Research

Isolation of native strains of entomopathogenic fungi from agricultural soils of northeastern Mexico and their virulence on *Spodoptera exigua* (Lepidoptera: Noctuidae)

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Abstract

Spodoptera exigua Hübner (Lepidoptera: Noctuidae) is considered a polyphagous pest distributed worldwide with a wide range of hosts, and is also considered a serious plague for vegetables and ornamentals. The objective of this study was to explore the occurrence of entomopathogenic fungi in agricultural soils from Mexico and to evaluate its virulence and effect on the life cycle of *S. exigua*. Selected isolates (HEB1 and γ01) were used to perform toxicity bioassays on neonatal larvae of *S. exigua*; additionally, the GHA (*Beauveria bassiana*), Pfr-612 (*Cordyceps fumosorosea*), Ma, and 2 isolates HIB-11 and HIB-12 (*Metarhizium anisopliae*) were included to obtain the median lethal concentration (LC_{50}) and the median lethal time (LT_{50}). Also, the life cycle of the surviving individuals was followed up after treatment to document the effects of the entomopathogenic fungi application. The isolates belong to 2 different species, which are *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and *Cordyceps fumosorosea* (Wize) Kepler, B. Shrestha & Spatafora (Hypocreales: Cordycipitaceae) (formerly *Isaria fumosorosea*). In toxicity tests, the isolate HIB-12 of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Cordycipitaceae) showed better mortality results against *S. exigua* than the other fungi evaluated with LC_{50} of 3.17×10^6 conidia per mL. The lethal time of LT_{50} oscillated between 3 to 5 d and the HIB-12 isolate had the lowest lethal time (3.15 d). During the monitoring of the biological cycle after treatment, the greatest interruption of the metamorphosis was caused by the GHA strain, while the Ma strain was the one that induced the slightest interruption of the biological cycle. These results show the feasibility of using strains of *M. anisopliae* for control of larval stages of *S. exigua* and their possible formulation for agricultural use, as well as opening a way for the exploration and research of mechanisms or metabolites involved in the interruption of metamorphosis due to the application of entomopathogenic fungi, specifically *B. bassiana*.

Key Words: beet armyworm; *Beauveria bassiana*; toxicity; *Metarhizium anisopliae*; metamorphosis

Resumen

Spodoptera exigua Hübner (Lepidoptera: Noctuidae) se considera una plaga polífaga, distribuida en todo el mundo que tiene una amplia gama de hospedadores, siendo una plaga grave de vegetales y ornamentales. El objetivo de este estudio fue explorar la aparición de hongos entomopatógenos en suelos agrícolas de México, y evaluar su virulencia y su efecto en el ciclo de vida de *S. exigua*. Los aislados seleccionados (HEB1 y γ01) se utilizaron para realizar bioensayos de toxicidad sobre larvas neonatas de *S. exigua*, adicionalmente se incluyeron las cepas de colección GHA, Pfr-612, Ma, y dos aislados HIB-11 e HIB-12, para obtener la concentración letal media (CL_{50}) y el tiempo letal medio (TL_{50}). Adicionalmente, los individuos sobrevivientes post tratamiento se le dio seguimiento a su ciclo de vida para documentar los efectos de la aplicación de los entomopatógenos. Se encontraron hongos entomopatógenos en el 60% de las muestras de suelo. Los aislamientos obtenidos pertenecen a dos especies diferentes, *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) y *Cordyceps fumosorosea* (Wize) Kepler, B. Shrestha & Spatafora (Hypocreales: Cordycipitaceae) (*Isaria fumosorosea*). En los ensayos de toxicidad, el aislamiento HIB-12 de *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Cordycipitaceae) mostró mejores resultados de mortalidad contra *S. exigua* que los otros hongos evaluados con una CL_{50} de 3.17×10^6 conidios por mL. Respecto al TL_{50} este osciló entre los tres y cinco días y fue el aislado HIB-12 el que obtuvo el menor tiempo letal (3.15 días). En el monitoreo del ciclo biológico después del tratamiento, la mayor interrupción de la metamorfosis fue causada por la cepa GHA, mientras que la cepa Ma fue la que indujo la menor interrupción del ciclo biológico. Estos resultados muestran la factibilidad de utilizar cepas de *M. anisopliae* para el control de estadios larvarios de *S. exigua* y su posible formulación para uso agrícola, así como también abren un camino para la exploración y búsqueda de los mecanismos o metabolitos involucrados en la interrupción de la metamorfosis por efecto de la aplicación de hongos entomopatógenos, específicamente *B. bassiana*.

