JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2015. Vol. **9**(1), p. 579-585

Preliminary Study of the Enzymatic Activity of Native Isolates of Entomopathogenic Fungi from the Citrus Area of Mexico in Solid Culture Media

Fatima Lizeth Gandarilla-Pacheco, Lilia H. Morales-Ramos, Maria del Socorro Flores-Gonzalez, Myriam Elias-Santos and Isela Quintero-Zapata*

Instituto de Biotecnologia, Facultad de Ciencias Biologicas,
Universidad Autonoma de Nuevo Leon (UANL). 66450 San Nicolas de los Garza, N.L., Mexico.

(Received: 06 Februray 2015; accepted: 10 March 2014)

Entomopathogenic fungi are among the agents of biological control most widely studied for their ubiquity, easy and fast growth on different substrates and its wide host range. In this study 30 entomopathogenic fungi isolated from soils where citrus is grown in different regions of Mexico to assess the production of chitinases, lipases and proteases on solid media by calculating the enzymatic index, a criterion that relates the growth rate and enzyme activity.We analyzed by ANOVA with mean separation by a Tukey test (± = 0.05). The results do not meet the criteria of normality were analyzed using a Kruskal-Wallis test. According to the results of this test for protease and lipase activity at least one of the isolated is different regarding their enzymatic index. Respect to chitinase activity the according to the ANOVA there is significative difference in chitinolytic activity (F = 2.44; p = 0.001) between the strains tested; It was observed that the chitinase activity was exhibited in a range 1.10-1.50; the highest enzymatic index value was for the isolate HIB-19, followed by the isolate HIB-1. These results show the presence of various hydrolytic enzymes from fungi evaluated.

Key words: Entomopathogenic fungi, Chitinase, Protease, Lipase, Enzymatic index value.

Entomopathogenic fungi are an important alternative for a wider context of insecticides within the schemes for insect pest management (Lord 2005; Roy *et al*., 2006). Physico-chemical aspects related to the development of the disease require the favorable interaction of the entomopathogen with external tissues your host. The pathogenesis involves: 1) the growth of the germ tube into the cuticle with the concomitant production of extracellular hydrolytic enzymes; 2) production of sticky mucilage; 3) the formation of appressoria on the surface of the cuticle; 4) the penetration of infectious plug into the epicuticle, then procutícula, and finally in the hemocele and hemolymph (Khachatourians and Qazi, 2008). One of the major virulence factors associated with successful penetration of the cuticle of insects depends mainly on various hydrolytic enzymes that degrade proteins, and lipids in the chitin insect's integument and provide nutrients for the fungus, even levels specific cuticle degrading enzymes, such as lipase, chitinase, protease, quimoelastase and chymotrypsin are related to variables of specific virulence (Hegedus and Khachatourians, 1995).Many pathogenic enzymes are important in determining virulence because they allow the pathogenic process coexist with metabolic changes associated with the disease state of the host (Qazi and Khachatourians, 2008). Regarding

* To whom all correspondence should be addressed.
E-mail: isela.quinterozp@uanl.edu.mx

entomopathogenic fungi are no results about the isolation, identification and purification of extracellular enzymes degrading of insect cuticle (James *et al*., 2003; Fang *et al*., 2005; Ali *et al*., 2011).Exist several studies of the in vitro production of extracellular enzymes of entomopathogenic. Entomopathogenic fungi are organisms widely distributed and their occurrence is practically ubiquitous. In nature, the different isolates exhibit different responses in terms of potential virulence and secretion of enzymes is a key determinant of this variation (Mustafa and Kaur, 2010;Subramanian and Punamalai, 2013). The aim of this study was to identify the natural enzyme activities in 30 native entomopathogenic fungi isolated from the citrus region of Mexico with potential for control of *Anastrephaludens* (Diptera: Tephritidae) and *Diaphorinacitri* (Hemiptera: Liviidae) (Gandarilla-Pacheco *et al.*, 2012;Gandarilla-Pacheco *et al.*, 2013 a, b) using solid media for secretion of proteases, lipases and chitinases and relate their possible potential for management of these pests of concern associated with the region.

