ISSN : 0974 - 7435 Volume 6 Issue 12

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 6(12), 2012 [386-395]

Isolation and identification of novel entomopathogenic fungal strains of the *Beauveria* and *Metarhizium* generous

Marily González Castillo[1], Ismael Amaya Rivera[2], Angélica Berlanga Padilla[3], Faustino Lara Victoriano[1], Cristóbal N.Aguilar[1], Raúl Rodríguez Herrera[1]*

[1]Food Research Department, School of Chemistry, Universidad Autonoma de Coahuila. Blvd. Venustiano Carranza y J. Cárdenas S/N, Col. Republica Oriente, C.P. 25280, Saltillo, Coahuila, (MÉXICO)

[2]Applied Microbiology Center, GreenCorp Biorganiks de México SA de CV. Saltillo, Coahuila, México. Brasilia N° 1000, Col. Latinoamericana, C.P. 25270, Saltillo, Coahuila, (MÉXICO)

[3]Agricultural Parasitology Department, Universidad Autónoma Agraria Antonio Narro. Calzada Antonio Narro, Nº 1923, Col. Buenavista CP.25315. Saltillo, Coahuila, (MÉXICO)

E-mail: raul.rodriguez@uadec.edu.mx

ABSTRACT

In this work, the isolation, morphological and molecular identification of new entomopathogenic fungal strains were performed. The fungal strains were isolated from 12 soils samples collected at three agricultural Mexican regions: Saltillo and Torreon, Coahuila and Mexico City using *Tenebrio molitor* larvae as host. Only four of all isolates strains were identified as entomophatogenic according to the morphological characterization. In addition, 11 entomopathogenic fungal strains were isolated directly from dead insects, in addition as control were used 5 fungal strains belonging to the Applied Microbiology Center (CEMAP) GreenCorp Biorganiks de México SA de CV. All fungal strains were identified through the 18S rRNA sequencing and comparing with those deposited in the NCBI database through BLAST tool. Three 3 strains from agricultural soils were identified as *Metarhizium anisopliae* and only one as *Cordyceps brongniartii*. From dead insects, 7 strains were identified as *Beauveria bassiana*, 2 as *C. brongniartii* and 2 more as *M. anisopliae*. The phylogenetic analyses confirmed the close relationship among the strains identified as *B. bassiana* and among those *M. anisopliae* strains and shows that some of these entomopathogenic fungi are novel strains which may have potential as biological insecticides for different insect pests.

KEYWORDS

Biological control;
18S rRNA sequencing;
Tenebrio molitor;
Cordyceps brongniartii.

INTRODUCTION

Actually, food production, in particularly vegetables production has the problem to preserve a high quality level, considering aspects like food safety, sustainable production systems and fair compensation for producers[1]. Therefore, synthetic chemical pesticides are applied in order to protecting crops but this practice pro-

motes environment pollution. Also some of these pesticides can contaminate soil and water and can be toxic to other organisms including human[2]. In Mexican agriculture is common and important, the plant and fruit damage for some pests like Spodoptera frugiperda (Smith), the most important pest for maize[3]; Bemisia tabaci (Gennadius) which attacks cotton, melon, watermelon and soybean crops[4] and Anthonomus grandis (Boheman), considering the major pest of cotton[5]. For this reason, the agricultural production systems have tended to the use of methods for pest control which are more rational and friendly with the environment[6]. Agricultural producers are replacing synthetic insecticides for more advantageous alternatives using an Integrated Pest Management (IPM). The IPM is based in cultural practices aimed to controlling pests, plant capacity to resist pest damage and pest mortality by natural factors, like parasitoids, predators and pathogens[7].

Biological control as a fundamental part of IPM has extended the use of microorganisms for pest control. The microorganisms used for this target are virus, bacteria and fungi[8]. These microorganisms may cause the direct death of the attacked insect species[9]. Particularly, fungi are one of the best alternatives for pest control. More than 750 fungal species have been documented infecting insects (National Academy of Sciences, 1979). The fungal species more used as biological insecticides include *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin[9,10]. *Beauveria bassiana* attacks over 200 insect species, including pests of agricultural importance like Hypothenemus hamperi[11], Plutella xylostella (Linnaeus)[12] and Cosmopolites sordidus (Germar)[13]. On the other hand, *Metarrizium anisopliae* attacks naturally more than 300 insect species, for example Aeneolamia varia (Fabricius), which attacks sugar cane plantations[10], although, fungal virulence and infection level may vary among fungal strains, by this reason is important to have different strains of each of the entomopathogenic fungal species.