Palabras Clave: gusano soldado; *Beauveria bassiana*; toxicidad; *Metarhizium anisopliae*; metamorfosis

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The beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), is considered as a polyphagous pest with worldwide distribution, and originally is native to southern China (Kolodny-Hirsch et al. 1997). The beet armyworm has a wide range of hosts, and is a pest of many agricultural and horticultural crops (Capinera 2002). In Mexico, *S. exigua* was found sporadically in cotton crops (*Gossypium* spp. L. [Malvaceae]) in 1990; however, in 1993 it became the main pest of this crop (Obando-Rodriguez & Blanco-Montero 1997; Garza & Teran 2007) and spread to other host plants. *Spodoptera exigua* has been reported as a pest of several important crops in Mexico, such as bean (*Phaseolus vulgaris* L. [Fabaceae]), tomato (*Solanum lycopersicum* L. [Solanaceae]), cotton, yucca (*Manihot esculenta* Crantz [Euphorbiaceae]), corn (*Zea mays* L. [Poaceae]), rice (*Oryza sativa* L. [Poaceae]), and sorghum (*Sorghum bicolor* L. Moench [Poaceae]) (Ruiz-Corral et al. 2013). *Spodoptera exigua* larvae feed on both foliage and fruit. The young larvae feed gregariously and skeletonize the foliage. As they mature, the larvae become solitary and cause large irregular holes in the foliage when they feed. In the wild, *S. exigua* has numerous natural enemies such as parasitoids and predators (Capinera 2002), but they are not considered an important mortality factor when the damage has exceeded the economic threshold. Regarding control methods of this pest, the use of organochlorine, organophosphate, carbamates, and pyrethroid type synthetic insecticides, which are applied by spraying methods, remains the main strategy against the larval stages of *S. exigua*; however, the indiscriminate use of these insecticides, such as chlorpyrifos, tebufenozide, chlorfluazuron, and metaflumizone has caused resistance (Barrientos-Gutiérrez et al. 2013; Che et al. 2013; Tian et al. 2014), whereas studies on the biocontrol of *S. exigua* have focused mainly on nuclear polyhedrosis viruses (Khattab 2013; Wan et al. 2016) and *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae), which now are available commercially in many countries. However, *S. exigua* has developed *B. thuringiensis* resistance (Hernández-Martínez et al. 2010; Qiu et al. 2015). Mass production of nuclear polyhedrosis viruses requires much time and cost, and productivity is very low. Therefore, it is important to develop alternative biocontrol agents to control *S. exigua* (Han et al. 2014). Entomopathogenic fungi are proven microbial control agents of arthropods (Douglas Inglis et al. 2012), so they can be an alternative to using insecticides to control insect pests. There are more than 700 species in 100 genera in the world, but few have been studied thoroughly. This type of microorganism is associated with insects that live in diverse habitats, such as water, soil, and aerial parts; because of its characteristic form of infection, they are the most important microorganisms that infect sucking insects such as aphids, whitefly, scales, leafhoppers, and bedbugs (Alatorre 2007). Entomogenous fungi have evolved mechanisms for adhesion and recognition of host surface cues that help direct an adaptive response that includes the production of (a) hydrolytic, assimilatory, or detoxifying enzymes including lipase/esterases, catalases, cytochrome P450s, proteases, and chitinases; (b) specialized infectious structures, e.g., appressoria or penetrant tubes; and (c) secondary metabolites and other metabolites that facilitate infection (Ortiz-Urquiza and Keyhani 2013).

