from 2001–2008.

A biological graduate's career alternatives include government (e.g. Ministry, Drug Org, Hospital), academia (e.g. University, College), industry (e.g. Biotechnology, Pharmaceutical, Chemical), self-employment (e.g. Private Practice), and…no employment (e.g. Sabbatical, enjoying the beach). While actually looking for a biotech job, the graduate's basic options include: a) graduate studies (e.g. biological sciences or business administration), an unpaid apprenticeship, or job hunting per se. Today, there is a large variety of commercial biotech/pharma courses around the world, for example at Columbia, Harvard, Berkeley, U Penn, Johns Hopkins, Northwestern, Kellogg, Carnegie Mellon, Duke, and others.

When in biotech/pharma, a biological graduate can expect job openings in research and development, manufacturing, marketing, or sales.

While in the sales department one can expect jobs such as national sales director, regional sales director, sales territory manager, or key account manager. Advantages include good remuneration, impressive incentives, constant training, a professional network, and international travel, while disadvantages include long hours, frequent traveling, pressure to achieve targets, internal competition, and tough customers.

In a biopharmaceutical marketing department one can expect jobs such as marketing manager, business unit manager, product manager or congress manager. Advantages include very good salaries and incentives, high responsibilities, fund management, constant training, and international travel, while disadvantages include pressure to achieve targets, professional insecurity, long hours, constant traveling, and internal competition.

In medical marketing one can expect jobs such as medical director, medical services manager, pharmaeconomist, pharmacovigilance manager, or scientific knowledge manager. Advantages include scientific focus, constant scientific training, direct contact with Opinion Leaders, participation in the innovation cycle, and relative professional stability. Disadvantages include very few innovative employers, significant responsibilities on clinical trials, external requests for basic research, less funding compared to marketing, and reduced career advancement.

In a regulatory affairs department one can expect jobs such as regulatory manager, pricing manager, reimbursement manager, or managed care director. Advantages include scientific focus, international intracompany contact, contact with Opinion Leaders, direct contribution into company's advancement, and professional stability. Disadvantages include standardized work, multiple work priorities, limited career advancement, reduced funding, and intracompany conflict.

There is evidence to suggest that, at least in the U.S., average wages within the biotechnology/pharmaceuticals sector were higher than the average wages in any other industrial sector.

Biological graduates interested in a red biotechnology career can find numerous information sources on the web, including web pages on the global biotech industry, the global pharmaceutical industry, biotechnology in academia, as well as a variety of country-specific information sources.

When it comes to biotechnology academics, they should constantly strive to: a) empower their biotech departments, b) attract talent, c) get known, and d) network with biopharma.

A planned commercial biotechnology curriculum should include courses on: bioeconomy, intellectual property regulation and management, entrepreneurship, financing, partnering, biologics research, bioinformatics/biostatistics, bioethics, biologics regulation, biomanufacturing, biologics marketing and selling, biologics pricing and reimbursement, biosupply chain management, biotechnology business models, biologics life cycle management, commercial biotechnology apprenticeship, and business plan writing.

Biological graduates looking for a biotechnology career should:Be meticulous, professional, deadline-minded, and informed on the professions that interest them; If they can't decide: 1) they should define their stamina (60-hour weeks, work 12/7, other city, foreign country), and 2) dream themself in ten years.

They should ask: their beloved ones, but decide THEMSELVES; Diversify, The global job market is and will remain competitive; Compare: 1) immediate job search, 2) post-graduate studies for an academic career, 3) specialization for a management / marketing career; Remember: if one has to enter the job market now, "job searching is a full-time job…"; Network! Link, communicate, meet, suggest, request (50 contacts weekly); Empower themselves! Use the Net, libraries, newspapers, journals, associations, congresses, career days; Identify an experienced career mentor (preferably multi-faceted); Sacrifice a full tropical summer for an unpaid apprenticeship; Learn interview techniques through dedicated books; Keep a digital calendar (e.g. in Outlook and on PC, stick, iPhone, iPad, Cloud) of: interesting classifieds, CV submission, employer responses, interview appointments, interview feedback, employer requests, next interviews, financial offers, etc.

Conclusions: Red biotechnology has evolved tremendously since its commercial inception in 1982. Today it represents an exciting and rewarding career prospect for all biological graduates

around the world. Interested applicants should take dedicated commercial biotechnology courses, be hard-working, inquisitive, and meticulus, compare their prospects, network intensively and empower themselves.
Finally, their professional career can not fully be predicted, will bring many sacrifices, changes and ups and downs, but rewards as well.

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IMMUNOGENIC SALIVARY PROTEINS OF *TRIATOMA DIMIDIATA*, A VECTOR OF CHAGAS DISEASE

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Abstract: The blood sucking bug*Triatoma dimidiata* is an important vector of Chagas disease. A wide range of salivary proteins are injected into the host during feeding and can elicit a humoral immune response in hosts. Immmunoassays using antibodies against salivary proteins show great promise to evaluate transmission risk of vector borne diseases in endemic areas. We need develop such immunoassays for an improved control of Chagas disease in the Yucatan Peninsula. To identify immunogenic proteins from *T. dimidiata* saliva, we used sera from four Balb/c mice previously exposed to triatomine bites to evaluate antibodies against salivary proteins by Western Blot. Immunogenic salivary proteins of 14, 21 and 79 kDa were recognized by all mouse sera. Previously, it has been demonstrated that the 14 and 21 kDa salivary proteins are specific oftriatomines. The Apyrase of 79 kDais frequently found in the saliva of other triatomines and blood-feeding insects such as *Aedesaegypti.* Immunogenic proteins of 14 and 21 kDa are good candidates for molecular markers of exposure to saliva of triatomines including *T. dimidiata*. We intend to clone and express both proteins for the production of recombinants that can be used in the development of anti-triatomine salivaimmunoassays.

Key Words: Proteins•*Triatoma dimidiata*•Chagas disease.

Introduction: Triatomines are vectors of *Trypanosomacruzi*, the etiological agent of Chagas disease in Latin America (1). *Triatoma dimidiata* is a hematophagous Hemiptera distributed from Southern Mexico to Northern Peru andis a main vector species of Chagas disease in these areas, including the Yucatan Peninsula (2, 3). We previously characterized the geografic distribution of this vector and developed predictive model of vector borne Chagas disease transmission risk to humans in the region. Our field data suggested that natural transmission occurs by transient and seasonal invasion of houses by sylvatic/peridomestictriatomines rather than through established resident domiciliated bug populations (3, 4). This transmission patterns implies high risk of re-infestation by non-domiciliated bugs after conventional indoor insecticide spraying. This was confirmed in a pilot study in wich we documented the re-infestacion of houses within only 3-4 months after insecticide application (5). Current methods to asses the prevalence and intensity of triatomine bug infestations in domestic and peridomestic sites involved timed manual collections, are costly, require skilled staff, and usually lack the sensitivity and precision necessary for detection of low-density populations. Thus, new methodologies are required to monitor bug populations in endemic regions of Chagas disease (6).

The saliva of hematophagous arthropods contains a complex mixture of proteins with biological activity. These include the modulation of the humoral and cell-mediated host immune response, as well as hemostatic responses such as vasoconstriction, blood coagulation and platelet aggregation, which help the bug to obtain its blood meal (7).

Moreover, salivary proteins can elicit an antibody response in their hosts, and this has been used as an epidemiological tool and biological marker of exposure to disease vectors including mosquitoes, ticks, tsetse flies and sand flies. Furthermore, the antibody response to the saliva can be also used as markers for transmission risk of infectious disease agents (7, 8).

Immmunoassays using antibodies against salivary proteins show great promise to evaluate transmission risk of vector borne diseases in endemic areas. We need develop immunoassays for the improved control of Chagas disease in the Yucatan Peninsula.

Materials and methods:

Obtaining serum of mice sensitized with saliva from *T. dimidiata.*

With the aim of identifying immunogenic proteins from *T. dimidiata* saliva we used sera from four Balb/c mouse previously exposed to triatomine bites to evaluate antibodies against salivary proteins by Western Blot.Mouse were exposed weekly to the saliva of 12 *T. dimidiata* fifth instar bugs (3 bugs/mice/exposure) during 4 weeks. Each mouse was anesthetized with sodium pentobarbital via intraperitoneal (50 mg/kg) and the insects were allowed to feed for 5 minutes in order to keep them hungry until the end of the experiment. At the end of 4 weeks of sensitization, mice were anesthetized and underwent a cardiac puncture to obtain whole blood; the animals were euthanized later by cervical dislocation. The samples were placed at room temperature until they coagulated and then serum was separated by centrifugation at 2000 x g. The samples were frozen at -20° C until use.

Preparation of salivary gland proteins of *T. dimidiata*.

We used two adult *T. dimidiate* wich were dissected to get salivary glands. The glands were macerated in 1 mL of sterile PBS and 100 μL of protease inhibitor cocktail (1mg/mL). The sample was centrifuged for 20 min. at 1000 x g. and the soluble fraction was collected. We determined the protein concentration of the sample using a standard curve by Bradford methodology and obtained a concentration of 2.4 mg/mL. The sample was stored at -20° C until use.

Western Blot

Proteins were separated by polyacrylamide gel electrophoresis (15%) and subsequently transferred from the gel to a nitrocellulose membrane in a transfer chamber with Tris-buffer methanol. Then the membrane was washed and blocked with a solution of 5% skim milk overnight. Then took place 3 washes with PBST buffer the membrane was incubated for 2 hours in mouse serum immunized with saliva (1:100). At the end of the incubation the membrane was washed 4 times with PBST and incubated for 1 hour with anti-IgG secondary antibody conjugated to alkaline phosphatase (1:4000). Finally the membrane was washed and incubated in NBT/BCIP solution (alkaline phosphatase substrate) to observe the immunogenic protein bands.

Results and discussion:

In this study, we used immune sera to identify *T. dimidata* immunogenic salivary proteins and made the first steps towards developing a novel immune-epidemiological tool to assess domestic and peridomestic infestations by *T. dimidata*.

In Western blots, antibodies to salivary proteins of *T. dimidiata* were detected in all four mice. Immunogenic salivary proteins of 14, 21 and 79 kDa were the main proteins recognized. Previously, croos-reactivity studies using saliva or salivary gland extracts from different hematophagous species, e.g. different triatomines, bed bugs, mosquitoes, sand flies and ticks have demonstrated that the 14 and 21 kDa salivary proteins are specific oftriatomines. (6,7).

Moreover, sera from peridomestic chickens and guinea pigs naturally exposed to triatomines in sites of known *T. infestans* challenge in Bolivia and Chile also recognized the 14 and 21 kDa proteins. (6-8). Therefore, these represent promising epidemiological markers to develop a new tool for vector surveillance in Chagas disease control programs.

The 79 kDa protein has been previously identified as Apyrase. It is a member of the 5`-nucleotidase family and also a platelet aggregation inhibitor. This protein is frequently found in the saliva of other triatomines and blood-feeding insects such as *Aedesaegypti.*(9-11) On the other hands, the analysis of the sialotranscriptome of the *T. dimidiata* reported a singleton (Td64) with homology with the 79 kDa salivary apyrase of *T. infestans*, but the sequence was truncated in the 5' region (11).

Conclusions: Immunogenic proteins of 14 and 21 kDa are good candidates for molecular markers of exposure to saliva of triatomines including *T. dimidiata*. We intend to clone and express both proteins for the production of recombinant proteins that can be used in the development of anti-triatomine salivaimmunoassays.

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PEPTIDE DETECTION OF PATHOGENS IN RECREATIONAL WATERS AND IRRIGATION OF MEXICO CITY

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Abstract: Contamination of drinking and recreational waters by pathogenic microorganisms associated with fecal waste has significant impact on public health. In this study we collected water samples in Mexico City and the surrounding areas in March 2008, August and September 2011, and January 2012. To investigate the most frequent contaminants, these samples have been analysed in the proteomic equipment MALDI-TOF and 3200 QTrap with an HPLC Agilent 1200. The proteomic analysis showed that the water is contaminated by human and animal pathogens such as: *Salmonella*, *Shigella*, *Vibrio*, and toxins, and others. Based on these results we obtained the DNA of 6 samples of water collected at different times, and designed specific primers labeled with fluorochromes for the detection of this pathogens though a DNA microarrays. Microarray design will also include specific primers to detect *Aedesaegypti*, *Culexpipiens* (vectors of dengue and West Nile virus respectively), and *Entamoeba histolytica*.

Keywords: *Flavivirus vectors* • *Entamoeba histolytica* • microarrays • proteomics.

Introduction: Contamination of drinking and recreational waters by pathogenic microorganisms associated with fecal waste has significant impact on public health. Rivers are exposed to a wide spectrum of potential contaminants as a result of the many recreational uses and the close proximity of the agricultural industry. These elevate the risk of accidental release and elevate concern of disease agents entering the rivers, making it an excellent testing ground to detect pathogens and vectors that transmit viral diseases.

Amebiasis is defined as the infection caused by the protozoan *Entamoeba histolytica*. This parasite infects the large intestine although may infects any tissue, and it is responsible for about 100,000 deaths per year, placing it after malaria in mortality due to protozoan parasites.

Culex complex mosquitoes transmit the WNV (West Nile Virus) in addition to other sickness. Birds serve as reservoir and amplification hosts of this mosquito-borne *flavivirus*, and the virus was introduced to Mexico in 2003. Our country, including Mexico City has ideal conditions to favor an outbreak of WNV.

The mosquito *Aedesaegypti* is the primary urban vector of dengue (DEN) and yellow fever flaviviruses. *Aedesaegypti* prefers to lay their eggs on rough, moist surfaces, just above the water line. Favor breeding conditions for mosquitoes includes dark, water holding containers in close proximity to a human population upon which the mosquitoes can feed. There is an estimated of 50 million infections with Dengue virus occurring in tropical and subtropical regions each year.

Materials and Methods: We are developing a method to determinate contaminants in this recreation and drinking waters. To start this study, we determined specific biomarkers to design a microarray by proteomic analysis of water samples collected in Mexico City and the surrounding areas in 2008, 2011 and 2012. To investigate the most frequent contaminants,

these samples have been analysed in the using the proteomic equipment MALDI TOFF and 3200 QTrap with an HPLC Agilent 1200.

DNA of 6 samples of the collected water at different periods of time was obtained to test the specificity of two labeled specific primers for *Ae. Aegypti*, *Cx. pipiens* and *E. histolytica*. DNA from laboratory cultures was use as positive controls in our assays. We design all the specific primer for *E. histolytica*, *Cx. Pipiens*, and *Ae. aegypti*. The specific primer for *E. histolytica* and *Cx. pipiens* were labeled with P1-Texas red and R22-Cy5 respectively. The primer specific for *Ae.aegypti* (ITS1A) was labeled with 6-FAM. The DNA in DMSO 50% was spotted in amino-silane slides to bind it covalently using the Robot Gere TAC™ G3.

Results and Discussion: The results showed that the water in 2008 contained *Ae. aegypti*, *Cx. pipiens*, and *E. histoytica* peptides (Table 1). These results are relevant since *Ae. aegypti* and *Cx. pipiens* are very important in the transmission of dengue and West Nile viruses respectively. We also detected the important pathogens: *E. coli*, *Samonella*, *Shigella*, *Vibrios* like *Vibrio cholera* and Enterobacteriace family in all water collected in 2008 and 2011; *Bacillus anthracis*, *Haemophilussomnus*, *Bordetellapertusis*, *Listeria monocytogenes*, *Streptococcus mutans*, and several poisonous rattlesnake in water collected in 2011 (Table 2).

Table 1.Proteins of *Ae.Aegypti*, *Cx.pipiens* y *E. histolytica* found in water collected in 2008.

Ae. Aegypti	*Cx. Pipiens*	*E. histolytica*
Juvenile hormone-inducible	Cytochrome P450 93A3	DHHC-zinc finger protein
Replication Factor C	Turtle protein	Metallo-lactamase
Steroid receptor-interacting	Adult cuticle protein	Hypothetical protein EHI_150410
	Leucyl-tRNAsynthetase	Exportin T
Rho-gtpase-activating	Multiple ankyrin repeats kh domain	Treoninedehydratase
Organicaniontransporter	Ubiquitin-1	Bromo domain protein
6-phosphofructo-2-kinase	Myosin-1	DNA-directed RNA pol II
Protease m1 zinmetalloprotease	Pre-mRNA-splicing factor ISY1	60S ribosomal protein L26
Synaptic Vehicle protein	Polynucleotide kinase-3'-phosphatase	Bromo domain protein

Table 2. Proteins of pathogen organisms found in water collected in 2011.

Protein names	Organism
6,7-dimethyl-8-ribityllumazine synthase	termite group 1 bacterium Rs-D17
50 kDa venom protease	*Proatherissuperciliaris* (snakevenom)
Periplasmic nitrate reductase	*Haemophilussomnus* (sudden death in cattle)
Alpha-conotoxin OIVB	*Conusobscurus* (snailvenom)
protein pXO2-14/BXB0013/GBAA_...	*Bacillus anthracis*
Chromosome partition proteína mukF	*Pasteurella multocida* (strain Pm70)
Gp19 tail tube protein	*Salmonella phage* Vi01
Gp19 tail tube protein	*Shigellaphage* phiSboM-AG3
Tail tubeprotein	*Serratia marcescens* phage KSP90
2-nonaprenyl-3-methyl-6-methoxy…	*Bordetella pertussis*
30S ribosomalprotein S6	*Vibrio tubiashii* ATCC 19109 (bivalvepathogen)
2-nonaprenyl-3-methyl-6-methox…	*Bordetella pertussis*
Aspartyl-tRNAsynthetase	*Listeria monocytogenes*
50S rib prot L5 OS=*Streptococcus mutans*	*Streptococcus mutans* (bacteria of the human oral cavity)
50S ribprot L5 OS=*Enterococcus faecalis*	*Enterococcus faecalis*
50S rib prot L5 OS=*Streptococcus uberis*	*Streptococcus uberis* (mastitis in cows)
50S rib prot L5 OS=*Bacillus cereus* cytotoxis	*Bacillus cereus* (found in contaminated food)
50S ribprot L5 OS=*Streptococcus suis*	*Streptococcus suis* (pathogen of swine)
Metalloproteinase 3	*Crotalusadamanteus* (poisonousrattlesnake)

Conclusions: Pathogen detection in water through proteomic analysis is a molecular method that is rapidly evolving in the field and has a wide range of applications for monitoring and rapidly diagnose microbial pathogens in water sea, lakes or rivers and even in drinking water. We demonstrate direct evidence of water contamination by using this method and displayed the wide variety of pathogens in the collected water samples.

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HERPES SIMPLEX VIRUS INFECTION IN OBSTETRIC PATIENTS: COMPARING SEROLOGY VS. REAL TIME PCR

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Abstract: Most of women infected with Herpes simplex (HSV) are not aware of it. The risk of neonatal herpes and/or death is higher in neonates born from mothers who had not seroconverted at the time of delivery. Therefore, the molecular diagnosis is a choice to be considered. In this work, 208 obstetric patients were included; in serum samples IgM antibodies against HSV were detected; and in cervicovaginal samples real time PCR detection of viral shedding was performed. We found 12.5% of IgM positive samples and 2.9%real time PCR positive samples. None of the patients was positive to both analyses simultaneously. The presence of viral DNA without seroconverting could indicate a recent infection or a reactivation with low viral load.A positive serological analysis with a negative real time PCR could indicate an active infection, although not necessarily viral shedding in the genital tract.

Keywords: Herpes ● Diagnosis ● Asymptomatic infection ● Viral shedding.

Introduction: Herpes simplex virus (HSV) is one of the most frequent sexually transmitted viruses. The most devastating consequence is neonatal herpes; 90% of neonatal infections are transmitted intrapartum, being at higher risk of disease and death the neonates born from mothers who had not seroconverted at the time of delivery (Broun et al., 2005). In addition, it has been reported that the molecular detection does not correspond to the presence of symptoms or lesions, or to serological diagnostic (Gardela et al., 2005). Therefore, we consider that molecular detection would be useful in prognosis and in the design of preventive strategies, especially in asymptomatic cases.

Materials and Methods: The studied population included obstetric patients attending to Hospital Regional Ignacio García Téllezin Mérida, Yucatán during 2010 - 2011.After informed consent, 5ml of venous blood and a cervicovaginal sample were obtained.

Cervical samples were processed for automated DNA extraction using MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche). For HSV DNA detection in cervicovaginal samples, realtime PCR was used (Harbecke et al., 2009). Serum samples were analysed in immunoassay (ELISA) to detect IgMatibodies against HSV1, 2(DRG, Germany) following the manufacturer's instructions. Each assay included the appropriate positive and negative controls. The clinical history was included in a database in SPSS.

Results and Discussions: None of the studied patients referred genital HSV episodes in the past, nor presented symptoms at the time of sampling. 98.6% referred having only one sexual partner the last six months. 48.6% referred full basic education; 38.4% referred medium-high education.

In IgM serological analysis, 12.5% (26/208) were positive; and 2.9% (6/208) were positive in Real time PCR analysis. The viral loads were 16-3567 copies/µl, with a mean of 658 copies/µl.

None of the patients was positive to both analyses simultaneously. The presence of viral DNA without seroconverting could indicate a recent infection or a reactivation with low viral load. It is interesting to notice that the patient with the highest viral load was negative to IgM and therefore might represent a recent infection; IgG test is needed to confirm this assumption.

A positive serological analysis with a negative real time PCR could indicate an active infection, although not necessarily viral shedding in the genital tract; it could represent shedding in oral mucosa. All studied patients were asymptomatic.

Conclusion: The presence of IgM antibodies did not correspond to actual viral shedding in the genital tract, and therefore viral DNA detection could aid to identify the patients in risk. Of most clinical interest are patients with a positive real time PCR result, with high viral loads, and negative serology.

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CHARACTERIZATION OF ARSENIC AND MERCURY RESISTANT BACTERIA FROM CONTAMINATED SITE IN NAYARIT, MEXICO

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Abstract: Heavy metal and antibiotic resistance have been shown to have a strong correlation in nature and its interrelationship is an important subject of study. Here we report an analysis of surface waters of the Mololoa river at the municipality of Tepic in the state of Nayarit, Mexico. This river has two distinctive sources of contamination: sewage waters and trash confinements. Our findings demonstrate the presence of bacteria resistant to antibiotics and heavy metal such as arsenic and mercury. Genes of ars operon an conjugative plasmids are present in isolates from the Mololoa river which provides a model to study the relationship between water flow and generation of biodiversity, and more importantly, it constitutes a model for studying genetic diversity of bacteria that affect human health.

Keywords: Heavy metal resistance ● Antibiotic resistance ● Ars operon

Introduction: Contamination of surface waters by industrial waste management has important effects in the life dynamics and the ecology of its surroundings. It has a direct impact on the public health of entire communities, such as heavy metal (HM) poisoning. In addition, the contaminants present in surface waters constitute a selective force in bacterial communities, which may become resistant to different xenobiotics (Tuckfield and McArthur 2008; Haferburg and Kothe 2007). Numerous reports have highlighted that the quality of the water that ends up in Mexican rivers is poorly monitored, and that the laws that regulate the monitoring are not properly applied (González et al. 2006; Graniel and Carrillo 2006; Guzmán et al. 2007; Jaúregui et al. 2007; Peña 2007; López 2008). Sewage water management is important since its contamination with potentially infectious bacteria can lead to their dissemination, and since the presence of certain HM can lead to the selection of bacteria resistant to either heavy metals or antibiotics, or both. The Mololoa river in the state of Nayarit (as shown in Figure 1A) is one of the biggest and most important superficial water flows in the state. Reports and evidence exist about how industrial, urban and agricultural contamination has considerably deteriorated the water quality of the river. The two main sources of contamination are municipal junk yard leaking and the discharge of considerable volumes of partially treated water from septic drains (Jaúregui et al. 2007). Furthermore, the Secretary of Environment and Natural Resources (SEMARNAT) has reported the presence of heavy metals in the river, originating in the industrial and municipal trash confinement and finally the Mololoa's river water is being used as an irrigating source for nearby agricultural fields (SEMARNAT 2002), thus having a direct impact in the quality of the natural products destined for human consumption.

Several multi heavy metal (Hg, As, Cr) and antibiotic (beta-lactamic and quinolone)-resistant microorganisms have been isolated and characterized from Mololoa river area (Mondragón et al 2011) and their roll is actually subject of study. Because microbial activities are directly or indirectly responsible for reducing the harmful effects of pollutants (Park et al. 2008; Sima et al. 2007), it may be important to examine the microbiology for preservation and restoration of the Mololoa river area. Mololoa river, provides a unique opportunity to study the

relationship between microorganisms and HM and metalloids especially for arsenic and mercury detoxification potential of microorganism in aquatic ecosystems. Therefore it may be worthwhile to examine and compare the resistance levels for arsenic and mercury from this environmental area. Here we reported the investigation of some bacteria, which could grow in the presence of mercury, arsenite and arsenate. We also present the screening and identification of the genes encoding arsenic transforming enzymes, involved in the arsenic resistance.

Materials and Methods: *Sampling*. Samples were collected at four different sites in one annual season, from the sampling sites as shown in Figure 1, and correspond as follows: site L3 corresponds to the site before the convergence of sewage and trash confinement; site L2 corresponds to the area where the trash confinement leaks the trash residues to the river. L3 site corresponds to another trash confinement, and site L4 to the site were municipality waters are discharged. Samples were collected in sterile bottles and were transported to the laboratory in ice to prevent growing after sampling.

Figure 1. Localization of the Mololoa River in Tepic municipality. The river flow and sampling points in yellow.

Bacteriological analysis. The HM resistant bacteria present the samples were isolated and analyzed by a method based on Mondragón et al. Additionally all the bacteria were recovered in three agar plates MacConckey (Bioxon) each containing either 2 and 100 mM $NaAsO_2$ and Na_2HAsO_4 respectively and 100 µM $HgCl_2$,. Plates were incubated over night at 37° C, afterwards bacteria were analyzed using the Microscan system for the identification according to the manufacturer's instructions (LaPro).
Bacterial growth. To test the resistance to HM or antibiotics, all the isolates were analyzed as described Mondragón et al. (2011).
HM resistance. The concentrations that render the minimal inhibitory concentrations, were determined by a method based on Mondragón et al. Briefly, Muller Hinton plates (BIOXON) were supplemented with the following chemical species: $HgCl_2$ (100, 150, 200, 250, 300, and 350 µM), $NaAsO_2$ (3, 5, 7, 9, 12, 15, 20 mM) and Na_2HAsO_4 (150, 200, 250, 300, 350, 400, 450, 500, 550 and 600 mM). In all experiments, a control plate without any HM was used as a positive control and maximum growing sample. All plates were inoculated with 2 X 10^3 CFU in 20 µl and incubated over night at 37° C. Minimal inhibitory concentration was assessed by the minimal concentration that gave 50% growth inhibition. As a control for metal sensitivity, *Escherichia coli*

strain J-53 (F-, Pro-, Met+ y Rifr and sensitive to Hgs<0.5 µM, Crs<0.5 mM and Cds<0.5 mM) was used.

*Screening for the genes from ars and mer operon***.** Total DNA isolation was carried out according to standard protocol as described by Sambrook and Russel2001. The detection of these genes was performed by PCR amplification of total bacterial DNA with specific primers. The primers used for the amplification of the genes in *ars* and *mer* operon were as follows (Table 1):

Table 1. Primer used for PCR amplification of *aox* and*mer*operon genes. [b]Tm, annealing temperature used in the PCR cycle.

Gen.	Primer sequence	[b] Tm (°C)	Fragment size (pb).	Reference
arsA		58	186	Saltikov*et al.* 2002.
sence	TCCTGGATTGTCGGCTCTTG			
antisence	ATCTGTCAGTAATCCGGTAA			
arsB		61	219	Saltikov*et al.* 2002.
sence	CGGTGGTGTGGAATATTGTC			
antisence	GTCAGAATAAGAGCCGCACC			
arsC		61	370	Saltikov*et al.* 2002.
sence	GTAATACGCTGGAGATGATCCG			
antisence	TTTTCCTGCTTCATCAACGAC			
merA		63	1695	Zeyaullah *et al.* 2009.
sence	CGGGATCCATGAGCACTCTCAAAATCACC			
antisence	TCCCCCGGG ATCGCACACCTCCTTGTCCTC			

The amount of genomic DNA of the arsenic-resistant bacteria added to the PCR mixture was approximately 50 ng in final volume of 25 µl. The PCR for each gene amplification consisted of 3 min of pre-denaturation at 94 °C, 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 58, 61 or 63 °C for eacg gene respectively , and 1 to 3 min extension for *ars*and *mer* genes respectively at 72 °C, and final 1 cycle of extension at 72 °C for 7 min. The PCR was conducted in a Mastercyclerthermalcycler (Eppendorf, Germany), and the PCR products were identified by gel electrophoresis using 0.7–1.2% agarose gels.

Plate matings. Parental strains that harbored a plasmid were grown overnight in 5 ml of Luria Bertani broth supplemented. Plate matings were performed as previously described (Miller, 1992). For these purpose *Escherichia coli* strain J-53 was used.

Verification of transconjugants. Plasmid transfer to the recipient strains was first verified by testing all plasmid-bound resistances with appropriate selective media. After this purification, transconjugants were screened for the presence of plasmids by plasmid isolation. Plasmids were isolated by a modified method of Kieser, 1984. Agarose gels electrophoresis was performed in a horizontal system for plasmid visualization.

Results and Discussion: It was determined a number of isolated samples coming from different areas in the Mololoa River flows (Table 2), in which discharges of waste from El Ixtete county's garbage collecting department are included.

Table 2. Characteristics of the strains isolated from Mololoa river area.

Specie	Site	MIC			Antibiotic resistance	Plasmids	Genotype		Mate
		Hg µM	As III	As V mM			*ars*	*mer*	
Achromobacter spp.	L3	S	12	400	CAZ, ESA	+	*arsA, arsB*		+
Achromobacter spp.	L3	S	12	400	AMP, CFZ	+	*arsA, arsB*		--
Acromobacter spp.	L3	150	9	500	S	+	*arsA, arsB*	-	--
Enterobacter spp.	L3	S	7	250	S	+	*arsC*		--
Achromobacter spp.	L3	S	12	400	P/T, T/S	+	*arsA, arsB arsC*		--
Serratia fonticola	L3	S	7	250	S	+	-		--
Klebsiella oxytoca	L3	S	7	250	S	+	*arsB*		--
Enterobacter aerogenes	L3	S	7	150	S	+	*arsB*		--
Klebsiella pneumoniae	L4	S	12	500	S	+	*arsA, arsC*		-
Klebsiella pneumoniae	L4	S	7	400	S	+	*arsA, arsB*		--
Actinobacillus ureae	L1	S	9	250	CFZ, CRM,	+	*arsB, arsC*		+
Escherichia coli	L4	300	15	500	S	+	*arsA, arsB arsC*	-	--
Klebsiella oxytoca	L3	300	3	S	S	+	NP	-	NP
Pseudomonas aeruginosa	L3	300	S	S	AZT, Pi CHL	+	NP	-	NP
Klebsiella pneumoniae	L1	300	3	S	S	+	NP	-	NP
Yersinia enterocolitica	L2	300	9	S	S	+	NP	-	NP
Klebsiella pneumoniae	L2	300	9	150	S	+	NP	-	NP
Yersinia enterocolitica	L2	300	9	S	S	+	NP	-	NP
Acromobacter spp.	L2	300	S	S	S	+	NP	-	NP
Enterobacter cloacae	L2	300	9	S	S	+	NP	-	NP
Klebsiella pneumoniae	L2	300	S	S	S	+	NP	-	NP
Acromobacter spp.	L2	300	S	S	AZT,CTX,CAX,PI	+	NP	-	NP
Acromobacter spp.	L2	300	12	300	S	+	NP	-	NP
Klebsiella pneumoniae	L2	S	15	300	S	+	*arsB arsC*		--
Klebsiella pneumoniae	L4	S	15	300	A/S, AMP, To, CFZ, GM, PI,TIM, T/S	+	*arsB arsC*		--
Klebsiella pneumoniae	L4	S	15	200	AMP, CFZ,CP, MXF, T/S,Pi, To (BLEE)	+	*arsB arsC*		--
Klebsiella pneumoniae	L4	S	15	600	S	+	*arsA*		--
Klebsiella oxytoca	L4	S	15	200	S	+	*Ars AarsB arsC*		--
Escherichia coli	L2	300	S	S	S	+	NP	-	NP

These isolated samples have been partially characterized and identified as well, up to now 31 isolated samples have been characterized and they belong to different species: 38.7% to *Klebsiella spp., 22.5%* to *Achromobacter ssp.* 9.67% to *Enterobacter spp.,* 6.45% to *Escherichia spp.,* and 16.1% correspond to other species. Minimal inhibitory concentrations tests showed that 45.16% (n=14) from the isolated samples resisted the presence of mercury at a metal concentrations of 300 uM. 35.5% (n=11) from the total, presented a unique resistance to mercury, 42% (n=13) to mercury-antibiotics and 3.2% (n=1) mercury-arsenate-arsenite. Equally, with the results from the minimal inhibitory concentrations tests, a resistance percentage was determined, 54.8 (n=17) for the arsenite, 51.6 (n=16) for the arsenate and 51.6 (n=16) of co-resistance to the arsenate-arsenite.

The maximum concentration of resistance to the arsenite was 15 mM for the CMI test, a greater variation for the arsenate was determined (200-600 mM). The determination from MicroScan allowed to find out the profile of the resistance to antibiotics and made the identification of a strain that produces BLEES possible. It also was determined that 25.8% (n=8) from the isolated samples presented multi-resistance to antibiotics, and 38.7% (n=12) presented co-resistance to MP-antibiotics. The MP-antibiotic resistance was present and was more linked to the resistance profile to arsenic rather than to the mercury. This differs from the information from Alonso et al., 2001 and Baker et al, 2006 who proposed that the genes resistant to mercury and antibiotics are frequently found in the same moving genetic element.

A greater pattern of resistance to arsenic in the isolated samples of contaminated water is observed, whereas the observed in the isolated samples from the leachate site 2 is greater than mercury, which can be related to a process of selective pressure due to the presence of these metals during long periods of time (Ayres, 1992; Vullo, 2003).

To verify if the plasmids are able to transfer among the bacterial populations some conjugation tests were developed, using the environmental strains listed in Table 2 as donators and the *Escherichia coli* strain J-53 as a receptor. Although all the strains present plasmid DNA, only two were able to conjugate, and these two belong to the group of arsenic-resistant, given that, it wasn't possible to develop conjugation tests with the mercury resistant strains due to the lack of a sensitive strain to be used as a receptor, as J53 showed a resistant behavior.

The PCR tests showed that the total of isolated samples (17) that presented positive resistance profiles to arsenic in 29.4% (n=5) the gene *arsA*was increased (codifies for an ATPasa), in 76.4% the arsB genes were increased (codifies for a arsenite pump expulsion) and arsC (codifies for arsenate reductase enzyme) (n=13).
The presence of gene *merA* wasn't determined in none of the isolated samples, it might be due to the intra and inter-specific variability presented in (NíChadhain et. al., 2006).

Conclusion: The analysis of the Mololoa River regarding the presence of *clinmical*bacteria that harbors dual resistance to HM and antibiotics. The presence of genetic determinats provides and opportunity to study the dynamic of horizontal transfer subjected to different selective pressures.

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STUDY OF A PHARMACEUTICAL SYSTEM CONSISTING IN NANOCARRIERS FOR INTERLEUKIN 2 AS ALTERNATIVE FOR CERVICAL CANCER TREATMENT

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Abstract: The lymphocyte growth factor Interleukin 2 (IL-2) has proven to be effective in the therapy against human tumors by activating the immune system, in particular the cytolytic T-lymphocytes (CTLs) and killer cells. Nevertheless, the systemic delivery of IL-2 has been limited by its high toxicity. In this respect nanocarriers constitute an interesting tool for in situ IL-2 delivery. Our work group has shown that several cervical cancer cells express, like lymphocytes, the IL-2 receptor (IL-2R) on their membranes[1]. Recently we have reported the construction of a nanocarrier structure that expresses non-covalently bound IL-2 on its external surface that renders the particle susceptible to be attached to IL-2R-bearing cells[2]. In fact these nanocarriers did bind to cervical cancer cells that express the IL-2R, making them in turn IL-2 presenters that attracted blood lymphocytes and induced tumor cell lysis[3]. In order to evaluate the stability of our IL-2 nanocarrier particles, their physical integrity in different conditions of temperature, light and pH was determined using a cytometer for particle size and complexity. Our results demonstrated that and that nanocarriers system stability was obtain at 5°C, at a pH of 4.0-4.5 and photostability under white light.

Keywords: Interleukin 2 ● IL-2 receptor ● Cervical cancer ● Nanotechnology

Introduction: According to the World Health Organization (WHO) each year 260,000 women die of cervical cancer worldwide and in México every two hours a woman dies of these pathology[4]. Currently conventional therapy for this disease is chemotherapy and radiotherapy; nevertheless, due to the non-selective nature of this approach, patients have an additional impact on their health with the consequent effect on their family and social relationships. In this regard biotechnology has presented alternatives to improve the drug targeting and reduce adverse effects. The lymphocyte growth factor Interleukin 2 (IL-2) has proven to be effective in the therapy against human tumors by activating the immune system, in particular the cytolytic T-lymphocytes (CTLs) and killer cells. Nevertheless, the systemic delivery of IL-2 has been limited by its high toxicity. In this respect nanoparticles constitute an interesting tool for in situ IL-2 delivery. Our work group has shown that several cervical cancer cells express, like lymphocytes, the IL-2 receptor (IL-2R) on their membranes. Recently we had reported the construction of a nanocarrier structure that expresses non-covalently bound IL-2 on its external surface that renders the particle susceptible to be attached to IL-2R-bearing cells. Our hypothesis for antitumor activity resides in the fact that the IL-2 bearing nanocarrier could promote close proximity of lymphocytes and tumor cells by the fact that both posses the IL-2R. Then if the close proximity of tumor cells with lymphocytes mediated by our nanoparticles could favor the recognition of a putative tumor antigen by the IL-2 activated lymphocytes a cytolytic activity could be induced.

In order to evaluate the stability of our IL-2 nanocarrier particles, their physical integrity in different conditions of temperature, light and pH was determined using a cytometer to evaluate particle size and complexity (roughness).

Materials and methods: For the preparation of nanocarriers[5] with IL-2, a mixture containing the synthetic lipid cholesteryl-spermidine and phosphatidylcholine in 1:1 molar ratio was used, the mixture of lipids (10µmol) was dissolved in chloroform, dried under nitrogen at reduced pressure, and the particles were produced by hydrating the thin lipid film in 330 µL phosphate buffered saline (PBS), with IL-2 by using three 5-sec sonication cycles followed by 30sec resting period in a Lab supply G112SOI sonicator. The nanoparticles formed were finally washed by sedimentation at 200,000 g for 40min, in PBS, and then resuspended to a final concentration of 100 IU/mL. For the stability testing procedures, the nanocarriers suspension were packed in 1.5 mL amber bottles and placed in stoves for thermal stability at 5 °C, 20°C and 37°C for 5 weeks. In addition nanocarriers suspensions were placed by one hand at different pHs: 4.0, 4.5 and 5.5 for 1 hour, on the other, were placed for 45 minutes under white light, samples were taken every 15 minutes. In order to evaluate the stability of our IL-2 nanocarrier particles, their physical integrity was determined using a Facs Aria® cytometer.

Results and discussion: In order to evaluate the effect of pH on the stability, nanocarriers suspensions were placed at pHs 4, 4.5 and 5.5 for one hour, then the suspensions were evaluated using a Facs Aria® cytometer. We obtained that the pH of maximum stability of the nanocarriers corresponded to a value between 4 and 4.5. This result is of particular interest since the physiological pH of the cervix is also acid. In consequence we can expect that our formulation could exert its expected activity when applied topically (Table 1).

Table 1. Percentage of liposomes bearing IL-2 that remain stable (roughness and size) at different pH.

pH	Percentage of stable liposomes	Average Coefficient of variation
4.0	84.1	86.1
	88.1	3.29
4.5	81.9	83.5
	85.1	2.71
5.0	77.0	77.25
	77.5	0.45

All measurements were performed by flow cytometry (FACSAria UB).

When we placed the nanocarriers suspension for 45 minutes under white light, we detected a conformational change of 3% at 20°C compared to the baseline (Table 2). In consequence the advantage of using translucent packaging material could reduce the production costs.

Table 2 Percentage of liposomes bearing IL-2 that remain stable (roughness and size) under white light /20°C.

Time (minutes)	Percentage of stable liposomes	Average Coefficient of variation
0	100,00	--------
15	95,2	96,60
	98,0	2,58
30	96,7	97,45
	98,2	1,34
45	97,7	97,25
	96,8	0,82

All measurements were performed by flow cytometry (FACSAria DB).

The nanocarriers were fairly stable in their physical integrity (complexity or roughness and size) at 5° C even after five weeks (Figure 1). In contrast the nanocarriers suspensions showed a significant change with respect to their size and roughness at 37° C (Figure 2). We think that the change in the physical integrity of our particles can compromise the availability of the drug for therapeutic purposes and thus these results provide confidence in the physiological use of the nanoparticles due to its stability when they are maintained in storage at 5°C.

Figure 1. Histograms overlap for initial condition and six weeks at 5ºC. A Roughness (SSC-A) and B Size (FSC-A).

Figure 2. Histograms overlap for initial condition and six weeks at 37ºC. A Roughness (SSC-A)and B Size (FSC-A).

Finally is important to mention that the final formulation is currently under development by our work group to propose a medicament safe, stable and efficient.

Conclusions: The stability of our nanocarrier system was obtained at 5° C, pH of 4.0 - 4.5 and it is photostable under white light. Thus we think that this system promises to offer a therapeutic alternative for cervical cancer.

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CUCURBITA FICIFOLIA FRUIT AS INSULIN SECRETAGOGUE IN RINM5F CELLS

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Abstract: Taking into account that the diabetes mellitus (DM) is an important public health problem globally, like part of a work to development a product added with an aqueous extract of *Cucurbita ficifolia* fruit with a possible use to the treatment of DM, a aqueous extract of this fruit was obtained and chemically characterized by its content of D-quiroinositol, the principal hypoglycemic compound of the fruit. To study the mechanism of hypoglycemic activity of this extract and of the D-quiroinositol alone, RINmF5 cells were exposed to different concentrations of both, and production insulin and Kir 6.2 channels were measured. Cells treated with D-quiroinositol and *C. ficifolia* increased mRNA expression of insulin and Kir 6.2 compared with control group, suggesting a mechanism of action throughout of increment in the expression of the gen of insulin. This research supports the idea of develop a new nutraceutic product from *C. ficifolia* fruits like a co-adjutant in the treatment of DM.

Keywords: DM ● Insulin ● Kir 6.2 ● *C. ficifolia*

Introduction: The DM is a set of metabolic disorders characterized by increased levels of blood glucose (hyperglycemia) due to defects in insulin production by the pancreas or inadequate use of insulin by the cells, which affects different tissues and organs (ADA, 2004).

One of the key factors in the process of insulin secretion are specific channels of ATP dependent K^+ (K^+_{ATP}) formed by two tetramers of the subfamily Kir6.2 and by a protein called sulfonylurea receptor 1 (SUR1), which together have the ability to induce changes in electrical excitability of the membrane, triggering the mobilization and release of insulin. Sulfonylureas, are antidiabetic agents that act by releasing endogenous insulin by binding to SUR1 specific receptor of the beta cell.

Despite the widespread use of these drugs, more than 70% of the world population uses traditional medicine as the only alternative available to solve their major problems (Farnsworth et al., 1995) and many medicinal plants have been reported with “anti-diabetic” properties. The fruit of *Cucurbita ficifolia* Bouché (Cucurbitaceae) has been extensively studied in Mexico as hypoglycemic agent in experimental animals and diabetic patients, and D-quiroinositol (DQI) has been proposed as responsible of this activity. Therefore, *C. ficifolia* represents a potential source of DQI, which may be used like coadjutants in diabetes mellitus control. (Alarcon et al., 2002; Acosta et al., 2001). The aim of this work was to determine the mechanism of hypoglycemic activity of this extract and of the D-quiroinositol alone in RINmF5 cells exposed to different concentrations of both, measuring insulin and Kir 6.2 channels production.

Material and methods: The fruit of *C. ficifolia* was collected in the municipality of Acolman, State of Mexico and, in order to prepare an aqueous extract, was cut and dehydrated. The dried material was ground and subjected to maceration with water (4 L) for 72 h in a laminar flow hood. The aqueous phase was filtered and centrifuged at 805 xg to obtain a precipitate, which was separated and lyophilized. The standardization of the extract was performed by measuring the content of DQI in *C. ficifolia* extract fruit by HPLC.

Culture of cell line RINm5F.The insulin producer cell line RINm5F from pancreatic islet tumor commercially acquired from American Type Culture Collection (ATCC) was grown in RPMI 1640

(GIBCO), 10% fetal bovine serum (2 mM 1-glutamine, 1 mM sodium pyruvate, 23.8 mM NaHCO 3 and 20 g/L of gentamycin) and kept at 37 ° C with an atmosphere of 5% CO2 and 95% humidity until reaching confluence (approximately 72 h). Cell viability (MTT test) was determined throughout using MTT by the method of Mosmann (1983). For the extraction of total RNA were seeded 1 x 106 cells per treatment in culture dishes of 6 wells to initiate treatments, at a concentration of 0.25 µmolar and 24 hours total RNA was extracted using Trizol adding, using the technique described by Chomczynski (1993).

Reverse transcription coupled to Reaction Chain Reaction (RT-PCR) in real time. The cDNA was synthesized from 2 µg of total RNA by reverse transcriptase PCR using ImProm II. The cDNA was amplified by the enzyme DNA polymerase kit "DNA master plus SYBR Green 1" for the gene: Insulin, Kir 6.2 and β actin as normalizing gene.

Results and Discussion: Figures 2 and 3 show the results obtained with treatment of the aqueous extract of *C. ficifolia* and DQI in RINm5F cells. The increase in gene expression of insulin and 6.2 Kir caused by both treatments was statistically significant compared with the control group.

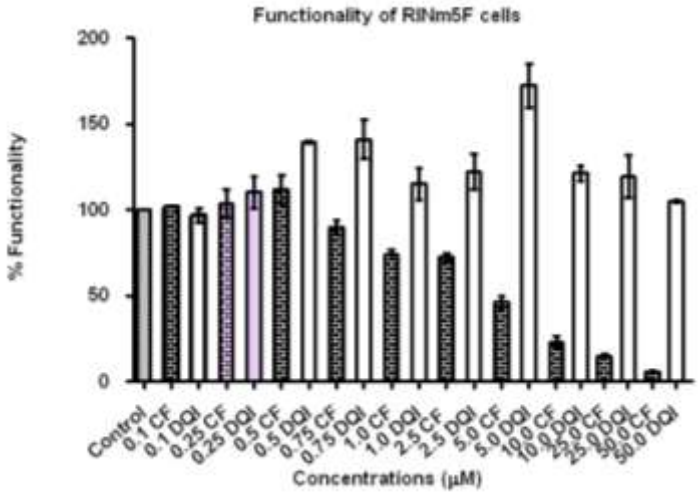

Figure 1.The functionality of mitochondria in RINm5F cells treated with the aqueous extract of C. ficifolia, and DQI.

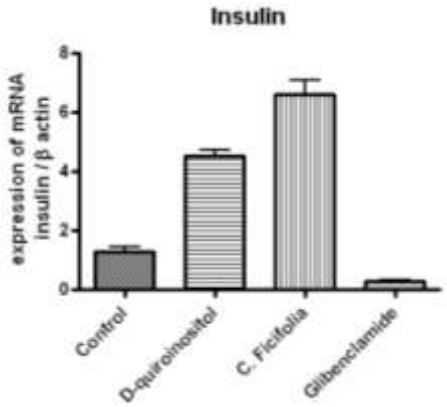

Figure 2. Gene expression of messenger RNA (mRNA) of insulin in RINm5F cells treated with *C. ficifolia* and D-quiroinositol.

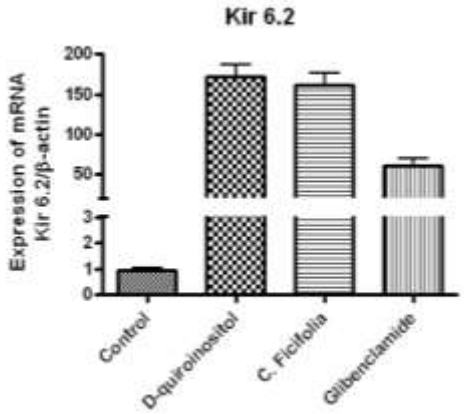

Figure 3. Gene expression of messenger RNA (mRNA) of Kir 6.2 in RINm5F cells treated with *C. ficifolia* and D-quiroinositol.

Cells treated with D-quiroinositol as well as the treatment of *C. ficifolia* extract showed a statistically significant increase in mRNA expression of insulin in comparison with the control group, suggesting that the mechanism of hypoglycemic action of both treatments is throughout an increase in the expression of the insulin gene in these cells. Cells treated with glibenclamide did not show significant differences (Fig. 2). Interesting, glibenclamide, a potent hypoglycemic, not acting on insulin mRNA level, but at the level of K^{+}_{ATP}channel.

This plant is a useful alternative for DM control and it is considered a potential source of raw material for obtaining new oral hypoglycemic drugs and this research supports the idea of develop a new nutraceutic product from *C. ficifolia* fruits like a co-adjutant in the treatment of DM.

Conclusion: The aqueous extract of *C. ficifolia* and D-quiroinositol increased mRNA expression of insulin and Kir 6.2 in comparison with the control group in the cell line RINm5F, suggesting a mechanism of action throughout increased expression of the insulin gene.

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GERMINATION OF SOYBEANS AND ITS EFFECT ON CHRONIC DISEASES

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Abstract: Soybean is an abundant source of proteins with high nutritional value and excellent physicochemical properties in foods, and also it is a rich source of non-nutritive components with potential health benefits. Soy consumption has beneficial effects in the treatment of obesity due to its proven ability to decrease several markers, such as the amount of lipids stored inside adipocytes. On the other hand, extensive epidemiological, *in vitro*, and animal data suggest that soybean consumption reduces the risk of developing several types of cancer. However, little is known about the effect of germination on the antiobesity and anticancer properties. The aim of this work is to study the influence of soybean germination time (0, 2 days) on some obesity markers and on tumor growth (0, 2, 6 days), both studies in mice. With the results obtained in the present study, it is possible to say that germination may help to increase antiobesity and anticancer activities. These effects can be correlated with the change in the protein pattern, and the increase of phytochemicals along the germination process.

Keywords: Soybean germination ● antiobesity properties ● anticancer properties

Introduction: Nutritional guidelines around the world recommend increased consumption of plant foods to combat life threatening ailments. Use of functional foods is an emerging trend in a diet based regimen against various lifestyle related disorders as obesity, diabetes mellitus, hypercholesterolemia, cardiac dysfunction, cancer, etc. (Naz *et al*., 2011). Obesity is a disease caused by an excess of fat tissue, due to the imbalance between consumption and energy expenditure and it is associated with diabetes, mellitus type II, metabolic syndrome, cardiovascular disease, among others (Gonzalez Espinosa de los Monteros *et al*., 2011). On the other hand, cancer is one of the chronic diseases with highest incidence in the world being the second leading cause of death worldwide and the third cause of mortality in Mexico (INEGI, 2010). González-Espinosa *et al*. (2011) reported that soybean consumption has beneficial effect on markers of obesity. Extensive epidemiological, *in vitro*, and animal data suggest that soybean consumption reduces the risk of developing several types of cancer (Barac *et al*., 2005). However, there is little research regarding the effect of germination on the chronic diseases as obesity and cancer. The objective of this work is to show the results obtained when testing soybean germinated for 0 to 6 days with and without ethanol soluble phytochemicals (ESPC) in reducing body weight and adipose tissue in obese mice C57/BL and on the tumor growth in nude mice (nu/nu) inoculated with cervical cancer cells.

Material and Methods: Seeds: Glycine max var. Crystalline was supplied by the National Research Institute for Forestry Agriculture and Livestock (INIFAP, Iguala, México). Seeds were germinated at 27°C in the dark and harvested at different intervals (0, 2 and 6 days). The sprouts were dried and ground. The effect of soy germination on obesity was tested using soy germinated with and without ESPC (2 days of germination). Protein isolates from soy germinated for 0, 2 and 6 days were used to study the anticancer effect.

Soybean protein isolation and extraction of ethanol-soluble phytochemicals (ESPC). The soy flour was defatted with hexane and was suspended in distilled water (1:15), pH was adjusted to 9.0 with 1N NaOH and the extraction was carried out under constant agitation for 45 min at room temperature. This suspension was centrifuged at 10,000 rpm for 30 min at 15°C. The pH of supernatant was adjusted to 4.5 with 1N HCl at 20 °C, and the precipitate collected by centrifugation at 10,000 rpm for 30 min at 4 °C. Isolates were lyophilized and stored at 4°C. Soy protein isolates can retain notable amounts of isoflavones associated with the proteins. These isoflavones have anti-obesity and antitumoral effects (González-Espinosa de los Monteros, et al.,2011; Robles-Ramírez, et al., 2011), that is why the isoflavones and other phenolic and saponin compounds were removed from the protein isolates according to Carrao-Panizzi et al. (2002). Phytochemical extraction efficiency was quantified by measuring total phenolic compounds with the Folin technique.

Animal studies. In order to carry out the study of antiobesity effect of germinated soy. Firstly, obesity was induced in C57/BL mice, by a high fat diet for 9 weeks. At the end of this time, the diet was replaced by isocaloric diets, containing germinated and ungerminated soy with and without ESPC. The mice were distributed in groups of 8. Each mouse was kept in individual cage. The diet and water was given *at libitum*. The mice were fed for 4 weeks. The weight of each mouse was recorded at the beginning of the experiment and then twice a week. The mice were killed and the subcutaneous, abdominal and epididymal adipose tissues were obtained.

To determine the effect of the protein isolates of germinated soy on the tumor growth of mice, twenty four female nu/nu mice were purchased. After one week of adaptation, mice were inoculated subcutaneously on the right flank with a suspension of 4×10^6 HeLa cells in 0.1 mL of saline solution and were then randomly assigned to one of the six dietary groups (n=4) and fed *ad libitum* with one of the experimental diets for 5 weeks. When tumors reached palpable volumes of 3-4 mm, two perpendicular diameters of the tumors were measured daily with a caliper and volumes were calculated according with the formula $0.5 \times L \times W^2$, were L and W were the largest and the smallest diameters respectively.

Results and discussion: In Table 1, it can see the results obtained from the effect of germinated soybean consumption in the weight of obese mice. It was found that mice body weight decreased by 10.22 and 12.64 in mice fed with soybean germinated for 2 days without and with ESPC, respectively; while those fed with ungerminated soybean did not lose weight, although did not continue to gain weight as those fed with the control diet (diet with casein). The increase in body weight was positively associated with weight gain of subcutaneous, abdominal and epididymal adipose tissues.

Table 2, shows the results obtained from the experiment where the effect of germinated soy proteins was tested in athymic mice. Relative tumor volumes from mice treated with diets containing 20% of soy protein isolates (SPI) from ungerminated soy, SPI from soy germinated for 2 days without ESPC, SPI from soy germinated for 2 days without ESPC, SPI from soy germinated for 6 days with ESPC and SPI from soy germinated for 6 days without ESPC, were reduced by 31.4% ($p<0.05$), 90.4% ($p>0.01$), 82.5% ($p<0.001$), 88.4% ($p<0.01$), respectively, at day 21, compared with those of casein-fed control mice (Table 2). Groups fed with germinated soy protein were significantly different ($p<0.05$) to the group fed with ungerminated soy protein.

Table 1. Effect of germinated soybean consumption in the weight of C57/BL obese mice.

Diet	Initial corporal weight (g)	Final corporal weight (g)	Difference of weight
Control	30.33±1.34[a]	31.21±2.76[a]	+ 0.88
High Fat (HF)	44.07±2.78[b]	49.66±0.52[b]	+ 5.59
HF + ungerminated soybean	44.43±2.07[b]	45.90±1.83[b]	+ 1.47
HF + soybean germinated for 2 days without ESPC	43.53±2.89[b]	33.31±2.35[a]	- 10.22
HF + soybean germinated for 2 days with ESPC	45.13±1.77[b]	32.49±2.94[a]	- 12.64

Similar letters in the same column means no statistical difference ($p<0.05$) according to Tuckey test, (n=4).

The differences among the germinated soy protein groups were not significant. Histological examination of the tumor tissues showed that consumption of soy products increases both apoptotic and necrotic death (Table 2). From day 21 of treatment the majority of mice of groups SPI 2+, SPI2- and SPI6+, began to suffer the degradation of tumors and showed only a thin rim of viable tumor tissue at the end of experiment.

Table 2. Effects of germinated soybean protein consumption on the tumor growth in female nu/nu mice.

Diet	RTV at day 21*	Tumor growth inhibition (%)	Apoptosis (%)	Necrosis (%)
Casein	48.7±5.6[d]	--	2.3±0.3[a]	21.7±6.3[a]
SPI0	27.0±3.6[c]	31.3±17.6[a]	23.8±1.8[a]	50.0±5.0[c]
SPI2+	0.6±0.2[a]	90.4±0.04[b]	36.5±3.0[a]	33.3±2.9[b]
SPI2-	1.1±0.1[a]	82.5±10.0[b]	46.5±1.8[a]	52.5±4.8[c]
SPI6+	2.6±1.5[ab]	88.4±3.3.6[b]	48.8±3.0[d]	45.0±5.0[bc]
SPI6-	3.6±0.1[b]	88.1±4.5[b]	40.7±2.2[a]	40.0±4.1[b]

RTV= relative tumor volume. *Values obtained before the degradation of tumors. SPI0= Soy protein isolates from ungerminated soybean, SPI2+= Soy protein isolates from soy germinated for 2 days with ESPC. SPI2- = Soy protein isolates from soy germinated for 2 days without ESPC. SPI6+= Soy protein isolates from soy germinated for 6 days with ESPC. SPI6- = Soy protein isolates from soy germinated for 6 days without ESPC.

Conclusion: This research demonstrates that the germination process could provoke important changes in some components as ESPC and proteins, affecting specific mechanisms related with energy expenditure and then contributing to the reduction of adipose tissue in obese mice. On the other hand, a biological process as simple as germination may help to increase the

anticancer activity of soy and, in this particular case, this effect can be correlated with changes in the protein pattern.

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EFFECT OF TEMPERATURE AND PROTEASE INHIBITORS ON THE PROTEASES OF SEA CUCUMBER (*ISOSTICHOPUS FUSCUS*)

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Abstract: There is limited use of marine proteases in the industry for the relative paucity of information, among other reasons. Therefore, through this study provided basic information on the proteases of the digestive tract of brown sea cucumber *Isostichopus fuscus*. Proteolytic enzymes had the significantly higher specific activity at pH 6 and 10 ° C and high activity at 37, 50 and 60 ° C. There was great resistance to temperature denaturation by incubating the protease to 40 and 60 °C. The enzymatic inhibition study showed that 2-β-mercaptoethanol causes the greatest percentage of inhibition (33.01%), followed by trypsin inhibitor (30.18%) suggesting the presence of cysteine proteases and serine proteases. These enzymes were characterized by high thermostability present what may be beneficial in various biotechnological applications.

Keywords: Sea cucumber *Isostichupus fuscus* ● Proteases ● Characterization ● Tract digestive

Introduction: There have been studies to purify and characterize digestive enzymes of aquatic organisms as they have adapted to different environmental conditions developing enzymes with unique properties compared with those of animals, plants or microorganisms. Some of these differences are a function of pH, temperature, pressure, salinity, light intensity and aeration (Haard and Simpson, 2000). However, there is limited use of marine proteases in the industry for the relative paucity of information, among other reasons. The objective of this study was to provide information on the effect of temperature and different specific inhibitors of proteases on proteolytic activity of the digestive tract of brown sea cucumber *Isostichopus fuscus* (Ludwig, 1875) from Gulf of California, Mexico. These organisms are distributed from Baja California to Ecuador. Sea cucumbers belong to the Phylum Echinodermata and Class Holothuroidea, in this phylum are also urchins, lilies, spider and starfish. Play an important ecological role in benthic feeding on suspended organic material (Bakus, 1973). *I. fuscus* is demanded in the Asian market (highlighting China) for consumption by its size, smooth texture of your skin (Caso, 1961) and protein content (50% dry weight). Furthermore, it goes to the food, food supplement since the wall of the body consists of insoluble collagen in addition to high levels of lysine, arginine and tryptophan (DeMoor et al., 2003; Taylor and White, 1997), and the pharmaceutical industry (FAO, 2004) for having several bioactive compounds as chondroitin sulfate (sulfated polysaccharide) used in the treatment of arthritis and other diseases of the joints, also used in medicine for its potent anticoagulant and antithrombotic action (Mourão et al, 1996; Volpi, 2006). Another important bioactive compound are the triterpenoids, which have hemolytic and cytotoxic activity (Popov, 2002). This resource represents a significant potential as income, if exploited rationally. However, Mexico is extracted and exported illegally since before granted the first commercial fishing permits in 1987. Currently, the measure of protection you have is the NOM-059-SEMARNAT-2001, which determines the species in the protected category. In the same context, some of the distinguishing characteristics of marine proteases are a greater catalytic efficiency at low reaction temperatures, the catalytic activity / stability at neutral or alkaline pH

(Hultin, 1980; Simpson and Haard, 1987; Asgiersson and Bjarnasson, 1991), and the response to specific inhibitors. There are no studies to date on proteases *I. fuscus*, most reports have used *Stichopus japonicus*, known as Japanese sea cucumber, distributed in the seas of Japan and northeastern China. Finally, more knowledge of the marine proteases may have a greater biotechnological use and efficient. For example, take advantage of its high catalytic activity in low temperature reactions in food processing, reducing energy costs and destruction of labile components essential (Simpson and Haard, 1987).

Materials and Methods: Enzyme extract was obtained from the digestive tract of 6 sea cucumbers (*I. fuscus*) adults of the Sea of Cortez by homogenization with 20 mM phosphate buffer pH 7 and centrifugation at 12 000 g at 4 °C for 30 minutes. We measured the protein concentration by the Lowry method (1951). Proteolytic activity was determined from 10 to 70 °C; casein was used (pH 6) as substrate and a reaction time of 10 minutes, the reaction was stopped with 25 % trichloroacetic acid, then centrifuged at 10 000 g was recorded absorbance at a wavelength of 280 nm. The results are reported as specific activity, proteolytic activity units per milligram of protein (UAP / mg). We performed a study of enzyme thermostability incubating the extract at 40, 50 and 60 ° C for 1 hour and recording the residual activity of 10 to 60 minutes, with the conditions mentioned above plus the calculated half-life. Finally we measured the effect of different specific inhibitors of proteases on the proteolytic activity. Was used 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM tosyl-L-lysine chloromethyl ketone (TLCK), 0.6 mg / mL trypsin inhibitor (TI), 1 mM 2-β-mercaptoethanol, and 50 µg/mL Pepstatin A. The extract was incubated with the inhibitor corresponding proteolytic relative 1:1 (v/v) for one hour at room temperature (25 °C) and specific activity was determined as mentioned above. Inhibition is reported as percentage of enzyme inhibition.

Results and Discussion: Statistical analysis (SPSS 15.0) showed that the digestive tract at pH 6 and 10 °C has the highest specific activity ($p<0.0001$) and high activity at 37, 50 and 60 °C (Figure 1). Fu et al., 2005, reported for the enzyme extract of the digestive tract of *Stichopus japonicus*, that the optimum temperature for proteases with optimum pH 13.5 at 37 °C, others as H. Qui et al., 2007 and Zhu et al., 2008, report optimal activity at 50 °C for cysteine proteases of the body wall of *S. japonicus*. Therefore it can be seen that the behavior of proteolytic enzymes is similar, in addition to which stress the uniqueness of proteases characterized in this study to have greater activity at low temperature.

As for the study of thermostability, there was a great resistance to denaturation temperature to 40 and 60 °C incubation (Figure 2). In addition, we observed increased proteolytic activity, possibly due to a restructuring of the enzyme for activation. Residual activity after incubating the extract obtained at 40 °C was 115 % up to 40 minutes and the half life time was 5.25 hours. Regarding the incubation at 60 °C, retained approximately 85.64 % of its original activity, with 3.98 hours half-life. In contrast, incubating at 50 °C, residual activity was 30.61 % at 40 minutes of reaction, with a half-life of 21.73 minutes. The results obtained differ with that reported by Fu et al., who mention that proteases with optimum activity at pH 5 were shown to be more temperature sensitive, since it was markedly reduced enzymatic activity when incubated at 40 °C with only 17.09 ± 1.16 % residual activity and showed no activity at 50 and 60 °C. However, it is similar to that reported by these authors for proteases with optimum at pH 2 and 13.5, also as obtained by Qui et al., And Zhu et al., who report for cysteine proteases of the body wall *S. japonicus* stability at 40 - 60 °C. Consequently, it is suggested that proteases with optimum at pH 6 of digestive tract *I. fuscus* have a behavior similar to that of proteolytic enzymes of *S. japonicus*.

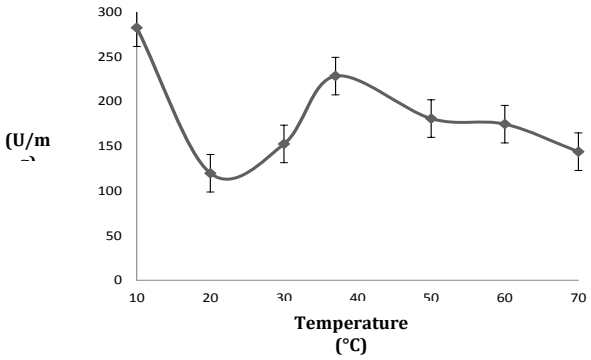

Figure 1. Effect of temperature on the proteolytic activity.

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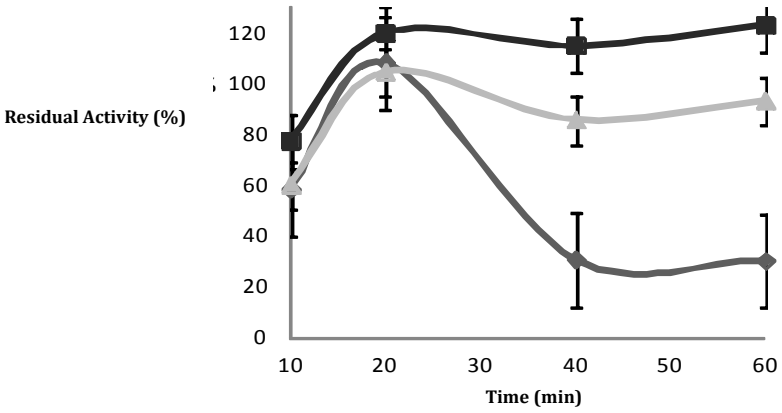

Figure 2. Enzyme thermostability. 1 hour incubation at 40 °C (■), 50 °C (◆) y 60 °C (▲).

In the study of enzyme inhibition (Figure 3) was determined that the inhibitor of cysteine proteases, mercaptoethanol, causes an inhibition percentage of 33.01 %. Trypsin inhibitor inhibit the enzymatic reaction in a 30.18 % followed by 25.92 % EDTA (metalloprotease inhibitor), 20.92 % PMSF (inhibitor of serine proteases) and 15.46 % TLCK a specific trypsin inhibitor which binds to the active site histidine residue. Lastly, Pepstatin A, an inhibitor of aspartyl proteases, had an insignificant value of percentage inhibition of 2.63 %. Fu et al., report that there was no inhibition with TI contrasting results, but reported similar inhibition with EDTA (33.82 ± 2.57 %) and PMSF (14.78 ± 1.72 %). Furthermore, these authors mention at pH 7 - 10, significant reduction in activity with PMSF and EDTA and IT suggesting the presence of a serine protease, probably a collagenase (Yoshinaka et al., 1986; Roy et al., 1996) with activity similar to the activity of trypsins and chymotrypsins, consistent with previous reports on proteases of the digestive tract of marine organisms with maximum activity at pH 6.5 to 8.0 and poor thermal stability (Kristjansson and Nielson, 1992.). The results suggest the presence of cysteine proteases and serine proteases.

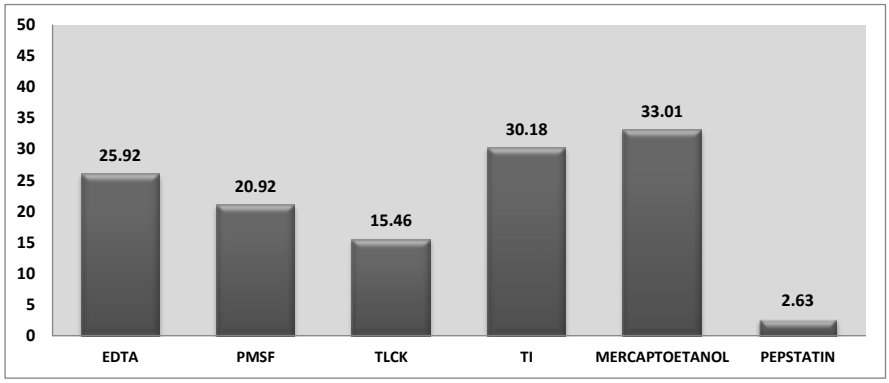

Figure 3. Percentage of enzyme inhibition.

Conclusions: Proteolytic enzymes of the digestive tract of *Isostichopus fuscus* had the highest specific activity at pH 6 and 10 °C, a high activity at 37, 50 and 60 ° C. Furthermore, they exhibited excellent resistance to denaturation temperature to be incubated at 40 and 60 °C for one hour. The study of enzyme inhibition suggests the presence of cysteine proteases and serine proteases, but the results of the effect of temperature on the proteolytic activity showed a similar behavior to that of cysteine proteases. Finally, based on the results and the literature suggests that these enzymes with high thermostability, possibly cysteine proteases, could be used in subsequent biotechnological applications for their particular characteristics.

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PHYSIC-CHEMICAL CHARACTERIZATION OF DRINKING WATER FROM NORTHERN MEXICO AND BIOTECHNOLOGICAL POSSIBILITIES TO IMPROVE ITS QUALITY

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Abstract: In this study, a total of 10 wells in northern Mexico were analyzed for a total of 36 water quality constituents to determine their suitability for human consumption. Parameters examined include pH, electrical conductivity, ammonia, nitrate, nitrite, sulfate, sulfite, orthophosphate, chloride, fluoride, bicarbonate, and others major/trace elements analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). The presence of these constituents in this type of water was investigated considering recent concerns about their quality and the lack of trace element information. The analytical results obtained in this study were compared to the drinking water quality guideline of 'Norma Oficial Mexicana NOM-127-SSA1-1994'. Results of this study have shown that at least one well is above the drinking water quality guideline of NOM-127-SSA1-1994 in chloride, fecal coliforms, total coliforms, total hardness, fluoride, N-nitrate, N-nitrite, sodium, total dissolved solids and/or sulfate, which some of them may have detrimental effects on human health. Even though there are some biotechnological advances to mitigate this problems, additional research is necessary.

Keywords: Heavy metals ● Groundwater ● Water quality ●Drinking water pollution.

Introduction: Drinking water quality is a global concern linked with human and environment health. It is well known that groundwater supplies most drinking water throughout the world, which the global population is 7 billions of people (UNFPA, 2011), and whereas about 1.1 billion of them worldwide lack access to improved drinking water supplies and use unsafe surface and groundwater sources.

Soils represents a major sink for HM ions, which can then enter the food chain via water, plants or leaching into groundwater (Nouri et al., 2008). HM toxicity can result in brain damage or the reduction of mental processes and central nervous function, lower energy levels, damage to DNA, alterations on the gene expression, skin, muscle, blood composition, lungs, kidneys, liver, heart, and other vital organs for humans and other living organisms. Long-term exposure to HM may result in slowly progressing physical, muscular and neurological degenerative processes that mimic Alzheimer's disease, Parkinson's disease, muscular dystrophy, multiple sclerosis), gangrene, diabetes mellitus, hypertension, and ischemic heart disease (Otles and Cagindi, 2010). Allergies are commons and repeated long-term contact with some HM or their compounds may even cause cancer.

For some HM, toxic levels can be just above the background concentrations naturally found in nature. However, HM have been excessively released into the environment due to rapid industrialization, manufacture of fertilizers and to the high production of industrial waste (Katsou et al., 2011) originated from metal plating, mining activities, smelting, battery manufacture, tanneries, petroleum refining, paint manufacture, pesticides, pigment manufacture, printing or photographic industries. This has created a major global concern (Ngah and Hanafiah, 2008),

because they are non-biodegradable and can be accumulated in living tissues, causing various diseases and disorders within the food chain.

Monitoring all drinking water sources for pollutants should be considered throughout the world, but good test methods must to be established, whereby measurement quality should include both sampling and analysis. The needed measurement quality can be achieved by validation that the test method is fit for the intended purpose and by establishing traceability of the results to stated references and an estimate of the uncertainly of measurement. However, to reach the requirements described above, technical knowledge, infrastructure, and analytical technologies are needed, which are not easy to get in low economic development areas or countries. World is currently facing critical water supply and drinking water quality problems, whereby drinking water quality policies, technologies, drinking water management strategies and human resources to satisfy water-quality standards are necessaries in many countries and cities throughout the world. Additional work to understand how to combine interventions and transition to greater level of service as incomes rise remain an important area of police-relevant work between governments, healthcare services, industries and drinking water-wells owners.The objective of this research is characterize the drinking water of ten wells from northern Mexico and identifies biotechnological possibilities to improve the drinking water quality.

Materials and Methods: The study was conducted at localities in the municipality of Saltillo, Arteaga, and Ramos Arizpe, Coahuila, Mexico (Figure 1). This area is located in the southeastern state of Coahuila, centered at 25°31' N, 101°37 ‘ W, 1600 m above sea level (MASL). Drinking water samples were collected during December 2011 from ten wells according to Schulze et al. (2011). The analytical methods to each characteristic are indicated in the Table 1.

Results and discussion: Results of this study have shown that at least one well is above the drinking water quality guideline of NOM-127-SSA1-1994 in chloride, fecal coliforms, total coliforms, total hardness, fluoride, N-nitrate, N-nitrite, sodium, total dissolved solids and/or sulfate, which some of them may have detrimental effects on humans health. It is well known that there are two main health issues about nitrate: the linkage between nitrate and (i) infant methaemoglobinaemia, also known as blue baby syndrome, and (ii) cancers of the digestive tract. However, the evidence for nitrate as a cause of these serious diseases remains controversial (Powlson et al., 2008), On one hand there is evidence that shows there is no clear association between nitrate in drinking water and the two main health issues with which it has been linked, and there is even evidence emerging of a possible benefit of nitrate in cardiovascular health (Machha and Schechter, 2011). There is substantial disagreement among scientists over the interpretation of evidence on the issue i.e. ninatre and nitrite concentration in drinking water. Given the lack of consensus, there is an urgent need for a independent study to determine whether the current nitrate limit for drinking water is scientifically justified or whether it could safely be raised. Meanwhile, it is necessary to developa technique to reduce the concentration of nitrates and nitrites in drinking water. Saeedi et al. (2012) stated that simultaneous removal of nitrate and natural organic matter (NOM) from drinking water was reached using a hybrid heterotrophic/autotrophic/BAC bioreactor (HHABB) which consisted of three compartments: ethanol heterotrophic part, sulfur autotrophic part, and biological activated carbon (BAC)-part (including anoxic and aerobic sections). Their study indicated that the HHABB without the anoxic BAC-phase could be a feasible alternative for simultaneous removal of NO_3^- and NOM from drinking water at full scale. However, sulfate production is the main disadvantage of the sulfur autotrophic denitrification process (Sahinkaya et al., 2011).

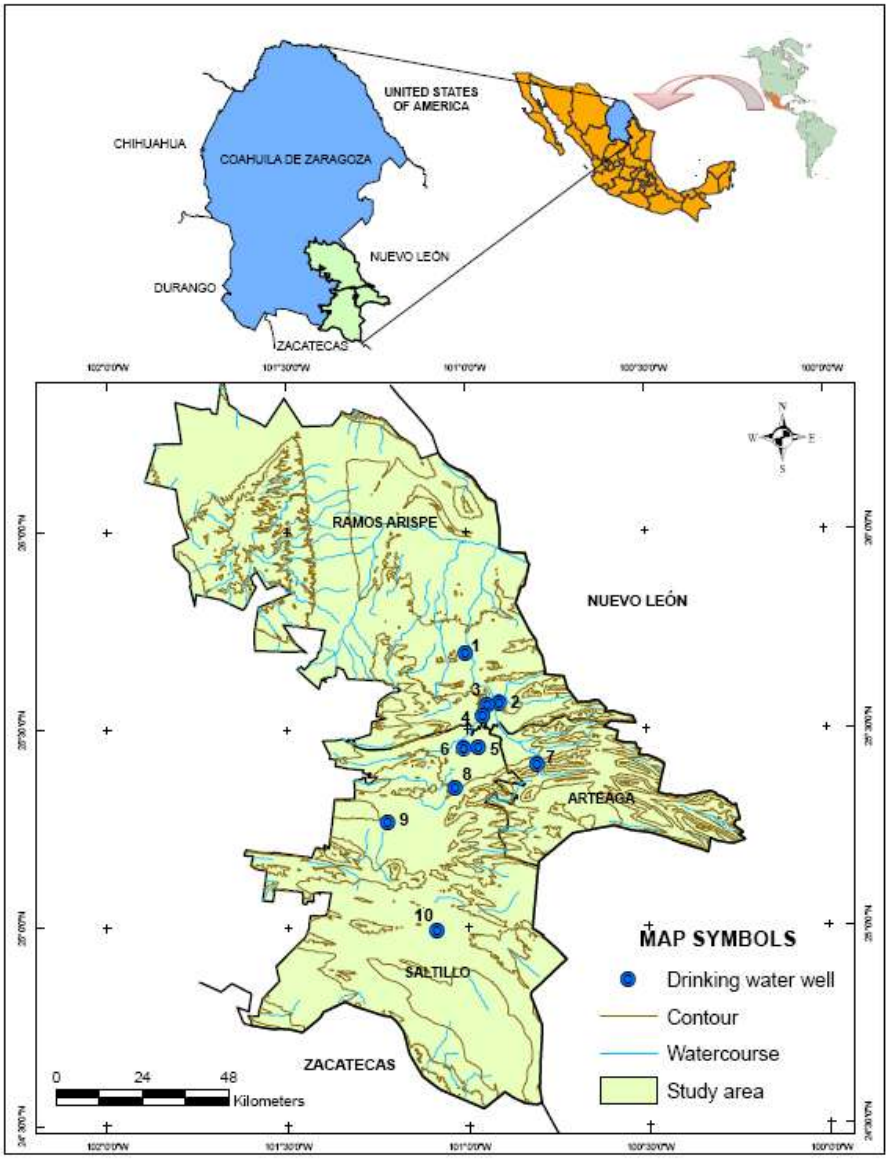

Figure 1. Geospatial localization of ten drinking water-wells from northern Mexico.

Table 1.Physic-chemical characteristics of ten drinking water-wells from northern Mexico.

Characteristics	Units	Analytical method	Number of well according to Figure 1									
			1	2	3	4	5	6	7	8	9	10
Temperature	◦C		21.5	40.7	22.7	40.7	19.6	21.8	18.1	21.4	21.5	22.4
pH	pH Units	NMX-AA-008-SCFI-2000	7.49	7.27	7.7	7.27	7.17	7.15	7.7	7.4	7.65	7.54
2,4 D (herbicide)	µg L^{-1}	EPA-8321B-2007	<25	<25	<25	<25	< 25	<25	<25	<25	<25	<25
Aldrin	µg L^{-1}	EPA-8081 B-2007	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Aluminium	mg L^{-1}	EPA-200.7-1994	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.08	<0.05	<0.05
Arsenic	mg L^{-1}	EPA-200.7-1994	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Barium	mg L^{-1}	EPA-200.7-1994	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.13	<0.05	<0.05
Benzene	µg L^{-1}	EPA-8260 B-1996	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Cadmium	mg L^{-1}	EPA-200.7-1994	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Cyanide	mg L^{-1}	NMX-AA-058-SCFI-2001	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025
Chlordane (Total isomers)	µg L^{-1}	EPA-8081 B-2007	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
Residual chlorine	mg L^{-1}	NMX-AA-108-SCFI-2001	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Chloride	mg L^{-1}	EPA-300.0-1993	57	31.2	345*	237.5	117	193	40	69.8	79	210
Copper	mg L^{-1}	EPA-200.7-1994	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Fecal coliforms	CFU¥ 100 mL^{-1}	NMX-AA-102-SCFI-2006	21	0	0	0	0	0	0	0	0	0
Total coliforms	CFU 100 mL^{-1}	NMX-AA-102-SCFI-2006	>100	0	0	0	0	0	0	0	0	0
Color	Scale Pt	NMX-AA-045-SCFI-2001	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Total chromium	mg L^{-1}	EPA-200.7-1994	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
DDT (Total isomers)	µg L^{-1}	EPA-8081 B-2007	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Dieldrin	µg L^{-1}	EPA-8081 B-2007	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Total hardness	mg L^{-1} $CaCO_3$	SM 2340B-2005	409.7	603.6	1291.8	793.1	728.2	807.7	344.3	420.6	728.1	773
Ethylbenzene	µg L^{-1}	EPA-8260 B-1996	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Iron	mg L^{-1}	EPA-200.7-1994	<0.05	0.06	0.07	<0.05	<0.05	<0.05	<0.05	0.07	<0.05	<0.28
Fluoride	mg L^{-1}	NMX-AA-077-SCFI-2001	<0.5	2.15	<0.05	<0.5	<0.5	0.61	<0.5	<0.5	0.53	2.44
Heptachlor	µg L^{-1}	EPA-8081 B-2007	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Heptachlorepoxide	µg L^{-1}	EPA-8081 B-2007	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
Hexachlorobenzene	µg L^{-1}	EPA-8081 B-2007	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lindane	µg L^{-1}	EPA-8081 B-2007	<0.05	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06	<0.06
Manganese	mg L^{-1}	EPA-200.7-1994	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Mercury	mg L^{-1}	NMX-AA-051-SCFI-2001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Methoxychlor	µg L^{-1}	EPA-8081 B-2007	<1.00	<1.00	<1.00	<1.00	<1.00	<1	<1	<1	<1.00	<1.0
N-nitrate	mg L^{-1}	EPA-300.0-1993	12.6	<1.5	23.6	39.4	11.7	26.1	<1.5	14.7	25	<1.5
N-Nitrite	mg L^{-1}	NMX-AA-099-SCFI-2006	<0.015	0.03	0.063	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015
Ammoniacalnitrogen	mg L^{-1}	NMX-AA-026-SCFI-2010	0.3	0.3	0.3	<0.1	<0.1	0.4	0.3	<0.1	0.4	0.3
Lead	mg L^{-1}	EPA-200.7-1994	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Sodium	mg L^{-1}	EPA-200.7-1994	61.4	28.2	210	118.9	94	155	16.6	47.8	58	200.8
Total disolved solids	mg L^{-1}	NMX-AA-034-SCFI-2001	748	1016	2498	1498	1198	1560	526	616	1280	1598
Sulfate	mg L^{-1}	EPA-300.0-1993	448	NDπ	1695	760	612.5	890	333	169.9	890	1210
Active substancesmethyleneblue	mg L^{-1} LAS£	NMX-AA-039-SCFI-2001	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
Toluene	µg L^{-1}	EPA-8260 B-1996	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Total trihalomethanes	mg L^{-1}	EPA-8260 B-1996	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Turbidity	NTU∞	NMX-AA-038-SCFI-2001	<1	<1	1.2	<1	<1	<1	<1	1.8	1.1	<1
Xylenes	µg L^{-1}	EPA-8260 B-1996	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
Zinc	mg L^{-1}	EPA-200.7-1994	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10

* Numbers typed in color red are above the drinking water quality guideline of 'Norma Oficial Mexicana NOM-127-SSA1-1994'
¥ Colony-forming units; £ Lauryl sulphate; ∞ nephelometric turbidity unit.Π Notdetected.