Use of microorganisms as bio-insecticides involves numerous laboratory and field tests to confirm their natural presence in the environment, virulence, environmental factors effect and their correct identification[9]. Actually, fungi identification is made using morphological traits as well DNA sequencing to establish phylogenetic relationships among organisms[14]. The most common morphological characteristics used for fungi identification are: growth type, shape and size of spore and reproductive structures type, as well as extracellular protein profiles and growth nutrient requirements, which sometimes are insufficient for an accurate identification to specie level[15]. The molecular characterization consist specifically in differentiate individuals of interest in accordance to their genetic variations or DNA polymorphisms though analysis of their sequences and nucleotide combinations[16]. The present work was carried out to performed morphological and molecular identification of new entomopathogenic fungal strains isolated from soil samples and dead insects and to determine the phylogenetic relationships among the isolated fungal in order to identify new and more effective entomopathogenic strains than current alternatives for use in the biological control of pest insect.

MATERIALS AND METHODS

Soil sampling

The fungal strains were isolated from 12 soil samples collected at three agricultural Mexican regions: two localized in the State of Coahuila (Saltillo y Torreon) and another one, near to Mexico City (TABLE 1). The sampling was performed at random based on the procedure described by Sanchez et al.[17]; for this, 1/3 kg of soil was taken from three different places of each agricultural plot. Then, the three soil samples were mixed and 1 kg of total weight was taken. This procedure was repeated for each agricultural soil sampling. The collected samples were moistening with water and placed in hermetic plastic containers, where 10 fresh larvae of *Tenebrio molitor* were placed. These containers were maintained closed during 7 days to 25 ºC. The infected larvae were placed in a Petri sterile dish on filter moistening paper to 25 ºC. The fungal growth on the larvae was monitored. The sporulated fungal strains isolated on the larvae were inoculated on Perti dish with PDA medium and incubated to 25 ºC during one week approximately. The fungal isolates were purified by monospore cultures on water-agar (AA: 18 g agar in 1000 mL distilled water), and increased on PDA. Later, the fungal strains were spread on liquid medium (Pontecorvo) in order to obtain spores and mycelium

FULL PAPER

production for DNA isolation. This biological material was conserved in a solution of skim milk and glycerol (9:1) at -17 ºC.

TABLE 1 : Soil samples tested, plant associated, origin and code

Code	Plant associated to the sample or soil code	Origin of the sample
Mez	Prosopis laevigata	Buenavista Coahuila
HV	Vegetables crops	Buenavista Coahuila
M	Zea mays	Buenavista Coahuila
Nop	Opuntia ficus indica	Buenavista Coahuila
Pin	Pinus sylvestris	Buenavista Coahuila
HJ	Garden soil	Saltillo Coahuila
S1	Soil 1	Distrito Federal Mexico
S2	Soil 2	Distrito Federal Mexico
S3	Soil 3	Distrito Federal Mexico
S4	Soil 4	Distrito Federal Mexico
ST1	Torreon Soil 1	Torreon Coahuila
ST2	Torreon Soil 2	Torreon Coahuila

Insect sampling

Amphidees latifrons and Musca domestica dead adult insects were collected in different locations of The State of Sinaloa, Mexico. In addition, dead adult insects belonging to the Coreidae family from Arteaga Coahuila, Mexico were collected (TABLE 2). The insects were sectioning into head, thorax and abdomen. Each section was disinfected used sodium hypochlorite (1.5%) by immersion for 3 min, after those insect sections were washed with sterile distilled water for 1 min and dried on sterile paper towels. Insect sections were placed on Petri dishes containing potato dextrose agar (PDA: 20 g potato, 20 g of dextrose, 18 g agar and 1000 mL distilled water) as culture medium, placing 4 pieces per plate. The Petri dishes were incubated to 25±1 °C and a continuous black light lamp 40 W was using during the day. The fungal isolates were purified by monospore cultures on water-agar (AA: 18 g agar in 1000 mL distilled water), and increased on PDA. In addition, for obtaining biomass for DNA isolation, the fungi were inoculated in 150 mL of liquid medium (Pontecorvo), which was incubated in agitation during 5-10 days at 25 ºC. The biomass generated was separated from the culture medium by filtration using a vacuum system. The samples were frozen at -17 ºC.

In addition, as control were used 5 microbial isolates identified as entomopathogenic fungal strains from the microorganisms bank belonging to the Applied Microbiology Center (CEMAP) GreenCorp Biorganiks de México SA de CV. Saltillo, Coahuila, México (TABLE 3).

TABLE 2 : Fungal strains isolated from insects pest adult dead and their code and origin

Code	Insect host	Origin of the isolate
Co1	Coreidae family	Sinaloa
Co2	Coreidae family	Sinaloa
Co3	Coreidae family	Sinaloa
Co5	Coreidae family	Sinaloa
Me1	Amphidees Latifrons	Arteaga Coahuila
Me3	Amphidees Latifrons	Arteaga Coahuila
Bb3	Amphidees Latifrons	Arteaga Coahuila
Bb4	Amphidees Latifrons	Arteaga Coahuila
Bb6	Amphidees Latifrons	Arteaga Coahuila
MD4	Musca domestica	Sinaloa
MD5	Musca domestica	Sinaloa

TABLE 3 : Fungal strains used as control, code, insect host and origin

Code	Insect host	Origin of the isolate
Ma	Tettigonia viridissima	Cuba
Pf	Bemisia tabaci	México
Pl	Unknown	México
Nr	Spodoptera frugiperda	México
Vl	Unknown	México

Morphological characterization

The macroscopic characterization of fungal strains was performed on the monospore cultures. The observed traits were: colony growth, appearance, texture and coloration in both faces of the Petri dish. The microscopic description was performed by staining the fungal spores and reproductive structures with lactophenol blue and then observed under a composed microscope and identified by morphology using taxonomic keys for the principal reproductive structures of fungi. Also, it was measured the length and diameter of these structures[18].