MATERIALS AND METHODS

Microorganisms

The isolates of entomopathogenic fungi used in this study were obtained from soils of the citrus region of Mexico (Table 1); in addition were selected seven strains from the Collection of the Biotechnology Institute FCB-UANL(GHA, Asdel 139,A-44 and A-48, *B. bassiana*; Pfr-114 and Pfr-612, *I. fumosorosea* y Met, *M. anisopliae*).The method used for the isolation and characterization of different entomopathogenic fungi recovered was described by Galán-Franco *et al* (2011). The stock cultures were maintained in the laboratory collection L6 Biotechnology Institute, FCB-UANL. Cryogenic vials for preservation were used (Corning, NY, USA) with 1 mL of 10% glycerol v/v and stored frozen at -80°C.

Activation and preparation of suspensions of conidia

Selected microorganisms were preserved in cryogenic state (10% glycerol -80°C) and thawed at room temperature (25 ± 2°C). Within about 30-60 min; were inoculated after in potato dextrose agar medium (Bioxon, NJ, USA) and incubated during 14 to 21 days to 28°C. After incubation, each fungus was added 10 mL of Tween 80 (Sigma Aldrich, St. Louis MO, USA) 0.1% (v / v) and the concentration was adjusted to 1 x 10^6 conidia mL^{-1}.

Composition of the culture media

Casein hydrolysis medium

The components of the hydrolysis of casein: 0.1% KH_2PO_4, 0.05% KCl, 0.02% $MgSO_4.7H_2O$, $CaCl_2.H_2O$ 0.01%, 1.0% glucose (Jalmek, San Nicolás de los Garza, NL, Mexico), 1.2% bacteriological agar (Bioxon, NJ, USA) per liter of bidistilled water; after was resuspended 15% skim milk agar (BD, NJ USA), in 25 mL of double distilled water and was added (adapted fromSubramanian and Punamalai, 2013).

Chitinases medium

The components of the chitinases medium: $NH_4H_2PO_4$ 0.13%, 0.02% KCl, 0.02% $MgSO_4.7H_2O$, 0.02% $CaCl_2$, 1.6% bacteriological agar (Bioxon, NJ, USA) in 750 mL of bidistilled water; after was added 20% chitin (Sigma Aldrich, St. Louis MO, USA) colloidal resuspended in 250 mL of double distilled water and was added (adapted from Chul-Kang *et al*., 1999 and Punamalai Subramanian, 2013).

Tween 20 medium

The components of the medium Tween 20: 3.9% potato dextrose agar (Bioxon, NJ, USA), 0.5% NaCl, 0.01% $CaCl_2$, 1.5% bacteriological agar (Bioxon, NJ, USA) per liter double distilled water; after was resuspended 10% Tween 20 (Sigma Aldrich, St. Louis MO, USA), in 90 mL of double distilled water and was added (adapted from Subramanian and Punamalai, 2013).

Inoculation and incubation

For inoculation of the petri dishes each containing of the mediums for observed the enzymatic activity was put a small circle of Whatman filter paper No.1 sterile in the central part of each of the boxes; then 1 mL of fungal suspension was added per box of each treatment and incubated at 25 ± 2 ºC.

Measurement of enzyme activity

The activity measurement was performed at 120 h of incubation. Using a vernier were measured the colony diameter and the halos formed around the treatment in millimeters.

Interpretation of enzyme activity on solid media

For the interpretation of enzymatic reactions on solid media (presence of halos around

the colony) was used a criteria relating the growth rate and enzymatic activity using the enzymatic index value which equals the total diameter of the colony more the halo divided between the diameter of the colony. Enzymatic index values > 1.0 indicate enzyme activity (Mustafa and Kaur, 2010).

Statistical analysis

Each treatment was performed in triplicate and all experiments were repeated at least twice.We analyzed by ANOVA with mean separation by a Tukey test (±= 0.05). All statistical tests were conducted by IBM ® SPSS ® v.19 Inc., N.Y., USA. The results do not meet the criteria of normality were analyzed using a Kruskal-Wallis test.