Mycopesticides are pesticides whose main active ingredient is composed of a fungus. *Beauveria bassiana* represents approximately 60% of the total market for mycopesticides, while *Lecanicillium lecanii* Zare & Gams (Hypocreales: Cordycipitaceae) and *Metarhizium* sp. account for 16% of this market (Zaki et al. 2020). Therefore, it is essential to continue the search for new isolates of entomopathogenic fungi that may have activity against insects, specifically Lepidoptera, which is of great importance in agriculture. To select or isolate a strain, the susceptibility of the insect and the state of development of the host must be taken into account because some insect entomopathogenic fungi have restricted their range of hosts, whereas other fungal species have a wide range of hosts, for example, *M. anisopliae*, *Metarhizium flavoviridae* Gams & Roszypal (Hypocreales: Cordycipitaceae), *Isaria farinosa* Persoon in Roemer, *B. bassiana*, and *Beauveria brongniartii* (Sacc.) (all Hypocreales: Cordycipitaceae) (Alatorre 2007). Hence, this work was aimed at exploring the occurrence of entomopathogenic fungi in agricultural soils from Tamaulipas State in Mexico, and to evaluate its virulence and effect on the life cycle of *S. exigua*.

Materials and Methods

SOIL SAMPLING

The sampling was performed in 10 agricultural fields located in the municipalities of Altamira and Aldama in southern Tamaulipas State. The size of each field tested was about 1 ha. The locations and altitudes of the sampled soils were recorded using global positioning system (GPS) equipment (Map 64sx, Garmin®, Olathe, Kansas, USA) (Table 1). Three hundred grams of superficial soil samples were collected with a garden spade taken from 5 randomly selected points from each area, which were then mixed and deposited in a plastic bag and transported to the laboratory.

ISOLATION OF ENTOMOPATHOGENIC FUNGI

For the isolation of entomopathogenic fungi, colonies of greater wax moth *Galleria mellonella* Fabricius (Lepidoptera: Pyralidae) were maintained in the L-6 Laboratory of the Institute of Biotechnology of the Autonomous University of Nuevo León, San Nicolás de los Garza, Nuevo León, Mexico. The moths were reared in plastic containers (14

Table 1. Sampled sites in agricultural soils from the southern region of Tamaulipas State, Mexico.

Location	Municipality	Elevation masl	Geographic location	
			Latitude (°N)	Longitude (°W)
Los Pepes	Altamira	30	22.284491	98.018979
Corpus Christi	Altamira	30	22.284450	98.134399
El catalán (Rancho de Piedra)	Altamira	40	22.273940	97.585557
Santa Rita	Altamira	40	22.275559	98.051994
El Alto	Aldama	30	22.403402	97.555129
Tabla Tirada	Aldama	20	22.403221	97.563399
Maclovio Herrera (Miradores)	Altamira	61	22.301479	98.549257
Benito Juárez	Altamira	36	22.294556	98.338534
El Chamizal	Altamira	30	22.340073	98.025585
Mata del Blanco (La Mata)	Altamira	40	22.314245	98.057157

L × 14 W × 16.5 H cm). These boxes were made in the laboratory with acrylic sheets, ventilated with a white tulle fabric lid and placed in total darkness at 28 °C. To achieve darkness, the plastic boxes were lined with brown paper and placed in a controlled area. An artificial diet for *G. mellonella* consisting of cereal mix (wheat, oats, rice, and barley) 600 g; honey bee 20 mL; glycerin 5 mL; bee pollen 5 g; vitamin solution 5 mL) and was used to feed both larvae and adults. The '*Galleria* bait method' was performed for fungal isolation (Zimmermann 1986). Soil samples were sifted in a strainer until obtaining 300 g of a fine powder after samples were placed in 14 L × 18 W × 6 H cm plastic containers. The samples were then dampened with sterilized distilled water, and 5 larvae of third instar *G. mellonella* were added per container. Containers were perforated laterally to allow soil aeration and incubated upside down at room temperature. Also, containers were flipped and shaken softly once every 2 d for 7 d to ensure maximum contact of larvae by soil (Ali-Shtayeh Mara'i & Jamous 2002). All larvae were removed at 7 d and their surface was sterilized with 0.05% sodium hypochlorite for 3 min, then washed 3 times in sterile distilled water, placed in a Petri dish of 60 × 15 mm containing a damp paper filter (Whatman No. 1, (Cytiva, Maidstone, United Kingdom) and incubated at 25 °C for 7 d to promote fungal development in a humidity chamber (Lab Companion, Seoul, South Korea) (Galán-Franco et al. 2011). Once mycelial growth was visible on the insect surface, samples were placed on potato dextrose agar supplemented with 500 ppm of chloramphenicol (Lezama-Gutiérrez et al. 2001). Plates were incubated at room temperature. Fungi isolates were subcultured to obtain axenic cultures. A pathogenicity test was carried out to discard opportunistic pathogens. Fungal isolates were sporulated on potato dextrose agar plates for 14 d. Five larvae of *G. mellonella* were placed with tweezers on top of each fungal culture for 30 s then transferred to Petri dish chambers containing an atmosphere with high humidity and incubated at room temperature in darkness until larvae death or pupation. The chambers were monitored daily. Mycelium growing out of dead larvae were analyzed to ensure it was the same as the inoculated. A pathogenicity test was carried out 3 times.