18S rRNA sequencing

To confirm the morphological identification of those fungi colonies and identify novel entomopathogenic strains that grown on insect larvae, DNA was isolated

using the methodology proposed by Ahrens and Seemüller[19]. Amplification of 18S rRNA was performed by PCR using the primers PN3 (5'-CCG TTG GTG AAC CAG CGG AGG GAT C-3') and PN10 (5'-TTC GCT TAT TGA TAT GCT TAA G-3'). PCR reaction was composed of sterile ultrapure water (14.5 μ L), 10X TBE buffer (2.5 μ L), $MgCl_2$ at 2.5 mM (2.08 μ L), dNTPs at 0.2 mM (2 μ L), primers PN3 and PN10 to 20 ρmol (2 μ L of each), DNA polymerase (Biogenic ®) 1U (0.2 μ L) and 80 ng of DNA sample (1 uL). PCR program consisted of an initial denaturing step of 95º C for 5 min, followed by 35 cycles of the following steps: 1 min at 94 ºC for denaturing, 1 min at 54º C for primers annealing and 1 min at 72º C for polymerization. Once the reactions were finished, the PCR products were resolved in agarose (1%) gel electrophoresis. The amplified products were sequenced in Perkin Elmer equipment by the Taq FS Dye Terminator Cycle Sequencing Fluorescence-Based Sequencing method. The sequences from the National Gene Bank Center for Biotechnology Information (NCBI) with the highest value of similarity were considered for comparison with the sequences obtained in this study. The 18S rRNA sequences obtained were aligned in the database of NCBI by the BLAST program (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST/). In order to reconstruct the phylogeny of the analyzed sequences, the MEGA 4.0 software with the UPGMA alignment option was used.

RESULTS

Isolation of entomopathogenic fungi strains

From the 12 soil samples were isolated 48 infected T. molitor larvae. For fungi isolation were chosen those larvae which presented an increased mycelial growth according with the morphology described for entomopathogenic fungi. In at least 5 soils was present more than one type of fungal growth. In the pine soil was found a greater number of infected larvae (TABLE 4). Fungal morphological characterization of the isolated and purified fungal strains from soil samples (TABLE 1) and dead insects (TABLE 2) were done according to the fungal taxonomical keys proposed by Domsch et al.[20]. Fungal strains isolated from soil samples were coded as follow; sample code plus initial of generous and specie per example: MezMa, *Metarhizium anisopliae* strain isolated from the Mez sample. Ten fungal strains were isolated from the soil samples, however only 4 out of 10 were selected (TABLE 5) for later analyses because the other 6 fungal strains were entomopathogenic but also have been identified as plant pathogens by this reason they were discarded.

TABLE 4 : Number of larvae infected (NIL) and number of larvae selected (NSL) of fungi isolation per sample

Soil	NIL	NSL	Soil	NIL	NSL
Mez	5	2	Soil 1	6	1
HV	5	1	Soil 2	4	1
M	3	1	Soil 3	2	0
Nop	3	0	Soil 4	3	0
Pin	8	2	ST1	3	1
HJ	4	1	ST2	2	0

TABLE 5 : Isolated fungal strains from cultivated soil samples

Code	Insect host	Origin of the isolate (Saltillo, Coahuila, México)
MezMa	Tenebrio molitor	Mesquite soil
PinMa	Tenebrio molitor	Pine tree soil
MezBb	Tenebrio molitor	Mesquite soil
HJMa	Tenebrio molitor	Garden soil

Beauveria bassiana (Bals.-Criv.) vuill

The Pf and Nr control strains; Co1, Co2, Co3, Co5, Bb3, Bb4, Bb6, MD4 and MD5 strains isolated from dead insect as well as MezBb isolated from soil samples were characterized as *Beauveria bassiana* in accordance with the following description: Macroscopic characteristics: flat cottony consistence, large growth, the colors vary between white and creamy white with the creamy yellow reverse. Microscopic characteristics: branched conidiophores, conglomerated conidiogenous cells, the conidia was observed rounded measuring at 1-2 μ of diameter and present septate and thin hyphae (Figure 1).

Metarhizium anisopliae (Metschnikoff) sorokin

The Ma control strain; Me1 and Me3 strains isolated from dead insects and HJMa, PinMa and MezMa strains isolated from soil samples were characterized

FULL PAPER

as *Metarhizium anisopliae* according with the following description: Macroscopic characteristics: cottony and powdery consistence, radial and plane growth, the color vary between olive green, yellow and white and present a reverse of color opaque yellow to light brown. Microscopic characteristics: branched conidiophores with cylindrical phialides which become thin toward the tip, the conidia are ovoid measuring 4-6 µ long and 1-2 µ width formed a chain and present septate hyphae (Figure 2).