RESULTS AND DISCUSSION

The results did not follow a normal curve for protease and lipase activities (Z = 2.21, *p* = 0.000) and (Z = 3.83, p = 0.000), respectively. Therefore, Kruskal-Wallis test was performed for these results.According to the results of this test for protease and lipase activity at least one of the isolated is different regarding their enzymatic index (protease: Ç2 = 70.91, p = 0.000; lipase: Ç2 = 56.79, p = 0.015).

Respect to chitinase activity the results were adjusted to a normal curve (Z =1.02, p = 0.25) and according to the ANOVA there is significative difference inchitinolytic activity (F = 2.44; p = 0.001) between the strains tested; therefore the comparison test of Tukey at 0.05 significance was performed. It was observed that the chitinase activity was exhibited in a range 1.10-1.50; the highest enzymatic index value was for the strain HIB-19, followed by the strain HIB-1 (1.45) (Table2).

Fungal infection on their hosts depends numerous biological events that are initiated by the adhesion of spores in the insect cuticle, germination and hyphal growth (Altre*et al*., 1999). Entomopathogenic fungi exhibit a variety of attributes or characteristics that determine virulence towards their hosts,such as the production of toxins and degradative enzymes (Dias *et al*., 2008). It is generally accepted that both the mechanical strength and enzymatic processes, including certain metabolic acids mediate initial interaction. Currently some of the genes responsible for the results of successful interaction with the host and the environment are known (Khachatourians and Qazi, 2008). The insect cuticle is the first barrier for the fungus thus a wide variety of extracellular enzymes are synthesized for the enzymatic processes involved in the degradation of protein, chitin and lipid, the main components of this structure (St. Leger *et al*., 1996). In vivo the enzyme secretion sequence corresponds to the polymers present in the cuticle. Lipases and proteases act before chitinases, chitin fibers are surrounded by a protein coat; therefore chitin can not be degraded until the proteins are removed (Bidochka*et al*., 1997); the significance of any enzyme depends on the cuticular characteristics and physiological state of the insect, as well as mechanisms of invasion by the fungus (Hegedus and Khachatourians, 1995). In vitro studies in plate, enzyme production is typically indicated by the formation of clear zones around the colony growth or the formation of colored products or by use of inducing substrates as insect cuticles or selective media such as chitin colloidal or casein. Some Studies suggest to proteases and chitinases as major determinants of virulence in the pathogen-host relationship (Fang *et al*., 2007; Yang *et al*., 2007).

Isolates and strains evaluated 51% presented the three activities (protease, lipase and chitinase), 46% only two and 3% only one (chitinase),while the isolates and strains evaluated that showed lipolytic, proteolytic and chitinolytic activity the 79% are *B. bassiana*, *I. fumosorosea*16% and 5% *M. anisopliae*.

Proteolytic enzymes secreted by different entomopathogenic have been attributed different roles in the pathogenesis of insects including degradation of the cuticle, prophenol oxidase activation and virulence in the hemolymph. Protein-degrading enzymes such as proteases, collagenases and quimoelastasashave been identified and characterized in *A. aleyrodis, B. bassiana, B. brongniartii, E. coronata, Erynia*spp., *Lagenidiumgiganteum, Nomuraearileyi, M. anisopliae*, y *V. lecanii*(Khachatourians, 1996; Sheng *et al*., 2006). The Pr1 protease is considered a key determinant in the virulence of entomopathogenic fungi, and is believed to be an increase in the formation of appressoria and conidiogenesis (Small and Bidochka, 2005). Campos *et al*. (2005) reported that