Additionally, Sun et al. (2011) stated that nanomaterials in combination with bacteria (*Clostridium* sp.) have potential for novel and effective treatment technologies for arsenic-contaminated ground water. According with the above statements, there are biotechnological possibilities which could decreases the concentrations of pollutants to improve the drinking water quality.

Conclusions: Ninety per cent of the wells studied had at least one chemical or element concentration above the drinking water quality guideline of NOM-127-SSA1-1994, which some of them may have detrimental effects on human health, but there are biotechnological possibilities which could decreases the concentrations of pollutants to improve the drinking water quality, however, additional research is necessary.

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MARINE BIOTECHNOLOGY. OPPORTUNITIES AND CHALLENGES FOR MEXICO´S XXI CENTURY

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Abstract: Continental México is ca 2 x 10^6square kilometers, while Marine México is ca 3 x 10^6sq km. Meaning that Marine México is two thirds of the total Country. Besides the size, Marine México is formed by a variety of disparate ecological niches. From estuarine waters, almost sweet water, to salt marshes with 6M NaCl; From two atmospheres to several hundreds; From subtropical or deep waters, to several hundreds °C in black hydrothermal vents at 3000 m deep in the Guaymas basin. All this causing specialized adaptations in organisms thriving in such environments. Leading to diverse metabolisms to be explored for biochemical mechanisms of production and transformation to become biotechnologies. Here we will define Biotechnology and how business based on biotechnologies is assessed and why basic research is needed to succeed and how success must be aimed; intellectual leadership. Also Biotechnology will be analyzed as a multidisciplinary science, what are the objectives of the science along with the tools of Biotechnology. Biotechnology is technology based on organisms, so organisms are quintessential in this paper. Benefits of exploring marine organism are plentiful. Like generating and attracting novel, clean, leading-edge technology industries to the Country; Jobs for the wellbeing of Mexicans. Increasing opportunities to keep Mexican- and abroad-trained professionals of science at the region. Sustain and restore both land and marine habitats. Potential products are: food for humans and feed for land and aquatic animals; Food and feed ingredients and supplements; pharmaceuticals, cosmetics, fertilizers and all sort of biological reagents, from enzymes to pigments. Of course there are potential benefits, as well as challenges. As long as we can keep a balance between them, México can achieve a better future based on science and biotechnologies.

Introduction: As we all know, the world's oceans comprise the biggest part of the biosphere and contain the most ancient and diverse forms of life. Only with the tools of modern science can these biological resources be studied in detail and thereafter be applied for human benefit and fundamental scientific progress. Reports have shown that the sea's resources remain largely unexplored and marine organisms represent a vast untapped resource with potential benefits in many different areas of life, including medicine, aquafarming and fisheries, industry, research tools and environmental applications; including new techniques to restore, protect and manage marine ecosystems. To bring together scientists, managers, faculties, decision makers among all organizations engaged in marine biotechnology and encourage the development of commercial marine biotechnology.

Marine biotechnology is a rapidly growing sector that encompasses a wide range of disciplines to develop novel or alternative products or services from the marine environment. Potential applications include new medicines, human health products and chemicals for use in the manufacturing and food industries.

A definition.

There are plenty definitions for Biotechnology, Here we have a functional one: “Biotechnology is the use of biochemical processing methods in productive activities” It is also called “Biologicalengineering” To fulfill its goal, Biotechnology is science as much as it is technology. The science part is aimed at understanding nature, while the technology part is aimed at

providing useful control of nature. Marine Biotechnology is the use of biochemical processing methods of marine organisms in productive activities. Marine Biotechnology uses molecular based biological processes; make a good use of the cellular machinery.

Biotechnology is a multidisciplinary science. Its "Formal object" is the study of biological processes, while the "Material object" is the use of biological processes in technologies. In this order of ideas, Biotechnology is achieved in successive steps. The most fundamental is to study the basic phenomena in living things to know what processes or products happen at organismal, tissular or cellular levels, this is called "The biological feasibility". When a process has been found and understood at certain grade, this is to know the most factors affecting the process possible in order to aspire to control the process in vitro we call the step of study "The Technical feasibility" in this step, scaling up happens and evaluation of inputs and output in vitro are done. When the process is reproduced systematically and the outputs are well known, the process can come to a commercial scale and the process or product is ready for commercialization, this "The Economic feasibility" of the process.

Because living systems cannot be exactly modeled or predicted, genuinely new products must be created, at least in part, by experimental research, that must be based on knowledge, creativity, and rigorous and systematic investigation, the hallmarks of good science in any context. This is why only the subtle knowledge of thephenomena processing processes will allow sustainable applications.

It is known that some, for not to say most biotechnologies high-profile failures are recognized as being due to companies pushing poor science in order to achieve funding goals.

Because Biotechnology is technologies derived from the deep understanding of biological phenomena and the use of the derived knowledge in technologies, it is important defining what criteria must be embraced to succeed in biotechnology enterprises. Biotechnology is a business and to be a business and to attract the interest of investors and users, so, it has to end in economic success. However Biotechnology is a particular kind of business; Economic success is only one part of the whole part of the business. A Biotechnology based company or NGO needs to succeed intellectually, knowledge of the biological phenomena supporting the technology is the capital of the company; Intellectual leadership. And intellectual leadership is not the same as company success. Success has to be defined based in intellectual leadership, competitive advantage, creating long-run activities (sustainable companies), and generating intellectual capital. Intellectual leadership can only be possible on knowledge, creativity, and rigorous and systematic investigation, the hallmarks of good science in any context.

Tools of Biotechnology.

Because Biotechnology is the study and application of biological phenomena, all disciplines of knowledge are eventually involved. Paramount are Biochemistry and its branches of study, like Enzymology and Enzyme technology, Genetic and protein engineering, Industrial microbiology, Cell and tissue culture.

A brief story of Marine Biotechnology.

Biotechnology is not a typical science derived from a Mother science, like Biochemistry that evolved from Microbiology at late XIX Century. Instead, Biotechnology was born as a concept meaning the use of several sciences, namely Biochemical Engineering, Biochemistry, Cell Biology, and later Molecular Sciences. For late 1970's and early 1980's the U.S.A. and Australia started looking to get a head-start in the activity. For 1989, Japan had 19 companies, like MITI and Marine Bio-technology Institutes. The first International Marine Biotechnology Conference was held in 1989 in Tokyo (IMBC'89), and the Japanese Biotechnology Society formalized activities. That was the year of the publishing of Journal of Marine Biotechnology. For the 1990's a new journal appeared, Molecular Marine Biology and Biotechnology. For 1994 The European Society for Marine Biotechnology (ESMB) started activities and in 1995, The Asian-Pacific

Marine Biotechnology Society. In 1999 Pan American Marine Biotechnology Association was founded in Halifax, Canada. Eventually Journal of Marine Biotechnology and Molecular Marine Biology and Biotechnology joined efforts becoming in Marine biotechnology, a journal by Springer publishers. It is the official journal of the The European Society for Marine Biotechnology (http://www.esmb.org/) and The Japanese Society for Marine Biotechnology (http://wwwsoc.nii.ac.jp/jsmb/index.html). The "International Marine Biotechnology Conference" has met nine times. 1st IMBC89 was held in Tokyo, Japan; 2nd IMBC91 in Baltimore, USA; 3rd IMBC94 in TromsØ, Norway; 4th IMBC97 in several cities of South Italy; 5th IMBC2000 in Sydney, Australia; 6th IMBC2003 in Japan, 7th IMBC2005 in St. John's, Newfoundland and Labrador, Canada, 8th IMBC2007 in Eilat, Israel, and IMBC2010 in Qingdao, China. Other regular meetings are happening. The Asia-Pacific Marine Biotechnology is in its 9th conference to be held in Kochi, Japan. Indicating the importance the Science-technology has become worldwide.

Mexico must invest capital and human resources to investigate biological resources; they can be eventually used in disparate disciplines to those originally intended. Like the example of *Xiphophorusmaculatus*, an endemic fish that, so far, is better studied abroad than in México. It is even an Institute in San Marcos, Texas: "The Xiphophorus Genetic Stock Center" (It is sponsored by the National Ocean Service, NIH National Center for Research Resources and Roy F. and Joanne Cole Mitte Foundation). The interest on the fish is based on its genetic physiology; it became a model to study human oncogenes. Same may happen for any other organism, the better we know them, the best we can use them. It is deep basic research than can show potential biotechnological uses. Domestic researchers must invest human and economic capital in biological marine processes for several reasons; to know what there is, descriptive science, how it came to be, evolution, how it is, biochemistry and molecular genetic, and how biological processes can be used, biotechnology.

Marine Biotechnology includes:

1. Marine bioproducts, making emphasis on drugs, bioadhesives, biominerals, and plenty otherbeneficial and economically important products. Based on the development of new models and screening for product discovery, the identification of new drug targets and mechanisms-of-action of marine-derivedrugs, the development of sustainable production methods for marine bioproducts and the development of novel methods for marine by-products utilization.
2. Aquaculture and Marine animal health,focused on marine pathogens, diagnostics, treatments, drug delivery systems, andimmunology, physiology, and pharmacology of both wild and cultured marine animals, cell and molecular techniques to improve size, growth rate, diseaseresistance, survivability, and reproductive yields of aquafarmed organisms.
3. Coastalhuman health risks, focused on the development of new diagnostic tools to assess seafoodpathogens and water-borne pathogens and pollutants like red tide ravages.
4. Coastal habitat restoration,focused on remediation, and molecular and cellular approaches to strain improvement, hybriddevelopment, and production technology for submerged and coastal aquatic vegetation.
5- Forensics and monitoring, focused on bioforensics for identification of threatened and endangered species, seafood identification, evaluation of health risks, and regulatory issues as related to economic fraud, and the development of new biosensors.

Biotechnology for the benefit of fisheries.

One of the most renowned marine economical activities is fishery. Some countries base it annual income on harvesting and processing marine products. When comparing México with countries with similar annual harvest volumes, like Canada and Spain, 1.4 x 10^6 ton, México yield 600 x 10^6 USD, while Spain makes 3000 x 10^6 USD and Canada 6000 x 10^6 USD.

Difference based on care and handling of catches and on processing. México sells for domestic and foreign markets scarcely processed products, raw materials. One example would be shrimp that is mostly exported to better income countries. Shrimp is processed by low-grade technology like beheading and freezing, while it is possible to improve revenues by exporting fresh product that demands for a different technology, even better, to export live product. Asian markets demand and pay suitable prices for it.

Biotechnology may transform waste products into by-products. What traditionally is considered wastes from fisheries, like heads, skin, guts, bones gonads and crustacean carapaces that are discarded contributing to environment tragedies, biotechnologies may make use of them making profit out of garbage. Lipids, mostly omega fatty acids, pigments, antioxidants, enzymes, biominerals, pharmaceuticals can be obtained by bio-processing processes and be value-added products.

Also, under exploited or non-commercial species may be seen as valued catches if devoted to industrialization. Low value fish, like the many found in Mexican waters can be processed. An example may be production of surimi, a muscle derived product used as ingredient for producing food. Surimi is a much-enjoyed food product in many Asian cultures and is available in many shapes, forms, and textures, and often used to mimic the texture and color of the meat of lobster, crab and other shell fish. Surimi quality is improved by Enzyme technology called cold binding, using a transglutaminase to improve gelling capacity.

Use, management and preservation of organisms and molecules (biological parts) for and by Biotechnology.

Biotechnology can and is making use of organisms belonging to the three main domains of Biology. Archaea, Bacteria and Eukarya, including animals and plants, and Viruses. Biotechnology looks in all kind of biology structures, Biome (Biota), Genome, Transcriptome, Spliceosome, Proteome including Enzymome, Metabolome and Interactome. Even is in the task of building genetically modified organisms and brand new organisms, synthetic life and Bio-bricks. The creation of a bacterial cell controlled by a chemically synthetized genome by the Craig Venter group is only the tip of the iceberg.

Transformation is the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surrounding and taken up through the cell membrane(s). Like *Agrobacterium sp.* That is used to transfer genetic material to plants and Lentiviruses used to transfer genes to animal cells. Marine viruses are under the lens of microscope to look for auto-assembling structures to produce nanoparticles to deliver pharmaceutical.

Patenting of life or life parts is an issue in Biotechnology. Ethical, economical and even political aspects must be considered. Countries don't agree yet on who must own rights on life and life parts. There are those, mostly companies, considering patent genomes and organisms, while, on the other end, mostly academicians who think patenting will affect the development of future science.

Finally, Bioinformatics.

Biotechnology is a comparative science. It takes advantages of better known organisms and biological processes to build hypothesis on less known ones. Advances in genomics are only possible based on robust and potent informatics systems.

SUSTAINABLE AGRO-INDUSTRIAL PRODUCTION IN CHIAPAS MÉXICO AS AN ALTERNATIVE TECHNOLOGY FOR FOOD SECURITY.

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ABSTRACT

Chiapas is one of world´s poorest region despite having a large pool of natural resources and biodiversity. The objective of this work was to develop a sustainable technology for mushroom production (*Pleurotus spp*) at the Lacandona Forest and Los Altos Regions in Chiapas Mexico for low-income women. The results show the comparison of three different systems for mushroom production in two regions of Chiapas, and transferring such technology to three target groups of low income women. This work is meant to contribute to the three specific United Nations Development Goals: end poverty and hunger, gender equality and environmental sustainability in one of the most beautiful regions in the world through the production of a high protein food using agro-industrial residues as raw material.

KEYWORDS: Sustainable development, • food security, • Low-income women

INTRODUCTION: Chiapas is the southernmost state of Mexico, adjacent to the states of Oaxaca and Veracruz to the east and Tabasco to the north and northeast. To the southeast and east of the state lies the border with Guatemala. Chiapas has an area of 75,634.4 square kilometers, which represents 3.8% of Mexico´s total area. Chiapas has approximately 1,250,008 indigenous language speakers which represent 29.1% of the state's population. The state of Chiapas is home to nine major ethnic cities and it was a major ceremonial center of the Mayan Empire. Chiapas is second only to Oaxaca in both indigenous population size and underprivileged socio-economic development levels of such groups caused largely by the discrimination towards these groups. The state of Chiapas is divided into nine different areas: The Central, Fraylesca, Isthmus Coast, Sierra and Soconusco regions are located on the Southwest areas of the state. The remaining 4 areas, the Highlands, Northern, Lacandon Jungle and Border are located on the northeast area of the estate. Over 70% of people living in the Highlands and Lacandona Jungle regions are indigenous language speakers who live in rural communities. Ironically, Chiapas is also one of the states with the most diverse base, generating 35% of Mexico electricity through hydropower. This estate also houses one of the most biodiverse zones in the world (INEGI, 2012).

In recent years, mushroom production has shown a vast improvement in rural communities of this State, particularly in the Highlands (42%) and Lacandona Jungle (18%) regions (Sánchez, 2007). The production of mushroom is accomplished largely by low income women of the tzotzil an tzeltal ethic group. Despite this vast improvement, higher yield crops have not being achieved

due to the lack the knowledge and skills required for this type of production. The objective of this work was to provide the skills and knowledge to three population groups at Lacandona Jungle and Highlands regions in Chiapas to develop such high yield crops by using a sustainable technology for mushroom production (*Pleurotus* spp).

MATERIAL AND METHODS

Research site. This study was carried out in two sites of the Lacandona Forest region in Ocosingo Chiapas: the first site was located at El Tumbo and Damasco. The second site was located at Los Altos region at Chalchihuitán . El Tumbo (17° 04''40' N and 91° 37'' 40' O) and Damasco (17° 13''N and 91° 33'' O) are two main locations of the Protected Natural area of flora and fauna "Nahá Metzabok" at the municipality of Ocosingo Chiapas. Chalchihuitán (16° 57''31' N and 92° 36'' 34' O) is the central village of the municipality that carries the same name.
Methodological techniques. The diagnostic of the mushroom production system at each location was carried out during up to five visits to the modules at each location. The analysis of the infrastructure was done through direct observations of the site, as well as an inventory of the materials and equipment locally available for the project. Once the diagnosis phase was completed, a sustainable system for mushroom production and a mechanism for technology transfer were specifically developed for each individual group according to the circumstances, environment resources surrounding each group. Additionally, two rounds of interviews for the women of the groups were carried out. Finally, the transfer of the production mechanism was done through conceptual and practical sessions.

RESULTS

Mushroom production system in Chiapas. Analysis of the mushroom production system was made during various visits to each locations. The first set of visits was conducted to the Damasco and el Tumbo in Ocosingo Chiapas at Lacandona Jungle region, and the second set of visits were conducted to the Chalchihuitan, Chiapas at Highlands region.
In the community of Damasco, municipality of Ocosingo, Chiapas mushroom cultivation was carried out in a wood rustic module of 20 m^2 (Fig. 1 A). This module was divided into four different areas: 1) the substrate treatment area (outdoor) (Fig. 2 A and B), 2) the spawning substrate area (Fig. 2 C and D), 3) and 4) the dark and light crop management areas (Fig. 2 E and F). Additionally, a high technology mushroom cultivation area was also located at this site (Fig. 1B). This building was divided into two areas, one for mushroom cultivation (downstairs) and one for mushroom spawn production (upstairs). All the required equipment for the activities mentioned above was present during the research at this location.
The Chalchihuitán, Chiapas location had no infrastructure for mushroom cultivation. A simple rural 30 m^2 room was built for this location (Fig 1C). This module was also divided into four areas, the same ones developed for the Damasco module.

Figura 1. Mushroom modules at Lacandona Jungle and Hignlands region in Chiapas. A. Damasco, Ocosingo, Chiapas. B. El Tumbo, Ocosingo Chiapas. C. Chalchihuitán, Chiapas.

Figure 2. Mushroom production system at Damasco municipality of Ocosingo, Chiapas. A. Sustrate pasteurization. B. Sustrate treatment. C. Spawning of substrate. D. Spawning of substrate area. E. Dark crop management area. F. Fruiting body of *Pleurotus spp* at light crop management area.

Technology transfer to low-income people. A specific plan was design by the research group to transfer this technology to each individual community according to their existing infrastructure, levels of skills in mushroom cultivation, and raw materials available throughout the area. The technology transfer was carried out with conceptual and practical sessions for the three population groups; a translator was required on all the cases.

Figura 3. Technology trnafer for mushroom production to low income women at Lacandona Jungle. A. Theorical session of the workshop at substrate production area. B. Translation of theorical session. C. Practical session. D. Sawning production area. E. Delivery of the diploma to low-income women. F. Work group.

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PERSPECTIVES TOWARDS OVERCOMING DROUGHT AND FLOODING CONSTRAINTS FOR SUSTAINABLE RICE PRODUCTION IN NIGERIA UNDER INCREASING CLIMATE CHANGE

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Abstract: Gene discovery initiatives have yielded knowledge and innovative technologies towards a much-more directed manipulation of plant genomes for desirable genes underlying yield, nutritional quality traits and resistance/tolerance to biotic and abiotic constraints. There is an urgent need to increase rice production in Nigeria, where a significant proportion of the agricultural environmental base is in marginal land that is further worsened by the environmental perturbations occasioned by global warming. The resource-poor farmers in Nigeria are more vulnerable to the harsh and erratically unpredictable agricultural conditions. An increasing drought and flooding threat prevails in Nigeria occasioned by the increasing climate change. The use of improved, inherently high yielding and tolerant rice varieties adaptable to the diverse rice production ecologies represents the most feasible option to combat these seriousconstraints. Progress in functional genomics has greatly accelerated the discovery and characterization of several genes underlying agronomically-important traits e.g. the *submergence tolerance* (*Sub1A)* gene of rice. The genomic region containing the *Sub1A* gene of rice, that has been reported to confer drought and flooding tolerance,could be usefully exploited to develop drought and flooding tolerant elite, high yielding and farmer-preferred introgression rice lines for Nigeria. Large-scale deployment of such drought resistant and submergence tolerant cultivars with high agronomic value and nutritional quality traits would lead to stable rice production in Nigeria where little or no attention has yet been given to the impact of increasing climate change on crop productivity.

Keywords: Sustainable rice production ● Nigeria ● *Sub1A* gene ● Flooding and drought stress ● Climate change.

Introduction: Unguided human activity and the resultant increase in the use of natural resources and industrialization are generating a series of adverse effects on the agricultural ecological systems e.g. pollution, loss of genetic diversity, soil fertility decline, drought, flooding desertification, etc (Monti, 1992). Agriculture in Nigeria- like in other parts of sub-saharan Africa - is expected to become more productive and simultaneously more sustainable, which implies meeting the food supply needs of the growing population without depleting the available resources – two seemingly contradictory needs (Monti, 1992). Increasing crop production can be achieved by expansion in the hectarage under cultivation and productivity in the unit area of cultivated land. Therefore, breeding of crop plants with higher agronomic worth including higher yield, superior quality traits and adaptive fitness without perturbations in the ecosystem are important to achieve the needed food supply especially in the world of increasing climate change that leads to extreme environmental stresses.

Improved crop yield has been influenced perhaps more by genetic improvement than by any other single factor (Fehr, 1984). Introgression of alleles accounts for differential survival to enhance plant fitness for adaptation to the different agricultural ecosystems. Evolutionarily, these was achieved by natural selection that preserved types with superior fitness to the given plant habitat and the destruction of those variants that are injurious (i.e. having less survival

capacity in the environmental niche). On the other hand, plant breeding, has traditionally sought for genetic variation in the gene pool and preferred adaptive traits transferred to the cultigens by different breeding methods. The breeder strives to strike a balance between selecting alleles with adaptive fitness to different environmental niches in order to stabilize crop productivity in those environments, as well as meet the requirements of both producers (profitability) and consumers (quality). Although conventional plant breeding is seemingly slow and usually involves several cycles of selection, it has significantly furnished the world with such enormous genetic materials (cultivars) for sustainable crop production over the years and helped to shift the "doomsday" predicted by Malthus as population growth increases geometrically. Despite the progress already achieved through breeding, additional gains in agricultural productivity are demanded at an ever-faster pace, to keep with increasing food demand, changes in agricultural practices (e.g. short fallow cycles), biotic and abiotic factors and consumer preferences.

Rapid advances in crop improvement including the development of plant types tolerant to abiotic constraints such as drought and flooding have also been made through agricultural biotechnology which has served as an innovative tool in the hands of plant breeders. Biotechnology, according to (OTA, 1989), is defined as "any technique that uses living organisms or substances from those organisms to make or modify a product, to improve plants or animals, or to develop micro-organisms for specific uses". In the perspective of plant improvement inherent in this all-encompassing definition of biotechnology, significant developments towards increasing crop productivity have been attained especially with the use of tissue culture (including development of somaclonal variant types), protoplast culture (e.g. somatic hybridization), molecular markers and recombinant DNA technology for accelerated transfer of desirable traits that was hitherto unattainable. Recent progress in genomics research have led to the availability of genomic sequences that have played an increasingly important role in the identification of genes that could be used to stabilize crop production, particularly rice, in the face of increasing climate change [e.g. the *Submergence tolerance –Sub1-* gene cluster of rice (Bailey-Serres et al. 2010; P. Ronald, Pers. Comm.; Jung et al. 2010)].

Urgent need for increased rice production in Nigeria in the face of increasing climate change: Rice is a major cereal of world-wide importance belonging to the genus *Oryzae*. In Nigeria, rice is ranked as the sixth major crop in cultivable unit area after sorghum, millet, cowpea, cassava and yam (Ukwungwu, 2000). There is an increasing trend in the per capita consumption of rice, as well as the urgent need to expand the area under rice cultivation in sub-saharan Africa (WARDA, 1997). Rice consumption is rapidly expanding especially in Nigeria due largely to urbanization. Rice is no longer considered a luxury food that is only eaten at Christmas, but has become a major source of calories for the urban poor. The availability of rice and prevailing prices for rice has, therefore, become a major issue of consideration for food security. However, there is a production deficit between a declining rice production and the increasing per capita consumption in Nigeria, which results in huge annual supplementation by importation (FAOSTAT, 2001). For instance, Nigeria spent 155 billion naira on rice importation alone in 2010 (Sanusi, 2011). Overcoming this production deficit by rice growers in order to meet present and anticipated demand for rice in Nigeria will necessitate the use of improved, high yielding varieties adaptable to the diverse rice production ecologies and significantly increase productivity in marginal farmlands. In the context of the increasing adverse effects of global warming, breeding rice with better adaptation to the drought- and flood-prone regions of Nigeria is important to meet the urgent need to increase, as well as stabilize rice production.

Climate change often results in erratic and uneven rainfall distribution which results in extreme environmental stresses that seriously hinders rice cultivation especially in the rain-fed upland and flood-prone low-land rice agro-ecologies.Global warming is associated with increased abiotic constraints to crop production (e.g. day length, solar radiation, temperature, flooding, drought, etc), which adversely affect both crop yield and plant distribution in natural

ecosystems. Of these abiotic stresses, drought is a major natural rice production constraint associated with food shortages and famine in severe cases. At the other extreme is flooding, which is also a serious rice production constraint in sub-saharan Africa. There is, therefore, an urgent need to increase rice production especially in these regions of marginal farmland in sub-saharan Africa where soils, water control and temperature are sub-optimal and further worsened by the environmental perturbations occasioned by the increasing global warming. An increasing drought and flooding (e.g. submergence caused by flash floods) threat occasioned by global warming prevails in most parts ofsub-saharan Africa including Nigeria with its huge economic cost. The deployment of tolerant rice varieties (genetic resistance), therefore, represents the most feasible options to combat these serious abiotic constraints.

The Sub1A – Key to flood and drought tolerant rice varieties: Plant improvement, whether as a result of natural selection or the efforts of plant breeders, has always relied upon creating and selecting the right combination of genes. The functional characterization and manipulation of a large number of genes is often required to improve even the simplest of characteristic and recognising the important alleles and determining their chromosomal location has hitherto been difficult or often impossible.

Recent advances in functional genomics research has greatly accelerated the discovery and characterization of several genes underlying traits of economic importance e.g. the *submergence tolerance* (*Sub1A)* gene of rice (Bailey-Serres et al. 2010; P. Ronald, Pers. Comm.; Jung et al. 2010), which has been known to underlie drought and flooding stress tolerance. The Sub1A-1 is reported as the primary determinant of submergence tolerance in rice (Xu et al. 2006). The Sub1A gene for drought and flooding tolerance, which are widely applicable in diverse genetic backgrounds and with no reported negative effects on agronomic performance and grain quality, are members of the *Sub1* gene cluster (Bailey-Serres et al. 2010). These Sub1 ERFs generally underlie diverse biological functions in growth and development, hormonal signaling (mediated by ethylene, cytokinin and brassinosteroid), as well as biotic and abiotic stress responses including pathogen infection, drought, submergence and freezing (Jung et al. 2010). The *Sub1A*-gene has now been identified as candidate genes mediating flooding-and drought tolerance of rice (UCDBP, 2011). The Sub1A gene is now being used in drought tolerance field tests (UCDBP, 2011). This project directed by Dr. Julia Bailey-Serres of the University of California, Riverside, California is expected to make a major impact towards producing high yielding rice crop that is both tolerant to flood and drought, two major rice abiotic constraints worthy of significant consideration in a world of increasing climate change especially in Nigeria .

Implications for resource-poor farmers in sub-Saharan Africa: A large proportion of farmers in Nigeria are resource-poor and dependent on subsistence agriculture to feed their growing populations. Unfortunately, however, breeding programs are characteristically less efficient in resource-poor areas (unlike in resource-rich areas) largely because genotype x environment interactions frequently pose serious problems particularly in subsistence agriculture (Allard, 1998). There is an urgent need to meet the demand gap for rice in Nigeria and research outcomes already achieved with the *Sub1A* gene are of fundamental importance to combat the growing drought and flooding threats in sub-saharan Africa and particularly in Nigeria.

Rice yields in the predominantly rain-fed conditions of Africa have become largely unpredictable especially due to drought stress. The new rice for Africa (NERICA) was developed to afford farmers the means of combating the growing drought threat (Jones et al. 1997). Recent studies have also focused on the screening of several other rice genotypes for drought tolerance including breeding lines and segregating progenies involving the African rice (*O. glaberrima*), the Asian rice (*O. sativa*) and different interspecific crosses (Ubi et al. 2011; Efisue et al. 2009). Most of these rice lines possessing tolerance to drought stress (e.g. the WBK lines)

also potentially have high agronomic worth to meet the requirements of producers and consumers. An example of rice genotype (WITA 9) that is resistant to drought stress is shown in Figure 1.However, given the poor research environment, funding and institutional bottlenecks in Nigeria, more time may still be required to get identified promising genetic materials (seeds) further developed and/or certified and placed in the hands of the resource-poor farmers.

Figure 1. A resistant genotype WITA 9 at the end of the drought stress experiment (22 days of maximum water stress) before resumption of watering. Left (L, water-stressed plant), and Right (R, non-stressed, constantly watered, control plant).

The *Sub1A* mega varieties have already made a tremendous impact for flooding tolerance in Asia. As further increases of rice production in the marginal farmlands of Nigeria are urgently needed, the deployment of such drought and flooding tolerant cultivars with high agronomic value and quality traits is urgently required. This would lead to stable rice production in Nigeria where little or no attention has yet been given to the impact of increasing climate change on crop productivity. The subsistence farmers in this region often face serious harsh and unpredictable agricultural conditions, yet they have to keep pace with rice production to meet the increasing consumption level. The use of this gene technology to develop Sub1 mega rice varieties that overcome the drought and flooding constraints is expected to bring about a quantum leap in rice production in Nigeria.

Conclusion: Collectively, the use of these emerging gene technologies in the development of Sub1 maga-varieties that retained their agronomic and nutritional superiority in addition to being tolerant to rice abiotic constraints (e.g. drought and flooding) and their large-scale deployment Nigeria will greatly lead to the expansion of the rice growing belt and assure food security, increased income and greatly enhance agricultural diversification among the resource-poor farmers who are more vulnerable to the effects of climate change.

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ASSESSMENT OF CUTICLE-DEGRADING ENZYMES PRODUCTION BY *BEAUVERIA BASSIANA* (BALS.) VUILLEMIN IN LIQUID MEDIA TO IMPROVE THE *EPILACHNA VARIVESTIS* (MULSANT) BIOCONTROL

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Abstract: Production of the cuticle-degrading enzymes (CDE) Pr1 and N-acetylglucosaminidase (NAGase) (Bidochka & Khachatourians 1988) by two selected Mexican *Beauveria bassiana* isolates was evaluated, supported by their recognized entomopathogenic fungi insecticidal activity. To achieve this, isolates were cultured in six liquid media, and blastospore and enzyme production as well as insecticidal activity was compared in with Sabouraud broth growth (Bidochka & Khachatourians 1990). All tested media improved the CDE production by both isolates compared with Sabouraud broth, but the maximum levels were observed by the C_1N_2 and C_2N_2. Only the blastosporas grown on the medium C_2N_2 were used for the insecticidal activity test. Were employed 3th instars larvae of Mexican bean beetle, at a dosage of $1X10^8$ blastospores/mL, using dipped larvae bioassays. There was no significant difference between the mortality values for blastosporas produced in C_2N_2 and Sabouraud cultures. However the median survival time (ST_{50}) values of blastosporas from C_2N_2 medium were significantly low than the values for blastosporas produced in Sabouraud medium. None difference was observed between the two isolate of *Beauveria bassiana.*
We may conclude that there is a relation between CDE and the ST_{50} of *B. bassiana* against Mexican bean beetle larvae.

Keywords: *Beauveria bassiana* ● liquid fermentation ● cuticle-degrading enzymes ● *Epilachna varivestis*

Introduction: *Beauveria bassiana* (Bals.) Vuill., is a cosmopolitan ascomycete fungus employed in the control of a wide insect pest range. Its mass production includes the diphasic fermentation production where the fungus's inoculum is produced in liquid culture, whose product is further used for solid substrate inoculums to achieve conidial production. Conidia are the principal infective unit in the commercial bioinsecticidal products intended for field application, due to its relative resistance under environmental conditions (de Faria & Wraight, 2007). Blastospores produced by liquid fermentation are less frequently infective unit in commercial products, but their production is easier, faster, and less expensive, and it is as efficient as conidia. The use of selected carbon and nitrogen source by liquid media for blastospores production has been described as a strategy to improve yield, tolerance to freeze-drying and propagules' virulence (Jackson, 1997; Jackson *et al*., 2010).

The Comité Estatal de Sanidad Vegetal de Guanajuato (CESAVEG) has two *B. bassiana* isolates effective against the Mexican bean beetle *Epilachana varivestis* (Mulsant) (Ocampo-Hernández *et al.,* 2011), but a higher speed of killing larvae might help to increase the acceptance by bean growers. The aim of the present investigation was to assess the efficacy of *B. bassiana* blastospores against *E. varivestis* larvae, produced in liquid media with selected carbon and nitrogen sources.

Materials and Methods:

Organism's source and inoculum preparation

Two *B. bassiana* isolates, Bb37 and Bb40, from the entomopathogenic fungi of the CESAVEG collection (Ocampo-Hernández *et al.,* 2011), were evaluated.

Isolates were propagated on potato dextrose agar (PDA) and incubated at 25ºC for 10 days. Conidia were collected by gently scraping the media surface with a spatula, re-suspending in Tween 20, 0.1% vol/vol solution, and vortexed for 5 min. Conidial concentration was determined by using a Neubauer hemacytometer (Ocampo-Hernández *et al.,* 2011). Spore viability was established before inoculation of the media by spreading 5 µL of conidia suspension on PDA and estimating the number of germinated propagules after 16 hours of incubation at room temperature. The conidia were considered viable when the germ tube lengths were two times the diameter of the conidia (Wraight *et al.*, 2007).

Culture media and growth conditions

Six different media were prepared combining 2 carbon (C) and 2 nitrogen (N) sources and were designated as C_1, C_1N_1, C_1N_2, C_2, C_2N_1, C_2N_2. Each medium with 10% and 5% weight/vol of carbon and nitrogen source, respectively, was mixed in a defined basal salt combination with KH_2PO_4, 1.5 g; $MgSO_4$-$7H_2O$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 1 mg; $ZnSO_4$, 1 mg; and distilled water for 1L. Sabouraud broth was used as a reference medium. Two hundred and fifty milliliters Erlenmeyer flasks containing 100 mL of medium were inoculated to a final concentration of $1x10^6$ conidia/mL. Inoculated flasks were grown for 5 d at 25ºC and 180 rpm (SEV^{MR} Mod 6040, Mexico DF). Each isolate was cultured in duplicate for each medium treatment.

Enzyme assays

Each 24 h, 10 mL of fungus-growing medium was obtained and centrifuged at 7000 rpm for 20 min at 4ºC. The supernatant was filtrated through Whatman # 1 filter paper and stored at -20ºC until use.

The Pr1 protease activity was determined using succinyl-$(alanine)_2$-proline-phenylalanine-*p*-nitroanilide (Sigma-Aldrich) as substrate. The assays were performed by mixing 20 µL of culture supernatant with 170 µL tris-HCl (pH 8) buffer and 1mM 10 µL substrate in 96-well microplates (Montesinos-Matias *et al*., 2008). The plates were incubated at 25ºC for 5 min, and the absorbance was measured at 405 nm using a spectrophotometer (BIORAD® 550 Microplate Reader). One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 nmol of nitroanilide per minute per mL under the assays condition. The assays were performed in triplicate for each sample.

The N-acetylglucosaminidase (NAGase) activity was determined using the substrate p-nitrophenyl N-acetyl-β-D-glucosamide (Sigma-Aldrich). The mix reaction contained 20 µL of culture supernatant, 140 µL phosphate-citrate (pH 4.8) buffer and 10 µL of 1 mg/mL substrate in microplates. Plates were then incubated at 37ºC for 10 min and optical densities were measured at 405 nm in a spectrophotometer (BIORAD® 550 Microplate Reader). The amount of enzyme that produced 1 nmol of nitrophenol per minute per mL under the assays condition was considered one unit of enzyme activity. The assays were performed in triplicate for each sample.

Bioassays

The blastospores media producing the maximum enzyme activity of the two selected CDE were selected to evaluate the insecticidal activity in a dipped larvae bioassay (Behle *et al.*, 2006), using a concentration of $1x10^8$ blastospores/mL. Insecticidal activity bioassay was performed against 3rd instar *E. varivestis* larvae (6 d-old), feeding on bean leaves. Before the bioassay, both larvae and bean leaves were disinfected using commercial sodium hypochlorite at 0.1% and 0.01% v/v in distilled water, respectively. The assays were performed three times

testing ten larvae every time. For this, ten larvae were dipped in 5 mL of $1X10^8$ blastospores/mL suspension and were gently shaken for 10 sec. After this, treated larvae were placed on sterile paper and individually transferred with a paintbrush in a vial containing a piece of sterile Whatman # 2 filter paper moistened with 700 µL of sterile distilled water and a bean leaf. If the bean-leaf was consumed by the larvae, a new disinfected leaf was placed inside the container to assure fresh food during all the bioassay. Vials were incubated at 25ºC, 55 % HR, and 12:12 (light: dark) photoperiod. Larvae were observed daily for 10 days after the exposure, and the numbers of live and dead larvae were recorded. The larvae were considered died when this didn't have any movement. Bioassays were done by triplicate in different days.

Data Analysis

Mortality records of each treatment were analyzed using SPSS 17 (2008). Median survival time (ST_{50}) was calculated by Kaplan-Meyer survival analysis, and compared with a long rank test (http://www.graphpad.com/prism/learn/survival%20analysis.pdf).

Results and discussion: All media selected in this study but Sabouraud broth improved the CDE production by the two *B. bassiana* isolates tested. Nevertheless, the CDE production by C_1N_2 and C_2N_2 media was better compared with all the other media (Fig. 1). Based on this, the nitrogen source may be responsible for the CDE production improvement by *B. bassiana*, similar than that as reported by Safavi *et al* (2007) and Dhar (2010), who demonstrated that yeast extract improved the Pr1 protease and chitinase production.

The blastopores grown on selected medium C_2N_2, that showed the highest CDE production, resulted in no survival (0%) when tested against 3rd instar *E. varivestis* larvae at a rate of $1x10^8$ blastospores/mL (Fig. 2). Testing *Paecilomyces fumosoroseus* at a same dose ($1x10^8$ blastospores/mL) against 3-d old larvae, Behle *et al* (2006) found survival of 10-14%, similar than that showed by our *B. bassiana* isolates grown in Sabouraud broth, but against older larvae (6-d old).

Differences were also observed when comparing ST_{50} by the two isolates cultured on the selected C_2N_2 media versus Sabouraud broth. Whereas the ST_{50} by *B. bassiana* blastospores cultured in C_2N_2 media was observed at of 48 h, in Sabouraud broth was of 96 h (Fig. 3). Moreover, aerial conidia from the same isolates, tested at a higher rate of $1x10^9$ conidia/mL, showed ST_{50} of 87 and 100 h by Bb37 and Bb40, respectively, also tested against 3rd instar *E. varivestis* larvae (Ocampo-Hernández *et al* 2011).

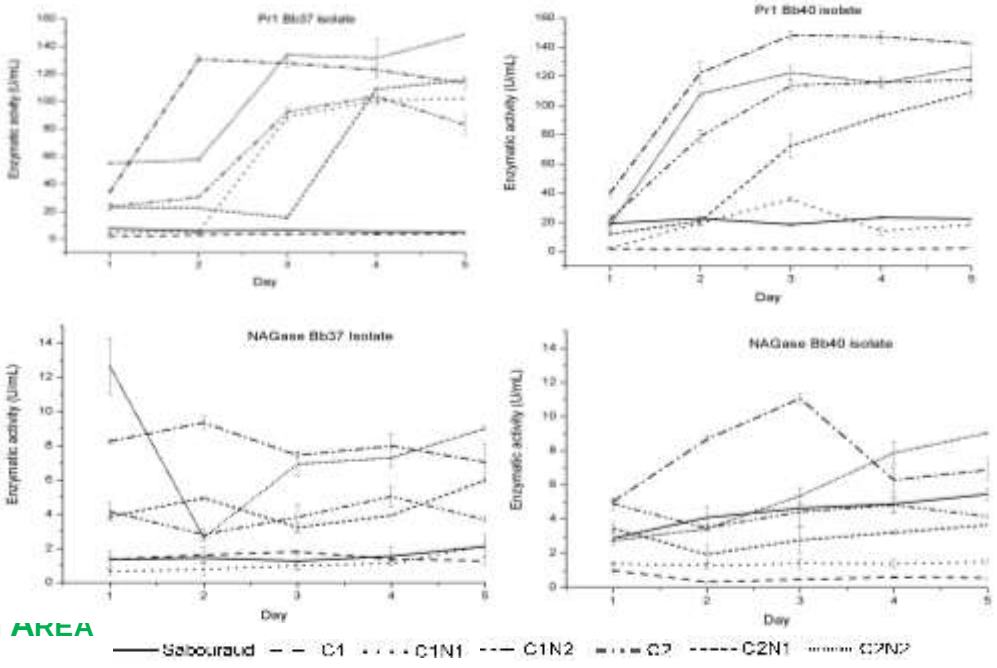

Figure 1. Kinetic of Pr1 and NAGASE production (U/mL) showed by two *Beauveria bassiana* isolates, cultured on seven media. Values are the three replications mean, where bars indicate standard deviation.

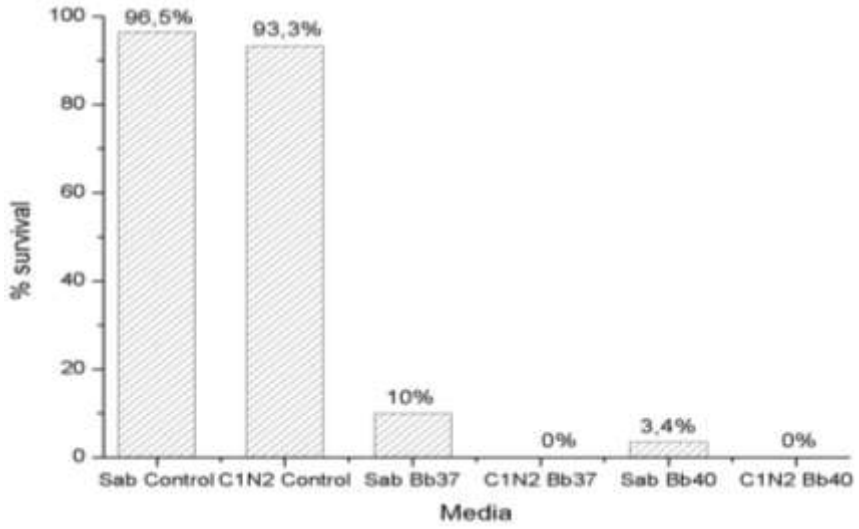

Figure 2. Survival percent of 3rd instar *Epilacha varivestis* larvae after exposed to *Beauveria bassiana* Bb37 and Bb40 isolates at a rate of $1x10^8$ blastospores/ mL. Values are the three replications mean.

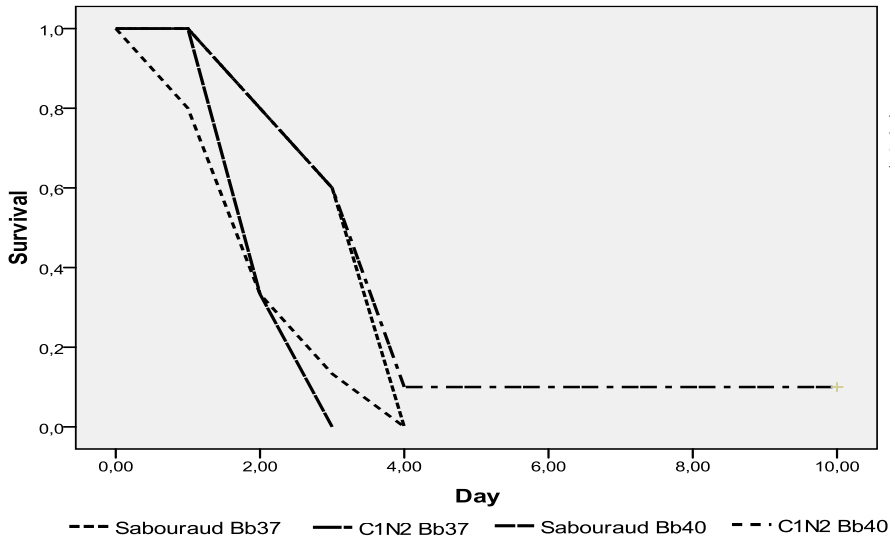

Figure 3. Survival time of 3rd instar *Epilacha varivestis* larvae after exposed to *Beauveria bassiana* Bb37 and Bb40 isolates at a rate of $1X10^8$ blastospores/ mL. Values are the three replications mean.

The increased speed of killing by blastospores cultured in our selected medium based on their higher CDE production, since has been associated with fungi pathogenicity (Joshi *et al* 1995).

Nevertheless, the main disadvantage of using blastospores as commercial product is their instability at room temperature (Jackson 1997). More studies focused on optimize blastospores storage conditions (temperature, formulation, etc.) are needed in order to potentiate their use for *E. varivestis* field biocontrol.

Conclusion: We determined the cuticle-degrading enzymes production by two isolate of *B. bassiana* (Bb37 and Bb40). Blastospores produced by isolates cultured in a selected nitrogen and carbon source medium, improved the *E. varivestis* biocontrol and showed an increased speed of killing.

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ANALYSIS OF THE INSECTICIDAL ACTIVITIES AND PERSISTENCE OF ESSENTIAL OIL OF AN AROMATIC PLANT (*CITRUS AURANTIFOLIA*)

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Abstract: Essential oils of aromatic plants with insecticidal properties are more and more considered as a safer alternative to protect stored products from insect damage. Many banned insecticides have high persistence which allows them to occur at several levels in the food chain. The insecticidal and persistence activity of essential oil extracted by steam distillation from the peel of an aromatic plant, *Citrus aurantifolia* (lime), was tested against the cowpea weevil (*Callosobruchus maculatus*) at concentrations of 16.8, 8.4, 4.2, 2.1 and 1.0 µl/L. The chemical composition of the essential oil used as the bio-insecticide was established by GC/MS analysis. The oil showed significant insecticidal activity even at 1.0 µl/L oil (LD_{50} 1.63 µl/L). However, at concentrations above 8.4 µl/L, more than 90 % of the test insects died. This study revealed the strong insecticidal activities of lime oil which can be used as a safer control measure for stored grain insect pests in order to decrease the current risk encountered with the use of synthetic insecticides.

Keywords: *Citrus aurantifolia* ● Insecticidal properties ● *Callosobruchus maculates* ● Stored grains

Introduction: The toxicity inherent in the use of conventional insecticides has been the bane of chemical control for stored grains especially in Nigeria. These chemical insecticides have serious drawbacks such as development of genetic resistance in the treated pest, toxic residue problems, toxicity to consumers, and increasing cost of application (Poirie and Pasteur, 1991). Over reliance on these compounds, as well as occasional misuse or abuse by farmers and households that consume these products has often resulted in fatalities. There is therefore need to adopt alternatives, such as bio-insecticides that are relatively safer and do not persist in the environment, in the control of these storage insect pests of grain crops. Essential oils from plant species are less toxic compounds with fewer environmental side effects and have been known as a good source of bio-insecticides in the control of storage pest. In this study, the bio-insecticidal activity and persistence of the essential oil extracted from the peel of lime (*Citrus aurantifolia*) was tested in the control of an important storage insect pest of cowpea, *Callosobruchus maculatus*.

Materials and Methods: The lime fruits (Figure 1) were purchased from Gwagwalada local market, Abuja (Nigeria) in December 2011 and neatly peeled with a sharp knife. The peel was then subjected to steam distillation for 4 hours in a Clevenger-type apparatus (Figure 2) according to the British Pharmacopeia (1980).

The extracted essential oil was recovered from the separating funnel & collected into falcon tube and stored at 4^0C for further use in the experiment. Infested cowpea seed were purchased from Gwagwalada local market and brought to the laboratory for the experiment. Tests were carried out to analyse the insecticidal activity of essential oil on freshly emerged adult insect (Figure 3). The persistence of the insecticidal efficiency of the essential oil was also tested.

Figure 1. Lime fruits from which peels were obtained and used for the extraction of essential oils with bio-insecticidal activity.

Figure 2. Steam distillation of the Lime peel in a Clevenger-type apparatus to produce the essential oils

Figure 3. Bioassay for the insecticidal activity of the essential oil. A 3MM Whatman paper was placed at the bottom of the container with the freshly hatched insect placed on it and the essential oil dropped on it.

Results and Discussion: The toxicity of the tested oil on the insects varied with the time of exposure. The essential oil had very short duration of toxicity. After 6 h of exposure, its toxicity decreased to 50 %, and after 24 hrs its toxicity on the insects was significant significantly high. The persistence of the insecticidal activity of the extracted essential oil is related to its chemical composition and the sensitivity of the target insect pest to the active compounds of the essential oil (Obey-Ofori *et al*., 1997). The insecticidal activity of this essential oils tested might have been translated into the *C. maculatus* by intoxication. This could cause the blocking of the transmission of the nerve impulse by inhibition of the hydrolysis of acetylcholin leading to the death of the organism. A LD_{50} of 1.63 µl/L correlated with that of other reported studies on bio-insecticides which suggest that the oil has great potential as insecticidal agent (Figure 4).

The activities of essential oils generally decrease with time because of their volatility. Oils with high content of hydrogenated compounds lose their activities quicker than those containing mainly oxygenated compounds (Huang & Ho, 1998; Reynault- Roger *et al.* 2002).

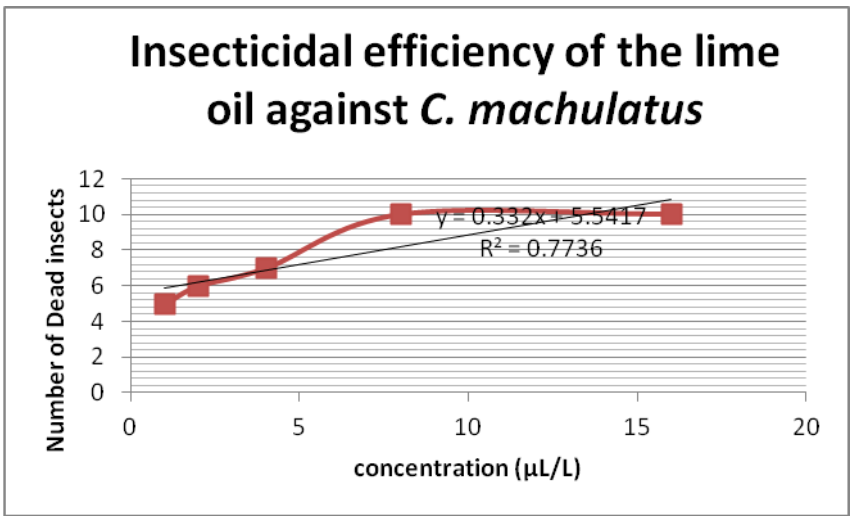

Figure 4. Insecticidal efficiency of the lime oil against *C. maculatus*

The speed of the oxidation of hydrogenated monoterpenes is greater for compounds such as sabinene, 1,8 Cineole-pinene (Kim *et al*., 2002). This oxidation leads to the reduction of the insecticidal efficiency of the oil.

Conclusion: In pest management strategies, aromatic plants with long lasting insecticidal efficiency should be considered. *Critrus aurantifolia* has an insecticidal activity on C. *maculatus* and should be considered as a candidate bio-insecticide for the control of insect pest of stored grain products to ensure safety to human health and the environment.

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PHENOLOXIDASE ACTIVITY BY *PERIPLANETA AMERICANA* (BLATTARIA: BLATTIDAE) EXPOSED TO *BEAUVERIA BASSIANA*

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Keywords: *Beauveria bassiana* metabolites ● *Periplaneta americana* ● phenoloxidase

Abstract: One of the most important insect's vector is the American cockroach *Periplaneta americana* L., because it carries human microbial pathogens. This pest is mainly controlled by chemical insecticide. Bioinsecticides, including entomopathogenic fungi, have been reported as potential control agents. Among arthropods, phenoloxidase (PO) activity is considered an innate mechanism of defense against microbial pathogens. In the present study, we tested the PO activity by *P. americana* when exposed to four *Beauveria bassiana* (Bals.) Villemin isolates, All isolates were tested as aerial conidia (AC), submerged conidia + blastospores (SC+B) or filtered supernatants (FS). For unexposed insects, late-instar nymphs and adult males produced the highest PO amounts, and were selected to compare mortality versus PO activity. Overall, nymphs were the least susceptible to *B. bassiana* isolates and SC+B treatment was the most virulent. The highest mortality was observed for adult females, which also showed the lowest PO activity. Pearson´s correlation analysis revealed significant association between increased *P. americana* mortality by *B. bassiana* treatments and reduced PO activity. The role of the *B. bassiana* infective unit (AC, SC+B or FS) on the virulence against *P. americana* versus its PO activity, is discussed.

Introduction: Insects play a major role in microorganism dissemination, and it is known that some carry human-disease pathogens (Tachbele *et al.* 2006). The American cockroach *Periplaneta americana* L. is associated with the transmission of enteropathogenic bacteria such as *Salmonella* spp., and studies have shown that this microorganism could survive up to 10 months in the digestive tract of this insect (Zarchi & Vatani 2008). Although chemicals are the first choice for roach control, *Beauveria bassiana* (Bals.) Villemin and *Metarhizium anisopliae* (Metschn.) Sorokın, have shown promising results as roach control (Zimmermann 2007), but field tests have shown conflicting results (Milner & Pereira 2007). Most *B. bassiana* products contain conidia produced by solid fermentation, followed by products containing hyphae combined with conidia, and the least used are blastospores produced by liquid fermentation (de Faria & Wraight 2007). Roaches have a well-developed immune system against infections and this could be one reason of the limited efficacy of bioinsecticides (Elliot & Hart 2010). PO activity is considered the first line of cellular defense and it is not only found in the hemolymph, but also in the integument, which along with the aromatic-L-amino-acid decarboxylase (DOPA), are part of the cascade-reaction associated with melanin production (Hiruma & Riddiford 2009). The assessment of PO activity throughout the life cycle of the cockroach relative to infection by the entomopathogenic fungus *B. bassiana*, might help elucidating the humoral immune defense in roaches. Different propagules produced by *B. bassiana*, either by solid fermentation (aerial conidia) or liquid fermentation (submerged conidia + blastospores or metabolites), may affect the PO activity, and might help explaining susceptibility differences of *P. americana* after *B. bassiana* exposure. In the present study, we reported PO activity by adult females, adult males,

and late-instar nymphs of *P. americana*, before and after exposure to various *B. bassiana* propagules produced by solid or liquid fermentation of four isolates. Two selected isolates are commonly used in phytosanitary programs for the control of the grasshoppers (Orthoptera: Acridoidea) *Melanoplus differentialis* (Thomas), *Sphenarium purpurascens* Charp., *Brachystola mexicana* Bruner, L., and Boopedon diabolicum Bruner, L. (http://www.cesaveg.org.mx/html/pboletines.htm).

Materials and Methods:

Organism's source

Periplaneta americana L. colonies were established from roaches collected in their natural habitat around the Universidad Autónoma de Nuevo León (UANL) campus. Before reared or added into the colony, they were kept in quarantine for 40 d to discard any ill or parasitized individual. Insects were reared at the FCB-UANL, and were placed inside of a plastic box with paper, under controlled conditions at 25 ± 4°C and 65–75% relative humidity and a 12-h light:dark photoperiod. Adults and nymphs diet consisted of dry pet croquettes (Purina, Nestlé México, S.A. de C.V, México, DF) and water. Adults and late-instar nymphs that had emerged in the established colony were used for the insecticidal activity and PO assays.

Culture media and growth conditions

Two strains of *B. bassiana* used in the present study (C1 and C2) were provided by the Comité Estatal de Sanidad Vegetal del Estado de Guanajuato, CESAVEG. Additionally, two *B. bassiana* isolates (C4 and C6) isolated from infected *P. americana* roaches were selected based on preliminary tests (data not shown). All strains were identified as *B. bassiana* by morphological characteristics according to Humber (2007). All four isolates were cultured on potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO), under the same conditions. All isolates were grown at 28°C until mycelia covered 85-90% of the agar surface. Isolates were grown from single conidia to obtain monoconidial cultures, as described by Choi et al. (1999). For preservation purposes, all isolates were stored in 5 mL vials as mycelial cultures in 20% glycerol at -20°C ± 4 °C. For each bioassay/replicate, a new vial with fungus was used.

Fungi treatments and bioassays

To determine the insecticidal activity by four *B. bassiana* selected isolates; treatments were tested at a dose of 1 x 10^6 spores per insect, or by 100 µL of sterilized supernatant from liquid fermentation (filtered). Liquid fermentation process was stopped generally after 11 to 13 d whereas solid fermentation was stopped after 8-12 d. The following treatments were used: 1) aerial conidia cultured by solid fermentation on PDA (AC); 2) submerged conidia and blastospores (SC+B) cultured by liquid fermentation on Sabouraud broth supplemented with yeast extract at 1% w/v; and 3) filtered supernatant (FS) without fungus collected from liquid fermentation. Conidial suspensions were made either by extracting aerial mycelia from PDA plates using a phosphate buffer solution (PBS), or directly from the filtered liquid fermentation, where supernatant fluids (produced metabolites) were included. Supernatants were obtained after three steps filtration, using sterile conditions. The first step consisted of pre-filtration of liquid fermentation culture by using a sterile gauze, in order to discard the major fungus mass. In the second step, the filtered liquid from the first step was paper-filtered (Whatman Inc., Piscataway, NJ) to remove residues of fungus mass. After this, the third filtration step consisted of a vacuum filtration, using 0.22-µm pore size cellulose acetate filters (Corning Gilbert Inc., Glandale AZ), to exclude all conidia or blastospores remaining in the liquid. For the treatment consisting on supernatant without fungi, 100 µL of undiluted supernatant were applied as previously described.

Insecticidal activity was performed with insects held for 20 min at 4°C to immobilize them to facilitate their manipulation and treatment application. All treatments / isolate combinations were tested by direct exposure (topically) on the first ventral abdominal segment, applying 100 µL of a 1 x 10^6 infective unit (conidia or conidia + blastospores)/mL dose abdominal area of either late-instar nymphs or *P. americana* adults (Pachamuthu et al. 1999). Males and females were tested separately. Controls consisted of application of PBS (by solid conidia comparison) or liquid fermentation medium only (for liquid fermentation comparison). All treatments (AC, SC+B or FS from each isolate) were transferred to 1 mL sterile vials and kept frozen at -20ºC, until use. Each treatment was tested against 12 roaches (late-instar nymphs, males and females), by three replicates performed on different days. After treated, roaches were transferred to individual 1-L plastic containers, containing pet bites, water and paper. Roaches were then incubated for 7 d under the same incubation conditions used by the insect colonies. Mortality was recorded on alternate days. Treatment mortality percentages were subjected to one-way analysis of variance (ANOVA) including Tukey HDS test, were significantly differences were determined at $P \leq 0.05$. Data were analyzed by using the SPSS 17.0 for Windows statistics software (2008).

Phenoloxidase Analysis

PO activity of *P. americana* development stages was determined spectro-photometrically by measuring the degree of dopachrome formation with a maximum absorption at 490 nm wavelength. PO activity in hemolymph was recorded from previously selected and frozen *P. americana* from controls or exposed to *B. bassiana* isolates treatments. Surviving insects were randomly selected and frozen before analysis. For early-instar nymphs, up to six roaches were selected; for middle-instar nymphs, three to four; and for late-instar nymphs or adults, only one roach was selected. Assay was done in triplicate. The hemolymph of P. americana was obtained by maceration of frozen individuals, homogenized with sterile pistils, and centrifuged at 14,000 rpm for 10 min at room temperature to obtain a cell-free hemolymph. Next, three 10 µL subsamples of the cell-free hemolymph for each replication of ground insects transferred to flat-bottomed 96-well plates. One hundred and fifty microliters of DL-dihydroxyphenylalanine (DL-DOPA) at 10 mM were added to each well as substrate. Microplates were then incubated in the dark, and absorbances were read at 490 nm every 10 min for 1 h, using a microplate reader (Multimode detector DTX 880, Beckman Coulter Inc., Austria). As a negative control, PBS with substrate only was monitored over the same time periods. The values obtained for each treatment were based on three replicates. Correlation analysis comparing insecticidal activity of each isolate / treatment versus PO activity and by each insect development stage of gender was performed by using Pearson's analysis (SPSS 2008).

Results and discussion: PO activity is a part of the humoral immune defense mechanisms in insects and has been related to entomopathogenic fungi infections (Vilmos and Kurucz 1998). There are reports where the presence of a microorganism induces PO activity, and that it varies during the life-cycle (Guyon et al. 2009). We found that unexposed late-instar nymphs and males produced more PO compared with early-instar nymphs or females (Fig. 1).

The mortality average of all treatments versus tested isolates showed that the highest mortality was against females (39.26%) versus males and late-instar nymphs (36.5% and 30.5% mortality, respectively), and that females showed lower PO activity compared with male and nymphs (0.265 versus 0.282 and 0.305, respectively). Correlation analysis of our data (Tukey-HDS test based on *Post Hoc* multiple comparisons and Pearson's correlation coefficient) showed a significant correlation between mortality and PO activity among treatments vs roaches nymphs or adults gender (SPSS Statistics 17.0). Results showed an inverse correlation between *B. bassiana* insecticidal activity and PO activity by *P. americana* late-instar nymphs and males, but not among females (Table 1), Values represent the average of three

replications. Correlation analysis of our data showed an inverse correlation between *B. bassiana* insecticidal activity and PO activity by *P. americana* late-instar nymphs and males, but no among females (Table 1). Similarly, Aoki and Yanase (1970 found that the PO activity was reduced as the *B. bassiana* infection increased in the Lepidoptera *Bombix mori*.

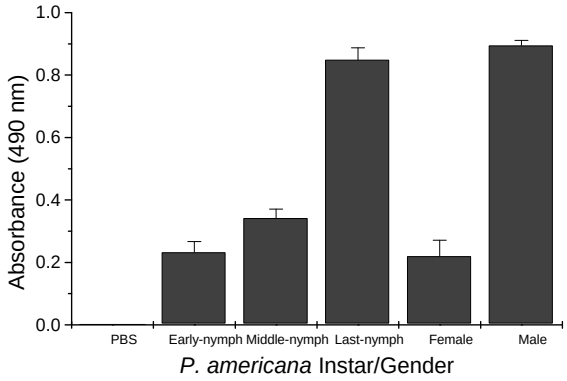

Figure 1. Phenoloxidase production by *Periplaneta americana* nymphal instars and adult genders. Bars on top of each column shows standard deviation values (SPSS 17.0).

Table 1. Insecticidal activity of *Beauveria bassiana* isolates (C1, C2, C4 and C6) against *Periplaneta americana* late-instar nymphs, females and males, and their correlation with the phenoloxidase activity.[1]

Roaches & *B. bassiana* isolates	**Treatments**					
	Aerial Conidia (AC)		**Submerged conidia + Blastospores (SC+B)**		**Filtered supernatant (FS)**	
Nymphs	**IA (%) ± SEM**	**PO± SEM**	**IA (%) ± SEM**	**PO± SEM**	**IA (%) ± SEM**	**PO± SEM**
Control	0.00±0.00 a	0.849±0.027 a	2.33±2.33 c	0.849±0.027 a	2.33±2.33 c	0.849±0.027 a
C1	0.00±0.00 a	0.469±0.007 bc	12.33±6.23 c	0.218±0.050 b	10.83±4.73 bc	0.454±0.027 b
C2	2.10±2.10 a	0.542±0.029 b	62.33±2.33 b	0.209±0.050 b	16.73±2.42 bc	0.314±0.056 b
C4	12.23±4.00 a	0.144±0.011 d	74.33±7.22 ab	0.059±0.008 c	50.00±10.0 a	0.408±0.031 b
C6	10.57±4.74 a	0.408±0.008 c	85.33±3.93 a	0.135±0.024 bc	29.16±5.07 ab	0.303±0.030 b
ANOVA	*F*= 4.13; *P* = 0.031	*F*= 302.07; *P* <0.001	*F* = 60.22; *P* <0.001	*F* = 88.19; *P* <0.001	*F* = 13.23; *P*=0.001	*F* = 51.36; *P* <0.001
Pearson's	*r*= -0.601; *P* = 0.023		*r*= -0.778; *P* = 0.008		*r*= -0.378; *P* = 0.202	
Females						
Control	1.75±1.75 c	0.218±0.029 c	1.67±1.67 c	0.218±0.029 a	1.33±1.33 c	0.218±0.029 bc
C1	7.01±0.80 c	0.634±0.045 a	10.85±3.14 c	0.211±0.007 ab	9.72±1.39 c	0.254±0.008 b
C2	11.24±1.72 bc	0.274±0.022 c	32.58±1.65 b	0.183±0.040 bc	18.05±5.01 b	0.160±0.019 c
C4	68.12±5.55 a	0.411±0.018 b	95.56±2.42 a	0.096±0.023 b	55.55±2.77 a	0.435±0.013 a
C6	21.96±3.22 b	0.212±0.003 c	91.93±1.20 a	0.064±0.005 c	48.61±5.00 a	0.243±0.007 b
ANOVA	*F*=75.21; *P*<0.001	*F*= 71.00; *P* < 0.001	*F* =437.58; *P*<0.001	*F* = 13.92; *P*< 0.001	*F* = 47.08; *P*< 0.001	*F* = 73.46; *P*< 0.001
Pearson's	*r*= 0.142; *P* = 0.615		*r*= -0.513; *P* = 0.107		*r*= 0.172; *P* = 0.557	
Males						
Control	2.67±2.67 c	0.889±0.011 a	4.74±2.47 c	0.889±0.011 a	4.00±2.31 b	0.889±0.011 a
C1	5.00±2.52 c	0.673±0.038 b	12.25±2.42 c	0.126±0.003 b	15.33±3.84 b	0.664±0.034 b
C2	9.67±1.67 bc	0.132±0.015 c	33.17±4.67 b	0.164±0.023 c	37.67±2.60 a	0.167±0.013 d
C4	38.33±2.73 a	0.196±0.023 c	87.74±2.42 a	0.045±0.001 c	50.00±4.62 a	0.573±0.021 b
C6	18.00±1.00 b	0.209±0.010 c	82.19±3.86 a	0.043±0.003 c	48.67±1.33 a	0.391±0.019 c
ANOVA	*F*=42.24; *P*<0.001	*F*= 287.59; *P*< 0.001	*F*=138.31; *P*<0.001	*F*=954.29; *P*<0.001	*F*= 42.30; *P*< 0.001	*F*= 269.87; *P*<0.001
Pearson's	*r*= -0.619; *P* = 0.014		*r*= -0.660; *P* = 0.007		*r*= -0.680; *P* = 0.005	

[1] IA (%) = percentage of insecticidal activity; PO = phenoloxidase activity (dopachrome formation detected at 490 nm wavelength absorbance);
SEM = standard error of the mean. Different letters in the same column/group (instar or gender) means significantly different at significance of *P*≤ 0.05, using Tukey

The relatively low susceptibility of *P. americana* to AC treatments might be related to *B. bassiana* metabolites produced during the liquid fermentation (Bidochka and Khachatourians 1991). In a mycoinsecticides product review by de Faria and Wraight (2007 they reported that 74.5% of the *B. bassiana* products contained conidia produced by solid fermentation (AC), and within that group, 75% contained conidia, while the other 25% contained a mixture of conidia + hyphae. SC+B represented the active ingredient in only 3.6% of the products. In the present study, we found differences among *B. bassiana* isolates and treatments. We observed that AC treatment was more virulent against females compared with males and nymphs (27.08% versus 17.75% and 6.23%, respectively); whereas SC+B showed similar activity against late-instar nymphs, females and males (58.58%, 57.73% and 53.84%, respectively) and with FS, mortalities were in between the other treatments (26.68% 32.98% and 37.92% mortality, respectively). Mortality average by *B. bassiana* isolates against nymphs by SC+B treatment was 58.58%, whereas with AC (6.23%) was not pathogenicity against *P. americana*. Although we did not find the factors by which nymphs survived the *B. bassiana* infection by AC exposure, but not from SC+B, it was clear that AC exposed nymphs possessed a higher PO activity (0.391), compared with SC+B (0.155) exposed ones (Table 1). The combined use of propagules and supernatant may provide a more effective bioinsecticide against roaches.

Conclusions: Roaches adults were more susceptible to *B. bassiana* compared with late-instar nymphs. *P. americana* phenoloxidase activity was negatively correlated with *B. bassiana* susceptibility.

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PATHOGENICITY OF *ISARIA FUMOSOROSEA* ON IMMATURE WHITEFLY *BEMISIA TABACI* (HEMIPTERA: ALEYRODIDAE)

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Abastract: The use of chemical insecticides to manage *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) has caused serious damage to the environment and has generated resistant populations to insecticides. The use of biological control agents such as entomopathogenic fungi is an excellent component for integrated pest management systems. In the present study the virulence of four native isolates (Pf-Tim, Pf-Tiz, Pf-Hal and Pf-Tic), as well as a commercial strain (Pae-sin) of *Isaria fumosorosea* (Wize) Brown & Smith was evaluated on eggs and second instar nymphs *B. tabaci*. All fungal isolates were more virulent on nymphs than on eggs. The most virulent native isolate for eggs was Pf-Tim, which had the same effect than the commercial strain Pae-sin. Regarding mortality of nymphs, the median lethal concentration (LC_{50}) was lower for the native isolate Pf-Tim (5.5×10^4), which was not significantly different than the observed for the commercial strain Pae-sin (2.6×10^4).

Keywords: Biological control ●Pest management ● Entomopathogenic fungi.

Introduction: The whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), is considered one of the most serious pest of field and greenhouse crops around the world (Musa and Shun 2005; Pineda et al*.,* 2007). *B. tabaci* causes direct plant damage by phloem-sap feeding, which can result in reduction of plant growth and yields, and induces plant physiological disorders due to the transmission of begomoviruses in a wide range of crop plants (Leshkowitz et al., 2006). The entomopathogenic fungus *Isaria fumosorosea* Wize (Deuteromycotina: Hyphomycetes) (formerly *Paecilomyces fumosoroseus*, designated as *Isaria* Clade) is a geographically widespread fungus infecting various orders of insects at all developmental stages (Wraight et al., 2000, Luangsa et al., 2005). The virulence of this fungus on *B. tabaci* and its potential use as a biological control agent have been reported in various regions where this pest causes severe damage to a large variety of valuable crops (Osborne and Landa 1992; Wraight et al., 2000; Pascual 2006). Several dozen isolates of *I. fumosorosea* have been evaluated in laboratory studies and greenhouse conditions against eggs, nymphs and adults of *B. tabaci* (Osborne and Landa 1992; Vidal et al., 1997; Negasi et al., 1998; Herrera et al., 1999; Wraigth et al., 2000; Saito and Sugiyama 2005). Some *I. fumosorosea* isolates are currently been produced commercially as biopesticides for *B. tabaci* in some countries as USA (PFR-97®, ECO-tec), Belgium (PreFeRal®, Biobest N.V.), Venezuela (Bemesin®,) and Mexico (Pae-sin®, Agrobiológicos del Noreste S.A. de C.V.) (Faria and Wraight 2001; Chan-Cupul et al., 2010). The search for new isolates to detect strains highly virulent against *B. tabaci* and environmentally adapted for a particular region of interest is a preponderant activity as the most successful market for entomopathogenic fungi has been local (Chan-Cupul et al., 2010).

In the present work we have investigated the virulence of *I. fumosorosea* isolates from the Yucatán Peninsula against eggs and second instar *B. tabaci* nymphs.

Materials and methods: Adult whiteflies were collected from field established habanero pepper (*Capsicum chinense* Jacq) in Conkal, Yucatan, Mexico. The colony was maintained in entomological cages made of anti-aphid mesh and aluminum frame, and kept in a greenhouse at 28±6°C with a natutal photoperiod (L:D) of approximately 12:12 hours. Insects were reared on *C. chinense* 40-70 day-old plants grown in 1 L plastic pots containing peat moss (Canadian Sphagnum Peat Moss, Canada) and fertilized daily with triple 19, 1 g L^{-1}.

Fungal isolates were obtained from *B. tabaci* collected on vegetable cropped fields where microbial pesticides has never been used; fungal identities were confirmed using morphologic characteristic according to Humber (1998) and Barnett and Hunter (2003). Fungi were isolated on Sabouroud Dextrose Agar (Difco, USA), cultured in the same medium in 90 x 15 mm plastic Petri dishes, and kept in total darkness at 25±2 °C. Isolates were obtained from different sites at the Yucatan state: Pf-Tim from Timucuy, Pf-Tiz Tizimin, Pf-Hal Halacho, and Pf-Tic from Ticul. The commercial strain Pae-sin was included in the study.

For bioassays, eggs and second instar *B. tabaci* nymphs were obtained using the procedure described by Muñiz and Nombela (2001). Experimental units were formed by groups of thirty eggs or nymphs. For egg bioassays, they were used immediately after obtaining them. For nymph bioassay, eggs laid in leaves were incubated for approximate 8 days.

Conidial suspensions for the bioassays were obtained from 15-day fungal colonies. Conidial concentrations were adjusted to 10^4 to 10^7 conidia mL^{-1} with the aim of a standard Neubauer chamber. Fungal inoculation was carried out as described by Gindin et al. (2000) and Saito and Sugiyama (2005). Briefly, individual *C. chinense* leaves bearing groups of eggs or nymphs were immersed in the respective conidial suspension for 10 seconds. Control leaves were immersed in water with 0.05% Tween 80 for the same length of time. Percentage of dead eggs and nymphs were scored daily.

Analysis of variance and mean comparison (Tukey, $P \leq 0.05$) of percentage of egg mortality were performed using GraphPad InStat (GraphPad Software Inc., 2000). To determine median lethal concentrations (LC_{50}) and median lethal times (LT_{50}) by Probit procedure, nymph mortality was analyzed using SAS (SAS Institute, 1998). LT_{50} for second instar nymphs were calculated using a fungal concentration of $1x10^7$ conidia mL^{-1}.

Results and discussion: All *I. fumosorosea* isolates caused egg mortality in *B. tabaci* under the tested conditions. The commercial strain Pae-sin and the native isolate Pf-Tim caused 61.3 and 55.5 percent mortality, respectively. These percent mortality values were significantly (F=17.28; n=5; $P<0.05$) higher than those showed by Pf-Rg, Pf-Tiz and Pf-Hal (Table 1).

Table 1. Percent mortality (mean ± s.e.m.) of *Bemisia tabaci* eggs after six days post inoculation ($1x10^7$ spores mL^{-1}) of *Isaria fumosorosea*.

Isolates	Mean± s.e.m
Pf-Tim	55.5±7.4 a
Pf-Tiz	33.3±1.6 bc
Pf-Hal	21.3±1.9 cd
Pf-Tic	48.8±5.1 ab
Pae-sin	61.3±3.8 a
Control	9.8±6.3 d

Means followed by the same letter are not significantly different (Tukey test, P < 0.05).

All *I. fumosorosea* isolates caused mortality on second instar *B. tabaci* nymph. The commercial strain Pae-sin and the native isolate Pf-Tim were considered the most virulent as they showed significantly lower values for CL_{50}, with $2.6x10^4$ and $5.5x10^4$ conidia mL^{-1} (Table 2). The LC_{50} values for the isolates Pf-Tiz and Pf-Hal were $3.5x10^5$ and $2.3x10^6$ conidia mL^{-1}, which indicates that these isolates were less virulent. The median lethal time (LT_{50}) for nymph mortality was significantly different among isolates (Table 3) when used a conidial suspension of $1x10^7$ conidia mL^{-1}. The commercial strain Pae-sin showed a significantly lower LT_{50} value (3.7 days) than the rest of the isolates. The TL_{50} values for the native isolates ranged from 4.3 to 6.3 days.

Table 2. LC_{50} values for *Isaria fumosorosea* isolates against second instar nymphs *Bemisia tabaci*.

Isolates	LC_{50} (conidia mL^{-1})	CI (conidia mL^{-1})	Slope ± s.e.m.	Pr>f
Pae-sin	2.6×10^4 a	$1.2x10^4$ - $4.7x10^4$	0.31 ± 0.029	<0.0001
Pf-Tim	5.5×10^4 a	$3.4x10^4$ - $8.2x10^4$	0.40 ± 0.029	<0.0001
Pf-Tic	1.5×10^5 b	$1.1x10^5$ - $2.0x10^5$	0.53 ± 0.032	<0.0001
Pf-Tiz	3.5×10^5 c	$2.0x10^5$ - $5.9x10^5$	0.28 ± 0.028	<0.0001
Pf-Hal	2.3×10^6 d	$1.6x10^6$ - $3.3x10^6$	0.53 ± 0.024	<0.0001

LC_{50}: Median lethal concentration; CI: Confidence interval; s.e.m.: standard error of the mean; Pr>f: Adjustment of the Probit analysis model for LC_{50} calculation.

Table 3. Median lethal time (LT_{50}) values for the isolates of *Isaria fumosorosea* against second instar nymphs *Bemisia tabaci*.

Strain	LT_{50} (Days)	IC (Days)	Slope ± ES	Pr>f
Pae-sin	3.72 a	3.41 - 4.04	3.47 ± 0.308	<0.0001
Pf-Tic	4.35 b	4.11 - 4.59	5.86 ± 0.484	<0.0001
Pf-Tiz	4.66 b	4.22 - 5.22	2.93 ± 0.308	<0.0001
Pf-Tim	5.04 b	4.70 - 5.45	3.70 ± 0.323	<0.0001
Pf-Hal	6.36 c	5.76 - 7.22	2.53 ± 0.242	<0.0001

LT_{50}: Median lethal time; CI: Confidence interval; s.e.m.: standard error of the mean; Pr>f: Adjustment of the Probit analysis model for LT_{50} calculation.

All native *I. fumosorosea* isolates from the Yucatan peninsula were found to be highly virulent to *B. tabaci* eggs and nymphs. Mortality of eggs caused by the two most virulent isolates Pf-Tim and Pf-Tic ranged from 48 to 55%. These values are relatively high when compared to those in other studies, such as the reported by Espinel et al. (2009), where the PC strain of *I. fumosorosea* caused no more of 50.5% mortality of *B. tabaci* eggs. In other study, Lacey et al. (1999) observed mortalities lower that 20% when using $1x10^3$ spores/cm^2 of *I. fumosorosea* against eggs of *B. tabaci*. Our study also demonstrated that the native isolate Pf-Tim has the same effectiveness on *B. tabaci* eggs than the commercial strain Pae-sin.

The virulence of all isolates on *B. tabaci* nymphs were higher than those reported by other authors. In the present study, the median lethal concentration of *I. fumosorosea* isolates on *B. tabaci* nymphs raged from $2.6x10^4$ to $2.3x10^6$ conidia mL^{-1}, values lower than the $1.7x10^8$ to $9.9x10^8$ conidia mL^{-1} reported by Vidal *et al.* (1997). Saito and Sugiyama (2005), however, found a LC_{50} of $1.1x10^4$ conidia mL^{-1} for a native *I. fumosorosea* isolate on *B. tabaci*, this value is similar to that observed in our study for the commercial strain Pae-sin. The native isolate Pf-Tim showed similar virulence that the commercial strain Pae-sin, which suggests that *I. fumosorosea* Pf-Tim may be considered a good candidate to develop a fungal biopesticide to manage *B. tabaci*. In is worth to point out that *I. fumosorosea* Pf-Tim was also one of the most virulent isolates for *B. tabaci* eggs. Others studies, such as those from Cabanillas and Jones (2009) and Wraigth et al. (2000) are difficult to compare with the present study as they reported the LC_{50} values in spores/mm^2. In the present work, the median lethal time (LT_{50}) for all fungal isolates were also calculated; in this regard, we have found that the commercial strain Pae-sin showed the lowest value for LT_{50} (3.7 day). The isolate Pf-Tim, however, showed a LT_{50} value of 5.0 days. The LT_{50} values for *I. fumosorosea* on *B. tabaci* reported by other authors are similar to those found in our most virulent isolates. For example, Gindin et al. (2000) and Saito and Sugiyama (2005) determined LT_{50} values of 4 days under similar experimental conditions. Similarly, Cabanillas and Jones (2009) reported a LT_{50} (3 days). These data highlight the potential of native *I. fumosorosea* isolates, particularly Pf-Tim, for microbial control agent against *B. tabaci* in the Yucatan Peninsula.

Conclusion: This study shows that *I. fumosorosea* isolates from the Yucatan Peninsula are highly virulent to eggs and nymphs *B. tabaci*. *I. fumosorosea* Pf-Tim may be considered a good candidate to be developed as fungal biopesticide to manage whitefly as its effect on nymphs was similar to that observed by the commercial strain Pae-sin.

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GERMINATION AND PLANT DEVELOPMENT OF PROTOCORM-LIKE BODIES OF *ENCYCLIA YUCATANENSE* AFTER MATURATION AND DESICCATION TREATMENTS

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Abstract: Orchid population in Yucatan peninsula has been considerably reduced environmental and anthropogenic causes. One tool for recover the orchid habitat and reduce this pressure is using plant biotechnologies as plant tissue culture and artificial seed technology. This research was conducted to develop a methodology to produce artificial seed of the orchid *Encyclia yucatanense* assesing the influence of the composition of maturation medium based in Murashige and Skoog (MS) medium added with abscisic acid (ABA), proline (PRO) and polyethylene glycol (PEG). MS medium added with plant growth regulators was used as control. We studied germination and plant development of protocorm-like bodies (PLBs) after maturation and desiccation. We observed that maturation medium improved in PLBs tolerance to desiccation. Germination of PLBs was not significantly different from the control however; plant development was significantly lower than control. After desiccation, PLBs showed humidity values between 48-57%, while humidity content of PLBs from control treatment was close to 87%, germination and plant development of PLBs of control treatment was significantly lower than maturation medium treatments. Best results were obtained with maturation medium added with 7.5 µM, 15 µM and 5% of ABA, PRO and PEG, respectively.

Keywords: Orchid ● Artificial seed ● Maturation

Introduction: Orchids show a wide range of diversity of flowers (color, size, shape, etc.). They exhibit a high price in both national and international markets. In Mexico, natural orchids habitat has been damaged by natural disasters, cities development, and also caused by people that take adult plants for sell them. *In vivo* vegetative propagation are time consuming and expensive because propagation of orchids from seeds is undesirable due to the heterozygosity of seed, the seed size, presence of reduced endosperm, also the requirement of an association with mycorrhizal fungi. However, Orchids can be multiplied by micropropagation. Artificial seed could be defined as an analogous of zygotic seed; it can be presented as desiccated somatic embryo without cover; desiccated somatic embryo coated with a water soluble resin; somatic embryo coated with alginate gel and somatic embryo into a gel called as fluid drilling (Fujii *et al*, 1987). Most limiting aspect of artificial seed technology is the production of vigorous somatic embryos also, for the market competition the artificial seed need to have a good and quick germination and develop in plant as a natural seed (Anandarajah & McKersie, 1990). The objective of this research was to asses conditions for preservation of the Yucatan's peninsula orchid's germoplasm as artificial seed, for the reestablishment of damaged ecosystems or for a commercial aim. We assessed the effect of addition abscisic acid (ABA), proline (PRO) and polyethylene glycol (PEG). in a basal MS medium defined as maturation medium on germination and plant development after maturation and desiccation of PLBs.