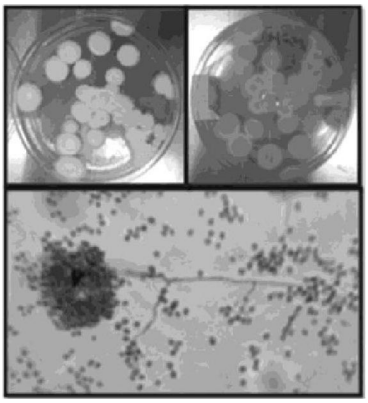

Figure 1 : Morphological characteristics of the *Beauveria bassiana* strains

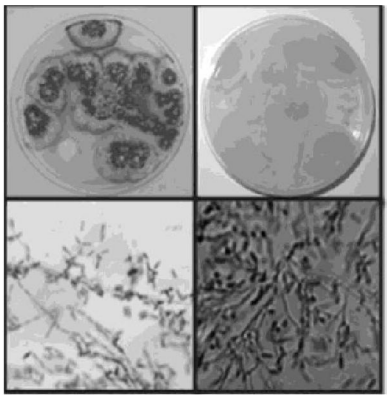

Figure 2 : Morphological characterization of the *Metarhizium anisopliae* strains

Paecilomyces lilacinus (Thom) samson

Only the Pl control strain was identified as *Paecilomyces lilacinus* according to the following description: Macroscopic characteristics: flat powdery consistence, extended radial growth, violet color and the reverse was observed from white to pink. Microscopic characteristics: verticillate branching conidiophores with broom form, the phialides are tapering towards the tip, the conidia are elliptical measuring 2.5-4 µ of diameter and observed septate hyphae (Figure 3).

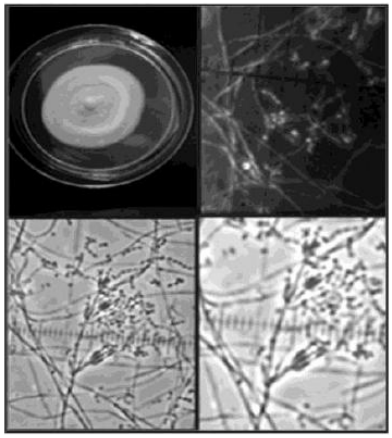

Figure 3 : Morphological characterization of the *Paecilomyces lilacinus* strain

Verticillium lecanii (Zimmerman) viegas

The Vl control strain was characterized as *Verticillium lecanii* according to the following description: Macroscopic characteristics: flat cottony consistence, extended radial growth and white color as well as reverse. Microscopic characteristics: the conidiophores present of 3 to 4 whorls of branching and growth of form apical on the hyphae. The conidia were observed ellipsoidal measuring 3 to 5 µ of diameter, and present septate hyphae with thin wall (Figure 4).

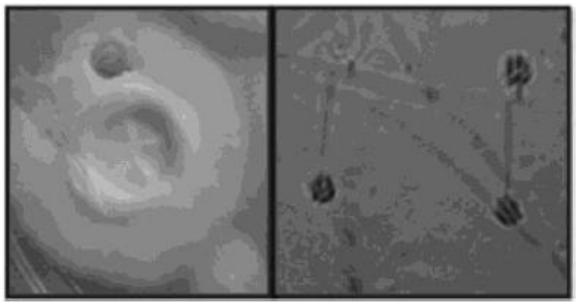

Figure 4 : Morphological characterization of *Verticillium lecanii* strain

18S rRNA sequences analysis

Most of DNA bands in the agarose gel were observed as defined and presented a high molecular weight. Some samples were treated with 15 µL of RNAsa (10 µg/ml) at 37 ºC for 30 min to eliminate RNA contamination. The DNA concentrations obtained in most of the cases were higher than those required concentrations for PCR amplification according to that described by Valadez y Kahl[21], therefore some samples were diluted in the proportions 1:10 and 1:20. The small subunit (SSU) 18S rRNA has repetitive ar-

rangement within the genome, providing excessive amounts of template DNA for PCR[22]. In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers. In this study, this region was amplified using the PN3 and PN10 primers which allowed obtaining uniform and defined bands of approximately 600 bp according to molecular marker of 100 bp used as reference (Figure 5).

The DNA sequences from the isolated entomopathogenic fungal strains were analyzed using the BioEdit program, where these sequences were aligned and compared with those deposited in the NCBI database. The fungal sequences must had a percent of maximum identity higher than 97 % according to described by Kruger et al.[23], so that these sequences were accepted as belonging to the same species. Therefore, according to the results shown in the TABLE 6, most of the analyzed sequences were identified similar to some sequence of the NCBI database.