the presence of cuticles of *Boophilusmicroplus* induces Pr1 in *B. bassiana*, while adding alanine to the culture medium suppressed the production of this enzyme. Recently, Ito *et al*. (2007) showed that a Brazilian strain of *B. bassiana* reactivated in a corn cobs based broth, produced a protease in a medium containing glucose and yeast extract. According to this study *B. bassiana* produced 80% of its total proteolytic activity in 48 h, with the highest activity on the fifth day of incubation; which coincides with the results of this work since the maximum activity was reported at 120 h of incubation, although not all strains and isolated evaluated showed proteolytic activity or was low.Bidochka and Khachatourians (1988) in a study with a strain of *B. bassiana* found that the presence of glucose, glycerol, trehalose or mannitol and ammonium together in a medium containing gelatin production suppressed serine protease (Pr1) in addition to has also been reported that protease production is influenced by the type of nitrogen source (Kucera, 1971); and it has been reported that regulation of the genes encoding isoforms cuticle degrading enzymes is probably complex and may involve a combination of induction of carbon / nitrogen and / or repression (Screen *et al*., 1998).This may explain that some of the strains were not detected activity because the medium used contained glucose which may have acted as a repressor.

The epicuticle lipids are the first barrier , so that lipases are important in the process of infection;the composition these lipid includes about 65% alkanes, 25% wax esters, triglycerides, alcohols, and fatty acids; lipid addition, the cuticle contains amino acids, and amino sugars that

Table 1. Entomopathogenic fungi isolated from cultivated soils of citrus orchards in different states of México

Key	Location of collection	Geographic location	Elevation (m asl)	Species
HIB-1	Guasave, Sinaloa	N 25°422 203 W 108°202 483	50	*B. bassiana*
HIB-2	Ahome, Sinaloa	N 25°552 13 W 109°102 423	10	*B. bassiana*
HIB-3	Ahome, Sinaloa	N 25°332 583 W108°262 443	10	*B. bassiana*
HIB-4	Ahome, Sinaloa	N 25°552 243 W 109°102 253	10	*B. bassiana*
HIB-5	Ahome, Sinaloa	N 25°482 213 W108°592 493	10	*B. bassiana*
HIB-6	Guasave, Sinaloa	N 25º342 313 W 108º282 183	50	*B. bassiana*
HIB-7	Ahome, Sinaloa	N 25°482 483 W 108°592 273	10	*B. bassiana*
HIB-8	Tamazunchale, San Luis Potosí	N 21°172 153 W 98°432 383	140	*B. bassiana*
HIB-9	Tanquián, San Luis Potosí	N 21°352 123 W 98°392 473	50	*I. fumosorosea*
HIB-10	Allende, Nuevo León	N 25°242 343 W 99°592 173	460	*B. bassiana*
HIB-11	Hualahuises, Nuevo León	N 25°242 343 W 99°592 173	400	*M. brunneum*
HIB-12	Allende, Nuevo León	N 25°242 343 W 99°592 173	460	*M. brunneum*
HIB-13	Hidalgo, Tamaulipas	N 24°142 523 W 99°262 113	400	*B. bassiana*
HIB-14	Hidalgo, Tamaulipas	N 24°142 523 W 99°262 113	400	*B. bassiana*
HIB-15	Hidalgo, Tamaulipas	N 24°142 523 W 99°262 113	400	*B. bassiana*
HIB-16	Ahome, Sinaloa	N 25°552 243 W109°102 253	10	*B. bassiana*
HIB-17	Ahome, Sinaloa	N 25°552 243 W109°102 253	10	*B. bassiana*
HIB-18	Padilla, Tamaulipas	N 24°52 03 W 99°72 303	153	*B. bassiana*
HIB-19	Linares, Nuevo León	N 25°092 133 W 99°512 103	350	*I. fumosorosea*
HIB-21	Tikinmul, Campeche	N 19º462 203 W 90º122 103	20	*I. fumosorosea*
HIB-22	Montemorelos, Nuevo León	N 25°102 033 W 99°572 113	430	*I. fumosorosea*
HIB-24	Montemorelos, Nuevo León	N 25°192 093 W 99°542 073	430	*B. bassiana*
HIB-25	Montemorelos, Nuevo León	N 25°102 033 W 99°572 113	430	*B. bassiana*
HIB-26	Hampolol, Campeche	N 19º552 253 W 90º232 263	10	*I. fumosorosea*
HIB-27	Hermosillo, Sonora	N 28°532 363 W 111°192 453	210	*I. fumosorosea*
HIB-29	Montemorelos, Nuevo León	N 25°182 123 W 99°532 133	430	*I. fumosorosea*
HIB-30	Montemorelos, Nuevo León	N 25°102 033 W 99°572 113	430	*I. fumosorosea*
HIB-31	Chencolli, Campeche	N 19º482 483 W 90º162 253	30	*I. fumosorosea*
HIB-32	Padilla, Tamaulipas	N 24°52 03 W 99°72 303	153	*I. fumosorosea*
HIB-33	Pocyaxum, Campeche	N 19º402 303 W 90º202 303	20	*I. fumosorosea*