Identification of fungal isolates

Isolates were characterized morphologically according to the taxonomic keys of Humber (1997) and Cañedo and Ames (2004). Microculture techniques were used for slide preparation according to Riddell (1950), then conidia and reproductive structures (conidiophores) were observed in an optical microscope (Olympus CX31, Tokyo, Japan) at 10× and 40×. Subsequently, monosporic cultures were obtained for molecular identification. Fungal DNA isolation was performed by a modified version of the Raeder and Broda (1985) protocol. Fungal biomass was taken from plate cultures and added to 1.5 mL microcentrifuge tubes. Three hundred µL of Mili-Q water (Merck KGaA, Darmstadt, Germany) and 0.5 mm diam glass beads were added to the microcentrifuge tubes, which were subsequently placed at −70 °C for 1 h, then frozen preparations were macerated with a micro-pistil for 5 min in a mini vortex stirrer (ICB, Jalisco, Mexico). A solution of 500 µL of extraction buffer (200 mM Tris-HCl [pH 8.5], 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) was added and mixed until achieving a homogenous sample. DNA was extracted with 1 v/v of chloroform-octanol [24:1]. The aqueous phase was recovered by centrifugation at 13,000 rpm for 30 min and transferred to a new tube, then 1 v/v of isopropanol was added to the precipitate and incubated for 30 min at −20 °C. Samples were centrifuged at 13,000 rpm for 5 min. Isopropanol was removed carefully and the pellet was washed gently with 70% ethanol, and finally was suspended in Mili-Q water. Polymerase chain reactions were performed to amplify complete internal transcribed spacer (ITS) sequences from the 5.8S ribosomal DNA. Polymerase chain reactions were performed using oligonucleotide primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (TCCTCCGCTTATTGATATGC-3′), first an initial denaturation at 95 °C for 10 min, after 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 52 °C for 30 s and elongation at 72 °C for 2 min. Polymerase chain reaction products were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), they were then sent to Eurofins MWG Operon (Huntsville, Alabama, USA) for sequencing and subjected to a Basic Local Alignment Search Tool (BLAST) to identify similar sequences deposited in GenBank of the National Center for Biotechnology Information, Bethesda, Maryland, USA. Finally, a phylogenetic tree was reconstructed with the internal transcribed spacer regions of both isolates and their respective reference sequences.

BIOASSAYS

Insects

The breeding of *S. exigua* that was used in this research is from the L-11 Laboratory of the Institute of Biotechnology of the Autonomous University of Nuevo León, San Nicolás de los Garza, Nuevo León, Mexico, where the large area of insect breeding is located. The larvae were fed artificial diet of Shorey (100 g sucrose, 100 g wheat germ, 100 g yeast extract, 8 g sodium benzoate, 2 mL vitamin solution, HCl 5 mL concentrated, 650 mL water) (Shorey & Hale 1965); adults had 15% honey syrup solution added to the diet. Brown paper was used as an oviposition substrate. Breeding conditions were 26 °C, 65 to 70% RH, and 14:10 h (L:D) photoperiod.