Material and methods:

Production and maintenance of Protocorm-Like Bodies (PLBs)

PLBs from *E. yucatanense* were obtained from previous works and these were reproduced using Murashige and Skoog (1962) media added with myo-inositol (100mg L^{-1}), thiamine (4mg L^{-1}), cysteine (50mg L^{-1}), sucrose (30g L^{-1}) and plant growth regulators: naphtaleneacetic acid, indoleacetic acid and bencilaminopurine used at 2 mg L^{-1}. Incubation for maintenance and experiments of PLBs were using a plant growth room at 25-27 ºC, relative humidity of 80%, fluorescents lamps as light source (45-60 µmol $m^{-2}s^{-1}$) and photoperiod light:dark (16:8h)

Experimental design

For maturation treatments, MS media was added with ABA, PRO and PEG in different levels (Table 1). Samples of 300 of PLBs were used in each treatment in triplicate.

Table 1. Levels of ABA, PRO and PEG used in the maturation medium.

Treatment	ABA(µM)	PRO(µM)	PEG (%)
Control	0	0	0
T-1	7.5	10	5
T-3	7.5	15	5
T-5	15	10	5
T-7	15	15	5

Control treatment was same medium used for the production of PLBs.

Maturation and Desiccation of PLBs

Maturation consisted in grow PLBs during 15 days in each treatment and after this time, PLBs were subjected to gradual desiccation by applying 6 stages using individually the hygroscopic salts K_2SO_4; Na_2CO_3; NaCl; NH_4NO_3; $Ca(NO_3)_2$ and $CaCl_2$. All experimental treatments remained 24h in each desiccation stage and humidity of PLBs was calculated. After maturation and desiccation, germination and plant development was assessed. Also, dry weight was determinate after desiccation treatment.

Results and discussion: After PLBs maturation treatments, detention of growth was visually observed in 87.7±5.3 % while control treatment did not affect differentiation and conversion to plant. Compared with zygotic embryogenesis, where the embryo into the seed is in quiescence and dormancy status and this grow up only if the external conditions are optimal, somatic embryos do not stop its growth (Senaratna *et al*, 1990)

The presence of PEG in maturation medium allowed the PLBs diminish its humidity content with a significant difference compared with control, humidity content of treatments was approximate 67-57%, after gradual desiccation of PLBs was close 57-48% (Figure 1).

Germination after maturation was similar in all treatments and values were between 65-70% and it was not significant differences between treatments and control (MS). After desiccation, germination was significantly affected in control and T-7 treatment, this may be caused by salt stress that could had an effect in viability of PLBs, because T-7 medium had highest ABA and PRO contents (15µM and 15µM, respectively) (figure 2).

PLBs reduced its growth and development process close to 15-40%, considerably lower than control, this effect could be caused by ABA presence in the maturation medium, it is know that ABA regulates key events during seed formation, such as the deposition of storage reserves, prevention of precocious germination, acquisition of desiccation tolerance, and

induction of primary dormancy (Kermode, 2005). After desiccation process, percentage of plant growth and development increased considerably compared to control (figure 3).

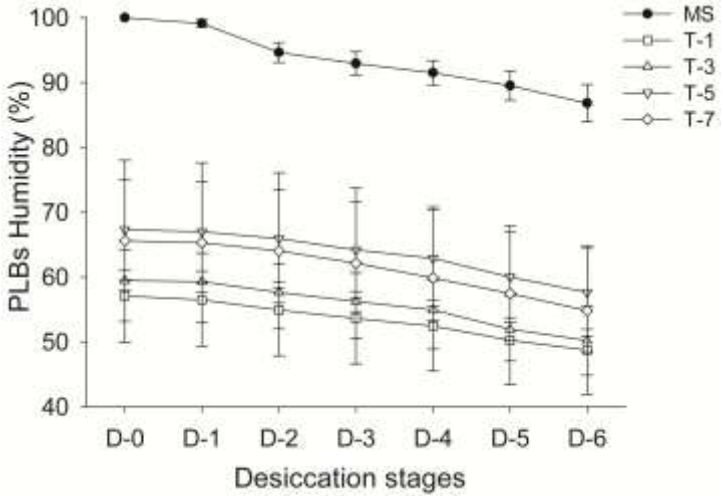

Figure 1. Humidity content after each desiccation stages of PLBs treated with 4 different maturation media and MS media used as control.

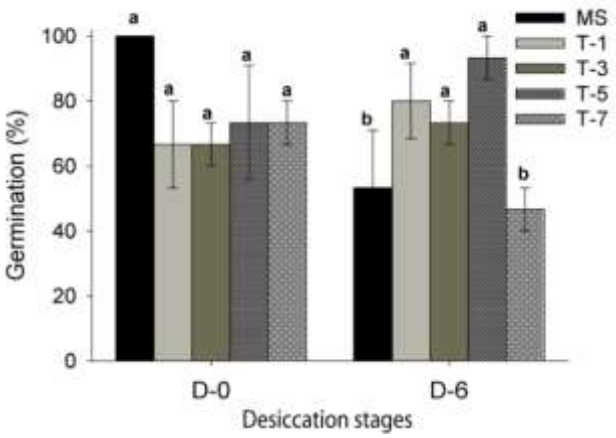

Figure 2. Germination (%) of PLBs treated with maturation media after 15 days maturation period (D-0) and after gradual desiccation (D-6). Different letter indicate significant differences using two-way ANOVA (α=0.05).

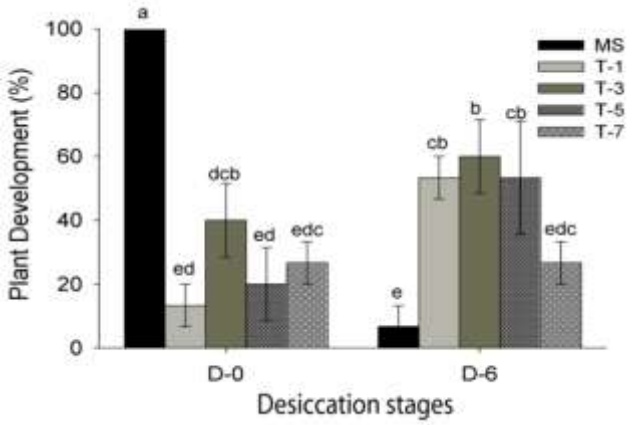

Figure 3. Plant development (%) from PLBs treated with maturation media after 15 days maturation period (D-0) and after gradual desiccation (D-6). Different letter indicate significant differences using two-way ANOVA (α=0.05).

Plant development after desiccation process was increased with the treatments. Also, dry weight of PLBs from treatments was significantly higher than control treatment; this effect was due to the presence of proline in maturation media. It is well known that proline have positive effects on enzyme and membrane integrity along with adaptive roles in mediating osmotic adjustment in plants grown under stress conditions (Ashraf & Foolad, 2007). Proline is accumulated during osmotic stress causing increment of dry weight (Hoekstra *et al*, 2001), as we observed in PLBs treated (figure 4).

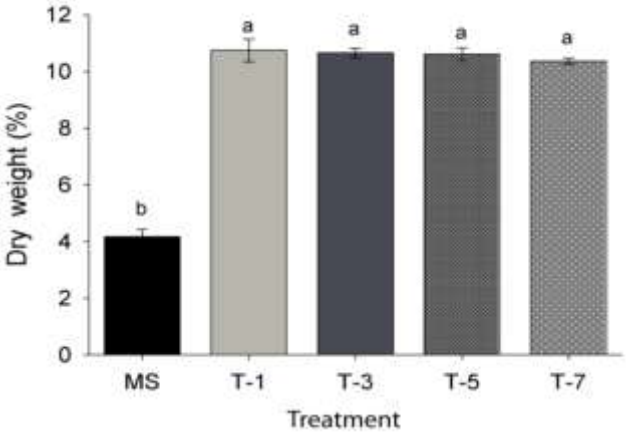

Figure 4. Dry weight of PLBs treated with maturation media added with ABA, PRO and PEG. Different letter indicate significant differences using one-way ANOVA (α=0.05).

Conclusions: Our finding shows the importance of define a maturation treatment to achieve a method for production of Yucatan's orchids artificial seed. The PLBs maturation was achieved with Murashigue and Skoog culture media added with ABA (10 or 15µM), PRO (7.5µM) and PEG (5 %) followed of gradual desiccation stages using hygroscopic salts.

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BACTERIAL DIVERSITY AND CONSTRUCTION OF A METAGENOMIC DNA LIBRARY FROM WHEAT RHIZOSPHERE

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Abstract: Rhizospheric soils contain a high diversity of microorganisms, and therefore, comprise a large reservoir for discovering genes with diverse agro-biotechnological applications. In this work, we have constructed an *E. coli* metagenomic library based on bacterial artificial chromosome (BAC) clones with large genomic inserts from metagenomic DNA of the rhizosphere of wheat plants (*Triticum aestivum*). Also, we analyzed the bacterial diversity associated with the rhizosphere of wheat plants by PCR amplification, construction of a library and sequencing of 16S rDNA genes. The average of the DNA cloned segments varies from 5 to 80 kb, with an average size of 38 kb. Random clones were end-sequenced and homology results shows that the clonation of metagenomic DNA codes mainly for metabolic and catalytic functions (40%), including amidohydrolase, hydrolase, peptidase, serine protease, endonuclease and exonuclease. Another interesting proportion of the clones revealed genomic sequences with hypothetical (17%) or unknown function (9%). The metagenomic sequences belonged mostly to Proteobacteria, Firmicutes, Archaea, Actinobacteria, Fungi, Virus, Unclassified Bacteria and Unknown taxa. Homology and phylogenetic analysis of 16S ribosomal genes suggests that there are different genera that can be associated with biocontrol and Plant Growth-Promoting Rhizobacteria. It is expected that the metagenomic library from rhizospheric soil of wheat plants keeps growing, while functional analysis are carrying out to search for genes of agrobiotech interest.

Keywords: Bacterial diversity ● Metagenomics ● Rhizosphere ● Wheat

Introduction: Metagenomics has revolutionized the way we study biology, in fact, there are currently more metagenomic sequences reported in databases such as NCBI than the total sequences of all completely sequenced organisms, representing a defiant challenge to biology (Moreno-Hagelsieb and Santoyo, unpublished results). Metagenomics has been investigated from various environments, including gut microbiota, aquatic ecosystems, mines, agricultural and forest soils, among others (Handelsman, 2004; Hernández-León et al., 2010). The relevant aspect of these studies is that each of them has shown different aspects to study and analyze, and in some cases, have provided innovative views of the microbial ecology of extreme environments for the development of life, while others have found novel genetic elements that could have applications in biotech industry.

One of the most complex environments is the rhizosphere, a microenvironment where lies a great microbial diversity. The rhizosphere is defined as the region of surrounding soil, which is influenced by the roots of plants (Ahmad et al., 2008). It is in this environment where various abiotic and biotic interactions take place, especially with microorganisms and plant roots. Abiotic interactions, such as temperature, soil type, climate, among other factors, are often involved in determining the population structure of the microbial community. However, these microorganisms have evolved various mechanisms that can be successful in the occupation of spaces, and thus obtain nutrients excreted by the plant root (Haas & Keel, 2003). The diversity of bulk-soils and rhizospheric soils of different plants has been studied extensively, reporting most studies a wide range of organisms (Daniel, 2005). It is estimated that there are about

$2x10^6$bacterial species in the marine environment, whereas in a sample of soil could contain up to $4x10^6$ different taxa (Curtis et al., 2002). This suggests that soils are a large reservoir for the discovery of several compounds that may have applications in agriculture, human health or industry (Handelsman, 2004; Hernandez-Leon et al., 2010). In this work we report the construction of a BAC metagenomic DNA library as well as the bacterial diversity by sequencing the16S rDNA genes from rhizosphere of wheat plants.

Materials and Methods: To study the soil microbial community, samples were taken from the rhizosphere of wheat plants near the city of Zamora, Michoacan, Mexico, with coordinates 19° 59' north latitude and 102° 17' west longitude at 1,560 altitude meter. 10 plants were collected along with their roots and rhizospheric soil when the plants had a month of being planted. Rhizospheric soil samples were taken at 10 cm depth and transported on ice to be stored at 4° C for immediate analysis in Lab. The rhizosphere was separated from the root and was stored at -4° C. The physico-chemical analysis of soil was as follows: the soil is a clay loam type, with the following percentages: sand 23.48%, clay 38.57% and silt 38%, with a neutral pH of 7.17.

Total DNA extraction from rhizospheric soil samples was done by using the MO-BIO PowerSoil® DNA Isolation Kit, following manufacturer instructions. Once DNA was pure enough, approximately 300 ng of DNA was digested with the HindIII enzyme for 4 hr at 37oC. The digestion was again observed in a gel electrophoresis at 1%. Then, the restriction enzyme was inactivated at 65 ° C in a Dry Bath Incubator Fisher Scientific for 20 min and purified by minicolumn (Wizard SV Genomic DNA Purification System, Promega) to be observed gain in agarose gel 1% (Fig2B).To perform the cloning of metagenomic DNA 50 ng were used plus 10 ngr of the vector pIndigoBAC-5 and incubating with the T4 ligase enzyme (4 units) during 12 hrs at 4oC. The ligation product was transformed by electroporation into electrocompetent E. coli cells in a 2510 eppendorf® electroporator. The transformed cells were plated onto LB solid medium with chloramphenicol (Cm, 10 µg/ml) and incubated for 20 to 24 hrs at 37°C. Clones were picked out again on LB medium plates containing 10 µg/ml of Cm plus 40 µl of X-gal (at a concentration of 60 mg/ml) and incubated for 24 hrs at 37°C.

Ribosomal 16S rDNA genes were amplified using the universal bacterial primers Fd1, 5'-CAGAGTTTGATCCTGGCTCAG-3' (forward) and Rd1, 5'-AAGGAGGTGATCCAGCC-3' (reverse), corresponding to positions 8 to 28 and 1526 to 1542 from the Escherichia coli 16S rDNA gene, respectively. The following Polymerase Chain Reaction (PCR) conditions were used: an initial denaturation at 95°C for 3 min; 30 cycles of 1 min at 95°C for denaturation, 1 min at 53°C for annealing, and 2 min at 72°C for extension, and a final extension step at 72°C for 5 min. The purified PCR fragments were then cloned into the pGEM-T Easy Vector (Promega) and the resulting ligation products were used to transform com¬petent E. coli cells. Positive white clones were detected on LB medium containing 80 µg/ mL X-Gal and 0.5 mM IPTG. 96 isolated plasmids from E. coli clones were isolated and corroborated for insert cloning, and were commercially sequenced. All sequences obtained were compared with sequences in the GenBank (NCBI) database by using the BLASTN program, to obtain the best matching sequences.

Results and Discussion: Metagenomic libraries can be divided into two classes: those with insert small or large insert, this latter is what we reported in this work. We isolated at random BACs of 26 recombinant *E. coli* clones and digested with *Hind*III to determine the approximate size of the cloned fragments. The cloned inserts in the clones analyzed ranges from 5 to over approximately 80 kb, with an average of 38 kb in size of the inserts. This range is essential to carry out the cloning of genes or operons that may encode complete new metabolic functions (Daniel et al., 2005).

To get a better idea what kind of genes or sequences were cloned in the metagenomic library, 75 recombinant clones were isolated and BAC-end commercially sequenced. It was

found that 23% of the sequences coded for metabolic functions, while another large percentage corresponds to catalytic functions (17%). Genes encoding amidohydrolase, hydrolase, peptidase, serine protease, endonuclease and exonuclease activities were found, among other. Interestingly, a significant percentage identity was not found with known sequences or genes are reported as hypothetical, indicating that might encode for functions still unknown. Moreover, identity was obtained for genes involved in basic cellular processes such as replication, transcription, translation and repair of DNA, as well as genes with identity to membrane proteins and transposons.

High quality sequences were obtained from 84 of the 16S rDNA gene clone library from wheat plants rhizosphere. According to the results of homology search, it was found species belonging to Classes, Alfaproteobacteria (7.1%), Betaproteobacteria (15.4%), Gammaproteobateria (36.9%), Deltaproteobacteria (4.7%), Actinobacteria (10.7%), Bacilli (10.7%) and Clostridia (2.3%). Also, some sequences (10.7%) showed high similarity to uncultured bacteria.

In this work it is reported the construction of a large insert size metagenomic DNA library from rhizosphere of wheat plants. Large inserts are important to isolate complete genes or even complete operons encoding functional pathways for synthesis of diverse compounds or metabolites. In this way, Chung et al. (2008) reported the cloning of a fragment of 40 kb DNA of forest soil metagenomic containing a complete operon encoding type II familypolyketide synthases, ACP synthases, aminotransferase and an ACP reductase, which showed antifungal activity in experiments. Nonribosomal peptide synthetases and polyketide synthases are multiprotein complexes, which are encoded by clusters of genes, transcriptional units usually involving operons with several kilobases of length. Other metabolites, such as siderophores, which are big peptide molecules with affinity for iron, are also synthesized by some nonribosomal peptide synthetases. Siderophores are important molecules to colonize and deprive the iron from pathogens from rhizospheric soils, thus inhibiting their growth in an indirect form (Rondon et al., 2004). Other compounds involved in the inhibition of pathogens such as lipopeptides from Pseudomonas and Bacillus species, also require clusters of genes for its synthesis. Therefore, it is desirable to clone large DNA if one wants to find antifungal activities of bulk-soils or rhizosphere soils.

The rhizosphere is defined as the portion of soil that is influenced by plant roots. In this microecosystem the root exudates are rich in nutrients, thus attracting a wide variety of microorganisms, including bacteria that can occupy those spaces, obtain nutrients to grow and limit the growth of pathogens (Weller, 1998). Such is the case of rhizospheric bacteria of the genera *Bacillus* and *Pseudomonas*, which are inhabitants of bulk-soil and rhizosphere (Tomashow, 1996), that this paper reports its presence as two of the most abundant in the 16S clone library from rhizosphere of wheat plants. Studies have shown that different strains of Bacillus, through direct or indirect mechanisms inhibit or control potential pathogens (Chen et al., 2009). It has been reported that diverse *Bacillus* species produce antibiotics with antifungal activity, including lipopeptides (Ongena & Jacques, 2008). Lipopeptides such as fengycin, surfactin and members of the iturin family have been studied in detail and have shown to be effective in suppressing different phytopathogenic organisms, including fungi, bacteria, nematodes, among other (Chen et al., 2009; Ongena & Jacques, 2008). On the other hand, it has also been suggested that some species of Bacillus may be plant-growth promoting through phytostimulation and volatile production (Velázquez-Becerra et al., 2011). In this sense, bacteria of the genus *Pseudomonas*, like *Bacillus*, also have the ability to be PGPR, in addition to show biocontrol activities (Compant et al., 2005; Haas & Defago, 2005). Pseudomonas has rapid growth and therefore is good colonizers in soil. *Pseudomonas* can use various substrates as nutrients and survive in different stressing conditions. Also the ability to produce various compounds, such as antibiotics, polysaccharides and siderophores are crucial to succeed. In recent works, Santoyo et al. (2010) showed that the strain *P. fluorescens* ZUM80 can restrict the growth of plant pathogenic fungi, such as *Fusarium oxysporum, Colletotrichum lindemuthianum,*

Colletotrichum gloeosporioides and *Phytophthora cinnamomi*, through the synthesis of iron chelators and other potential antibiotics. Another interesting biocontrol indirect mechanism employed by Bacillus and Pseudomonas is the ability to Induce Systemic Resistance (ISR) in plants, thus protecting them from diverse phytopathogen infections (Ongena & Jacques, 2008).

Another genus found in the 16S rDNA library from plant wheat rhizosphere was Stenotrophomonas. This genus also contains rhizospheric habitants, playing an interesting ecological roles on plant-growth promoting activities (Velázquez-Becerra et al., 2011).Other works on studies of diversity in rhizosphere of wheat plants have also shown a wide variety of bacteria (Germida & Siciliano, 2001). Importantly, they analyzed the diversity by using the fatty acid methyl esterified method, but not involving isolation DNA sequences. The authors report that found mainly bacteria belonging to Gammaproteobacteria and Bacilli Classes, and the predominant genera were *Pseudomonas, Arthrobacter, Bacillus, Flavobacterium, Micrococcus, Xanthomonas, Agrobacterium* and *Enterobacter*, among others. This is in agreement with our results, where phylogenetic analysis shows that some representative 16S genes were grouped in clusters with sequences from species that are inhabitants of the rhizosphere of plants. *Cellulomonas, Microbacterium* and *Geobacter* are other genera found in the 16S rDNA clone library. These genera have been found as endophytes of different plants, such as rice, soybean, sweet potato, grapevine, Mexican husk tomato and maize, playing important ecological roles (Marquez-Santacruz et al., 2010). Other genera such as *Xanthomonas* and *Pantoea* are also endophytes of plants; however, some species are plant pathogens, which mean that potential phytopatogenic microorganisms are also resident of the rhizosphere of wheat crops. Another major portion of the library was the presence of ribosomal sequences belonging to non-culturable bacteria. This suggests that even though the vast majority of sequences belong to rhizospheric bacteria, there are still a significant proportion of organisms that could not be detected by culture methods.

Conclusions: The analysis of the Shannon-Wiener diversity of the 16S rDNA library from rhizosphere of wheat plants suggests that this environment is highly diverse, which is a prerequisite for other metagenomic studies to discover genes of interest with some application in agricultural biotechnology. Also, the pursuit of relevant metabolic activities are currently carrying out in the library reported in this work, as well as ongoing genomic cloning of more genetic material from the metagenome of wheat rhizospheric soils from crops of Mexico, which represent a relevant option to study due to its high microdiversity. Finally, functional analyses are carrying out to search for genes of agrobiotech interest.

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BIOENERGY AS AN EXCELLENT CHOICE OF RENEWABLE ENERGY

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Abstract: Environmental pollution and global warming associated with the use of fossil fuels has forced the search for alternative fuels, the Biomass is considered the renewable energy source with the highest potential to contribute to the energy needs of modern society for both the industrialized and developing countries worldwide. The most important biomass energy sources are wood and wood wastes, agricultural crops and their waste byproducts, municipal solid waste, animal wastes, waste from food processing, and aquatic plants and algae. Currently, much research has been focused on sustainable and environmental friendly energy from biomass to replace conventional fossil fuels. Biomass yield and its potential differs between countries, however, many technologies have been studied in recent years for their possible use with biomass, such as combustion, pyrolysis, gasification and liquefaction. The objective of this research is determinate the potential and the uses of biomass energy and their contribution to the sustainable energetic development throughout the world.

Keywords: Bioenergy ● Renewable energy ● Sustainability.

Introduction: The environmental pollution and global warming associated with the use of fossil fuels, has forced a search for alternative fuels. Renewable energy is energy generated from natural resources such as water, sunlight, wind, rain, tides, geothermal and biomass sources. Renewable energy sources are continually and naturally replenished in a short period of time. Solar cells, wind turbines, biofuels and emerging technologies are poised to become major energy sources throughout the world. Renewable energy has an important role in the industry, business and, households as providing modern energy access to the billions of people in developing countries that continue to depend on more traditional sources of energy. Worldwide there is a growing interest in the use of solid, liquid and gaseous biofuels for energy purposes. There are various reasons for this, such as: i) political benefits (for instance, the reduction of the dependency on imported oil); ii) employment creation (biomass fuel create up to 20 times more employment than coal, gas and oil; and iii) environmental benefits such as mitigation of greenhouse gas emissions, reduction of acid rain and soil improvements (van Loo and Koppejan, 2008).

Biomass is a term for all organic material that stems from plants (including algae, trees and crops). Biomass is produced by green plants converting sunlight into plant material through photosynthesis and includes all land- and water-based vegetation, as well as all organic wastes. The biomass resource can be considered as organic matter, in which the energy of sunlight is stored in chemical bonds. When the bonds between adjacent carbon, hydrogen and oxygen molecules are broken by digestion, combustion, or decomposition, these substances release their stored chemical energy. Biomass has always been a major source of energy for mankind and is presently estimated to contribute of the order 10–14% of the world's energy supply (McKendry 2002).The objective of this research is determinate the potential and the uses of

biomass energy and their contribution to the sustainable energetic development throughout the world.

Materials and Methods: Some papers were studied to determinate the trade-offs between different source of bioenergy and its environmental impacts. Energy source, technology, water footprint and gas emissions were considered. All the reported data about biomass generation or about greenhouse gas emissions were obtained from greenhouse experiments. The details of the experimental design and the methods used to characterize the soil and biomass can be found in the next papers: Fernández-Luqueno et al. (2009), Fernández-Luqueno et al. (2010), Mendez-Bautista et al., (2010), and Juarez-Rodriguez et al., (2012). The amounts of wastewater or sludge added to soil varied although they were according to traditional fertilization rates.

Results and Discussions: Anaerobic digestion, also called methane fermentation, offers practical solutions for the treatment of high-strength organic wastes such as sewage sludge (Appels et al., 2008), municipal solid waste (Ismail and Abderrezaq, 2007) livestock wastewater (Cantrell et al., 2008), food waste (Kim et al., 2007) and dedicated energy crops (Amon et al., 2007). It is well known that carbohydrates, lipids and proteins are among the main components of biodegradable matter. Of these, carbohydrates are known to be easily and rapidly converted via hydrolysis to simple sugars, and subsequently fermented to volatile fatty acids (VFAs) (Miron et al., 2000).

Additionally, second generation microalgal systems have the advantage that they can produce a wide range of feedstocks for the production of biodiesel, bioethanol, biomethane and biohydrogen. Algae are an extremely diverse group of organisms, and it is not surprising that different species of algae produce different compounds that could be used as alternative fuel feedstock. Five commonly studied algal components or products useful for alternative fuels are: lipids for petroleum fuel substitutes, carbohydrates for ethanol, hydrogen, methane via biomass gasification, and biomass for direct combustion, anaerobic digestion, or thermochemical conversion.

Biodiesel is a renewable energy produced from algae, seeds or legumes. Assessments of the uptake of biofuels range between 20% and 25% of global transport road fuels by 2050 (Francisco et al., 2009).Biodiesel is currently produced from oil synthesized by conventional fuel crops that harvest the sun's energy and store it as chemical energy. This presents a route for renewable and carbon-neutral fuel production. However, current supplies from oil crops and animal fats account for only approximately 0.3% of the current demand for transport fuels. Increasing biofuel production on arable land could have severe consequences for global food supply. In contrast, producing biodiesel from algae is widely regarded as one of the most efficient ways of generating biofuels and also appears to represent the only current renewable source of oil that could meet the global demand for transport fuels. The main advantages of second generation microalgal systems are that they: (1) have a higher photon conversion efficiency (as evidenced by increased biomass yields per hectare); (2) can be harvested batch-wise nearly all-year-round, providing a reliable and continuous supply of oil; (3) are able to utilize salt and waste water streams, thereby greatly reducing freshwater use; (4) can couple CO_2-neutral fuel production with CO_2 sequestration; (5) Produce non-toxic and highly biodegradable biofuels. However, current limitations exist mainly in the harvesting process and in the supply of CO_2 for high efficiency production (Schenk et al., 2008). Recent wareness of CO2 emissions has resulted in a shift from less environmental friendly fossil fuels to renewable and sustainable energy alternatives (Gil et al., 2010). Among these, biomass is considered to be one of the few viable replacement options (Munir et al., 2009). Biomass can be grown in a sustainable way through a cyclical process of fixation and release of CO_2, there by mitigating global warming problems (McKendry, 2002). Biomass fixes CO_2 in the form of lignocellulosics during

photosynthesis and the CO_2 emitted from the combustion of these materials makes no net contribution to the accumulation of CO_2 in the atmosphere or to the greenhouse effect.
World production of biomass is estimated at 146 billion metric tons a year, mostly wild plant growth. Some farm crops and trees can produce up to 20 metric tons per acre of biomass a year. Types of algae and grasses may produce 50 metric tons per year (Demirbas, 2001). The conversion of biomass into energy can be achieved in a number of ways. To provide a fuel suitable for direct use in spark ignition gas engines, the fuel must be provided in either a gaseous, or a liquid form. Production of a gaseous fuel from biomass can be achieved by the application of a number of technologies, each with its specific requirements, advantages and disadvantages.

In general, the characteristics of the ideal energy crop are: a) high yield (maximum production of dry matter per hectare), b) low energy input to produce, c) low cost, d) composition with the least contaminants, e) low nutrient requirements. Desired characteristics will also depend on local climate and soil conditions. Water consumption can be a major constraint in many areas of the world and makes the drought resistance of the crop an important factor. Other important characteristics are pest resistance and fertilizer requirements.

Some 1.5 billion people worldwide still lack access to electricity, and approximately 2.6 billion are reliant on wood, straw, charcoal, or dung for cooking their daily meals (REN21, 2010), which shows that a cheap, friendly environmental source energy is necessary. Social and economic development production of renewable energy, particularly biomass, can provide economic development and employment opportunities, especially in rural areas, that otherwise have limited opportunities for economic growth. At this time there are many renewable energy technologies which supplies energy from sun, wind, water, waves, subsoil, microorganisms, plants, manure, sludge, domestic organic wastes, etc., biofuels is a wide area which produces energy such as power, biogas, biodiesel and, bioethanol. However, biofuels will be a viable alternative only if they provide a net energy gain, have environmental benefits, be economically competitive, and be producible in large quantities without reducing food supplies (Hill et al., 2006).

Fossil fuels provide 85% of the US energy requirements, a figure that is similar in most countries. Energy demands are increasing with population growth and economic development. This situation is not sustainable for several reasons; oil reserves are limited, and the increasing use of oil and coal leads to ever increasing CO_2 emissions, which carry the risk of climate change (Schelleret al., 2010). The use of fossil fuels is now widely accepted as unsustainable due to depleting resources and the accumulation of greenhouse gases in the environment that have already exceeded the “dangerously high” threshold of 450 ppm CO_2-e. To achieve environmental and economic sustainability, fuel production processes are required that are not only renewable, but also capable of sequestering atmospheric CO_2 so that biofuels are rapidly being developed.

Biomass encompasses among others, vegetation, energy crops, as well as biosolids, animal, forestry and agricultural residues, the organic fraction of municipal waste and certain types of industrial wastes. Its appeal is due to its potential worldwide availability, its conversion efficiency and its ability to be produced and consumed on a CO_2-neutral basis (Cebrucean et al., 2010). Such as waste-to-energy plants offer both generation of clean electric power and environmentally safe waste management and disposal (Iakovou et al., 2010).

Many research efforts document the current and potential role of biomass in the future global energy supply (e.g. Parikka, 2004; Yamamoto et al., 2001). Theoretically, the total bio-energy contribution (combined in descending order of theoretical potential by agricultural, forest, animal residues and organic wastes) could be as high as 1100 EJ, exceeding the current global energy use of 410 EJ (Hoogwijk et al., 2003). (Berndes et al. 2003) further reinforce this potential of biomass in the future global energy supply by analyzing and synthesizing earlier studies on the subject. However, a careful analysis of all the related literature reveals that there

is no consensus regarding the biomass potential among the researchers, but rather their assessments differ strongly. One of the most critical bottlenecks in increased biomass utilization for energy production is the cost of its logistics operations.

Conclusions: It is evident from the study that biomass can only replace conventional energy to some extent due to economic, social and other constraints, this is true for developed countries due to their huge energy demand compared with what biofuel options are able to supply as well as for developing countries due to the low yield of their agriculture and competition for land and water for food production. However, biomass may contribute to optimizing the energy and resource balance of agricultural, livestock, or industrial production systems at an appropriate scale. The beneficial effect of regenerating energies not only regarding the environment protection, but also in economical and social domain, thus biomass could be recommended as a good renewable-energy source throughout the world.

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IMMUNITY RESPONSE BY SECOND- AND FOURTH-INSTAR LARVAE OF FOUR LEPIDOPTERAN PESTS AFTER *BACILLUS THURINGIENSIS* EXPOSURE

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Abstract: Insect's immune response against microbial pathogens includes phenoloxidase activity, but its relationship with microbial bioinsecticides efficacy is little known. This study was undertaken to evaluate phenoloxidase activity of second- and fourth-instar larvae of the tobacco budworm *Heliothis virescens* Fabricius, Indian meal moth *Plodia interpunctella* (Hübner), beet armyworm *Spodoptera exigua* (Hübner), and cabbage looper *Trichoplusia ni* (Hübner). Larvae were exposed to the LC_{50} value of the *Bacillus thuringiensis* (Bt) spray Biobit®. Biobit mortality by fourth-instar exposed larvae was not significantly different compared with unexposed larvae. Unexposed insects had a significantly higher phenoloxidase activity in pre–pupae and pupae than early–instar larvae and adults, whereas in adult females the phenoloxidase activity was higher than in males but exposed *P. interpunctella*, which second instar had 10 times more phenoloxidase activity than unexposed larvae. Correlation analysis between mortality percentage and phenoloxidase activity revealed significant *r*–values ($p < 0.01$) in 2nd instar *H. virescens* ($r = 0.979$) and *P. interpunctella* ($r = 0.930$).Similarly, the amount of total protein was lower in 4th instar Biobit–exposed *H. virescens* and higher in *S. exigua*. This information may be useful if the Biobit application period is timed in development stage with low phenoloxidase activity.

Keywords: *Heliothis virescens* ● *Plodia interpuctella* ● *Spodoptera exigua* ● *Trichoplusia ni* ● phenoloxidase activity

Introduction: Arthropods immunity relies on diverse mechanisms, both passive and innate. Innate immunity includes the prophenoloxidase (proPO) system, which is manifested in a series of cascading enzymatic reactions by the stimulation of peptidoglycan, β–glucans or lipopolysaccharides, and phenoloxidase (PO) enzyme activation. proPO is activated to PO by serine proteases and is responsible for initiating the biosynthesis of quinones to melanin (Ashida and Dohke 1980; Bidla et al. 2009). Melanin is a brown–black pigment that inhibits entomopathogenic bacterial and fungal enzymatic activity by encapsulation, as has been observed in Lepidoptera (Jiang et al. 1998), and may be related to the efficacy of certain bioinsecticides. An alternative to chemicals for Lepidopteran pests is the application of bioinsecticides, including *Bacillus thuringiensis* (Bt) commercial products. However, the immune response to biopesticides in arthropods may be related to their lack of efficacy. In the present study, Bt susceptibility differences were evaluated in the innate immune response based on PO activity in second- and fourth-instar larvae of four species including the tobacco budworm *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), Indianmeal moth *Plodia interpunctella* (Hübner) (Pyralidae), beet armyworm *Spodoptera exigua* (Hübner) (Noctuidae), and cabbage

looper *Trichoplusia ni* (Hübner) (Noctuidae). Two of these species, *P. interpunctella* and *T. ni*, were selected because they have demonstrated differences in their susceptibility to Bt in our laboratory (Rubio-Cota et al. 2010; Tamez-Guerra et al. 2006).

Materials and Methods

Insects

S. exigua and *H. virescens* colonies were established from field collected insects in Northeast Mexico in 2000, whereas the *T. ni* colony was obtained from Dr. Howard T. Dulmage (USDA-ARS, Weslaco, TX) and reared since 1982 in León on artificial diet as described in Tamez-Guerra et al. (2006). The *T. ni* colony has been crossed with field–collected Mexican populations every 5-7 years to avoid homocygamy–related problems. The *P. interpuctella* colonies were from the Center for Grain and Animal Health Research (Manhattan, KS) and reared in León on a previously described cracked wheat artificial diet (McGaughey and Beeman 1988). Insects were incubated at 25 ± 2ºC, 55-60 ± 10% RH, and 16:8 L:D photoperiod.

Biobit insecticidal activity

All substrates and chemicals were from Sigma–Aldrich (www.sigmaaldrich.com) unless otherwise specified. Initial tests were conducted to determine the fifty percent lethal concentration (LC_{50}) for insects exposed to Biobit HP 32,000 IU/mg potency (Valent Biosciences Corporation, www.valentbiosciences.com), produced from a Bt var. *kurstaki* strain from DuPont (www.dupont.com) using an overlayer bioassay (Tamez-Guerra et al. 2006). Bioassays were performed in triplicate by exposing 30 neonates of each insect to six Biobit concentrations, prepared as serial doses (diluted 1:2) in distilled water. For *H. virescens*,the highest concentration tested was 0.16 IU/cm^2; for *T. ni*, 0.19 IU/cm^2; and for *S. exigua*, 1.9 IU/cm^2. Thirty-five µL of each dose or distilled water only used as a control, were applied to 5 mL wheat germ artificial diet, 7.1 cm^2 surface area. Doses were air dried for 30 min and then infested with two neonates per cup with *S. exigua* or *T. ni*, or one per cup with *H. virescens*. The insecticidal activity of Biobit against *P. interpunctella* was determined using a diet–incorporated bioassay with six doses (0, 0.6, 1.2, 2.4, 4.8, 9.6, and 19.2 µg/g of diet) (McGaughey and Johnson 1992). For this assay, Biobit doses were prepared by incorporating 1.5 mL of each Biobit dose into 5 g of wheat-germ diet and allowed to air dry, and then infesting with 10 *P. interpunctella* neonates in triplicate. Treatments were incubated at 28°C and 14:10 L:D photoperiod. To calculate LC_{50} values for Biobit, mortality data for each lepidopteran species were evaluated after five days and analyzed using POLO-Plus (LeOra 2007).

PO activity in unexposed and Biobit–exposed larvae

PO activity was measured from the hemolymph of different developmental stages of *P. interpunctella*, *H. virescens*, *S. exigua*,and *T. ni* using a technique first described by Ashida (1971) and Seed et al. (1978), and modified by Harizanova et al. (2004). The bioassay was conducted with 2nd or 4th instar larvae of each lepidopteran species, using the LC_{50} values for Biobit–exposed neonates (Table 1), and using the overlayer bioassay for *H. virescens*, *S. exigua*, and *T. ni*, or the diet–incorporation bioassay for *P. interpunctella* as previously described. In these bioassays, 40 larvae of each insect species, either 2nd or 4th instar, were incubated for 24 hours on either the control or Biobit–treated diet; 20 larvae were used for insecticidal activity (IA) determination and the other 20 for PO activity and protein analysis.

For PO and protein analyses, hemolymph was collected by gently removing an anterior proleg, using a 14 cm sterile entomological dissection scissor. Hemolymph was collected directly into a chilled 1.5 mL microcentrifuge tube on ice (Shelby and Popham 2006) and was diluted 1:24 with ice–cold PBS. Hemolymph was frozen for 48 h to lyse the hemocytes and release the inner–cell plasma. Samples were thawed and centrifuged at 5000 rpm for 1 min to separate the

plasma containing PO. Fifty microliter aliquots of plasma sample were placed in a microplate well, and 150 µL of 10 mM DL-DOPA were added to each well as substrate. PO activity was measured and calculated as previously described. Two hundred microliters of substrate 10 mM DL-dihydroxyphenylalanine (DL-DOPA) were added to each well. Microplates were incubated in the dark at room temperature, and absorbance was read at 490 nm every 5 min for 30 min, using a microplate reader (Multimode detector DTX 880, Beckman Coulter Inc., Austria). As a negative control, phosphate buffered saline (PBS, 1.48 g of Na_2HPO_4, 0.43 g of NaH_2PO_4, 7.2 g NaCl, 1000 mL distilled water, pH 7.2) with substrate only was monitored over the same time periods and was subtracted as background. PO-specific activity was defined as the change in optical density over time. Tests were in triplicate with insects from different rearing lots. Data were analyzed using ANOVA posthoc Tukey α = 0.05 SPSS version 17.0 (SPSS, 2008). This bioassay was performed in triplicate.

Protein determination was performed in triplicate using the diluted hemolymph from the PO activity assay (Bradford 1976). A standard curve was prepared with standard concentrations of 12 serial BSA dilutions, from 0.0 to 2.0 mg/mL, using PBS as diluent. For treatment analysis, 5.0 µL of diluted hemolymph from each sample and 200 µL of Bradford reagent were mixed and transferred to a 96–well plate. Absorbance was read in a spectrophotometer (Beckman Coulter Inc.) at 595 nm 2-10 min after mixing. The reaction setting time was selected to allow adequate reaction development, but no longer than 10 min to prevent oxidation. Total protein was calculated by comparison to the standard curve value.

Correlation analysis

Correlation analyses comparing insecticidal activity versus PO activity, total protein versus PO activity, or insecticidal activity versus total protein of 2nd and 4th instar larvae from unexposed or Biobit–exposed insects was performed by using Pearson's analysis (SPSS, 2008) using a cutoff for significance of $p < 0.05$.

Results and discussion: In the present study, we evaluated the relationship among Bt susceptibility and PO activity levels (as representative of the innate immune response) among laboratory colonies of four selected Lepidoptera. Samples included hemolymph from unexposed or Biobit-exposed 2nd- and 4th-instar larvae. Of the species tested, *H. virescens* was the most susceptible lepidopteran to Biobit, whereas the least susceptible was *P. interpunctella*. The percent mortality of 2nd instar larvae exposed to the LC_{50} value of Biobit was similar to the predicted 50% in all of the insects tested, except *S. exigua* in which mortality was only 20% (Table 1).

Table 1. Fifty percent lethal concentrations (LC_{50}) for Biobit in 4 lepidopteran pests.[1]

Species	LC_{50}	C.I. (95%)	X^2	Slope	S.E.
Heliothis virescens	0.023 µg/cm^2	0.001-0.024 µg/cm^2	4.57	0.33	0.18
Plodia interpunctella	1.24 µg/g	0.829-6.184 µg/g	4.87	1.05	0.22
Spodoptera exigua	0.402 µg/cm^2	0.318-0.623 µg/cm^2	6.6	2.6	0.3
Trichoplusia ni	0.046 µg/cm^2	0.023-0.089 µg/cm^2	4	1.9	0.23

Although the product recommendation dose is similar for *H. virescens* and *S. exigua* (http://www.pro-agro.com.mx/prods/valent/valent02.htm), we observed that *H. virescens* susceptibility was 17-fold higher than that of *S. exigua* in the laboratory. Biobit contains a mixture of Cry toxins 1Aa, 1Ab, 1Ac, 2Aa, and 2Ab). According to previous bioassays, *H. virescens* and *T. ni*were more susceptible to Cry1A and 2A toxins, and *S. exigua* was not susceptible to any of

the Biobit toxins (Table 2), whereas *P. interpunctella* was susceptible to all Cry1A toxins.The different bioassay method, in which the toxin was incorporated into the diet instead of concentrated on the surface, may have contributed at least in part to an increased LC_{50}. In our study, Biobit was not effective with 4th instar larvae. Similarly, Kwon and Kim (2008) reported that 5th instar *S. exigua* larvae exposed either to Bt svar. *kurstaki*(Btk, Thuricide®) or to Bt svar. *aizawai* (Bta GB413, GreenBioTech Chungju, Korea) showed no differences in mortality compared with that of unexposed controls.

Table 2. Insecticidal activity, phenoloxidase activity, and total protein (mg/mL) of 2nd or 4th instar *Heliothis virescens, Plodia interpunctella, Spodoptera exigua* and *Trichoplusia ni* larvae, either control or exposed to Biobit for 24 h.[1]

Insect Species	Treatment	2nd Instar Larvae[2]			4th Instar Larvae[2]		
		IA ± SEM	PO ± SEM	Protein ± SEM	IA ± SEM	PO ± SEM	Protein ± SEM
H. virescens[3]	Control	1.11 ± 1.11 b	0.38 ± 0.02 b	1.42 ± 0.03 c	0.00 ± 0.00 b	1.45 ± 0.16 a	1.99 ± 0.00 a
	Biobit-exposed	50.0 ± 3.33 a	0.76 ± 0.02 b	1.46 ± 0.11 c	3.88 ± 2.00 b	0.64 ± 0.02 b	1.78 ± 0.09 b
P. interpunctella[4]	Control	2.22 ± 2.22 b	0.07 ± 0.00 c	1.35 ± 0.01 c	0.00 ± 0.00 b	0.83 ± 0.02 a	1.95 ± 0.01 a
	Biobit-exposed	44.4 ± 4.44 a	0.67 ± 0.05 b	1.59 ± 0.01 b	4.44 ± 2.22 b	0.92 ± 0.03 a	1.92 ± 0.07 a
S. exígua[5]	Control	1.11 ± 1.11 b	0.42 ± 0.01 a	1.43 ± 0.02 b	0.00 ± 0.00 b	0.36 ± 0.07 a	1.45 ± 0.01 b
	Biobit-exposed	20.0 ± 5.77 a	0.46 ± 0.05 a	1.42 ± 0.03 b	1.11 ± 1.11 b	0.36 ± 0.03 a	1.74 ± 0.10 a
T. ni[6]	Control	2.22 ± 1.11 b	0.09 ± 0.04 b	1.66 ± 0.04 b	1.11 ± 1.11 b	0.23 ± 0.01ab	1.99 ± 0.01 a
	Biobit-exposed	45.6 ± 4.00 a	0.21 ± 0.04 b	1.73 ± 0.08 b	5.55 ± 0.55 b	0.38 ± 0.03 a	1.99 ± 0.01 a

[1]Larvae were exposed to the Biobit LC_{50} for each insect species for 24 h (Table 1).

[2]IA (%) = insecticidal activity as defined by percent mortality; PO = phenoloxidase activity (dopachrome formation detected at 490 nm); Protein = mg total protein per mL of hemolymph; SEM= standard error of the mean. Different letters in the same rows/group (IA, PO or protein) means that are significantly different at significance of $P \leq 0.05$, using Tukey HDS test based on *Post Hoc* multiple comparisons, by ANOVA analysis (SPSS, 2008). Values represent the average of three replicate determinations.

[3]ANOVA: IA, F =143.47, df=3.8, P<0.001; PO, F =29.72, df=3.8, P<0.001; Protein, F = 39.71, df=3.8, P<0.001

[4]ANOVA: IA, F =60.61, df=3.8, P<0.001; PO, F =177.70, df=3.8, P<0.001; Protein, F = 208.79, df=3.8, P<0.001

[5]ANOVA: IA, F =10.39, df=3.8, P =0.004; PO, F =1.62, df=3.8; P =0.27; Protein, F =12.11, df=3.8, P =0.002

[6]ANOVA: IA, F =97.12, df=3.8, P <0.001; PO, F =12.49, df=3.8; P =0.003; Protein, F =40.60, df=3.8, P <0.001

However, if they applied the immune suppressor benzylideneacetone, mortality increased to 60 and 80% with Bta– and Btk, respectively. Previous reports have indicated that the earlier stages of Lepidoptera were more susceptible to Bt toxins (Huang et al. 1999). In our study, this was also true in all species except *S. exigua*. PO activity was significantly lower in 2nd instar *P. interpunctella* larvae compared with that of *H. virescens*, *S. exigua*, and *T. ni*. However, exposure to Biobit resulted in significantly increased PO activity only in 2nd instar *P. interpunctella*, and may correlate to the relatively higher LC_{50} for this lepidopteran. Nwanze et al. (1975) found that a dose of 25 mg/kg of Dipel® diet in a diet–incorporation whole wheat bioassay resulted in 100% mortality of 1st instar *P. interpunctella* larvae, whereas 200 mg/kg was needed to obtain the same mortality level in larvae 18-21 days old. Furthermore, the most Biobit–sensitive insect in our test, *H. virescens*, had significantly lower PO activity when 4th instar larvae were exposed to Biobit (Table 2). PO levels increased in succeeding larval instars of *S. littoralis* and *P. interpunctella* (Ishaaya et al. 1974; Hartzer et al. 2005; Valadez-Lira et al. 2010). With *P. interpunctella*and *S. exigua*, an increase in immune response was related to decreased susceptibility to entomopathogens (Gassmann et al. 2009). However, increased tolerance to Btk resulted in a reduced immune response and lower PO activity in Bt–susceptible vs resistant *T. ni* (Ericsson et al. 2009). We found that PO activity and total protein in Lepidoptera species were affected differently by Bt exposure. In general, the total protein was higher in 4th instar compared with 2nd instar larvae, and increased in Biobit–exposed 2nd instar *P. interpunctella*and 4th instar *S. exigua* larvae, whereas total protein was lower in Biobit–exposed 4th instar *H. virescens* larvae (Table 2).

Conclusion: We conclude that phenoloxidase protects Lepidoptera larvae from *Bacillus thuringiensis* more effectively during later instars, but this is variable among species.

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BIOTECHNOLOGY-DERIVED PRODUCTS FOR INSECT PEST CONTROL

Patricia Tamez-Guerra and Robert W. Behle

Abstract: Efforts to produce microbial-based insecticides have resulted from development of new and improved methods in biotechnology. Microorganisms, metabolites from plants and microorganisms, and transgenic crops have been used to make biotechnologically-derived products for control of insects. New biotechnologies regularly confront major challenges to develop biological agents into pest control products by reducing the costs of production, and demonstrating efficacy comparable with current chemical pesticides. Although wide-spread adoption of biological insecticides is rare, each successful product supports continued research for the development of biological-based pest controls.

Agents of Bioinsecticides. Many entomopathogens are used directly as bioinsecticides including bacteria, viruses, fungi and nematodes. Most commonly, a selected agent is produced in mass and applied to a specified environment for control of a target insect pest using a conventional insecticide paradigm. However, several agents provide highly effective insect control by indirect applications made available by biotechnology manipulation of production system (e.g. transgenic crops).

Advantages of Bioinsecticides. Biological insecticides that utilize microbial agents as the active ingredient have an obvious safety advantage over chemical insecticides. Microbial agents are highly specific to infect insects, although individual microbes differ widely among host ranges. Microbial agents impart a low hazard risk to humans and non-target animal species. When compared with chemical insecticides, host specificities of bioinseticides result in less adverse impact on the environment, required less rigorous evaluations for human and ecosystem safety, produce non-hazardous waste during production, and are often easier to register as pesticide products. As a result of these advantages, the costs associated with developing microbial agents into insecticide products are often less than cost for developing chemical agents (Tamez-Guerra & Behle 2011).

Bacteria. Over 100 species of bacteria have been recognized that infect and kill insects. Insect death may be a result of either sepsis or toxins produced by the microbe. The most important bacteria for controlling insect pests are from the Bacillaceae family, particularly, from the *Bacillus* genera. Among *Bacillus* bacteria, *Bacillus thuringiensis* (Bt) has provided more products than any other biological agent and remains a model for development of future microbial insecticides. During the sporulation process of the bacterium, a crystal-shaped protein (Cry) is produced. At the end of the sporulation stage, both spores and Cry protein (a protoxin) are released. Once a susceptible immature insect (larva) eats the Cry protein, specific gut enzymes, which only function in the alkaline conditions of the caterpillar gut, dissolve the protein producing the active ingredient or toxin. The toxin binds to specific receptors in the larva's gut, which disrupts the pest's digestive tract. Infected larvae stop eating and die 12 hours to 5 days after ingestion. It must be noted that Bt products were not an immediate success in terms of economical production and consistent field efficacy. First academic and government research, and then agroindustry research focused development programs to overcome these problems (Srivastava *et al.* 2009). Still, extensive list of commercial Bt products attests to the success of this research and development.

Biotechnology played a significant role in the development of Bt as an insecticide. A major step was the development of liquid culture production techniques that were efficient and inexpensive. Also, techniques were developed to distinguish among strains with varied Cry

proteins and demonstrating the differences in efficacy to target pests. Bt Cry proteins identification and description allowed for tailor-made products. The flexibility afforded by selection among Cry proteins and the ease of genetic manipulation of bacteria has allowed for development of a multitude of products. At least one bioinsecticide was produced by a transgenic *Pseudomonas fluorescens* (MPV®, Mycogen Corporation, San Diego, CA) to produce Cry protein, perhaps paving the way for subsequent use of Cry proteins in transgenic crops. Some of the first transgenic plants were made to express Bt genes and still are known as transgenic Bt-plants (i. e. Bt-maize, Bt-cotton, Bt-tobacco). These genetically modified plants produce their own "insecticide" (Cry proteins) without changing the crop product (grain, fiber, tobacco, etc.), nutritional nature, or representing any threat to the environment (Evans, 2004, Toenniessen *et al.* 2003, Sankula & Blumenthal 2004). Commercial adoption of transgenic Bt-crops opened the gates for development of transgenic plants for a wide variety of benefits including herbicide resistance.

Spinosad is another widely commercialized bacterial metabolite. Spinosad is the result of a mixture of two metabolites isolated from a naturally occurring soil actinomycete *Saccharopolyspora spinosa* (Crouse & Sparks 1998). The mixture of these two compounds (spinosyn A and spinosyn D which give insecticidal activity of the fermentation products) was first observed to kill mosquito larvae. These compounds were then characterized and traced to a family of novel macrocyclic lactones called spinosyns (Thompson *et al.* 2000). Spinosyns are produced by liquid fermentation. Vegetative inoculum of the bacteria is grown by a submerged aerobic fermentation process. Metabolites in the supernatant are collected, re-crystallized, dissolved in methanol, centrifuged or filtered (to remove solids wastes), concentrated by distillation and converted to salt by mixing with acidified water. Insoluble crystallized spinosad is dissolved by adding enough alkali to neutralize the solution (Sparks *et al.* 1998), and then formulated into pest control products.

Fungi. Entomopathogenic fungi are an effective tool for use as biological insecticides. Recently, 170 mycoinsecticide products were available commercially worldwide (Faria & Wraight 2007). Most of the products are based on fungi classified as Anamorphic Hypocraeles and more specifically on *Beauveria bassiana* (34%), *Metarhizium anisophilae* (36%), *Lecanicillium* spp. (9%) and *Isaria* (formally *Paecilomyces*) *fumosorosea* (6%) (Faria & Wraight 2007). In contrast with bacterial and viral pathogens, fungal agents have a different mode of action, target pests, and production techniques. These differences allow the fungal pathogens to target arthropod pests that are not susceptible to other biological insecticides. The mode of action of mycoinsecticides is characterized as contact activity because viable spores that contact a susceptible insect will germinate and infect the target insect directly through the cuticle. This differs from bacteria and viruses that must be ingested to initiate infection through the intestine. Zimmermann (2007) describes the infection process in six steps: 1) attachment of the spore to the cuticle, 2) spore germination, 3) penetration through the cuticle, 4) overcoming the host's immune defense response to infection, 5) fungus proliferation within the host (formation of hyphal bodies/blastospores), and 6) saprophytic outgrowth from the host cadaver and production of new conidia. Entomopathogenic fungi are known to produce secondary metabolites that are classified as toxins. Li *et al*. (2003) classified fungal toxins into two groups according to their structure and function: low molecular weight compounds including coylcodepsiptides, pigments, organic acids, and others, and higher molecular weight proteins including proteases and protein toxins. Examples of low molecular weight cyclic peptide toxins include Beauvericin and Oosperein (from *Beauveria*), and Destruxin (from *Metarhizium*).

Biotechnology has contributed to the use of fungi as biological insecticides primarily by developing efficient methods of production. Both solid and liquid substrate systems have been developed. Solid substrates generally utilize grain to grow the fungus until sporulation (production of conidia). These conidia are collected and formulated into products of use.

However, production of fungal agents in liquid media allows for production of several alternative structures for use as bioinsecticides including blastospores and microsclerotia (Jackson & Jaronski 2009). The ability to produce a variety of structures adds to the potential of developing products to target insect pests in a variety of environments including on plants, in soil and in structures. Also, fungus/insect interactions for development, pathogenicity, and diverse fungal lifestyles are being studied by utilizing targeted gene knockout techniques (Zhang *et al*. 2011).

Baculovirus. More than 500 insects are susceptible to infection by viruses. Members of the pathogenic family Baculovirus have been isolated from nearly every moth registered as a pest (Van Beek 2007). Based on this vast diversity, it is possible that viruses could be developed as a major strategy for future pest control. When a susceptible insect ingests active virus, virions (containing the virus's genes) initiate infection of intestinal cells. Once infected, the insect cells replicate the virus and these replicates infect additional insect cells, repeating the process until the insect is overcome by the infection. A successful infection often results in death of the insect within 5 to 15 days, depending on environmental conditions.

Perhaps the greatest commercial success of a virus-based insecticide to date has been the control of the velvet soybean caterpillar, *Anticarsia gemmatalis* (Hübner), in Brazil by the application of *Ag*NPV. Beginning in the early 1980's, a government supported program developed *Ag*NPV as an insecticide, and has successfully expanded the program to treat about 1.5 million hectares annually (Moscardi 1999). The manufacture of *Ag*MNPV insecticide products is the result a unique industry/government partnership. Companies collect infected larvae from the field to use as the active agent for the commercial insecticides. Samples of subsequent products are sent to EMBRAPA (Brazilian Agricultural Research Corporation) for verification of quality assurance. Once certified, these products are available for application by growers. The number of hectares treated annually distinguishes the use of *Ag*MNPV insecticides as a highly successful control program.

Development of virus-based insecticides has provided a platform for debate about the application of genetically modified organisms to the field environment. Currently, all viruses used for commercial insecticides are from natural sources. Yet, the simplicity of these DNA viruses coupled with the current understanding and ability to manipulate genetic material has lead to research efforts to improve the efficacy of natural viruses. Specific efforts were directed at improving the speed of kill by engineering genes encoding insect specific hormones, enzymes, or toxins (Hawtin *et al*., 1992). Also efforts were aimed at expanding the host range of specific viruses by transferring genes to improve infection rates in otherwise marginal target pests. Although technically successful, genetically modified viruses have not been commercialized due to ethical criticism and negative public perceptions. Biotechnology has contributed to successful *in vitro* production of baculovirus. Unfortunately, high media costs for current cell cultures contribute greatly to prevent commercialization of these techniques for bioinsecticide products.

Nematodes. The use of entomopathogenic nematodes (EPN) as bioinsecticides has been broadly studied since 1930´s. However, significant production, sales, and application as bioinsecticides began 15 years ago. Insecticidal activity of EPN's is caused by the symbiotic bacteria carried by the nematodes. Once a nematode enters a susceptible host, the bacteria are released to infect and kill the insect. Nematodes reproduce rapidly in the cadaver, producing many nematodes after a single infection. Ultimately, the body wall of the dead host insect ruptures and releases thousands of juvenile EPN, which represents the insect-infective stage (Kaya 1985). There are 3 known genera of EPN from the order Rhabdita (previously known as Neoaplectana), *Steinernema, Heterorhabditis,* and *Phasmarhabditis*. They contain the most important species of EPN. *Steinernema* juveniles enter the insect through natural openings, such as the mouth, spiracles and anus, and then penetrate into the body cavity, whereas *Heterorhabditis* can also enter by directly crossing the body wall. For Steinernematidae, the

bacterial symbiont is usually of the bacterial genus *Xenorhabdus,* and for Heterorhabditidae the symbiont genus is *Photorhabdus*. Both Steinernematid and Heterorhabditid nematodes are currently marketed for pest management programs against soil dwelling insect pests.

Biotechnology has been important in the development of *in vitro* production techniques for nematodes. Production in liquid medium can be done in small containers or in large fermentation tanks, similar to Bt. Greater numbers of juveniles can be produced per unit area in fermentation tanks, which makes this method especially suited for large-scale commercial production. Compared with other biological agents, fermentation of nematodes is a slow process and resulting in higher production costs simply due to the added time requirement in addition to the increased potential for contamination.

Conclusion: Microbial agents have demonstrated great versatility and capacity for control of target pest insects. Biotechnology has provided new tools for study and development of these agents by providing greater understanding of disease cycles and the ability to manipulate production and application systems for pest control.

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STUDY OF THE FERMENTATIVE CAPACITY AND ETHANOL PRODUCTION OF TWO MICROORGANISMS ISOLATED FROM BOVINE RUMEN

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Abstract: The aim of this work was to determinate the fermentation conditions for two wild yeast isolated from bovine rumen. A factorial design 3x3x2 evaluating temperature (35, 40, and 45° C), agitation (0, 100, and 200 rpm), and strain type (LR2 and LR4) was used. The analyzed responses were growth and fermentation parameters. Results showed that the best growth conditions were 35° C and 200 rpm for the development of the strains LR2 and LR4. On the other hand, the best results obtained in alcoholic fermentation were observed at the temperature of 45° C with no agitation for the strain LR2. The thermal stability of the strain LR2 may have a viable application in industrial fermentative processes in tropical climates.

Keywords: Fermentation ● Yeast ● Bioethanol.

Introduction: The ethanol production as an energetic alternative has been the mayor interest subject from the beginning of the oil crisis in the 70's. As a result, the necessity to produce ethanol with high yields using low cost materials and energy consumption has taken interest in recent years. In order to solve this problematic, several methodologies have been proposed. The use of microorganisms with the capacity to produce high concentration of ethanol or to metabolize 5 and/or 6 carbon sugars, the use of mixed cultures to increase sugars consumption and ethanol yields are some of the strategies reported (Tao et al., 2005). Some of the microorganisms capable to metabolize carbohydrates have been isolated from soil, decaying vegetable materials, industrial effluents, municipal wastes, manure, and rumen. However, their fermentative ability is related to their isolation environment (Arellano et al., 2008; Ten et al., 2004). This fermentative ability may also be affected by other factors such as temperature and agitation. Changes in temperature and/or agitation may negatively affect the process and as a result low yields or absence of the interest metabolite are observed (Arellano et al., 2008). The aim of this work was to determinate the fermentative conditions in synthetic medium for two wild yeast isolated from bovine rumen.

Materials and methods: Two wild yeasts (LR2 and LR4) isolated from fistulated bovine rumen from Yucatan, Mexico and in process of identification were used in this study. The factorial design 3x3x2 with 18 treatments is showed in Table 1. The evaluated factors were temperature (35, 40, and 45° C), agitation (0, 100, and 200 rpm), and strain (LR2 and LR4). A seed culture for each strain was carried out in a nine-milliliter test tube for 24h containing YPD medium (20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone of casein) and pH of 4.5 units. Temperature and agitation were set as mentioned in Table 1 for each essay. Seed cultures were inoculated in 250 mL Erlenmeyer flasks containing 90 mL of YPD medium. Cultures lasted 24 hours and kinetics parameters were obtained every 2 hours. Total microbial population was determined by direct method using a microscope (objective 40x) and a Neubauer chamber. Biomass quantification was obtained by dry weight method. The Dinitrosalicylic acid (DNS) method established by Miller (1959) was used to quantify the free reducing sugars. Ethanol

concentration was quantified by the technique of potassium dichromate of Bohringer and Jacob (1964). Maximal growth rate (μ_{max}), doubling time (td), and biomass yield (Yx/s) were the growth kinetic parameters estimated and the ethanol production kinetic parameters were product yield (Yp/s), maximal productivity (P_{max}), and fermentation efficiency. All results were statistically analyzed using the software Statgraphics Centurion XV.I.

Table 1. Factorial design 3x3x2.

	Encoded factors			Non-coded factors		
Treatments	A	B	C	Temperature (°C)	Agitation (rpm)	Strains
1	-	-	-	35	0	LR2
2	0	-	-	40	0	LR2
3	+	-	-	45	0	LR2
4	-	0	-	35	100	LR2
5	0	0	-	40	100	LR2
6	+	0	-	45	100	LR2
7	-	+	-	35	200	LR2
8	0	+	-	40	200	LR2
9	+	+	-	45	200	LR2
10	-	-	+	35	0	LR4
11	0	-	+	40	0	LR4
12	+	-	+	45	0	LR4
13	-	0	+	35	100	LR4
14	0	0	+	40	100	LR4
15	+	0	+	45	100	LR4
16	-	+	+	35	200	LR4
17	0	+	+	40	200	LR4
18	+	+	+	45	200	LR4

Results and discussion: Kinetic results of growth and alcohol production of every treatment are listed in Table 2. Highest values of dry weight and biomass yield were observed in treatment 16 and 7 (35° C and 200 rpm) with the strains LR4 and LR2 respectively (Table 2). For the alcohol production, the highest values of product yield, fermentation efficiency and alcohol production efficiency were observed in treatment 8 with the strain LR2 (40° C and 200 rpm) followed by the results obtained with the same strain in treatment 3 (45° C and 0 rpm).

Data were analyzed using ANOVA and the results are summarized in Table 3. These results indicate that temperature ant agitation had a significant effect in all the analyzed responses. They also suggest that the strain used had a significant effect in the ethanol production and doubling time. The interaction between temperature and agitation had a direct effect to all responses and the interaction of the three factors (temperature, agitation, and strain) had a significant effect to the ethanol production and doubling time.

In order to explain these effects, Figure 1(a) represents that the temperature, agitation, and their interaction have an effect statistically significant in the dry weight response (biomass). Contrary to that, there was no effect when the strain was changed. Figure 1(b) presents the fermentation efficiency between the strains tested. This chart exhibits a statistically difference between the strains, LR2 had better results than LR4.

Table 2. Kinetic results for growth and production for the strains LR2 and LR4.

Treatments	Max. Pop. ($x10^6$ el/mL)	Dry weight (g/L)	μ_{max} (h^{-1})	Td (h)	$Y_{x/s}$	Ethanol (g/L)	$Y_{p/s}$	Efficiency (%)	P_{max} (g/Lh)
1	179	1.87	0.34	2.10	0.10	7.94	0.43	84.30	0.79
2	129	1.73	0.27	2.53	0.09	7.53	0.38	74.50	0.63
3	227	1.53	0.26	2.63	0.08	8.70	0.45	88.24	0.54
4	293	2.80	0.60	1.15	0.15	8.20	0.43	84.30	1.03
5	248	2.83	0.38	1.81	0.15	7.99	0.42	82.40	0.80
6	272	2.70	0.58	1.20	0.14	8.04	0.43	84.30	0.67
7	439	4.22	0.57	1.22	0.22	7.62	0.40	78.40	0.76
8	124	3.90	0.45	1.55	0.20	9.40	0.50	98.03	0.94
9	0	0	0	0	0	0	0	0	0
10	53	1.70	0.34	2.04	0.09	8.00	0.42	82.40	0.80
11	31	1.70	0.41	1.70	0.09	7.40	0.39	76.50	0.62
12	0	0	0	0	0	0	0	0	0
13	130	2.99	0.69	1.00	0.16	7.62	0.42	82.40	1.10
14	95	2.14	0.41	1.70	0.11	6.88	0.36	70.59	0.57
15	105	3.30	0.55	1.25	0.18	5.90	0.32	62.70	0.42
16	219	4.72	0.55	1.26	0.25	7.80	0.41	80.40	0.78
17	142	3.12	0.43	1.63	0.17	6.20	0.33	64.70	0.52
18	0	0	0	0	0	0	0	0	0

Table 3. Results from ANOVA

	Dry weight (g/L)	μ_{max} (h^{-1})	Td (h)	$Y_{x/s}$	Ethanol (g/L)	Efficiency (%)	P_{max} (g/Lh)
Temperature (°C) - **A**	*	*	*	*	*	*	*
Agitation (rpm) - **B**	*	*	*	*	*	*	*
Strains - **C**			*		*	*	*
AB	*	*	*	*	*	*	*
AC			*	*	*	*	*
BC			*		*	*	
ABC			*		*	*	*

* Statistically significant difference at 95% confidence

Table 4. Most suitable conditions obtained from LSD test at 95%

Stage	Temperature (°C)	Agitation (rpm)	Strains
Growth	35	200	LR2, LR4
Alcoholic fermentation	45	0	LR2

During the fermentation, both strains showed similar results during their development (biomass concentration); however for the ethanol production the strain LR2 exhibited better results.

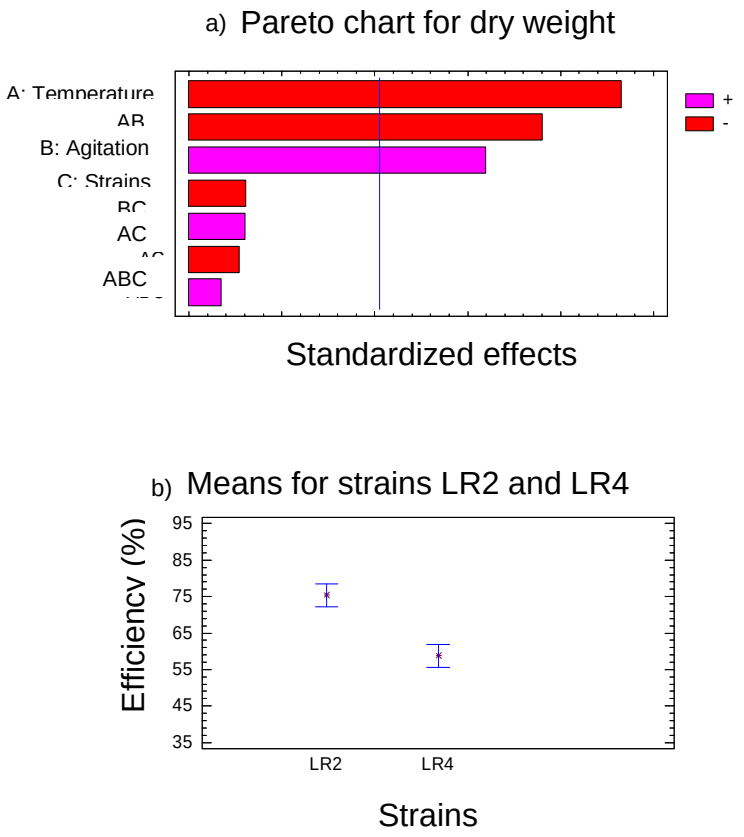

Figure 1. Pareto chart and mean for answers: dry weight and efficiency of alcoholic fermentation.

According to the obtained results and the statistical analysis, it is possible to suggest that the best fermentation conditions (growth and ethanol production) for the strains testes in this experiment are showed in Table 4.

Conclusions: The wild strains isolated from bovine rumen showed the ability to metabolize glucose to produce ethanol. These strains are statistically different concerning the ethanol production, however in the growth parameters they seem to be similar. Temperature and agitation were observed to have a significant effect in all the analyzed responses. Under the conditions of 45° C and no agitation the results obtained with the strain LR2 were the best for the ethanol production.

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ANTIMICROBIAL EFFECT OF A METHANOL EXTRACT OF A PLANT OF THE RUTACEAE FAMILY AGAINST *STREPTOCOCCUS MUTANS*

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Abstract: Because dental caries is one of the most common oral diseases and is considered a major public health problem, our study evaluated the *in vitro* antimicrobial potential of the methanol extracts of a plant of the Rutaceae family, against the major etiologic agent of dental caries, *Streptococcus mutans*. We measured the bacterial growth in liquid medium using the colorimetric technique (reduction of the tetrazolium bromide, MTT) and in solid medium by counting colony forming units (CFU). We found that the minimum inhibitory concentration (MIC) of the methanol extracts was 250 µg / mL ($p<0.05$) in liquid medium and 3.9 µg / mL ($p<0.05$) in solid medium.

Key words: Antimicrobial • plant extracts • dental caries• *Streptococcus mutans*

Introduction: It is recognized the importance of plants to modern medicine; for a long time natural remedies and medicinal plants were the main or even the only resource for the physicians. For all cultures and in all times, medicinal plants have been used as the basis of their own medicine (Núñez, 1982). Among the many diseases afflicting the world's population, infections, both bacterial and fungal diseases with inflammatory processes, which in some cases incapacitate the sufferer, represent a major group (Choi et al., 2003). There are several plants of the Mexican medicinal plants that exhibit antimicrobial activity and are used intreating various human diseases (Pushpam, 2004).

Oral diseases are still a major health problem worldwide (Petersen, 2005). Dental cariesis a transmissible infectious disease remains a major public health problem in many developing countries and in disadvantaged populations in developed countries (Mattos et al., 1998; Ramos et al., 2002). She was selected a plant of the Rutaceae family that might be important for the development of alternative treatments such as health strategy in general and dental health in particular.

In the present study, a plant of the Rutaceae family that might be important for the development of alternative treatments as a health strategy in general and dental health in particular was selected for.

Materials and Methods: The methanol extract was adjusted to a concentration of 1 mg/mL and was serially diluted, making three independent experiments. The evaluation of the antimicrobial effect *in vitro* was tested by the reduction method (MTT) assay in liquid medium in microtiter plates at an optical density of 570 nm, and by using the technique of counting colony forming units (CFU).

Results and discussion: The methanol extractobtained from a plant of the Rutaceae family, was observed to possess an MIC against *S.mutans* of 250 µg/mL ($p<0.05$), where the ranges of growth inhibition ranged from 35% to 63% (Fig. 1).

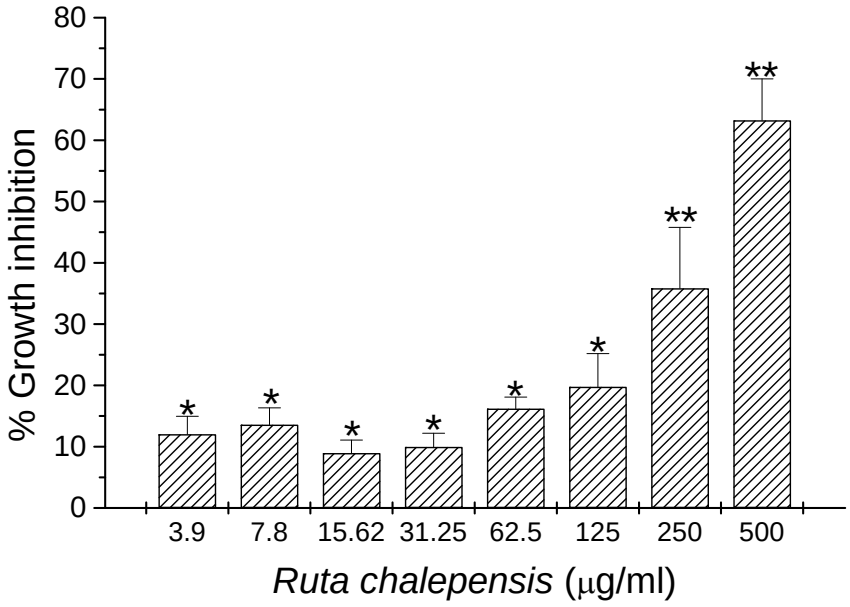

Figure 1. Effect of *R. chalepensis* on *S. mutans* growth by the MTT reduction technique.

By counting CFU it was observed that the MIC against *S. mutans* was 3.9 µg/mL ($p<0.05$), where the ranges of growth inhibition were 52% to 92% (Fig.2).

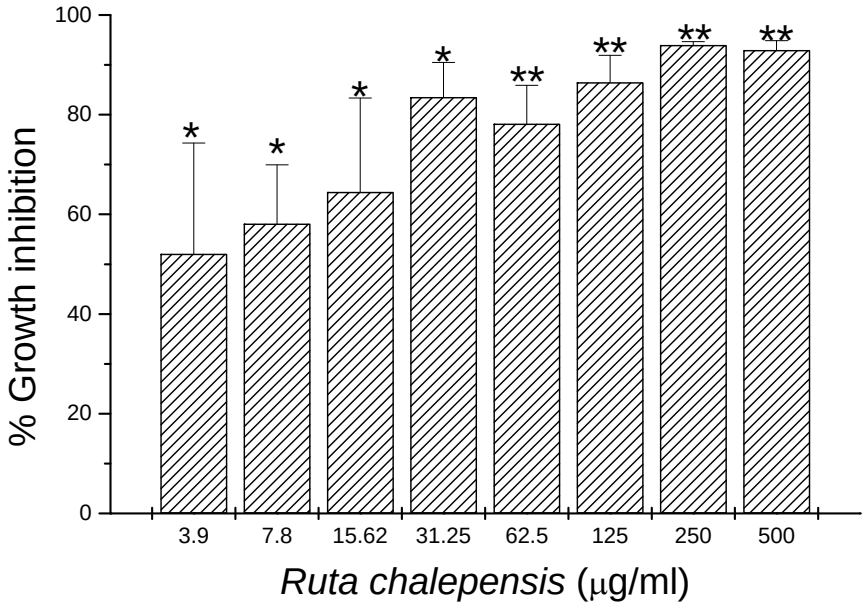

Figure 2. Effect of *R. chalepensis* on *S. mutans* growth by the CFU technique

Medicinal plants have been used as traditional treatments for many diseases that have affected humans for many years and in different parts of the world. Natural products derived from medicinal plants have proven to bean important source of biologically active compounds, many of which have been the basis for the development of new pharmaceuticals.

With regard to the diseases caused by microorganisms, because of the increasing resistance of many pathogens to common therapeutic agents used today, such as antibiotics and antiviral agents, a renewed interest in the discovery of new compounds to treat systemic and oral diseases.

Conclusion: In this study, we evaluated the inhibitory activity of a methanol extract of a plant of the Rutacea family against the *in vitro* growth of *S. mutans* (dental caries), and we observed that the plant showed significant *in vitro* inhibitory activity against *S. mutans*.

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ISOLATION OF CELLULOSE-HYDROLYTIC BACTERIA CAPABLE OF HYDROLYZING CITRUS PEEL WASTE

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Abstract: A cellulose-hydrolytic bacterium isolated from the rumen of Bos indicus was examined for their ability to hydrolyze citrus peel waste. Cellulose-hydrolytic ability was screenedusing microcrystalline cellulose as a carbon source and Congo Red Assay. The cellulose-hydrolytic bacterium was identified by 16 RNA like *Klebsiella* sp. This strain is a cellulolytic microorganism that produces large extracellular multienzyme complexes called cellulosomes in culture broth of citrus peel.

Keywords: Klebsiella sp, ● Citrus peel ● Hydrolysis

Introduction: For several decades the production of ethanol from biomass has been considered a laudable goal because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity. Cellulosic waste-materials including agricultural, forestry, and municipal wastes are among the Earth's most abundant and available renewable resources. The biological treatment for hydrolysis of cellulosic waste-materials involves the use of the whole organisms or enzymes in pretreatment. In order to reduce capital and operating costs, it is desirable to accomplish the fermentation of cellulose to ethanol in one step. Few species of bacterial microorganisms are capable of this conversion. In a previous study, microorganisms from the rumen of *Bos indicus* were isolated and the ability of these organisms for hydrolyze cellulose and citrus peel waste were tested. In this work a *Klebsiella* sp strain, capable of hydrolyzing citrus peel waste is reported. Orange peel is an organic fruit waste which is a completely waste and not used anywhere so we showed more interest in upgrading this waste for some value added products along with solving the disposal problems of waste.

Materials and methods:

Bacterial isolation and activity test. The bacterial strain used in the present study was isolated from rumen and identified as B-*20B*. The organism was maintained on agar slant with a composition in g/L of yeast extract 2, carboxymethyl cellulose CMC 5, $NaNO_3$ 0.5, K_2HPO_4 1, $MgSO_4$ $7{\cdot}H_2O$ 0.1, KCl 0.5, congo red 0.01 and agar 15. The pH of the medium was adjusted to 6.5 units. One loop of cells of the bacterial strain was transferred to 50 ml of nutrient broth medium prepared with distilled water in 250 ml flask and cultivated for 24 h under microaerophilic conditions. The culture was centrifuged at 10000 g and 4.0°C, for 10 min and cells were washed three times with sterile saline water. The washed cells were transferred to 50 ml of the cellulosome production medium which contained 2.0% orange peel flour, additions with phosphates; pH was adjusted to alkalinity and grown by shaking at 120 rpm and 37 °C, for two days. Biomass quantification was determined by UFC through time during growth. Cells from culture during growth were suspended in 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7) and broken twice in a fresh press using a mortar**.** The crude extract was centrifuged at 8,000 g for 20 min and then the bacteria were lyophilized. The resulting 100mg of protein per g of crude extract for cellulase assay.

Cellulase assay. The substrate used for measuring 1,4-β endoglucanase was 1% CMC in 0.05M sodium phosphate buffer (pH 7.0). The enzyme action was arrestedusing DNS. The absorbance was measured at 540 nm. One enzyme unit was defined as the amount of enzyme which releases 1 µM of glucose equivalent from substrate per minute.β-glucosidase was assayed using the previously described procedure (Oberoi et al. 2012).

Exopolygalacturonase, the major component of pectinase was assayed using citrus pectin as substrate. 0.5 ml of suitably diluted enzyme and 0.5 ml of 1% (w/v) pectin prepared in citrate buffer (50 mM, pH 4.8) were mixed by constant agitation and incubated at 50°C for 30 min. Galacturonic acid (GA) released was measured using the method described by Miller (1959) and its concentration was quantified from the GA standard curve. One international unit (IU) of enzyme activity was defined as the quantity of enzyme required to liberate 1 µmol of D-galacturonic acid per minute under standard assay conditions and was reported as (IU)

Results and discussion:

Congo Red Assay: Performing a congo red assay, yellow zones were obtained around the isolated colonies which were indicative of extracellular cellulase production by the cultures. (Figure 1)

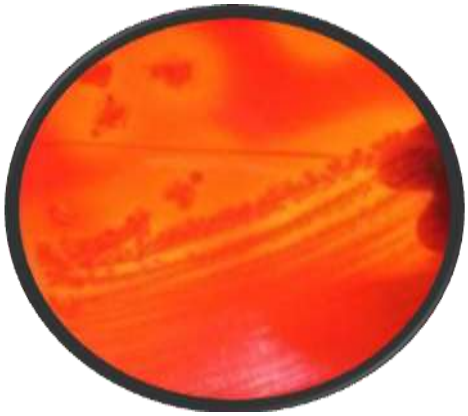

Figure 1. Screening for cellulase-producing microorganisms on carboxymethylcellulose (CMC) plates

The more active strain, originally isolated from the rumen of *Bos indicus*on cellulose medium, was selected for further study and was designated strain B-20B. This strain grew well on cellulose in both liquid and solid medium. Strain B-20B was found to have a fast growth. Under scanning electron microscope (SEM), cellulosamal structure is observed in culture broth of citrus peel (Figure 2). These observations suggest that B-20B strain is a strain of *Klebsiella* sp. sequencing of the intergenic spacer region ITS1 and subsequent BLAST search indicated 100% sequence identity to several strains of *Klebsiella*.

Maximum growth was observed at around 27 h, and the maximum activity was observed for 1,4-β endoglucanase at 36 h and pectinase at 24 h (Figure 3). The strain grew well on cellulose in both liquid and solid medium, although no zone of cellulose clearing could be seen on solid medium suggesting that large amounts of extracellular cellulase were not secreted by this strain. The late activity the standardization of the cellulosamales preparations was in the

Figure 4. The hydrolytic activity is reduced in 40 % as a result of Freeze dried, in relation to the fresh preparation.