function as nutrients for germination of the fungus (Bidochka*et al*., 1997).

It has also been reported that production of lipases may be influenced by the type and concentration of carbon and nitrogen sources. In previous work it was reported that the physiology in the production of lipases shows that the mechanisms regulating the biosynthesis varies widely in different microorganisms (Sharma *et al*., 2001). In lipolytic activity also can influence other factors such as metal ions, changes in pH and temperature to name a few. Divalent metal ions such as the ferric can inhibit the lipase activity, whereas magnesium ions may slightly increase activity at low concentrations (Ali *et al*., 2009). In this work the culture media Tween 20 was used and its composition includes potato dextrose agar, a culture medium widely used in the isolation of fungi and yeasts. This means that in its formulation contains potato infusion that mainly supplements carbohydrates, such as starch but may also contain small amounts of minerals in the composition that may influence the secretion of these enzymes in culture media.

Table 2. Enzymatic Index Values of enzymes produced by entomopathogenic fungi at 120 h of incubation under laboratory conditions at 25 ± 2 ° C

Isolates /strains	Enzymes		
	Lipase	Protease	Quitinase*
HIB-1	1.24	1.05	1.45 [ab]
HIB-2	1.00	1.02	1.41 [ab]
HIB-3	1.20	1.02	1.36 [ab]
HIB-4	1.25	1.00	1.12 [a]
HIB-5	1.00	1.04	1.35 [ab]
HIB-6	1.00	1.05	1.30 [ab]
HIB-7	1.13	1.02	1.38 [ab]
HIB-8	1.09	1.05	1.33 [ab]
HIB-9	1.00	1.06	1.34 [ab]
HIB-10	1.56	1.04	1.29 [ab]
HIB-11	1.00	1.03	1.26 [ab]
HIB-12	1.00	1.03	1.20 [ab]
HIB-13	1.12	1.04	1.36 [ab]
HIB-14	1.04	1.04	1.33 [ab]
HIB-15	1.13	1.03	1.33 [ab]
HIB-16	1.37	1.02	1.21 [ab]
HIB-17	1.13	1.06	1.26 [ab]
HIB-18	1.00	1.05	1.43 [ab]
HIB-19	1.23	1.01	1.50 [b]
HIB-21	1.00	1.04	1.31 [ab]
HIB-22	1.00	1.14	1.20 [ab]
HIB-24	1.36	1.02	1.18 [ab]
HIB-25	1.14	1.05	1.16 [ab]
HIB-26	1.00	1.13	1.37 [ab]
HIB-27	1.06	1.08	1.28 [ab]
HIB-29	1.14	1.01	1.34 [ab]
HIB-30	1.11	1.00	1.20 [ab]
HIB-31	1.00	1.14	1.25 [ab]
HIB-32	1.00	1.05	1.16 [ab]
HIB-33	1.00	1.07	1.24 [ab]
GHA	1.06	1.13	1.27 [ab]
Met	1.02	1.01	1.10 [a]
Pfr-612	1.00	1.00	1.38 [ab]
Pfr-114	1.00	1.14	1.38 [ab]
Asdel 139	1.23	1.03	1.32 [ab]
A-44	1.22	1.00	1.41[ab]
A-48	1.22	1.02	1.15 [ab]
Mean ±SD	1.10 ± 0.20	1.04 ± 0.04	1.29 ± 0.13