Strains

For this study, we selected isolates of the previously isolated fungi with keys HEB (renamed HEB1, *B. bassiana*) and HKR (renamed γ01, *C. fumosorosea*) because they were the only ones that showed the capacity to sporulate in sufficient quantity for the preparation of the bioassay suspensions, 3 collection strains (GHA; *Beauveria bassiana*, Pfr-612; *Cordyceps fumosorosea* and Ma; *Metarhizium anisopliae*), and the native isolates HIB-11 and HIB-12 of *M. anisopliae* from the collection of the Institute of Biotechnology of the Autonomous University of Nuevo Leon, San Nicolás de los Garza, Nuevo León, Mexico, were stored cryogenically in a chest freezer Revco ULT 2090-9-A-31 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (10% glycerol at −80 °C). The strains were thawed at room temperature, inoculated on potato dextrose agar and incubated at 28 °C for 14 d.

Preparation of conidial suspensions

After each strain had been incubated for 14 d, 10 mL of Tween® 80 solution at 0.1% (v/v) was added, then the surface of each plate was scraped with a spreader to obtain a concentrated suspension of conidia. A Neubauer chamber (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to determine the concentration of these suspensions, and each suspension was adjusted to different concentrations (1.00×10^5; 1.00×10^6; 1.00×10^7; and 1.00×10^8 conidia per mL). The conidia suspensions were used immediately for bioassays.

Conidia viability

Spore viability was determined at the time of treatment by spraying 100 µL aliquots of conidia suspension at 10^6 conidia per mL onto potato dextrose agar plates (Petri dish of 10 cm in diam) and incubating at 25 °C for 18 h. Spore germination was halted by placing 3 separate drops of lactophenol cotton blue (Merck KGaA, Darmstadt, Germany),

then adding a coverslip over the stain droplet. The proportion of viable conidia was determined by examining 100 spores in each of these 3 different fields of view at 400× magnification with a compound microscope (Olympus, Tokyo, Japan), and determining the proportion of spores that possessed a distinct germ tube was defined by germ tube lengths that were 2 times the diam of the spore (Douglas Inglis et al. 2012).

Toxicity tests

For the inoculation, neonatal larvae of *S. exigua* were used. In a Petri dish of 90 × 15 mm, 3 mL of the treatment to be applied was added for 30 s. After this time, the suspension containing the larvae was poured carefully onto blotting paper to remove excess moisture; once they showed activity (movement), specimens were selected and each larvae was placed with a brush in cell culture plates of 24 wells (Corning Industries, Corning, New York, USA) containing the artificial diet of Shorey. Once the larvae were placed on the plate, the cap was placed and the sides were sealed with tape. The plates were incubated at 26 °C, at 65 to 70% RH for 14:10 h (L:D) photoperiod for 10 d, and were reviewed every 24 h for 10 d to record mortality. The dead larvae were placed in Petri dishes (60 × 15 mm) on a Whatman No. 2 filter paper and incubated in a humid chamber (> 85% RH) at 25 ± 2 °C to favor the development of fungi. Additionally, an absolute (untreated) control and 1 with 0.1% Tween 80 solution (v/v) also were placed. The bioassay was performed in quintuplicate on at least 3 occasions. The larvae that showed no signs of infection were incubated under the initial conditions (26 °C, 65–70% RH, at 14:10 h [L:D] photoperiod) and were monitored until their life cycle was completed.

Monitoring of the biological cycle after treatment

The bioassay with *S. exigua* neonatal larvae was developed for 10 d during which mortality was recorded daily for each concentration and fungus used. Once the incubation time of the bioassay was completed, the surviving larvae of each treatment were kept under the same conditions to document the post-treatment development. The follow-up period after treatment was 10 d. During this time, the daily review of the instars and the behavior were recorded, which included the aspects of survival and mortality including change of the biological cycle. In this sense, an analysis was performed to determine the mortality, survival, and interruption rates of the metamorphosis of the treated individuals.