TABLE 6 : Fungal DNA sequences analysis for all fungal strains tested in this study

Code	Description	Coverage	E. value	Max. Identity
Strains from dead insects				
Co1	*Beauveria bassiana*	100%	0.0	99%
Co2	*Beauveria bassiana*	96%	0.0	100%
Co3	*Beauveria bassiana*	99%	0.0	100%
Co5	*Beauveria bassiana*	100%	0.0	99%
Bb3	*Isaria farinosa*	68%	5e-100	92%
Bb4	*Cordyceps bassiana*	100%	0.0	100%
Bb6	*Cordyceps brongniartii*	98%	0.0	99%
Me1	*Metarhizium anisopliae*	49%	0.0	99%
Me3	*Metarhizium anisopliae*	99%	0.0	99%
MD4	*Cordyceps bassiana*	100%	0.0	99%
MD5	*Cordyceps brongniartii*	99%	0.0	100%
Strains from soil samples				
MezMa	*Metarhizium anisopliae*	100%	0.0	100%
MezBb	*Cordyceps brongniartii*	99%	0.0	100%
PinMa	*Metarhizium anisopliae*	100%	0.0	100%
HJMa	*Metarhizium anisopliae*	96%	0.0	99%
Control strains				
Ma	*Metarhizium anisopliae*	100%	0.0	100%
Pl	*Paecilomyces lilacinus*	100%	0.0	100%
Vl	*Beauveria bassiana*	100%	0.0	100%
Pf	*Nomuraea rileyi*	100%	0.0	100%
Nr	*Cordyceps bassiana*	100%	0.0	97%

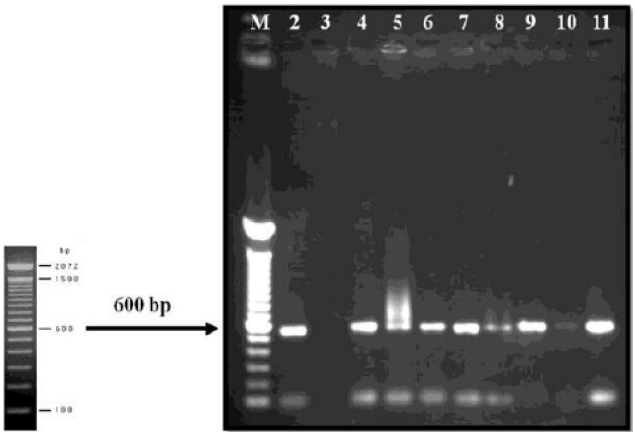

Figure 5 : 18S rRNA amplification from different entomopathogenic fungal strains: lane 1 molecular marker 100 bp, lines 2-7, PinMa isolate; lanes 8-9, Bb4 isolate; lane 10, MD4 isolate; and lane 11, MezBb isolate

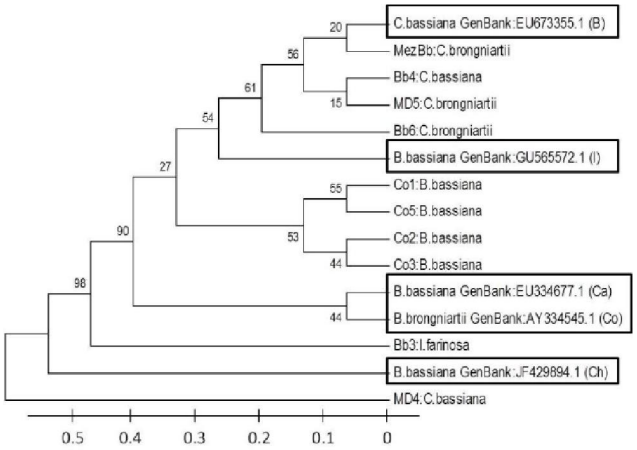

Figure 6 : Phylogenetic tree of DNA sequences from the fungal strains identified as belonging to the beauveria (Cordyceps) generous compared with those sequences reported in GenBank of NCBI (Brazil, India, Canada, Colombia and China)

Phylogenetic analyses

Phylogenetic analyses To analyze the degree of genetic relativeness among the identified fungal strains belonging to the two most common species (*B. bassiana and M. anisopliae*) found in this study, a phylogenetic tree using the MEGA 4.0 program with UPGMA alignment option and performing 1000 repetitions per alignment was built. According to the Figure 7, most of the strains identified as belonging to the *Beauveria* generous are related because these lie in the same group of the phylogenetic tree. However, according the comparison between sequences of fungi insolated and sequences reported in the GenBank of NCBI from several countries can be observed the dif-

FULL PAPER

ferences in the genetic distances into the phylogentic tree. In the Figure 8 is shown the phylogenetic tree of the samples identified as *Metarhizium anisopliae*, which belong to same group and are highly related, but some of them presented differences in the phylogenetic distances.