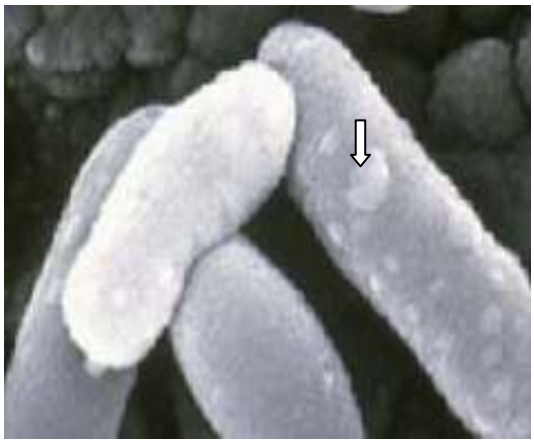

Figure 2 Scanning electron microscope of *Klebsiella* sp in culture broth of citrus peel

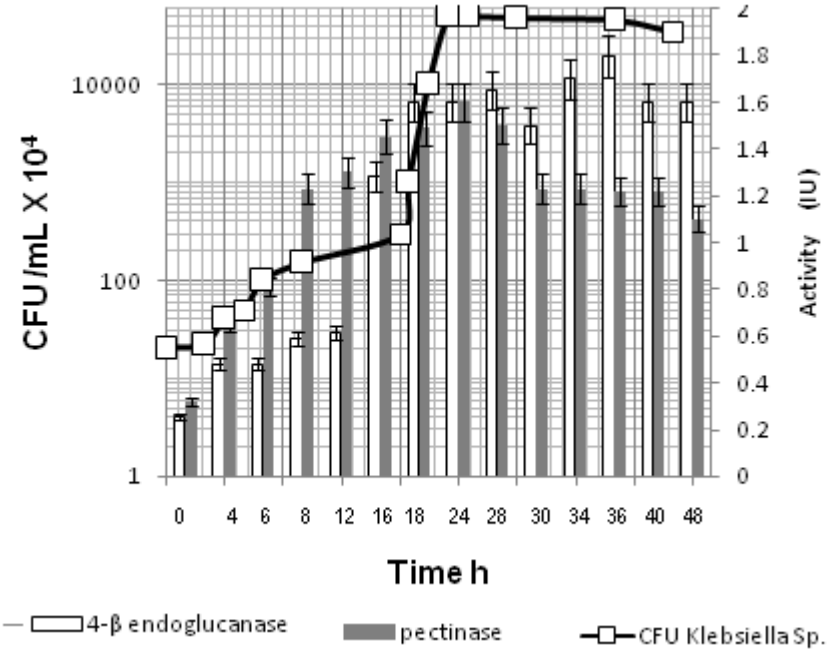

Figure 3.Growth kinetics and hydrolytic activity of *Klebsiella* sp in citrus peel waste

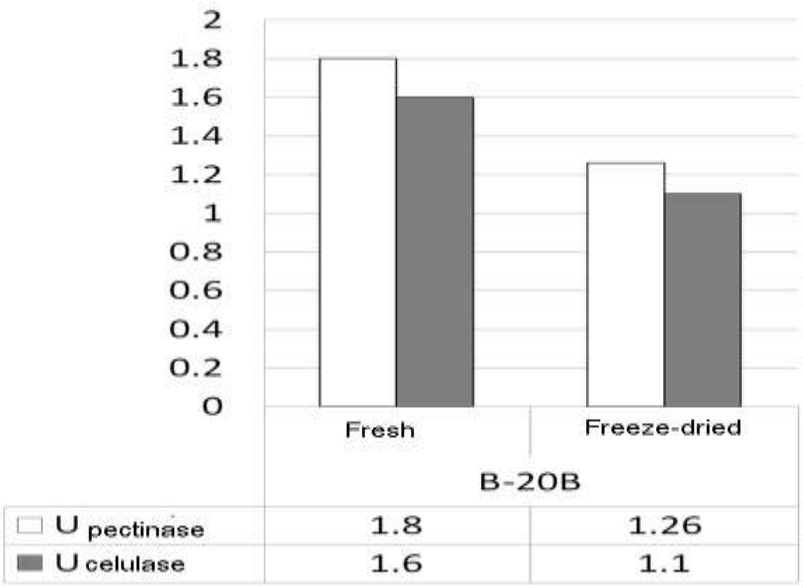

Figure 4. Hydrolyzing activity.

Conclusions: The ability of this new bacterial strain to use a wide variety of carbohydrates (including crystalline cellulose) combined with its minimal nutrient requirements and the availability of a genetic system suggests that the strain merits further investigation of its ability to convert biomass to ethanol. An optimal growth was observed in CMC and citric wastes.During the development of this strain a direct relation of a high hydrolytic activity of pectinase was observed at the beginning of the lag phase and during the cellulase activity was observed to increase in function to the number of cells and this later activity remains stable even after the dead phase.

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HUMORAL IMMUNE-RESPONSE AND ENZYMATIC ACTIVITY BY *HELIOTHIS VIRESCENS* AFTER *BACILLUS THURINGIENSIS* EXPOSURE

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Keywords: *Heliothis virescens* ● *Bacillus thuringiensis* ● Phenoloxidase ● Tysozyme ● Heliomicin

Abstract:

Heliothis virescens susceptibility to *Bacillus thuringiensis* (Bt) could be reduced by changes of the Cry toxin receptor aminopeptidase-N (APN) and/or enzymatic activity, but there is little information on the insect's immune response. The aim of the present work was to correlate the enzymatic activity and APN, along with humoral response components such as heliomicin (HEL) and lysozyme (LYS) and phenoloxidase activity of three *H. virescens* strains, after being cyclically exposed (1:3 generations) to the commercial Bt-product Biobit® DF. After two years, increased survival of larvae exposed to Biobit was observed, where the HvS laboratory strain showed the highest susceptibility and the US field-collected HvA strain was the least susceptible (LC_{50}=0.42, versus 1.178 UI/ cm^2, respectively). Enzymatic analysis revealed changes in the activity profile after strains exposure to trypsin- or chymotrypsin-like substrates and casein. A positive correlation between the PO activity and Biobit-susceptibility during pupa stage was determined. Results showed a correlation between the differences in susceptibility to Biobit with the enzymatic activity, the APN expression, and the humoral immune response mediated by HEL, LYS and PO activity among HvAX versus HvSX and HvMX *H. virecens* strains.

Introduction

Heliothis virescens (Lepidoptera: Noctuidae) is an important pest of several crops, including maize and cotton. *Bacillus thuringiensis* is a soil bacterium which produced delta endotoxins (Cry protoxins) effective in the Heliothines control, among other lepidopteran (ref). After Cry protoxins are ingested, insect´s enzymes selectively activate them to toxins by midgut enzymatic proteases, whereas midgut receptors define the Cry toxin host range (Schnepf *et al*. 1998). Changes in Cry receptors such as aminopeptidase-N (APN), cadherin and alkaline phosphatase, along with enzymes and other physiological and biochemical factors, have been associated with Bt resistance development by several Lepidoptera species, including *H. virescens* (Pigott & Ellar 2007, Jurat-Fuentes *et al.* 2011), but studies on the relationship between Bt-susceptibility and the insect' immune response are scarce (Ma *et al.* 2005, Tamez-Guerra *et al.* 2008, Shrestha *et al.* 2009). The aim of the present study was to determine the relationship between differences in susceptibility to the Bt bioinsecticide Biobit® DF, with their enzymatic activity, the presence of receptor APN, and the humoral immune response of three *H. virescens* strains, one from laboratory (used as susceptible control), and two US field-collected stains from the Bt-cotton belt area.

Materials and Methods

Insects

In the present study, eight *H. virescens* strains, four unexposed and four Biobit®-exposed were evaluated. *H. virescens* strains included a Mexican laboratory strain reared since 1975 which was provided in 2002 by José L. Martínez-Carrillo form the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) from Sonora, Mexico. This strain has been used as chemical-susceptible control in resistance-development by field populations in México (Terán-Vargas *et al.* 2005), and was coded HvS. Two *H. virescens* strains were kindly provided in 2003 by Dr. Carlos Blanco (United States Department of Agriculture, Agricultural Research Service, Southern Insect Management Research Unit, Stoneville, MS, USA), and coded HvA and HvM, and were collected from Mississippi Bt-cotton fields. Adults from these three strains were mixed in the same container and their progeny was coded Hvmix, using the same methodology previously described by *Trichoplusia ni* (Tamez-Guerra *et al.* 2008), and reared as a different strain. By 2008, all *H. virescens* strains were separated in unexposed and Biobit-exposed strains. Biobit-exposed strains were labeled with an "X" after the strain code to differentiate them from the unexposed ones, and were named HvSX, HvAX, HvMX and HvmixX.

Insecticidal activity

Bioassays performed in 2008 were conducted to determine the fifty- and ninety- percent lethal concentration (LC_{50} and LC_{90}) against neonates of unexposed or exposed to Biobit (Biobit HP, 32,000 IU/mg potency, Valent Biosciences Corporation, Libertyville, Illinois, USA, produced from a Bt var*. kurstaki* strain distributed by DuPont México, S.A. de C.V.), using an overlayer bioassay (Tamez-Guerra *et al.* 2006). To calculate LC_{50} and LC_{90} values, mortality data were analyzed using POLO-Plus (LeOra 2007). After that, 75 larvae of each strain were exposed to Biobit as neonates, using the LC_{50} values. When surviving larvae reached the pupae stage, about 15 females and 10 males were pooled to initiate the Biobit-exposed colonies (HvSX, HvAX, HvMX and HvmixX). After two generations of being unexposed, *H. virescens* neonates from each colony were again exposed to Biobit, and reared the surviving insects, making a cyclic exposure of 1:3 generations. After two sets of four 3:1 cycles (2009 and 2010), LC_{50} and LC_{90} for neonates of unexposed and Biobit-exposed *H. virescens* strains was calculated using a dose-response bioassay by triplicate.

Enzymatic activity

Neonates surviving from of each dose after cycling Biobit-exposure were tested to determine changes in the enzymatic activity among *H. virescens* strains due to Biobit exposure. Insect's midgut was obtained to evaluate the enzymatic activity from crude protease extracts. In this test and due to fitness cost (García-Careño *et al*. 1993), the number and size of used larvae to remove the midgut was different by each treatment (data not shown). Enzymatic activity was measured *in vitro* using the chromogenic synthetic substrates of trypsin and chymotrypsin, N-α-benzoyl-L-arginine-*p*-nitroanilide (BApNA) and N-succinyl-ala-ala-pro-phe-*p*-nitroanilide (SAAPFpNA), respectively, along with casein. Activity blots were performed as previously reported by Oppert *et al.* (1996). In brief, protease activity in gut extracts from each unexposed strain (HvS, HvA and HvM) or Biobit-exposed (HvS, HvA and HvM) and the progeny of adults from exposed strains (Hvmix) were mixed with loading buffer, and were subjected to electrophoresis in precast 12% Tris-glycine SDS polyacrylamide gels (BioRad) at a constant 35 mA at 4ºC, using a Hoffer mighty small 250 (Hoffer Scientific, San Francisco, CA). Pre-stained markers (Multimark, Invitrogen, Carlsbad, CA) were included to estimate the relative molecular masses of proteases. After electrophoresis, gels were transferred to nitrocellulose membranes, following methodology as previously described by Tamez-Guerra *et al* (2006). Protein substrate hydrolysis tests were performed using Dipel or casein as gel-incorporated substrates for the enzymatic activity in *H. virescens* larval midgut extracts. Enzymes in midgut extracts of each strain (unexposed or Biobit-exposed) were separated by 8% Tris-glycine SDS-PAGE in a Hoffer

mighty small 250 electrophoretic chamber, following methodology as previously described by Tamez-Guerra *et al* (2006).

APN, HEL, LYS and HvL4 molecular detection

This study was designed to evaluate the association between *H. virescens* susceptibility to Biobit and their humoral immune response along with the enzymatic activity and APN receptor changes. RT-PCR was used to detect APN, HEL, LYS and HvL4 genes as transcript amplification. To identify transcripts of the constitutive ribosomal protein HvL4 (as positive internal expression gene), PCRHvL4 was amplified using the primers HvL4A and HvL4B (Gene Bank: EU5231116.1) for an expected product of 400 bp; APN was amplified with AP1 and AP2 (Gene Bank: AF378666), for an expected product of 274 bp; HEL using the primers HEL1 and HEL2 (Gene Bank: 10092609), for an expected product of 221 bp; and LYS using the primers LYZ1, and LYZ2 (Gene Bank: U50551), for an expected product of 925 bp. APN, HEL, LYS and HvL4 transcripts detection was performed as previously described by Tamez-Guerra *et al.* (2008).

PO activity in unexposed and Biobit-exposed larvae

PO activity was measured from the hemolymph from different developmental stages of *H. virescens* strains, using a modified technique described by Ashida (1971). Tests were in triplicate with insects from different rearing lots. Data were analyzed using ANOVA posthoc Tukey α = 0.05 SPSS version 17.0 (SPSS 2008).

Results and discussion

In the present study, the susceptibility of three strains of *H. virescens* to Biobit and its relationship to the humoral immune response, enzyme activity and expression of APN were evaluated. None of the *H. virescens* strains not exposed to Biobit showed differences in susceptibility during the two years of exposure cycles. However, after two years of cyclic exposure of one generation and observing increased survival of larvae exposed to Biobit, we found that there were significant differences at confidence limits of 95% of the LC_{50} and LC_{90} values of Biobit required among the three strains tested, where the one showing the highest susceptibility to the commercial product was the HvS laboratory (Table 1), whereas the one showing the least susceptibility to this treatment was the HvA strain.

Table 2. Insecticidal Activity of Biobit (UI/cm^2) against *Heliothis virescens* neonates[1]

Strain/year	LC_{50}	CL_{95}	LC_{90}	CL_{95}	Slope	X^2	SEM	t
HvS09	0.187	0.173-0.587	6.711	5.985-455.2	0.87	0.587	0.196	2.246
HvS10	0.318	0.128-0.516	3.106	1.498-26.21	1.213	0.687	0.171	2.13
HvA09	0.152	0.011-0.391	6.232	5.418-385.6	0.90	0.663	0.194	3.451
HvA10	0.559	0.350-0.807	2.469	1.502-7.407	1.98	0.228	0.175	2.877
HvM09	0.206	0.011-0.543	1.607	0.985-4.702	0.91	0.685	0.189	5.201
HvM10	0.485	0.251-0.827	4.844	2.063-65.08	1.282	0.463	0.165	2.443
Hvmix09	0.355	0.221-0.496	1.825	1.157-4.482	1.801	0.760	0.167	4.843
Hvmix10	0.318	0.183-0.455	1.579	1.012-3.825	1.842	1.82	0.181	5.082
HvSX09	0.423	0.131-0.816	11.48	3.2-988.2	0.852	0.388	0.135	2.366
HvSX10	1.504	0.581-2.539	23.722	8.14-3712.5	1.070	0.586	0.159	-1.196
HvAX09	1.178	0.868-1.544	7.957	5.20-1559.1	1.545	2.795	0.099	-1.109
HvAX10	3.045	1.877-9.341	32.113	10.1-1196.3	1.253	2.873	0.132	-4.597
HvMX09	0.310	0.115-0.509	3.188	1.519-29.80	1.265	0.166	0.166	3.885
HvMX10	0.966	0.358-2.674	8.237	2.881-547.8	1.377	12.17	0.094	0.216
HvmixX09	0.321	0.118-0.532	2.229	1.182-12.86	1.523	0.655	0.197	3.818
HvmixX10	0.383	0.194-0.580	2.440	1.287-15.50	1.592	0.919	0.198	3.354

[1]POLO-Plus (LeOra, 2007), SEM = standard error of the median. Year 09= 2009; 10 = 2010.

The enzymatic activity of larvae exposed to different concentrations of Biobit showed that the only strain with differences in activity patterns was the HvM, with a less intense proteolytic activity observed compared with other strains, being more evident in the bands corresponding to ~ 27 and 37 kDa and 50 kDa for chymotrypsin (Fig. 1). Similar results were observed when repeating a chymotrypsin- or casein-type substrate zymogram plus Dipel (another Bt-based product widely used in Mexico with similar Cry toxins (data not shown).

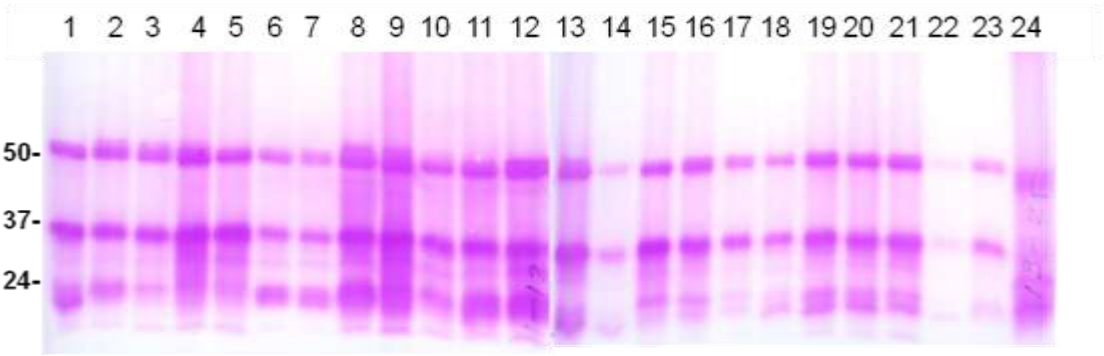

Figure 1. Activity blots of three *Heliothis virescens* strains showing chymotrypsin-like enzymatic activity, using SAAPFpNA as substrate. Left side indicates molecular weights. Lanes 1-12 shows Hvmix; lanes 13-21, HvM; lanes 22-24 shows HvS strains.

APN, HEL, LYS and HvL4 transcripts detection analysis showed the presence of APN in the gut of 3rd instar larvae of HvA strain not exposed to Biobit, but the HvAX strain did not amplify it. APN was detected in the Mexican strain HvS before and after Biobit-exposure (Table 2).

Table 2. Aminopeptidase-N (APN), heliomicine (HEL), lysozyme (LYS) and *Heliothis virescens* ribosomal protein L4 (HvL4) transcript amplification by gut or body (no gut) of three unexposed or Biobit-exposed *H. virescens* strains[1]. DNA relative amplification compared with the positive control HvL4 (ng)[2]

Strain /Sample	DNA (ng)[2]		
	APN	HEL	LYS
HvS-body	0	100	0
HvS-gut	100	0	0
HvSX-body	0	86	0
HvSX-gut	100	102	89
HvM-body	0	100	0
HvM-gut	132	0	110
HvMX-body	0	132	0
HvMX-gut	135	63	118
HvA-body	0	76	0
HvA-gut	154	92	102
HvAX-body	0	73	0
HvAX-gut	0	76	145

[1]Average of three replications
[2]Calculated values from densitometry software ImageMeter versión 1.1.1.

HEL is one of the few defensins that have been reported in *H. virescens* (Shrestha *et al.* 2009). In our study, it was observed the presence of the HEL transcript in the unexposed strains. Nevertheless, an increased amplification was observed in the gut treatments of the most Bt susceptible strains, the HvS and HvM, after being exposed to Biobit, whereas the HvA showed

no differences. We observed no LYS amplification in any body sample, and with the most Bt-susceptible strain (HvS) we amplified the transcript only after cyclic Biobit-exposure (Table 2).

In the present study, we did not observe an inhibition of PO activity after Bt exposure. In fact, for the *H. virescens* strain HvS it was observed that increasing the concentration of Bt induced an increase of PO activity (Table 3). With regard to the development phase of the insect, the prepupae from larvae exposed to Biobit showed significantly higher values of PO compared with non-exposed prepupae of insects (Table 3), whereas it was shown that the HvA strain (the one less susceptible to Biobit) had higher PO activity for both treatments. In contrast, pupas PO activity showed that unexposed HvS strain produced more PO than HvA pupae, whereas Biobit-exposed pupae from both strains showed no differences between them.

Table 3. Phenoloxidase production (dopachrome-DO- formation detected at 490 nm wavelength absorbance) by prepupae and pupae of two *Heliothis virescens* strains after Biobit exposure as neonates in 2010.[1]

Biobit doses (IU/cm^2)	Phenoloxidase production			
	HvS prepupae	HvS pupae	HvA prepupae	HvA pupae
0.0	0.61±0.01 b	1.26±0.02 a	0.66±0.01 c	0.78±0.03 c
0.16	Nd	Nd	0.63±0.00 c	0.82±0.05 c
0.32	0.66±0.02 b	0.83±0.00 d	1.25±0.02 a	0.88±0.11 bc
0.64	0.59±0.01 b	1.18±0.03 ab	Nd	1.14±0.02 ab
1.28	0.69±0.01 b	0.67±0.03 e	0.59±0.01 c	0.94±0.03 bc
2.56	1.03±0.05 a	1.00±0.03 c	0.89±0.03 b	1.28±0.03 a
5.12	0.61±0.02 b	1.07±0.04 bc	0.66±0.01 c	0.95±0.07 bc

[1]Tukey HDS test where different letters in the same column are different values at a significance of $P \leq 0.05$. Evaluation was based on multiple comparisons by *Post Hoc* analysis (SPSS Statistics 17.0). Mean is the average of three replications.

Conclusion

Taken together, our results showed a correlation between differences in susceptibility among *H. virecens* after cyclic exposure of Biobit, with the midgut enzymatic activity, the Cry gut receptor APN, and the humoral immune response mediated by HEL, LYS and PO activity.

Acknowledgements

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COMPARISON OF THE EFFECTIVENESS OF CONIDIA OF *BEAUVERIA BASSIANA* (BALS.) VUILLEMIN AGAINST *EPILACHNA VARIVESTIS* MULSANT PRODUCED IN 2 SUBSTRATES

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Abstract: The production of conidia of the entomopathogenic fungus *Beauveria bassiana* (Balsam) Vuillemin is usually done by biphasic fermentation, using a liquid whose product serves as inoculate to a solid substrate, such as rice grain, but it is not known if the product's effectiveness is the optimum. This study focused on the effectiveness of conidia of an isolation (Bb01) previously selected by its pathogenicity against *Epilachna varivestis* Mulsant produced in Sabouraud dextrose agar (SDA) with 0.5% of yeast extract, against biphasic fermentation in fried rice (BFR). In addition one evaluated the Bb37 isolation (monoconidial generated from Bb01) and commercial stock GHA (Mycotrol), as a positive control. The insecticide activity at $1x10^6$ and $1x10^7$ conidia/mL, was determined, inoculating 2 µL on larvae of the third instar of *E. varivestis*. Although differences between dose were not observed, after 10 days the ADS treatments caused 87, 73 and 50% of mortality, whereas those of BFR averaged 30, 30 and 0% for Bb01, Bb37 and GHA, respectively. These results indicate that the Bb01/Bb37 isolation is effective and has potential for biocontrol of *E. varivestis*, but it's necessary to establish a production methodology that allows to obtain great amounts (similar to the BFR) without reducing its effectiveness.

Keywords: *Epilachna varivestis* ● *Beauveria bassiana* production ● biphasic fermentation in rice ● biological control.

Introduction: In Mexico, of 1,887,177 hectares planted of bean only 1,630,224 are harvested with a grain production of 1,156,257 ton, with a yield of 0.71 ton/hectares (SIAP-SAGARPA, 2010). These decreases in the cultivation of beans are largely due to insect pests, the most important being the Mexican bean beetle (MBB) *Epilachna varivestis*, both in Mexico and the United States (Singh, 2001). MBB is an important defoliator because both adults and larvae feed on the underside of the leaves (Hammond *et al*, 2009). Entomopathogenic fungus like *Beauveria bassiana* (García-Gutiérrez *et al,* 1999) and *Isarea fumosoroseus* (Behle et al. 2006) have been reported as biological control of this insect. On the other hand, it has been seen that the effectiveness of these fungi is dependent on the environmental conditions (nutrients and temperature) during its growth. Generally, the production of *B. bassiana* is usually done by biphasic fermentation in a liquid medium, whose product is used to inoculate rice (de Faria and Wright, 2007), but it's necessary to find the conditions of growth that optimize the effectiveness of conidia thus produced. The objective of this study was to determine the effectiveness of conidia produced in Sabouraud Dextrose Agar (SDA) with 5% of yeast extract, against biphasic fermentation in rice (BFR), evaluating three isolates: two previously selected for its pathogenicity

against *E. varivestis*, the multiconidial Bb01, a generated monoconidia culture of the Bb01, the Bb37 (Ocampo-Hernandez *et al.,* 2011) and commercial stock GHA, used like positive control.

Materials and Methods:

Rearing of E. varivestis: Adults and larvae of diverse stages of *E. varivestis* were collected from bean fields in Ocampo, Guanajuato, MX, breeding was established in bean plants in greenhouse conditions: 27±3 °C, 70±10 % relative humidity and 12:12 hour light:darkness (Ocampo-Hernández *et al.* 2011).

Production B. bassiana conidia: The Entomopathogenic fungus used was provided by the Centro de Reproducción de Organismos Benéficos, Comité Estatal de Sanidad Vegetal de Guanajuato (CESAVEG), which they named Bb01 and its monoconidial culture Bb37, as well as the commercial stock of *B. bassiana* GHA (Mycotrol). The isolates were inoculated in plates with Sabouraud dextrose agar (SDA) with 0.5% of yeast extract, they were left incubating at 25ºC, in the dark until they displayed sporulation; to obtain conidia in biphasic fermentation in rice (BFR) Sabouraud dextrose broth was inoculated, then incubated at 25°C for 72 h in orbital agitation later 20 mL were inoculated in previously sterilized rice, it was incubated at 25°C and 70% relative humidity for 20 days. Once sporulation appeared, the conidia was mechanically sifted and left drying at room temperature until a 7-8% of humidity was obtained.

Viability of conidia: The viability of conidia was determined by the method of microculture in potato dextrose agar (PDA). A small sample of conidia of each of the fungi was placed in an Ependorff tube with sterile water with a dispersing agent (Inex A, 0.05%) and was mix for 1 min. A $1cm^2$ square of agar was placed on a slide on which a drop of conidial solution was added; finally a cover-slip was placed. The slide was incubated at 25°C with humidity for 17 h. The germinated conidia (viable) and the not germinated (non-viable) were counted in 5 fields in a optical microscope (40X), to calculate the percentage of viability. An average from the 5 fields and the used equation was: *% viability = [V/(NV+V)] X 100*.

Bioassays: In order to determine the insecticide activity against *E. varivestis*, 11 treatments were done, each consisting in ten of third instar larvae (9 days). The larvae were previously disinfected with a solution of hypochlorite of sodium 0.1%, they were rinsed twice in sterile water and dried. 2µL of a solution with Inex A 0.05% was applied along with 1) $1x10^7$ conidias/mL of Bb37 from SDA, 2) $1x10^6$ conidia/mL of Bb37 from SDA, 3) $1x10^7$ conidia/mL of Bb37 from BFR, 4) $1x10^6$ conidia/mL of Bb37 from BFR, 5) $1x10^7$ conidia/mL of Bb01 from SDA, 6) $1x10^6$ conidia/mL of Bb01 from SDA, 7) $1x10^7$ conidia/mL of Bb01 from BFR, 8) $1x10^6$ conidia/mL of Bb01 from BFR, 9) a solution of Inex A 0.05% like control, 10) $1x10^7$ conidia/mL of GHA from SDA as positive control, 11) $1x10^6$ conidia/mL of GHA from SDA as positive control. Each larva was individually placed in a Petri dish with leaves of bean as food (which was disinfected using sodium hypochlorite at 0.01% in distilled water and replaced on a daily basis) and moist cotton. They were incubated at 25°C for 10 days and was evaluated daily looking for mortality by the fungus (aerial mycelium or mummification). Repeats were conducted in three different occasions.

Results: Highest mortality was obtained in treatments where conidia were produced in SDA with 0.5% yeast extract. The mortalities observed were 87% and 73% for Bb01, 73% and 73% for Bb37, 50% and 47% for GHA using concentrations of $1x10^6$ and $1x10^7$ conidia/mL, respectively. While Bb01 and Bb37 from BFR using both concentrations gave mortality values around 30% as shown in Fig 1.

Discussion: The isolations of the entomopathogenic fungus of the CESAVEG turned out to be effective for the control of Mexican bean beetle as mention by Ocampo-Hernandez *et al.* (2011)

when they selected these isolates for biocontrol of *E. varivestis*. Nevertheless, in order to use these isolates in greater amount it's necessary to develop a suitable method of biomass production as is the mixed Fermentation (liquid-solid) in Rice (Jenkins and Goettel, 1997). In this work we compared the effectiveness of conidia of *B. bassiana* obtained by BFR and SDA against *E. varivestis* and when evaluating the treatments, SDA was significantly more effective. It is possible that the process and the conditions of obtaining conidia in BFR were not the optimal ones, since the ideal humidity of conidia is 5%, as Posada-Flórez (2008) mentions, whereas in this work the relative humidity reached a 7-8% in conditions not controlled. The difference in the percentage of mortality was not directly proportional with the concentrations. A possible explanation to this is that the chemical and mechanical interactions between cuticle of the fungus and conidia are diverse, as García-Gutiérrez *et al* (1999) proposed, when they examined the effectiveness of the isolated entomopathogenic fungus of diverse insects and was challenged against *E. varivestis*. Perhaps the addition of yeast extract has influence the effectiveness, as Safavi and collaborators propose (2007). In addition, in the future the use of desiccant agents could be implemented to reduce the relative humidity of the conidia, and establish its impact in the effectiveness. However, this could increase the production costs for the development of a commercial product.

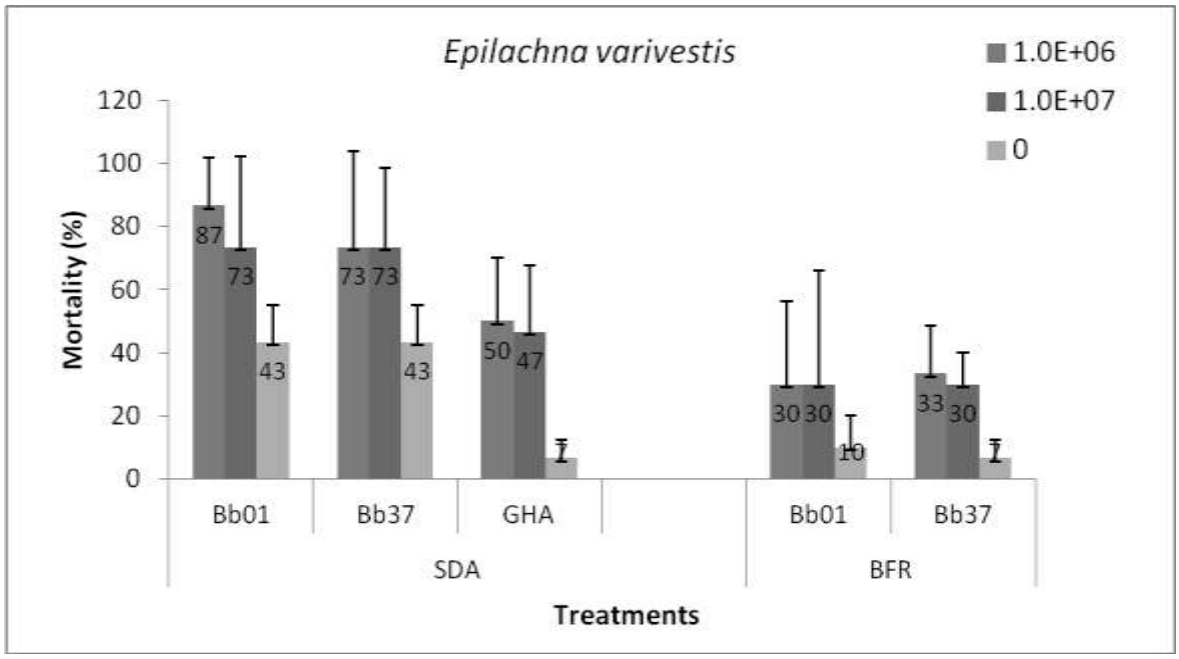

Figure 1. Percentage of mortality of *Epilachna varivestis* 3 instar larvae at 10 days exposed to topic solution of conidia of Bb01, Bb37 and GHA of *Beauveria bassiana* obtained in Sabouraud dextrose agar and of Bb01, Bb37 obtained by biphasic fermentation in rice. The bars on the columns show the values of standard deviation of the average of three repetitions.

Conclusions: Conidia of Bb01 and Bb37 produced in Sabouraud dextrose agar (SDA) with yeast extract showed greater effectiveness for the control of larvae of *E. varivestis* at the concentration of $1x10^6$ conidia/mL, this when comparing them with 50% of mortality of GHA. We can conclude that the isolate is effective and has a future as a biological control agent; nevertheless, now the challenge is to find the conditions to produce it in great amounts without decreasing its effectiveness.

Acknowledgments: We thank Fundación Guanajuato Produce, A.C., SIFP 11-2009-2760; FGP 542/09, and Consejo Nacional de Ciencia y Tecnología (CONACyT) CB-155771 grants to PTG.

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ACTINOMYCIN-D-INDUCEDAPOPTOSIS IN *DROSOPHILA* S2 CELL LINE *IN VITRO* VERSUS *VANESSA CARDUI* L. PUPAE AND LARVAE *IN VIVO*

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Abstract: Apoptosis represents one of the homeostasis mechanisms in multicellular organisms to endure normal metabolism and cellular components regulation. Apoptosis activation results in nuclear DNA fragmentation, leading to nuclear disruption into small DNA sub-fragments. Research on apoptosis to better understand the mechanisms to induce this event has leaded to evaluate several mechanisms that may be involved in this process. Actinomycin-D induced apoptosis in Lepidoptera cell lines *in vitro* as been demonstrated. Nevertheless, the apoptosis effect *in vivo* on Lepidoptera is unknown. The aim of this study was to evaluate the role of actinomycin-D (Act-D) *in vitro* against the Diptera *Drosophila* cell line S2, and *in vivo* against the Lepidoptera Painted Lady *Vannesa cardui* L. pupae and larvae. *In vitro* treatments were tested by using the CaspGLOW^{TM} red active caspase staining kit; whereas *in vivo* and were then stained withacridine orange*/* ethidium bromide (AO/EB). *In vitro* results showed caspases activation by positive fluorescence linked with apoptotic bodies. Similarly, *in vivo* test using Act-D treated pupae, the characteristic nuclear DNA fragmentation apoptosis was detected by using 1.2% agarose. Results demonstrated that, as well as *in vitro*, Act-D is an apoptosis activator in *V. cardui* pupae *in vivo*.

Keywords: Apoptosis ● Actinomicin-D ● *Vanessa cardui*

Introduction: Cellular number homeostasis in multicellular organisms is regulated by cell proliferation and programmed cell death via apoptosis (Yefu *et al.,* 2003). The apoptosis instead representing a treat is an important mechanism for healthy cellular-tissue development regulation (Ribble *et al.,* 2005). The apoptosis event induces the nuclear DNA fragmentation. As a result, nuclear disruption evolves to the formation of small DNA sub-fragments (Yefu *et al.,* 2003). Since the apoptosis represent a process related with the healthy cellular regulation, the investigation of the biochemical mechanisms that participate in such event is needed. Several *in vitro* bioassays using cell-lines involve fluorescence-detection staining components/ methods such as the acridine orange*/* ethidium bromide (AO/EB), the 4,6-diamino-2 phenyl-indole (DAPI), the Hoechst staining, the Annex in V staining, the DNA ladder staining markers, the terminal deoxynucleotidyl-transferase mediated dUTP Nick-end labeling (TUNEL), the Caspase-3/7 activity, and the ssDNA-staining methods (Ribble et al., 2005).

The Painted Lady butterfly *Vanessa cardui* L. is an ecologically important insect since their distribution is cosmopolitan with high migration ability throughout the Northern Hemisphere, participating in wild plants pollination (Otaki 2007).Although early butterfly wing development appears to be fairly typical for holometabolous insects, there is one interesting aspect of the process that is possibly unique to Lepidoptera: the differentiation and eventual degradation of the ‘‘peripheral tissue’’ around the developing wing margin by apoptosis, as reported by the butterfly *Pieris rapae* (Kodama *et al.,* 1995). Recently, more studies related to apoptosis molecular mechanisms in lepidoptera have been reported (Macdonald *et al*., 2010), but little is known on regard to the biochemistry mechanisms. The aim of this study was to determine

apoptosis induction by Act-D *in vitro* (testing the S2 cell line) versus *in vivo* apoptosis (among pupae versus larvae by the Painted Lady butterfly).

Materials and Methods:

Insects

Vanessa cardui (Fig. 1) colony was established from insect brought from the American Butterfly Association (http://www.naba.org/), and reared with a Unidad de Manejo para la Vida Silvestre del Gobierno del Estado de Nuevo León permit (PVSNL-UMA-IN-0305-NL) granted to PTG, since 2008 on artificial diet as described in Tamez-Guerra et al. (2006), but using bigger plastic containers (50 mL) with 10 mL artificial diet each. The Insects were incubated at 25 ± 2ºC, 55-60 ± 10% RH, and 16:8 L:D photoperiod.

Figure 1. Adult and pupa view of *Vanessa cardui* L.reared in the FCB-UANL

In vitro apoptosis bioassay

In vitro treatments were tested by using the *Drosophila* cell line S2 (R690-07, Invitrogen™). Cells grown on Schnider's *Drosophila* medium (Cat No. 11720-034, Invitrogen®) supplemented with bovine fetal serum at 10% v/v. Cells were treated with Act-D (SigmsAldrich) serial increasing concentration, ranging from 0.25 to 5.0 µg/mL. Cells were then incubated at 25ºC by 24 h under dark condition. Apoptosis by the Act-D treated cells were then analyzed by adding the CaspGLOW™ (Biovision K-190-25) red active caspase staining kit stained withacridine orange (AO) / ethidium bromide (EB) (Ribble *et al.,* 2005). Treated cells with apoptosis were confirmed by fluorescent microscopic (Olympus®) analysis, by using a rhodamine filter at an excitation point of 480/30 nm by AO and EB, respectively.

In vivo apoptosis bioassay

In vivo bioassays consisted of 5 µL injection (insulin syringe BD Ultra-Fine®) of Act-D serial concentration: 25, 50, 100, and 200 ng/mL**,** into *V. cardui* fourth-instar larvae or pupa. Five insect per concentration per insect stage, were tested. Treated insects were kept in individual plastic cups with 10 mL artificial diet by 24 h, under the same rearing conditions as the laboratory colony. After that, treated insects were immobilized on ice and hemolymph was extracted as described by Shelby & Popham (2006). DNA was extracted from the hemolymph samples by using the Wizard® Genomic kit (Promega®). Samples were then analyzed in 1.2% agarose to determine the apoptosis process displayed as DNA fragmentation (Gavrieli*et al.* 1992, Zhang *et al.* 2011).

Results and discussion: Apoptosis-induced by Act-D *in vitro* bioassays was observed by using the staining kit withacridine orange (AO) ***/*** ethidium bromide (EB), as reported by Ribble *et al.* (2005) (Figure 2). S2 cell line treated with Act-D induced apoptosis *in vitro* was verified with florescent microscopy, where intracellular nuclear condensation and fragmentation were observed (Figure 3).

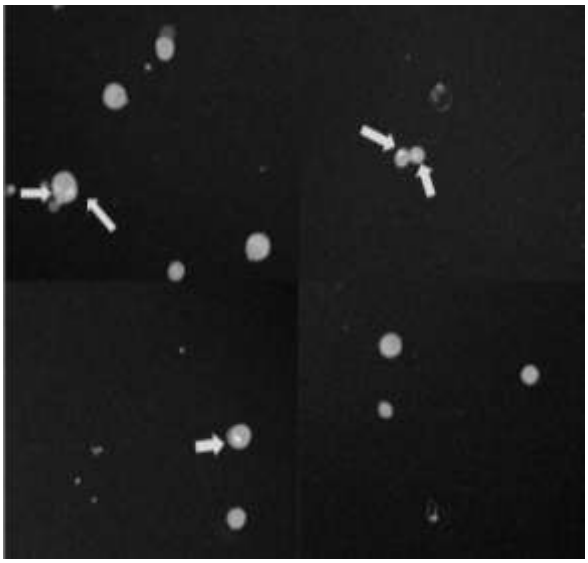

Figure 2. Actinomycin-D S2 cell treated stained with acridine orange / ethidium bromide

Caspases releasing, also associated with apoptosis, was observed when 0.2 to 1.0 µg/mL of Act-D were tested (Figure 3), similar to that reported by Georgel *et al.* (2001).

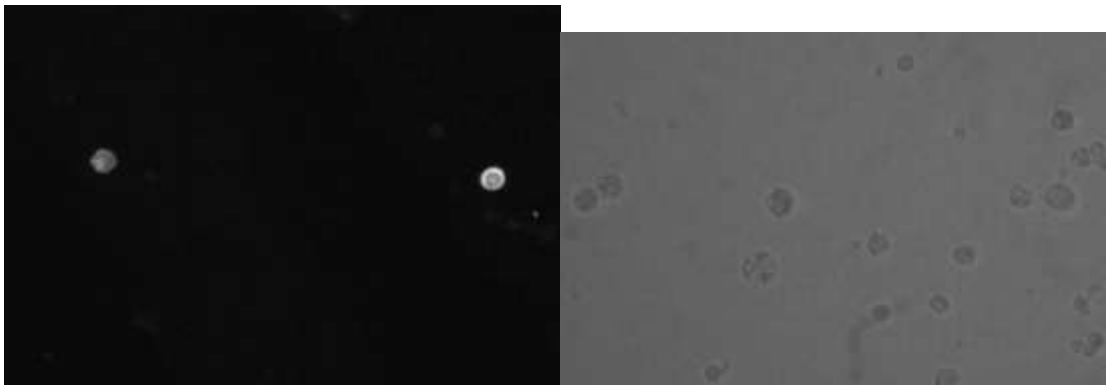

Figure 3. Actinomycin-D S2 cell treated: caspases detection by fluorescence microscopy.

In vivo assays showed that apoptosis-induced by Act-D treated larvae inconclusive results. When pupae were tested, a clear DNA fragmentation induced by apoptosis was observed when 5.0 µL of 200 ng/mL of Act-D were injected to each pupa (Figure 4), similar than that reported by LaCount & Friesen (1997) when exposing SF21 cell line to baculovirus.

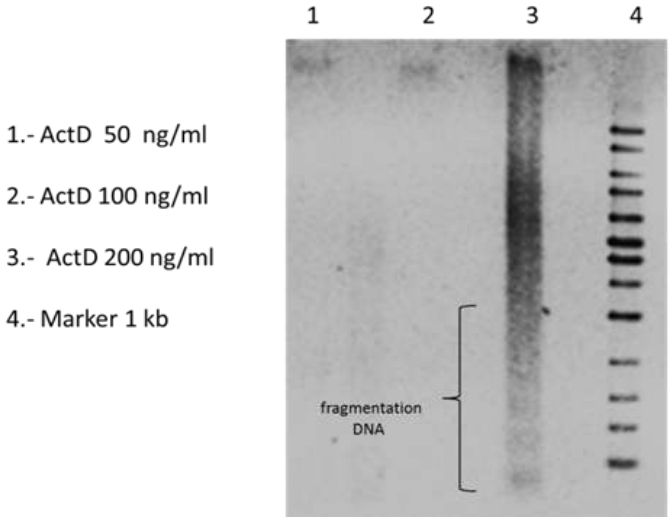

Figure 4. DNA fragmentation induced by Actinomycin-D injection on *Vanessa cardui* pupae.

Previous studies evaluating apoptosis have been performed on *Spodoptera frugiperda* cell line SF21. For instance, Vucic *et al* (1997) reported that the expression of the reaper gene (*rpr*) in the dipera *Drosophila* cell line S2 induced apoptosis. When testing apoptosis in the Lepidoptera *Spodoptera frugiperda* cell line SF-21, they also found that apoptosis process displayed nuclear condensation and fragmentation. More recently, apoptosis evaluation were performed in a *Spodoptera litura* cell line treated with actinomycin-D using fluorescence-detection staining components. Results demonstrated apoptosis by showing nuclear fragmentation and apoptotic body formation (Zhang *et al.* 2011), similar to ours *in vivo* results with *V. cardui* pupae.

Conclusion*:* This study demonstrated that actinomycin-D can be used as apoptosis-inducer in Lepidoptera, tested *in vivo* bioassays, to compare toxicity by bio-molecules or entomopathogenic microorganisms, based on DNA fragmentation.

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PRODUCTION OF INOCULUMS FROM INDIGENOUS BACTERIA FROM THE RHIZOSPHERE OF *CARICA PAPAYA*

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Abstract: The agricultural sector is interested on the research or microorganisms that promote the plant's growth and development. Since the soil has beneficial microorganisms, it is necessary to identify and characterize microorganisms useful for the production of inoculants. Since papaya is a tropical fruit with a high national and international demand and México is one of the main producers and exporters of the world, this research carried out the development of Inoculants suitable for *C. papaya.* Seven bacteria were isolated, characterized and identified from the rhizosphere of *C. papaya*. The identified microorganisms were: *Bacillus sp., Pseudomonas sp., Acinetobacter sp., Stenotrophomonas sp., Staphylococcus sp., Arthrobacter sp*. and *Nocardioides sp.* In *C. papaya* cultures, eleven treatments were tested in laboratory under conditions with average values of 25.5°C, 45% HR and daily time was fixed at 16 h, to assess its role as inoculants: Seven were related to bacteria isolated from the rhizosphere of *C. papaya*, one microbial consortium (CMP) similar to a commercial organic product (PHC BioPak), one positive chemical treatment (nitrofoska), one more positive treatment category (PHCBioPak) and finally a negative one. Treatments with the highest results regarding plant growth and development were treatment 1 (*Bacillus sp.*) and treatment 8 (CMP).

Keywords: Inoculants • Indigenous bacteria • Rhizosphere • *Carica papaya*

Introduction:

Most of the microorganisms that are present in the soil are beneficial to plant cultures. These microorganisms constitute the living part of soil and contribute to the development of the plant as well as being involved in nutrient transformation (Bashan, 1998). In recent years there has been an increased interest in the use of organic products, such as inoculums, as an alternative for soil rehabilitation and as a fertilizer aid (Díaz, 2008).

Papaya is a tropical fruit with a high national and international demand. In the international market, Mexico is one of the main producers and exporters (SAGARPA, 2007; FAOSTAT, 2007). The aim of this work is to isolate, identify and characterize biochemically and molecularly the indigenous bacteria from the rhizosphere of *C. papaya* and these bacteria tested in laboratory in *C. papaya* cultures.

Materials and methods:

Bacterial isolates. Soil samples were collected from the rhizosphere of *C. papaya* in the town of José María Morelos, located in the municipality of Santa Maria Huazolotitlán in the state of Oaxaca. The isolation of microorganisms was carried out by the method of dilutions and rod extension. Then the pure culture was obtained by cross streak on nutrient agar (NA) and soil extract agar (SEA) (Sánchez, 2004).

Macroscopic and microscopic analysis. For the macroscopic analysis of the microorganisms the morphology of the colonies was described. The microscopic analysis was carried out by Gram staining (Bergey, 1994).

Biochemical characterizations. Biochemical tests were conducted as suggested by MacFaddin (2004) for the identification of bacterial genus and species.

Extraction and purification of genomic DNA. Extraction and purification of bacterial DNA was carried out by the Charge Switch gDNA kit minibacteria (Invitrogen, USA).

16S rDNA sequencing. DNA was extracted from the isolated strains and used for the amplification and sequencing of the 16S rDNA gene. Universal primers to amplify region from the *E. coli* 16S were used.

Inoculants tests in laboratory. Isolated bacteria were tested in laboratory under conditions with average values of 25.5°C, 45% HR and daily time was fixed at 16 h, to assess its role as inoculants: Seven were related to bacteria isolated from the rhizosphere of *C. papaya*, one microbial consortium (CMP) similar to a commercial organic product (PHCBioPak), one positive chemical treatment (nitrofoska), one more positive treatment category (PHCBioPak) and finally a negative one. The treatments were distributed in a completely randomized design. The response variables were measured during 65 days: percentage of seedling emergence, days to germination, plant height, number of leaves (Chilo et al., 2009; Quiñonez et al., 2003; Salguero, 2006).

Results and discussion: The identified microorganisms were: *Bacillus sp., Pseudomonas sp., Acinetobacter sp., Stenotrophomonas sp., Staphylococcus sp., Arthrobacter sp*. and *Nocardioides sp.* The result of Biochemical and molecular characterization are show in Table 1. The different genera of bacteria isolated from the rhizosphere of *C. papaya* as inoculums have proven biofungicide functions and promote absorption of nutrients, nitrogen fixation and growth promoters and encompasses beneficial microorganisms that can solubilize soil phosphorus to make it available to the plant (Rai, 2006).

Table 1. Comparative results of biochemical and molecular characterization.

Strain	Biochemical characterization	Molecular characterization
MMRL1	*Bacillus sp.*	*Bacillus sp.*
MMRL2	*Pseudomonas sp.*	*Pseudomonas sp.*
MMRL3	*Acinetobacter sp.*	*Acinetobacter sp.*
MMRL4	*Stenotrophomonas sp.*	*Stenotrophomonas sp.*
MMRL5	*Staphylococcus sp.*	*Staphylococcus sp.*
MMRL6	*Arthrobacter sp.*	*Arthrobacter sp.*
MMRL7	*Nocardioides sp.*	*Nocardioides sp.*

In *C. papaya* cultures, eleven treatments were tested in laboratory to assess its role as inoculants. The treatments with the highest results regarding plant growth and development are shown in Table 2. With respect to the time of germination and percentage of seedling emergence data obtained were better than those reported (CONABIO, 2007). With respect to

the number of plants didn't found data reported at 65 days of growth of *C. papaya.* Finally the plant height data were similar to those reported (CONABIO, 2007).

Table 2. Response variables of the different treatments tested in *C. papaya* cultures.

Response variables	Treatment	Result
Percentage of seedling emergence	*Bacillus sp*	*53.3%*
Days to germination	*Bacillus sp.*	*25 days*
Plant height	CMP (*Bacillus sp.* and *Pseudomonas sp*.)	0.097 m
Number of leaves	CMP (*Bacillus sp.* and *Pseudomonas sp*.)	6.5 leaves

Conclusions: The strains identified from the rhizosphere of C. papaya were *Bacillus sp., Pseudomonas sp., Acinetobacter sp., Stenotrophomonas sp., Staphylococcus sp., Arthrobacter sp*. and *Nocardioides sp.* The inoculant formulated with *Bacillus sp* (treatment 1) showed a better response in the percentage of seedling emergence (53.3%) and shorter time of germination (25 days) that commercial fertilizers tested. The inoculant formulated with *Bacillus sp.* and *Pseudomonas sp*. (treatment 8) showed a better leaf development (6.5 leaves) and better growth of plants (0.097 m) that commercial fertilizer tested.

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GREENHOUSE GAS EMISSIONS FROM SOIL RECEIVING WASTEWATER, SLUDGE OR COW MANURE FOR BIOMASS CULTIVATION

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Abstract: Nowadays the human dimensions of greenhouse gas emissions and global warming attract considerable attention, while biomass production has been increased considerably in the last years to use it as bioenergy source.The objective of these experiments was determinate the greenhouse gases emissions from soils receiving organic amendments for biomass cultivation. Three experiments were done in greenhouse condition to determinate the greenhouse gas emissions from soils cultivated with maize and common bean and amended with wastewater, sludge or cow manure. CO_2 and N_2O emissions were significantly largest in soils amended with sludge, compared with the other organic amendments, while CH_4 increased significantly when soil was irrigated with wastewater. Wastewater, sludge or cow manure increased the biomass; however, from a holistic point of view, using this organic amendment as fertilizer to produce biomass and/or bioenergy should be viewed with concern.

Keywords: Biomass ● CO_2 emissions ● Renewable energy ● Growth and yield.

Introduction: Energy demands are increasing with population growth and economic development. This situation is not sustainable for several reasons; oil reserves are limited, and the increasing use of oil and coal leads to ever increasing CO_2 emissions, which carry the risk of climate change (Scheller et al., 2010). Theoretically, the total bio-energy contribution (combined in descending order of theoretical potential by agricultural, forest, animal residues and organic wastes) could be as high as 1100 EJ, exceeding the current global energy use of 410 EJ (Hoogwijk et al., 2003). The use of urban wastewater in agriculture is a centuries-old practice that is receiving renewed attention with the increasing shortage of freshwater around the world. Irrigation of crops with wastewater is already a common practice in urban and peri-urban farming communities of the developing world. Wastewater is often the only water source for agriculture and its use will increase with an increased demand for fresh water. Additionally, wastewater contains important nutrients, such as inorganic N, and organic material, which favours crop growth.

Sludge originating from wastewater treatment contains nutrients and organic material. Upon mineralization of the organic material more nutrients are released and wastewater sludge is therefore widely used to improve soil fertility or restore degraded soils. The N mineralization rate of the organic material in the sludge when added to soil depends on the application rate, sludge C:N ratio, soil characteristics and temperature (Er et al., 2004). Sludge, however, can also contain contaminants such as metals, pathogens and organic or inorganic pollutants.

Wastewater sludge is therefore often dumped in landfills are incinerated, thereby loosing a potential source rich in plant nutrients.

Nitrous oxide (N_2O) is present in the atmosphere at a low concentration (310 parts per billon), but the amount is increasing at rate of 0.25 percent per year. Despite its low concentration, N_2O is an important greenhouse gas because of its longer lifetime (150 years) and much greater warming potential, i.e. about 30 times, than that of carbon dioxide (Lu et al., 2006). Although the N_2O budget remains poorly understood, fertilized agricultural soils where N_2O is produced through nitrification and denitrification, are believed to be a major source of N_2O emission (Mosier et al., 1998). The atmospheric concentration of CH_4 (1.75 ppm) is much lower than that of CO_2 (379 ppm), but the amount of CH_4 is increasing by 4.9 ppb y^{-1}, while that of CO_2 1.9 ppm (IPCC, 2007). Methane from agricultural origin is emitted by methanogenic bacteria living in anaerobic soils, e.g. rice production, manure management and from the rumen of cattle and sheep (Johnson et al., 2007). Concentrations of CO_2 in the atmosphere have increased by 36% from 280 parts per million (ppm) in 1850 to 380 ppm in 2005 (Christopher and Lal, 2007).The objective of these experiments was determinate the greenhouse gases emissions from soils receiving wastewater, sludge or cow manure for biomass cultivation.

Material and Methods: The soils used in the experiments reported here were collected from different arable lands or from the former lake Texcoco in the State of Mexico, Mexico, (N.L. 19° 42', W.L. 98° 49'; 2349 m above sea level). The climate is sub-humid temperate with a mean annual temperature of 14.8 °C and average annual precipitation of 577 mm mainly from June through August (http://www.inegi.gob.mx). The arable soils are generally low in organic matter and N depleted. The area is mainly cultivated with maize and common bean, receiving a minimum amount of inorganic fertilizer without being irrigated (http://www.inegi.gob.mx). The soil of Texcoco is characterized by a high pH and salinity. Details of the arable Mezquital soil used in the experiment can be found in Fernandez-Luqueno et al. (2010), of Otumba soil in Fernandez-Luqueno et al. (2009), and of the Texcoco soil in Juarez-Rodriguez et al. (2012). Soil was sampled at random by augering the top 0-15 cm soil-layer of three plots of approximately 0.5 ha. The soil from each plot was pooled and as such a total three soil samples was obtained.

All the reported data were obtained from greenhouse experiments. The details of the experimental design and the methods used to characterize the soil can be found in each of the mentioned papers i.e. Fernandez-Luqueno et al. (2009, 2010), and Juarez-Rodriguez et al. (2012). The amounts of wastewater or sludge added to soil varied although they were according to traditional fertilization rates.

A cylindrical PVC chamber (length 50 cm and ∅ 16 cm) was placed on the PVC tube and air-tied sealed. Zero, 3, 15 and 30 min after the upper cylindrical chamber was sealed, 20 cm^3 air was injected in the PVC chamber headspace and an equal amount was sampled and injected into 17-mL evacuated vials. The amount of CO_2 and N_2O was determined with an Agilent 4890D gas chromatograph fitted with an electron capture detector. A J&W Scientific GS-Q column was used to separate CO_2 and N_2O from the other gases; the carrier gas, N_2, flowing at a rate of 5 ml min^{-1}. Injection, detection and column-oven temperatures were set at 100 ^oC, 225 ^oC, and 35 ^oC, respectively. The amount of CH_4 was determined with an Agilent 4890D gas chromatograph fitted with a flame ionization detector. A Porapak Q column (80/100 12' × 1/8'' × 0.085'') was used to separate CH_4 from the other gases with the carrier gas He flowing at a range of 25 mL min^{-1}. Injection, detection and column-oven temperatures were set at 100 ^oC, 310 ^oC, and 32 ^oC, respectively. For each analysis, an aliquot of 1 cm^3 was injected into the chromatograph using a Teflon sealed glass syringe (Hamilton®, USA).

Significant difference between plant and soil characteristics as a result of the different treatments were determined by analysis of variance (ANOVA) and based on the least significant difference using the General Linear Model procedure (PROC GLM, SAS Institute, 1989).

Significant differences between treatments for production of CO_2, N_2O and CH_4 were determined using PROC MIXED considering repeated measurements (SAS Institute, 1989).

Results and Discussions: Biosolid had the lowest pH as witnesses in Table 1, and it had the largest conductivity i.e. 5.7 dS m^{-1}. The total N concentration in wastewater was 33 mg L^{-1}, 27.7 g kg^{-1} in biosolid and 34 g kg^{-1} in cow manure. Organic carbon content was 509 in biosolid and 57 g kg^{-1} in cow manure (Table 1). Organic C content was 38, 27 and 7 g kg^{-1} in Texcoco, Mezquital and Otumba soils, respectively. pH values ranked from 7.6 in Otumba soil to 9.3 in Texcoco soil. Total N content was 2.0, 1.9 and 1.0 g kg^{-1} in Texcoco, Mezquital and Otumba soils, respectively (Table 2). It is known that addition of different forms of organic or inorganic fertilizer had little effect on pH compared to the unamended soil.

Table 1.Organic amendments used as fertilizers.

Characteristics	Wastewater	Biosolid	Cow manure
pH_{H2O}	8.4 [a]	6.4	8.6
Conductivity (dS m^{-1})	1.2	5.7	NM
Organic C (g kg^{-1})	NM [b]	509	57
Total N (g kg^{-1})	33 mg l^{-1}	27.7	34
Total P (g kg^{-1})	NM	1.7	NM
Extractable PO_4^{3-} (mg kg^{-1})	NM	600	600
Water content (g kg^{-1})	NM	793	855

Wastewater: Fernández-Luqueno et al. (2010); Biosolid: Fernandez-Luqueno et al. (2009); Cowmanure: Juarez-Rodriguez et al. (2012). [a] Mean of ninereplicates. [b] Not measured. All values are on a dry matter base.

In soils cultivated with maize and irrigated with wastewater the daily CO_2 emission rate ranged from very low (0.04 µg C kg^{-1} soil h^{-1}) to a maximum of 30.99 µg C kg^{-1} soil h^{-1}. Adding urea to soil had no significant effect on the mean CO_2 emission rate compared to the unamended soil, but cultivating maize in the urea-amended soil increased it 6.7 times ($P<0.05$; Table 3). When soil was cultivated with common bean and amended with sludge, at the onset of the experiment and until 15 days, large amounts of CO_2 were emitted from the soil (Data not shown). Between days 15 and 30, emissions of CO_2 were low and increased towards the end of the experiment. Application of urea had no significant effect on the emission of CO_2 compared to the unamended soil (0.043 mg C kg^{-1} dry soil). The mean emission of CO_2 was larger when wastewater sludge was added to soil (0.064 mg C kg^{-1} dry soil) compared to the unamended soil, but the application rate had no significant effect (Table 3).

Table 2.Characteristics of the Mezquital, Otumba, and Texcoco Soil.

Characteristics	Mezquital	Otumba	Texcoco
pH_{H2O}	8.2 [a]	7.6	9.3
Conductivity (dS m^{-1})	0.8	1.2	9.5
Organic C (g kg^{-1})	27.3	7.2	38
Inorganic C (mg kg-1)	NM [b]	661	5600
Total N (g kg^{-1})	1.9	1.0	2.0
Total P (g kg^{-1})	NM	NM	NM
Extractable PO_4^{3-} (mg kg^{-1})	NM	NM	NM
Water holding capacity (g kg^{-1})	NM	564	920

Mezquital: Fernández-Luqueno et al. (2010); Otumba: Fernandez-Luqueno et al. (2009); Texcoco: Juarez-Rodriguez et al. (2012). [a] Mean of ninereplicates. [b] Not measured. All values are on a dry matter base.

When maize was growth in soil fertilized with cow manure the CO_2 emission rate was highly variable over time and ranged from undetectable amounts of CO_2 to a maximum of 14.8 mg C kg^{-1} soil day^{-1}. The CO_2 emission rate increased significantly (1.6 times) when soil was cultivated with maize compared to the uncultivated soil (P <0.05). The CO_2 emission rate increased significantly 3.1 times when cow manure was added to soil and 3.5 times in cow manure-amended soil cultivated with maize compared to the unamended soil (P <0.05).The N_2O emission rate was highly variable over time and ranged from −0.9 μg N kg^{-1} soil day^{-1} to 17 μg N kg^{-1} soil day^{-1}. The mean N_2O emission rate was −0.0004 μg N kg^{-1} soil day^{-1} in the unamended uncultivated soil and increased to 0.27 μg N kg^{-1} soil day^{-1} when maize was cultivated (Table 3). Application of cow manure resulted in an average production of 4.59 μg N_2O-N kg^{-1} soil day^{-1}, but cultivation the sludge-amended soil with maize nearly halved it. Meijide et al. (2007) found that emission of N_2O increased in the field when untreated pig slurry or composted pig slurry plus urea were added to soil, but not when digested thin pig slurry fraction or municipal solid waste plus urea were added. They stated that denitrification was the most important process responsible for N_2O emissions when organic fertilizers were applied to soil. Mackenzie (1998) stated that wastewater increased the amount of N_2O emitted due to microbial transformation of the nitrogen contained in the wastewater i.e. oxidation of NH_4^+ under aerobic conditions or reduction of NO_3^- under anaerobic conditions.

Addition of wastewater increased the emissions of CO_2 and production of CH_4 upon application compared to the urea-amended soil, but not emissions of N_2O. Irrigating soil or maize cultivated soil with wastewater increased the global warming potential >2-fold compared to the urea amended soil according with Khalil and Inubushi (2007). Environmental and economic implications must to be considered to decide how many, how often and what kind of organic fertilizer could be used to increase yields and produce biomass to bioenergy production, while limiting soil deterioration and greenhouse gas emissions. The application of anaerobic digested cow manure stimulated maize development in an alkaline saline soil, but increased emissions of CO_2 and N_2O. Additionally, it is known that although the increase in EC due to application of anaerobic digested cow manure was small, prolonged application should be linked toother measures to reduce soil salt content.

Table 3. Emissions of greenhouse gases during the growing of crops.

Treatment	CO_2 (mg C kg^{-1} soil day^{-1})	N_2O (µg N kg^{-1} soil day^{-1})	CH_4 (µg C kg^{-1} soil day^{-1})
Maize cultivated and wastewater irrigated[a]	0.13	0.066	3.9
Maize cultivated and urea fertilized[a]	0.37	0.107	0.2
Beans cultivated sludge-amended soil [b]	0.16	0.95	NM
Beans cultivated urea-amended soil[b]	0.15	0.1	NM
Maize cultivated unamendedsoil[c]	2.48	0.27	NM
Maize cultivated cow manure-amended soil[c]	5.21	2.42	NM

[a]: Fernández-Luqueno et al. (2010); [b]: Fernandez-Luqueno et al. (2009); [c]: Juarez-Rodriguez et al. (2012). [a] Mean of ninereplicates. [b] Not measured. All values are on a dry matter base.

Conclusions: It was found that application of wastewater or sludge stimulated the growth and yield of crops and also increased the greenhouse gas emissions, but not always. This implies that wastewater, cow manureor sludge increased the biomass; however, from a holistic point of

view, using wastewater or sludge as fertilizer to produce biomass and/or bioenergy should be viewed with concern.

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BISECTION AND EMBRYO TRANSFER IN HAIR SHEEP

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Abstract: To assess the viability of bisected embryos transferred into hair sheep treated with GnRH on the diestrus, 39 embryos at stage of compacted morula grade 1, were transferred into 39 recipient ewes (19 with whole embryos and 20 with bisected embryos), distributed in four treatments: complete embryos with GnRH (EEG), complete embryos control (EET), bisected embryos with GnRH (EBG) and bisected embryos control (EBT). EEG and EBG treatments received a dose of 100µg of GnRH during diestrus (7 days from sponges withdrawal). Pregnancy diagnosis by RIA at 17 days after sponge withdrawal, showed differences ($P < 0.05$) in pregnancy rates of treatments EEG, EET and EBG respect to EBT, being 70, 66 and 70 vs 30% respectively. Pregnancy diagnosis by ultrasound at 45 days after sponge withdrawal resulted higher ($P < 0.05$) in treatments EEG (70%) and EET (63%) compared to treatments EBG (10%) and EBT (25%), a result that was maintained until delivery. The total number of offspring per treatment was 7, 6, 2, 9 and EEG, EET, EBG and EBT, respectively. A 37.5% of twins was obtained and a level of profitability of 55% for the bisecting technique. The application of GnRH in the diestrus of ewes, has a favorable effect on survival of complete embryos, but not in bisected embryos

Keywords: GnRH ● Diestrous ● Embryo ● Bisection

Introduction: Multiple ovulation and embryo transfer (MOET) and techniques derived from them, are important tools in animal reproduction to contribute, from the genetic and productive point of view to improvement and conservation of populations (Baril et al., 1995). However, this technique is limited in sheep because of the reduced number of viable embryos produced, since normally 4 to 6 embryos per wash are collected (Cognie, 1990). At commercial level it is necessary to increase the level of efficiency of the superovulatory treatments. From this standpoint embryo micromanipulation, is a technique aimed to increase profitability of superovulatory treatments. Thus, embryo bisection allows duplicating number of originally collected embryos and obtaining identical twins, eliminating genetic variability among individuals rendering each one an exact replica of the other (Chesné et al., 1987). On the contrary, embryo manipulation is limited by embryo mortality (Ramón, 1997), a factor that in tropical areas becomes especially important due to environmental effects, which increase losses possibilities. Because of the formerly addressed, strategies to increase possibilities of embryo survival either complete on bisected, become necessary. Aplication of GnRH in the diestrus of ewes receiving complete embryos has demonstrated a significant increase in embryo survival as compared to those untreated receptor ewes (Cervera et al., 2011). However, little is known about the possible effects on ewes receiving bisected embryos, where fertility rate is reduced as compared to complete embryos (Ramón et al., 2005). The aim of the present study was to evaluate the viability of bisected embryos transferred to hair ewes treated with GnRH during diestrus.

Materials and methods: The study was carried out during summer at the Selection y Reproduction Center Ovine (CeSyRO) of Technological Conkal Institute, located in Conkal in the

northeast of the state of Yucatán (20°59' North latitude and 89°39' west longitude) with climate Aw0 (García, 1987).

Fourteen adult, multiparous, non-lactating Katahdyn ewes were selected as donors and 45 Black belly ewes as receptors. Estrous induction and synchronization in donor and receptor ewes was achieved by insertion of intravaginal sponges with 20 mg of Fluorogestone (FGA; Chronogest®, Intervet) for 12 days. Sponge withdrawal was carried out simultaneously in both groups. Additionally, on sponge withdrawal the receptor group received a dose of 200 IU of eCG (Folligon®, Intervet). Superovulatory stimulus in donors was performed by injection of 8.8 mg of FSHo (Ovagen™; Inmuno-Chemical Products LTD; 17.6+/-2 mg equivalents to NIADDK-oFSH-17 bioactive FSH/vial in 20 ml) in decreasing doses at 12 h intervals (2, 2, 1.5, 1.5, 1, 1, 0.5, 0.5 ml) initiating with the first application 72 h before sponge withdrawal (Sponge withdrawal=Hour 0), plus one dose of 10 mg of $PGF_{2\acute{a}}$; (Dinoprost, trometamina: Lutalyse®, Pfizer). Ewes received an IM dose of 100µg de GnRH at 36 h (Ovalyse®; Pfizer). Intrauterine insemination was carried out with fresh semen ($100x10^6$ spz) at 56 h.

Embryo recovery and transfer was carried out on day 7 by the technique described by Ramón et al., (2008). Only ewes that developed at least 3 corpora lutea were perfused. Once recovered, embryos were classified according to morphological criteria as described by Witenberger-Torres and Sevellec, (1987) adapted by Cocero (1992). Bisection of 20 embryos was performed with a microblade mounted on a hydraulic arm observed in an inverted microscope (Leica®, Modelo MMN-1). From all recovered embryos, 39 in morula stage grade 1 were selected and transferred into 39 receptor ewes (19 with complete embryos and 20 with bisected embryos distributed into four treatments: Complete Embryos with GnRH (EEG), complete Embryos without GnRH (EET), Bisected Embryos with GnRH (EBG) and Bisected Embryos without GnRH (EBT). At time of transfer, 20 ewes (10 of each treatment: EEG and EBG) received a dose of 100µg de GnRH in diestrus (Day 7 of sponge removal). Fertility rate (F) was measured on Days 17 and 45 after sponge withdrawal. Pregnancy diagnosis on Day 17 was measured by determination of circulating concentrations on progesterone in plasma by RIA (RIA commercial kit DPC No. Catalog TKPGX). Assay sensitivity was 0.05 ng/ml, variation coefficient intra and inter assay for the referred simple 6.64 and 2.94 %, respectively. Pregnancy diagnosis on day 45 was performed by real time ultrasonography with an Aloka® 500 SSD provided with a transrectal 7.5 MHz linear array probe. Results were analyzed X^2 (Analitycal software, 2003).

Results and discussion: Superovulatory response in donor ewes was a total of 102 corpora lutea (CL) (7.28 CL/treated ewe), with a recovery rate of 68.6 % (n=70), which a viability rate of 78.5 % (n=55), from which only 70.9 % (n=39) resulted in compact morula grade 1. Results obtained are lesser than those reported by Cervera et al. (2011) with this same breed, who obtained 11.4 CL/treated ewe, 85 % of recovery rate and 76 % of viability rate. This difference may be due to the season in which the study was carried out (summer), as opposed to the authors mentioned above, who performed their studies during the reproductive season.

Pregnancy diagnosis at Day 17 of sponge withdrawal, showed differences ($P<0.05$) in gestation rate of treatments EEG, EET and EBT as compared to treatment EBT. Pregnancy diagnosis by ultrasonography at Day 45 resulted greater ($P<0.05$) in treatments EEG and EET, as compared to treatments EBG and EBT, values that were maintained until lambing (Table I).

Results of lambing of ewes that received complete (EE) and bisected (EB) embryos without taking into account GnRH treatment, show a higher ($P<0.05$) lambing rate favoring those treatments with EE. Reduction in gestation rates in some treatments at Day 45 with regards those observed in Day 17 may be due to embryo mortality. Under the conditions of the present study, in the peninsula of Yucatán during summer, temperatures above 35º C are observed as well as abundant rains, which brings along stressful conditions for animals. From this standpoint, it is known that exposure of animals to conditions of cold, rain, heat or handling stress, reduces

possibilities of the embryo in the first three weeks of (Ramón, 1997). Losses observed in treatments EBG and EBT are similar to those reported by Roche et al. (1981) and Gustafsson (1985). The latter authors indicate that embryo losses occur during this period with ranges going from 30 to 40 %. Similarly, the inevitable damage caused to embryo when bisecting also exists, this is considered a factor that reduces pregnancy rate after transferring half embryos (Shelton, 1992). On the other hand, the poor results obtained in this study when transferring half embryos, may be also due to embryo stage, since most authors report a greater pregnancy rate when half embryos from precocious blastocysts (Williams et al., 1984; McEvoy y Sreenan, 1990) or expanded blastocysts (Arave et al., 1987) are transferred.

Table I. Pregnancy Diagnosis at 17 and 45 days after sponge withdrawal

Treatment	Transferred ewes	RIA %	Ultrasonography %
EEG	10	70.0a	70.0a
EET	9	66.6a	55.5a
EBG	10	30.0b	10.0bd
EBT	10	70.0a	30.0bc

a-b; c-d (P< 0.05).

As it has been observed in the present study, the effect of GnRH favoring survival of half embryos (EBG) is not as efficient as that observed in treatment EEG, which results are similar to those reported by Cervera et al., (2011) with a fertility rate of 80% in receptor ewes of complete embryos, that have been treated with GnRH in diestrus. On the contrary, results obtained in group EET (55.5 %) are slightly greater than that obtained by the latter authors (46 %). Results obtained in the treatment EEG, confirms the hypothesis that GnRH prolongs the CL lifespan through luteinization of follicles, which should cause either luteolysis or induce an accessory CL (Thatcher et al., 1989; Taponen et al., 2003). However, it seems that this factor is not determining in embryo survival when this is subject to bisection, due to the fact that it can loose up to 6.4 of the total cells (Skrzyszowska and Smorag, 1989). Hence, the greater the number of viable cells, the stronger the maternal recognition of pregnancy (Heyman, 1985) It also can be due to half embryos needing a different uterine environment to that for complete embryos (Beckett et al., 1999). Twinning percentage and profitability of the technique can be observed in Table II. Meanwhile percentage of twinning observed is similar to that reported for sheep, (Chesné et al., 1987) and cattle (Ozil et al., 1982; Ozil; 1983). Profitability is lower as compared with that obtained by other authors (Rorie and Godke, 1987). The present study demonstrates that under tropical conditions, it is possible to obtain identical twins after bisecting ovine embryos in stage of compacted morula.

Table II. Profitability of the technique embryo bisection ovine.

	Total	%
Bisected Embryos	20	
Total of half embryos obtained	40	
Total receptors	20	
Pregnant Receptors	8	40
Receptors with twin lambs	3	37.5
Receptoras with single lambs	5	62.5
Total offsprings	11	
Technique profitability	11/20	55

Conclusion: Injection of GnRH in the diestrus of embryo receptor ewes exerts a favorable effect on complete embryos survival but not in bisected embryos survival.

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FERTILITY RESPONSE IN HAIR EWES WITH DIFFERENT PROGESTAGEN TREATMENT

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Abstract: It was studied the fertility (F) of cervical inseminated ewes synchronized in estrus using 136 hair sheep in 6 treatments: Long Cycle (CL: 14 days): CLMPA (n = 24), 60 mg of MAP (Medroxi Progesterone Acetate) CLFGA (n = 31) 40 mg of FGA (Fluorogestone Acetate), CLP (n = 20) with 0.3 g progesterone CIDR. Short cycle (SC: 7 days): SCMPA (n = 21); SCFGA (n = 20), SP (n = 20). Day 0 (removal of devices) a intramuscular dose of 200 IU of eCG. Estrus onset detected by vasectomized male. At 36 h after on day 0, were cervical inseminated with semen cooled ($150x10^6$ spz). The average hourly heat output was between 24-40 h (CL: 28-40, SC: 28-32). Only 73% of the all showed in estrus (CL: 89, SC: 54, P <0.05). The F average was 64%. The F in the SC treatment showed differences (P<0.05) between SCMPA vs SCP (57 vs 85%) and SCFGA vs SCP (55 vs 85%), and CL treatments no differences were found. The F among treatments SC vs CL, only showed differences (P<0.05) between SCP vs CLP (85 vs 60%) and positive trends (P<0.1) between SCP vs CLMPA (85 vs 69%) and SCP vs CLFGA (85 vs 71%). Prolificacy (P) averaged 1.43, being higher (P <0.05) in the treatment of CL compared to SC (1.58 vs. 1.25). P was better (P<0.05) in SCFGA to compare SCMPA and SCP (1.45 vs. 1.25 and 1.11). The duration of progestagen treatment to the control of cycle affects the estrus onset time without influence on F, the CIDR for 7 days will get a greater F compared to vaginal sponges, progestagen treatment of CL give greater P respect to treatments SC.

Keywords: Ewes ● CIDR ● MAP ● FGA

Introduction: The use of artificial insemination (AI) in sheep and goats is linked to breeding programs, independent of their ability: milk, wool or meat. Currently, AI programs perform the induction and synchronization of oestrus by progestagen impregnated intravaginal devices, followed by administration of gonadotropins. These devices are usually used for 12 to 14 days, although there is the possibility of using them for shorter periods (Menchaca and Rubianes, 2004) or longer (Martinez-Tinajero et al., 2007). Moreover, in nontropical breeds, cervical AI is performed after 48 h of removal devices (Lopez-Sebastian, 1991), on the contrary, in tropical breeds, ovulation typically due to the environment ahead (Chemineau , 2004) therefore seems appropriate to make the AI at 36 h after removal of the devices (Dominguez et al., 2007). The aim of this study was to evaluate the fertility of ewes inseminated via cervical and synchronized estrus induced by various treatments progestativos and their duration.

Materials and Methods: The work was conducted in summer at the Selection and Reproduction Center Ovine (CeSyRO) of Technological Conkal Institute, located in the municipality of Conkal northeast of Yucatan (20° 59' north latitude and 89° 39' west longitude) with a climate Aw0 type (Garcia, 1987). 136 Pelibuey hair sheep were distributed by weight (38 ± 1.2 kg) and body condition score of 3 (Russel et al., 1969) in 6 treatments: Long cycle (14 days): CLMAP (n = 24): vaginal sponge with 60 mg of MPA (Medroxy Progesterone Acetat, Sincro-Gest®; Ovejero España); CLFGA (n = 31) sponge with 40 mg of FGA (Fluorogestone Acetate, Chronogest®; Intervet), CLP (n = 20) uterine device with 0.3 g progesterone (CIDR®, Pfizer). Short cycle (7

days): CCMPA (n = 21); CCFGA (n = 20); CCLP (n = 20). All the sheep were fed on pasture (Cynodon nlemfluensis) + 250 g/animal/day of a commercial feed (14% CP). A removal of the uterine devices (day 0) was applied dose intramuscular (IM) of 200 IU equine chorionic gonadotropin (eCG; Folligon®: Intervet; eCG: Ovejero, España). The heat output was detected by vasectomized males from 20 h on day 0. At 36 h on Day 0, were inseminated with cooled semen cervical approach, at a dose of $150x10^6$ spz/ewe. Fertility (F) and prolificacy (P) were measured at birth and the results were compared using the X^2 test with Analytical Software (2003).

Results and Discussion: The average hourly heat output was between 24-40 h (CL: 28-40; CC: 28-32). The average F of all treatments was 64%, similar to that obtained by Dominguez et al., (2007). In CL treatments showed no differences in F (Table I) which resulted in a 66.3% average. By contrast, the F in CC (Table II) showed differences (P<0.05) CCMAP vs. CCP (57 vs 85%) and CCFGA vs CCP (55 vs 85%). The F observed in CCP was higher than that obtained by Ungerfeld and Rubianes (1999) with a similar protocol, thus, the results were higher than those obtained by Bartolomeu et al., (2008), and lowest in both CC and CL than those reported by Iwamura et al., (2008) in hair sheep. The F between CC vs. CL, only showed differences (P<0.05) between CCP vs CLP (85 vs 60%) and trends (P<0.1) between CCP vs CLMAP (85 vs 69%) and CPC vs CLFGA (85 vs 71 %), while the fertilities observed in treatments with MAP and FGA CC are similar to those reported by Ungerfeld and Rubianes (1999).

Prolificacy (P) averaged 1.43, being higher (P<0.05) compared to CC vs. CL (1.58 vs 1.25), highlighting the P obtained in the treatment CCFGA which was better (P<0.05) than CCMAP and CCP (1.45 vs 1.25 and 1.11), possibly due to follicular dynamics present at the time of insertion devices (Menchaca and Rubianes, 2004).

Table I. Fertility and prolificacy in progestative treatments long cycle (CL: 14 days)

Treatments	No.	Fertility (%)	Simple birth (%)	Multiple birth (%)
MAP	23	69.0	56.3	47.3
FGA	31	71.0	36.3	63.7
CIDR	20	60.0	42.0	58.0

Table II. Fertility and prolificacy in treatments progestative short cycle (CC: 7 days)

Treatments	No.	Fertility (%)	Simple births (%)	Multiple births (%)
MAP	21	57.2b	75.0	25.0b
FGA	20	55.0b	54.5	45.5a
CIDR	20	85.0a	88.2	11.7b

a,b: P<0.05

Conclusion: The duration of a progestagen treatment cycle for controlling estrus affects without influencing the F; the CIDR for 7 days allows further regarding vaginal sponges F; progestative treatment of 14 days give a higher P relative to treatments 7 days.

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EVALUATION OF *CAPSICUM ANNUUM* GERMOPLASM FOR SOURCES OF RESISTANCE TO WHITEFLY (*BEMISIA TABACI*)

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Abstract: The whitefly (*Bemisia tabaci*) is a serious plant pest that represents a constraint in the development of a wide variety of horticultural crops due to the damage caused by sap feeding and transmission of begomoviruses. To explore possible sources of resistance to *B. tabaci*, adult attractiveness and oviposition preference of *B. tabaci* to local *C. annuum* genotypes collected in southeast Mexico were evaluated. Low adult attractiveness to the genotype Maax relative to the commercial genotype Jalapeño was observed. Similarly, *B. tabaci* showed significantly lower oviposition preference to the genotype Maax relative to that of Jalapeño. The genotype Maax might be used as source of resistance to *B. tabaci* in breeding programs for *C. annuum.*

Keywords: *Capsicum annuum*, ● *Bemisia tabaci*, ● plant resistance, ● chili pepper,

Introduction: Chili pepper (*Capsicum annuum* L.) is a widely cultivated crop and is one of the most consumed vegetable worldwide. In America *C. annuum* is used as fresh or processed product, as well as food flavoring, coloring agent, pharmaceutical ingredient, and human health care (Casseres, 1981). One of the main pest that affects seriously the field production of this vegetable crop is the whitefly (*Bemisia tabaci*, Hemiptera: Aleyrodidae) (Brown, 1996). This pest causes direct damage to plants by sap feeding and indirectly by begomovirus transmission (Ortega, 2002). In México, this pest has affected the production of pepper in various regions by virus transmission, particularly in Jalisco, Guanajuato and San Luis Potosí, where big areas of commercial pepper crops are found (Garzón et al., 2002). The control of *B. tabaci* in pepper has been typically carried out by the application of synthetic insecticides, which has had negative impact on the environment and human health (García, 1999). The use of resistant genotypes to manage *B. tabaci* has shown promising results in various crops (Toscano et al., 2002; Fancelli et al., 2003). On pepper, however, no detailed study has been carried out on this respect.

The exploration of wild and domesticated local genotypes of peppers offers an excellent opportunity to find sources for *B. tabaci* resistance. A recent study on genetic diversity and structure of wild and semi-domesticated *C. annuum* in Mexico showed that southeast Mexico is an important center for domestication and diversity of pepper as a wide variety of genotypes was found, some of them were unique (Aguilar-Meléndez et al., 2009).

In the present work, local genotypes of *C. annuum* were collected in southeast México and their resistance to *B. tabaci* was evaluated based on adult attractiveness and oviposition preference.

Materials y methods: The experiment was carried out in a greenhouse at the Instituto Tecnológico de Conkal, Yucatán. Fourteen genotypes were collected from rural markets and back gardens in southeast Mexico (Yucatán, Tabasco and Chiapas). The commercial genotype "Jalapeño" was used as susceptible genotype. To homogenize germination, previous to sowing seeds were immersed for 24 h in a solution of 250 mg L^{-1} gibberellic acid. Seedlings were maintained with 60% humidity in the substrate and fertilized twice a week with Triple 17® (2 g L^{-1})

in the watering. Twenty five-day-old plants were planted individually in 1 L styrofoam cups. Bioassays were carried out in 45-day-old plants.