* Treatments with the same letter are not significantly different according to Tukey test (p d" 0.05)

Entomopathogenic fungi synthesize a diverse range of chitinases. This class of enzymes act synergistically with enzymes such as proteinases and ²-1,3glucanases, which are closely involved in the insect cuticle. Chitinases are also involved in fungal development and pathogenesis (Khachatourians and Qazi, 2008). Feng *et al*. (1994) reported achitinase in *Beauveriabassiana* that increased its virulence. Several studies have reported a positive correlation between the production of extracellular enzymes and their virulence. The expression of chitinase genes in different organisms is controlled by a system induction/suppression in which the chitin or other degradation products act as inductors. In yeast, it has been observed that glucose and other carbon sources can act as system repressors(Felse and Panda, 1999); while chitinase production in liquid culture medium was suppressed by the presence of an assimilable carbon source and induced colloidal chitin or yeast extract (Dhar and Kaur, 2010).

In the present study all fungi evaluated reported chitinase activity in media colloidal chitin which is consistent with previous studies. Most strains and isolates selected were evaluated previously in work against two citrus pest (Gandarilla- Pacheco *et al*., 2012, Gandarilla-Pacheco *et al*., 2013 a,b) where some of them showed levels of important virulence, since it has been reported that specific enzymes or various more of them in an enzymatic cascade can serve as

virulence determinants since enzymes are differentially expressed in culture media, is also possible that may be involved in determining the host range (Gupta *et al*., 1994); and it is also clear that the entomopathogenic fungi seek to adapt to suit their ecological niches (St. Leger *et al*., 1997) which may also explain the variability in production of hydrolytic enzymes.

CONCLUSIONS

Analysis of the results shows that most entomopathogenic used in this study secreted in solid media, proteases, lipases and chitinases, the latter are detected in all tested fungi. This study presents new perspectives and helps to complement other studies in which the pathogenicity of these isolates with two important citrus pests evaluated.

ACKNOWLEDGEMENTS

The projects PAICYT 2011-2012 KEY CN 1008-1011 and "Network for the use of biodiversity in Mexico through biotechnological tools", key 103.5 / 13/9254.