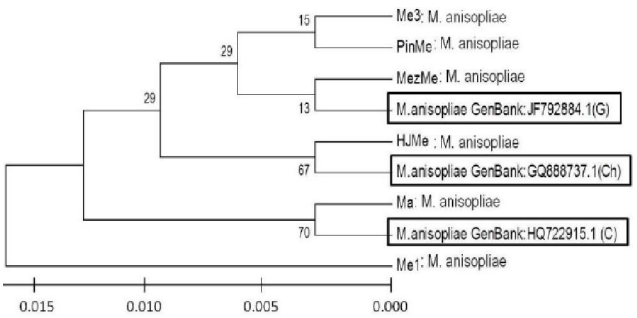

Figure 7 : Phylogenetic tree of DNA sequences from the fungal strains identified as belonging to the *Metarhizium anisopliae* specie compared with those sequences reported in GenBank of NCBI (Greece, China and Colombia)

DISCUSSION

Isolation of entomopathogenic fungal strains from agricultural soils is a process which may be influenced by different factors, among them, environmental factors. These microorganisms can live forming part of the natural flora of the ecosystem, as unwanted contamination or acting as antagonists of others harmful organisms for the ecosystem[24]. According to the morphological and molecular characterization, the isolated strains from agricultural soils using T. molitor larvae as host, HJMa, PinMa and MezMa strains were identified as *Metarhizium anisopliae* and MezBb strain was identified as *Cordyceps bassiana*.

Metarhizium anisopliae is an entomopathogenic fungus that naturally attacks more than 200 insect-pest species[25]. This is a fungus with asexual reproduction. Infections of arthropods by *Metarhizium* species are easily recognized few days after insect death, when the fungus grows out of the arthropod integument and form reproductive structures. Initially, one only sees fungal hyphae that appear white, but, as conidia form and mature them often take a characteristic olive green color. However, depending on the species and strain of *Metarhizium*, spores can range in color from white to yellow to brown and green[26].

On the other hand, the *Cordyceps* generous is the anamorph (sexual form) of *Beauveria* in agreement with that reported by Zheng et al.[27]; therefore, it is the same generous and specie than *B. bassiana*. The remaining isolated strains presented morphologic characteristics according to the entomophthorales and mucorales orders, which also are classified as entomopathogenic fungi according to Tanada and Kaya[28]. However, the use of these types of fungi for biological control has been controversial because some of them also are phytopathogens[29] by this reason these strains were discarded in this study.

The isolated strains from dead insect and coded as Co1, Co2, Co3, Co5, Bb4 and MD4 were identified as *Beauveria bassiana*. In this case, these fungal strains were isolated from different insect hosts which were collected in different Mexican regions, which suggest that the place of collect and the host insect were not determinants for *B. bassiana* isolation. When *B. bassiana* spores come in contact with the cuticle of susceptible insects, they germinate and grow to the inner body of their host and may produce toxins[30]. Also, the Bb6 and MD5 strains were identified as belonging to the *Beauveria* generous according to their 18S rRNA sequence. However, these strains were identified as *Cordyceps* (*Beauveria*) brogniartii. Although, this specie is more similar to *B. bassiana*, present some microscopic differences in shape and size of conidiogenous cells[31]. In this case, it is necessary to performance a more specific biochemical analysis to determine the differences of this strain with those belonging to *B. bassiana*. The Bb3 strain was morphologically similar to the *B. bassiana* strains, but molecular identification was not entirely specific because the identity percent was lower than 93%. For this reason, it is suggested that this strain belong to the *Beauveria generous*, but, it maybe a specie non-previously reported in the NCBI data base. Finally, the Me1 and Me3 strains were identified as *Metarhizium anisopliae* according with the morphological and molecular characterization.

The control strains proportionated by CEMAP, were morphological and molecularly identified as entomopathogenic strains according to the characteristics reported by Badii[6]. However, only Ma and Pl strains agreed with the previously identification as *Metarhizium anisopliae* and *Paecilomyces lilacinus*.

The Vl, Pf and Nr strains were identified according to their 18S rDNA sequences as *Beauveria bassina*, *Nomuraea rileyi* and *Cordyceps bassiana* respectively. These differences may be attributed to cross contamination by the handling of samples.