STATISTICAL ANALYSIS

The experiment was a completely randomized design with 7 entomopathogenic fungi, 5 replications per fungi, and 24 larvae per replication. The experiment was done at least twice under the same conditions. Median lethal time (LT_{50}) and median lethal concentration (LC_{50}) values were estimated through a Probit Regression Analysis (SPSS version 19, IBM, New York, USA). The monitoring results of the biological cycle after treatment were expressed as a percentage with a confidence interval of 95%. The Kolmogorov-Smirnov test was used to verify the normality of data, 1-way ANOVA, was used to compare means and a Tukey test ($P \leq 0.05$). For bioinformatic analysis, sequences alignment with clustal W module and construction of a Maximum Likelihood tree were performed in the MEGA 6® software (Pennsylvania State University, University Park, Pennsylvania, USA).

Results

Isolation of entomopathogenic fungi

Twenty isolates were initially obtained; only 6 isolates were established finally as real entomopathogens. Entomopathogenic fungi were found in 60% of the soil samples (6 of 10 localities). The isolates belong to 2 different species: *B. bassiana* and *C. fumosorosea* (Table 2).

Identification of isolates

The conidial bodies of the isolates corresponded to both species *B. bassiana* and *I. fumosorosea* according to taxonomic keys through observations using the optical microscope. Polymerase chain reaction amplification generated fragments of about 600 base pairs. Internal transcribed spacer sequences of the isolates were aligned by BlastN searches with internal transcribed spacer sequences of references uploaded in GenBank, finding 98% identity between isolate HKS and *Isaria fumosorosea* isolate SKCH-1, 99% identity between isolates HGB and HKR, and the same reference strain SKCH-1, 99% identity between HEB and *B. bassiana* strain IIFT-B10, and HKB and *B. bassiana* strain CCTCC M 2017045, and 100% identity between HBB and *B. bassiana* isolate SASRI BB444. A phylogenetic tree was created with the internal transcribed spacer sequences of both isolates and reference strains in the bioinformatics program MEGA 6® (Pennsylvania State University, University Park, Pennsylvania, USA) (Fig. 1).

Dose evaluation to obtain lethal concentrations

As shown in Table 3, seven entomopathogenic fungi were assessed to determine their lethal concentrations for control of *S. exigua* larvae that showed toxicity values in which LC_{50} ranged from 10^6 to 10^8 conidia per mL. The isolate HIB-12 showed better mortality results to control *S. exigua* than the other fungi evaluated. In regard to the LT_{50}, it oscillated between 3 to 5 d and the HIB-12 isolate had the lowest lethal time (Table 4).g

Table 2. Different fungal isolates of sampled sites in agricultural soils from the southern region of Tamaulipas State, Mexico.

Location	Total samples	Positive samples	*Beauveria* sp.	*Isaria* sp.	*Aspergillus* sp.	*Fusarium* sp.	*Rhizopus* sp.
Los Pepes	5	2	0	1	0	0	0
Corpus Christi	5	5	1	0	2	2	0
El catalán (Rancho de Piedra)	5	1	1	0	0	0	0
Santa Rita	5	1	0	0	1	0	0
El Alto	5	3	0	1	1	0	1
Tabla Tirada	5	1	0	0	0	1	0
Maclovio Herrera (Miradores)	5	3	0	1	0	0	0
Benito Juárez	5	1	1	0	0	0	0
El Chamizal	5	2	0	0	1	0	0
Mata del Blanco (La Mata)	5	1	0	0	0	0	0

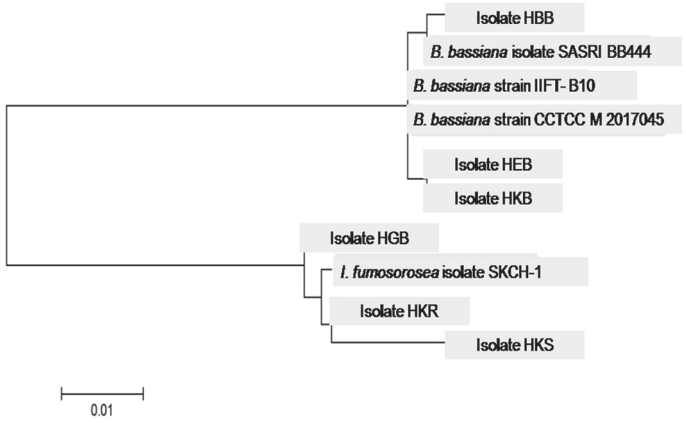

Fig. 1. Phylogenetic tree reconstructed from internal transcribed spacer sequences of the isolates compared with referenced internal transcribed spacer sequences deposited in the NCBI GenBank. The phylogram size bar represents a 1% sequence divergence. Labelled branches represent referenced internal transcribed spacer sequences.