REFERENCES

1. Lord, J. C. From Metchnikoff to Monsanto and beyond: the path of microbial control. *J. Invert.Pathol*.,2005; **89**: 19-29.
2. Roy, H. E., Steinkraus, D. C.,Eilenberg, J.A., Hajek, E., Pell, J. K. Bizarre interactions and endgames: Entomopathogenic fungi and their arthropod hosts. *Annu. Rev. Entomol*.,2006; **51**: 331-357.
3. Khachatourians, G.G., Qazi, S.S. Entomopathogenic Fungi: Biochemistry and Molecular Biology. In:*The Mycota VI Human and Animal Relationships*(Brakhage AA, ZipfelPF, eds.). Berlin, Alemania. Springer,2008; pp 34-53.
4. Hegedus, D.D., Khachatourians, G.G. The impact of biotechnology on hyphomycetous fungal insect biocontrolagentes. *Review Biotechnology Advances*.,1995; **13**: 455-490.
5. James, R. R., Buckner, J. S., Freeman, T. P.Cuticular lipids and silverleaf whitefly stage affect conidial germination of *Beauveriabassiana* and *Paecilomyces fumosoroseus*. *J. Invert. Pathol.*, 2003; **84**: 67-74.
6. Fang ,W., Leng, B., Xiao, Y., Jin, K.,Ma, J.,Fan, Y., Feng, J., Yang, X.,Zhang, Y., Pei,Y. Cloning of *Beauveriabassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. *Appl. Environ. Microbiol*.,2005; **71**: 363-370.
7. Ali, S., Huang, Z., QeZang, W., Xiang Ren,S. Production and regulation of extracellular proteases from the entomo pathogenic fungus *Metarhiziumanisopliae* (Cordycipitaceae: Hypocreales) in the presence of diamondback moth cuticle. *Pakistan J. Zool*., 2011;**43**: 1203-1213.
8. Mustafa, U., Kaur,G.Studies on extracellular enzyme production in *Beuaveriabassiana* isolates. *International Journal of Biotechnology and Biochemistry*., 2010; **6**: 701-713.
9. Subramanian Ch., Punamalai, G. Production of hydrolytic enzymes by *Beauveriabassiana* isolates on solid medium**.** *Indian Streams Research Journal*.,2013; **10**: 1-5.
10. Gandarilla-Pacheco, F. L., Nava- González, H. D., Arévalo-Niño, K., Galán-Wong, L. J., Elías-Santos, M., Quintero-Zapata, I.Evaluation of native strains of *Isariafumosorosea* (Wize) against *Anastrephaludens* (Loew) (Diptera: Tephritidae). *Journal of Life Sciences*.,2012; **6**: 957-960.
11. Gandarilla-Pacheco, F. L, Galán- Wong, L. J., López-Arroyo, J. I., Rodríguez-Guerra, R., Quintero-Zapata,I. Optimization of pathogenicity tests for selection of native strains of entomopathogenic fungi isolated from citrus-growing areas of México on adults of *Diaphorinacitri*Kuwayama (Hemiptera: Liviidae). *Fla. Entomol*.,2013a; **96**: 187-195.
12. Gandarilla-Pacheco, F.L., López-Arroyo, J.I.,Galán-Wong, L.J.,Quintero-Zapata, I.Patogenicidad de hongos entomopatógenos nativos de la zona citrícola de México sobre ninfas de *Diaphorinacitri* Kuwayama (Hemiptera: Liviidae). *Southwest. Entomol*., 2013b; **38**: 325-338.
13. Galán-Franco, L.A., Morales-Loredo, A.,Álvarez-Ojeda, G., López-Arroyo, J. I.,Arévalo-Niño, K.,Sandoval-Coronado, C., Quintero-Zapata,I.Isolation and characterization of entomopathogenicfungiobtainedfrom citrus-growingareas of Mexico.*Southwest. Entomol*.,2011; **36**: 443-449.
14. Chul-Kang, S., Park, S., Gyu Lee,D. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhi ziumanisopliae*. *J. Invert. Pathol.*,1999; **73**: 276-281.
15. Altre, J. A., Vandenberg, J. D.,Cantone,F. A.

Pathogenicity of *Paecilomycesfumosorosea* isolates to diamondback moth, *Plutellaxylostella*: correlation with spore size, germination speed, and attachment to cuticle. *J. Invert. Pathol.*,1999; **73**: 332-338.