The small subunit (SSU) 18S rRNA ribosomal is highly conserved presenting common regions in all microorganisms, but has variations which are concentrated in specific areas[22]; by this reason, this sequence is one of the most frequently used genes in phylogenetic studies. This region is exposed to similar selective forces in all living beings[32]. In this study, a phylogenetic analysis was performance based in the 18S rRNA sequences of *B. bassiana and M. anisopliae*. Only these two generous were used because they were the principal fungal groups isolated and considering that they are two of the most important entomopathogenic fungi used in biological control, and these fungal species seem not to infect humans or other animals and are considered safe as an insecticide[33]. All samples identified as belonging to the *Beauveria* generous are related, in particularly Co1, Co2, Co3 and Co5 samples (Figure 7), although they have genetic differences to be considered as distinct. However, it is interesting to test the entomopathogenic potential of some *Beauveria* strain like Bb3, Bb4, Bb6, MD4, MD5 and MezBb because these strains are more different, and it has long been recognized that many entomopathogenic isolates are insect-specific. Less genetic variation was found in the phylogenetic analysis of the *M. anisopliae* strains, it is interesting also test these strains for their entomopathogenic potential. A large number of *M. anisopliae* isolates that are adapted to certain groups of insects have been isolated, but they have now been assigned as new *Metarhizium* species, such as *M. anisopliae*, *M. majus* and *M. acridum*. For example *Metarhizium taii* was placed as *M. anisopliae* var. *anisopliae*[32]. The differences between the insolated strains were corroborated with the comparison of sequences reported in GenBank of NCBI. Although the strains insolated in this study and strains insolated in others countries belong to the same species these are differentiated in the nucleotides arrangement even when they have a common ancestor, since these have adapted to different ecosystems and environmental factors may determinate their characteristics. The isolated and identified new strains in this study can be used in the biological control of insect pest, according with the isolation criteria; however, before including these microorganisms in commercial preparations as insecticides, different pathogenic and field test must be performed.

CONCLUSIONS

In this work, we were able to isolated 10 entomopathogenic fungi strains from cultivated soils of three different regions of Mexico, however only four of them were identified as entomopathogenic fungi. The 15 fungal strains isolated from dead insects and soil samples were identified as follow: six fungal strains as *B. bassiana* (*C. bassiana*), three as *C. brongniartii*, five as *M. anisopliae* and only one was was not identified at the specie level but belonging to the *Beauveria* generous according to the molecular identification. The phylogenetic analysis confirmed the close relationship between Co1, Co2, Co3 and Co4 strains identified as *B. bassiana* as well as all strains identified as *M. anisopliae*. This information shows that some of these entomopathogenic fungi are novel strains which may have potential as biological insecticides for different insect pests.

ACKNOWLEDGEMENTS

This investigation was supported by a collaborative funding grant to ABC SA de CV. Project M0005 208-C06-138917 from the National Council of Science and Technology of Mexico (CONACYT). MGC thanks to CONACYT, for the financial support, during her BSc degree studies.

REFERENCES

[1] C.Garcia, M.González; Use of bio-insecticides for the control of vegetables pest in rural communities, Ra Ximhai, **6**, 17-22 **(2010)**.

[2] W.Aktar, D.Sengupta; A.Chowdhury; Impact of pesticides use in agriculture: their benefits and hazards, Interdisc Toxicol, **2**, 1-12 **(2009)**.

[3] S.Perez, M.A.Zavala, M.M.González, N.C.Cárdenas, M.A.Ramos; Bioactivity of carica papaya (Caricaceae) against spodoptera frugiperda (Lepidoptera: Noctuidae), Molecules, **16**, 7502-7509

(2011).

[4] M.R.V.Oliveira, T.J.Henneberry, P.Anderson; History, current status, and collaborative research projects for Bemisia tabaci, Crop Protection, **20**, 709-723 **(2001)**.

[5] T.Stadler, M.Buteler; Migration and dispersal of Anthonomus grandis (Coleoptera: Curculionidae) in South America, Rev Soc Entomol Argent, **66**, 205-217 **(2007)**.

[6] M.H.Badii, J.L.Abreu; Biological control a sustainable way of pest control, International Journal of Good Conscience, **1**, 82-89 **(2006)**.

[7] R.Dufour; Bio-intensive Integrated Pest Management (IPM). Fundamentals of Sustainable Agriculture. ATTRA., **52**, 2-12 **(2001)**.

[8] M.M.El-Husseini; Microbial control of insect pests: is it an effective and environmentally safe alternative. Arab J.Pl Prot, **42** 162-169 **(2006)**.

[9] L.A.Rodriguez, H.C.Arredondo; Theory and application of biological control. Mexican Society of Biological Control, México, 1-303 **(2007)**.

[10] A.Monzon; Production, use and quality control of entomopathogenic fungi in Nicaragua, Advances in the promotion of non-synthetic phytosanitary products, Integrated Pest Management, **63**, 95-103 **(2001)**.

[11] J.Jaramillo, C.Borgemeister, P.Baker; Coffee berry borer hypothenemus hampei (Coleoptera: Curculionidae): searching for sustainable control strategies, Bulletin of Entomological Research, **96**, 223-233 **(2006)**.

[12] M.Chong, M.Maldonado, J.Hernández, L.Galan, C.Sandoval; Study of *Beauveria bassiana* growth, blastospore yield, desiccation-tolerance, viability and toxic activity using different liquid media, African Journal of Biotechnology, **10**, 5736-5742 **(2011)**.

[13] V.Tumuhaise, C.M.Nankinga, C.S.Gold, S.Kyamanywa, W.K.Tushemereirwe, P.Ragama, D.Moore, S.R.Gowen, D.Moore, S.R.Gowen; Kairomone trapping for delivery of *Beauveria bassiana* to control the banana weevil, Cosmopolites sordidus (GERMAR), African Crop Cience Conference Proceedings, **6**, 346-351 **(2003)**.