The colony of *Bemisia tabaci* was obtained from individuals collected in *Capsicum chinense* that was established in greenhouse at the Institute. Adults were confined in entomological cages that contained 30-day-old *C. chinense* plants. For adult attractiveness and oviposition preference, 45-day-old plants were set in entomological cages and *B. tabaci* adults were liberated with a proportion of 30 insects per plant. Number of adults and number of eggs laid in two young fully-expanded leaves were evaluated 24 and 48 h after liberation. Adult attractiveness was calculated as indicated by Baldini and Lara (2001): IA=2G/(G+P), where G is the number of adults attracted to the local genotype and P is the number of adults attracted to the commercial susceptible genotype (Jalapeño). Values of IA range from 0 to 2, IA=1 indicates similar attractiveness between the regional and the commercial genotypes, IA>1 indicates higher attractiveness to the local genotype relative to the commercial genotype, and IA<1 indicates lower attractiveness to the local genotype relative to the commercial genotype. For oviposition preference, the index was obtained as indicated by Oriani et al. (2005): IPO=[(T-P)/(T+P)]*100, where T indicates the number of eggs laid in the local genotype and P is the numbers of eggs observed in the commercial susceptible genotype. IPO values range from +100 (full preference) to -100 (no preference).

Experiment was set in a randomized block design. The analysis of variance and the mean comparison (Tukey, $p \leq 0.05$) were run in GraphPad InStat (GraphPad Software Inc., 2000). For the data analysis original values were transformed to root square to homogenize variance.

Results y discussion: Adult attractiveness. Attractiveness of adults to the genotypes of *C. annuum* varied significantly in both evaluation periods. The genotype Maax showed lower number of adults (0.6 and 0.0 adults per cm^2, respectively) and lower attractiveness index (0.14 and 0.0) compared to Jalapeño (Table 1).

Oviposition preference. Oviposition preference of *B. tabaci* on the pepper genotypes varied significantly on both evaluation periods. *B. tabaci* showed lower oviposition preference on the genotype Maax (0.5 and 0.7 eggs cm^2 at 24 and 48 h, respectively) compared to that observed in the genotypes Jalapeño (12 y 16 eggs cm^2) and Parado (13 y 16.3 eggs cm^2). The IPO values for the genotypes Maax were -92.0 and -91.9 at 24 and 48 h, respectively (Table 2).

To feed phytophagous insects select their plants based on visual y olfactive cues (Prokopy and Owens, 1983; Visser, 1988). In this context, the genotypes that attracted low number of adults might have certain traits in the leaves, such as color or odors that made leaves less attractive to *B. tabaci*. The liberation of volatile compounds as defense against phytophagous insects has been well characterized in other vegetable species such as tomato where substances like alcaloids, glucosides and phenolic compounds have been found to protect or repel noxious insects from foliage (França and Castelo, 1987).

The oviposition preference of *B. tabaci* is influenced by various factors, such as plant age and leaf position (De la Lima and Campos, 2008). Quality and chemical composition of leaves are also traits of enormous influence for oviposition preference (Walker and Perring, 1994). In a previous study, Muñiz and Nombela (1997) found significant variation on *B. tabaci* fecundity when fed on different commercial genotypes of *C. annuum*. This precedent is clear evidence that it is possible to find genotypes with some degree of resistance, specifically based on oviposition preference.

In the present study, *B. tabaci* showed less oviposition preference on the genotype Maax. This genotype also attracted fewer adults, which suggests that this genotype might have morphological or chemical traits in the foliage that deters or repel *B. tabaci*. This resistance mechanism might aid to suppress population levels of this pest on peppers.

Table 1. Mean number of *B. tabaci* adults per cm^2 leaf and attractiveness index (IA) 24 and 48 hours after adult liberation in free-choice tests.

Genotype	Adults/cm^2		Attractiveness index (IA)	
	24 h	48 h	24 h	48 h
Jalapeño	8.2 ± 1.7 a [z]	4.2 ± 1.9 a	1.00	1.00
Huero	6.7 ± 2.8 abc	4.6 ± 1.4 ab	0.90	1.05
Parado	7.1 ± 0.8 ab	2.5 ± 0.9 ab	0.93	0.75
Pozol	4.0 ± 1.1 abcd	3.7 ± 1.4 ab	0.66	0.94
Pico paloma	5.2 ± 1.1 abcd	1.7 ± 0.7 ab	0.78	0.58
Payaso	4.5 ± 1.6 abcd	2.3 ± 1.0 ab	0.71	0.71
Bolita	5.0 ± 1.0 abcd	1.2 ± 0.7 ab	0.76	0.44
X´cat ik	2.8 ± 1.0 abcd	1.1 ± 0.3 ab	0.51	0.42
Yaax ik	2.5 ± 0.6 abcd	1.5 ± 0.6 ab	0.47	0.53
Blanco	2.6 ± 0.7 abcd	1.3 ± 0.8 ab	0.48	0.47
Amaxito	3.2 ± 0.9 abcd	0.5 ± 0.3 ab	0.56	0.21
Chawa	1.5 ± 0.8 bcd	0.6 ± 0.3 ab	0.31	0.25
Simojovel	1.1 ± 0.4 cd	0.2 ± 0.2 ab	0.24	0.09
Maax	0.6 ± 0.9 d	0.0 ± 0.0 b	0.14	0.00

[z] Means followed by the same letter within the same column are not significantly different (Tukey, $P < 0.05$).
IA: Attractiveness index. IA=2G/(G+P), where G is the number of insects attracted to the local genotype, and P is the number of insects attracted to the commercial genotype (Jalapeño).

Table 2. Mean values for the number of eggs on the leaves of pepper genotypes and oviposition preference index (IPO) for *B. tabaci* in free-choice tests.

Genotypes	Eggs cm^2 24 h	48 h	IPO values 24 h	48 h
Jalapeño	12 ± 4.35 ab [z]	16.5 ± 6.49 a	00.0	00.0
Parado	13 ± 4.75 a	16.3 ± 4.05 a	04.0	-0.6
Huero	5.7 ± 1.67 abc	9.7 ± 2.33 ab	-35.6	-26.0
Pozol	3.1 ± 1.06 bc	9.0 ± 3.25 ab	-58.9	-29.4
Bolita	5.8 ± 2.09 abc	7.5 ± 2.11 ab	-34.8	-37.5
Pico paloma	5.8 ± 2.09 abc	6.1 ± 1.27 ab	-34.8	-46.0
Yaax ik	4.0 ± 0.77 abc	5.3 ± 1.91 ab	-50.0	-51.4
Payaso	2.2 ± 0.86 bc	4.8 ± 2.00 ab	-69.0	-54.9
Amaxito	2.6 ± 0.96 bc	4.1 ± 1.61 ab	-64.4	-60.2
Blanco	2.0 ± 0.71 c	3.5 ±1.21 b	-71.4	-65.0
X´cat ik	1.7 ± 0.64 c	2.6 ± 0.49 b	-75.2	-72.8
Simojel	1.5 ± 0.84 c	2.2 ± 0.97 b	-77.8	-76.5
Chawa	1.5 ± 0.98 c	2.2 ± 0.95 b	-77.8	-76.5
Maax	0.5 ± 0.32 c	0.7 ± 0.31 b	-92.0	-91.9

[z] Means followed by the same letter within the same column are not significantly different (Tukey, $P < 0.05$).
IPO: Oviposition preference index. IPO=[(T-P)/(T+P)]*100, where T is the number of eggs observed in the local genotype and P is the number of eggs observed in the commercial genotype (Jalapeño).

Conclusions: The local *C. annuum* genotypes collected in southeast México showed significant differences on adult attractiveness. There was also difference on oviposition preference by *B. tabaci*. Low adult attractiveness and low levels of oviposition preference by *B. tabaci* on the genotype Maax was observed. This genotype might be a good candidate as source of resistance to *B. tabaci* in *C. annuum* breeding programs.

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MICROPROPAGATION OF *CARICA PAPAYA* L. VAR. MARADOL

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Abstract: All previous studies related to *in vitro* propagation of *Carica papaya* L. reveal that the main drawbacks to propagate this species are the multiplication *in vitro*, rooting *ex vitro* and acclimatization of plants in greenhouses. Therefore, we became interested in developing an efficient protocol for *in vitro* micropropagation of papaya plants cv Maradol micropropagation via 2 different systems: direct organogenesis and somatic embryogenesis. As plant material we used elite plant axillary buds, which were introduced *in vitro* and certified seeds of papaya var. Maradol, for somatic embryogenesis.

Keywords: • Direct Organogenesis, • Somatic embryogenesis, • Micropropagation.

Introduction: Papaya (*Carica papaya* L.) belongs to the family Caricaceae. This species grows in tropical and subtropical American and African countries (Cronquist, 1981). Globally, the papaya is a fruit Maradol high consumption and demand has increased not only by having good sensory characteristics and nutritional, but also for the benefit they bring certain biomolecules, such is the case of carotenoids (Yamamoto, 1964). The increase in production and marketing of papaya Maradol in Mexico, particularly in the state of Yucatan, create the need to generate knowledge to improve their production. The cultivation takes a lot of agricultural inputs (seeds, fertilizers, insecticides and fungicides), a good irrigation system, also labor for the daily work and harvest, now, is necessary to apply appropriate technologies to obtain yields that can come to exceed standards of performance per cycle (Mishra et al., 2007).

The spread of the papaya is by seed and results in considerable variability in the final characteristics of the fruit (Litz and Conover, 1978). In 1983 our country began developing protocols to propagate plants of agronomic interest (Herrera-Estrella et al., 1983). These techniques include the use of *in vitro* culture of plant tissues, which is necessary to promote the formation of phenotypically normal plants through organogenesis and somatic embryogenesis.

The importance of this work is to improve the production of papaya solving the problem of heterogeneity and sexual diversity in the cultivation of this species as hermaphroditic plants are the most commercial interest to the producer. By planting papaya seed by known until the third month after planting, the type of flower you want, which is in many cases an extra expenditure of production during this period, because only 70% could be plants hermaphrodites. Therefore, by the benefit to the producer plant organogenesis plantations 100% hermaphrodites, reducing costs and achieving high production of papaya per acre, the same way by somatic embryogenesis is used as an alternative for the propagation, breeding and germplasm conservation of papaya.

This work focused on improving strategies for developing micropropagation protocols in seedlings of papaya var. Maradol at different stages of cultivation: shoot multiplication, growth, maturation and acclimatization of plantlets. So plant biotechnology through tissue culture techniques is proposed as a reliable procedure for the propagation and conservation of plants *in vitro* papaya.

Materials and Methods: The work was carried out at the Laboratory of Molecular Physiology and Biotechnology Unit of the experimental greenhouse belonging to the Scientific Research Center of Yucatán, A.C., (Merida, Yucatan, México).

Direct organogenesis. To set the protocol used micropropagation plantlets obtained from lateral buds of adult plants of papaya Maradol hermaphrodite, which were taken from the nursery of the institution. The buds were washed and sterilized with 2% chlorine. For multiplication of shoots vitroplants established in culture media with MS salts (Murashige and Skoog, 1962) supplemented with different concentrations of plant growth regulators (Table 1).

Table 1. Different concentrations of plant growth regulators used to promote shoot regeneration of *Carica papaya* L. var. Maradol via direct organogenesis.

SHOOT INDUCTION								
Treatment	Number of plants	Initial Height (cm)	Duration (days)	Growth regulators (%)				
				BAP	GA3	IBA	ANA	Kin
Control	15	1.5	21	-	-	-	-	-
A	15	1.5	21	100	-	-	-	-
B	15	1.5	21	-	100	-	-	-
C	15	1.5	21	50	-	-	-	50
D	15	1.5	21	-	-	20	-	50
E	15	1.5	21	-	-	-	20	50

For the elongation, we use shoots from the best treatment from the shoot induction stage. These shoots were sown on MS medium supplemented with different concentrations of plant growth regulators (Table 2), after 84 days (4 consecutive cultures) we evaluated the following variables: height and number of leaves per seedling.

Table 2. Varying concentrations of growth regulators used to promote the elongation of shoots from *Carica papaya* L. var. Maradol.

GROWTH OF SHOOTS					
Treatment	Number of plants	Duration (days)	Growth regulators (%)		
			BAP	GA3	Kin
Control	15	21	-	-	-
C1	15	21	-	-	50
C2	15	21	50	-	-
C3	15	21	-	50	-
C4	15	21	25	-	50
C5	15	21	-	-	50

For the maturation stage, we use plantlets from the best treatment from the growth stage, these plantlets were planted on MS medium under different concentrations of NAA (25, 50, 75 and 100 %), the plantlets were transferred to rooting medium stimulation *in vitro*, according to the protocol proposed by Drew (1988), which contained half strength MS salts, supplemented with different concentrations of IBA (33.3, 66.6 and 100 %).

Somatic embryogenesis. To optimize the micropropagation protocol, certified seeds from papaya var. Maradol, plants were used. Seeds were washed with water and detergent, and then they were sterilized with 96% alcohol.

For induction of somatic embryos 4 explants were used: zygotic embryo, hypocotyl, cotyledon and root. These explants were placed in a medium to induce somatic embryogenesis, according to the protocol established by Cabrera-Ponce et al. (2000). These explants were placed in darkness for 3 months and subcultured every 21 days at 27 °C.

After 3 months, embryos were regenerated on media containing MS salts, according to the protocol developed by Solis et al. (unpublished), continuous light conditions and subcultured every 21 days for a period of 2-3 months, and these germinated embryos were placed in a maintenance medium containing MS salts, according to the protocol developed by Solis et al. (unpublished) for 16 h photoperiod at 27 °C.

For the 2 micropropagation protocols (direct organogenesis and somatic embryogenesis) the same techniques for acclimatization and *ex vitro* rooting were used.

For *ex vitro* rooting stage, plants were washed at its base to remove traces of culture medium with sterile distilled water. Tissue from 3 mm from the base was removed to prevent decay. Subsequently, we applied a solution with fungicide Benlate by soaking plants for 5 minutes. A commercial rooting Radifarm was also applied by soaking plantlets for 1 hour. In the solution. Plants were then transplanted to plastic trays containing a mixture of peat moss-vermiculite substrates: perlite (2:1) previously sterilized and moistened with sterile distilled water.

In the greenhouse phase the trays were covered with a plastic dome to maintain the relative humidity as high as possible to 100% and sprinkling irrigation was applied to the substrate using sterile distilled water + 2.5 ml L^{-1} of commercial rooting Radifarm, every five days. After 30 days, the plastic domes were removed overnight and covered in the hottest hours of the day in order to promote acclimatization. After 45 days, the plastic domes were removed and plants were exposed greenhouse under natural conditions. For the last stage of acclimatization in the greenhouse phase, the parameter to be evaluated was: percentage of surviving plants after 50 days of being exposed under greenhouse conditions.

Results and Discussion: *Direct organogenesis.* For shoot multiplication rate, the MS medium (Murashige and Skoog, 1962) supplemented with the growth regulators of treatment C, proved to be the most suitable as it produced 5.46 shoots per seedling. For the elongation phase the best results were obtained using the C5 treatment, plantlets reaching a height of 3.35.

Figure 1 shown: A) Vitroplants obtained from lateral buds of adult plants of papaya Maradol hermaphrodite. B) Preparation of shoots with cytokinins. C) Quantification of the number of shoots per seedling. D) Height of plants at the stage of growth with cytokinin. E) Obtained in step seedling ripening, with a height of about 3 cm having 3 to 4 leaves. F) *In vitro* stimulation of root, seedling roots small provided at the base of the stem. G) Plants with plastic dome. H) First true leaf of plants at 30 days after planting *ex vitro* rooting and I) plant with root system functional at 45 days after planting and transplanted into polyethylene bags (Figure 1).

*Somatic embryogenesis***.** The protocol established by Cabrera-Ponce et al. (2000) was successfully reproduced at different stages *in vitro*. It was observed that when using zygotic embryos and hypocotyl embryogenic callus were obtained after 3 months When cotyledons were used a s source of explants a higher percentage of somatic embryos (63.88%) were obtained after 3 months of culture. Plants are regenerated after 4 months of culture under conditions of photoperiod with subcultures of 21 days. Embryo regeneration was achieved 2-3 month period obtaining 67.55% germination.

Figure 1. Micropropagation protocol via direct organogenesis.

Conclusions: It was possible to optimize two protocols for micropropagation of hermaphrodite papaya (*Carica papaya* L.) var. Maradol plants, obtained via direct organogenesis and somatic embryogenesis. For plants obtained via direct organogenesis it was observed that the use of the protocol 1 (Estrella et al., unpublished) proved to be the best treatment to produce 5.46 shoots.
In terms of the somatic embryogenesis protocol, by using cotyledons as explants, we were able to obtain 63.88% of somatic embryos after 3 months.
In the acclimatization phase, after 50 days of plants being exposed to greenhouse conditions 68% survival was achieved by plants obtained via direct organogenesis while 84% of survival for plants obtained via somatic embryogenesis.

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MORPHOLOGICAL, BIOCHEMISTRY AND MOLECULAR CHARACTERIZATION AND SELECTION OF GENOTYPES WITH FLESH COLOR RED ORANGE IN *CARICA PAPAYA* L.

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Abstract : This crop was developed during the period October 2009 to May 2010 in a protected area antiáfidos mesh in the experimental field of Scientific Research Center of Yucatán (CICY). The aim was to characterize and select small fruit and flesh color red orange (*Carica papaya* L.) hermaphrodites genotypes of crosses criollo7 x Maradol 22 by morphological, biochemistry and molecular characterization. The fruit characteristics variability was evaluated to use it later as a basis to continue studying the selection of potentially useful *Carica papaya* L. genotypes. Multivariate analysis grouped the genotypes of papaya yellow flesh color with large and small fruits, and genotypes orange-red flesh color with large and small fruits. Molecular analysis with molecular marker CPF1 confirmed that 82 percent of the genotypes that are phenotypically and genotypically of orange-red flesh color.

Keywords: *Carica papaya* L. ● Flesh color ● Morphological biochemistry and molecular characterization ● Selection

Introduction: The specie *Carica papaya* L. is believed to be native to southern Mexico and neighboring Central America. Actually, it is distributed widely throughout tropical and subtropical regions of the world (Yeh *et al*., 2007). So that Brazil, Mexico, Nigeria, India and Indonesia yield more than 70% of the total world production. Although Mexico is one of the leading producers of papaya, it has no alternatives for the choice of hybrids and cultivars this crop, being Maradol the most widely used cultivar, which has lost in size and flesh color quality of the fruit. Flesh color of the papaya is indicative of nutritional content and is the result of the accumulation of carotenoids in chromoplasts of fruit cell, mainly lycopene in the red flesh and β-carotene in yellow flesh, which provide antioxidant activity (Blas, 2008). Also, the fruit is a good source of vitamin A and C (Manshardt, 1992). The color of papaya pulp is an aspect that determines its acceptance by the consumer, whose trend is the preference for orange-red flesh fruit (Miranda *et al*., 2002). This can only be characterized phenotypically and biochemically of 7 to 9 months after planting, reason why we tested the strategy of marker-assisted selection and thus reduce the waiting time for selection. DNA markers can be used efficiently in breeding to know information about genes of agricultural importance and as a tool to accelerate plant breeding traditional processes, to facilitate the work to breeder. There on, Demey *et al*. (2003) report that those studies incorporating morphological descriptors and molecular markers provide a better description and interpretation of genetic diversity of individuals. In this regard, Blas *et al*. (2010) reported a molecular marker associated with the flesh color in *Carica papaya* L. Hawaiian type. Using this molecular marker is looking to start a program of marker-assisted selection, so that the correlation with the morphological characterization is required the selection of promising and exemplary programs useful in breeding the species.

Materials and Methods: This crop was developed during the period October 2009 to May 2010 in a protected area antiáfidos mesh in the experimental field of Scientific Research Center of Yucatán (CICY). We perform a cross between the parent native 7 and the pollen donor parent Maradol 22, which have contrasting characteristics in flesh color and fruit size. Of crosses was developed forty-two F1 adult plants, 14 female and 28 hermaphrodite, only the latter were

considered for characterization and selection. Were collected three fruits per plant at physiological maturity, this maturity is identified by a yellow-orange in a quarter of the fruit surface (Sañudo *et al*. 2008). Three fruits per plant at physiological maturity were collected, this maturity is identified by a yellow-orange in a quarter of the fruit surface. The fruits were transported to the laboratory of Molecular Physiology CICY which reached ripeness. The weight, length and diameter of the fruit were evalued based on the descriptors reported by UPOV, 2010. Flesh color was assessed visually and the color components *L, a, b, h* and *C* using the colorimeter (Konica Minolta Sensing, INC.). The β-carotene and lycopene content was obtained following the protocol reported Nagata and Yamashita (1992). Also, pH, total solubes solids and acidity were measured. Data obtained were analized with the UNIVARIATE procedure of SAS 9.0. Then cluster multivariate analysis was performed using the method of clustering based on Ward and Euclidean distance, corresponding dendrogram was constructed (InfoStat/L). The management analysis used the principal components with the correlation matrix between standardized characters, using the numerical taxonomic system (NTSYS 2.1). For the molecular characterization established a DNA extraction method based on CTAB suggested by Doyle and Doyle, 1990, and modified by Vázquez, 2010. The amplification of molecular markers (CPF1) associated with characters of flesh color fruit was performed by PCR, and the presence or absense of bands were visualized by 5% agarose electrophoresis.

Results and Discussion: Figure 1a shows that papaya genotypes evaluated, with a squared Euclidean distance of 35.0 defines six groups. The genotype Maradol 22 formed a group characterized by having red-orange fruit of large size, while the second group joined the genotype H70B which has orange-red fruits of small size and the third group joined nine genotypes with orange fruits with intermediate fruit size. On the other hand, group four joined with four genotypes that have yellow flesh color with large fruits, group five consisted of six genotypes with yellow flesh color and fruit size smaller, and group six formed with nine genotypes whose flesh color is yellow but its fruits are of intermediate size (Table 1). In this regard, Santamaria *et al* (2009) reported similar data of group one, two and three from this study for the coordinates *L, a, b* and h in a pulp of 51, 23, 36 and 60 in mature fruits Maradol.

Figure 1b shows that genotypes were concentrated in four groups: one group was composed of four genotypes that have reddish orange flesh and small size of the fruit. On the other hand, group two has thirteen genotypes that produce fruits with yellow flesh and a small size. Group three consists of five genotypes develop large fruit with yellow flesh and group four consists of seven genotypes that have large fruit but orange-red flesh color. In this study focused on the selection of genotypes H6B, H18B, H70B and H77B, which are parts of group one. Mean values of the variables were compared individually (data not shown) and was the genotype H77B being better characteristics according to flesh color, fruit size, content, total soluble solids, β-carotene and lycopene. De Morais *et al* (2007) reported that the fruits of papaya type 'SOLO' with average weight of 460 to 690 g are highly preferred for export, due to its ease of handling, packing and transportation, this feature was observed in the H77B.

Maradol 22 presented orange-red flesh and displayed an amplified band only. While the native 7 features two alleles amplified from 650 bp, which is yellow flesh color, the same condition is seen in Hawaiian papaya. These data are consistent with those reported Blas *et al*. (2010) because they indicate that hybrids and Richter Rainbow (yellow flesh) are heterozygous and contains the two alleles, demonstrating amplification of 500 bp double bands.

The molecular marker CPF1 is useful because it facilitates the detection of genotypes with orange-red flesh color. Compared the result of amplification of the molecular marker CPF1visualizado in figure 2 with the genotypes of group one and four principal components analysis and found that 82% of the F1 genotypes are phenotypically and genotypically grouped with reddish orange flesh color.

Table 1. Mean values of the traits evaluated in fruit for group formed by cluster analysis in F1 population of intraspecific crosses of *Carica papaya* L. native 7 x Maradol 22.

Grupo	PF	LF	DF	Li	ß-C	SST	AT	PH	*L*	*a*	*b*	*h*	*C*
1	2230.0	27.0	13.0	3.75	0.35	11.00	0.02	5.00	54.48	23.45	42.15	60.91	48.23
2	449.2	15.3	8.00	0.62	5.13	13.92	0.17	4.95	49.45	22.23	40.85	60.59	46.74
3	691.4	18.3	9.4	0.80	3.05	13.13	0.03	5.13	51.95	20.10	40.86	63.74	45.63
4	858.7	19.6	10.8	0.08	5.06	12.29	0.03	5.28	62.37	14.81	56.64	75.38	58.55
5	469.5	16.2	8.0	0.05	3.46	8.98	0.04	5.30	61.27	14.91	54.16	74.64	56.19
6	542.40	16.7	8.67	0.27	4.58	11.76	0.04	5.25	54.39	14.79	55.57	71.88	58.00

PF = Fruit weight (kg), **LF** = Length of fruit (cm), **DF** = Diameter of fruit (cm), **SST** = total soluble solids (º Brix), titratable acidity **AT** = (% citric acid), **pH** = potential hydrogen. **Li** = Lycopene (mg.g-1 fresh weight), **ß-C**: carotene = (mg.g-1 fresh weight). Scale for pulp color components: ***L***: 0 to 100 dark to light), ***a***: -100 to +100 (green to red), ***b***: -100 to +100 (blue to yellow), ***h***: 0 to 90 (red to yellow), ***C***: color intensity (for e - + yellow to yellow).

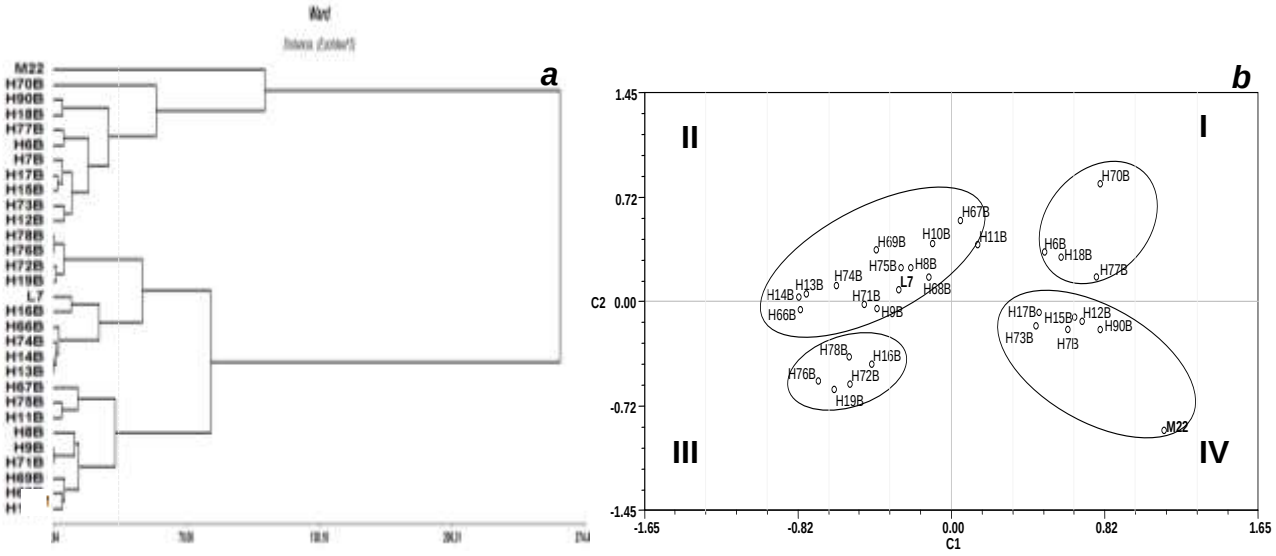

Figure 1. a) Dendogram of clustering based on squared euclidean distances using Ward's method for variables evaluated in fruit of *Carica papaya* L. genotypes. **b)** Dispersion Biplot of 28 genotypes of *Carica papaya* L. based on principal components 1 and 2.

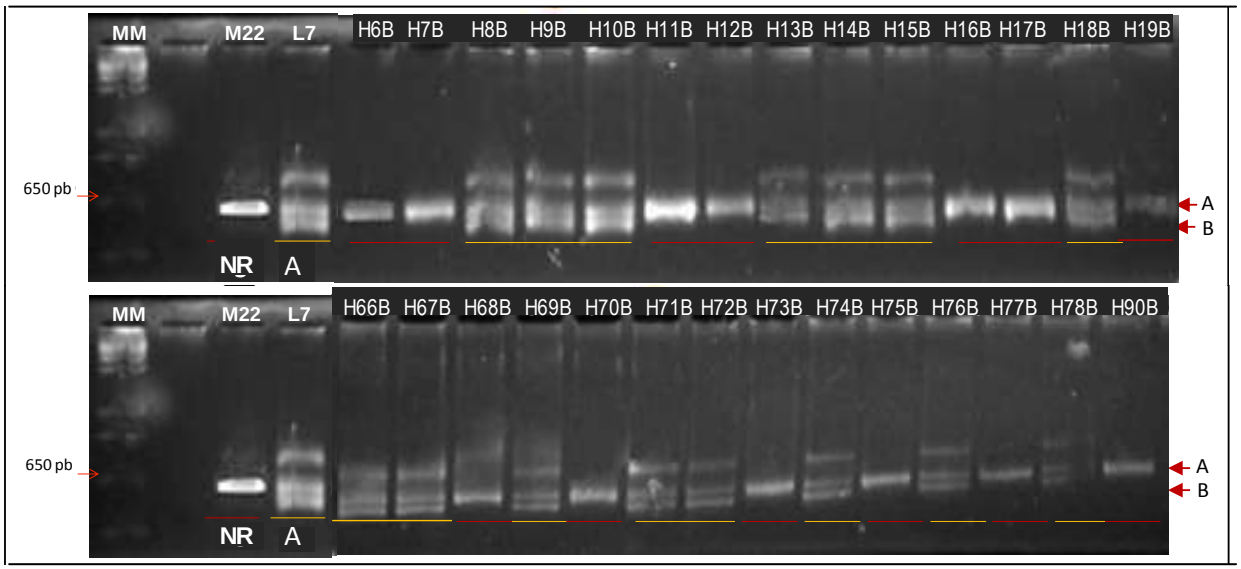

Figure 2. Electrophoresis on 5% agarose color marker (CPF1) in parentals and F1 progeny (crosses native 7 x Maradol 22) of *Carica papaya* L. M22: maradol 22, L7: native 7, H6B-H90B: F1 hermaphrodite progeny. The A allele corresponds to orange-red flesh color (NR) and the B allele corresponds to the yellow flesh color (A).

Conclusions: Multivariate analysis grouped the genotypes of papaya yellow flesh color with large and small fruits, and genotypes orange-red flesh color with large and small fruits. Genotype H77B meets the phenotypic and genotypic characteristics most important to be an exemplary elite and be incorporated into the program selection and breeding. Molecular analysis with molecular marker CPF1 confirmed that 82% of the genotypes that are phenotypically and genotypically of orange-red flesh color. Molecular marker CPF1 is useful because it facilitates the early detection of those plants of *Carica papaya* L. who will develop fruits with reddish orange flesh, which can be used in the genetic improvement of this character

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DIFFERENTIAL INDUCTION AND REPRESSION PATTERNS OF BETA-FRUCTOFURANOSIDASES OF *ASPERGILLUS NIGER* IN SUBMERGED AND IMMOBILIZED CULTURE

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Abstract: Fructofranosidases induction and repression patterns were studied for *A. niger* N402 strain cultured in Submerged and Immobilized Fermentation. A mineral minimal media with 100 g/l of sucrose, inuline, sucrose-glucose or inulin-sucrose in an 80-20 % proportion as carbon source was used. Invertase and inulinase activity were determined for each of condition in both culture systems. In both SmF and ImF the maximal production of invertase was obtained with sucrose and inulinase maximal production was obtained when inulin was used as only carbon source. Both culture systems shown crossed induction for inulinase with sucrose and invertase when inulin was used as carbon source. When glucose was added to the cultures showed almost a complete repression with both sucrose as inulin. ImF showed resistance to catabolic repression with sucrose and inulin in the presence of glucose. ImF culture system allowed the reproduction of SSF advantages as over production of enzymes and resistance to catabolic repression and at the same time it allowed the complete analysis of fermentation variables as SmF. When ImF study has been complete it is possible that this culture system represent a very good alternative for production of homologous but especially heterologous protein production by *Aspergillus niger*.

Keywords: *Aspergillus niger* ● Fructofuranosidases ● Induction-repression ● Immobilized culture.

Introduction: Filamentous fungi have been widely used for the production of homologous and heterologous proteins (Viniegra *et al*, 2003). Main production system used is Submerged Fermentation (SmF), the choice of this culture system is based in the very good control of fermentation variables it makes possible; but protein production normally is lower to the reported in any other culture system. In the other hand, Solid State Fermentation has reported to improve production and secretion of both homologous and heterologous proteins by *Aspergillus niger*, unfortunately the presence of edible supports in this culture system makes control and analysis of this fermentation system very hard and sometimes even impossible (Pandey, 2003). Immobilization of *Aspergillus* can replicate many of the advantages of Solid Substrate Fermentation with the straightforward control and analysis of Submerged Fermentation (Romero *et al*, 2000).

Invertase (b-D-fructofuranoside fructohydrolase, EC 3.2.1.26) and Inulinases (2,1- b-D-fructan fructanohydrolases, EC 3.2.1.7) are synthesized and secreted by *Aspergillus niger* and can be used as a good model for the study of extracellular glycoprotein secretion (Wallis *et al*, 1997). Invertase production in ImF has been reported to be as high as 4 times bigger than in SmF (Romero *et al*, 2000). Previous results of zymography analysis of SmF and ImF invertase extracts obtained in this lab produced two activity bands for SmF extracts and only one for ImF; isoelectric focusing analysis of the same extracts is consistent with zymography showing two peaks of invertase activity for SmF and only one again for ImF.

Fructofuranosidase (Ffase) activity in *Aspergillus niger* is related to the production of invertase from *suc*1 and *suc*2 genes and inulinase from *inu*A, *inu*B and *inu*E genes (Yuan *et al*, 2006). It has been reported that for SmF, invertase and inulinase activities are regulated by sucrose and inulin in a complex way; per example it is known that sucB invertase shows inulinase activity in an invertase: inulinase ratio of 1:4. In a first approach to elucidate the induction-repression patterns of Ffases for ImF, invertase and inulinase production and regulation have been studied for ImF using polyurethane foam as inert support.

Materials and methods:

Aspergillus niger strain and culture conditions. *Aspergillus niger* ATCC 1640 strain was obtained from a commercial source and maintained at 4º C on PDA slants that were re-cultured every three months. Basic mineral medium (BMM) containing ($g*L^{-1}$): $NaNO_3$, 15.0; KH_2PO_4, 1.76; KCl, 0.76; $MgSO_4$, 0.76; $FeCl_2$, 0.001; $CuSO_4$, 0.001; $MnCl_2$, 0.001; $ZnCl_2$, 0.001 was used; sucrose, inulin (100 $g*L^{-1}$) or sucrose/glucose, inulin/glucose (80/20 $g*L^{-1}$) were used as carbon source. Inoculation was performed with $2*10^7$ spores per g of carbon source. Seed cultures were propagated on PDA culture medium for 72 h.

Culture conditions. Submerged fermentations were done in 125 ml flasks. Immobilized fermentation was performed in 250 ml flasks with one gram of polyurethane foam as inert support. 25 ml of inoculated BMM were added to each flask and both culture systems were incubated at 30º C and agitated at 200 rpm in an orbital shaker for the time of the fermentation to avoid differences on culture conditions. One flask for each culture system was retired from the incubator every 12 hours, extracts were obtained by filtration trough filter paper for SmF and mild pressure and paper filtration for ImF.

Enzyme activity determination. Invertase or inulinase activity was determined by estimating the release of reducing sugars from the proper substrate (sucrose or inulin) using DNS technique according to Miller (1959). One unit of either enzyme activity was defined as the necessary to release one micromole of reducing sugar per minute.

Results and discussion. Figure 1A shows previous results obtained in this lab; SmF extracts show two activity bands of beta-fructofuranosidase while one band is obtained for ImF extracts. The two bands on SmF lane have been reported for *A. niger* and *A. nidulans* (Vainstein & Peberdy, 1991; Boddy *et al*, 1993; Chen *et al*, 1996); The presence of only one Ffase activity band in ImF extracts is consistent with reports about the modifications in the expression profile of enzymatic families that has been reported previously for ImF (Parra *et al*, 2004; Villena & Gutierrez-Correa, 2006).

Figure 2B shows the resistance of ImF to catabolic repression; while in SmF the addition of 25 $g*L^{-1}$ completely close the expression of Ffase activity, ImF produces a level that is very similar to the one obtained without the addition of glucose. Those results correspond to the reports of several authors that report the higher resistance of Solid State fermentation (SmF) and ImF to catabolic repression (Pandey, 2003; Viniegra & Favela, 2006).

Table 1 shows maximal production level for invertase and inulinase using sucrose, inulin, sucrose plus glucose or inulin plus glucose as carbon source; it is shown that in both SmF and ImF the maximal production of invertase is obtained with sucrose as only carbon source and the inulinase maximal production is obtained when inulin is used as only carbon source. Inulinase production is about a 30 percent of invertase production when sucrose is used as the only carbon source; while invertase shows a similar level related to inulinase when inulin is used as unique carbon source. That both culture systems show the bigger production level with its correspondent inducer and crossed for inulinase with sucrose and invertase when inulin is used as carbon source are correspondent to previous reports of Ffases induction in SmF (Yuan *et al* 2006). Higher production levels of both enzymes by ImF are correspondent to the idea that this culture system allows the replica of SSF advantages, being the most important the over

production of enzymes (Pandey, 2003; Viniegra *et al*, 2003). When glucose was added to the cultures in a 20 %; SmF shows almost complete repression with sucrose as with inulin, this has been reported several times as is the normal behavior for enzyme production in SmF (Yuan *et al*, 2006); that the repression was not complete despite the previous finding of this or other reports can be attributed to variation in the response of different fungal strains to catabolic repression. ImF shows resistance to catabolic repression with sucrose and inulin in the presence of glucose as has been reported previously (Aranda *et al*, 2006; Viniegra & Favela, 2006). In fact invertase and inulinase production are a 10% higher when glucose is added to the culture media, it can be related to a faster biomass accumulation in the beginning of fermentation that allows a higher production of enzymes for that biomass. When inulin is used in combination with glucose in ImF there is decrease of about the 20 % for inulinase and invertase production, but in both cases the maximal production time passed from 36 to 24 h, this change in the maximal production time can be related to the same biomass accumulation effect due to glucose faster incorporation rate.

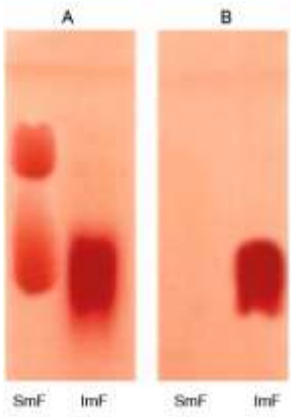

Figure 1. Beta-fructofuranosidase activity zymogram of *A. niger* N402 extracts produced by SmF and ImF using 100 g*L^{-1} of sucrose (A) and 75/25 g*L^{-1} of sucrose/glucose as carbon source.

Table 1. Invertase and inulinase activity of *A. niger* ATCC1640 extracts produced by SmF and ImF using different carbohydrates as carbon source.

Carbon source		Invertase (IU*L^{-1})	Inulinase (IU*L^{-1})
Sucrose 100 g*L^{-1}	SmF	3661	1080
	ImF	4924	1731
Suc/gluc 80/20 g*L^{-1}	SmF	719	217
	ImF	4611	1948
Inulin 100 g*L^{-1}	SmF	1984	3399
	ImF	2807	7082.
Inu/gluc 80/20 g*L^{-1}	SmF	267	906
	ImF	2185	5289

Several authors have reported that higher production level of enzymes, shorter production times, resistance to catabolic repression and the change in enzymatic production profile are advantages of SSF over SmF; however, the changes in culture conditions between both culture systems are so extreme that any comparation of SmF results to SSF results is near to impossible (Pandey, 2003). The use of PUF as inert support ensures that there are no change in culture conditions or media composition; therefore, the differential enzyme expression can be attributed only to the presence of the support and its effect on the way *Aspergillus* grow over it, the geometry of growth has been reported to have a very important effect on enzyme production and protein secretion (Papagianni *et al*, 2002; Talabardon & Yang, 2005).

Conclusions: The ImF culture system allows the reproduction of SSF advantages as over production of enzymes and the resistance to catabolic repression and at the same time allows the complete analysis of fermentation variables of SmF. When ImF study has been complete it is possible that this culture system represent a very good alternative for production of homologous but especially heterologous protein production by *Aspergillus niger*.

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ACTIVITIES AND METABOLIC PROFILES OF SOIL MICROORGANISMS AT KILN SITES IN *QUERCUS SPP*. TEMPERATEFORESTS OF CENTRAL MEXICO

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Abstract: Temperate forest dominated by *Quercus* spp. L. cover large parts of central Mexico and rural communities depend on these forests for wood and charcoal. A KILN site, used for charcoal production, is prepared by clearing all vegetation, removing litter and soil from the surrounding area. The wood is piled up in the centre of the KILN site and covered with litter and soil. The charcoal production had a negative effect on the functional diversity and enzymatic microbial activity in soil. Community level physiological profiles (CLPP) analyses showed lower substrate richness, and functional diversity in soil at the KILN sites compared to the undisturbed soil. Canonical discriminant analysis (CDA) indicated that substrate utilization at the KILN sites was different from undisturbed forest soil. The activity of six enzymes, i.e. CM-cellulase, □-glucosidase, N-acetyl- □-d-glucosaminidase, nitrate reductase, urease and proteinase, decreased from 44% to 90% at the KILN sites compared to the undisturbed forest soil. It was found that charcoal production at a KILN site showed lower functional diversity and enzymatic microbial activity than in the surrounding forest soil as a result of loss of litter and organic matter and changes in microclimatic conditions.

Keywords: BIOLOG ● Charcoal production ● Functional diversity ● Soil quality.

Introduction: Charcoal is used as fuel in rural communities in many places around the world and is made by burning wood in a KILN, creating internal suppressed combustion with the least possible flame (Vázquez-Marrufo et al., 2003). During the production lasting 12–14 days, a proportion of the biomass is transformed to charcoal. In the central highlands of Mexico charcoal is still produced in the Santa Rosa forest (Guanajuato). Typical KILN sites have a diameter of 7–10 mand up to 20 can be found per ha. Ex-KILN sites have different climatic conditions, such as higher solar radiation, exposure to wind and water erosion, extreme temperatures and a greater UV light incidence, than in the surrounding forest (Vázquez-Marrufo et al., 2003). In a previous study in the Santa Rosa forest, the concentrations of exchangeable Ca^{2+}, K^{+} and Mg^{2+} increased >1.6 times at KILN sites and the pH increased from 4.5 in undisturbed soil to 7.0 at the KILN sites (Gómez-Luna et al., 2009). In the Santa Rosa forest the soil organic C at the KILN site was <50% of that found in the undisturbed forest and the microbial biomass C decreased 1.3 times in the rainy season and >2 times in the dry season.

The functional diversity of heterotrophic microbial communities in soil has been examined using the BIOLOG® system (Garland,1997). This method determines the structure and metabolic capacity of microbial communities to use sole carbon sources. This provides a potential tool to monitor changes in microbial functional diversity in soil subjected to some kind of disturbance (Calbrix et al., 2005). The metabolic profiling technique is used for qualitative and quantitative characterization of microbial communities and it allows the use of multivariate statistical techniques to calculate and analyze diversity indices (Bending et al., 2002).Enzyme

activity in soil is also used as an indicator of changes in quality and productivity of soil (Eivazi and Bayan, 1996). It is applied to study short and long-term effects of a disturbance, as the biochemical reactions in soil are mediated by microorganisms (Burns, 1982; Eivazi and Bayan, 1996). The objective of this study was to determine the impact of charcoal production on enzymatic and metabolic profiles of soil microorganisms at a KILN site in *Quercus spp*. temperate forest in central Mexico during the dry and rainy season.

Materials and methods: The study area is located in Santa Rosa in the state of Guanajuato, Central Mexico (N.L. 20°50´59´´–21°55´05´´and W.L. 100°59´28´´–100°33´09´´). The altitude of the study area is 2660 m above sea level, with mean annual temperature ranging from 12 to 18 ◦C and an average rainfall of 1100 mm. The rainy season starts in June and ends in September, while the driest part of the year is between March and May.

Three different points were sampled at each treatment and location: (i) non disturbed soil that was never used to produce charcoal (the CONTROL treatment), (ii) soil where charcoal was produced for one year in 1999 (the KILN treatment), and (iii) a transition zone located between the area where charcoal was produced and the control soil. In the transition zone, the vegetation was cleared, but the litter layer was retained (the TRANSITION ZONE treatment). Soil was sampled from the 0–15 cm layer from the CONTROL, KILN and TRANSITION ZONE treatment in the dry (May) and rainy (July) season.

The community level physiological profiles (CLPP) were determined with the BIOLOG® system. Substrate utilization patterns of the soil microbial community were determined using BIOLOG® ECO and MT microplates BIOLOG®. Substrates in ECO microplates were added with BIOLOG® and substrates in MT microplates were: glucose, protocatechuic acid, tartaric acid, cellobiose, cadaverine, maleic acid, palmitic acid, arginine, oxalic acid, succinic acid, urea, xylose, citric acid, fumaric acid, glutamic acid, tryptophan, thiamine, quinic acid, gluconic acid, pantothenic acid, stearic acid, 1-aminocyclopropane-carboxylic acid, heptadecanoic acid, pentadecanoic acid, putrescine, syringicacid, cinnamic acid, benzoic acid, 2-benzoic acid, ferulic acid and carboxymethyl cellulose.

Diversity indices (S), (H) and (E) were calculated with (S) defined as Richness, (H) as Shannon's index and (E) as Evenness Shannon. Shannon's diversity: H =−□□pilnpi, where pi is the ratio of the activity on a particular substrate to the sum of activities on all substrates. Shannon's evenness: E =H/lnS, where H is Shannon's diversity and S richness (Staddon et al.,1997).

Soil enzyme activity. The carboxymethylcellulase (EC 3.2.1.4) activity in soil was determined using methods developed by Tabatabai (1994). Sub-samples of 5 g soil were mixed with 15 mL(0.7% w/v) carboxymethyl cellulose substrate solution (pH 5.5) and stored at 50 ◦C for 24 h. The glucose released was determined by absorbance at 535 nm. □-Glucosidase (EC 3.2.1.21) activity in soil was measured by the Eivazi and Tabatabai (1988) method. Sub-samples of 1 g soil were mixed with 1 mL 25 mMPNG (p-nitrophenyl- □-d-glucoside) pH 6 at 37 ◦C for 1 h. The N-acetyl-□-d-glucosaminidase (EC 3.2.1.30) activity was determined as described by Parham and Deng (2000). Sub-samples of 1 g soil were mixed with 1 mL(10 mM) pNNAG p-nitrophenyl-N-acetyl-□-dglucosaminide pH 5.5 at 37 ◦C for 1 h. The p-nitrophenol released was measured at 405 nm. Nitrate reductase activity in soil was measured with the Abdelmagid and Tabatabai (1987) method. Sub-samples of 5 g soil were mixed with 10 mL(5 mM) KNO_3 pH 6 at 25 ◦C for 24 h. The NO_2–N produced was determined with the Griess–Illosvay reaction. Urease (EC 3.5.1.5) activity in soil was measured with the Klose and Tabatabai (1999) method. Subsamples of 5 g soil were mixed with 1 mL (0.2 M) urea pH 9 at 37 ◦C for 2 h. The NH_4^+–N released was determined at 660 nm. Protease activity of soil was measured with the Yao et al. (2006) method. Sub-samples of 5 g soil were mixed with 5 mL (1%w/v) gelatin solution pH 7.4 at 38 ◦C for 24 h. The amino acids produced were measured at 570 nm.

Results and discussion: The CDA showed that PC2 was similar for the dry season, but PC1 was different for the different treatments. The PC2 for the rainy season was different between treatments, except for KILN-r PC1 that was highly variable in the rainy season. Along the PC1 axis, the KILN treatment was found on the right-hand side, the CONTROL and TRANSITION ZONE treatment in the rainy season were in the middle and TRANSITION ZONE in the dry season on the left. Along the PC2 axis (dispersion percentage of 37%), the three treatments in dry season were found in the upper side quadrants while those in the rainy season in the lowers quadrants. The cumulative proportion of total dispersion for the two factors was 82% Figure 1.

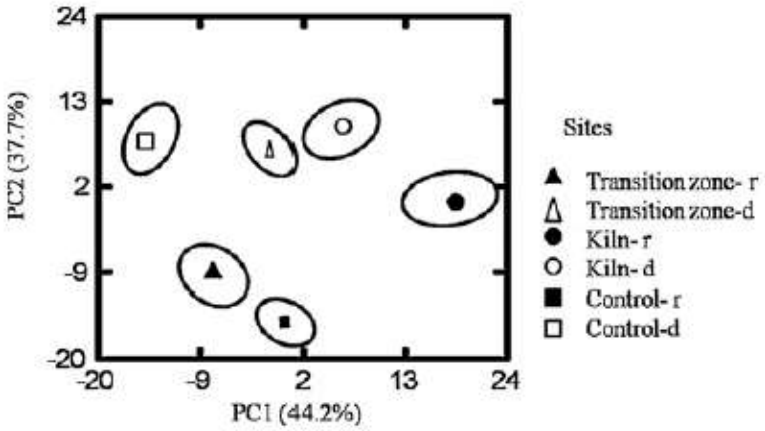

Figure1. Canonical discriminant analysis.

Treatment had a significant effect on richness measured with BIOLOG® ECO and MT in the rainy and dry season ($P < 0.05$).Richness was lower in the KILN treatment than in the other treatments. The Shannon diversity index was significantly affected by treatment as measured with BIOLOG® ECO and MT in the rainy and dry season ($P <0.05$). The Shannon diversity index was lowest for the KILN site and largest in the CONTROL site. The evenness index was significantly lower at the KILN site as measured with BIOLOG® ECO and MT in the dry season, but not in the rainy season ($P < 0.05$). The highest correlation was found between the TRANSITION ZONE and CONTROL sites in the rainy season (0.937) and the lowest between the TRANSITION ZONE site in the dry season and the KILN site in the rainy season (0.707) Table 1.

In the two seasons, the enzyme activity in soil of the TRANSITION ZONE soil was higher than in the KILN soil, but lower than in soil of the CONTROL site. In the rainy season, the cellulase activity was 10 times lower at the KILN site compared to CONTROL site, the □-glucosidase 4 times and N-acetylglucosaminidase, nitrate reductase and urease 2 times. In the dry season, the activity of N-acetylglucosaminidaseactivity and proteinase activity was 7 times lower at the KILN site compared to the CONTROL site; while the nitrate reductase was 2 times lower Table 2.

Some studies have shown that the functional diversity of soil microbial communities can be altered by changes in land use, forestry practices or disturbing factors, such as fire (Staddon et al., 1997; and Graham and Haynes, 2005).Charcoal production induced significant changes in selected soil characteristics at the KILN site compared to the CONTROL site. The CLPP measures the metabolic capacities of microorganisms in soil and is an indicator of functional diversity (Graham and Haynes, 2005).In this study, production of charcoal at the KILN site reduced the metabolic capacity of the soil microbial community and resulted in lower substrate use.

Table 1. Richness, diversity and eveness Shannon index of microbial community at the kiln sites (KILN treatment) and boundary sites (TRANSITION ZONE treatment) compared to an undisturbed forest with *Quercus* spp as the dominant species (CONTROL treatment) at Santa Rosa (Guanajuato, Mexico) sampled in the rainy or the dry season.

Treatment	ECO microplate		MT microplate	
	Rainy	Dry	Rainy	Dry
Richness (S)				
KILN	24 (2.0)[a] B[b]	26 (1.9) B	17 (1.9) B	13 (1.9) B
TRANSITION ZONE	30 (0.4) A	27 (1.1) A	23 (1.2) A	14 (2.0) B
CONTROL	29 (0.6) A	28 (1.0) A	23 (0.9) A	18 (1.3) A
LSD	1.256	1.382	1.363	1.839
Diversity Shannon index (H)				
KILN	3.00 (0.07) B	3.00 (0.02) C	3.02 (0.58) A	2.09 (0.09) C
TRANSITION ZONE	3.26 (0.04) A	3.14 (0.04) B	3.22 (0.41) AB	2.49 (0.22) B
CONTROL	3.26 (0.15) A	3.20 (0.04) A	3.67 (0.37) A	2.70 (0.10) A
LSD	0.1010	0.0389	0.4525	0.1504
Evenness (E)				
KILN	0.95 (0.02) A	0.92 (0.02) B	0.87 (0.02) A	0.82 (0.03) B
TRANSITION ZONE	0.96 (0.01) A	0.95 (0.01) A	0.95 (0.02) A	0.94 (0.09) A
CONTROL	0.96 (0.02) A	0.96 (0.02) A	0.94 (0.01) A	0.94 (0.03) A
LSD	na	0.0181	na	0.0569

[a]Values between parenthesis are standard deviations.
[b]Values with the same letter are not significantly different between treatments (P<0.05) (n = 9).
LSD = Least significant difference
na = not applicable

Table 2 Enzyme activities of microbial community at the kiln sites (KILN treatment) and boundary sites (TRANSITION ZONE treatment) compared to an undisturbed forest with *Quercus* spp as the dominant species (CONTROL treatment) at Santa Rosa (Guanajuato, Mexico) sampled in the rainy or the dry season.

Treatment	Enzymes											
	CM-Cellulase μmol glucose g^{-1} d^{-1}		β-glucosidase μg *p*-nitrophenol g^{-1} h^{-1}		N-acetylgluco-saminidase μg *p*-nitrophenol g^{-1} h^{-1}		Nitrate Reductase μg $N\text{-}NO_2^-$ g^{-1} d^{-1}		Urease μg $N\text{-}NH_4^+$ g^{-1} h^{-1}		Proteinase μg N-glycine g^{-1} d^{-1}	
	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry	Rainy	Dry
KILN	106 (5.3) C	419 (6.05) C	9.77 (0.34) C	38.47 (0.56) C	47.58 (0.59) C	8.71 (0.25) C	16.55 (0.63) C	16.11 (0.49) C	47.58 (0.59) C	44.54 (0.17) C	52.16 (0.65) C	92.13 (1.06) C
TRANSITION ZONE	467 (4.42) B	841 (7.66) B	21.8 (0.29) B	92.92 (0.98) B	68.59 (1.20) B	41.73 (0.79) B	22.07 (0.61) B	21.95 (0.77) B	68.59 (1.20) B	74.37 (1.25) B	93.77 (0.73) B	381.41 (1.34) B
CONTROL	1247 (4.17) A	1689 (9.01) A	42.68 (0.28) A	112.95 (0.97) A	87.72 (1.05) A	58.89 (0.65) A	29.57 (0.89) A	27.47 (0.97) A	87.72 (1.05) A	103.76 (1.90) A	332.72 (3.52) A	609.19 (2.57) A

Value between parenthesis is the standard error of the estimate,
Value with the same letter are not significantly different between the treatments (P<0.05) (n = 9).

The multivariate statistical analysis indicated differences in metabolic capacity of the soil microbial communities between the sites, and also seasonal differences between the sites. The importance of organic C influencing catabolic diversity has been reported by others researchers (Degens et al., 1998; Graham and Haynes, 2005). The KILN site showed a lower diversity, richness and activity compared to the CONTROL site. These results are similar to those reported by Staddon et al. (1997) who investigated the effect of clear-cutting and prescribed burning on forest soil microbial community. They found lower diversity and a changed microbial community structure after clear cutting and burning. The effect of pre-harvest burning has been studied by Graham and Haynes (2005). They found that evenness, the richness and functional diversity was lower in soils treated with fire than in untreated soils. This reduction is related to the loss of organic matter and subsequent changes in soil moisture content (Graham and Haynes, 2005). In a forest soil without disturbance, the litter layer favors a high metabolic diversity as a high diversity of substrates favors a diverse microbial community (Graham and Haynes, 2005). Generally, a loss in organic matter reduces the functional diversity of the microbial community and the microorganisms are less resilient to environmental stress, such as temperature and moisture fluctuations (Graham and Haynes, 2005). The enzyme activity is affected by soil organic matter quality and quantity, nutrient availability, substrate (labile or

recalcitrant), humidity, temperature and pH. The enzyme activity of soil as an indicator of disturbance showed significant differences between sites in the order CONTROL> TRANSITION ZONE > KILN. This suggests a change in microbial population or a change in their activity.
Soil enzyme activity showed a seasonal effect at the three sites except for nitrate reductase. Microclimatic conditions during the dry season (temperature, humidity) favored CM-cellulase, □-glucosidase, urease and proteinase activity, while the rainy season N-acetylglucosaminidase activity.

Conclusions: In a previous study, it was found that soil characteristics (lower soil organic C content, and higher Ca^{2+}, K^{+} and Mg^{2+} concentrations and pH) changed at KILN sites after charcoal production. The study reported here found substantial changes in the ability of the microbial communities to metabolize a range of C substrates in soil collected at the KILN sites compared to the undisturbed forest. In addition, charcoal production reduced the functional diversity in soil and had a negative effect on the enzymatic activities involved in the C and N cycle. Differences in microbial functionality indicators observed at the KILN site were presumably due to changes in organic matter content and seasonal effects. As such, improved forest management is necessary and strategies to restore soil fertility must be promoted.

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AXENIC ESTABLISHMENT AND *IN VITRO* FORMATION OF ADVENTITIOUS SHOOTS IN NARDO (*POLIANTHES TUBEROSE* L.)

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Abstract: The nard (*Polianthes tuberosa* L.) is a plant endemic to Mexico that is used in the pharmacological and fragrances industries, as well as ornamental plant (Herrera, 1990). In ornamental exploitation, the species offers little or no genetic variability, which reduces the opening of new markets, limiting their profitability. In this context, biotechnology through genetic transformation or induced mutagenesis, offer possibilities for breeding of new varieties. However, both techniques depend for their success of micropropagation protocols that enable there generation and mass propagation of new varieties. Therefore in the present study wee valuated three disinfectant agents (H_2O_2, Bioaxénico® and NaClO) for establishing aseptic tissue bulbs and 11 combinations ANA/BA to form adventitious shoots, in a basal medium. It was determined that using NaOCl (3% a.i) the axenic tissue percentage was 65% and the formation adventitious shoots via direct organogenesis was achieved with a frequency of up to 4 shoots per explants in the treatment of 7mM BA in the absence of ANA.

Keywords: Axenic ● Benzyladenine ● Naphthalenaceticacid ● Organogenesis.

Introduction: World wide, the nard (*Polianthes tuberose* L.) is used as ornamental in gardens and floral arrangements as well as raw material for the extraction of essences for perfumes and cosmetics (Gonzatti, 1981). Besides having a pleasant aroma, it has opened up the ability to export to foreign markets mainly to the United States and the European Union (Herrera, 1990). In Mexico is grown primarily for flower arrangements. The main producing states of this species are Guerrero, Mexico, Morelos, Puebla and Veracruz (Camino *et al*., 2001). The genus *Polianthes* is endemic to Mexico and since prehispanic times is important in scientific, economic, and cultural activities (Solano and Patricia, 2007). Among its species is *P. tuberosa*, known by the common name of spikenard, considered as a garden ornamental and important to the pharmaceutical and perfume because they present a production of secondary metabolites and compounds of commercial value (Gonzatti, 1981, Sangavai and Chellapandi, 2008). The tuberose has little genetic variability that only propagates through bulbs (Shillo, 1992), so the in vitro culture techniques can be a valuable tool for cell regeneration and subsequent mass propagation of material processed or artificially mutated. As mentioned above the aim of this study was to obtain a method of establishing aseptic tissue of tuberose and determine a hormonal balance for the direct formation of shoots in vitro, to serve as a basis for future biotechnological applications in this species.

Materials and Methods:

Selection and management of plant material. Bulbs of spikenard cultured in Chocholá, Yucatán, México, was selected. The bulbils were separated and washed with water and commercial detergent to eliminate the soil before work on the laboratory.

Disinfectants tested for in vitro establishment of bulblets of nard. This experiment consisted of 6 treatments disinfection test, using 3 disinfectants in two concentrations and a control (Table 1). Prior to treatment, the bulbs were washed with a soft brush, water and commercial detergent. After washing, in aseptic conditions, bulbs were exposed for 20 min a teach treatment and

rinsed three times with sterile distilled water, after rinsing, using steriles surgical instruments were removed layers of cells damaged by the process disinfection, planting 4 bulbs in petri dishes (9x 1.5cm) with potato-dextrose agar (PDA). For this experiment was used a completely randomized design with 7 treatments (including control) and 4 replicates (each replicate consisted of Petri dish with 4 bulbs). The variables evaluated were: number of bulbils axenic, contaminated and necrotic, with data from the respective percentages were calculated.

Table1. Disinfection treatments for bulbils of nard (*Polianthes tuberose* L.)

Agent	Concentration (%)
H_2O_2	20 (T1)
	50 (T2)
Bioaxenic©	20 (T3)
	50 (T4)
NaClO	1.2 (T5)
	3.0 (T6)
Control	0 (T7)

Effect of NAA/BA combination in morphogenesis of nard shoots (Polianthes tuberosa). After achieving the proceedings for the establishment of axenic explants, with a completely randomized design with 4x3 factorial arrangement (4 levels of BA and 3 levels of ANA). We evaluated the effect of auxin / cytokinin combination in organogenesis of tissues bulbils. To this was prepared culture medium of Murashige and Skoog (MS, 1962) with vitamins Morel and Wetmore (1951), 3% sucrose, treatment of growth regulators (ANA / BA) corresponding (Table 2) and 0.25% of Gelrite®, adjusting the pH to 5.8 with the aid of a pH meter. Were poured 25 mL of culture medium in baby food jars (110 mL) and sterilized by autoclaving at 121 ^{o}C for 15 min. The seeding procedure was performed under aseptic conditions in laminar flow chamber and the incubation was done in an incubation room at 27 ± 2 °C and 40 μmoles·m^{-2}·$s^{-1}$of light.

Table2. ANA/BA combination tested for *in vitro* morphogenesis, in tissues of bulbils of *Polianthes tuberose* L

	BA (μM)			
ANA (μM)	**0**	**7**	**13**	**22**
0	T1	T2	T3	T4
2.5	T5	T6	T7	T8
4.5	T9	T10	T11	T12

NAA=naphthaleneaceticacid; BA =benzyladenine, T = Treatment.

Statistical analysis. The data obtained were analized by ANOVA and multiple range tests (Tukey, α= 0.05)

Results and discussion: *Establishment of aseptic culture.* Because the plants constantly interact with microorganisms, aseptic cultures in vitro is essential because fungi and bacteria have a greater growth rate than the plants in the culture medium and these can be completely invaded (Obledo-Vázquez *et al.* 2004). Using the NaClO 3% was obtained axenic explants 65% (Figure 1A), 19% of the contamination was caused by fungi and 44% by bacteria and microorganisms appeared between three and 10 days after sowing. This percentage was

statistically same than with 50% H_2O_2 (T2) and both higher than the control and other treatments, however with T2 had more necrotic tissues (Figure 1B).

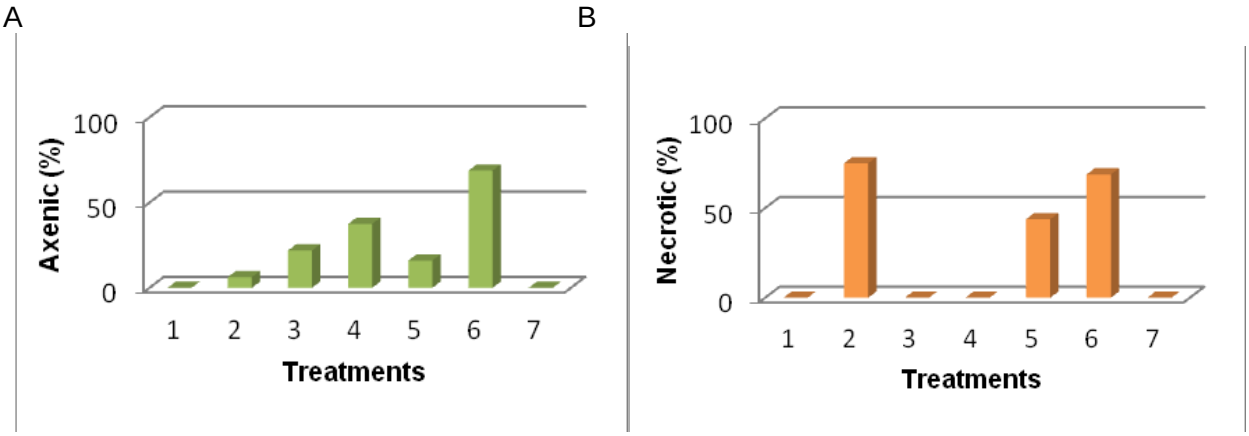

Figure1. Effect of six disinfection treatments in tissues of *Polianthes tuberose* L. A) Establishment axenic. B) Necrotic tissues.

Effect of NAA/BA combination in morphogenesis of Polianthes tuberose. Although we found no differences in the interaction NAA/BA, T2 of 7μM BA in the absence of NAA showed a frequency of four shoots per explant compared to the control with a shoot per explants (Figure 2). Nazneen *et al*. (2003) obtained a similar response to achieve shoot regeneration in callus of the same species (*Polianthes tuberosa*) with 13 μM BA in the absence of auxin.

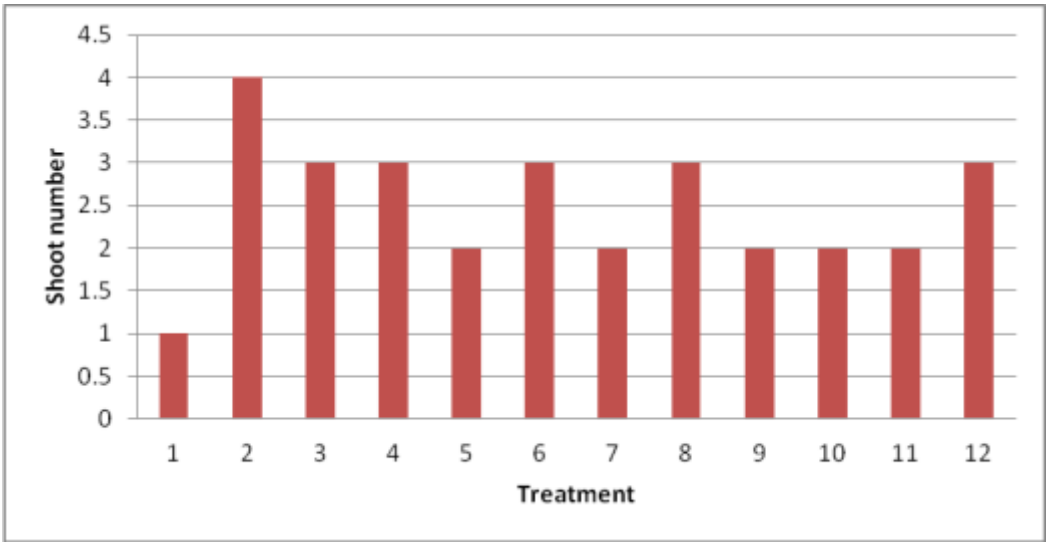

Figure2. Effect NAA/BA the interaction in adventitious shoot formation of *Polianthes tuberosa.*

Conclusions: The disinfection of bulbils of nard (*Polianthes tuberose* L.) with sodium hypochlorite (NaClO) to 3% (50% commercial bleach) for 20 minutes, achieved the *in vitro* establishment of the axenic explants in 65 %. The addition of between 7 and 13 μM benzyladenine without the presence of naphthalenacetic acid in the culture medium induces the direct formation of shoots in tissues of bulbils of *Polianthes tuberose* L.

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ETHYLENE INHIBITORS AND *IN VITRO* GROWTH OF SHOOTS OF *CAPSICUM CHINENSE* JACQ.

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Abstract: An evaluation was made of the effect of 1.5 and 3 µM of AVG, 10 and 100 µM of AOA, and 20 and 40 µM of STS, on the growth and quality of shoots of H163. The STS (20 and 40 µM) and the AVG (3 µM) improved by 65% in shoot growth, leaf formation (an average of 14 against 9 in the other treatments and 6 in the control) and the chlorophyll content (50% higher with inhibitors than without them), avoiding the symptoms of chlorosis and epinasty. These results confirm the presence and negative effects of ethylene during the *in vitro* culture of Chile Habanero.

Keywords: Ethylene inhibitors ● *in vitro* culture ● Shoot growth.

Introduction: Chile habanero (*Capsicum chinense* Jacq.) is the most pungency species of its genus and one of the most important vegetables of the Yucatan Peninsula. Its production now extends to other states of Mexico and to other countries that exploit it not only for alimentary purposes, but also for medicinal and industrial uses. Therefore, it is important to implement research programs that guarantee the conservation and improvement of this Mexican vegetable, by means of procedures that combine the methods of conventional improvement with those of modern biotechnology, such as tissue culture and genetic engineering.

In chile habanero, Pinzón *et al.* (article in preparation) were able to obtain shoots directly in cotyledons and hypocotyls of seedlings of 20 d. They emphasized that this shoot formation consistently began in the part of the explant that was immersed in the culture medium, a zone that has limitations of O_2, and therefore a lower synthesis of ethylene, according to Chang (1980). In addition, there were difficulties in the development *in vitro* generated shoots, presenting symptoms linked to the presence of ethylene in the form inhibition of growth, epinasty and leaf senescence, according to Biddington (1992) and Buddendorf-Joosten and Woltering (1994).

The objective of the present study was to examine the effect of ethylene inhibitors on quality and shoot growth *in vitro* of chile habanero.

Materials and Methods: Shoots with a length of 2.5 cm (genotype H163), were placed under aseptic conditions in a MS culture medium that was previously sterilized in an autoclave (121 °C). The inhibitors described above were later added after being filter sterilized. Each of the four treatments had six replicates, and each replicate consisted of a jar with 25 ml of medium which contained one shoot.

Thirty days after sowing, the following parameters were measured: length of shoots in cm; diameter of the stem, with a vernier; number of leaves; number of roots; length of the main root in cm. To determine symptoms of epinasty and yellowing, the angle of the third leaf was measured with respect to the stem, with a protractor and the chlorophyll content was determined with a SPAD (Minolta). The treatments were distributed in a completely random design, and variance analysis was made for each variable and Tukey tests were performed when there were statistical differences in the F tests.

Results and Discussion: The shoots cultivated with 40μM STS had superior growth to that of the control (p≤0.05) with values of 8.5 cm length against 3 cm of the control (Figure 1). Previous reports show that the addition of 30μM $AgNO_3$ was responsible for the fact that 50 % of the shoots of *Capsicum annuum* having a rosette-like appearance lengthen satisfactorily (Hyde and Phillips, 1996). Similarly, the presence of 23.5 μM $AgNO_3$ in the culture medium produced greater lengthening of the stems of Cassava (*Manihot esculenta* Crantz) compared to the control (Zhang *et al.*, 2001).

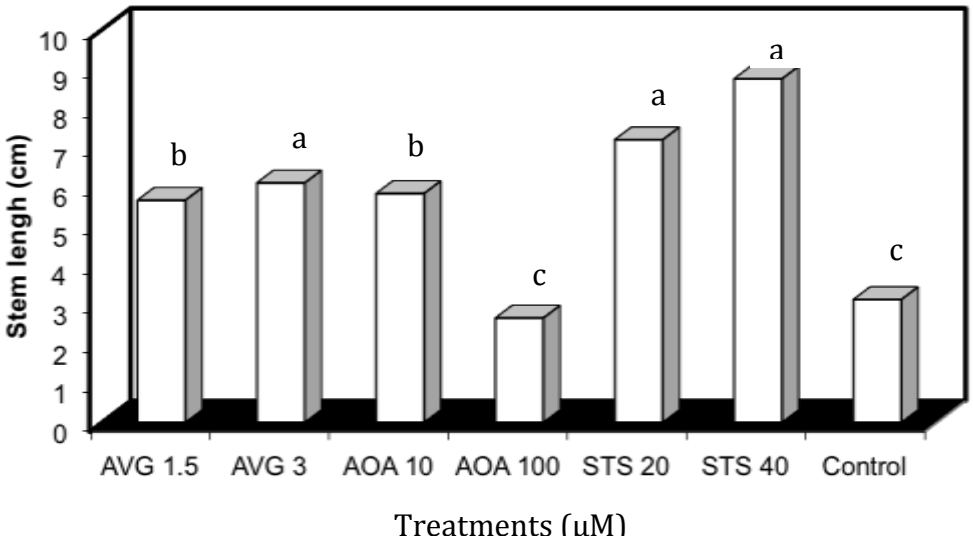

Figure 1. Effect of ethylene inhibitors on stem growth of chile habanero shoots at 30 days of culture *in vitro*. Treatments with different letter are statistically different (Tukey 0.05).

Adding 40 μM STS improved leaf production (14 against 6 of the control) and the chlorophyll content of the leaves (48 against 30 SPAD U. of the control). The epinasty ethylene-induced (positive geotropism in the leaves; leaf angles greater than 70°), was also repressed by the inhibitors, with the exception of the shoots cultivated in 100 μM AOA. However at this high scale of AOA it was consistently found significantly lower values than the control, drastically increasing epinasty, thus repressing leaf formation and the synthesis of chlorophyll (Figure 3). It is possible that AOA at high concentration resulted toxic to the plans.

To this respect, Robledo and Carrillo (2004) observed that the addition of 11.8 μM $AgNO_3$ to the culture medium prevented leaf abscission in chile (*Capsicum annuum;* cv. Mirasol and Árbol) shoots obtained *in vitro*.

In the case of the conservation *in vitro* of potato microplants (*Solanum tuberosum* L.), the addition of STS to alginate capsules improved the size of the microshoots, reducing leaf senescence and improving the quality of green leaves, and increasing their fresh mass (Sarkar *et al.*, 2002).

As for the root system (Table 1), with the exception of the treatments of 3 μM AVG that showed results superior to those of the control (p≤0.05) and 100 μM AOA which completely inhibited root formation, all of the other treatments were similar.

These results show that the use of inhibitors can help to obtain shoots of chile habanero with normal growth, robust stems, a good quantity of leaves without symptoms of chlorosis or epinasty, and with a root system adequate for increasing the probabilities of success in transplanting from artificial (*in vitro*) to natural conditions.

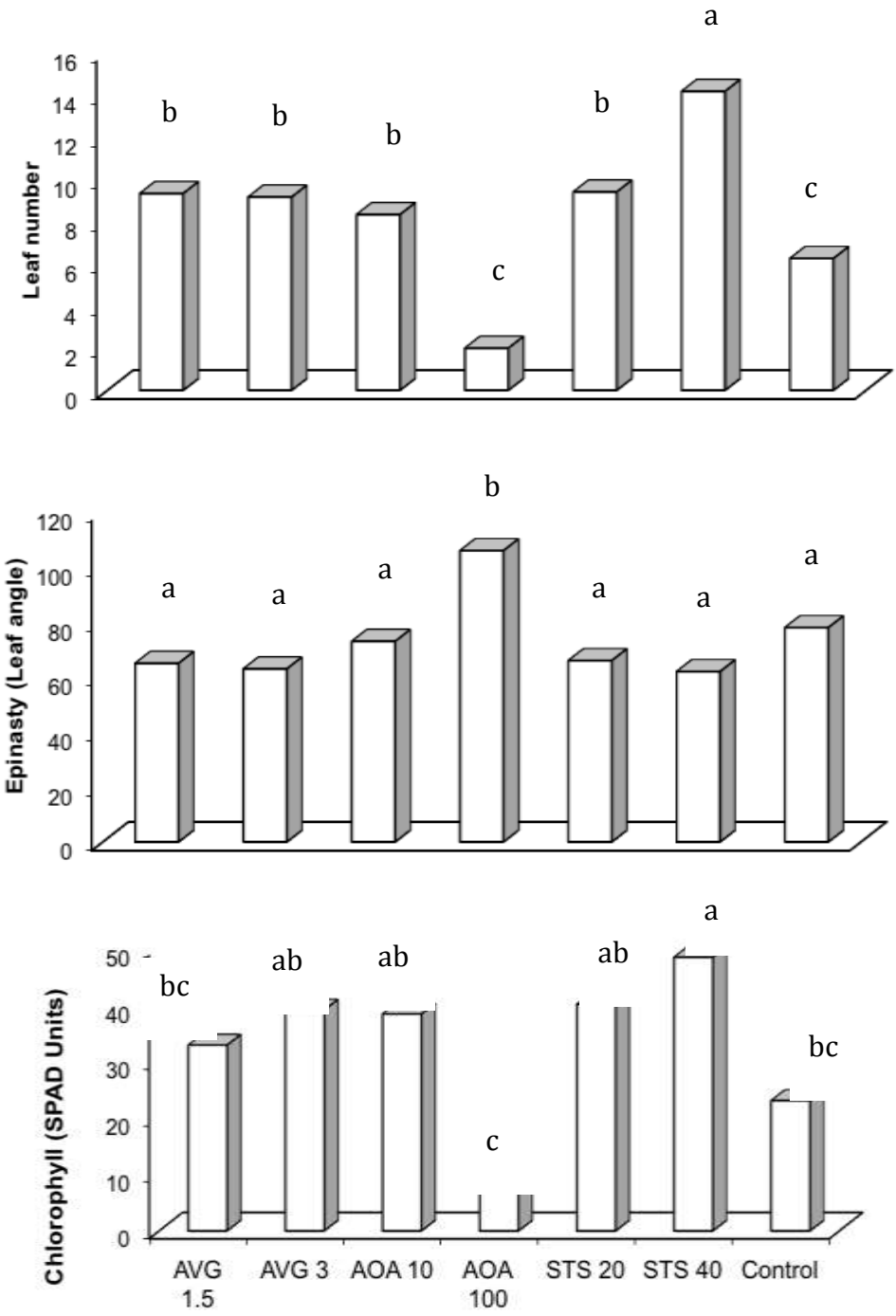

Figure 3. Effect of ethylene inhibitors on a) leaf number, b) epinasty and c) chlorophyll content in leaves of chile habanero shoots cultivated *in vitro* for 30 days. Columns with different letter are statistically different (Tukey 0.05).

Table 1. Effect of three ethylene inhibitors on the root growth of shoots of chile habanero cultivated *in vitro* for 30 days. Treatments with different letter in each row are statistically different (Tukey 0.05).

	Treatment (µM)						
	AVG		AOA		STS		Control
Variables	1.5	3	10	100	20	40	
RN	2.8a	2.5a	5a	0b	3a	2.8a	2a
RL (cm)	11.5ab	14.1a	9.6abc	0d	9.5abc	8.4bc	5.63bc

RN= Root number; RL= Root lengh; AVG=2-(aminoethoxyvinyl)-glycine; AOA=(aminooxy)-acetic acid; STS= silver thiosulfate.

Conclusions: The use of 40 µM STS increased: growth (283 %); leaf formation (43 %) and higher chlorophyll content (63 %), avoiding epinasty in the chile habanero shoots cultivated *in vitro*. The results of the experiment carried out in the present paper together with reports found in the literature, provide evidence that ethylene is the cause of inhibition of growth *in vitro* of chile habanero shoots.

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AEDES AEGYPTI INNATE IMMUNE RESPONSE AFTER *BACILLUS THURINGIENSIS* CRY11AA EXPOSURE

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Abstract: *Aedes aegypti* innate immune response is regulated in hemocyte cells by different receptors and effectors, whose major mechanisms of action involve antimicrobial peptides production, melanization, nodulation and phagocytosis. This study was undertaken to evaluate the *A. aegypti* innate immunity defense mechanisms after *Bacillus thuringiensis* var. *israelensis* (Bti) Cry11Aa toxin exposure. Exposed larvae were incubated with the Cry11Aa toxin LC_{50} for 5 and 24 h and analyzed for phenoloxidase activity (using DL-DOPA as substrate) or antimicrobial peptide expression (using the semi-quantitative RT-PCR analysis). Phenoloxidase activity results showed no significant differences between bioassay and lots, however, whereas phenoloxidase activity was lower over the incubation time among the unexposed larvae, activity increased among Cry11Aa-exposed larvae after longer time of toxin ingestion. In contrast, Cry11Aa-exposed larvae showed lower amplification of antimicrobial peptides, compared with the unexposed ones, particularly the defensin. The implication of diminished immune response by Bt Cry toxins exposure is discussed.

Keywords: *Aedes* aegypti ● *Bacillus thuringiensis* ● Cry11Aa ● phenoloxidase activity.

Introduction: All insects are naturally exposed to a variety of microorganisms. Their microbial defense mechanism relays on the innate immune response. Innate immunity is regulated by Toll receptors, which is involved in the defense against fungi, virus and mainly Gram (+) bacteria. When exposed to microorganisms, an increase in phenoloxidase activity has been demonstrated in mosquitoes after microbial pathogens challenge (Cerenius *et al* 2008). In addition to phenoloxidase activity, the innate immune response of mosquitoes has been related to antimicrobial peptide decreased expression to control Gram (-) bacteria (Sim and Dimopulos, 2010); in contrast, activation of antimicrobial peptides transcription within minutes after mosquito-parasites challenge has been reported, where gene transcript has increased to 1-100 M for specific peptides, mainly for the defensin (Lowenberger *et al* 2001) or cecroprin (Vizioli *et al* 2000) families. The antimicrobial peptide activation was related to a reduced parasites development in mosquitoes was reduced. In addition to chemicals, mosquitoes can be controlled by entomopathogenic microorganisms, including the soil bacteria *Bacillus thuringiensis* var. *israelensis* (Bti) (Bravo *et al* 2011). Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba are some of the crystal (Cry) and cytolytic (Cyt) toxins produced by Bti, with insecticidal activity against mosquitoes. Of these, only Cry11Aa (65kDa) toxin has shown the capability to bind three different receptors in the mosquito´s midgut brush-membrane cells (Likitvivatanavong *et al* 2011), including a glycosylphosphatidyl-inositol-anchored alkaline phosphatase receptor (Bravo *et al* 2007). Less susceptibility against Bt commercial products has been related to diminished (Ericsson *et al.,* 2009) or increased (Tamez-Guerra *et al.,* 2008) lepidoptera immune response. Increase phenoloxidase has been related to Cry1Ac toxin resistance by Lepidoptera species as well (Ma *et al* 2005), but little information about mosquitoes immune response to Bt products or crystals has been reported. This study was

undertaken to evaluate innate immune changes in mosquito larvae after being exposed to Cry11Aa toxin.

Materials and Methods: *Insect source and bioassay. Aedes aegypti* larvae were provided by Alejandra Bravo (Departamento de Biología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, MX). Fourth instar larvae were exposed to Cry11Aa at the given LC_{50} dose of 454.11 (312.7–760.6) ng/mL or to buffer (untreated control) for 5 h or 24 h in 100 ml water. Surviving larvae were removed and frozen at -20ºC. Frozen larvae were shipped to FCB-UANL to continue the immune response evaluation. Two lots of larvae from different bioassays were collected.

Phenoloxidase activity. To determine the phenoloxidase activity levels between the two lots of *A. aegypti* larvae, a modified technique from Harizanova *et al*. (2004) was used. Phenoloxydase activity was measured from the whole body by using 100 mg of *A. aegypti* larvae. In brief, insects were surface sterilized in ethanol, rinsed with sterile water, homogenized by using a pistile by 2 min, centrifuged at 13000 g by 10 min and then, 10 µL of the cell free soluble fraction present in the supernatant was collected and directly transferred into a chilled 1.5 mL microcentrifuge tube. Next, 200 µL of DL-DOPA (L-hydroxyphenylalanine) at 5 mM was added as substrate, and absorbance values were recorded after 15 min of incubation, using a wavelength of 490 nm. Tests were done in triplicate, testing insects from different bioassay lots. Data were reported as means $\pm$ standard deviations and analyzed using ANOVA Posthoc Tukey $\alpha = 0.05$ (SPSS, 2008).