16. Dias, B. A., Neves, P. M. O. J.,Furlaneto-Maia L., Furlaneto,M. C.Cuticle-degrading proteases produced by the entomopathogenic fungus*Beauveriabassiana*in the presence of coffee berry borer cuticle. *Brazil. Jo. Microbiol*., 2008; **39**: 301-306.
17. St. Leger, R. J., Joshi, L., Bidochka,M. J.,Roberts,D. W. Characterization and ultrastructural localization of *Metarhiziumanisopliae, M. flavoviride*and *Beauveriabassiana*during fungal invasion of host (*Manducasexta*) cuticle. *Appl. Environ. Microbiol.*,1996; **62**: 907-912.
18. Bidochka, M. J., R. J. St. Leger, and D. W. Roberts. Mechanisms of deuteromycete fungal infections in grasshoppers and locusta: an overview. *Memoirs Entomol. Soc. Can.*,1997; **171**: 213-224.
19. Fang, W. G., Gou, S. J.,Pei, X. Q.,Zhang, Y. J.,Xiao, Y. H.,Li, D. M.,Jin, K.,Bidochka, M. J., Pei. Y. Increased insect virulence in *Beauveriabassiana*strains overexpressing an engineered chitinase. *Appl. Environ. Microbiol*.,2007; **73**: 295-302.
20. Yang, J. K., Tao, N., Liang, L. M., Mi, Q. L.,Li, J., Zhang,K. Q. Cloning of the gene *Lecanicilliumpsalliotae*chitinaseLpchil and identification of its potencial role in the biocontrol of root-knot nematode *Meloidogyne incognita*. *Applied Microbiology and Biotechnology.*,2007; **76**: 1309-1317.
21. Khachatourians, G.G. Biochemistry and molecular biology of entomopathogenic fungi. In: *The Mycota VI. Human and animal relationship*. (Howard, DH, Miller JD,eds.).Springer Berlin, Alemania. 1996; pp. 331-364.
22. Sheng, J., An, K.,Deng, C., Li, W.,Bao, X., Qiu,D.Cloning a cuticle-degrading serine protease gene with biologic control function from *Beauveriabrongniartii*and its expression in *Escherichia coli*. *Curr.Microbiol*.,2006; **53**:124-128.
23. Small, C. L. N., Bidochka,M. J. Up-regulation of Pr1, a subtilisin-like protease, during conidiation in the insect pathogen *Metarhiziumanisopliae*. *Mycol Res.*,2005; **109**: 307-313.
24. Campos, R. A., Arruda, W., Boldo, J. T.,Silva, M. V., Barros, N. M.,Azevedo, J. Schrank, L. A.,Vainstein,M. H.*Boophilusmicroplus* infection by *Beauveriaamorpha*and *Beauveriabassiana*: SEM analysis and regulation of subtilisin-like proteases and chitinases. *Curr. Microbiol*.,2005; **50**: 257-261.
25. Ito, E. T., Varéa-Pereira, G., Mitagui, D. T., Pinotti, M. H. P.,Neves,P. M. O. J. Production of extracellular protease by a Brazilian strain of *Beauveriabassiana*reactivated on coffee berry borer, *Hypothenemushampei*. *Braz. Arch. Biol. Technol*.,2007; **50**: 217-223.
26. Bidochka, M. J., Khachatourians,G. G. Regulation of extracellular protease in the entomopathogenic fungus *Beauveriabassiana*. *Exp. Mycol.*,1988; **12**: 161-168.
27. Kucera, M. Toxins of the entomogenous fungus *Beauveriabassiana*. Effect of nitrogen sources on formation of the toxic protease in submerged culture. *J. Invertebr. Pathol.*,1971; **17**: 211-215.
28. Screen, S., Bailey, A.,Charnley, K., Cooper, R.,Clarkson,J. Isolation of a nitrogen response regulator gene (nrr1) from *Metarhiziumanisopliae*. *Gene.*,1998; **221**: 17-24.
29. Sharma, R., Chisti, Y., Banerjee,U.C. Production, purification, characterization and applications of lipases. *Biotechnol. Adv*.,2001; **19**: 627-662.
30. Ali, S., Huang, Z., Xiang Ren.,S. Production and extraction of extracellular lipase from the entomopathogenic fungus *Isariafumosoroseus* (Cordycipitaceae: Hypocreales). *Biocontrol Sci. Technol*.,2009; **19**: 81-89.
31. Feng, M. G., Poprawski, T. J.,Khachatourians,G. G.Production, formulation and application of the entomopathogenic fungus *Beauveriabassiana*for insect control. *Biocontrol Sci. Technol*1994; **4**: 3-34.
32. Felse, P. A., Panda, T. Regulation and cloning of microbial chitinases genes. *Appl. MicrobialBiotechnology.*,1999; **51**:141-151.
33. Dhar, P., Kaur,G. Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Beauveriabassiana*isolates. *Afr. J. Biotechnol.*,2010; **9**: 8092-8099.
34. Gupta, S. C., Leathers, T. D.,El-Sayed, G. N.,Ignoffo,C. M. Relationship among enzyme activities and virulence parameters in *Beauveriabassiana* infections of *Galleria mellonella*and *Trichoplusiani*. *J. Invert. Pathol*.,1994; **64**: 13-17.
35. St. Leger, R. J., Joshi, L., Roberts,D.W. Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology.*,1997; **143**: 1983-1992.