The results show the mortality rates for each concentration and fungus evaluated, where higher average mortality can be observed in the highest concentrations (10^7–10^8 conidia per mL), and according to the results of the statistical analysis, there was no significant difference between the mortality according to the concentration used ($F = 0.422$; df = 3; $P = 0.740$) or fungus evaluated ($F = 2.61$; df = 3; $P = 0.099$). Regarding the survival of individuals, this was higher in the least tested concentration (1×10^5 conidia per mL), and according to the results of the statistical analysis, there was no significant difference between survival according to the concentration used ($F = 0.402$; df = 3; $P = 0.754$) or fungus evaluated ($F = 2.80$; df = 3; $P = 0.085$) after treatment. Finally, the interruption of the metamorphosis was observed slightly higher on average in the highest concentration used (1×10^8 conidia per mL), and according to the results of the statistical analysis, no significant difference was found in the interruption of the metamorphosis according to the concentration used ($F = 0.283$; df = 3; $P = 0.837$), but there was a significant difference ($F = 10.05$; df = 3; $P = 0.001$) in each fungus (Table 5). The greatest interruption of the metamorphosis was caused by the GHA strain, while the Ma strain was the one that induced the slightest interruption of the biological cycle (Fig. 2).

Finally, the largest number of individuals with interruption in the metamorphosis (i.e., which stayed in the larval stage), were those treated with *B. bassiana* (GHA and HEB1), since up to 94% of them died at this stage, and where only 6% survived as larvae without reaching the pupal stage. Those who reached the pupal stage and later became adults were in a range of 48 to 59%; however, adults only managed to survive 2 to 5 d more after the emergence. In the case of *M. anisopliae*, the totality of individuals that reached pupal stage was converted into adults, but as in the case of *B. bassiana*, they survived only 5 days after their emergence (Fig. 3).

Discussion

A first step to develop a microbial control program is the knowledge of the occurrence of insect pathogens to use them as a component of an integrated pest management scheme (Lezama-Gutiérrez et al. 2001). In this sense, the objectives of this study were to determine the occurrence of entomopathogenic fungi in agricultural soils from the state of Tamaulipas, Mexico, one of the main producers of soybean and sorghum in the country. In this study, we selected 2 municipalities, Altamira and Aldama, Tamaulipas, which are located in the southern part of the state. Sub-humid and humid climates predominate in this area. In these regions, agricultural activities are carried out in rainfed, irrigated, and cultivated pastures where the most important annual, perennial and semi-perennial crops are corn, tomato, chili, onion, sorghum, beans, safflower, and some vegetables. Entomopathogenic fungi are cosmopolitan and can be found in cultivated and uncultivated soils, forest environments, and orchards, to name a few examples (González-Baca et al. 2019).

Due to these agricultural activities and the presence of preferred hosts of *S. exigua*, these sites were selected for the search of new strains of entomopathogens with some toxic activity on the insect. According to the results obtained, the occurrence of entomopathogenic fungi was relatively low compared to some previous studies where a large number of entomopathogenic fungi species in cultivable Mexican soils have been obtained (Galán-Franco et al. 2011; Hernández-Velázquez et al. 2011; Sanchez-Peña et al. 2011) and around the world as well (Hemasree 2013). These remarkable differences in obtaining new isolates can be due to several factors. One of the limitations when carrying out explorations in this type of land for agricultural use, besides the geographical location and agricultural practices, as well as pesticide use, is that it may have an impact on the occurrence of natural control agents in the host population or the soils (Vanninen 1996; Mietkiewski et al. 1997). It is likely that the wide use of chemicals in these regions for the control of pests will contribute to a high percentage of residuals in these soils, which will influence the small presence of entomopathogenic strains in these sites. On the other hand, it had been assumed that fungal population genetics are closely related to host insects. However, recent research shows that *M. anisopliae* population structure may be driven by habitat selection, not insect host selection (Bidochka et al. 2001). This was proven in this study because although *S. exigua* hosts are cultivated in these soils, the amount of fungi recovered does not seem to be related to the host. Similarly, *B. bassiana* has adapted to selected habitats, and any evidence of an insect-host-related population structure should be viewed primarily as coincidental and not as a result of co-evolution (Meyling & Eilenberg 2006; Meyling & Hajek 2009). To determine the virulence of the isolates obtained from these localities, they were cultured in conventional laboratory media (potato dextrose agar, Sabouraud dextrose agar) to obtain inoculum, specifically spores, for the bioas-