Antimicrobial peptide detection. Antimicrobial peptides transcript amplification was evaluated by semi-quantitative RT-PCR analysis. RNA from unexposed and Cry11Aa exposed whole larvae was extracted following the technique provided by Ambion® using the Tri-reagent. In a Eppendorf plastic tube, 1.5 mg of *A. aegypti* larvae were mixed with 1 mL of the Tri-reagent. Samples were then incubated 5 min at room temperature. In each sample, 0.2 mL of chloroform was added and vigorously mixed for 15 sec and incubated at room temperature for 2 to 3 min. Samples were then centrifuged at 12,000g for 8 min at 2 to 8ºC. 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 to 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 7500g. 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 mM of dNTPs, 2 µL of 2.5 mM of primer dT12-18, and 1 µL of 200 U of Maloney murine leukaemia virus (MMLV) reverse transcriptase (PROMEGA) were 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 S3a (RPS7, as positive internal expression gene) and AMPs (defensin, cecropine and lysozyme), specific internal gene primers were used (Table 1). 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 of 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: *Phenoloxidase activity.* Results showed no significant differences between bioassay and lots, and PO production. However, phenoloxidase activity was significantly lower in Cry11A exposed-larvae after 5-h exposure compared with untreated control

(average of 0.436 and 0.461 versus 0.493 and 0.521, respectively); after 24 h Cry11Aa-exposure, PO activity was significantly higher compared with untreated control (average of 0.624 and 0.657 versus 0.303 and 0.321, respectively) (Fig. 1). Innate phenoloxidase activity has been related with insect stage and adult sex. Reports have shown that the PO activity increases during the insect larvae growth (Valadez-Lira *et al* 2012). In addition, PO activity was higher in Cry1Ac toxin resistant Lepidoptera compared with a susceptible population, probably due to that coagulation induced by PO may avoids the interaction of the toxin with the midgut receptors (Ma *et al* 2004). Since Cry11Aa toxin has shown similar mode of action than that reported by Cry1A toxins (Bravo *et al* 2007), and that we observed an increased PO activity by exposed larvae over incubation time, our findings may indicate a similar stimulation by mosquitoes.

Table 1. Primers used for *Aedes aegypti* antimicrobial peptide transcripts amplification[1].

PRIMER	SEQUENCE	ACCESS Genbank	Expected Size
S7 ribosomal	F GGG ACA AAT CGG CCA GGC TAT C R TCG TGG ACG CTT CTG CTT GTT G	AY380336.1	270pb
Cecropine	F ATG AAC TTC ACA AAG TTA TT R CTA CTT TCC TAG AGC TTT AG	AF3874.661	160pb
Defensin	F ATG CAG TCC CTC ACT GTC AT R TCA ATT CCG ACA GAC GCA CA	AF387467.1	307pb
Lysozyme	F CTT TGC TGG CTC AAG GAT TC R TTG TTT GTC CAT CCG TAC CA	AY693973.1	281pb

[1]Lowenberger *et al* 1999

Antimicrobial peptide detection. Out of the primers selected for the antimicrobial peptide transcript amplification, only defensin was successfully amplified with the RNAm from 4th instar mosquito larvae (Fig. 2). Semi-quantitative analysis showed that, at 24h incubation with Cry11Aa the expression of defensin transcript was lower than in the control larvae.

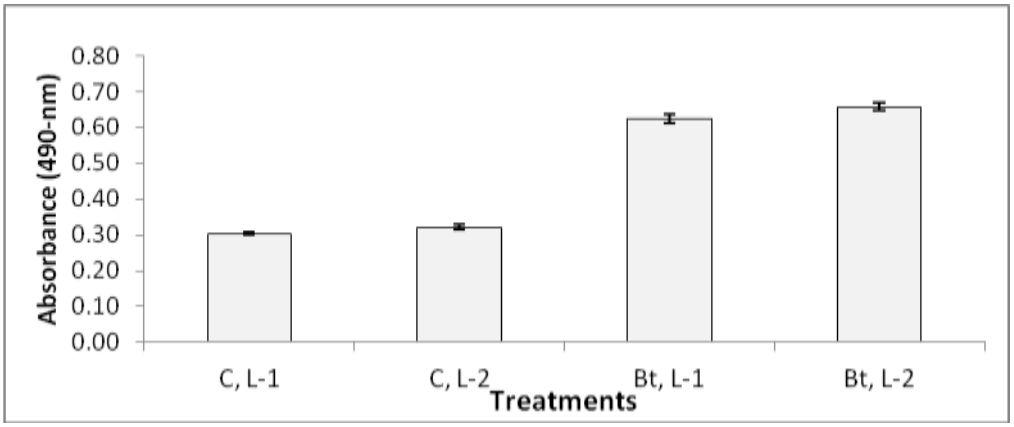

Figure 1. Phenoloxidase activity showed by two lots (L-1 and L-2) of fourth instar *Aedes aegypti* larvae unexposed (C) or exposed by 24-h to the Bti crystal toxin Cry11Aa (Bt) and incubated with DL-DOPA. Bars at the top of the column represent the standard deviation of three different tests.

The production of antimicrobial peptides has emerged as an important mechanism of innate immunity, where defensins contribute to the antimicrobial action of granulocytes, mucosal host defense in the small intestine (Ganz 2003). Similar than that reported by Ericsson *et al* (2009), we observed a diminished defensin expression in the mosquito larvae after intoxication with Cry11Aa toxin. Nevertheless, our results are preliminary and more bioassays and molecular analysis are needed in order to confirm that this immune response model is the most important in *A. aegypti.*

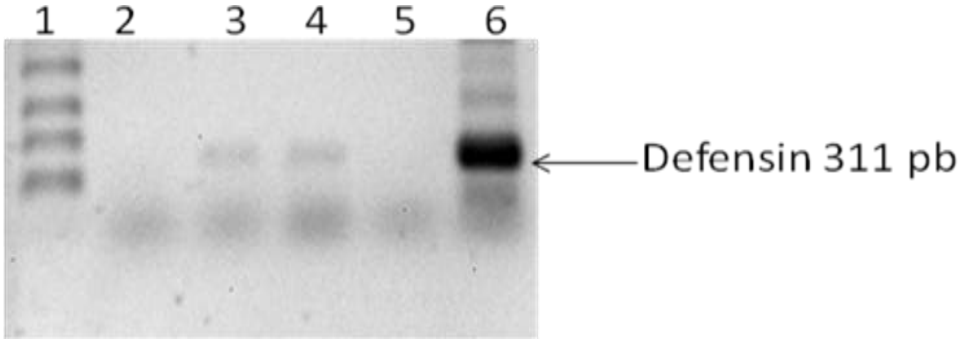

Figure 2. Defensin transcript amplification by *Aedes aegypti* whole larvae using semi-quantitative RT-PCR analysis in 1% agarose gels. Lane 1: weight marker; lane 2, negative control; lane 3, unexposed *A. aegypti* incubated by 5-h; lane 4, 24-h incubated *A. aegypti* with Cry11Aa-Bti toxin; lane 5, 5-h incubated *A. aegypti* with Cry11Aa-Bti toxin; lane 6, unexposed *A. aegypti* incubated by 24-h.

Conclusion: *Aedes aegypti* larvae exposed to the *Bacillus thuringiensis* var. *israelensis* Cry11Aa toxin showed an increased phenoloxidase activity after 24 of Cry11Aa intoxication and a diminished antimicrobial peptide defensin expression

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ISOLATION OF NATIVE STRAINS OF *BACILLUS* SPP. AND EVALUATION OF THEIR ANTAGONISTIC ACTIVITY AGAINST PHYTOPATHOGENIC FUNGI

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Abstract: Native strains from *Bacillus* spp. were isolated and their antagonistic activity in different fungal pathogens was evaluated. The strains of *Bacillus* spp. were isolated from soil in different localities of the Yucatan Peninsula. The antagonistic activities of isolates were evaluated by direct confrontation againstthe phytopathogenic fungi *Macrophomina* sp., *Colletotrichum* sp., *Helminthosporium* sp. and *Alternaria* sp. In general, the isolates caused 42-88% of inhibition of fungal colony growth. The isolate CBCK46 caused 80% inhibition of radial growth in at least two of the fungi tested. In the evaluations of inhibition zones, the isolate CBCK47 produced an inhibition zone of 0.30, 0.72, and 0.50 cm in *Colletotrichum* sp., *Helminthosporium* sp. and *Alternaria* sp., respectively. The presence of zones of inhibition suggests that the antagonistic activity of some isolates is due in part to the production of antifungal substances.

Keywords: Growth inhibition, ● Antagonism

Introduction: The phytopathogenic fungi are the principal agents that cause diseases in plants worldwide; with losses amounting to billions of dollars a year (Agrios, 1988). Some of the most important pathogens affecting foliage in tropical zone are *Macrophomina* sp., *Colletotrichum* sp., *Helminthosporium* sp. and *Alternaria* sp. (Agrios, 1988, Wallerand Brigde, 2000, Beas-Fernandez, 2006.). The use of chemical fungicides has caused considerable negative consequences, such as pollution, and development of resistance of pathogen populations(Lewis and Papavizas, 1991). The use of the *Bacillus* is an excellent alternative, due to its antagonistic activity through the production of antibiotics (Weller, 1988; Podile and Laxmi, 1998), production of lytic enzymes such as chitinases (Shoda, 2000), and competition for space and nutrients (Rovira, 1965). The genus *Bacillus*has been widely used to control soil-borne pathogens (Larrea, 2001). Little is known, however, on the activity on *Bacillus*on foliarpathogenic fungi. In this context, Fravel and Spurr(1977) reported that the use of *Bacilluscereus* inhibited germination and germ tube development of *Alternaria alternata*. Meanwhile, Saleem and Ulaganathan (2002) found that *Bacillus* sp. BC121, isolated from the rhizosphere of sorghum, showed antagonistic activity against *Curvularia lunata*, where a zone of growth inhibition of 0.5-1 cm was evident. The goal of this study was to isolate strains of *Bacillus* spp. from the Yucatan Peninsula and to evaluate the antagonistic activity on phytopathogenic fungi.

Material and Methods: *Colonies of phytopathogenic fungi.* Four species of fungal pathogens were used: *Colletotrichum* sp. was obtained from papaya fruits (*Carica papaya*), *Macrophomina* sp. was obtained from bean plants (*Phaseolus vulgaris*), *Helminthosporium* sp. and *Alternaria* sp. were obtained from palm kerpis (*Adonidia Merrilli*). The fungi were maintained on Potato

Dextrose Agar (PDA) in the laboratory of phytopathology of Institute Technology Conkal, in Yucatan.

Isolation of strains of Bacillus sp. Soil samples were taken in different locations in the states of Yucatan and Campeche. The soil samples were taken according the method of Escobar *et. al.* (2004). Soil samples were taken 10 cm depth, near the rhizosphere of plants in different areas of cropped and non-cropped areas. Samples were processed following the methodology of Artiom (2002), briefly, 1 g of soil was added to 9 mL distilled water and exposed to heat treatment at 80 °C for 30 min. An aliquot was taken and diluted 10x in sterile water. A sample of 100 □L of this mixture was added to Nutrient Agar (NA) in 10 cm diameter Petri dish. The Petri dishes were incubated at 28 °C. Colonies grew morphologically similar to *Bacillus* sp. were re-isolated in AN and kept to the tests of antagonism.

Morphological and biochemical characterization of Bacillus sp. Morphological description of each one of the selected isolates on NA based on the appearance, shape of the edge, color, shape cell and presence of spore was recorded. This description was made after 48 h of culture growth, incubated at 28 °C. Tests for catalase and Gram stain were also carried out (Koneman, 2003).

Evaluation of antagonistic activity. The antagonistic activity of the isolates was carried out following the procedure described by Lopez-Sosa *et al*. (2011). Fungal colony disks containing fungal mycelia were set in the center of 10 cm diameter Petri dishes with PDA. The isolates were inoculated (6 □L of suspensions of $1x10^7$ CFU mL^{-1}), approximately 2 cm away of the fungal colony disk. In each dish four bacterial isolates were inoculated. The Petri dishes were incubated at 27 °C. The radial growth of fungal colonies in the presence and absence of bacterial isolates was evaluated. Percent inhibition of fungal growth was evaluated using the formula proposed by Orberá *et al*. (2009). The zone of inhibition of fungal growth between the edge of the bacterial colony and the edge of the fungal colony was also evaluated. Was performed an analysis of variance and means comparison test (Tukey, $P<0.05$) of the percentage of inhibition of fungal growth and growth inhibition zone were carried out in SAS (Institute Inc. Cary, NC 27513, USA) version 8.1 for Windows.

Results and Discussion: *Isolation of Bacillus spp.* Isolates obtained were selected based on their ability to inhibit more than 70% the radial growth of at least two fungal pathogens (Table 1). The identity of the strains was confirmed base onthe colonial morphology, microscopic and biochemical characteristics, such as Gram positive, endospore-forming and catalase positive. The selected strains were coded and grouped according to the location where the sample was taken: The isolates CBRF6, CBRF8, CBRF12, CBRF15, CBRM9, CBRM14 were obtained from Conkal;the isolates CBSN67 from Cansahcab,and the isolate CBCC2 from San Crisanto, Yucatan; in addition the isolates CBCK36, CBCK41, CBCK44, CBCK46 and CBCK47 were obtained from Calakmul, Campeche.

Percent inhibition of radial growth of fungi. Significant differences were observed in the antagonistic activity of the isolates (Table 1). For *Macrophomina* sp., the inhibition of colony growth were 48 to 73%, the isolates CBRF15, CBCK36, CBCK41, CBCK46 and CBCK44 showed the highest percentages of inhibition (70% or higher). For *Colletotrichum* sp., all isolates caused more than 70% of fungal growth inhibition. For *Helminthosporium* sp., the isolates showed a wide range of fungal growth inhibition ranged from 39 to 79%, only four isolates showed inhibition greater than 70%. For *Alternaria* sp. the isolates caused 70% or more of fungal growth inhibition. The isolates CBCK36, CBCK41, CBCK44 and CBCK46 showed 71% to 82% inhibition on the four fungi. Previous work report antagonistic activities similar to this work. Benitez et al. (2007) isolated five strains of *Bacillus* sp. that inhibited the *in vitro* growth of *Botrytis cinerea* and *Sclerotina sclerotiorum*, with inhibition percentages of 83 to 87%, Orberá et al. (2009) evaluated *Bacillus* sp. against *Curvularia lunata*, *Fusarium oxysporum* and *Colletotrichum* sp., and found that five strains produced 52 to 72% inhibition of fungal growth.

Growth inhibition zone. Most isolates showed the presence of inhibition zones, with values of 0.02 to 0.76 cm (Table 2). In *Macrophomina* sp., *Helminthosporium* sp. and *Alternaria* sp. 76% of the isolates showed fungal inhibition zones, and in *Colletotrichum* sp. 92% of isolates were active. The isolates CBCC2 and CBCK47 showed fungal inhibition zones of 0.70 cm on *Helminthosporium* sp. colony. The results of this study suggest that these isolates have a broad spectrum of antifungal activity, probably due to the presence of extracellular metabolites that affect the mycelial growth of fungi. Saleem and Ulaganathan (2002) reported that strains of *Bacillus* sp. showed high antagonistic activity against *Curvularia lunata*, showing an inhibition zone of 0.5-1 cm. Some isolates that showed percentages of mycelial growth inhibition did not produce fungal inhibition zones, where the mechanism of antagonism is probably by direct competition for space or nutrients (Rovira, 1965).

Table 1. Mean percentage (± standard error) of fungal growth inhibition produced by *Bacillus* spp. in four phytopathogenic fungi.

Isolates	*Macrophomina* sp.	*Colletotrichum* sp.	*Helminthosporium* sp.	*Alternaria* sp.
CBRF6	54.22 ± 2.59 d e	81.57 ± 0.0 a b c	70.66 ± 5.90 a	62.85 ± 1.78 b c
CBRF8	66.22 ± 0.83 a b c d	86.34 ± 0.59 a	42.74 ± 2.65 c d	76.15 ± 1.88 a
CBRF12	61.33 ± 1.50 a b c d	83.41 ± 0.48 a	39.60 ± 4.03 d	78.46 ± 1.96 a
CBRF15	73.77 ± 1.63 a	82.58 ± 1.64 a b	65.60 ± 5.30 a b	79.45 ± 4.32 a
CBRM9	64.88 ± 4.62 a b c d	84.50 ± 0.93 a	60.55 ± 4.92 a b c	77.85 ± 0.71 a
CBRM14	60.44 ± 3.32 b c d e	84.50 ± 0.93 a	50.00 ± 3.40 b c d	71.42 ± 0 a b
CBCK36	71.55 ± 2.57 ab	81.63 ± 2.22 a b c	77.41 ± 3.97 a	79.55 ± 1.08 a
CBCK41	72.44 ± 2.06 ab	77.04 ± 1.03 b c	74.58 ± 4.36 a	78.66 ± 0.88 a
CBCK44	71.11 ± 2.43 abc	75.73 ± 1.67 c d	70.35 ± 4.50 a b	76.88 ± 2.17 a
CBCK46	72.44 ± 2.06 ab	82.29 ± 1.31 a b	79.76 ± 2.30 a	80.44 ± 1.08 a
CBCK47	58.66 ± 1.13 c d e	84.00 ± 1.08 a	67.77 ± 3.23 a b	75.33 ± 1.33 a
CBCC2	56.44 ± 3.26 d e	84.00 ± 1.08 a	68.88 ± 2.22 a b	76.66 ± 0 a
CBSN67	48.00 ± 2.28 e	70.13 ± 1.55 d	71.34 ± 4.07 a	54.78 ± 2.94 c

Means with the same letter within the same column are not significantly different (Tukey, P<0.05).

Table 2.Mean percentage (± standard error) of fungal growth inhibition (cm) produced by *Bacillus* spp. in four phytopathogenic fungi.

Isolates	*Macrophomina* sp.	*Colletotrichum* sp.	*Helminthosporium* sp.	*Alternaria* sp.
CBRF6	0.18 ± 0.08 a b c	0.06 ± 0.04 d e f	0.24 ± 0.11 b c	0.32 ± 0.05 a b
CBRF8	0.28 ± 0.02 a	0.14 ± 0.04 b c d e f	0.04 ± 0.02 c	0.42 ± 0.03 a b
CBRF12	0.10 ± 0.03 a b c	0.34 ± 0.05 a b c	0.20 ± 0.08 c	0.56 ± 0.21 a
CBRF15	0.08 ± 0.02 a b c	0.32 ± 0.11 a b c d	0.26 ± 0.09 b c	0.34 ± 0.11 a b
CBRM9	0.10 ± 0.03 a b c	0.06 ± 0.05 d e f	0.32 ± 0.11 a b c	0.32 ± 0.02 a b
CBRM14	0.26 ± 0.14 a b	0.10 ± 0.05 c d e f	0.12 ± 0.09 c	0.32 ± 0.02 a b
CBCK36	0.18 ± 0.03 a b c	0.46 ± 0.06 a	0.38 ± 0.18 a b c	0.72 ± 0.19 a
CBCK41	0.00 ± 0 c	0.02 ± 0.02 f	0.00 ± 0 c	0.00 ± 0 b
CBCK44	0.02 ± 0.02 b c	0.04 ± 0.04 e f	0.00 ± 0 c	0.00 ± 0 b
CBCK46	0.00 ± 0 c	0.00 ± 0 f	0.00 ± 0 c	0.00 ± 0 b
CBCK47	0.04 ± 0.04 a b c	0.30 ± 0.03 a b c d e	0.72 ± 0.13 a b	0.50 ± 0.08 a
CBCC2	0.00 ± 0 c	0.04 ± 0.04 e f	0.76 ± 0.11 a	0.30 ± 0.08 a b
CBSN67	0.08 ± 0.02 a b c	0.40 ± 0.04 a b	0.44 ± 0.11 a b c	0.40 ± 0.04 a b

Means with the same letters within the same column are not significantly different (Tukey, P<0.05).

Conclusion: Highly active antagonistic bacteria of the genus *Bacillus* were obtained from different locations in the state of Yucatan and Campeche. A big group of isolates caused at least 70% inhibition of growth of at least two of the four fungal pathogens used. Strains CBCK36, CBCK41, CBCK46 and CBCK44 showed 70% or more fungal growth inhibition in the four fungi. The presence of inhibition zones indicates that the isolates produce metabolites as antagonistic mechanism against pathogenic fungi.

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COLLECTS, ISOLATION AND IDENTIFICATION OF ARBUSCULAR MYCORRHIZA FUNGI IN DIFFERENT AGRO ECOLOGICAL REGIONS OF MÉXICO

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Abstract: Soil collects from high cool valleys, arid-semiarid, and dry-humid tropics regions, was carry out, corresponding to 20 states of Republic of Mexico. From a total of 100 random samples of agricultural or none disturb soils, spores of arbuscular mycorrhizal fungi were isolated, identified and propagate pots were set. Regions of Quintana Roo, Campeche and Veracruz, were registered high number of diferent spores. Was made 104 isolates, six genus were found (*Glomus, Sclerocystis, Entrophospora, Gigaspora, Scutellospora* y *Acaulospora*), from which major predominance was *Glomus* with an incidence of 73%.

Keywords: Arbuscular mycorrhiza ● Biodiversity ● Ecological regions

Introduction: Arbuscular mycorrhizal (AM) are defined as symbiotic associations between fungi in soil and absorption of plant organs are found in all ecosystems covering up to 90% of the territory of the planet (Modjo & Hendrix, 1986; Sieverding, 1991). The AM are classified within the Glomeromycota phyllum (Redecker *et al*. 2006), belong to the order Glomales, suborder Glomineae which comprises two families: Glomaceae consisting of the genera *Sclerocystis* and *Glomus* (*Glomus* the more predominant in soils) (INVAM, 2004) and the family Acaulosporaceae comprising the genera: *Acaulospora*, *Archaeospora*, *Entrophospora* y *Paraglomus.* The suborder Gigasporineae has a family: Gigasporaceae with two genera: *Gigaspora* and *Scutellospora* (Morton & Bentivenga, 1994). Biermann & Linderman (1983) they mention that the morphology of mycorrhizal is crucial for identification if there is large amount of similarities between genders thus hindering their classification.

The most important effect that produces AM in plants is an increase in the absorption of nutrients from the soil and water which translates into greater growth and development of plants. The expansion of the external mycelium of the fungus on the rizospheric soil is the main cause of this effect, allowing the uptake of water and nutrients over the zone of depletion that is created around the roots (Sanders & Tinker, 1973). Haussler *et al.* (1992) mentioned that the role of the symbiosis between AM and plant is essential in the uptake of mineral elements of slow diffusion in soils, as phosphorous, Zn y Cu, the external mycelium extends to greater distance than the radical hair, improve the soil aggregation by the formation of glomalina, increase the photosynthetic rate, resistance to pests and environmental stress and stimulate the activity of growth regulatory substances (Blanco & Salas, 1997). Methods of identification and extraction of arbuscular mycorrhizae are increasingly innovative, e.g., with molecular methods it is possible to identify the taxa of mycorrhizae regardless of the morphological criteria, techniques such as wet screening and decanted are widely used for the extraction of spores, as well as the gradient of sucrose (Van der Heidjen & Sanders, 2002; Gerdemann & Nicolson, 1963; INVAM, 2011).

Materials and methods: *Sampling.* Two types of soil were collected: agricultural soils and soil without disturbing, excavated at a depth of 20 to 30 cm in the arable area, approximately 1 kg of soil were collected by site which they stored in a cool place and then transported to the laboratory for further analysis.

The collected sites were: cool valleys (Tlaxcala, State of Mexico, Puebla, Morelos, Hidalgo, Michoacan, Guanajuato and Queretaro); arid-semiarid (Nuevo Leon, Tamaulipas, San Luis Potosi, Coahuila) (Chihuahua, Durango and Sonora); dry Tropics (Veracruz, Tabasco, Campeche and Quintana Roo); and dry-humid tropics (Veracruz and Chiapas).

Sample processing. Samples were processed by the humid and decanted screening method, the procedure was to take 100 g of sample and mix with a 1 lt of water it is then allowed to sit up to form two phases (sediment and supernatant), aqueous phase passed through sieves of pore size 20, 60 and 400 microns. The sample is collected from the last two you screens and is recovering in a beaker of 40 ml, later were observed the 40 ml of sample using a stereoscope and identified the different types of microorganisms present.

Morphological identification and planting of mycorrhizae. The isolated strains were identified using the taxonomic keys proposed by INVAM. The morphological characters that were considered were: diameter of the spore, color, length of the hypha, sacular, presence of auxiliary cells, type of arbúsculos and formation of intraradicales hyphae. The spores after identified planted directly in vessels of 1 lt with substrate and used as plant host sorghum seed var. "Patrón".

Results: *Development of crops monosporic, isolation and collections.* Were obtained 100 collections of 20 states of the Mexican Republic (Figure 1).

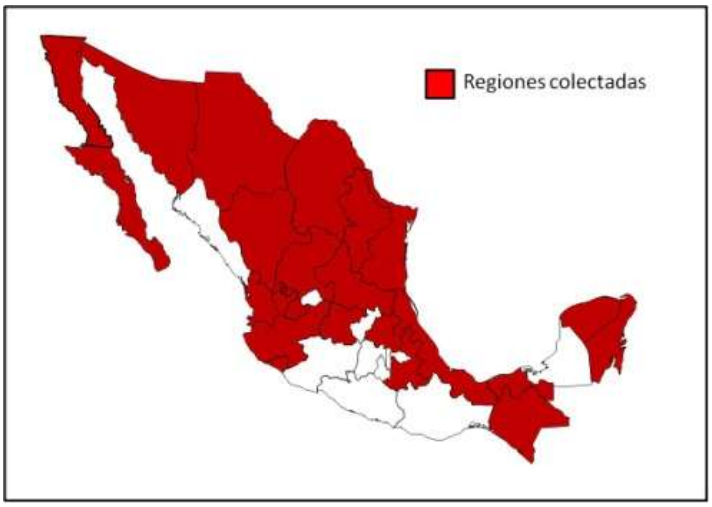

Figure 1. Collections made in the different states of the Mexican Republic.

As noted in Table 1 of the collections carried out a total of 104 AM isolates were obtained, identified species were: *Glomus intraradices, G. fasciculatum, G. mosseae, G. albidum, G. glomerolatum, G. rubiformi, Gigaspora albida, Gigaspora gigantea, Gigaspora nigra, Sclerocystis coremioides, Sclerocystis sinuosa, Scutellospora* sp*, Scutellospora coralloidea, Sclerocystis clavispora, Entrophospora* sp.*, Entrophospora colombiana, Acaulospora* sp.*, Scutellospora rubra* and *Glomus geosporum.*

Table 1. Isolates AM obtained from 20 States of the Mexican Republic and collections.

States of the Mexican Republic	N° of collections	N° isolated AM fungus
Sonora	4	2
Chihuahua	6	8
Durango	8	6
Tamaulipas	6	9
Nuevo León	3	6
Veracruz	10	11
Campeche	14	13
Yucatán	3	2
Hidalgo	1	0
México	5	6
Tlaxcala	6	4
Morelos	3	4
Querétaro	5	4
Puebla	2	3
Chiapas	1	5
Michoacán	1	1
Coahuila	6	6
Quintana Roo	17	13
Guanajuato	1	0
San Luis Potosí	1	1
Total	100	104

There were various features of different arbuscular mycorrhizal spores (Table 2), among them we can high light the size, color, type of supresor hyphae; presence or absence of bulb, sacular or if they are sporocarps, aggregate or individually on the ground; as well as the generation of auxiliary cells (Figure 2).

Table 2. Main features of the genera of AM.

Genus	Sporocarp // individual aggregate	Size (µm)	Different shades colors	Hypha	Bulb	Auxiliary cells
Glomus	Individual	40-140	Brown	Present	Absent	Absent
Sclerocystis	Sporocarp	200-350	Brown	Present	Absent	Absent
Gigaspora	Individual	150-350	Yellow	Present	Present	Present
Acaulospora	Individual	100-250	Cream	Present	Absent	Absent
Scutellospora	Individual	100- 350	Maroon	Present	Present	Present
Entrophospora	Individual	100-200	Brown	Present	Absent	Absent
Paraglomus	Individual	60-110	Hyaline	Present	Absent	Absent
Archaeospora	Individual	100-180	Brown	Present	Absent	Absent

Figure 2. Spores of arbuscular mycorrhizae (A) *Acaulospora*, (B) *Glomus* and (C) *Gigaspora.*

The spores of mycorrhizae are generally found in any ecosystem; however, not all are present in the same proportion. The genus *Glomus* turned out to be the largest group while that genus of *Entrophospora* and *Acaulospora* they are showing a lower incidence in made insulation (Table 3).

Table 3. Percentage of total incidence of spores of AM 104 isolates obtained from 20 states of the Mexican Republic.

Genus of AM	Incidence (%)
• *Glomus*	73%
• *Gigaspora*	12%
• *Sclerocystis*	9%
• *Scutellospora*	5%
• *Entrophospora*	0.6%
• *Acaulospora*	0.4%

Discussions: Samples from the majority regions were found arbuscular mycorrhizae fungi. The states of Quintana Roo, Veracruz and Campeche where met greater diversity of arbuscular mycorrhizal fungus, these results coincide with the above were found in most of the states of the Mexican Republic sampled by Sieverding (1991) who mentions that AM fungi are native of tropical soils and terrestrial ecosystems.

Were extracted isolates of different genus of mycorrhizal spores, each with distinctive morphological characteristics of genres, thus coinciding with the proposed descriptions for the INVAM (2004).

Van der Heidjen & Sanders (2002) mention that the molecular methods are another alternative that identifies the taxa of arbuscular mycorrhiza regardless of the morphological criteria, so it can be ensured that the identification through morphological key is an effective method, but can be supplemented by molecular methods.

While wet and decanted screening was used for processing samples, it is not a technique that facilitates the observation of the spores of AM as already processed samples contained a large amount of artifacts and debris. Another technique that can use for a good extraction of spores of soil is the proposal by INVAM (2004), which consists in a sucrose gradient, which are retained lots of artifacts which allows an extraction and tidiest observation of the spores of AM.

The incidence showed high percentages as in the case of *Glomus* which garnered a 73% incidence in the sampled soil, these results are consistent with those mentioned by INVAM (2004) who mentions that the spores of the genus *Glomus* are those that most frequently occur in any type of soil. Guadarrama *et al.* (2007) made collections of soil in Oaxaca and registered to the family Glomeraceae as the most frequent. Redecker *et al*. (2006) mentioned that *Glomus* it is the larger group of spores with 70 different morpho–species. The same, Tapia *et al.* (2008)

determined that *Glomus mosseae*, was the most dominant species in saline soils of San Luis Potosí.

Conclusions: We managed to carry out the isolation of microorganisms of mycorrhizae arbuscular with potential activity of plant growth of the different agro-ecological zones of Mexico. Mycorrhizae are variety of morphologies that by morphological codes can be identified in a fast and easy way. Quintana Roo, Campeche and Veracruz were states which were obtained a greater number of different spores of arbuscular MA. The genus *Glomus* is the largest group of arbuscular mycorrhiza.

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THE "OMICS" APPROACH FOR SOLVING THE PRE-HARVEST AFLATOXIN CONTAMINATION PROBLEM: UNDERSTANDING THE GENOMICS AND METABOLOMICS OF THE FUNGUS AND PROTEOMICS OF THE AFFECTED CORN CROP

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Abstract: Aflatoxins are highly carcinogenic secondary metabolites produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus.* Aflatoxin contamination of food and feed has been of particular concern over the last four decades because of the toxicity of these compounds. Regulations exist in over 100 countries of the world against sale of contaminated commodities, causing severe economic burden on the farmers. Therefore, this problem is both a food safety and an economic issue. Now, for the first time control measures for this problem appear within reach. For practical and sustainable control of pre-harvest aflatoxin contamination to be realized, however, additional information is needed about the fungus, the affected crops and the specific molecular factors (both in the plant and the fungus) involved during host plant-fungus interaction. The information derived from the use of novel tools such as genomics, proteomics and metabolomics provides us with the best and the quickest opportunity to achieve a clear understanding of the survival of toxigenic fungi in the field, the ability of the fungus to invade crops, and the process of toxin contamination under various environmental conditions. Significant progress has been made recently in understanding the genomic makeup of the most significant aflatoxin producing field fungus, namely *Aspergillus flavus*. Progress also has been made in the study of host crop resistance to fungal invasion through the use of proteomics. The information available on production of aflatoxin and other metabolites by *Aspergillus flavus* is reasonably extensive, although the application of metabolomics as a tool in this study is relatively new. In this presentation, the use of genomics, proteomics and metabolomics in deriving the requisite information for developing effective strategies to interrupt the machinery in the fungus for production of these toxins, as well as to enhance host-resistance against fungal invasion and aflatoxin contamination of crops will be discussed.

Introduction: Mycotoxin contamination of food and feed is an age old problem lasting over four decades. For the first time it appears that control measures for this problem are within reach. To hasten this process, additional information is needed rather rapidly, particularly for understanding the specific molecular factors (both in the plant and the fungus) during host plant-fungus interaction. The information derived from the use of tools such as genomics, proteomics and metabolomics provides us with the best and the quickest opportunity to achieve a clear understanding of the survival of toxigenic fungi in the field, the fungal invasion of crops, and toxin contamination process under various environmental conditions. Significant progress has been made by many researchers around the world in understanding the genomic make-up of some of the most significant toxin producing fungi namely *Aspergillus flavus*, *Fusarium graminearum* and *Fusarium verticillioides.* Progress has also been made in the study of fungal-host crop interactions for these and other fungi. For this presentation, the information available on aflatoxin production by *Aspergillus flavus* will be used as a model system in the use of genomics, proteomics and metabolomics in deriving the requisite information required for developing effective strategies to interrupt the machinery in fungus required to produce these toxins, as well as to assist in the development of host-resistance against fungal invasion and aflatoxin contamination of crops.

Aflatoxin contamination: Aflatoxins are highly toxic and carcinogenic secondary metabolites produced mainly by three anamorphic species of the genus *Aspergillus*: *A. flavus*, *A. parasiticus*

and *A. nomius.* They are the most potent, naturally-occurring carcinogens known and have been linked to liver cancer and several other maladies in animals and humans. Among the aflatoxin-producing fungi, the ubiquitous *A. flavus* is the known pathogen of cotton, corn, peanuts and other oil-seed crops, producing toxin both in the field and during storage. Aflatoxin contamination continues to be a serious problem in many parts of the world (reviewed in Richard and Payne, 2003). Initially studied because of their negative impact on human and animal health, aflatoxins are perhaps the most well-known class of mycotoxins, serving as a model system for the study of the genetics of mycotoxin biosynthesis and secondary metabolism in general. Due to the acute and chronic toxicity of aflatoxins, nearly a hundred countries are known to have regulations limiting mycotoxin levels, with 61 having specific regulatory levels for total aflatoxins in foodstuffs and 39 having regulations for aflatoxins in feedstuffs. The U.S. Food and Drug Administration has set limits of 20 ppb total aflatoxins for interstate commerce of food and feed and 0.5 ppb of aflatoxin M_1 for sale of milk. Chronic, as well as sporadic, aflatoxin contamination in a variety of field crops and agricultural commodities worldwide have had a serious impact on the economics and food safety of these products. It has been estimated that the annual cost from aflatoxin contamination in the USA at roughly $500 million through two categories of loss, market rejection and animal health impacts. However, the total economic impact of aflatoxins is much larger when taking into account many other factors such as export market loss, sampling and testing costs, costs to food processors, grocery markets and consumers, and human health effects. Therefore, it is imperative upon researchers across the globe to develop strategies for effective control of aflatoxin contamination of crops. New biotechnologies such as, 1) the use of disarmed, nontoxigenic biocompetitive strains of *A. flavus* in biocontrol of aflatoxin contamination, and 2) identification of plant constituents that disrupt aflatoxin biosynthesis or fungal growth and their use in new biochemical marker-based breeding strategies to enhance resistance in crops to aflatoxin, are saving and could potentially save the agricultural industry in the U.S. alone hundreds of millions of dollars.

Understanding the complex interrelationships of plant and fungal gene products during the host plant-*Aspergillus flavus* interaction is key in developing strategies to interrupt the aflatoxin contamination process. *Aspergillus flavus* genomics and proteomics of seed-based resistance provide 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. Functional genomics is needed to speed up our understanding of the field biology of the fungus in relationship to its interaction with the host plant.

Proteomics of host plants. Proteomics is the study of a complete set of proteins in a cell and their structures and functions in the physiological pathways of cells. The term "proteomics" is analogous to genomics, the study of the complete set of genes. The word “proteome” is derived from a combination of “*prote*in” and “gen*ome*”. The proteome of an organism is the set of proteins produced by it during its life, based on its genome, i.e. its set of genes. Plant factors have been discovered through the use of proteomics and natural product chemistry that may influence fungal processes involved in invasion and aflatoxin contamination. The discovered factors can be divided into three categories: 1) seed proteins/inhibitors of fungal cell wall degrading enzymes, 2) seed/kernel natural products which may influence fungal growth and/or aflatoxin synthesis, and 3) plant stress responsive proteins.

Table 1. Examples of resistance-associated proteins (RAP) identified through proteomics.

Protein	Resistant genotype[a]	Putative Function
Trypsin inhibitor (14 kDa)	MI82; CI2; T115	Antifungal; anti-amylase
Glyoxalase I	Mp420; Mp313E	Stress-related; antitoxigenic
Pathogenesis related protein (PR-10)	Mp420; Mp313E; GT-MAS:gk	Antifungal; ribonucleolytic
Peroxiredoxin antioxidant (PER1)	Mp420; Mp313E	Peroxidase; stress related
Zeamatin	GT-MAS:gk	Antifungal

[a]Maize genotypes in which an association with resistance has been identified; other resistant genotypes are involved as well.

Identification of specific proteins and their genes that affect critical pathways in plants is essential for conventional and transgenic improvement programs. Analysis of gene function in native plant environment is possible using efficient genetic transformation technology and it has been practiced routinely for cotton, peanut and corn. In conventional breeding, marker-assisted selection (MAS) may not be preferable for traits controlled by a large number of QTLs, because the individual gene's contribution may be too small to measure since many genes with small effects are involved.

***Aspergillus flavus* genomics**:
Aspergillus flavus genomics is aimed at understanding the genetic control and regulation of toxin production by this fungus as well as the evolutionary process in *Aspergillus* section *Flavi*. More importantly, the mechanism of toxin production in response to environmental influences on the fungus, i.e., simultaneously to environmental conditions like nutrition status of crops, temperature, water stress, pH, and volatile compounds from plants must be understood. The ecological/ evolutionary significance of *A. flavus* propagation, fungal virulence, and aflatoxin formation as manifested by changes in gene expression profiles and global signal transduction within the fungus need to be ascertained. These parameters are now being rapidly analyzed using the genomic information that has been recently obtained.

***Aspergillus flavus* expressed sequence tags:** Sequencing and annotation of *A. flavus* expressed sequence tags (EST) identified 7,218 unique EST sequences. Genes that are involved in or potentially involved in aflatoxin formation were identified from these EST's and have been categorized as involved in global regulation, signal transduction, pathogenicity, virulence, fungal development and toxin formation.

Whole genome sequencing of *Aspergillus flavus:* The *flavus* whole genome sequencing project was funded by a USDA, National Research Initiative grant awarded to Professors Gary A. Payne and Ralph Dean, North Carolina State University, Raleigh, North Carolina. The Food and Feed Safety Research Unit of Southern Regional Research Center, USDA/ARS, provided

funding for fine finishing and gene calling. The sequencing has been completed at The Institute for Genomic Research (TIGR) under the supervision of Dr. William C. Nierman by a shotgun approach and Sanger sequencing protocol. Primary assembly indicated that the *A. flavus* genome consists of 8 chromosomes and the genome size is about 36.8 Mb with over 12,000 functional genes in the *A. flavus* genome similar to that found in related *Aspergillus*. The annotation of the *A. flavus* genome sequence data has been achieved by using the *A. flavus* EST database, *A. oryzae* EST database, and the *A. oryzae* whole genome sequence.

Results from the whole genome sequencing project 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 a more complex pattern of secondary metabolites. The expanded genome of *A. flavus* over other *Aspergillus* species suggests that *A. flavus* is adapted to growing in complex environments. An analysis of the function of these extra genes may reveal those genes that make this fungus a successful saprophyte as well and a pathogen of plants and animals. Interestingly, Comparative genomic studies show that *A. flavus* is highly similar to *A. oryzae* (37.6 Mb) with respect to genome size and number of genes for secondary metabolism. These results support the conclusions of others that *A. oryzae* is not a separate species, but rather is a domesticated ecotype of *A. flavus*.

Microarrays as tools for functional genomics studies: Several types of microarrays for *A. flavus* have been constructed within the last few years : (1) The first cDNA microarray consisting of 753 gene features including known aflatoxin pathway genes and regulatory gene *aflR*; (2) A 5,031 gene-elements *A. flavus* EST based amplicon microarray; (3) Using genomic DNA as template, a comprehensive whole genome *A. flavus* oligo microarray has also been constructed; (4) All of the 11,820 genes unique to *A. flavus* form the basis for an Affymetrix microarray, also containing *A. oryzae* unique genes, plus additional genes of interest from corn, *Fusarium* species, mouse and human genomes. Expression profiling of genes involved in aflatoxin formation using those microarrays, performed at USDA labs, the labs of North Carolina State University, TIGR and JCVI, identified hundreds of genes that are significantly up or down regulated under various nutritional and environmental conditions that either support or prevent aflatoxin formation. Further studies using these microarray resources for a genome-wide gene profiling and functional analysis in relation to aflatoxin formation are expected to reveal the regulatory elements (global or specific) required for aflatoxin production.

Metabolomics of *Aspergillus flavus*

Metabolomics can be defined as the “systematic study of the unique chemical fingerprints that specific cellular processes leave behind” i.e., the study of their small-molecule metabolite profiles. The metabolome represents the collection of all metabolites in a biological organism (such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites), which are the end products of its gene expression. Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell. In metabolomic studies with *A. flavus*, comparisons of the chromatographic fingerprints of the wild type and a specific atoxigenic mutant revealed large differences in the small molecule compositions of the two strains. Although the mutant strain appeared to have some unique components when compared to the wild-type, chromatograms of the mutant samples lacked many of the prominent peaks present in wild type. Further experiments are in progress, using mass spectrometry and pulse/pulse-chase technique, to identify specific compounds and pathways impacted by the mutation. Additionally, this metabolic profile, when compared with a transcriptional profile (using an EST microarray) to determine the changes in gene expression resulting from the mutation will provide insights into the signaling pathways in the fungus that control not only toxin formation but also secondary metabolism as a whole.

Conclusion: The fundamental strategy in a Functional genomics, Metabolomics and Proteomics approach is to expand the scope of biological investigation from studying single genes, individual compounds (fungal secondary metabolites) or plant proteins to studying all genes, metabolites and proteins at once in a systematic fashion. The results from genomics are expected to provide information for developing novel strategies to control aflatoxin contamination by identifying targets for inhibiting fungal growth or toxin production, as well as developing "designer" biological control agents. With the large volume of information being reported with respect to proteomics of host and genomics and field ecology of the fungus, novel strategies will also emerge based on a clear understanding of the aflatoxin contamination process, especially at the molecular level. If the interaction between the plant and the fungus can be better understood through use of proteomics, genomics and natural product chemistry, particularly in how plant factors may influence fungal processes contributing to virulence and aflatoxin contamination, this information could accelerate development of breeding through marker selection and/or gene insertion technologies for enhancing host plant resistance.

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BIOTECHNOLOGICAL APPROACH TO GLOBAL WARMING EFFECTS ON AGRICULTURE

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Abstract
Global warming is a serious threat to food production worldwide. Most plant species die after a short exposure to temperatures close to 50°C. Summer temperatures at sites around the world are reaching 45°C lately. If the experimental data proves right at the level of whole crop, a scenario of desertification might occur at various regions of the world. Plants have evolved mechanisms to deal with excessive temperatures. Among those mechanisms at the cell level, the transcription factors called Heat Shock Factors (HSF) might play a key role in preventing cell death. From the Biotechnological view point, it is possible that the over-expression of this HSF might result in increased heat resistance in crops. In our preliminary experiments, papaya plants did not die even after 1 hour exposure to 50°C. This appears to be related with an increased expression in most of the HSF evaluated. Experiments are in progress now, to evaluate if plants that over-express this HSF, prove to show increased heat tolerance.

Keywords: Carica papaya L. ● *hsf* ● Temperature stress ● Gene Expression

Introduction

The global warming nowadays is a real problem based on scientific facts and one of these consequences as a result of the growing accumulation of greenhouse gases is the expansion of the tropical area for the world agriculture and the water supplement. But however, the increase of the temperature is the most important problem for the cultivars growing. Estimating the effect of global warming on the world economy indicates that the increase of one degree Celsius in average temperature would reduce the income in the world between 2.7 and 4.2% of the total (Horowitz, 2009). Evidence suggests that climate change will prevail in the coming decades, as a direct result of these changes, the reduction in the performance of important cultivars contribute to the deterioration in the quality of life of people in the world. It is therefore essential to develop improved varieties can be grown in environments less propitious to wild varieties. Worldwide there are about 13 commercial plants such as corn, soybeans, alfalfa, cotton, papaya, improved by genetic engineering, and there are many papers as model species as Arabidopsis, tobacco, tomato and rice that demonstrate the high potential and utility of plants genetically modified.

The search for genes that mitigate the negative effects of abiotic factors (temperature) and biotic factors in plant is constant. As usual biotechnology strategy to characterize genes coding for transcription factors, these are proteins that regulate the expression of other genes. During stressful events, primarily due to high temperatures has been observed activation of specific transcription factors collectively known as heat shock transcription factors (hsfs) that promote the expression of genes coding for chaperones, whose activity is on counteract the negative effects of temperature high.

Given the few studies on plants resistant to high temperatures, the need for studies based on biotechnology tools, aimed first to characterize genes involved in counteracting and secondly the production of plants resistant to thermal stress by high temperatures. According to Ming (et al, 2008) Papaya (*Carica papaya* L.) is one of the most important tropical fruits on a commercial basis, is the first genetically modified fruit (resistance to PRSV) and its genome has recently been fully sequenced and because it is a relatively fast growing with a small genome size, papaya can potentially be a model for molecular studies to tropical fruits.

This work should complement the few studies on characterization of heat shock genes transcription factors (hsfs) in plants and generate new knowledge in *Carica papaya* var Maradol for biotechnological purposes, because molecular studies of this cultivar varieties now focus on the generation transgenic plants resistant to papaya ringspot virus, pesticide, against *Colletotrichum gloesporioides* and the generation of heavy metal accumulating plants.

Materials and methods

All this work was made at CICY. The treatments were made inside the heating box (Figure 1). Papaya plants from 21 days-old were used. The experimental temperature was: 25 (control), 30, 40 and 50°C for 1h.

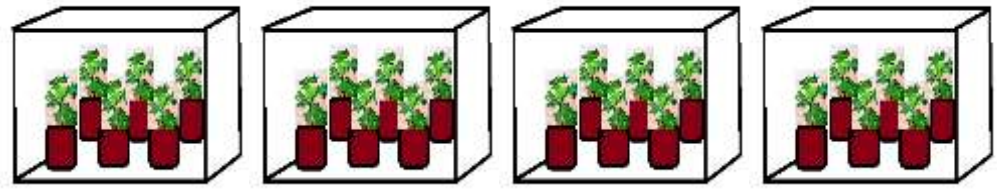

Figure 1. View of temperature treatments. The papaya plants from 21 days-old were exposed at different temperatures during 1 h at box maintained light and relative humidity at same condition.

Parameters analyzed

Temperature parameters. During all the experiment the temperature was monitored 1) inside the box, 2) the leave, 3) the substrate, and the relative humidity (RH). All experiments were conducted at 60 μmol m^{-2} s^{-1} of light and at 50% of (RH).

Physiological parameters.

The chlorophyll and carotenoids content was made according to Wellburn protocol (1994).

Molecular parameters.

Bionformatics characterization were done using programs like TAIR (Swarbreck et al., 2008), PlantaTFDB (Plant Transcription Factor databases, version 2.0 and version 3.0), BioEdit (version 7.0.9.0 (Hall, 1999), Mega 5 .

Results and Discussion

Temperature treatments at box were maintained at the same at experimental temperature (Figure 2). The light was maintained

	Treatments			
	25 °C	**30 °C**	**40 °C**	**50 °C**
AirBox Temperature (°C)	24.7	30.2	39.8	49.9
Leaf Temperature (°C)	24.9	30.07	39.13	50.6

Figure 2. Temperature analysis (°C) at box experimental treatment. The temperature treatment (25, 30, 40 and 50°C) was almost the same that foliar temperature.

Physiological and Growth Determinations.

The papaya plants showed different response according the temperature treatment. Those plants exposed at 25°C were the control treatment (Figure 3A). Those plants exposed at 50°C for 1 h showed curl of old leaves (Figure 3B). Moreover, the leaves and petioles showed decreased water content (Figure 3B). However, when these plants were back at 25°C for 1 h, those plants recovery the phenotype only showed a small necrotic spots at old leave (Figure 3C).

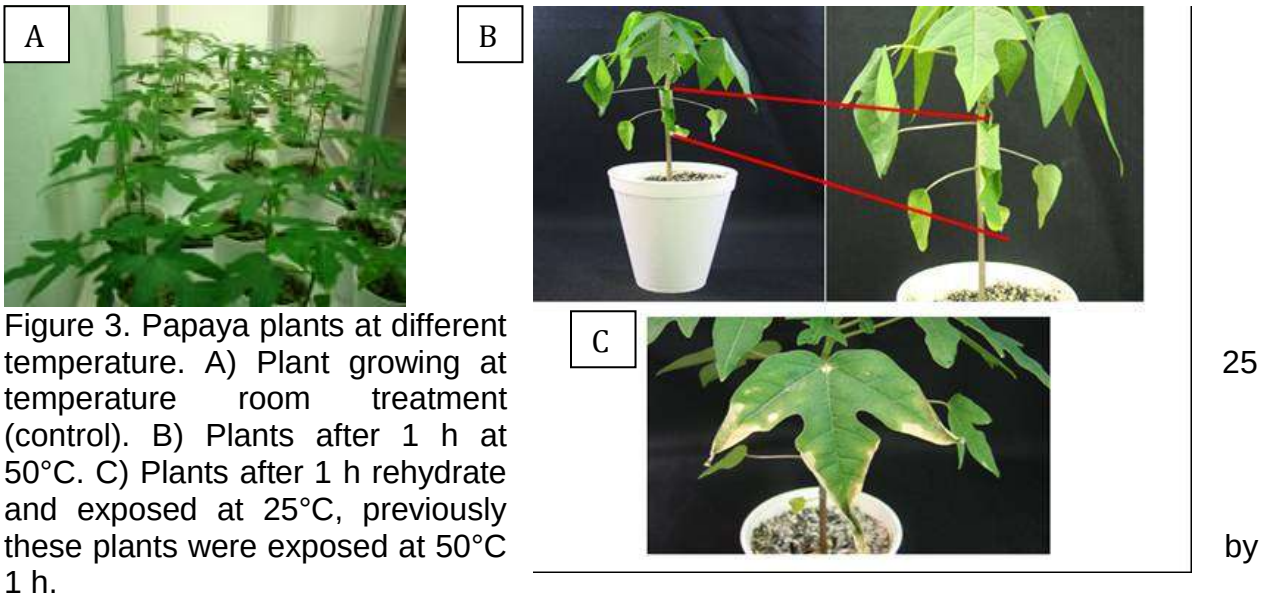

Figure 3. Papaya plants at different temperature. A) Plant growing at temperature room treatment (control). B) Plants after 1 h at 50°C. C) Plants after 1 h rehydrate and exposed at 25°C, previously these plants were exposed at 50°C 1 h.

The content of total chlorophylls and carotenoids at papaya plants, in bases of fresh weight (FW), were different depending of the tissue (Figure 4). The leaves showed higher total chlorophyll and carotenoids content than those petioles and stem tissues. However, those plants exposed at 50°C shoed more chlorophyll content than those

plants exposed at 25°C (Control) (Figure 4). This chlorophyll increased response to the protection mainly to reactive oxygen species (ROS).

Table 1. Chlorophyll and Carotenoids content at papaya plants. Those plants exposed at different temperature treatment were analyzed at different tissue plants.

Tissue	Temperature Treatment (°C)	Carotenoids (µg/g FW)	Total chlorophylls (µg/g FW)
Leaves	25 C	451.8	2999.56
	30 C	221.99	3222.03
	40 C	179.13	3726.14
	50 C	115.06	4008.87
Petioles	25 C	0	154.20
	30 C	0	794.69
	40 C	0	1414.48
	50 C	0	808.72
Stem	25 C	16.88	94.73
	30 C	19.86	129.04
	40 C	0	1320.72
	50 C	0	1406.77

Molecular analysis.
The *Arabidopsis thaliana* genome showed 24 hsf homologues sequences in *Carica papaya* var. Sun Up genome. According to the sequence alignment comparison, we found conserved domains probably related with transcription regulation. In preliminary results, the phylo-genetic tree showed three clades were A, B and C hsf are grouped. The molecular analysis showed finally 6 homologous sequences that was study in *Carica papaya* L. cv. Maradol. We showed the expression of 3 this sequences at different temperature treatments (25, 30, 40, 50°C).

Basal Expression. The *CphsfC* transcription factor showed different basal expression at different papaya tissue (Figure 5). Leaves, petioles and petals showed the band, but those other tissue like fruit immature and mature not showed this band.

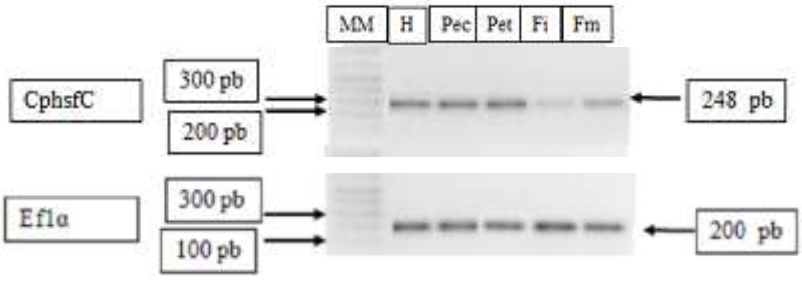

Figure 5. Basal expression of *CphsfC* at different papaya plant tissues H) Leaves, Pec) Petiols, Pet) Petals, Fi) Immature fruits, Fm) Mature fruits. The Ef1α is a housekeeping gene. The amplicon size is right of the picture (pb). Gel dye with ethydium bromide. The molecular marker is 1 Kb Plus.

The *CphsfA1*a transcription factor expression was showed at the different temperatures treatment. The band was more intense at those papaya plants exposed at 50°C than the plants exposed at others experimental temperatures (Figure 6).

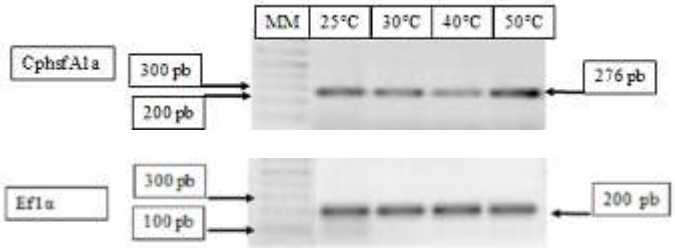

Figure 6. *CphsfA1a* expression at leaves papaya tissue. Treatments were done at different temperature at indicated in boxes. The Ef1α is a housekeeping gene. The amplicon size is right of the picture (pb). Gel dye with ethydium bromide. The molecular marker is 1 Kb Plus.

The *CphsfA1b* transcription factor expression was detected at different temperatures treatment. The band was high in those papaya plants exposed at 40 and 50°C. But was less intense in those plants exposed at 25 and 30°C (Figure 7).

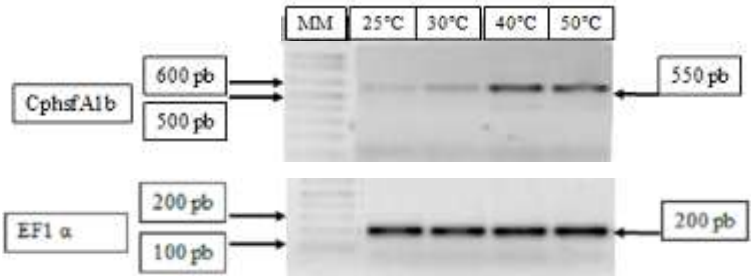

Figure 7. *CphsfA1b* expression at stem papaya tissue. Treatments were done at different temperature at indicated in boxes. The Ef1α is a housekeeping gene. The amplicon size is right of the picture (pb). Gel dye with ethydium bromide. The molecular marker is 1 Kb Plus.

These transcription factors showed different expression in response to temperature treatments. But this expression was different in different tissues of papaya plants. This is important because from these results we could select the transcription factor(s) for over-expression study.

Conclusion

We are currently working on the cloning of this Transcription Factor in order to transform plants with this gene and evaluate if the over-expression do result in increased heat tolerance in this important tropical plant, moreover the over-expression of this gene in other temperate crops might be an important contribution to increase heat tolerance in food production temperate crops threatened by global warming in the coming years.

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GERMINATION OF SOYBEANS AND ITS EFFECT ON CHRONIC DISEASES

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Abstract

Soybean is an abundant source of proteins with high nutritional value and excellent physicochemical properties in foods, and also because it is a rich source of non-nutritive components with potential health benefits. Soy consumption has beneficial effects in the treatment of obesity due to its proven ability to decrease several markers, such as the amount of lipids stored inside adipocytes. On the other hand, extensive epidemiological, *in vitro*, and animal data suggest that soybean consumption reduces the risk of developing several types of cancer. However, little is known about the effect of germination on the antiobesity and anticancer properties. The aim of this work is to study the influence of soybean germination time (0, 2, 6 days) on some obesity markers and on tumor growth both studies in mice. With the results found it is possible to say that a process as germination may help to increase antiobesity and anticancer activities. This effect can be correlated with the change in the protein pattern, and the increase of phytochemicals along the germination process.

Keywords: Soybean germination, antiobesity properties, anticancer properties.

Introduction

Nutritional guidelines around the world recommend increased consumption of plant foods to combat life threatening ailments. Use of functional foods is an emerging trend in a diet based regimen against various lifestyle related disorders as obesity, diabetes mellitus, hypercholesterolemia, cardiac dysfunction, cancer, etc. (Naz, *et al*., 2011). Obesity is a disease caused by an excess of tissue, due to the imbalance between consumption and energy expenditure and it is associated with diabetes, mellitus type II, metabolic syndrome, cardiovascular disease, among others (Gonzalez Espinosa de los Monteros, *et al*., 2011). On the other hand cancer is one of the chronic diseases with highest incidence in the world being the second leading cause of death worldwide and the third cause of mortality in Mexico (INEGI, 2010). González-Espinosa *et al*. (2011) reported that soybean consumption has beneficial effect on markers of obesity. Extensive epidemiological, *in vitro*, and animal data suggest that soybean

consumption reduces the risk of developing several types of cancer (Bara *et al*., 2005). However there are few researches regarding the effect of germination on the chronic diseases as obesity and cancer. The objective of this work is to show the results obtained when testing soybean germinated for 0 to 6 days with and without ethanol soluble phytochemicals (**ESPC**) in reducing body weight and adipose tissue in obese mice C57/BL and on the tumor growth in nude mice (nu/nu), inoculated with cervical cancer cells

Material and Methods

Seeds: Glycine max var. Crystalline was supplied by the National Research Institute for Forestry Agriculture and Livestock (INIFAP, Iguala, México). Seeds were germinated at 27°C in the dark and harvested at different intervals (0, 2 and 6 days). The sprouts were dried and ground. The effect of soy germination on obesity was tested using soy germinated with and without ESPC (2 days of germination). To study the anticancer effect were used isolated of soy germinated for 0, 2 and 6 days.

Soybean protein isolation and extraction of ethanol-soluble phytochemicals (ESPC). The soy flour was defatted with hexane and was suspended in distilled water (1:1.5), pH was adjusted to 9.0 with 1N NaOH and the extraction was carried out under constant agitation for 45 min at room temperature. This suspension was centrifuged at 10,000 rpm for 30 min at 15°C. The pH of supernatant was adjusted to 4.5 with 1N HCl at 20 °C, and the precipitate collected by centrifugation at 10,000 rpm for 30 min at 4 °C. Isolates were lyophilized and stored at 4°C. Soy protein isolates can retain notable amounts of isoflavones associated with the proteins [14]; these isoflavones have anti-obesity and antitumoral activities (González-Espinosa de los Monteros et al., 2011; Robles-Ramírez et al., 2011) and then, the isoflavones and other phenolic and saponin compounds were removed from the protein isolates according to Carrao-Panizzi et al. (2002). Phytochemical extraction efficiency was quantified by measuring total phenolic compounds with the Folin technique.

Animal studies. Firstly, obesity was induced in mice, by high fat diet for 9 weeks, at the end of this time; the diet was replaced by isocaloric diets, containing germinated and ungerminated soy with and without ESPC. The mice were distributed in groups of 4. Each mouse was kept in individual cage. The diet and water was given *at libitum*. The mice were fed for 4 weeks. The weight of each mouse was recorded at the beginning of the experiment and then twice at week. The mice were killed and it was obtained de subcutaneous abdominal adipose tissue.

To determine the effect of isolate of germinated soybean on tumor growth of mice, twenty four female nu/nu mice were purchased. After one week of adaptation, mice were inoculated subcutaneously on the right flank with a suspension of 4×10^6 HeLa cells in 0.1 mL of saline solution and were then randomly assigned to one of the six dietary groups (n=4) and fed *ad libitum* with one of the experimental diets for 5 weeks. When tumors reached palpable volumes of 3-4 mm, two perpendicular diameters of the tumors were measured daily with a caliper and volumes were calculated according with the formula $0.5 \times L \times W^2$, were L and W were the largest and the smallest diameters respectively.

Results and discussion

In Table 1, it can see the results obtained of the effect of germinated soybean (0 to 2 days) consumption in the weight of obese mice. It was found that mice body weight decrease 10.22 and 12.64 in mice fed with soybean germinated by 2 days without and with phytochemicals, respectively; while those fed with ungerminated soybean did not lose weight, although did not continue to gain weigh as those fed with the control diet (diet with casein). The increase in body weight was positively associated with weight gain of adipose tissue subcutaneous, abdominal and epididymal.

Table 2, shows the results obtained from the experiment where it was tested the effect of germinated soy proteins in athymic mice. Relative tumor volumes from mice treated with diets containing 20% de soy protein isolates (SPI) from ungerminated soy, SPI from soy germinated for 2 days without ESPC

Table 1. C57/BL mice weight fed with isocaloric diets of soybean germinated by 0 to 2 days.

Diet	Initial corporal weight (g)	Final corporal weight (g)	Difference of weight
Control	30.33±1.34[a]	31.21±2.76[a]	+ 0.88
High Fat (HF)	44.07±2.78[b]	49.66±0.52[b]	+ 5.59
HF + Soybean ungerminated	44.43±2.07[b]	45.90±1.83[b]	+ 1.47
HF + soybean germinated by 2 days without phytochemicals	43.53±2.89[b]	33.31±2.35[a]	- 10.22
HF + soybean germinated by 2 days with phytochemicals	45.13±1.77[b]	32.49±2.94[a]	- 12.64

Similar letters in the same column means no statistical difference ($p<0.05$) according to Tuckey test, (n=4).

Table 2. Effects of germinated soybean protein on the growth of tumor induced by HeLa cells, in female nu/nu mice.

Diet	RTV at day 21*	Tumor growth inhibition (%)	Apoptosis (%)	Necrosis (%)
Casein	48.7±5.6^{d}	--	2.3±0.3^{a}	21.7±6.3^{a}
SPI0	27.0±3.6^{c}	31.3±17.6^{a}	23.8±1.8^{a}	50.0±5.0^{c}
SPI2+	0.6±0.2^{a}	90.4±0.04^{b}	36.5±3.0^{a}	33.3±2.9^{b}
SPI2-	1.1±0.1^{a}	82.5±10.0^{b}	46.5±1.8^{a}	52.5±4.8^{c}
SPI6+	2.6±1.5^{ab}	88.4±3.3.6^{b}	48.8±3.0^{d}	45.0±5.0^{bc}
SPI6-	3.6±0.1^{b}	88.1±4.5^{b}	40.7±2.2^{a}	40.0±4.1^{b}

RTV= relative tumor volume. *Values obtained before the degradation of tumors. SPI0= Soy protein isolates from ungerminated soybean, SPI2+= Soy protein isolates from soy germinated by 2 days with phytochemicals. SPI2- = Soy protein isolates from soy germinated by 2 days without phytochemicals. SPI6+= Soy protein isolates from soy germinated by 6 days with phytochemicals. SPI6- = Soy protein isolates from soy germinated by 6 days without phyotchemicals.

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VIRUS-FREE PLANTS

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Abstract: One of the problems of agriculture strong, principally in fruit trees and flowers, is the presence of diseases caused by different microorganisms, but mostly by plant viruses. These are responsible for reducing the force, the growth of many plant organs (leaves, roots, stems, flowers) and lower yields and crop quality, reduced longevity, limit the use of certain patterns and can result in greater or lesser extent the death of the plants. For removal of viruses have been developing various strategies such as thermotherapy, chemotherapy and the use of micrografting. The results indicate that the best method is the application of thermotherapy. However, the micrografting technique has been used for disposal, in citrus, viruses are not eradicated by heat. Finally we can conclude that the success of therapy depends on the type of virus and characteristics of plant material.

Keywords: Meristems • thermotherapy • chemotherapy • virus

Introduction: Since Morel and Martin (1955) started dahlia virus removal using the apex meristem culture, the technique has been widely used and adopted in horticulture. It is noteworthy that the incidence of diseases caused by viruses on a number of important crop plants, has found a significant response by certain special forms of propagation that provide virus-free plants. Unlike diseases caused by fungi and bacteria that can be controlled and eradicated by the application by spraying, chemicals, viruses vegetatively propagated plants have been eliminated by using tissue culture, thermotherapy, chemotherapy or a combination of both methods and by micrografting.Virus removal should not be used as a method of propagation, just to get healthy plants from a plant infected mother. The technique has been used with great success when it has been used in various tissues such as calluses, nucellus, ovules, meristems of floral primordia, apical meristems (Walked, 1978) and protoplasts. However, the apical meristem culture is the most effective for producing virus-free plants in a large group of plants of economic and commercial interest (Quak, 1977, Walkey, 1978). Many ornamental plants and fruit trees are propagated vegetatively.

Materials and methods: This is a review of methods that can be employed to eradicate plant diseases, especially those caused by viruses

Results and discussion: Transmission of plant viruses. Viruses can be spread by direct transfer of sap by contact of a wounded plant with a healthy one. Such contact may occur during agricultural practices, as by damage caused by tools or hands, or naturally, as by an animal feeding on the plant (mechanical transmission). Generally TMV, potato viruses and cucumber mosaic viruses are transmitted via sap. The viruses are transmitted by insect vectors, mites and nematodes. The virus cannot be eliminated by pesticides and once the infection is spread by vegetative propagation and for years the crops remain infected.

Methods of eradication of virus

Meristem-tip size: The term meristem culture, generally, is not properly used, since in most cases inoculating the meristematic dome accompanied by one or two leaf primordia (figure 1). The dome is a structure of less than 0.1 mm in diameter very difficult to remove with success in isolated form, and which is often difficult to obtain complete plants. Mori (1977) notes that penetration depends on the type of virus and the host plant. Thus we have the snuff mosaic virus and potato virus X; both are more widely distributed than the cucumber mosaic virus. Therefore the successful eradication of the virus is depending on the initial size of the meristematic tissue to serve as explant (Stone, 1968). Apices vary in size from 0.1 mm to 2 mm in diameter; however, most studies indicate that the size of 0.5 to 1 mm is the most employed. In these dimensions the apex is the meristematic dome and two or more leaf primordia. According to experience, the number of virus free plantlets produced is inversely proportional to the size of cultivated apex, and we can also conclude that the smaller size of the apex, probability of survival is lower, which may mean that to succeed in a high number of healthy plants should be cultivated to ensure a high having good production. However, it may be possible to remove a healthy meristem of a diseased plant and regenerate healthy.

Characteristics of the meristems

There is no definitive explanation can be given to understand that this virus eradication meristem (Wang and Charles, 1991). However, there have been several explanations:

- The absence of plasmodesmata in the dome of the meristem,
- Competition between synthesis of nucleoproteins for cell division and viral replication,
- Substances inhibitory
- Absence of enzymes present only in cells of the zones of meristem,
- Removal by excision of small domes meristem.

The latter proposal could explain why some potato plant showing virus particles in the meristematic domes, could regenerate a plant virus-free (Mellor and Stace Smith, 1977).

Thermotherapy: Thermotherapy involves the application of high temperatures to whole plants or isolated parts. Thermotherapy is a treatment which has been widely used in the process for the production of virus-free plants. Generally, the treatment can be applied to a part or all of the infected plant, but is preferred to select, in part of the whole plant to propagate, the new shoots to give sufficient material to start or meristem culture grafting bud (apple trees infected with chlorotic mottle virus leaf). The procedure is performed in a temperature controlled chamber at temperatures between 30-40 °C for periods of 6-12 weeks. As an alternative thermotherapy has been applied in meristematic tissue infected cultured at temperatures between 30-40 °C so that virus has been eradicated as cucumber mosaic virus (CMV) virus and alfalfa mosaic (Walkey, 1976) However, Kassanis (1957) mentions that the ability of viruses to infect and multiply on plants is not correlated to their thermal inactivation point. The duration of high temperature treatment is often critical, since there is a risk of, on one hand, if the treated tissue is returned to lower temperatures before the virus has been inactivated completely, the particles remaining concentrations can occur, even higher than in the original infected plant or tissue. Apparently, the reason given is the consequence of the high temperature factor that affects the resistance to the virus by the plant host. Temperature inactivation studies have shown that daily cycles of low and high temperatures are often preferred for continuous high temperature treatments (Walkey and Freeman, 1977).

Chemotherapy: Because the multiplications of virus and host metabolism are closely associated, attempts have been made to interfere selectively viral replication have not

been successful. However, in recent years is being used successfully Ribavirin (1-b-dribofuranosyl-1,2,4-triazole-3-carboxamide). In trials using ribavirin the meristem culture medium in vitro has been improved significantly the production of virus-free plants. Quack (1961) suggests that eradication of the virus can be performed by applying the culture medium for chemical apical meristem growth promoters, however, has no consistent evidence to support this. However, the addition to the culture medium of auxins and cytokinins in various concentrations showed that these growth regulators, sometimes, can reduce the viral concentration, without eliminating the virus. Subsequent studies suggest that incorporation, into the culture medium, of a chemical known as anti-metabolite or rubavirinvirazol was more effective. It is presumed that this compound can be supplemented by high temperature treatments, block viral replication in infected tissues, probably stops the synthesis of the virus and degrades existing viruses eradication occurs. In this sense, Shepard (1977) indicates that the virazol was able to eliminate potato virus X in cultured shoots.

Micrografting: This technique consists using of the apical dome and leaf primordia, and then plant it on the surface of the decapitated epicotyl of a seedling 2 weeks of age (pattern) from seed grown *in vitro*. The technique isbased on the fact that the apical meristem contains little or no virus titre and meristematic cells grows faster than all viruses. Therefore, the production of disease-free foundation plants by micrografting remains the only mean to supply disease-free bud sticks to the growers (DeLange, 1978). Studies on the potential of this technique suggest that certain pathogens that are difficult to eliminate by thermotherapy e.g., citrus exocortis and stubborn, can be successfully eliminated by a method of shoot tip grafting *in vitro* (Murashige *et al*., 1972).According to Ben Abdallah *et al*. (1997) when it is perfectly realized, the micrograft could be a rapid screening method,

Virus eradication program: The four stages in therapeutic operations are: Identification of viruses present in the parental clones infected; therapy; testing of treated plants and propagation and continued testing of cured plants under condition that avoid reinfection. A scheme for virus-free plant production is given in figure 2.

Conclusions: The success of therapy depends on the virus to be eliminated and on characteristics of the plant material. Heat treatment is usually effective only against isometric and thread-like viruses and against diseases now known to be caused by mycoplasmas. Successful sanitation is higher the smaller the inoculum. Meristems are more stable area, genetically speaking, a plant tissue

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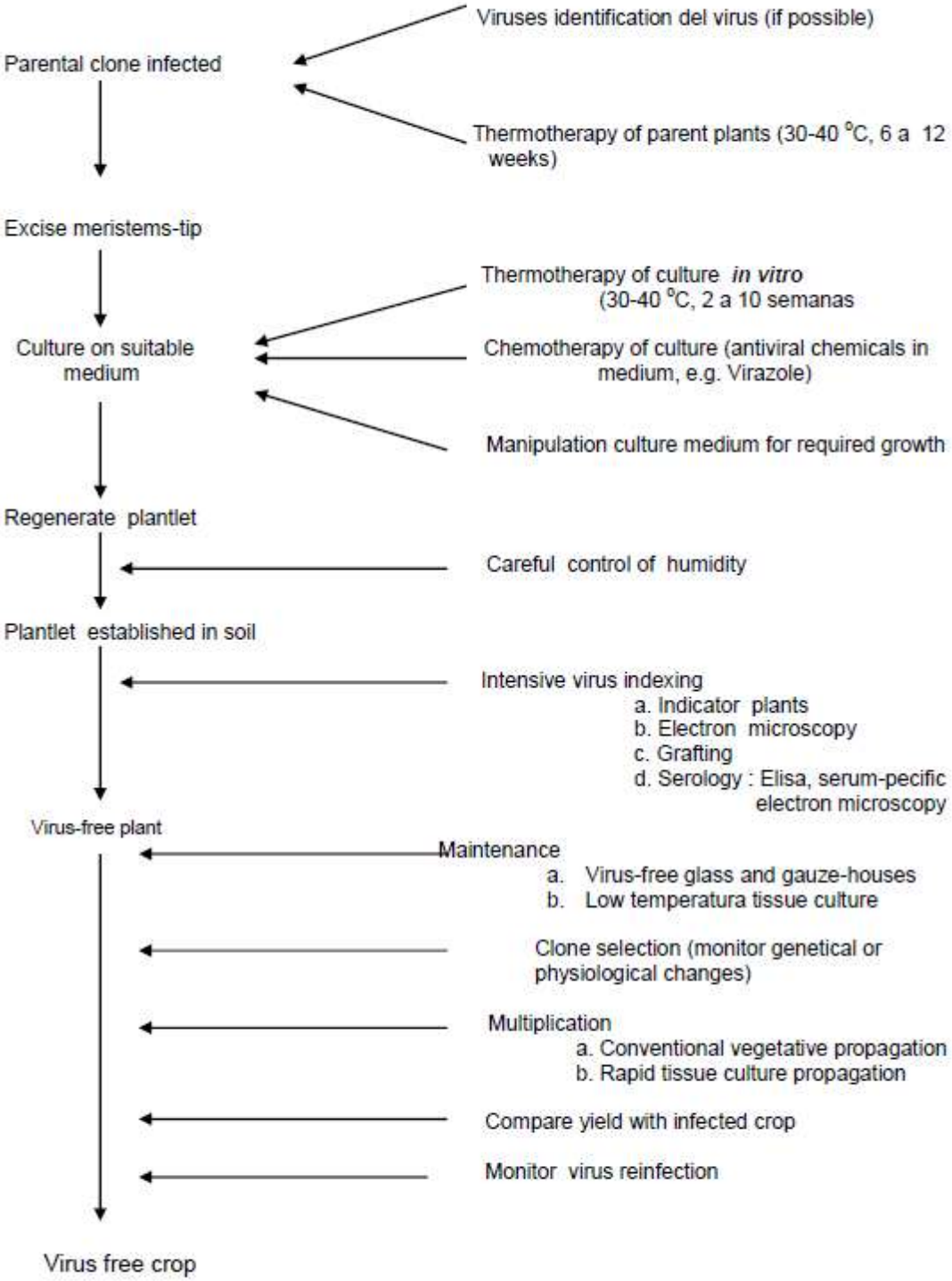

Figure 2. Scheme for the production of virus-free plants production by culturing meristem tip culture

DESIGN AND SIMULATION OF A LOW OFFSET VOLTAGE AMPLIFIER FOR BIOMEDICAL PURPOSE

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Abstract: This paper presents the design and simulation of a low offset voltage pre-amplifier (PA) for biomedical purpose which will be integrated into an instrumentation amplifier. This design supports up 500 mV of offset voltage. Two stages form the biomedical amplifier; first stage is a differential amplifier with channel length of the loads longer than those of the input differential transistors to achieve low noise and second stage is two similar common source amplifiers, ones is the output amplifier and another is used as a unit gain filter. The voltage gain is 70.53 dB @ 100 nF, cut-off frequency at unity gain equal 770.8 Hz in low-pass filter, flicker noise equal 55.9 μV @ 100 nF and 5.59 μV @ 1μF, and the power consumption is 47.5 μW. The design has been simulated in 0.8 μm CMOS process.

Keywords: Biomedical ● Amplifier ● Offset Voltage.

Introduction: Nowadays biotechnological Instruments for ambulatory use for monitoring of biomedical signals into the human body are crucial for quality life. Electromyogram (EMG), Electrocardiogram (ECG) and Electroencephalogram (EEG) needs conditioning circuits to acquire manipulated signals from the human body. Instrumentation amplifier (AI) of three amplifiers is the most common circuit to achieve high CMRR in a conditioning signal circuit (Hwang 2007 and Jin 2009). Moreover, there exist low-power biomedical amplifiers where the chopper technique is used to lower offset voltage and low flicker noise requiring an extra filter to discriminate spikes generated by modulation and demodulation circuits (Qiang 2010 and Yazicioglu 2008). Another widely used technique is current mode instrumentation amplifier which is an amplifier with low input impedance and higher output impedance not being restricted by the power-supply voltages so that wider signal dynamic ranges may be possible at low power supply voltage and -3 dB bandwidth using negative feedback independent of the closed-loop gain (Allen 2002). Previous designs suffer of offset voltage because of electrodes interface skin-electrode; in the proposed design an amplifier of two single-ended outputs reduces offset voltage, as follow: the output of single-ended differential amplifier and the output of the unity gain filter, which is fed back to inverted input, are subtracted, which results input voltage amplified without offset voltage.

Materials and Methods: The preamplifier presented here is a conventional differential amplifier shown in figure 1 and its voltage gain at node V_{o1} is represented by equation (1). In the new design has included a second output stage fed back to inverting input loading it with a capacitor C_{ext} which works as a unity gain filter. In figure 1, PMOS transistors Q7-Q8 of differential stage

have been used at input as they exhibit lower flicker noise compared to NMOS transistors (Nanda and Qu, 2010). Transistors from Q1 to Q6 and from Q9 to Q11 exhibit a constant current, while drains of transistors Q12 and Q13 deliver the output voltages at nodes V_{o1} and V_{o2}. Transistors Q5-Q12 and Q6-Q13 work as source followers. With the intention does not charge the output stage of the differential amplifier a second output stage was included fed it backs to the inverting input to shape a low-pass filter. Equation (3) shows the output voltage V_{o1} takes in account feedback loop.

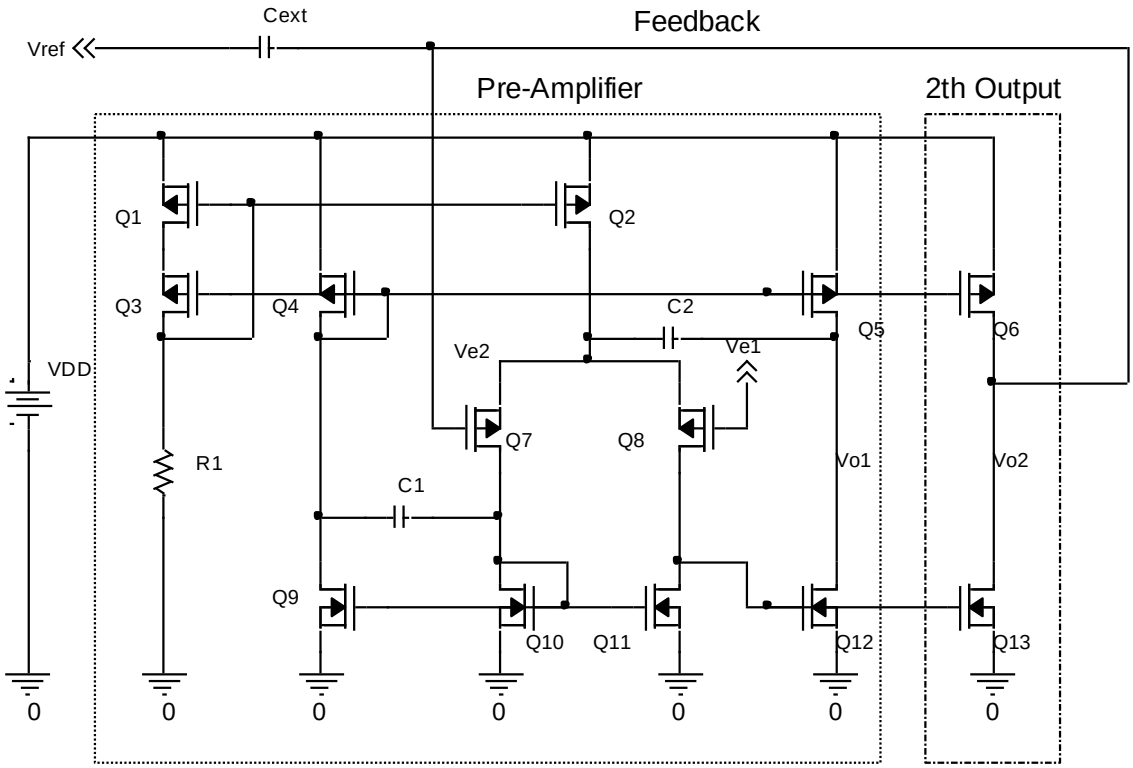

Figure 1. Biomedical Preamplifier.

$$\frac{V_{o1}}{V_{e1}-V_{e2}} = -g_{m8}(r_{o8} \parallel r_{o11}) \cdot \frac{g_{m12}}{g_{m5}} \quad \text{....ec. (1)}$$

Offset Cancelation: Considering offset voltage in the preamplifier, we have:

$$V_{o1} = A\,(V_{e1} \pm V_{off}) \quad \text{....ec. (2)}$$

where $A = g_{m8}(r_{o8} \parallel r_{o11}) \cdot \frac{g_{m12}}{g_{m5}}$ and V_{off} is offset voltage, while:

$$V_{o2} = \pm A V_{off} \quad \text{....ec. (3)}$$

Subtracting equations (2) and (3) offset voltage is cancelled; equation (4) shows resultant gain:

$$V_o = g_{m8}(r_{o8} \parallel r_{o11}) \cdot \frac{g_{m12}}{g_{m5}} V_{s1} \quad \text{....ec. (4)}$$

Results and discussion: Simulations were carried out using spice parameters at 0.8 μm CMOS technology. For time response was applied an offset voltage in the input V_{e1} from 0 mV to 500 mV every 100 mV, figure 2 shows how is holding the output voltage Vo along reference voltage, which is according to the equation (4) where offset voltage is canceled.