[14] P.Inglis, M.Tigano; Identification and taxonomy of some entomopathogenic *paecilomyces* spp (Ascomycota) isolates using rDNA-ITS Sequences, Genetics and Molecular Biology, **29**, 132-136 **(2006)**.

[15] L.Bielikova, Z.Landa, L.Osborne, V.Curn; Characterization and identification of entomopathogenic and mycoparasitic fungi using RAPD-PCR Technique, Plant Protection Science, **38**, 1-12 **(2002)**.

[16] A.Azevedo, M.Furlaneto, D.Sosa, M.Fungaro; Molecular characterization of *Paecilomyces* fumosoroseus (Deuteromycotina: Hyphomycetes) isolates. Scientia Agricola, **57**, 729-732 **(2000)**.

[17] S.Sanchez, J.San-Juan, R.Medina; Occurrence of entomopathogenic fungi from agricultural and natural ecosystems in Saltillo, México, and their virulence towards thrips and whiteflies. Journal of Insect Science, **11**, 1-10 **(2010)**.

[18] V.Cañedo, T.Ames; Laboratory Manual for Entomopathogenic Fungi Management, Potato International Center (CIP), Lima, Perú, 62 **(2004)**.

[19] U.Ahrens, E.Seemüller; Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene, Phytopathology, **82**, 828-832 **(1992)**.

[20] K.Domsch, W.Gams, T-H.Anderson; Compendium of soil fungi, Academic Press, **2**, 1264 **(1980)**.

[21] E.Valadez, K.Günter; DNA fingerprinting in plant genomes (theory and laboratory protocols). Mundi-Prensa (Ed); México, **(2005)**.

[22] A.Meyer, C.Todt, N.Mikkelsen, B.Lieb; Fast evolving 18S rRNA sequences from solenogastres (Mollusca) resist standard PCR amplification and give new insights into mollusk substitution rate heterogeneity, BMC Evolutionary Biology, **10**, 1-12 **(2010)**.

[23] D.Kruger, D.Kapturska, C.Fischer, R.Daniel, T.Wubet; Diversity measures in environmental sequences are highly dependent on alignment quality-data from ITS and New LSU primers targeting Basidiomycetes, Plos one, **7**, 1-21 **(2012)**.

[24] A.Turbe, A.De Toni, P.Benito, P.Lavelle, P.Lavelle, N.Ruiz, W.Van der Putten, E.Labouze, S.Mudgal; Soil biodiversity: functions, threats and tools for policy makers, Bio Intelligence Service, IRD, and NIOO, Report for European Commission (DG Environment), 254 **(2010)**.

[25] L.Liu, R.Zhan, L.Yang, C.Liang, D.Zeng, J.Huang; Isolation and identification of *Metarhizium anisopliae* from chilo venosatus (Lepidoptera: Pyralidae) cadaver, African Journal of Biotechnology, **11**, 7609-7617 **(2012)**.

[26] V.Gouli, S.Gouli, M.Brownbridge, M.Skinner, B.Parker; Manual for mass production of entomopathogenic fungi in developing countries with particular reference to *Beauveria bassiana* and

Metarhizium anisopliae. Insect Pest Management with fungi: A mass Production Technique for Farmers, 50 **(2005)**.

[27] P.Zheng, Y.Xia, G.Xiao, C.Xiong, X.Hu, S.Zhang, H.Zheng, Y.Huang, Y.Zhou, S.Wang, G.Zhao, X.Liu, R.Leger, C.Wang; Genome sequence of the insect pathogenic fungus *cordyceps* militaris, a valued traditional chinese medicine, Genome Biology, **12**, 1-21 **(2011)**.

[28] Y.Tanada, H.Kaya; Insect pathology, Academic Press, San Diego, California, USA. 666 **(1993)**.

[29] E.Garces, M.Orozco, G.Bautista, H.Valencia; Fusarium oxysporum, the fungus that we need to know, Acta Biológica Colombiana, **6**, 1-20 **(2001)**.

[30] E.-J.Scholte, B.Knols, R.Samson, W.Takken; Entomopathogenic fungi for mosquito control: A review, Journal of Insect Science, **4**, 19-24 **(2004)**.

[31] J.-M.Sung, J.-O.Lee, R.Humber, G-H.Sung, B.Shrestha; *Cordyceps bassiana* and production of stromata in vitro showing *Beauveria* anamorph in Korea, Mycobiology, **34**, 1-6 **(2006)**.

[32] J.F.Bischoff, S.A.Rehner, R.A.Humber; A multilocus phylogeny of the *Metarhizium anisopliae* lineage, Mycologia, **101**, 512-530 **(2009)**.

[33] A.Sevim, M.Hofte, Z.Demirbag; Genetic variability of *Beauveria bassiana* and *Metarhizium anisopliae* var. Anisopliae isolates obtained from the Eastern Black Sea Region of Turkey. Turk J.Biol, **36**, 255-265 **(2012)**.