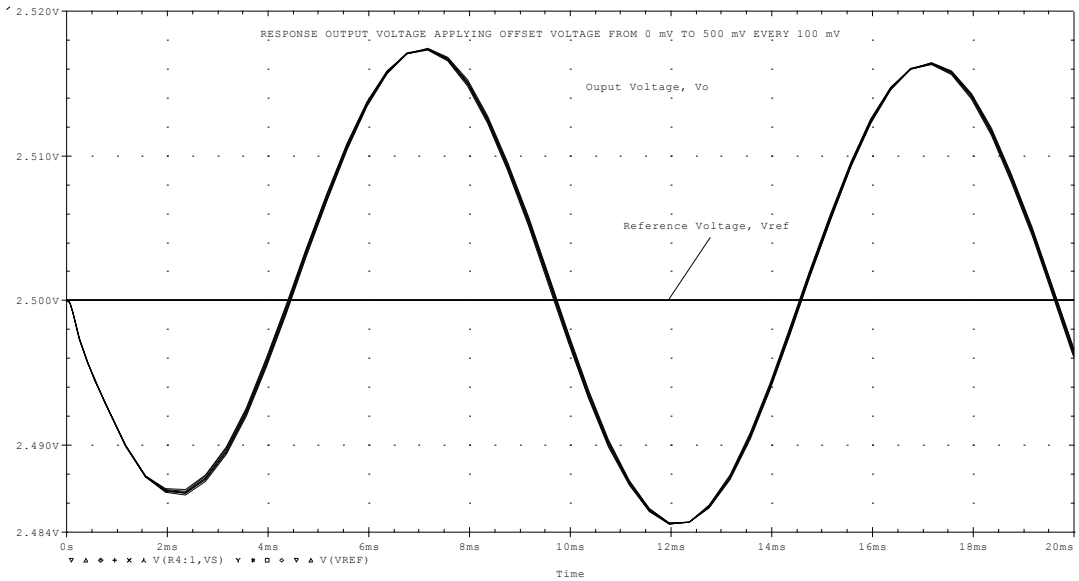

Figure 2. Output voltage: Response time applying offset voltage.

Frequency response in figure 3 shows output voltage gain V_{o1} and output voltage V_{o2} at unity gain filter at different cut-off frequency using capacitive values of: 10nF, 100nF and 1uF. It is important noticed that the second output in figure 1 works as a low-pas filters and as feed it back the differential amplifier works as a high-pass filter. Simulations in Figure 3 confirm this result changing waves form: filter at high frequency and preamplifier at low frequency. Because of is used feedback loop was necessary compensate the differential amplifier put in capacitors C_1=100pF and C_2=20pF in different branches of the circuit look after does not attenuate higher frequency, as is shown in figure 1 and dotted waves form in figure 3, where 135 degrees is the higher margin phase.

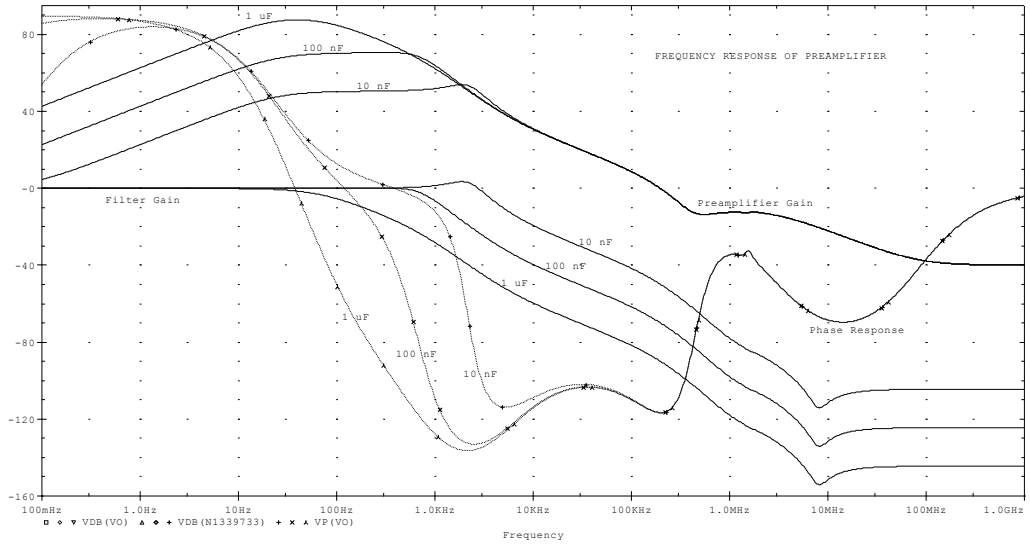

Figure 3. Frequency response of preamplifier applying capacitive loads of 10 nF, 100 nF and 1uF.

One main problem in biological signal acquisition system is the flicker noise owing to that these signals are around hundreds of microvolts to millivolts. Figure 4 shown flicker noise responses at 10nF, 100nF and 1uF; obviously, each time capacitive value is increased the flicker noise reduced is. Therefore, more appropriate capacitive value is 1μF achieving an input noise voltage equal 5.59 μV. Moreover, 55.9 μV of input noise voltage using a capacitive value of 100nF in some cases is appropriate, depends all about biomedical signal value (Shuenn 2009).

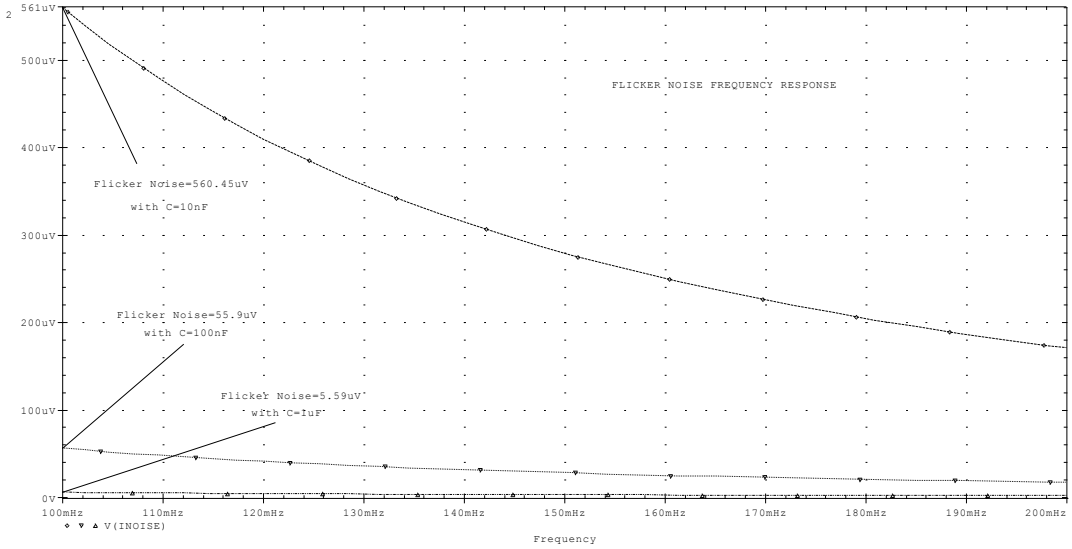

Figure 4. Flicker noise frequency response with capacitance C=10nF, 100nF and 1uF.

Conclusions: This biomedical preamplifier offers simple design including filter and preamplifier in the same circuit with advantage of remove up 500mV of offset voltage. However, it suffers from large capacitor to obtain lower flicker noise voltage and phase compensation.

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ISOLATION, MOLECULAR CHARACTERIZATION AND EXPRESSION OF A NEW *CRY*3A GENES FROM NATIVE ISOLATES OF *BACILLUS THURINGIENSIS* AND ITS TOXICITY AGAINST ASIAN GRAY WEEVIL *MYLLOCERUS UNDECIMPUSTULATUS UNDATUS* MARSHALL (COLEOPTERA: CURCULIONIDAE)

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Abstract: The genetic diversity and distribution of cry genes in *B. thuringiensis* strains vary based on geographical location. Each habitat may contain novel *B. thuringiensis* isolate that have more toxic effects on target spectra of insects. To obtain novel *B. thuringiensis* strains for the production of Cry proteins, isolation of numerous new *B. thuringiensis* strains is becoming a routine activity in many industries. The *B. thuringiensis* cry3A gene is regulated by a different mechanism from that of most of the other cry genes. 1.935-kb DNA fragment of cry3A gene was PCR amplified using gene specific primers, cloned in expression vector pQE-80L, and then used for transformation of *E. coli* M15 cells. The optimum expression was obtained with 1 mM IPTG at 37^0C for 3 h. Nucleotide sequencing of the cry3A gene revealed an open reading frame of 1935 bp, encoding a protein of 645 amino acid residues in length, with a predicted molecular mass of 77.4kDa. These full length gene sequences were deposited at NCBI GenBank with accession number JQ038134 and JQ038135. Present work is now being directed towards the isolation and expression of the new Cry3A gene to access its effect against Asian Gray Weevil *Myllocerus undecimpustulatus undatus* (Coleoptera: Curculionidae). The availability of the recombinant protein will also allow the isolation and identification of its receptor in the insect midgut. Additionally, novel activities/specificities based on the sequence of the new Cry3A gene can be explored by the use of techniques such as DNA shuffling or point mutations. This system offers an additional method for potentially improving the efficacy of Bt insecticidal proteins efficiently, stably and safely particularly against anti lepidopteran insect pests. In other words, Bt technology will be a key to future growth of agriculture.

Introduction: Insect pests, diseases and weeds inflict enormous losses to the potential agricultural production. Anecdotal evidences also indicate rise in the losses, despite increasing use of chemical pesticides. At the same time, there is a rising public concern about the potential adverse effects of chemical pesticides on the human health, environment and biodiversity. These negative externalities, though, cannot be eliminated altogether; their intensity can be

minimized through development, dissemination and promotion of alternative technologies such as biopesticides and bioagents as well as good agronomic practices rather relying solely on chemical pesticides. India has a vast flora and fauna that have the potential for developing into commercial technologies [1]. *Bacillus thuringiensis (Bt)* is a crystalliferous spore-forming bacterium, which typically contains both a spore and a crystal toxin within its cell wall. *Bt* has a long and safe history of use as a microbial insecticide and this has prompted research into the nature of the toxin moiety that is central to mortality of target organisms. Recognition that *Bt* has a diverse genetic structure within the range of toxin genes, referred to as *cry* genes, has led to research into the interactions between crystal toxins and potential hosts. Although *Bt* was already the dominant microbial insecticide globally, the ability to identify and incorporate toxin genes into plants has accelerated interest in the agent and has expanded both research and uptake in integrated pest management (IPM). The unique feature of these *cry* genes, the *cry3Aa* and *cry3Bb* gene is that it is sporulation -independent and it may be induced or depressed during stationary phase, probably by transition - phase regulators. This sporulation -independent promoters may be useful in improving the production of sporulation –dependent Cry proteins. Heterologous recombination may be used, not only to integrate *cry* genes into a resident plasmid, or into the chromosome, but also to disrupt genes of interest [2]. The *Bt cry3A* gene is regulated by a different mechanism from that of most of the other *cry* genes. Its expression begins during late-exponential growth and not during sporulation as for the other classes of *cry* genes [6] because of its non-sporulation- dependent *cry3A* promoter. Many studies were conducted and indicated that the *cry3A* promoter was able to enhance the expression of other *cry* genes and significantly increase the production of those Cry proteins [3, 5, 6, and 7].

Climate change will have direct and indirect impacts on pests and their natural enemies. Farm management responses to these changes are also starting to affect pest complexes. Transboundary plant pests, animal diseases and invasive alien aquatic species are a constraint to food security due to their impacts on food availability, food access, food safety and food stability. The discovery rate of new Bt toxin is more because of its genome diversity. Therefore, it is reasonable to search for new Bt toxins which are more effective against insect pests. Previous studies on screening of indigenous Bt strains for toxicity and molecular characterization of new Bt strains revealed difference in nature and composition of Bt toxins, between native and standard Bt strains. Hence, cloning of toxin genes from new Bt strains and their characterization are essential to expand the use of Bt and for the management of resistance development in insects. Searching for novel sources of cry genes encoding new Cry toxins active against coleopteran insect-pests is important; a cry3A-type gene was isolated from the Andaman and Nicobar Island and Jammu Kashmir Bt strains. Herein, we report the cloning and characterization of the cry3A gene, its expression in *E. coli* cells and also its insecticidal specificities and activities towards Coleopteran insect-pests.

Materials & Methods: Isolation of a *cry*3A gene from the *B. thuringiensis* strain. *B. thuringiensis* strains were grown in 30 mL Luria-Bertani (LB) broth for 12 h at 200 rpm and 30^{0}C, cells were centrifuged and the pellets were kept at –20^{0}C for DNA extraction. DNA extraction was done using bacterial genomic DNA kit (HiMedia) following the manufacturer's instructions. In order to identify *cry3A*-type genes among the *cry3*- type genes detected in *B. thuringiensis* total DNA, primers to amplify complete *cry3A*-type genes (i.e. from the start codon till the stop codon) were designed on the basis of multiple alignment of all previously described *cry3A* and the consensus sequences of their N- and C-terminal coding regions. PCR were performed using two sets of primers designed to amplify complete *cry3A* genes (forward primer: 5'-ATGAATCCGAACAATCGAAGTGAACATGAT-3', reverse primer 5'-

TTAATTCACTGGAATAAATTCAATTTTGTC-3'), and (forward primer: 5'-GGATCCATGAATCCGAACAATCGAAGTGAA-3', reverse primer 5'-AAGCTTTTAATTCACTGGAATAAATTCAAT-3'), 30 cycles of amplification at 62^{0}C annealing temperature and high fidelity LATaq DNA polymerase (Sigma). The resulting PCR fragment was excised from the gel and purified using the Nucleospin® Extraction II Kit (MN), following the manufacturer's instruction.

Cloning, sequencing and sequence analysis of the *cry*3A gene from the *Bt* strain. The purified PCR fragment putatively corresponding to a *Bt* strain S811 *cry3A* gene was cloned into the pTZ57T/R- (Fermentas, USA) and used to transform *E. coli DH5α* cells. The clone was sequenced using an automated sequence analyzer (MWG). Computer analyses of the cloned DNA sequence were done using the BioEdit software package. Databank comparisons of the cloned DNA sequence with other published cry sequences were made using the BLASTx software from the NCBI databank (http://www.ncbi.nlm.nih.gov). The Conserved Domain Database search (CDD-search) from the NCBI site was used to compare motif identity and similarity with the clone DNA sequence and known conserved domains. Sequence alignments and dendrograms were obtained by using CLUSTAL W software and were edited with the BioEdit software. Dendrograms were edited using the TreeView software (http://darwin.zoology.gla.ac.uk/ ~rpage/treeviewx). The molecular mass and predicted pI of the clone deduced protein sequences were determined by the Protein Machine software available at the EXPASY site (http://us.expasy. org/tools/).

Construction of *E. coli* expression vector pQE80-L *cry3A.* *E.coli* M15 cells containing the cry3A gene cloned into the pQE 80-L expression vector were grown in LB medium in the presence of 100 µg/mL ampicillin, 25 µg/mL kanamycin for 14-16 h at 37^{0}C and the plasmid DNA was isolated using GeneJet plasmid Miniprep kit (Fermentas) following the manufacturer's instruction. PCR was performed using the isolated plasmid as template, (forward primer: 5'-GGATCCATGAATCCGAACAATCGAAGTGAA-3', reverse primer 5'-AAGCTTTTAATTCACTGGAATAAATTCAAT-3') 30 cycles of amplification at 50^{0}C annealing temperature and high fidelity LA Taq DNA polymerase (Sigma). The resulting PCR fragment was excised from the gel and purified using the Nucleospin® Extraction II Kit (MN), following the manufacturer's instruction. The purified PCR fragment was sub cloned into the expression vector pQE80-L (Qiagen), following the manufacturer's instructions. The resulting construction, encoding the recombinant *cry3A* gene to be expressed fused to a C-terminal His-tag, was named pQE806-cry3A.

Recombinant *Cry*3A toxin expression and purification. *E. coli* M15 cells transformed with pQE80L-*cry3A* were grown at 37^{0}C at 200 rpm agitation, in the presence of 250 µg/mL ampicillin, 75 µg/mL Kanamycin until O.D.600= 0.6. Cry3A expression was induced by addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when an O.D.600 = 0.6 was reached. Four hours after induction the cells were harvested by centrifugation at 4,000 rpm for 20 min. The pellet containing the cells expressing the Cry3A His-tagged protein was then resuspended in Lysis buffer (50 mM Sodium Phosphate buffer, 300 mM NaCl, 1% Glycerol and 0.5% Triton X-100, pH 7.0). Thaw the cell pellet from an *E. coli* expression culture on ice (if frozen). Resuspend 1 g of pelleted, wet cells in 2-5 ml Lysis-Equilibration-Wash buffer **(**LEW) buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). Add lysozyme to a final concentration of 1 mg/ml. Stir the solution on ice for 30 min. Sonicate the suspension on ice according to the instructions provided by the manufacturer (e.g. use 10 x 15 s bursts with a 15 s cooling period between each burst). Centrifuge the crude lysate at 10,000 x *g* for 30 min at 4°C to remove cellular debris. Carefully transfer the supernatant to a clean tube without disturbing the pellet. Store

supernatant on ice. Purification under native conditions using Protino® Ni-TED (MN) packed columns as per the manufactures guidelines. Finally Use protein assay and/or SDS-PAGE analysis to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

Bioinformatics analysis. Nucleotide and deduced amino acid sequences were analyzed with the Blast tools (www.ncbi.nlm.nih.gov/BLAST). Signal peptide sequence was analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). Related sequences obtained from databases using the software GENSCAN online tool (www.genes.mit.edu/GENSCAN.html) were used for identification of gene features such as exon and splice sites in genomic DNA. BioEdit (version 7.0.4.1) was used for sequence editing and analysis. Conserved Domains and Protein Classification (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, version CDDv2.32-40526 PSSMs) and the predicted structure was validated using protein structure validation software suite (PSVS) tool. Determination of protein functional analysis obtained from databases using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and ProFunc (http://www.ebi.ac.uk/thornton-srv/databases/profunc/index.html). COBALT is a multiple sequence alignment tool that finds a collection of pairwise constraints derived from conserved domain database, protein motif database, and sequence similarity, using RPS-BLAST, BLASTP, and PHI-BLAST.

Bioassays of the recombinant *Cry*3A toxicity against insect larvae. The activity of the Cry3A-type protein obtained from the recombinant *E. coli* strain was tested against coleopteran insect pest *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae). A preliminary test was performed to determine the specificity of this protein at a relatively high protein concentration (1200 µg/ml). Bioassays with *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae) were carried out by dipping leaf disks prepared from mango into the protein solution using second instar larvae (grubs) and adults. Negative controls for all the insects tested were included using the same conditions but without any toxin. Bioassays were repeated at least three times. Bioassays were conducted at 25°C, 60 to 70% relative humidity (RH), and a 16:8 (light/dark [h]) photoperiod. For all insects bioassayed, mortality was evaluated after 5 days.

Statistical methods. For all investigated parameters, the analysis of variance (ANOVA) was performed using the GraphPad Prism5 statistical software. The measurements of treatments were compared and grouped using Duncan's multiple range tests at the 0.01 significance level.

Results: *cry3A* gene. The *cry*3A gene was amplified, cloned and sequenced. Total DNA isolated from all native *Bt* isolates from Andaman and Nicobar Islands [7], Jammu and Kashmir (Unpublished data) was amplified (Fig. 1A and 1B) using gene specific primers. The optimum conditions for amplification of *cry*3A gene were 250 ng of DNA, 200 lM of dNTPs, 1.5 mM of MgCl2 and 50 pmoles of primers and annealing temperature of 62^0C. Gene cloning was confirmed by colony PCR and restriction digestion with *BamHI* and *HindIII* of recombinant plasmid DNA containing *cry*3A gene (Fig. 2 and fig. 3). The total length of *cry*3A gene was 1,935 nucleotides which encode protein of molecular weight 77.4 kDa, approximately. All the sequences of full length *cry*3A gene of *Bt* isolates were submitted to GenBank database with accession numbers JQ038134 and JQ038135. The 1.935 kb *cry3A* gene sequence from SITAB and SLK Bt isolates, showed 91% homology with *Bacillus thuringiensis* serovar *tenebrionis* Cry3A gene when aligned with already known sequences using BLAST (basic local alignment search tool).The deduced amino acids sequence of toxic region of Cry3A proteins of two Bt

isolates was aligned with Cry3A like protein of public database. A total of 117 variations found in toxic domains.

Composition of *Cry*3A of *Bt* isolates and phylogenetic analysis. The deduced amino acid sequence similarity varies from 31-82% of *cry*3 like sequences. The amino acid residues ranged between 36.49% (Ala), 28.17% (Thr), 18.19% (Gly) and 17.16% (Cys). A total of 645 amino acids were found in Cry3A proteins in which the negatively charged amino acids ranged between 32 and 34 (66), whereas positively charged amino acid ranged between 10 and 37 (70). The phylogenetic relationship of *cry3A* gene with other *cry3A* genes reported earlier on from the present study and other laboratories is shown in Fig.2. *cry*3A reported from present study are distinctly different from the ones reported elsewhere. The cluster of *cry*3A genes reported present study seems to have evolved differently from the other reported *cry*3A genes.

Recombinant *cry*3A: expression in *E. coli*, purification and evaluation of toxicity. The *E. coli* M15 strain harboring the pQE80L-*cry*3A constructs was induced with 1 mM IPTG to express the recombinant His-tagged Cry3A protein. SDS-PAGE analysis of *E. coli* extracts after IPTG induction, showed a differential protein band corresponding to the expected 77.4-kDa recombinant His- tagged Cry3A protein. No additional bands were identified within extracts of non-induced cells with empty pQE80-L vector (Fig. 1C). In these conditions, a large amount of the recombinant His-tagged Cry3A protein was produced and, subsequently, purified using Ni-TED affinity chromatography from sonicated *E. coli* crude extract (Fig. 1D). The toxicity of purified recombinant His-tagged Cry3A was tested in leaf dip bioassays against Asian Gray Weevil *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae). The bioassays revealed that the insect pest adults were susceptible to the purified recombinant His-tagged Cry3A (Fig. 1E). The purified recombinant His-tagged Cry3A was toxic to Asian Gray Weevil *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae), being 1200 µg/mL and 920 µg/mL the highest concentrations tested to achieve the maximum toxicities,

Conclusion: This study led to the identification of a novel *cry3A* gene, which is known to effective, against coleopterans. These new genes served as candidate gene against different coleopteran insect pests and also new encounters due to climate change. Present study data is strengthened by an efficient expression, selection or screening system, hopefully this allows us to reach into the full potential of these environmentally friendly pesticides.

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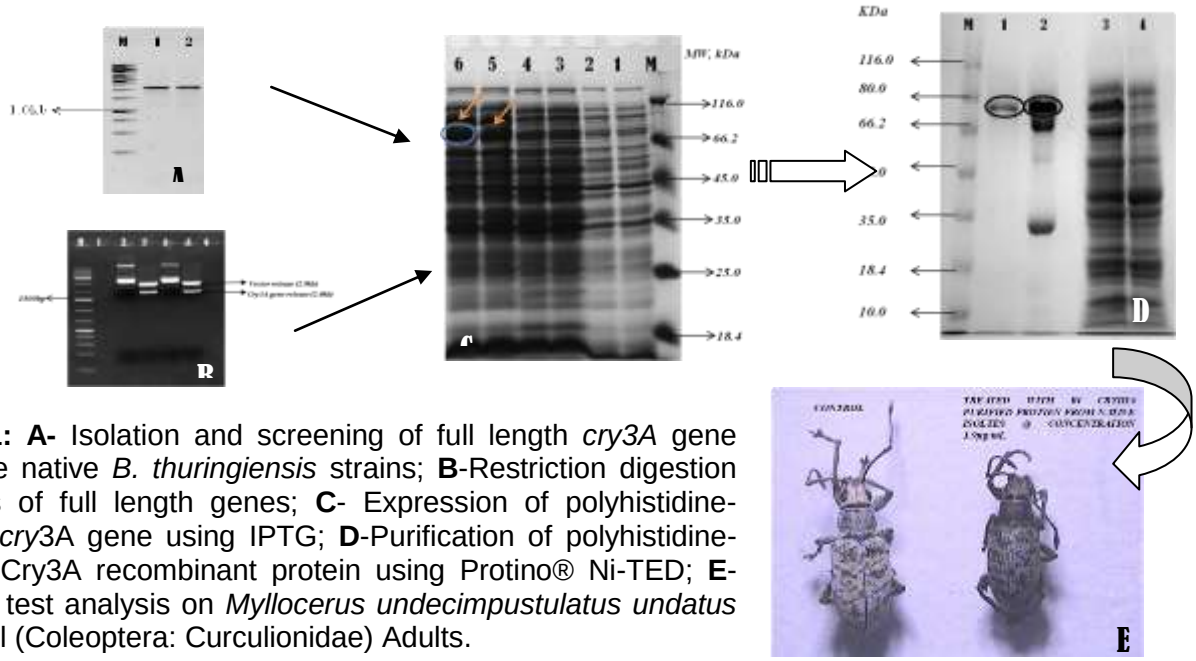

Figure1: A- Isolation and screening of full length *cry3A* gene from the native *B. thuringiensis* strains; **B**-Restriction digestion analysis of full length genes; **C**- Expression of polyhistidine-tagged *cry*3A gene using IPTG; **D**-Purification of polyhistidine-tagged Cry3A recombinant protein using Protino® Ni-TED; **E**-Toxicity test analysis on *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae) Adults.

DIFFERENTIAL INDUCTION AND REPRESSION PATTERNS OF BETA-

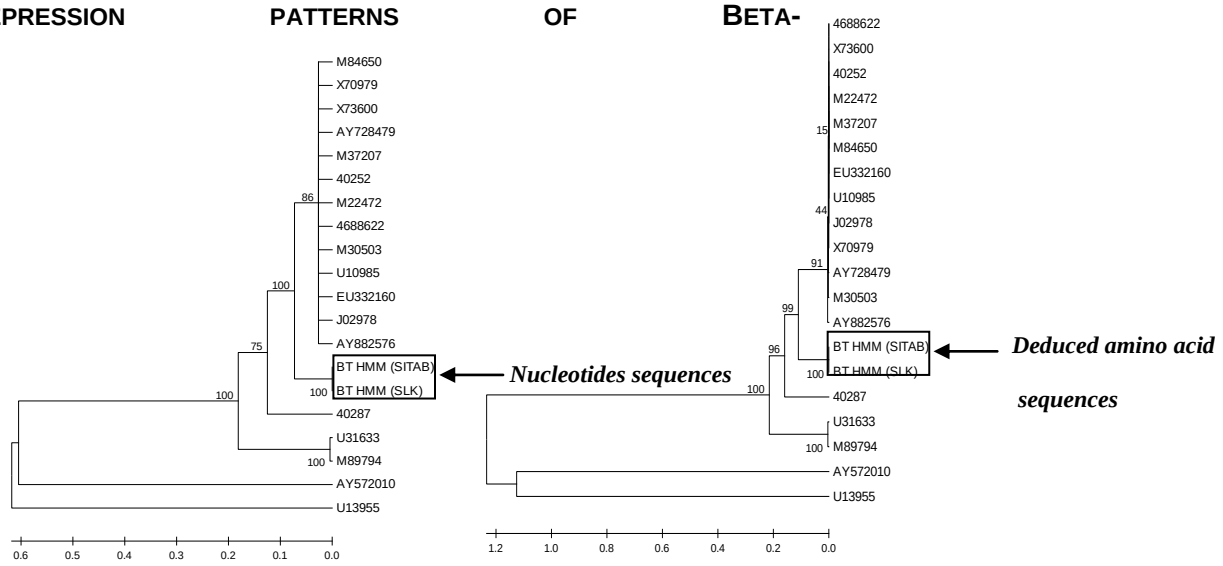

Figure 2: Neighbour-joining tree showing phylogenetic relationship amongst different *Bacillus thuringiensis cryIIIA* genes based on nucleotide and deduced amino acid sequences using NCBI GenBank accession number U13955 (*cry14* gene) as an out-group. The NJ tree was constructed using CLUSTAL W with default parameters are indicated at the nodes. Genetic distances between *cryIIIA* gene sequence profiles were calculated by using Kimura 2-parameter. Bootstrapped data set were generated using phylogeny reconstruction (2000 replicates), and analyzed by using MEGA (version 5.4) software. Sequences were obtained from the databases of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov). The gene sequences were responsible for geographic separation for divergence within *cry* serotypes, consistent with the evaluation of distinct bacterial population. Despite the geographical distances, *Bt* strains have originated from common ancestors. Some strains have evolved to be quite distinct and others remain as members of closely related groups. Arrow Position of the novel Cry sequence, numbers at nodes percentages of bootstrap resamplings. The values shown at the base of divergent lines are bootstrap values. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

IDENTIFICATION AND CHARACTERIZATION OF (1,3)-ß-GLUCAN SYNTHASE GENE FROM *GANODERMA LUCIDUM*

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Abstract: *Ganoderma lucidum* is a macromycete economically and culturally important in the globe, because of its medicinal properties. This species contain different bioactive compounds, including polysaccharides that have demonstrated immunomodulatory, anticancer and radioprotective effects. These polysaccharides have been isolated and characterized, being β-(1,3)(1,6)-D-glucans the most representative structure. The metabolic pathway for the synthesis of β-(1,3)-D-glucans has been proposed for various organisms, including *G. lucidum*. Based on these background the enzymes involved in the synthesis of β-(1,3)-D-glucans from sucrose are: α-glucosidase, β-fructofuranosidase, hexokinase, glucose-6-phosphate isomerase, phosphoglucomutase, UDP-glucose pyrophosphorylase and (1,3)-β-glucan synthase. Nonetheless these genes have not been identified in *G. lucidum*.

The aim of this study was to design primers, identify and characterize the sequence of the (1,3)-β-glucan synthase gene from *G. lucidum*. The primers were designed by searching for this gene in organisms similar to *G. lucidum*. Then, with the application of PCR, primers were tested using DNA extracted from *G. lucidum s. l.* strain 382. The fragments were sequenced and analyzed in the GenBank. We identified two fragments of the enzyme (1,3)-β-glucan synthase of this fungus.

Key words:(1,3)-β-glucan synthase● gene ● primers.

Introduction: *Ganoderma lucidum* is a white rot fungus, belonging to the Polyporales order and Ganodermataceae family, is a macromycete economically and culturally important in the world, principally in China, Korea and Japan because of its medicinal properties. The fruiting body, mycelium in liquid culture and spores of this species contain different bioactive compounds, including polysaccharides that have demonstrated immunomodulatory (Berovic *et al*., 2003), anticancer (Sone *et al.,* 1985) and radioprotective effects (Kim y Kim, 1999). Polysaccharides from *G. lucidum* such as β-D-glucans and heteropolysaccharides have been isolated and characterized (Wasser, 2005), having β-(1,3)(1,6)-D-glucans as the most representative structure (Paterson, 2006). The synthesis pathway of β-(1,3)-D-glucans has been proposed for various organisms, including *G. lucidum* (Zhong and Tang, 2004). Based on these background the enzymes involved in the formation of β-(1,3)-D-glucans from sucrose are: α-glucosidase, β-fructofuranosidase, hexokinase, glucose-6-phosphate isomerase, phosphoglucomutase, UDP-glucose pyrophosphorylase and β-(1,3)-D-glucan synthase. The genes involved in the synthesis of these polysacharides have not been identified in *G. lucidum*. Previous works have identified some of these genes for other fungi like *Laccaria bicolor (*Martin *et al.,* 2008*), Malassezia globosa (*Xu *et al.,* 2007*),* as well as *Ustilago maydis (*Birren *et al.,* 2006*)*.

Materials and methods: *Ganoderma lucidum s. l*. strain 382 was maintained on MY agar (30 g/l of dextrose, 3 g/l of malt extract, 5 g/l of yeast extract, 3 g/l of casein peptone and 15 g/l of agar) at 4°C.

DNA was isolated from mycelia grown on MY agar. Extraction was performed with Charge Switch plant gDNA Kit from Invitrogen (EUA) following the manufacturer instructions. The primers were designed by searching for the (1,3)-β-glucan synthase gene in *Cryptococcus neoformans* (Thompson *et al*., 1999), *Ustilago maydis* (Birren *et al*., 2006)and *Saccharomyces cerevisiae* (Ohyama *et al*., 2004) considering the conserved sequences along the gene. The sequences of the designed primers are shown in the table 1 and these were tested by PCR using the isolated DNA. The final concentrations of the PCR mix components were: 0.2 mM dNTP's mix, 3 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase per 20-µl reaction (Invitrogen, EUA) and 0.2 µM of each of the two primers used in a particular reaction. Temperature cycling was carried out using a gradient thermal cycling (Palm-cycler, Corbett). For the primers BGS2113UmF, BGS3097UmR and BGS547UmF, BGS2113UmR an initial denaturation step of 94°C for 30 s was followed by 35 amplification cycles, the temperature and times for the denaturation, annealing and extension steps of each cycle were 94°C for 30 s, 53°C for 30 s and 72°C for 90 s. After the 35 cycles were completed, the samples were incubated 10 min at 72°C. For the primers BGS2947UmF and BGS3561UmR an initial denaturation step of 94°C for 30 s was followed by 35 amplification cycles, the temperature and times for the denaturation, annealing and extension steps of each cycle were 94°C for 30 s, 57°C for 30 s and 72°C for 60 s. After the 35 cycles were completed, the samples were incubated 10 min at 72°C. Negative controls (No DNA template) were used in all experiments.

Table 1. Designed primers.

Primer	Sequence
BGS2113UmF (Forward)	CAAGGCYACYGGBGGYATC
BGS3097UmR (Reverse)	CACATTGAYTTCAARWCRGCYACC
BGS547UmF (Forward)	CCGTGMGKCAGATTGAGCAY
BGS2113UmR (Reverse)	ATCCTSATGAMCTCKTTCAACACG
BGS2947UmF (Forward)	TTGAYGTCDGCYTTSGTCTTGTC
BGS3561UmR (Reverse)	GAARTGCTTGATRGCMGCWCC

PCR products were analyzed on an agarose gel (0.8%). These products resulted in two or more fragments of DNA, hence, a purification step was carried out, where we selected the corresponding bands of DNA to the expected molecular weight. Purification was performed using QI Aquick Gel Extraction Kit de QIAGEN (Germany). Once purified these fragments were sent to sequencing. All PCR products obtained were sent to the sequencing unit of the Biology Institute of UNAM.

Results and discussion

Only two of the three PCR products analyzed on an agarose gel showed fragments of expected molecular weight (984 bp and 1566 bp for BGS1 and BGS2 respectively) and when these PCR products were sequenced, favorable results were obtained. Figure 1 shows the fragments of DNA obtained using the BGS2113UmF and BGS3097UmR primers (BGS1) and the BGS547UmF and BGS2113UmR primers (BGS2).

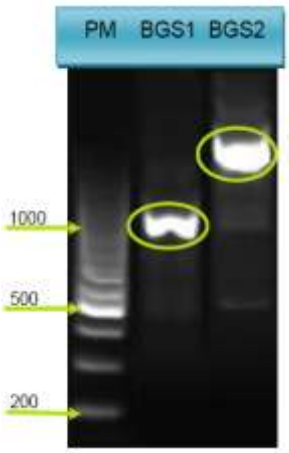

Figure1. PCR products obtained.

The nucleotide sequences were traduced to amino acid sequences and then were compared against the database of GenBank that used BLAST algorithm (blast p). We found that both had strong similarity with the (1,3)-β-glucan synthasegene of other basidiomycetes. Amino acid sequences of 169 and 293 residues were obtained for BGS1 and BGS2, respectively (Table 2). Table 3 summarizes the BLAST results, where the coverage and percentage of similarity of each fragment obtained with other organisms.

Table 2. Amino acid sequences.

Sample (primers)	Sequence
Fragment BGS1	RKWYFAPQLNLDDAVGQVQNPGLQRLRSVK GKPQTAGSKSLDSALNRWRNAMNSMSQYDR LRQVALYLLCWGEAGNVRFTPECLCFIFKC ADDYYRSPECQNRIDPVPEGLYLETVVKPL YRFMRDQYEVVDGKFVRREKDHDQIIGYDD INQLFWYPEGIAKIVLQDN
Fragment BGS2	HVQRLLYHQVDSPDGRRTLRAPPFFTANNG NESQFFPVGGEAERRLSFFASSLTTALPEP LPVDAMPTFTVLVPHYSEKILLSLREIIRE EDQNTRVTLLEYLKQLHPVEWDNFVKDTKI LAEESETTFDATQSTNEKSGNKRTDDLPFY CIGFKTAAPEYTLRTRIWASLRAQTLYRTV SGMMNYSKAIKLLYRVENPQIVQRFAGNTD RLERELERMSRRKFKFTVSMQRYAKFNKEE LENAEFLLRAYPDLIAYLDEEPAPKGGDPR LFSTLIDGHSEIDEQTGKRKPKF

Tabla 3. Comparison of the sequencesobtained with amino acid sequencesdepositedin GenBank.

Fragment	Putative gene	Organism	Coverage (%)	Similarity (%)
BGS1	(1,3)-β-glucan synthase	*Laccaria bicolor* *[XP_001875386]	100	86
		Coprinopsis cinérea *[XP_001833273]	99	83
		Ustilago maydis *[XP_757786]	99	71
		Cryptococcus neoformans *[BAC15535]	100	76
BGS2	(1,3)-β-glucan synthase	*Moniliophthora perniciosa* *[XP_002392175]	100	85
		Laccaria bicolor *[XP_001875386]	100	85
		Coprinopsis cinérea *[XP_001833273]	100	83
		Ustilago maydis *[XP_757786]	100	77
		Cryptococcus neoformans *[BAC15535]	100	76

* GenBank accesion number.

In other hand, the gene for the enzyme (1,3)-β-glucan synthase in *C. neoformans* (AF102882) is characterized completely and it is constituted by 8023 nucleotides (Thompson *et al.,* 1999), while the sequence of this gene is incomplete for the rest of the fungi listed in the Table 3. For this reason, we chose to use *C. neoformans* as align template. With this alignment, it was determinated the location of fragments BGS1 and BGS2 over the gene (1,3)-β-glucan synthase from *C. neoformans*; fragmentBGS1 extends from nucleotide 2391 to 2921, while fragment BGS2 covers the region 4025 to 4918 (Figure 2).

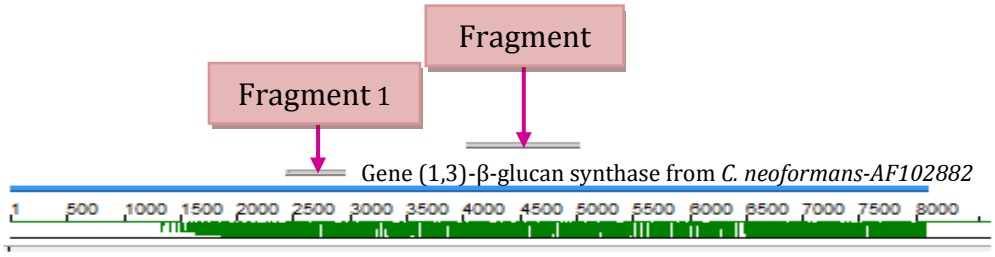

Figure3. Location of fragments 1 and 2 from *G. lucidum s. l.* strain 382.

Conclusions

Degenerate primers were designed to identify the (1,3)-β-glucan synthase gene from *G. lucidum* and we obtained amplification with two of three pair of primers proved.

The amino acid sequence obtained by using of the BGS547UmF and BGS2113UmR primers, as well as the amino acid sequence obtained with the BGS2113UmF and BGS3097UmR primers, showed strong similarity to the sequence corresponding to the (1,3)-β-glucan synthase of some basidiomycetes such as *Coprinopsis cinerea*, *Moniliophthora pernicious*, *Laccaria bicolor*, *Ustilago maydis* and *Cryptococcus neoformans*.

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STUDY OF BMP-15 AND BMPR-1B GENE POLYMORPHISM IN WEST AFRICAN SHEEP OF YUCATÁN

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Abstract

This study was carried out for detection of possible polymorphisms in BMPR-IB and BMP15 gene. Blood samples were collected from 50 ewes of West African sheep. All samples have the wild-type allele for both genes. Considering the phenotypic records in this breed, the obtained result indicates that the genetic factor responsible for twining or multiple lambing rate is not related to reported mutated alleles at Boorola major gene and must analyzed the complete precursor for these two genes and also find other genes that possibly are connected with this character.

Keywords: West African sheep ● prolificacy● BMPR-1B● BMP15

Introduction

One of the breeds used in grazing systems by good toughness, resistance to parasites and highly adaptable to different environmental conditions is the hair sheep breeds West Africa, whose comparative advantages have allowed him to have a wide distribution throughout the country. However, these characteristics of the races have been lost mainly due to uncontrolled crossbreeding with other breeds such as synthetic dorper and katahdin (Hinojosa-Cuellar et al., 2009). An alternative to genetic rescue of this breed, you should consider the identification of genes associated with economically important traits and could be of interest as new targets of selection (Legarra et al., 2007). One of these characters is prolificacy in various breeds of sheep has identified the presence of genes with major effect prolificacy (Davis, 2005), so that selection for these genes would allow a significant increase in the number of lambs born in no time.

The West African sheep have up to 1.5 lambs in the autumn months (Martinez-Rojero 2011). In recent years, many studies on the genetics of prolificacy in sheep (*Ovis aries*) lead to highlight the importance of three major genes: BMPR-1B, BMP15 and GDF9, which have been shown to affect ovulation rate and litter size through different mechanisms (Davis, 2005). However it is known that these genes are more affected by environmental conditions of each region, and is therefore mutations in these genes fertility for these races do not always show the same effect on litter size for other race as West African Sheep. The identification of new genes or new variants of fertility can be a tool to increase production efficiency in herds with low production levels in the mexican tropics.

For West African sheep, identification of mutations in major genes and their subsequent use under strict control with tech could contribute to improved economic performance and therefore you determine the viability of sheep farms, thanks to increase litter size without resorting to synthetic crossing with another breed, improving the competitiveness of production.

In this study, we evaluated the presence of mutations known as FecB and $FecX^G$ in BMPR-IB and BMP-15 genes respectively in 50 females of West African sheep, this study did not revealed the presence of this mutation and concluded that prolificacy possibly that occurs due to another mutation in another part of these genes, making it necessary to obtain the reading frame for these genes.

Materials and methods

Sample collection

Approximately 5 ml of blood was collected from jugular vein of 50 West African sheep, in a sterile *Vacutainer*® tube containing anticoagulant 500 □l EDTA (0.5 M, pH= 8.0).

Isolation of genomic DNA and restriction enzyme

Genomic DNA was extracted from blood preserved in EDTA, using the Wizard Genomic DNA Kit (Promega), according to the manufacturer's procedure. Primers were synthesized by Integrated DNA Technologies, Inc. DNA test were carried using forced PCR-RFLP based method described by Wilson *et al.* (2001) and Davis *et al* (2006). The primers were designed as follows:

Name	Mutation	Sequence primer
BMPR-IB	FecB	AF 5´-GTCGCTATGGGGAAGTTTGGATG-3´
		AR 5´- CAAGATGTTTTCATGCCTCATCAACACGGTC-3´
		CR-5´-CAAGATGTTTTCATGCCTCATCAACACGGTC-3´
BMP-15	$FecX^G$	BF-5´-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3´
		BR 5´-GATGCAATACTGCCTGCTTG-3´

For the identification of FecB mutation in BMPR-IB gene, we used two pairs of reverse primers (AR and CR), was deliberately introduced a point mutation resulting in PCR products with FecB carrier sheep containing an AvaII restriction site (G|GACC), whereas the products from the noncarriers lack the site. The amplification was carried out using 35 cycles at 94 °C for 15 s, 60 °C for 30 s and 70 °C for 30 s, followed by 72 °C for 5 min and 99 °C for 15 min. For $FecX^G$ mutation in BMP-15 gene, we used pairs of primer (BF and BR). The amplification conditions for primers of the BMP-15 gene were as follows: denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 63°C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72°C for 10 min on a iCycler IQ (Bio-rrad ™).
The 5-7 µl of product of PCR de gen BMPR-IB, was mixed with 1 U AvaII restriction enzyme (Promega) and the same volume of product de PCR of gene BMP-15 was mixed with 1 U *Hinf* I restriction enzyme (Promega). Separately were mixed with 2.0 µl 10× R buffer specific for the enzyme. The digestion mix was incubated at 37°C 4 hour. The digested products were separated by electrophoresis in 3 % (w/v) agarose gel in 1X TAE. A 100-bp DNA ladder marker (Promega) was run alongside the digested products to determine the size of the bands. The gel was visualized with ethidium bromide under UV light and the RFLP patterns were documented.

Results and discussion

The forced PCR of the *FecB* gene produced a 190 pb band. After digestion with *Ava*II (Promega), according to Wilson (2001) expect to observe the following genotypes: the *FecB* gene homozygous carriers had a 160 bp band (BB), the non carrier had a 190 bp band (++), whereas heterozygotes had both 160 and 190 bp bands (B+). When all PCR products of animals under study where digested AvaII restriction enzyme, digestions resulted in 190 bp band in all the animals studied revealing the absence of this restriction site of Ava II in those animals. All samples are homozygous (++) (Fig 1). The second pair of primer for FecB gene amplified a 140 bp band. After digestion with Ava II (Promega), expect to observe the BB animals had a 110 bp band, the B+ animals had 140 pb and 110 bp bands, and the ++ animals had a 140-bp band (Wilson *et al*., 2001). Only genotype ++ (140 bp/140 bp), were detected in all samples of West African sheep (Fig 2).

The wild type allele of this gene (B+) with one restriction site resulted DNA fragments with 30 and 111 were detected for BMP-15 heterozygote shapes. Restriction enzyme did not find the mutation $FecX^G$ in the BMP-15 gene. All samples of West African sheep, were monomorphic for genotype (B+) (111 pb/ 30 pb) (Fig 3).

Recent research has shown that mutations in these genes typical of a race does not always have an effect on litter size in other races and species, for example in the race of goat Iranian, here was no evidence of mutation in $FecX^B$ and $FecX^G$ in these goats, all of which were monomorph for exon 2 BMP-15 gene Tajangookeh et al. (2009). In Egyptian Sheep Abulyazid *et al*. (2011), did not identify the mutation Booroola (FecB) in BMPR-1B gene reveals and do further study to identify the gene or genes affecting prolificacy in this breed egyptian. In the Iranian sheep Sangsari Kasiriyan *et al*. (2011), analyzed the polymorphism in BMP-15 and GDF9 genes, where most of the sheep were homozygous and only a small part were heterozygous, which means that the population is a copy of the mutant gene GDF9. However, all samples were monomorphic for the BMP-15 gene, the high prolificacy of this breed is possibly associated with other factors such as age, season, nutrition, factors that may affect levels of expression of this gene is regulated or the other major gene unknown.

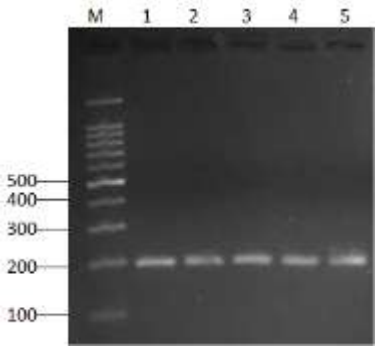

Fig 1. PCR-RFLP of BMPR-IB analysis of West African sheep Samples; M= Molecular weight marker, 1,2=product PCR of 190 pb; 3,4,5 = enzyme-treated AvaII.

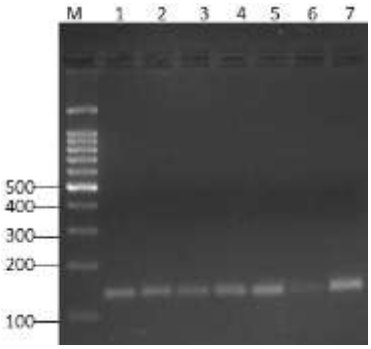

Fig 2. PCR-RFLP of BMPR-IB analysis of West African sheep samples; M= Molecular weight marker, 1,2=product PCR of 140 pb; 3,4,5,6,7 = enzyme-treated AvaII.

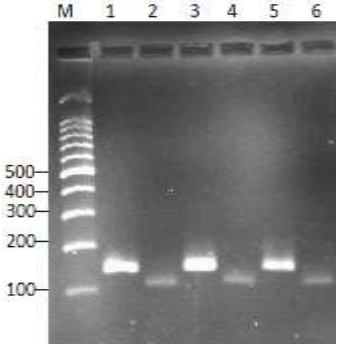

Fig 3. PCR-RFLP of BMP-15 analysis of West African sheep samples; M= Molecular weight marker,1,3,5=product PCR of 141 pb; 2,4, 6,=enzyme-treated Hinf I.

Conclusions

West African sheep lacks the FecB and FecX^{G} mutation. These mutations have positive effect on reproduction in European and Asian breeds, where there is a marked seasonality. It is possible that the absence of these mutations in this race is because they are not the same genes or mutations that regulate the mechanism of folliculogenesis in these sheep with low reproductive seasonality. So it is necessary to analyze other genes involved in the litter size and identifying new mutations in the full precursor of these two genes.

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BACILLUS THURINGIENSIS CRY1I AND CRY3A COLEOPTERAN-ACTIVE INSECTICIDAL CRYSTAL PROTEINS: HOMOLOGY-BASED 3D MODELLING AND IMPLICATIONS FOR TOXIN ACTIVITY

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Abstract: Determining the structure and function of a novel protein is a cornerstone of many aspects of modern biology. Three-dimensional (3D) models for the 79.2-kDa activated Cry1I and 77.4 kDa activated Cry3A δ-endotoxins from *Bacillus thuringiensis* native isolates that are specifically toxic to Coleopteran insect pests were constructed by homology modeling. They were structurally similar to the known structures, both derived 3D models displayed a three domain organization: the N-terminal domain (I) is a seven helix bundle, while the middle and C-terminal of anti-parallel β-sheets. Significant structural differences within domain II in this model among all Cry protein structures indicates that it is involved in recognition and binding to cell surfaces. Comparison of Coleopteran-active cry toxins predicted structure with available experimentally determined Cry structures reveals identical folds. The collective knowledge of Cry toxin structures will lead to a more critical understanding of the structural basis for receptor binding and pore formation, And will allow to appreciate the scope of diversity .Taken together, these studies provided promising evidence that domain swapping, epitope-mapping and protein-engineering under the guidance of molecular modeling can serve as a rational and useful tool in understanding the mode of action of Cry toxins, and ultimately in producing better toxins. Structural insights from these molecular modeling studies would therefore increase our understanding of the mechanic aspects of these two closely related Coleopteran-active insecticidal crystal proteins. These proteins are of interest for agriculture, as they offer a means for control of beetles and other insect crop pests.

Introduction: Currently the Cry δ-endotoxins have been shown to be highly active against a wide variety of insect larvae in the Diptera (mosquitoes and flies), Lepidoptera (moths and butterflies), Coleoptera (beetles and weevils), and Hymenoptera (wasps and bees) (Schnepf et al., 1998; de Maagd et al., 2001) orders. Insect pests that belong to the order Coleoptera are mainly tissue borers & other methods of management are generally ineffective. Their presences have been reported throughout the world and the magnitude of the problems they causes has been widespread over the past years. In general, the management strategy depends primarily on the use of highly poisonous poor graded chemical pesticides. The uses of bio-control agents in general and bacterial based Bacillus thuringiensis have been largely ignored. Applications towards non-lepidopteran insects are not as common as applications towards lepidopteran ones. Intensive screening programs are leading to a broader activity spectrum of toxins as the result of isolation and characterization of new strains with different combinations of crystal proteins, as well as the discovery of new toxins. δ-endotoxins are of great interest for development of new bioinsecticides and in the control of agriculturally important insect pests. Strains possessing novel *cry1I* genes have been evaluated as a source of new proteins with a broad host range

(Shin *et al.,* 1995; Song et al., 2003). Cry1I proteins were initially characterized by their dual activity towards Lepidoptera and Coleoptera (Tailor et al., 1992). The Cry3A toxin, produced by Bt var. *tenebrionis* (Krieg et al., 1983, Krieg et al., 1987) and other strains (Donovan et al., 1988, Lambert et al., 1992), is toxic to coleopteran species including the yellow mealworm, *Tenebrio molitor* (Krieg et al., 1983, Donovan et al., 1988), the Colorado potato beetle, *Leptinotarsa decemlineata* (Krieg et al., 1984, Lambert et al., 1992) and the cottonwood leaf beetle, *Chrysomela scripta* (Bauer 1990). The comparatively simple structure of the Cry3A toxin (Li et al., 1991), which is remarkably similar to Cry1Aa (Grochulski et al., 1995), makes it a useful model for exploring the structure and function relationship between ligand and receptor. The three-dimensional structure of the Cry toxins consists of three functional domains: (I) a cluster of seven K-helices predicted to be involved in membrane interaction (Li et al., 1991) ; (II) three antiparallel L-sheets involved in receptor binding (Li et al., 1991, Grochulski et al., 1995, Wu et al., 1996); and (III) a L-sandwich implicated in receptor binding (Lee et al., 1995 & 1999, DeMaagd et al., 1996, Burton et al., 1999) and ion channel activity (Chen et al., 1993, Wolfersberger et al., 1996, Schwartz et al., 1997) in related Cry toxins.

Comparative or homology modelling can provide a useful three-dimensional (3D) model for a protein based on its aligning to one or more proteins of known structure. The prediction process consists of fold assignment, target–template alignment, model-building and evaluation of models. The number of protein sequences that can be modelled accurately is increasing steadily because of the growth in the number and variety of experimentally determined structures and because of improvements in the modelling software (Marti-Renom**,** 2003). Homology based modelling is the most reliable method to predict the 3D structure of a protein accuracy, experimentally determined structure. Even models with errors can be useful because some aspects of function can be predicted from coarse structural features (Marti-Renom**,** 2003). To gain molecular structural knowledge of the two closely related *Bt* Coleopteran-active insecticidal crystal proteins, Cry1I and Cry3A, we generated plausible 3D models of these two toxins by homology modeling. Implications for the molecular basis of proteolytic activation, membrane-pore formation, and specificity determination of these Coleopteran-active insecticidal crystal proteins are discussed.

Materials and methods: Nucleotide and deduced amino acid sequences were analyzed with the Blast tools (www.ncbi.nlm.nih.gov/BLAST). Signal peptide sequence was analyzed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP). Related sequences obtained from databases using the software GENSCAN online tool (www.genes.mit.edu/GENSCAN.html) were used for identification of gene features such as exon and splice sites in genomic DNA. BioEdit (version 7.0.4.1) was used for sequence editing and analysis. The 3D structures was predicted using phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/), Conserved Domains and Protein Classification (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, version CDDv2.32-40526 PSSMs) and the predicted structure was validated using protein structure validation software suite (PSVS) tool. Determination of protein functional analysis obtained from databases using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and ProFunc (http://www.ebi.ac.uk/thornton-srv/databases/profunc/index.html).

Results and Discussion: Crystal structures of the active toxins have been analyzed for Cry1Aa5, Cry2A protoxin6, Cry3A7, Cry3B8, Cry1Ac9, Cry4Ba10, and Cry4A11 by X-ray diffraction crystallography and Cry1Ab17, Cry11Bb12, Cry5Aa13 and Cry5Ba by homology modelling method. These reports have shown that the toxin has three structural domains. Domain I is an α-helical bundle made up of seven α helices. Domain II is comprised of antiparallel β sheets, and Domain III is made up of a β sandwich. Cry1 toxins have been extensively used in studies of Lepidopteran insect control and have attracted less attention to their coleopteran activity either alone or in combination. A complete understanding of 3D

structures of all the coleopteran-active Cry family members is desirable for a comprehensive understanding of mechanisms underlying toxicity.

3D structure and functional prediction of Cry1I and Cry3A protein. The structure and function of a new Cry1I and Cry3A protein of native isolates of *Bt* is determined by using conserved domain database search service at the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and the protein homology/analogy recognition engine (Phyre^2) computational tool (http://www.sbg.bio.ic.ac.uk/phyre2) fig. 1 and 2. These results show that the query protein sequence indeed belongs to *Bt*. Three dimensional (3D) structure is developed with a high confidence match (>90% confidence) and descriptors of the fold and superfamily of the template used. The overall fold of the model certainly correct and the central core of the model will be tending to be accurate. Consensus prediction score a confidence value for the secondary structure predicted (0=low confidence, 9=high confidence) whether regions of the query are structurally ordered (O) or disordered (d). Such disordered regions have often been found to be involved in protein function and should be taken into account when analyzing predicted functional sites. The practical applications of protein structure prediction are varied, including improving phasing signals in crystallography, selecting sites for mutagenesis and the rational design of hybrid toxins/domain swapping (Tuli et al., 1989). A better understanding of the 3-D structure of Cry1I and Cry3A will be helpful in designing domain swapping experiments to improve its insecticidal toxicity.

The reported structural models corresponds with residues 720 (*Cry*1I) and 645 (*Cry*3A) of the primary structure of CryV (X62821) and Cry3A (U10985). Alignment of Domain I was straightforward and the highly conserved nature of helix 5 in the *Cry*1Ib9 and *Cry*3A and Cry3A toxin suggest the presence of other residues in this domain. Alignment of Domain II was also reliable. Domain III of the protein is quite well conserved at the N- and C-terminal sides. Domain I was composed of 267 (60–282) amino acid residues folded into a bundle of 9 amphipathic α-helices and two small (Data not shown). These features are highly conserved among the Cry toxins7 and have been proposed to be involved in 'pore formation' by analogy with the helical bundle pore forming structures of Colicin A toxin (Parker et al., 1989) and diphtheria toxin (Choe et al, 1992). Evidence from several studies has shown that the central helix (α5) is specifically involved in pore formation (Ahamad et al, 1990, Gazit et al, 1993, Wu et al, 1992). All the helices in the *Cry*1Ib9 model were similar to those in *Cry*V. Helix α1 probably does not play an important role in toxin activity after the protoxin has been cleaved. It is possible that mutations proposed to increase the amphiphilicity in these helices could be involved in the improvement of pore forming activity of Cry1 type toxins. Cry3A domain I was composed of N-terminal 284 amino acid residues folded into a bundle of 9 amphipathic α-helices and two small -strands (Data not shown). As with other Cry toxins, Domain II of Cry1Ib9 and Cry3A consist of three Greek key β sheets arranged in β prism topology. It comprises residues 387–487, with one helix and 11 β strands in *Cry*1Ib9 and 12 β strands in *Cry*3A. Domain II consists of three anti-parallel β sheets, each ending at exposed loop regions. These loops are thought to participate in receptor binding and hence in determining the specificity of the toxin for attachment on different insect receptors. Domain III is composed of highly conserved residues 507–644 (with reference to *Cry*1Ib9) whereas scattered variation was observed in *Cry*3A (Fig 4).

The loops (β2-β3 and β4-β5) probably interact with the receptor through both hydrophobic and electrostatic interactions. This probably helps in receptor binding by providing more mobility to glycine and other similar residues that may interact through salt bridges with the receptor. Loop β4-β5 is mostly hydrophilic and the charged residues at the tip of the loop are probably important determinants for insect specificity. Aromatic amino acids within and adjoining the vicinity of apical loops 2 and 3 of Domain II have been postulated for protein-protein, protein-ligand interactions and have been reported to interact specifically with the outer envelope of the lipid membrane. It has been proposed that these residues interact with hydrophobic lipids tails.

The exposed loop architecture has structural affinity to glycoprotein receptors of the target insect membrane. The absence of α12a and presence of additional α9b components in comparison with CryV. A few of the components α2b, α9a, β2, α10b, β3, β4, β5, β6, β7, β8, β9, β11, β17, β18 and β20 differ in locations (*Cry*1Ib9). Whereas in the case of Cry3A the absence of β3, β24 and the presence of β12a components have been noticed. A few of the components α2a, α3, α4, α5, β1a, α6, α8a, α8b, β2, β4, β5, β6, β7, β8, β9, β10, β11, β12, β13a, β13b, β14, β15, β16, β17, β18, β19, β20, β21 and β23 differ in their positions. Like all the known Cry structures, the core and domain interfaces of both model molecules are built from sequence blocks that are highly conserved motifs of all Cry toxins (Höfte and Whiteley, 1989; Crickmore *et al*., 1998).

The recognition of artefacts and errors in experimental and theoretical structures remain a problem in the field of structure modelling. Web-based software tools like PROSA have a large database and are deployed for the validation of developed models (Wiederstien et al. 2007). The software evaluates the model by parsing its coordinates and energy using a distance-based pair potential (Sippl 1990, 1995) and capturing the solvent exposed protein residues (Sippl 1990, 1995). The results are displayed in form of a Z-score and a plot of residues energy. The Z-score shows overall model quality and provides deviations from the random conformation (Sippl 1993, 1995). The plot checks whether the Z-score of the protein is within the range of similar proteins (NMR and X-ray derived structures) as in Fig. 5. The value -8.53 (*Cry*1Ib9) close to the native conformation and the overall residues energy was largely negative. The Ramachandran plot showed that most of the model residues (84.2%) have φ and ψ angles in the core regions and 11.7% are in allowed regions, except for some proline and glycine residues (0.3%) that fall in the outlier region. The value -6.24 (*Cry*3A) is close to native conformation and the overall residues energy was largely negative. Number of residues in favoured region (~98.0% expected): 594 (92.5%; Number of residues in allowed region (~2.0% expected): 37 (5.8%; Number of residues in outlier region: 11 (1.7%). The results for most bond lengths, bond angles, and torsion angles were among the expected values for a naturally folded protein (Fig. 2).

Conclusion: Evidences presented here, based on the identification of structural equivalent residues of *Cry*1Ib9 and *Cry*3A toxin through homology modelling indicate that, they share a common tridimensional structure. *Cry1I*b9 and *Cry*3A contain the most variable regions in the loops of domain II, which determine the toxin specificity. These are the first models of Coleopteran-active protein and its importance can be perceived since members of this group of toxins are potentially important candidates for pest control programs. These models will serve as a starting point for the design of domain swapping and mutagenesis experiments aimed to the improvement of toxicity, and to provide a new tool for the elucidation of the action of mechanism of these Coleopteran-active proteins. The Cry toxin models constructed by us, will be valuable for protein engineering.

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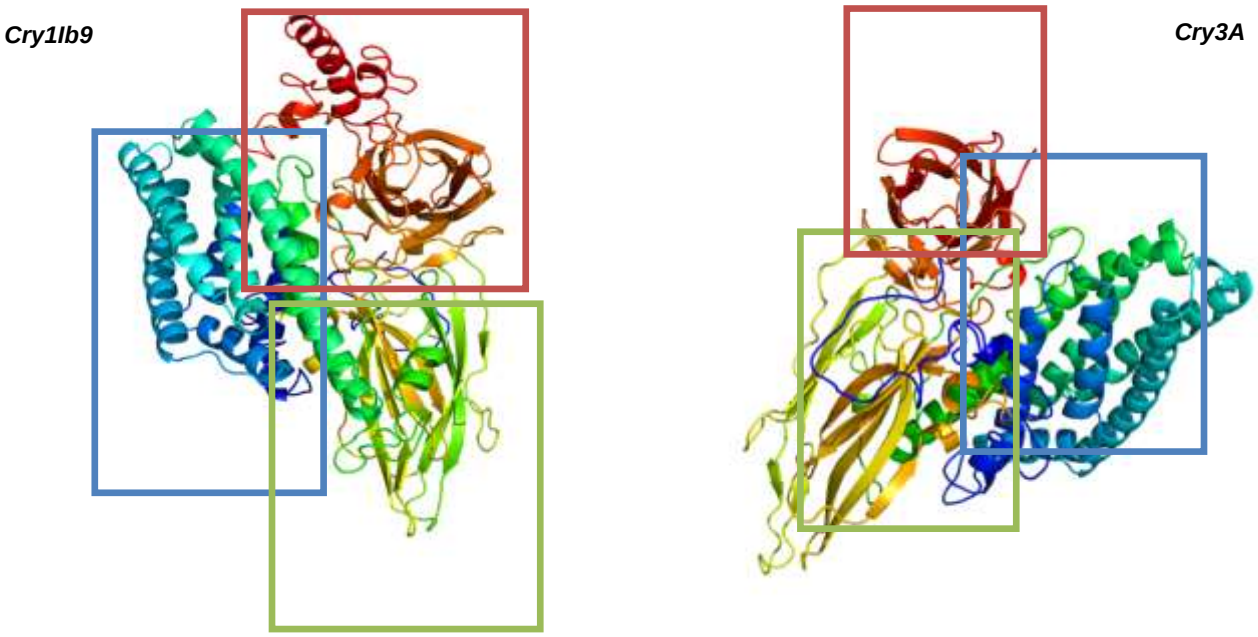

Fig. 1. Ribbon representation of the crystal structure of Cry1Ib9 and Cry3A toxins. The colored boxes denote the positions of the different domains.

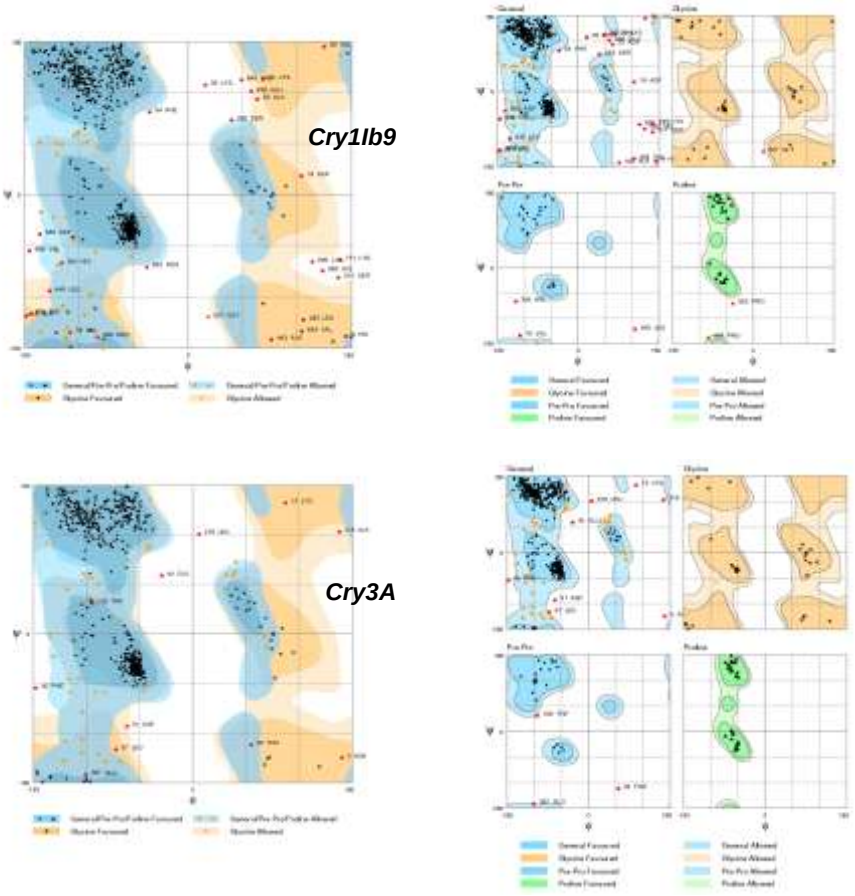

Fig. 2. Ramachandran plot analysis of the *Cry*1Ib9 and *Cry*3A toxin oligomer showing placement of residues in deduced model. The structure orientation residues are separately considered for angle and torsions. General plot statistics are: Number of residues in favoured region (~98.0% expected): 594 (92.5%; Number of residues in allowed region (~2.0% expected): 37 (5.8%; Number of residues in outlier region: 11 (1.7%).Other plots are evaluated for specific residues as showed at the top left corner of each plot.

Early stimulation on science and technology in elementary school

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Abstract:

In recent decades, Mexico has made significant progress with regard to the coverage of elementary education, which has been achieved by important advances in the education system, by the annual implementation in Mexico of the test called The National Assessment of Academic Achievement in Schools test (for its acronym in Spanish ENLACE). This test recognizes work strategies that allow the child to have better ability intellectual, social and physical. For example, nowadays, the use of the computer as a basic tool from an early age begins to stimulate the child´s mind as it activates his imagination and attention span; for this reason it is important to basically outline the material resources available to the School. Activities that can be made through the Information and Communication Technology (TIC´s) in education are, in general, motivating to the students for their playful character, by the use of visuals, colors and three dimensional figures and hearing, among other advantages. We used National Institute of Statistics, Geography and Informatics for its acronym in Spanish (INEGI) data to make a situation analysis of social, educational and technology issues available on the Mexican population. Early stimulation is a set of actions to promote the physical, mental and social aspects of any child, and even helps preventing child psychomotor retardation, cure and rehabilitates motor impairment, sensory deficits, intellectual disabilities, and language disorders. This supports the integration of these children to society, replacing the burden of feeling useless for the joy of becoming a useful individual and reducing feelings of aggression, indifference or rejection, and stimulating solidarity, cooperation and hope. Educational backwardness and poverty are the main factors that exist in the rural states, which affect the child population, and leads to obtain a high index of infant mortality.

Keywords: TIC´s ● childhood ● improvement ● achievement.

Introduction:

For years, researchers have studied and contributed to the knowledge of children's learning, we briefly mention those we consider the most important. Maria Montessori 1870-1952. The system Montessori separates the difficulties that arise in the learning of literacy so that children can move forward from simple to complex. It also continues the process of each student individually and will successively isolate specific difficulties at every stage you can ask. William Fritz Jean Piaget 1896-1980, creator of the genetic epistemology and famous for his contributions in the field of genetic psychology, for his studies on children and his theory of cognitive development. Piaget defined a sequence of 4 epistemological stages including sensorimotor, preoperational, the concrete operational and formal operational. (lberto Munari. 1994). Edouard Claparede (1873-1940) Swiss psychologist and educator, after university studies in Switzerland, Germany and France, he returned to his hometown, where he began his educational career at the University of Geneva. In his theories, he related pedagogy with child psychology. He took ideas and concepts to apply psychology to pedagogy, and proposed that teachers learn to observe their students and work and investigate from these observations. His study of the development of childhood intelligence found continuity in the work of Jean Piaget. (Daniel Hameline. 1993). Skinner and his theories of behaviorism were the basis for programmed instruction, and these in turn, served to design the first computer education systems currently used in children, such as clapping when they give a correct answer and put a cross through or sound "booo" when

there is a mistake. (Louis M. Smit. 1994.) Effects of early simulation: Burton Withe published in 1975 a book entitled: First Three years of life, which states that parents are teachers, not just creators of babies. Glen Doman is a pioneer in working with children with brain disorders, and early development in healthy children. He is known worldwide for promoting early literacy through "cards" or "flash cards". He said that every child has shown a better attitude than adults because the child recognizes that learning is a means of survival. Benjamin Spock studied about children over intellectualized, with emphasis on that over achievement may actually hamper emotional, physical, or creative aspects of development. (IAHVideos. 2011.)

Material y methods: We used in this study a data base from National Institute of Statistics, Geography and Informatics (INEGI) of Mexico City about child population, infant mortality, educational backwardness and rural locations, and causes of death, including total general deaths of infants under one year by main causes of mortality, and natality percentage, natality. Literature search was conducted at the web page http://www.google.com.

Results and discussion: The consensus of population and housing unit of measurement for thousands of inhabitants in 2010 registered a value of 112,337.00. 7.

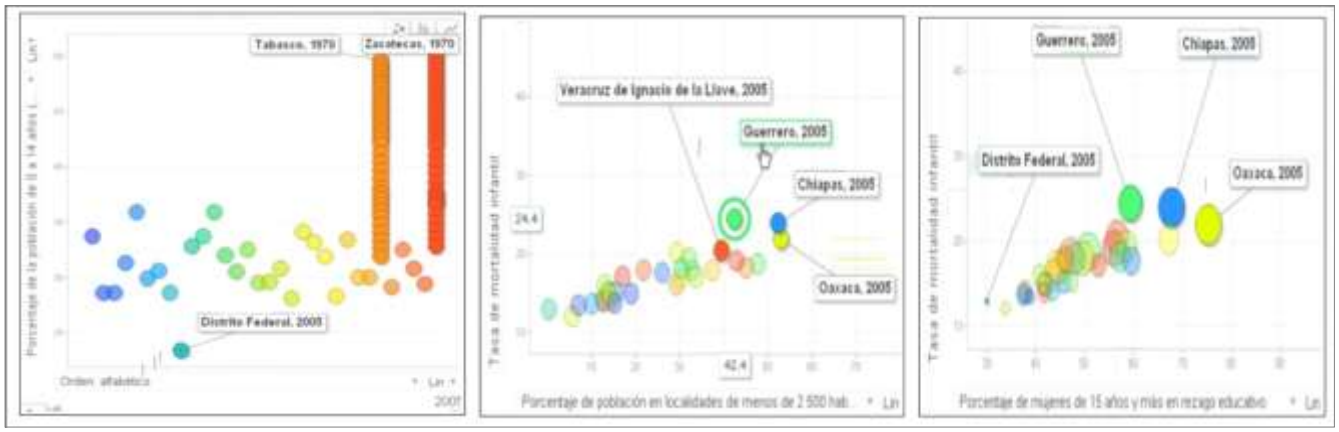

Figure 1**. A.** Child population in 1970. **B.** Infant mortality in 1990. **C.** Educational backwardness and rural locations.

Child Population: As you can observe in Figure No. 1 A considering the percentage of the population aged 0 to 14 years in 1940 Tabasco is the entity that registered the highest value with 48.6% of its population in this age range, while the record of Federal District is 34.5%, lower with all entities in an increasing trend was observed up to 1970 years, in which the maximum value in all of them emphasizing Tabasco, Zacatecas and the highest values of 50.6 and 49.7% respectively. **Infant mortality:** Figure No. 1 B To compare the states for 1990 the mortality rate and percentage of population in rural areas considering these as less than 2500 inhabitants, there is a direct relationship between both indicators to percentil higher rates of rural population are more high infant mortality being greater the difference in extreme values of each of these indicators, to display the changing values in the entities we see a general decrease in mortality of children under one year or less marked decreases in the percentage rural population since the 1990-2005 period. **Educational Backwardness and rural locations:** Figure No. 1 C The percentage of women over 14 who have not completed the 9 grades of compulsory basic education is expressed and localized mainly in the states with the highest proportion of people in rural areas. In recent decades the gradual depletion of the educational lag kept a close relation to the decline in mortality rates of children under one year. The schooling offers women a new larger social network, reference groups and greater identification with the modern world of health institutions, educational gap reduction between women could be related to an increased interest in health services. than is possible with greater approval preventive behaviors and new remedies which contributes to increased survival of less than one year. Reducing the

educational gap between women could be related to an increased interest in health services, allowing greater approval of preventive behaviors and new medicines also contributing to increased survival of children under one year. (http://www.youtube.com/watch?v=RntBb2m-Tks&f accessed 28 february 2012)

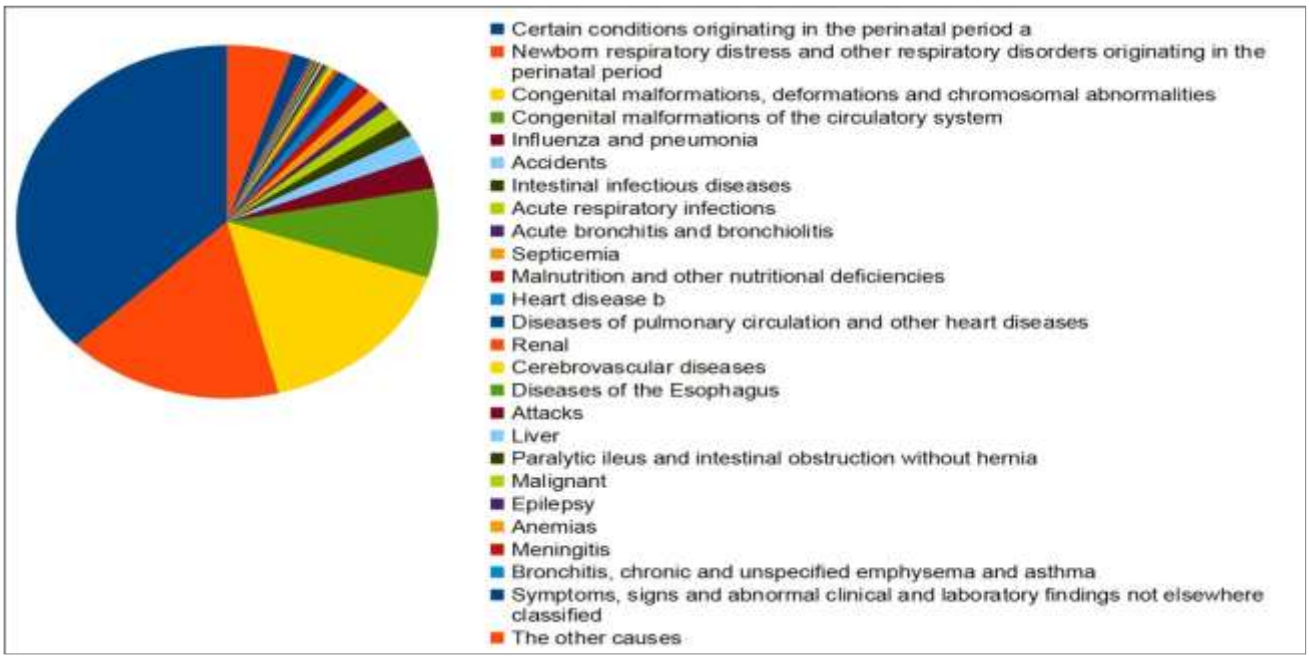

Figure 2. Mortality Statistics

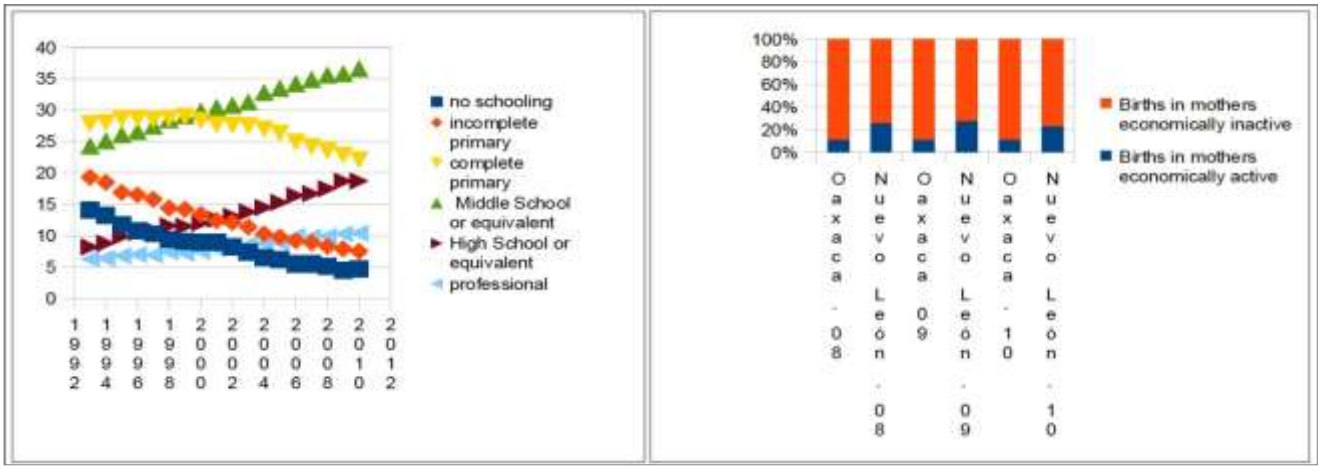

Figure 3. Natality Percentage distribution of births by mother's education, from 1993 to 2010

(Source: INEGI. Birth Statistics)

Figure 4. Natality: Percentage distribution of births according to economic activity status of the mother for each state, 2008, 2009 & 2010 (Source: INEGI. Birth Statistics)

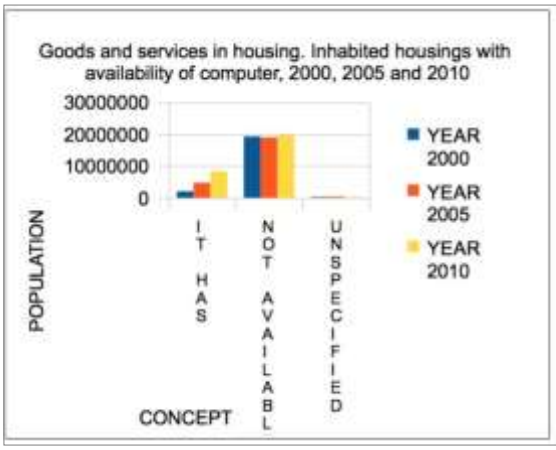

Figure 5 Availability of computer

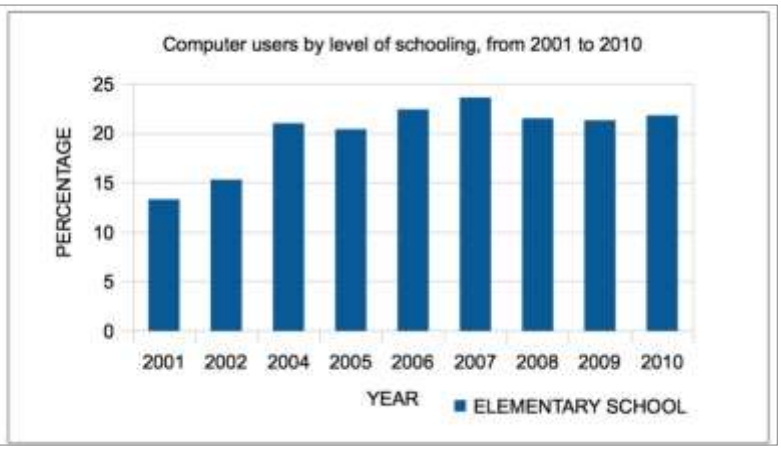

Figure 6 Computer use in elementary school

Information and Communication Technologies (TIC´s):

Households with computer (as a proportion of total households) in 2008 was 25.7 in 2009 was 26.8 and in 2010 was 29.8. INEGI. Censos de Población y Vivienda, 2010.

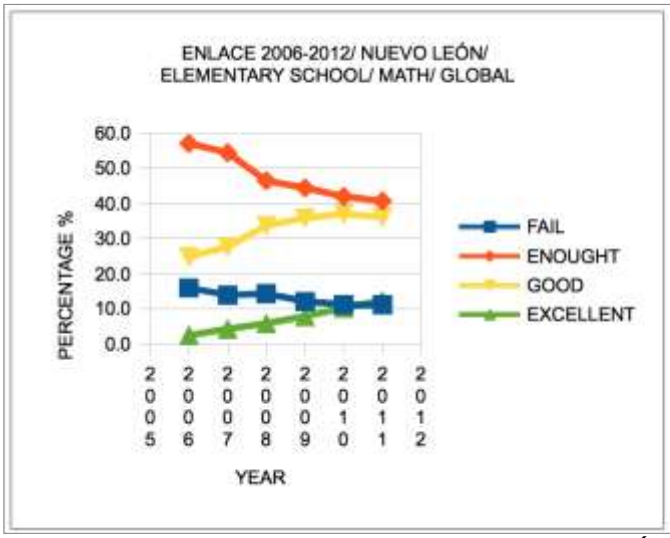

Figure 7. ENLACE 2006/2011 NUEVO LEÓN

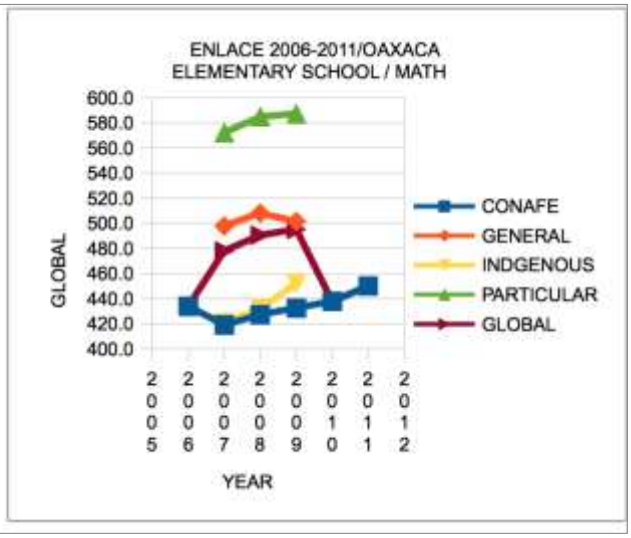

Figure 8. ENLACE 2006/2011 OAXACA

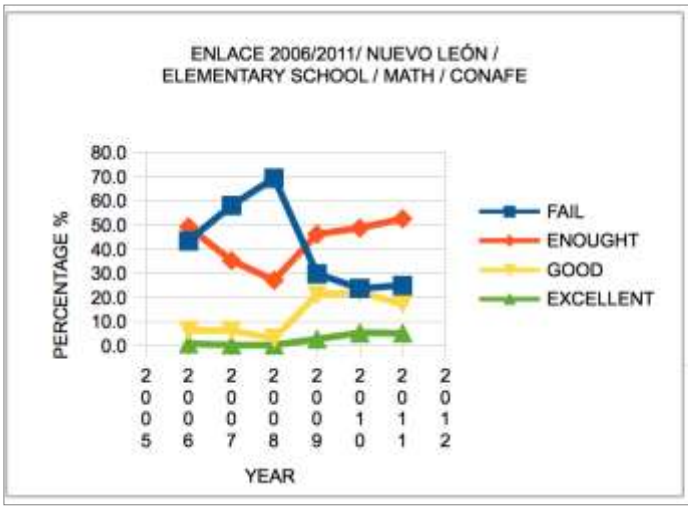

Figure 9. ENLACE 2006/2011 NUEVO LEON.

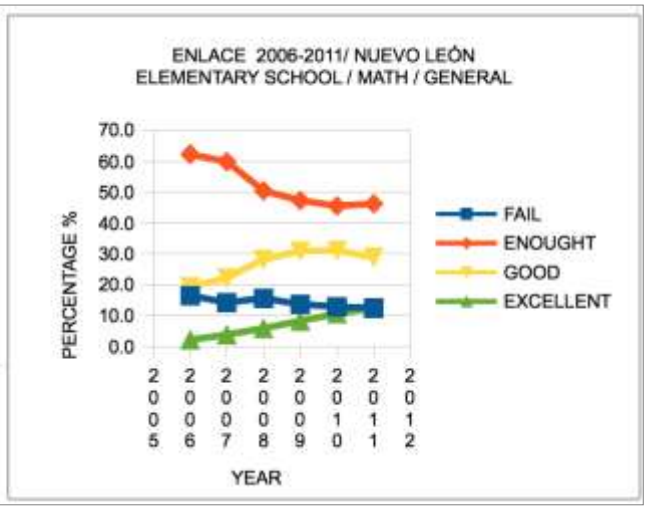

Figure 10. ENLACE 2006/2011 NUEVO LEÓN

Conclusion:

Early stimulation is a set of actions to promote the physical, mental and social aspects of any child, and even helps preventing child psychomotor retardation, cure and rehabilitates motor impairment, sensory deficits, intellectual disabilities, and language disorders. This supports the integration of these children to society, replacing the burden of feeling useless for the joy of becoming a useful individual and reducing feeling of aggression, indifference or rejection, and stimulating solidarity, cooperation and hope. Educational backwardness and poverty are the main factors that exist in the rural states, which affect the child population, and lead to obtain a high index of infant mortality.

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INNOVATION IN BIOTECHNOLOGY AND THE SOCIOECONOMIC IMPACT OF BIOTECHNOLOGY RESEARCH

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Abstract: In this presentation I will discussed how biotechnology has shown to be a disruptive innovation, which has generated new products and services, has displaced products and producers already in the market, has created immense wealth and many new companies have emerged. To illustrate the disruptive nature of biotechnology three areas of application will be analyzed:

-Human healfth: bioterapeutics, biogenerics and biosimilars
-Agriculture: transgenic plants
-Energy: biofuels (ethanol and biodiesel)

Also it will be discussed how the socioeconomic impacts of its applications have been distributed unequally among industrialized countries and developing countries, and the trends that are emerging of food versus energy or food versus industry (bioplastics and new biomaterials) will be described.
The innovation process that biotechnology follows in industrialized countries (open innovation) will be compared with the process that is used in most developing countries (lineal model), and Mexico will be reviewed in more detail:

-Present situation of academia/research
-Negative impacts of biotechnology (loss of markets and producers; increase of bioproducts importation)
-Development of new bioproducts

A new way to approach innovation related to biotechnology for developing countries will be presented, based on the following model:
where: = innovation, = ideas, = resources, risk and rewards.
Some examples for Mexico and Latin America described; also some recommendations about future international collaboration will be done.

Introduction

Biotechnology has shown in the last 30 years (the first recombinant product, human insulin reached the market in 1982), that it has all the characteristics of a disruptive technology (Christensen 1997): it has created new products and services, has displaced products and producers already in the market, has generated immense wealth and many new firms have emerged. To illustrate its disruptive nature, the following areas of application will be reviewed:

- Human health. Until 2010, the number of biopharmaceuticals on the market was just over 200 products, with the global market value of 99 billion dollars, 61 billion corresponding to recombinant therapeutic proteins, and 61 billion to mAb-based products. From 2006 to 2010, 58 biopharmaceutical gained approval in the European Union and/or the United

States, 25 of them were genuinely new biopharmaceuticals, and 28 products were biosimilars, reformulated or me-too versions of previously approved substances. Additionally, 5 of the approved for the first time in one region had previously been approved in a different region before 2006 (Walsh 2010).

- Agriculture. Transgenic plants were introduced in 1996, and in 2010 reached 148 million hectares of biotech crops (mainly corn, soybean, cotton, canola and potato), in 10 industrialized countries and 19 developing countries (James 2011). According to our estimates by 2015 global hectares will exceed 184 million hectors and developing countries will have almost 42% of them.
- Energy. Biofuels have been developed in several countries as a response to the climate change due to increasing concentration of GHG in the atmosphere. The main biofuels are ethanol (from corn in USA and sugarcane in Brazil) and biodiesel (from soybean oil). In 2010 total amount of ethanol produced was 101.4 billion liters and 59.6 billion liters of biodiesel, but it is expected that by 2015 it will have increase more than 63% (Hart 2010).

There are many more examples of new or improved products and services based on biotechnology, and every day we know of more incremental innovations in different sectors (health, food, environment, biomaterials, etc). Another characteristic of the innovation in biotechnology has been that its large socioeconomic impacts have not been distributed equality among countries and regions. Now, it is clear that some countries are winners because they have received more benefits than the rest, the most outstanding example is United States, followed by some European countries and few of the so called emerging countries.

In developing countries the situation is different, today there are more bioproducts and bioservices, most of them are imported and very few are produced endogenously and even a smaller number is based on technology developed by them. Other countries have been impacted in a different way, because they used to be producers and exporters of a particular product, v. gr. animal insulin in Argentina, or sugar in Caribbean countries, and now they have to import bioproducts to cover their own needs.

For the future some new trends are emerging, bioproducts of large volume and medium price are being developed and produced but, they required plenty of agricultural biomass as the main raw material. This demand has created a competition for land, water and other resources; in fact the controversy is food vs biofuels and more recently food vs industry (bioplastics and new biomaterials). There are already examples of this situation in USA (corn starch for ethanol), Brazil (sugar for ethanol and bioplastics), Argentina (biodiesel from soybean oil), etc. So, the questions that we would like to ask and try to answer in this document are why this is occurring and how it can be changed.

Bio-innovation in industrialized countries

There are several models that try to explain how innovation works out in these countries, the innovation process is divided in two stages (Figure 1) the first one, covering mostly the research part and the second involves business aspects. But, also it has been widely reported (Afuah 2008) that there are fractures which are considered to be essential to have and efficient and successful innovation system: government plans and programs to support and promote

biotechnology development; a set of resources to finance ideas, projects both in academia and firms; laws to protect intellectual property rights and adequate climate and regulations for bio-business.

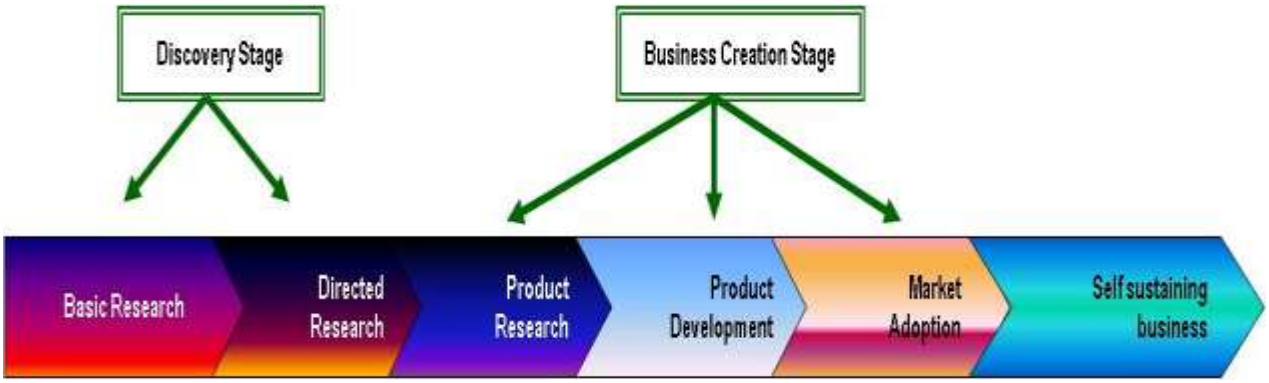

Figure 1. Classical innovation process model.

USA, which is undoubtedly the leader in biotechnology innovation, has even established specific systems for particular areas of application v. gr. health, biofuels and environment. New procedures and schemes of collaboration between universities and private sector and government laws have been key elements and now the open innovation process is been accepted widely (Chesbrough 2003). In this process (Figure 2), the flow of ideas is the opposite to the traditional process, companies collaborate with different groups, from academia, of different countries or with other companies, seeking new ideas, products in different stages of development, with this process it is expected that the efficiency of generating innovation will increase dramatically.

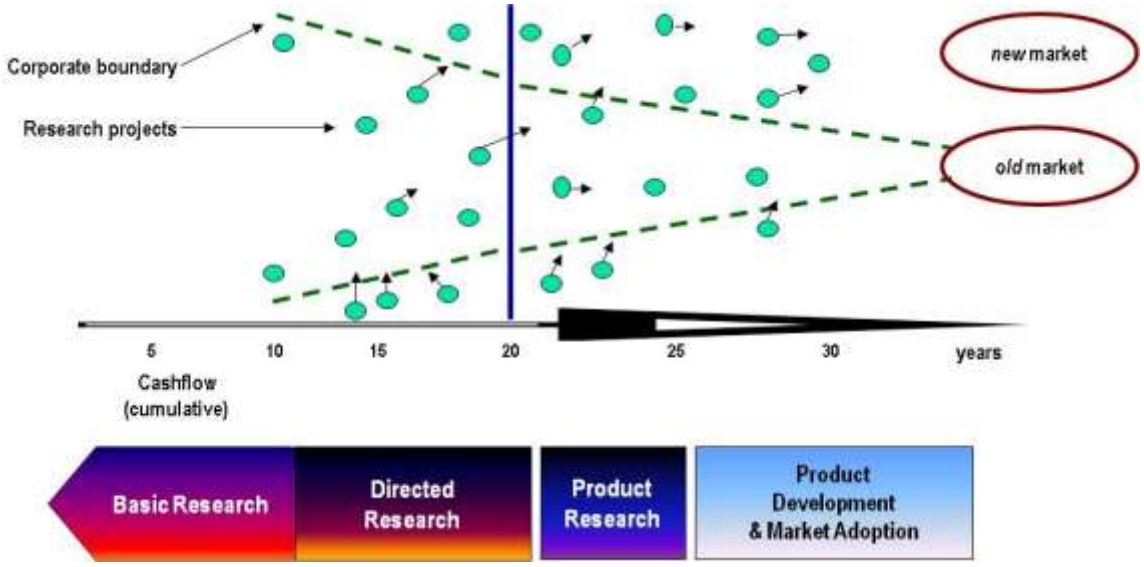

Figure 2. Open innovation process.

Bio-Innovation in the developing countries: The case of Mexico

In order to analyze what has happened in the developing countries, we have chosen Mexico as a good case of how bio-innovation has had socioeconomic impacts in development of the country. Fortunately biotechnology development has been followed and there have been several publications covering the last 25 years (Quintero 1985, Bolivar 2001, Quintero 2007, Trejo 2010) that are summarized as follows:

- Present situation of academia research: biotechnology is considered one of the most developed research areas, there are more than 2000 full time academic researchers in about 150 institutions, some of very high level, several projects have received national and international recognition, and there is already a strong education system from undergraduate to Ph. D. level.
- Negative Impacts of biotechnology: traditional biotechnology (aminoacids, antibiotics, organic acids and vaccines) have been the losers in the bioindustry, large firms have closed their productions facilities and it is recognized that the main causes have been price increase of raw materials (sugar cane) and the displacement by new and better biotechnology in order parts of the world (Quintero, González 2008).
- Development of new bioproducts: new biotechnology is only carried out in few Mexican companies; there are two biogeneric producers and recently a pharmaceutical lab started producing and exporting a new anti scorpion serum to USA and Africa. In the agricultural sector most of the effort is concentrated in plant tissue culture and transgenic plants (corn resistant to insects) and are not allowed to be cultivated and there is a very strong opposition for the commercialization of these crops. In the environment sector, mostly bioremediation, several projects form academic research have been scaled up and even exported to USA and other Latin American countries, the best examples are anaerobic

waste water treatment systems (integrated with biogas utilization), by infiltration of contaminated air and bioremediation of soils after oil spills.

Mexico has to certain extent already established the previously mentioned requirements to support biotechnology. What is lacking is more participation and interest of the private sector.

A proposal

A new approach to innovation related to biotechnology for developing countries is required; our proposal is based on the following simple model:

$$I = f(i, r_n)$$

Where I = innovating

i = ideas

r_n = resources, risk, rewards

- With regard to ideas is needed to have a more profound knowledge of the economic potential of research projects, its value not only in terms of new knowledge but also how it might impact social and economically our society, in order to do this, the educational system will have to be changed incorporating new ways of looking and assessing research.
- Financial resources to support projects moving into pilot plant scale or demonstration facility must be set up and the time to allocate funds should be reduced. Also the integration of teams is important, they should be multidisciplinary, accepting members coming from different institutions, but efficiency is a concern because there is little experience in developing projects with this kind of characteristics.
- The concept and culture of considering risk in research and business is lacking in most of the innovation system, again educational change is needed. During a certain period it should be accepted that there is no previous experience in evaluating risk and new procedures should be tried and tested.
- Rewards. It is a conflicting matter but also fundamentals for any innovation system to be successful (Scotchmer 2004) institutions of different type: federal, local, private or public, should recognize and share the benefits of innovation setting regulations that are clear and accepted, right now there is already happening in a few institutions, mostly universities.

Finally we think that networking should be emphasized in this effort, through formal and informal activities in order to construct new capacities, share facilities, exchange experiences between researchers, entrepreneurs, public servants, non-governmental organizations, international organization, etc. etc.

For international collaboration our recommendation is that the emphasis must change, the priorities should be set up first, then the research team should be assembled and a continuous evaluation system established. As an example of this procedure, we propose the following projects:

- *Latin-American aim*. A project to produce transgenic seeds for important crops (corn, potato, sugar cane, coffee, rice, etc.) should involve the international agricultural centers located in the region (CIMMYT, CIP, CIAT, CATIE), as well as the best universities oriented towards agricultural biotechnology, and a new firm to cover the Latin American market should be founded.
- *Regional aim*. For Central American countries, a project to diversify the products based on sugar cane, through biotechnology, could bring wealth and job creation. The technology already exists and what is needed is a market approach.

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CLONING EMBRYOS: APPLICATION TO WILDLIFE SPECIES AS ENDANGERED BIGHORN SHEEP

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La clonación a través de sus técnicas, es una de las biotecnologías más relevantes debido a sus múltiples aplicaciones tanto a nivel de la investigación científica básica como la aplicada en diversas áreas tales como la conservación de especies animales en peligro de extinción. Sin embargo, para comprender lo que es la clonación, primero necesitamos definirla.

Embryo cloning techniques are some of the most important ways to get animal embryos production and have many applications at basic scientific research as well at applied in many areas such as endangered animal species conservation programs.

A nivel de biotecnología, la clonación incluye algunas técnicas que permiten obtener individuos que posean la misma carga genética lo cual asegura que en ellos se expresarán aquellas características previamente seleccionadas, por ejemplo, la clonación por transferencia nuclear de animales altamente productivos (de leche, carne, lana, piel, etc.) asegura que las características deseables se expresarán en toda la población de individuos clonados. Gracias a esta técnica se obtuvo a la borrega “Dolly”, como uno de los ejemplos más conocidos.

As a biotechnology, cloning includes some techniques that allow obtaining individuals charring genetic charge which ensures the expression of previously selected characteristics, for example, cloning of highly productive animals (milk, meat, wool, skin, etc.) by means of nuclear transfer, ensures that, desirable characteristics will be expressed in the whole population of cloned individuals. Thanks to this technique it was possible to clone Dolly the ewe, as one of the most well known examples.

La transferencia nuclear consiste en retirar el núcleo de una célula somática embrionaria, fetal, juvenil o adulta (carioplastos que por lo general son fibroblastos) para luego introducirlo en un ovocito enucleado al que se le llama citoplasto o receptor de núcleo. Otra forma es fusionando la célula somática completa con un ovocito enucleado. A la célula así reconstruida, se le dan estímulos químicos, físicos o biológicos mediante los cuales se activará como si el ovocito hubiera sido fertilizado por un espermatozoide y entonces llevará a cabo las divisiones mitóticas propias de un embrión, hasta desarrollarse en un blastocisto que podrá ser transferido a una hembra receptora (subrogada) para que lo geste. Así nacerá un individuo genéticamente “idéntico” a aquel del que se obtuvo el núcleo.

Nuclear transfer implies withdrawal of the nucleus of an embryo, fetal, young or adult somatic cell (karyoplasts commonly fibroblasts) to introduce it into an enucleated oocyte (cytoplasts) which receives it. Also means the cell-cell fusion, where the fibroblast is electrically fused with the enucleated oocyte. Once reconstructed these cells, they are located into an electrical

chamber which fuses them and converts them into a zygote that will be mitotically divided to form a preimplantational embryo known as blastocyst. At this level, the embryo can be transferred into a surrogated female in which their development will end; giving birth to an individual genetically identical to that from it was obtained the nucleus or the fibroblast (karyoplast).

Para enuclear al ovocito se espera a que éste haya alcanzado la Metafase II (MII), es decir, que haya madurado ya sea *in vivo* (en el ovario) o *in vitro* (en un medio de cultivo). La enucleación se hace introduciendo una pipeta de micromanipulación que succione tanto al primer cuerpo polar (liberado del citoplasma del ovocito durante la MII y que queda en el espacio perivitelino del mismo), como al huso metafásico con sus cromosomas maternos asociados que sale junto con aproximadamente 10% del citoplasma del ovocito. El ovocito enucleado (citoplasto) recupera la apertura efectuada a su citoplasma y zona pelúcida, y está listo para recibir el núcleo o carioplasto. La ausencia de señal de ADN en el citoplasto se confirma mediante una tinción con fluorocromo específico para ADN visualizado bajo luz ultravioleta, sin embargo permanece el ADN mitocondrial.

To enucleate an oocyte this must have reached the Metaphase II (MII), this means that must have maturated *in vivo* (within the ovary) or *in vitro* (in a culture media). Enucleation occurs when a micromanipulation pipette is introduced into the oocyte to withdraw the first polar body and the metaphase spindle with its maternal chromosomes associated and 10% of the ooplasm. The enucleated oocyte (cytoplast) recovers and it is ready to receive the nucleus or karyoplast. The absence of DNA in the cytoplast is confirmed using a DNA specific fluorocrome visualized under ultraviolet light, yet, mitochondrial DNA remains.

En la partición de embriones lo que se hace es partirlos con una micro aguja o micro navaja, proceso que se efectúa en las etapas más tempranas del desarrollo embrionario (mórula o blastocisto) con la intención de obtener individuos a partir de la mitad o la cuarta parte del número de células presentes en el embrión original. Las mitades o cuartos así obtenidos usualmente se colocan en zonas pelúcidas (cubiertas externas de los embriones) vacías y se transfieren a hembras receptoras hasta el término de su desarrollo, así nacerán individuos idénticos entre sí, dando lugar a gemelos o cuádruples monocigóticos. De ahí que los gemelos son en realidad clones. Entre los animales se conoce que el armadillo de nueve bandas también llamado mulita grande, tatú o toche (*Dasipus novemcinctus*) y el armadillo de siete bandas, mulita pequeño o tatú-eté (*Dasipus septemcinctus*) se reproducen de manera natural como cuádruples monocigóticos.

A second way for embryo cloning is embryo splitting, in which an embryo normally developed *in vivo* (inside the female reproductive tract) or *in vitro* (into a culture media) to the mórula or blastocyst stages, is splitted into two or more pieces using a microneedle or a razor blade, making possible to divide the embryo in pieces, each one of them capable to develop as a single embryo. Each piece is then introduced into an empty pellucid zone (the external membrane of an oocyte-embryo) and transferred into a surrogate female to end the embryo development, so, it will be born identical individuals as monozygotic twins or monozygotic quadruplets. Then, twins are indeed, clones. Among animals there are two species that reproduce themselves this latter way, the nine rings armadillo or big mulita or tatù or toche (*Dasipus novemcinctus*) and the seven rings armadillo or small mulita or tatù-etè (*Dasipus septemcinctus*).

Esta técnica consiste en tomar un embrión en etapa temprana de su desarrollo (previo a la etapa llamada de compactación) y separar por métodos físicos o químicos cada una de sus células ó blastómeros. Dado que cada blastómero es totipotencial hasta un determinado punto

de su desarrollo (varía según la especie pero en general hasta la etapa de ocho células), es decir que contiene toda la información genética necesaria para formar un nuevo individuo, se cultivan *in vivo* o *in vitro,* ya sea aislados o en pares de blastómeros, hasta que se desarrollen en blastocistos que serán transferidos a hembras receptoras. Al igual que con la partición de embriones, los individuos así clonados serán gemelos idénticos. En los mamíferos (entre ellos el ser humano) existe la teoría de que los gemelos que se producen de manera natural, son el resultado de la separación de los blastómeros de embriones en etapa de desarrollo de dos células y cada uno genera un organismo independiente.

Finally, there is a third way for embryo cloning, blastomere separation. This involves the physical or chemical separation of one or more blastomeres (cells that compose a preimplantational embryo) of an embryo in a development stage previous to the compacted morulae. As each blastomere is totipotential (depending on the specie in general until the 8 cells stage), that is that contents all the genetical information needed to form a new complete individual, it is *in vivo* or *in vitro* cultured, isolated or in couples of blastomeres, until they develop as blastocysts that will be transferred into surrogated mothers. As in embryo splitting, cloned individuals will be identical twins. In mammals (among them human beings) there is a theory that explains twins as the result of the natural separation of blastomeres of a two cell stage embryo.

El hecho de que estas técnicas imiten procesos naturales nos demuestra que la clonación de embriones no es algo “sobrenatural” como se presupone, sino que el ser humano ha tenido la capacidad de imitar fenómenos biológicos existentes mucho antes de su descubrimiento.

The fact that these techniques imitate natural processes shows that embryo cloning is not something over natural as is presupposed, but that human being is capable to imitate existent biological phenomena.

La clonación de embriones tiene diversas aplicaciones, tales como:

- La salud.
- La terapia celular.
- La investigación científica.
- El comercio.
- La reproducción asistida.
- La conservación de especies de fauna silvestre en peligro de extinción o aún las ya extintas.
- La reproducción de mascotas.

Embryo cloning has several applications, such as:

- Health.
- Cellular therapy.
- Scientific research.
- Commerce.
- Assisted reproduction.
- Conservation of endangered wild animal species or even those already extinct.
- Pet reproduction.

En conclusión, la clonación de embriones con cada una de las técnicas que involucra, abre un horizonte de posibilidades muy grande y prometedor; pero el cómo, cuando y por qué utilizarla, requiere consciencia, responsabilidad, ética, principios y valores humanos.

In conclusion, embryo cloning with each one of the involved techniques, opens a huge, promising horizon of possibilities; but how, when and why use it, requires consciousness, responsibility, ethics, human principles and values.

MARINE BIOTECHNOLOGY. OPPORTUNITIES AND CHALLENGES FOR MEXICO´S XXI CENTURY

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Abstract: Continental México is ca 2 x 10^6 square kilometers, while Marine México is ca 3 x 10^6 sq km. Meaning that Marine México is two thirds of the total Country. Besides the size, Marine México is formed by a variety of disparate ecological niches. From estuarine waters, almost sweet water, to salt marshes with 6M NaCl; From two atmospheres to several hundreds; From subtropical or deep waters, to several hundreds °C in black hydrothermal vents at 3000 m deep in the Guaymas basin. All this causing specialized adaptations in organisms thriving in such environments. Leading to diverse metabolisms to be explored for biochemical mechanisms of production and transformation to become biotechnologies. Here we will define Biotechnology and how business based on biotechnologies is assessed and why basic research is needed to succeed and how success must be aimed; intellectual leadership. Also Biotechnology will be analyzed as a multidisciplinary science, what are the objectives of the science along with the tools of Biotechnology. Biotechnology is technology based on organisms, so organisms are quintessential in this paper. Benefits of exploring marine organism are plentiful. Like generating and attracting novel, clean, leading-edge technology industries to the Country; Jobs for the wellbeing of Mexicans. Increasing opportunities to keep Mexican- and abroad-trained professionals of science at the region. Sustain and restore both land and marine habitats. Potential products are: food for humans and feed for land and aquatic animals; Food and feed ingredients and supplements; pharmaceuticals, cosmetics, fertilizers and all sort of biological reagents, from enzymes to pigments. Of course there are potential benefits, as well as challenges. As long as we can keep a balance between them, México can achieve a better future based on science and biotechnologies.

Introduction: As we all know, the world's oceans comprise the biggest part of the biosphere and contain the most ancient and diverse forms of life. Only with the tools of modern science can these biological resources be studied in detail and thereafter be applied for human benefit and fundamental scientific progress. Reports have shown that the sea's resources remain largely unexplored and marine organisms represent a vast untapped resource with potential benefits in many different areas of life, including medicine, aquafarming and fisheries, industry, research tools and environmental applications; including new techniques to restore, protect and manage marine ecosystems. To bring together scientists, managers, faculties, decision makers among all organizations engaged in marine biotechnology and encourage the development of commercial marine biotechnology.

Marine biotechnology is a rapidly growing sector that encompasses a wide range of disciplines to develop novel or alternative products or services from the marine environment. Potential applications include new medicines, human health products and chemicals for use in the manufacturing and food industries.

A definition: There are plenty definitions for Biotechnology, Here we have a functional one: “Biotechnology is the use of biochemical processing methods in productive activities” It is also called “Biological engineering” To fulfill its goal, Biotechnology is science as much as it is technology. The science part is aimed at understanding nature, while the technology part is aimed at providing useful control of nature. Marine Biotechnology is the use of biochemical processing methods of marine organisms in productive activities. Marine Biotechnology uses molecular based biological processes; make a good use of the cellular machinery.

Biotechnology is a multidisciplinary science. Its “Formal object” is the study of biological processes, while the “Material object” is the use of biological processes in technologies. In this order of ideas, Biotechnology is achieved in successive steps. The most fundamental is to study the basic phenomena in living things to know what processes or products happen at organismal, tissular or cellular levels, this is called “The biological feasibility”. When a process has been found and

understood at certain grade, this is to know the most factors affecting the process possible in order to aspire to control the process in vitro we call the step of study "The Technical feasibility" in this step, scaling up happens and evaluation of inputs and output in vitro are done. When the process is reproduced systematically and the outputs are well known, the process can come to a commercial scale and the process or product is ready for commercialization, this "The Economic feasibility" of the process.

Because living systems cannot be exactly modeled or predicted, genuinely new products must be created, at least in part, by experimental research, that must be based on knowledge, creativity, and rigorous and systematic investigation, the hallmarks of good science in any context. This is why only the subtle knowledge of the phenomena processing processes will allow sustainable applications. It is known that some, for not to say most biotechnologies high-profile failures are recognized as being due to companies pushing poor science in order to achieve funding goals.

Because Biotechnology is technologies derived from the deep understanding of biological phenomena and the use of the derived knowledge in technologies, it is important defining what criteria must be embraced to succeed in biotechnology enterprises. Biotechnology is a business and to be a business and to attract the interest of investors and users, so, it has to end in economic success. However Biotechnology is a particular kind of business; Economic success is only one part of the whole part of the business. A Biotechnology based company or NGO needs to succeed intellectually, knowledge of the biological phenomena supporting the technology is the capital of the company; Intellectual leadership. And intellectual leadership is not the same as company success. Success has to be defined based in intellectual leadership, competitive advantage, creating long-run activities (sustainable companies), and generating intellectual capital. Intellectual leadership can only be possible on knowledge, creativity, and rigorous and systematic investigation, the hallmarks of good science in any context.

Tools of Biotechnology. Because Biotechnology is the study and application of biological phenomena, all disciplines of knowledge are eventually involved. Paramount are Biochemistry and its branches of study, like Enzymology and Enzyme technology, Genetic and protein engineering, Industrial microbiology, Cell and tissue culture.

A brief story of Marine Biotechnology: Biotechnology is not a typical science derived from a Mother science, like Biochemistry that evolved from Microbiology at late XIX Century. Instead, Biotechnology was born as a concept meaning the use of several sciences, namely Biochemical Engineering, Biochemistry, Cell Biology, and later Molecular Sciences. For late 1970's and early 1980's the U.S.A. and Australia started looking to get a head-start in the activity. For 1989, Japan had 19 companies, like MITI and Marine Bio-technology Institutes. The first International Marine Biotechnology Conference was held in 1989 in Tokyo (IMBC'89), and the Japanese Biotechnology Society formalized activities. That was the year of the publishing of Journal of Marine Biotechnology. For the 1990's a new journal appeared, Molecular Marine Biology and Biotechnology. For 1994 The European Society for Marine Biotechnology (ESMB) started activities and in 1995, The Asian-Pacific Marine Biotechnology Society. In 1999 Pan American Marine Biotechnology Association was founded in Halifax, Canada. Eventually Journal of Marine Biotechnology and Molecular Marine Biology and Biotechnology joined efforts becoming in Marine biotechnology, a journal by Springer publishers. It is the official journal of the The European Society for Marine Biotechnology (http://www.esmb.org/) and The Japanese Society for Marine Biotechnology (http://wwwsoc.nii.ac.jp/jsmb/index.html). The "International Marine Biotechnology Conference" has met nine times. 1st IMBC89 was held in Tokyo, Japan; 2nd IMBC91 in Baltimore, USA; 3rd IMBC94 in TromsØ, Norway; 4th IMBC97 in several cities of South Italy; 5th IMBC2000 in Sydney, Australia; 6th IMBC2003 in Japan, 7th IMBC2005 in St. John's, Newfoundland and Labrador, Canada, 8th IMBC2007 in Eilat, Israel, and IMBC2010 in Qingdao, China. Other regular meetings are happening. The Asia-Pacific Marine Biotechnology is in its 9th conference to be held in Kochi, Japan. Indicating the importance the Science-technology has become worldwide.

Mexico must invest capital and human resources to investigate biological resources; they can be eventually used in disparate disciplines to those originally intended. Like the example of *Xiphophorus maculatus*, an endemic fish that, so far, is better studied abroad than in México. It is even an Institute in San Marcos, Texas: "The Xiphophorus Genetic Stock Center" (It is sponsored by the National Ocean Service, NIH National Center for Research Resources and Roy F. and Joanne Cole Mitte Foundation). The interest on the fish is based on its genetic physiology; it became a model to study human oncogenes. Same may happen for any other organism, the better we know them, the best we can use them. It is deep basic research than can show potential biotechnological uses. Domestic researchers must invest human and economic capital in biological marine processes for several reasons; to know what there is, descriptive science, how it came to be, evolution, how it is, biochemistry and molecular genetic, and how biological processes can be used, biotechnology.

Marine Biotechnology includes:
1. Marine bioproducts, making emphasis on drugs, bioadhesives, biominerals, and plenty other beneficial and economically important products. Based on the development of new models and screening for product discovery, the identification of new drug targets and mechanisms-of-action of marine-derived drugs, the development of sustainable production methods for marine bioproducts and the development of novel methods for marine by-products utilization.
2. Aquaculture and Marine animal health, focused on marine pathogens, diagnostics, treatments, drug delivery systems, and immunology, physiology, and pharmacology of both wild and cultured marine animals, cell and molecular techniques to improve size, growth rate, disease resistance, survivability, and reproductive yields of aquafarmed organisms.
3. Coastal human health risks, focused on the development of new diagnostic tools to assess seafood pathogens and water-borne pathogens and pollutants like red tide ravages.
4. Coastal habitat restoration, focused on remediation, and molecular and cellular approaches to strain improvement, hybrid development, and production technology for submerged and coastal aquatic vegetation.
5- Forensics and monitoring, focused on bioforensics for identification of threatened and endangered species, seafood identification, evaluation of health risks, and regulatory issues as related to economic fraud, and the development of new biosensors.

Biotechnology for the benefit of fisheries: One of the most renowned marine economical activities is fishery. Some countries base it annual income on harvesting and processing marine products. When comparing México with countries with similar annual harvest volumes, like Canada and Spain, 1.4 x 10^6 ton, México yield 600 x 10^6 USD, while Spain makes 3000 x 10^6 USD and Canada 6000 x 10^6 USD. Difference based on care and handling of catches and on processing. México sells for domestic and foreign markets scarcely processed products, raw materials. One example would be shrimp that is mostly exported to better income countries. Shrimp is processed by low-grade technology like beheading and freezing, while it is possible to improve revenues by exporting fresh product that demands for a different technology, even better, to export live product. Asian markets demand and pay suitable prices for it.

Biotechnology may transform waste products into by-products. What traditionally is considered wastes from fisheries, like heads, skin, guts, bones gonads and crustacean carapaces that are discarded contributing to environment tragedies, biotechnologies may make use of them making profit out of garbage. Lipids, mostly omega fatty acids, pigments, antioxidants, enzymes, biominerals, pharmaceuticals can be obtained by bio-processing processes and be value-added products.

Also, under exploited or non-commercial species may be seen as valued catches if devoted to industrialization. Low value fish, like the many found in Mexican waters can be processed. An example may be production of surimi, a muscle derived product used as ingredient for producing food. Surimi is a much-enjoyed food product in many Asian cultures and is available in many shapes, forms, and textures, and often used to mimic the texture and color of the meat of lobster, crab and other shellfish. Surimi quality is improved by Enzyme technology called cold binding, using a transglutaminase to improve gelling capacity.

Use, management and preservation of organisms and molecules (biological parts) for and by Biotechnology: Biotechnology can and is making use of organisms belonging to the three main domains of Biology. Archaea, Bacteria and Eukarya, including animals and plants, and Viruses. Biotechnology looks in all kind of biology structures, Biome (Biota), Genome, Transcriptome, Spliceosome, Proteome including Enzymome, Metabolome and Interactome. Even is in the task of building genetically modified organisms and brand new organisms, synthetic life and Bio-bricks. The creation of a bacterial cell controlled by a chemically synthetized genome by the Craig Venter group is only the tip of the iceberg.

Transformation is the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surrounding and taken up through the cell membrane(s). Like *Agrobacterium sp.* That is used to transfer genetic material to plants and Lentiviruses used to transfer genes to animal cells. Marine viruses are under the lens of microscope to look for auto-assembling structures to produce nanoparticles to deliver pharmaceutical.

Patenting of life or life parts is an issue in Biotechnology. Ethical, economical and even political aspects must be considered. Countries don't agree yet on who must own rights on life and life parts. There is those, mostly companies, considering patent genomes and organisms, while, on the other end, mostly academicians who think patenting will affect the development of future science.

Finally, Bioinformatics, Biotechnology is a comparative science. It takes advantages of better known organisms and biological processes to build hypothesis on less known ones. Advances in genomics are only possible based on robust and potent informatics systems.

THE NEED OF BIOTECHNOLOGY TO COUNTER-BIOTERRORISM

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Abstract: Bioterrorism (BT) is the threat of release and/or intentional dissemination of, contagious and/or non-contagious Biological agents (Ba) with the purpose to harm and cause terror to civil populations, and introduce animal or crop diseases. A BT attack can result in deaths of susceptible population. Furthermore, panic among populations can overcome entire health services, vaccines and therapeutics stockpiles. An efficient surveillance network of diseases and major Ba -listed as BT agents-, and stockpiles or rapid production platforms of proper vaccines and therapeutics are required to counter-bioterrorism. Prophylactic molecules such as recombinant antigenic proteins for subunit vaccines, monoclonal antibodies, adjuvants and immunomodulator molecules can be produced by the biotechnology industry in a rapid, cost-effective, scalable and safety manner. Platforms to produce these biotech-products include green plants, algae and virus-insect systems. Because in principle, the nature of a Ba used during a BT attack is unknown, counter-bioterrorism actions should count on biotechnology in order to be prepared and to rapidly manufacture therapeutic molecules for the affected population.

Keywords: ▪bioterrorism ▪biotechnology ▪recombinant proteins ▪surveillance network.

Introduction: Bioterrorism (BT) is the threat of release and/or intentional dissemination of Biological agents (Ba), contagious (viruses, bacteria, fungi, protozoa) and/or non-contagious (toxins derived from living organism such as botulin toxin, snake venom and saxitoxin), with the purpose to harm and cause terror to civil populations. BT attacks can affect crops or livestock as well, causing major economic problems and food crisis (Rebert, 2006; Foxwell, 1999; Dudley and Woodford, 2002). The main purpose of BT events is to scare governments and societies for the profit of particular ideological causes. Ba has been used as bioweapons throughout history of humankind. Typically, they were used on human populations. An example was the Tatars' hurling of plague victims' bodies over the wall of the Crimean city of Kaffa in 1346, which probably helped spread the Black Death, caused by the gram-negative bacterium *Yersinia pestis* (Wheelis, 2002; Armstrong, 2010). Although there are documented reports regarding the use of Ba as bioweapons during war, concern about BT attacks grew up since the 9/11 terrorist attacks in the USA and the shortly after mailing letters-containing anthrax spores. The envelopes containing a highly virulent formulation of anthrax spores resulted in less than 22 cases of clinical anthrax and 5 deaths and, as important as this, the attack provoked an enormous fear, panic, disruption of the mail service, distrust in the US government's ability to protect its citizens and hundreds of millions of dollars in remediation expenses (Lindler et al, 2005). So, the lesson learned was that bioterrorist attacks go farther than one expect.

The Centers for Disease Control and Prevention (CDC) in USA recognize three categories for the highest priority Ba. Of these, all are human and zoonotic pathogens (www.cdc.gov). Then, surveillance of Ba is the main task in counter-bioterrorism (notably, leaving livestock, poultry, fishes, crops and biodiversity richness aside). For counter-bioterrorism is mandatory to have proper vaccines and therapeutics supplies. Here is where problems begin because, as we have seen during epidemic outbreaks, supplies can be insufficient to cover all the susceptible population.

Biotechnology industry has shown its capacity to produce therapeutic molecules and vaccines in a rapid, cost-effective, scalable and safety manner (Arzola et al, 2011; Phoolcharoen et al., 2011; Mena and Kamen, 2011; Mett et al., 2011; Hempel et al., 2011).

Bioterrorism agents

Biologic agent refers to any living organism or substance produced by an living organism that can be used as a weapon to cause harm to humans, like viruses, bacteria, and toxins (Swearengen, 2006). So the organisms that can be used as biological weapons are countless. Nevertheless, CDC in USA identifies high-priority agents based on their ease of dissemination or transmission, potential impact on major public health, the potential for public panic and social disruption, and how ready public health systems are to cope. Table 1 summarizes these bioterrorism agents/diseases in which category A refers to the most high-priority agents. They require special action for public health preparedness because its ease of dissemination or transmission from person to person, they may result in high mortality rates and have the potential for major public health impact, and might cause public panic and social disruption (www.cdc.gov). Between them, anthrax and botulism toxin have caused special concern because they can be quickly inhaled upon release and its ease of production and transport (Jain et al., 2011). Another high risk Ba is variola major virus because its stability, high mortality and airborne transmission (Hoffmann et al., 2002). Legitimately, stock of this virus exists only in two laboratories in the world: one in the Centers for Disease Control and Prevention in Atlanta, Georgia, USA, and the other in the State Research Centre of Virology and Biotechnology in Novosibirsk, Russia (Bossi et al., 2006). So, any new case of smallpox would be caused by an accidental or intentional delivery. Because prophylaxis campaigns for smallpox finished in the late 1970s and previous vaccination against this Ba does not confer lifelong immunity, concerns about the possible use of the virus as a weapon of BT have increased in recent years (Pennington, 2003; Hoffmann et al., 2002).

Strangely, there is little attention to other bioterrorist agents such as fungi, which are less selective Ba. Infection and disease with fungi or fungal toxins can affect equally to humans and animals through inhalation, ingestion, and contact with skin and mucous membranes. Also, bioterrorist attacks can target crops which can be fatal when a group of people is dependent on a single crop as its staple food supply. The tragic potato blight in Western Europe during 1845 to 1848 was caused by *Phytophthora infestans* fungus. It resulted in the deaths of 1 million people (Klassen-Fischer, 2006). Trichothecenes, fumonisins, ergot alkaloids and aflatoxins are some of the highly dangerous mycotoxins that can be delivery by aerosol. The major threat of fungi as Ba relies in its toxicity and the nonspecific or delayed symptoms they produce. So it is difficult to identify the sources of disease outbreaks. Furthermore, fungal contamination may have serious adverse economic and health effects.

Biotechnology to counter-bioterrorism

Therapeutic and prophylactic molecules such as recombinant antigenic proteins for subunit vaccines, monoclonal antibodies, adjuvants and immunomodulator molecules can be produced by the biotechnology industry in a rapid, cost-effective, scalable and safety manner (Brower, 2003; Mett et al, 2011; Marzi et al, 2011; Hempel and Mena, 2011; Kamen et al, 2011; Phoolcharoen et al, 2011). Platforms to produce these biotech-products include bacterial suspensions, cultures of insect, yeast or mammalian cells, virus-insect systems, green plants and algae. Because in principle, the nature of a Ba used during a BT attack is unknown, counter-bioterrorism demand cost-efficient and scalable manufacturing technologies that can rapidly deliver effective therapeutics into the clinical setting.

Advances in biotechnology have positioned plant-made pharmaceuticals as a viable option in the market. Advantages of using this platform includes cost-effectiveness, scalability, safety (for production of human therapeutics, because plants do not harbor or propagate human pathogens), rapid time frames for the production of gram quantities of new proteins, production of large and complex proteins with correct processing and assembly into multi-subunit complexes with human-like glycosylation patterns (Rigano 2009; Mett et al., 2011; Arzola et al., 2011; Phoolcharoen et al., 2011). An example is the plant-produced antibody pp-mAb^{PANG} targeted to the protective antigen of anthrax using transient expression using agroinfiltration of *Nicotiana benthamiana* (Hull et al., 2005; Mett et al, 2011). Actual scheme of vaccination with the current licensed anthrax vaccine, AVA (Anthrax Vaccine Adsorbed), requires repeated administrations prior to the predicted exposure to anthrax spores (Mett

et al, 2011). However, a single dose pp-mAb^{PANG} resulted in protection against an aerosolized *B. anthracis* spore challenge in mice and macaque monkeys. This could be useful in both prophylactic treatment of individuals who could potentially be exposed to anthrax and therapeutic treatment of individuals already infected with anthrax.

Table 1. Bioterrorism agents/diseases by category*

Category A	Category B	Category C

• Anthrax (*Bacillus anthracis*)
• Botulism (*Clostridium botulinum* toxin)
• Plague (*Yersinia pestis*)
• Smallpox (variola major)
• Tularemia (*Francisella tularensis*)
• Viral hemorrhagic fevers
  ○ Filoviruses [Ebola and Marburg]
  ○ Arenaviruses [Lassa, Machupo])
	• Brucellosis (*Brucella* sp.)
	• Epsilon toxin of *Clostridium perfringens*
	• Food safety threats:
	  ○ *Salmonella* sp.
	  ○ *Escherichia coli* O157:H7
	  ○ *Shigella*
	• Glanders (*Burkholderia mallei*)
	• Melioidosis (*Burkholderia pseudomallei*)
	• Psittacosis (*Chlamydia psittaci*)
	• Q fever (*Coxiella burnetii*)
	• Ricin toxin from *Ricinus communis*
	• *Staphylococcal* enterotoxin B
	• Typhus fever (*Rickettsia prowazekii*)
	• Viral encephalitis (alphaviruses [e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis])
	• Water safety threats:
	  ○ *Vibrio cholera*
	  ○ *Cryptosporidium parvum*
		• Nipah virus
		• Hantavirus

*According to CDC, USA.

In another work, Phoolcharoen and co-workers developed an Ebola immune complex (EIC) in *Nicotiana benthamiana*. Mice immunized with EIC showed production of anti-Ebola virus antibody. Their results showed potential use for the plant-expressed EIC as a human vaccine (Phoolcharoen et al., 2011).

Also, algae are proposed as high-efficient biofactories for pharmaceutical molecules. Hempel and co-workers showed that *Phaeodactylum tricornutum* diatom produce higher amounts highly-specific HBsAg antibody than the current yeast system (Hempel et al., 2011). HBsAg is used as the approved vaccine for Hepatitis B, so microalgae seems as very promising systems to produce therapeutic molecules because of their feasibility, low cost and fast growth rates without the risk of human pathogenic contaminations.

Finally, the baculovirus-insect expression system is a well known approved system for the production of viral antigens with vaccine potential for humans and animals and has been used for production of subunit vaccines against parasitic diseases as well (Aucoin et al., 2010; van Oers, 2006). There are various vaccines currently in the market produced by this platform. Baculovirus-insect systems improve production of recombinant proteins in short periods of time at low costs. Moreover, final products are safe because baculovirus only infect insects. This makes baculovirus-insect system a good candidate to respond during possible BT attacks.

Conclusions: Bioterrorist agents/diseases are major cause of concern. Most biological agents considered as candidates for weaponization are not cause of national public health problems. So, in

case of BT attacks, most of the world's institutes of health are unprepared and have limited capacity to deal with a mass casualty. Surveillance is considered the most important public health instrument for identifying public health events of global concern (Kman and Bachmann, 2012). However, counter-bioterrorist measures can't count only in a good surveillance network because during a BT attack (as during an epidemic outbreak) casualties and social panic can overwhelm health services. Moreover, BT attacks can produce losing of livestock, poultry and entire crop fields, which can cause famine and great economical looses. Biotechnology has offered multiple platforms to produce therapeutic molecules as well as vaccines for different pathogen/diseases. These biotech-systems for rapid, low-cost and scalable production of pharmaceutical molecules can help before, during and after a BT attack.

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INNOVATIVE INVENTIONS AND PATENTS

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Innovations deeply impact not only areas related to medicine, engineering, biotechnology, physical sciences, and information technology, but also, education, business and social sciences, some of which stimulate research and development, commercialization, and technology transfer derived from products improvement, creation of novel products or disruptive technologies. It is well known that investing in research and development are more likely to compete or take leadership in areas of opportunity. The parties involved include universities, companies and research centers. Innovations provide the advantage of creating new products or services that give vitality to those involved; this encourages more competitive advantage. However, innovation is considered not a general feature of all individuals, but rather an elitist aspect among individuals with a talent for seeing beyond the picture presented to them daily, and who have the vision of prosperity through the implementation of changes in their environment and transformation of matter or energy; nevertheless, there exists systematic innovation through TRIZ which can be thought and used for those who want or are required to innovate and that does not require a special talent or mental gift. If innovation is not protected, it is at risk of being duplicated or plagiarized and this would lead to losing any right to it. The intellectual property protection in the form of patents, utility models and copyrights is a legal tool for the inventor that entitles him (her) to define the way of exploiting it.

According to the Mexican Institute of Industrial Property (IMPI), in 2009, Mexico requested 14.281 patents, of which only 822 were Mexican, the rest were foreigners who mainly work in Mexican companies. Compared with the U.S., for example, Mexico usually records between 500 and 800 patents a year, while IBM and Sony in that country in one year generated more than 4000 and 1400 patents, respectively. This patent apathy is multifactorial; there are cultural and political factors that have prevented our country advancing in this area as other countries do. Many Mexican inventors believe that if they disclose their inventions they can be plagiarized, and those who trust in the authorities often lack the financial capacity to cover the costs of patenting and prefer to "give away" his invention and "know-how" to the highest business bidder.

These expenses include filing the patent to IMPI, the cost of international protection by the so-called PCT (Patent Cooperation Treaty), national phase payments in those countries of interest, payment to get support for market search and commercialization of the invention or for business incubation which includes preparing business plans, market research, pre-commercial evaluation, field research and marketing, among others. The current yearly cost of a patent in Mexico is about 900 dollars and including the 20-year maintenance spending of the patent it results in about 3000 dollars; if PCT is needed, then the inventor must also cover an expenditure of about 5000 dollars plus the costs of national phase in countries where protection is required. For example, if you want protection in the U.S., the sole registration cost is about 10 thousand dollars, in France would be about 300 dollars, and 120 dollars in Spain and including the 20 annuity cost it would sum to a total of 6000 dollars. If the inventor has the economic capacity, he can face the little interest shown by companies in acquiring his invention, because companies prefer to resolve their specific technology needs to license a technology.

Then, patenting is not just an inventive problem because Mexicans have it. Some of Mexican inventions have greatly contributed to improving the quality of life of many societies. In addition to having invented the "zero" (Mayan culture) and the ball game (Aztecs), we invented the color television, the flying belt, the tridilose, the first tortilla making machine, the translucent concrete, the first oral contraceptive. Hector Garcia Molina, Mexican professor at the University of Stanford, directed the doctoral thesis of Larry Page and Sergey Brin, Google co-founders; and Armando Fernandez redesigned the PC mousepad for commercial use. All of this has been given in a political and social system that is not very encouraging for the development of inventions and innovations and where only 0.5 of the gross internal product (PIB) is engaged to scientific research.

The traditional university model where research spending only results in publications, must adapt to global competitiveness and become a university where in addition to generating publications, patents are produced and licensed to companies or used in companies incubated in the university, with the consequent creation of value, using the innovations embodied in intellectual assets. Nowadays, researchers are evaluated for their production of scientific publications, and recently, by their generation of patents and products licensed. Royalties generated by this process are distributed among the university, laboratories and inventors. Laboratories where the inventors work receive this incentive to promote new research or to continue with the projects that originated the patented inventions. The university researcher or inventor also has the opportunity to create and incubate his own company with the products of their patented inventions/innovations.

Inventions are not useful if they are of no use to others, while the useful ones become innovations. An innovation differs from an invention in that the former can be produced and marketed, and so the society can use it to its advantage to improve its quality of life. Continuous innovation enables companies to constantly adapt to changing conditions resulting from globalization; this makes companies stronger, more competitive and highly cost-efficiency. For an invention to be patentable it must be new, the result of an inventive step and susceptible to industrial applicability for economic development.

Once the Center for Business Incubation and Technology Transfer (CIETT), center of patenting at Universidad Autónoma de Nuevo León (UANL), receives a technology or invention for protection and business transfer, we proceed to evaluate its relevance in terms of originality, usefulness, not obviousness to experts, and market potential. If the proposal fails this stage it may be considered for abandonment or amendment to obtain a utility model, and restart the process. The approval of this phase involves filling out forms for which the inventors receive our advice and personalized support. When the documents are ready, they are sent to the IMPI for initial assessment and filing of the patent.

A center such as the CIETT is very strategic because it can meet academic, research and business demands to encourage these sectors to promote projects and initiatives that allow the generation of value through:

1. Advice in the process of protecting intellectual property through trademarks, patents, utility models, industrial designs, plant variety rights and copyright.
2. Negotiations for patent licensing, copyrights and technological assistance.
3. Advice on business incubation and entrepreneurship.
4. Advice on product marketing, and
5. Offering of courses and workshops on intellectual property and marketing.

This improves the identity of companies based on trademarks and licensing negotiation through contracts or agreements, increases the market value of companies by protecting and encouraging the use of innovations to increase competitiveness, promotes and enhances access to funding sources and acquisition of venture capital, and limits or prevents the activity of plagiarism or imitation of products and services of a company. With the acculturation on processes of intellectual property from a business perspective, we use the patents and trademarks arising from technological or pre-commercial developments as a source of information for the exploitation of intellectual property generated at UANL by industrial licensing and business formation.

The registration of the first patent to UANL was granted in July of 1991 to Dr. Alfredo Piñeyro Lopez for the isolation of anthracene compounds from the plant *Karwinskia humboldtiana* with antitumor activity. Since then and particularly since 2002, when the first Center of Patenting at UANL (Technology Transfer Center) was founded, and to date, through the CIETT, there has been a significant increase in the number of patent applications generated by university researchers, thanks to the initiative and drive of the former Presidents Dr. Reyes Tamez Guerra, Dr. Luis Galán Wong, Ing. José Antonio González Treviño and currently Dr. Jesús Ancer Rodríguez. To February 2012, UANL possesses 79 patent applications, 19 granted patents, 277 copyright materials, 40 intermediate technology business incubators and 29 high technology business incubators.

The UANL uses the assets of its intellectual property as a tool for leading growth to increase

the number of patents and to promote technology transfer in a solid and efficient manner, making our approach with our Intellectual Property Protection attractive to inventors and other universities. Patents are a key tool for protecting technologies and play a key role in the process of knowledge transfer, as facilitators of the exchange between universities and industry. Scientists contribute to these technologies in an indirect way, by expanding the science basis, and directly by producing industrially applicable patented innovations. Patents of university inventions and discoveries are essential as a basis to start companies. These companies create jobs and profits for inventors and their research units. The process of obtaining, patent, promoting, and licensing a patent to the industry is a highly effective way to develop a lasting interaction between universities and businesses, including increased opportunities to support research and employment to university researchers.

The present moment requires the productive sector of our society to develop innovative capabilities that allow it to compete in the global market, supported by rising productivity, derived from a scientific, technological and intellectual property system and a strong and continuously developing university-business interaction. Given this reality, and limited to investing in research and development, the productive sector is in the need for reliable sources of knowledge and innovations that respond to current and future problems, to improve its competitiveness and increase its viability in the global market.

The primary objective of CIETT is to ensure that technologies emanating from UANL are incorporated into the market for the benefit of society. Efficient and effective transfer of university research results to the productive sector of society, turning them into new products and services, is vital to stimulate economic growth and job creation.

CIETT supports academics in protecting the intellectual heritage of our institution and transferring technology to the productive sector of society with the consequent creation of value that contributes to the greatness of the university and its academic and scientific programs supporting the substantial objectives of the Vision 2020.

The transfer of technology is defined as "the exchange of research results, *know-how* and *expertise* between the Institutions of Higher Education and the Productive Sector leading to the commercialization of new products and processes". UANL has been linked to various companies through the transfer of some of our technologies, which is expected to progressively increase in the coming years in order to validate the usefulness of university and industrial intellectual assets in the productive sector of our society. Thus we have for example, the technology "a modified antigen for detection of antibodies to *Brucella* and method of use", which has been licensed to the National Program of Veterinary Biologics (PRONABIVE); the technology "genetically modified methylotrophic yeasts for the production and secretion of hormone human Growth (HGH) ", which was licensed to the company Probiomed; and the software "Nutris", which has been distributed for sale to the Mexican Social Security Institute (IMSS) and private clinics and nutrition center in Mexico.

Universities have individuals with higher education and training, and ideas that can be converted into innovative products and processes generating value (assets). Although the primary mission of universities is to create, preserve and disseminate knowledge, they can and must contribute to the processes of industrial development through innovations generated in their units and research centers and in areas such as Health, Engineering, Biology and Biotechnology, Chemistry, Physics, Agriculture and Agrobiotechnology, Nutrition and Environment, among others. From these niches arise investigations that have the potential to be commercialized. Universities contribute to the formation of human capital at undergraduate and graduate levels, spread the knowledge generated through the publication of scientific articles and patents, and conferences, and produce specialized software.

In addition, they support the community through social service programs or internships, provide consultants for creating technology-based firms through entrepreneurship programs and business incubators, research projects with industry and transfer technology by licensing to the industry. It is well recognized that research and innovation, technological development and business engagement with the marketing of innovative products, converge on an ideal of corporate economic competitiveness, through university assets (innovations), to contribute to a more sustainable world and improve the quality of life of our Society.

NUTRIGENETICS AND NUTRIGENOMICS: IMPLICATIONS IN TYPE 2 DIABETES

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Nutritional genomics is a broad term that includes the nutrigenomics which study the role of nutrients in gene expression, and nutrigenetics that is the study of how genetic variants can affect responses to nutrients (Castle, 2008). This field provides powerful approaches to decipher the complex relationship between bioactive molecules, genetic variation and biological system and can develop personalized nutrition and dietary recommendations. The data and high-throughput molecular technologies generated through the international genomic projects are generating an unprecedented opportunity to facilitate the understanding of diet-related diseases (Morine, 2008). The impact of nutrients can be evaluated through a multitude of 'omic' technologies and biomarkers (Fenech, 2011).

In monogenic diseases, the diet can influence phenotypic expression and nutrigenomics will improve the prevention or treatment through early identification of specific variants that modulate dietary response in affected subjects. In complex diseases including obesity, the metabolic syndrome (MetS), type 2 diabetes *mellitus* (T2DM), the nutrigenomics approach has begun to reveal that are susceptible to dietary intervention and may modulate the onset and progression of disorders (Gorduza, 2008). Developing individual risk factors acknowledging the genetic diversity of human population, food complexity, culture and lifestyle, and the variety of metabolic processes that lead to health or disease is a significant challenge for personalizing dietary advice for healthy individuals as well as ones with chronic disease like T2DM (Kaput, 2008); nutrigenetics and nutrigenomics enable individuals to obtain better nutritional advice, as well as genetic subgroups, but it is often difficult due to the complexity of the area, to appreciate their relevance of preventive approaches for optimising health, delaying onset of disease and diminishing its severity (Ferguson, 2008; Fenech, 2011).

There is a significative familiar aggregation in T2DM and related phenotypes, among them are obesity, hypertension and dislypidemia. The aggregation of metabolic characteristics is probably due to genetic effects that interact with unique life style/environmental shared factors. The metabolic syndrome (MetS) is a very common condition that often precedes T2DM (Isomaa, 2001). It is characterized by abdominal obesity, insulin resistance, dyslipidaemia, hypertension and a chronic sub-acute proinflammatory state that modifies insulin signalling (Ghanim, 2004; Roche, 2005). The high family aggregation suggests the importance of screening MetS, especially in early onset families (Li, 2006). Recently genetic studies show genomic regions associated to metabolic adverse effects; close to 100 of variations associated to metabolic characteristics, among which reduction of beta cell function, obesity, increase of lipid and glucose levels. These findings are transforming the view of how these features develop and interact for the development of T2DM (De Silva, Frayling, 2010).

Fatty acids are central metabolic regulators that may interact with genetic factors and influence glucose metabolism (Cuda, 2011). It has been reported several interactions between genotypes and MetS risk. For example, the citokines LTA, TNF-alpha, IL-6 and plasma fatty acid level modulate risk for the MetS and its components (Phillips, 2010). On the other hand, long-chain acyl CoA synthetase 1 (ACSL1) play an important role in fatty acid metabolism and triacylglycerol (TAG) synthesis. Alteration of these pathways may result in dyslipidemia and insulin resistance, major characteristics of the MetS. Dietary fat is a fundamental environmental factor that may interact with genetic determinants of lipid metabolism to impact MetS risk, ACSL1 genetic variation modify MetS risk, most likely via changes in fatty acid metabolism, which was modulated by dietary fat consumption, particularly PUFA (polyunsaturated fatty acids) intake, suggesting novel gene-nutrient interactions (Phillips, 2010).

Other genes previously associated to T2DM have been reported to interact with dietary fat. For example, the PPAR-gamma variant genotype is responsive to different types and levels of lipids (Ferguson, 2008), transcription factor 7-like 2 (*TCF7L2*) is the strongest genetic determinant of T2DM and insulin-related phenotypes to date. The allele rs7903146 influences MetS risk, which is augmented by both gender and dietary SFA intake, suggesting novel gene-diet-gender interactions (Phillips, 2011). Calpain-10 protein (intracellular Ca(2+)-dependent cysteine protease) may play a role

in glucose metabolism, pancreatic β-cell function, and regulation of thermogenesis. Genetic variation at the *CAPN10* gene locus may modify insulin sensitivity by interacting with the plasma fatty acid composition in subjects with MetS (Perez-Martinez, 2011).

The discovery of genetic factors in T2DM will contribute to the knowledge of its pathophysiology and may help understanding of gene-nutrients interactions in this disease. Personalized interventions according to genotype may be considered. Furthermore, the future progression of nutritional genomics in T2DM needs diverse "omics" studies in a systems-biology-orientated approach, and also studies in diverse animal and in vitro models.

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ABSTRACTS

GENOMICS OF WHEAT UNDER SALT STRESS

José Luis Díaz De León Álvarez

Mapping of quantitative trait loci (QTL) was carried out in a set of 114 RILs of the International Triticeae Mapping Initiative (ITMI) mapping population under salt stress. Seedling population was grown during 8 days under salt treatment (Hoagland's ½ strength + 110 mM NaCl, EC 12.4 mS/cm) and normal treatment (Hoagland's ½ strength, EC 0.9 mS/cm). We calculated starch degradation, measuring the dry weight of the grains on the 4th, 6th, and 8th days of culturing. Formation of biomass was calculated measuring leaf and root length on the 4th, 6th, and 8th days of culture. Interval mapping resulted in 13 QTLs, 2 major QTLs (LOD >3) and 11 minors QTLs (LOD >2). A total of 10 QTLs were associated with saline treatment and 3 QTLs at normal treatment. The data show that a high percentage of QTLs were in chromosomes 2B (3, 23.0%) and 1A (3, 23.0%), followed by 4D (2, 13.6%).

The differential response to field salinity of the parents of the ITMI wheat mapping population (cv. Opata 85 and the synthetic hexaploid W7984) was exploited to perform a QTL analysis of the response to salinity stress of a set of agronomic traits over two seasons. The material was irrigated either with potable water (EC of 1.0 dS m1) or with diluted seawater (12.0 dS m1). Grain yield was positively correlated with tiller number, plant height, percentage survival, ear weight, ear length, grain number per ear, grain weight and thousand grain weight, and negatively with time to booting, anthesis and physiological maturity, under both the control and salinity stress treatments. In all, 22 QTL were detected under control conditions, and 36 under salinity stress. Of the latter, 13 were major loci (LOD >3.0) and eight were reproducible across both seasons. Chromosome 2D harboured 15 salinity stress associated QTL and chromosome 4A six such QTL. The remaining loci were located on chromosomes 2A, 5A, 6A, 7A, 1B, 4B, 3B, 6B, 7B and 6D.

The parents (the landrace Chinese spring (CS) and a synthetic hexaploids (S6x)) and 17 derived single chromosome substitution lines (SL) were grown in parallel in the field under non-saline (1.0 dSm^{-1}) and saline (12.0 dSm^{-1}) conditions, and evaluated for a set of phenotypic traits. The performance of CS indicated it to have borderline salinity tolerance with respect to all of the traits except for leaf area (for which it behaved in as a salinity sensitive type). The SL 4D was early in booting, ear emergence, flowering and maturity, while 5D and 2B SLs were both late. The 2B SL produce 33% more ears than CS. The 5D SL under-performed with respect to ear weight, grain number per ear, grain weight per ear and 1000 grain weight both under non-saline and saline conditions. Under saline conditions, four SLs (1A>5A>1D>2B) outperformed Cs for ear length, and six SLs (1D>6A>4B>3A>3B>3D) showed an improved grain weight. The grains produce by the 2B SL were smaller than those of CS. Leaf area developed better in four SLs (4D>2B>1A>7D) than in CS.

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CURRENT STATE OF RED BIOTECHNOLOGY: MOLECULES, THERAPEUTIC AREAS, AND MANUFACTURERS

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The international healthcare (red) biotechnology arena is an exciting intersection of science and business, that has grown dramatically since its inception three decades ago. The present review summarizes the state of bioeconomy, compares red biotechnology with pharmaceutical companies, and discusses the major bionations, manufacturers, therapy areas and molecules.

Summary: In 1953 Watson and Crick published a paper in Nature describing the double helix. In 1976 Swanson and Boyer founded Genentech, eventually succeeding in launching the first biosynthetic insulin in 1982. Today there exist several commercial sectors, each given its own corresponding coding color, e.g. red, green, blue, and white. Healthcare biotech is color-coded red (from the red blood cells) and includes the biosynthetic production of medicines and vaccines, stem-cell research, DNA sequencing and more. The pharmaceutical lifecycle includes the pre-discovery, discovery, preclinical, clinical, regulatory approval, and post-marketing phases. In total, it usually scans through 10,000 promising molecules, tests thousands of human volunteers and patients, and requires 10-15 years and in excess of one billion USD to bring a new pharmaceutical to the marketplace.

Conclusion: Red biotechnology has evolved tremendously since its commercial inception in 1982. To date, it has introduced more than 130 biotherapies, it has a positive global net income, it has helped save the lives of millions of patients around the world and it is on a pace to surpass the traditional pharmaceutical products at the top of the global sales list. The next decade will show whether the 21st century can finally be named the biotechnology century.

THE USE OF BIOTECHNOLOGY CROPS IN ARGENTINA

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Abstract: Argentina is a mainly agricultural country. During last time, lands considered marginal were incorporated to the production, impulses by technological changes and incorporation in 1996 of genetically modified crops. Actually, 21.3 million ha are cultivated with transgenic soybean, corn and cotton. The transgenic maize use contributes to control of mainly Lepidoptera pests, *Spodoptera frugiperda* and *Diatraea saccharalis*, although others such as *Heliothis zea* and *Agrotis ipsilon*, especially in the northwest were tropical corn are cultivated. Other traits give herbicides tolerancy (gliphosate and ammonium gluphosinate). The last transgenic events approved in Argentina, were stacked. This type of events is incorporated into cultivation similarly to the simple events and arises from the accumulation of events developed and approved previously. These are to belong to the same company or competency companies. In these cases plants are modified with many traits which give an integral protection to the crop (Lepidoptera and root worms, herbicides). About soybean, around 90% of total cultivated are gliphosate resistant, what caused a true revolution in this crop. Transgenic cotton, BT and gliphosate resistant, is less cultivated, compared than soybean and corn. Actually, companies are working in order to get new traits that solve wide spectrum herbicides sensibility; more efficient in the water and critical nutrients use; salt, flooding and high temperatures tolerance. In conclusion, in Argentina, the rate of transgenic crops adoption is high because growers are satisfied. Motivates by low costs, decrease in insecticide use; high crop yields with high quality. However faces challenges such as responsible management of these crops to avoid pests resistant and damages to native biodiversity.

CLONING EMBRYOS: APPLICATION TO WILDLIFE SPECIES AS ENDANGERED BIGHORN SHEEP

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Abstract: Embryo cloning is a biotechnology that has been applied since the 30s, to reproduce different animal species. There, we have some works from Hans Spemann, who divided a fertilized salamander oocyte using a baby hair, obtaining more than two viable embryos; to Wilmut and Campbell who, working with domestic sheep, transferred a mammary gland cell into an enucleated oocyte, giving birth to the very first cloned sheep from adult cells "Dolly". What to say about the uses that cloning biotechnologies have, for the reproduction of selected productive animals, for the economical profits that this represents and for the ecological interest in attempt to preserve or avoid the species extinction, as it happened for Dr. Lanza, who cloned the first wild gaur "Noah". It had occurred several works in which this biotechnology has won supporters and enemies, rumors and scientific evidences, everything around the need and pertinence of its application in humans. In this conference it will be explained the different ways for embryo cloning, its applications and will see some examples, giving emphasis to its application to wild animal still in existence and that already extinct.

THE "OMICS" APPROACH FOR SOLVING THE PRE-HARVEST AFLATOXIN CONTAMINATION

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Abstract: Aflatoxins are highly carcinogenic secondary metabolites produced primarily by the fungi Aspergillus flavus and Aspergillus parasiticus. Aflatoxin contamination of food and feed has been of particular concern over the last four decades because of the toxicity of these compounds. Regulations exist in over 100 countries of the world against sale of contaminated commodities, causing severe economic burden on the farmers. Therefore, this problem is both a food safety and an economic issue. Now, for the first time control measures for this problem appear within reach. For practical and sustainable control of pre-harvest aflatoxin contamination to be realized, however, additional information is needed about the fungus, the affected crops and the specific molecular factors (both in the plant and the fungus) involved during host plant-fungus interaction. The information derived from the use of novel tools such as genomics, proteomics and metabolomics provides us with the best and the quickest opportunity to achieve a clear understanding of the survival of toxigenic fungi in the field, the ability of the fungus to invade crops, and the process of toxin contamination under various environmental conditions. Significant progress has been made recently in understanding the genomic makeup of the most significant aflatoxin producing field fungus, namely Aspergillus flavus. Progress also has been made in the study of host crop resistance to fungal invasion through the use of proteomics. The information available on production of aflatoxin and other metabolites by Aspergillus flavus is reasonably extensive, although the application of metabolomics as a tool in this study is relatively new. In this presentation, the use of genomics, proteomics and metabolomics in deriving the requisite information for developing effective strategies to interrupt the machinery in the fungus for production of these toxins, as well as to enhance host-resistance against fungal invasion and aflatoxin contamination of crops will be discussed.

SUSTAINABLE AGRO-INDUSTRIAL PRODUCTION IN CHIAPAS MÉXICO AS AN ALTERNATIVE TECHNOLOGY FOR FOOD SECURITY.

Peggy E. Alvarez-Gutiérrez

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Abstract: The State of Chiapas is located in the south region of Mexico. Despite of having vast natural resources and an immense biodiversity, this state is one of the world's poorest regions. The main purpose of this study was to develop a sustainable technology for mushroom production (Pleurotus spp) for low-income women living in the Lacandona Rainforest Area and also Los Altos Region area in Chiapas Mexico(The results of this study showed the comparison of three different systems for mushroom production in two different regions of the state of Chiapas, and the mechanism of technology transfer to three groups of low income population, with a special focus on the female population. This study is meant to support the United Nation's Development Goals: end poverty and hunger, gender equality. Additionally, this study elucidates how a sustainable environment can be possible by producing a high protein food using agro-industrial residues as raw material.

BIOPROCESS INTENSIFICATION THROUGH HIGH CELL-DENSITY CULTIVATION IN BATCH MODE OF METABOLLICALY ENGINEERED *E. COLI* STRAINS

Alvaro R. Lara

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High cell-density cultivation (HCDC) is the preferred option for industrial bioprocesses using *Escherichia coli*. Despite its wide use, HCDC present several difficulties. Attaining high cell-densities require the use of elevated amount of carbon source (usually glucose). Due to the production of acetate by overflow metabolism in *E. coli*, glucose has to be fed to the bioreactor in order to keep the specific uptake rate below critical levels for overflow metabolism triggering. This leads to extended cultivation times and substrate gradients in the large-scale. To avoid overflow metabolism, we developed a strain with an alternative glucose transport system and decreased acetate production. The modified strain has been cultivated using up to 130 g/L of glucose in batch mode with a very low acetate accumulation. Compared to the parent strain, superior plasmid DNA and recombinant protein production have been achieved. Another challenge of HCDC is the large oxygen demand, which can not be easily satisfied in conventional bioreactors. In order to improve oxygen transfer, we evaluated the use of high pressure cultivations. A total pressure of up to 8 atm in the bioreactor allowed maintaining aerobic conditions whereas the kinetic behavior of the strains was not affected. A molecular alternative to contend with oxygen-limiting conditions is the expression of a recombinant hemoglobin in the engineered strain, which has improved its performance under microaerobic conditions in HCDC in batch mode.

CHLOROPLAST TRANSFORMATION: A TOOL TO MINIMIZE HORIZONTAL GEN TRANSFER

Quintín Rascón-Cruz

Abstract: Due to the growing concern that genetically (GM) modified crops can transfer genes through pollen to related plants (wild), it is necessary to propose strategies for containment of these. The use of genetic engineering of the chloroplasts to promote maternal inheritance of the transgenes is highly desirable for those instances that involve a potential risk of flow of genes between GM crops or plants wild type and GM crops. Genetic transformation of chloroplast genome can reduce or avoid unwanted gene transfer because the chloroplast genes are through maternal inheritance in most plants. This project used three delivery plasmids for genetic transformation of the chloroplasts of maize (Zea mays). Plastid transformation offers advantages over conventional nuclear genome transformation which includes high levels of protein expression, introduction of the transgene into the genome by homologous recombination cloroplastídico (site specific), absence of position effects and gene silencing expression of various transgenes in tandem (polycistronic) and dramatically decreased gene flow through pollen since chloroplasts are maternally inherited. Currently, there is a real concern that genetically modified crops can transfer their transgenes to other plants, therefore, in our laboratory have constructed vectors for chloroplast transformation by homologous recombination of B. gracilis and Zea mays. Transplantomic plant could contribute to maximize the benefits of transgenic plants to significantly reducing the potential risk of horizontal gene transfer to other crops.

NON-CONVENTIONAL METHODS FOR PROTEIN CRYSTALLISATION: USING PHYSICAL PARAMETERS TO CONTROL DE CRYSTAL QUALITY FOR X-RAY CRYSTALLOGRAPHY

Abel Moreno Cárcamo

Instituto de Química, UNAM

Abstract: Medium-sized single crystals with near-to-perfect habits made of molecular arrangements with no defects that produce well resolved and intense diffraction patterns are the dream of every protein crystallographer. Four basic crystallisation methods are presently at the disposal of crystal growers to grow such crystals. As half a century ago in chemistry labs, crystallisation assays can be set up using either (i) in batch, (ii) by the diffusion of vapour between hanging (or sitting) droplets and a reservoir of precipitant, (iii) by dialysis across a semi-permeable membrane or (iv) free interface diffusion at the interface of two liquids. In this plenary talk an overview of crystal growth methods from Nature grown crystals until conventional and non-conventional (using electric and magnetic fields) experimental methods of crystal growth of proteins will be presented. In order to control the kinetics of the crystallisation process, it is also presented what physical and chemical parameters allow us to control the nucleation, and crystal growth of biological macromolecules. Finally, a short overview of the main counter-diffusion methods, and new strategies for enhancing the crystal quality for high resolution X-ray crystallography will be also revised.

ENZYMES AS BIOTECHNOLOGICAL TOOLS FOR THE SYNTHESIS OF NATURAL FOOD ADDITIVES

Dolores Reyes-Duarte

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Abstract: The modification of natural antioxidants to improve their chemical, oxidative and/or thermal stability, or to alter their hydrophilelipophile balance (HLB), yields a series of "semisynthetic" antioxidants with a great impact in industry. On the other hand, the synthesis of carbohydrate fatty acid esters has become of interest due to the wide range of applications in the food, cosmetics, oral-care, detergent and pharmaceutical industries. The chemical synthesis of these derivatives generally uses harsh conditions with strongly corrosive acids or also bases at high temperatures, resulting in poor selectivity and the formation of undesirable by-products. To overcome these shortcomings, new approaches based on the use of biological catalysts are being evaluated. Biocatalysts are biodegradable, use mild reaction conditions, low energy requirements and display chemo-, regio- and/or stereospecificity resulting in decreased by-product formation thus avoiding the need for functional group protection and activation usually required in the chemical synthesis. In this ocassion, I will review the applications of enzymes such as lipases and esterases in the synthesis and modification of food additives as potential nutraceuticals like fatty acid esters of antioxidants, sugar esters and other natural products.

METABOLIC ENGINEERING TO INCREASE THE PRODUCTION OF PLASMID DNA VACCINES BY *ESCHERICHIA COLI*

Alvaro R. Lara

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Abstract: Plasmid DNA (pDNA) is the base for upcoming vaccines and gene therapy drugs. As the demand of pDNA is expected to grow, better *Escherichia coli* (the usual host for pDNA synthesis) strains for industrial production need to be developed. We have tested *E. coli* strains with a very low overflow metabolism for the production of pDNA in high cell-density cultivations in batch mode. The engineered strain has shown a superior performance compared to its parent. Further modifications in the activity of the pyruvate kinase resulted in improved pDNA production and reduced oxygen consumption. A second generation engineered strain was developed by mutating genes related to plasmid regulation and nucleotides synthesis and transport. Three mutations additional to the modification of the glucose transport system allowed increasing the pDNA yield nine times compared to the wild type strain. The second generation mutant produced more than twice as much pDNA than the commonly used DH5alpha strain. The mentioned results show the potential of rational modifications for increasing pDNA production by *E. coli*.

PROTEIN RESEARCH IN THE XXI CENTURY

Eduardo Armienta-Aldana

Abstract: Protein research has shown a great diversification in the late twentieth century. It is no longer enough to know and understand the features and functions of proteins; today want to know how it works in vivo, understand their interactions and structural conformations in an active organism. This will allow us to have a better understanding of what they really are and make proteins, both those structural and enzymatic functions. Progress has been made about the study and research of proteins, not only just the traditional electrophoretic and chromatographic analysis routinely performed by laboratories dedicated to the study of proteins. We have now covered more than 10 years of the new millennium and expectations in the study of proteins has grown exponentially, key examples, the Proteomics Unit of CINVESTAV-IPN, Campus Guanajuato (Mexico), which is one of the most modern country and also the Biostructural Nuclear Magnetic Resonance Unit of the Leloir Institute (Argentina), to name a few. What else can we expect in the coming years?, Perhaps only our imagination can give us that answer.

GENOMICS OF AIRBORNE BACTERIA IN MEGACITIES

Jaime García Mena

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Abstract: Megacities all over the world congregate huge amounts of human beings in high density populated areas with activities imposing deleterious consequences for the environment and all aspects of their life. One of such problems are the outdoor bioaerosols which disperses high amounts of microorganisms during certain periods of the year. Megacities such as Mexico City, with a population of *ca*. 20 million inhabitants in its metropolitan area, reports data for several respiratory and gastrointestinal diseases, in which the infectious agent could be an airborne bacteria. We performed a continuous systematic analysis of the bacteriological content of the low outdoor atmosphere in Mexico City, following the airborne bacteria by microbiological and molecular biology methods, during the spring, summer, fall, and winter seasons. Bacterial diversity has been determined by metagenomic analysis of massively isolated DNA from the samples, and also by identification of single isolated bacteria. Our data recollection shows a reasonable expected diversity of the bacterial community in the analyzed bioaerosols but an unexpected variation in the type and abundance of the identified bacteria which suggest a correlation with the prevalence of certain diseases in this megacity during the year. This work has been financed by Cinvestav and ICyTDF.

MOLECULAR STRUCTURAL BIOINFORMATICS OF INTEGRAL MEMBRANE PROTEINS: THE H3 RECEPTOR, A TARGET FOR COGNITIVE DISEASES

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Abstract: G protein-coupled receptors (GPCRs) are a subclass of the seven-transmembrane helix proteins (7TM) that transmit signals to cells in response to stimuli, thus mediating physiological functions through interaction with hetero-trimeric G proteins. Many diseases involve the malfunction of these receptors, making them important drug targets. The histaminergic system plays a major role in cognition and the H3 GPCR plays a regulatory role in the pre-synaptic release of histamine by inhibiting its release in brain and making it an attractive target for CNS indications including cognitive disorders, narcolepsy, ADHD and pain. H3R inverse agonists, by suppressing this brake, enhance histamine neuron activity. In spite of serious difficulties, the 3D structures of several GPCRs have recently been determined experimentally. Nevertheless, hundreds of sequences of interesting "druggable" GPCRs are still awaiting structure determination. Molecular modeling and simulation methods attempt to fill this gap by generating reliable 3D models of GPCRs. In order to study its behavior, once a receptor model is obtained, it is embedded in a phospho-lipid bilayer and surrounded by aqueous solvent and counter-ions, so as to constitute a realistic system comprising hundreds of thousands of atoms. Here, we will describe and illustrate the modeling and simulation of the H3 receptor.

GERMINATION OF SOYBEANS AND ITS EFFECT ON CHRONIC DISEASES

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Abstract: Soybean is an abundant source of proteins with high nutritional value and excellent physicochemical properties in foods, and also because it is a rich source of non-nutritive components with potential health benefits. Soy consumption has beneficial effects in the treatment of obesity due to its proven ability to decrease several markers, such as the amount of lipids stored inside adipocytes. On the other hand, extensive epidemiological, *in vitro*, and animal data suggest that soybean consumption reduces the risk of developing several types of cancer. However, little is known about the effect of germination on the antiobesity and anticancer properties. The aim of this work is to study the influence of soybean germination time (0, 2, 4, 6 days) on some obesity markers and on tumor growth both studies in mice. The results found it can say that a process as germination may help to increase antiobesity and anticancer activities. This effect can be correlated with the change in the protein pattern, and the increase of phytochemicals along the germination process.

MOLECULAR MECHANISM OF HISTAMINERGIC SIGNAL TRANSMISSION BY THE H3 RECEPTOR, A TARGET FOR COGNITIVE DISEASES

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+ UAM, Mexico City, Mexico. * CIRB, Collège de France, Paris, France

Abstract: G protein-coupled receptors (GPCRs) are a subclass of the seven-transmembrane helix proteins (7TM) that transmit signals to cells in response to stimuli, thus mediating physiological functions through interaction with hetero-trimeric G proteins. Many diseases involve the malfunction of these receptors, making them important drug targets. In spite of serious experimental difficulties, the 3D structures of several GPCRs have recently been determined. Nevertheless, hundreds of sequences of interesting “druggable” GPCRs are still awaiting structure determination. Molecular modeling and simulation methods attempt to fill this gap. The histaminergic system plays a major role in cognition and the H3 receptor, a GPCR, plays a regulatory role in the pre-synaptic release of histamine by inhibiting its release in brain and making it an attractive target for CNS indications including cognitive disorders, narcolepsy, ADHD and pain. H3R inverse agonists, by suppressing this brake, enhance histamine neuron activity. In order to study the behavior of H3R in a realistic fashion, we obtained 3D models of the receptor and of its ligand-bound complexes through homology modeling and docking calculations. The models correspond to the apo form, to the complex with histamine, the endogenous agonist, and to the complex with ciproxyfan, a powerful, selective inverse agonist. We then embedded the models in a phospho-lipid bilayer surrounded by aqueous solvent and counter-ions and then performed molecular dynamics calculations. Analysis and comparison of the corresponding molecular dynamics trajectories should give us an insight into the molecular mechanism of histaminergic transduction by receptors of the same family.

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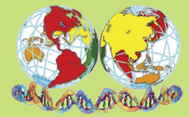

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