Table 3. Mean lethal concentration obtained for each treatment using Probit regression analysis.

Treatments species	LC_{50} (conidia per mL)	Fiducial limits (95%)	χ^2	R^2	Probit model
Ma *M. anisopliae*	6.54×10^6	0.047(0.013–0.081)	4.36	0.894	y = −1.34 + 0.198x
GHA *B. bassiana*	7.75×10^7	0.064(0.021–0.107)	2.66	0.908	y = −1.31 + 0.167x
HEB1 *B. bassiana*	2.05×10^7	0.064(0.024–0.104)	2.03	0.918	y = −1.16 + 0.160x
HIB-12 *M. anisopliae*	3.17×10^6	0.037(0.010–0.064)	5.12	0.803	y = −0.962 + 0.171x
γ01 *C. fumosorosea*	5.34×10^7	0.060(0.019–0.101)	3.34	0.869	y = −1.18 + 0.153x
Pfr-612 *C. fumosorosea*	5.38×10^8	0.053(0.019–0.087)	0.063	0.958	y = −4.29 + 0.513x
HIB-11 *M. anisopliae*	1.85×10^7	0.051(0.015–0.087)	0.322	0.921	y = −1.07 + 0.151x

Table 4. Mean lethal time obtained for each treatment using Probit regression analysis.

Treatments species	LT_{50} (d)	Fiducial limits (95%)	χ^2	R^2	Probit model
Ma *M. anisopliae*	3.29	0.0022 (0.018–0.026)	23.48	0.931	y = −7.15 + 3.76x
GHA *B. bassiana*	3.26	0.007 (0.0044–0.0096)	24.11	0.953	y = −0.618 + 0.008x
HEB1 *B. bassiana*	3.22	0.006 (0.0038–0.0082)	18.88	0.945	y = −5.33 + 2.82x
HIB-12 *M. anisopliae*	3.15	0.0044 (0.0028–0.0060)	19.14	0.811	y = −13.57 + 7.22x
γ01 *C. fumosorosea*	5.39	0.0084 (0.0047–0.0121)	49.06	0.851	y = −9.00 + 4.26x
Pfr-612 *C. fumosorosea*	3.87	0.0066 (0.0040–0.0092)	73.62	0.846	y = −2.62 + 1.66x
HIB-11 *M. anisopliae*	4.39	0.0073 (0.0043–0.0103)	55.24	0.846	y = −3.57 + 2.05x

say. However, most of the isolated fungi showed low rates of sporulation, which were insufficient for the bioassays and were therefore discarded. As mentioned by Feng et al. (1994), a key factor in the selection of new isolates as potential biocontrol agents, specifically in entomopathogenic fungi, is the capacity of mass production (i.e., high sporulation rate), rapid growth, and maintaining viability and infectivity. In this sense, only 2 isolates with these characteristics (HEB1 and γ01) were selected, which were tested with collection strains, and 2 native isolates of *M. anisopliae* were included from the strains collection of our laboratory to integrate a panel that included the 3 main species of entomopathogens of interest (*B. bassiana*, *I. fumosorosea*, and *M. anisopliae*). Multiple-dose bioassays of selected isolates to control *S. exigua* showed that the isolate HIB-12 of *M. anisopliae* obtained the best values of LC_{50} and LT_{50} with 3.17×10^6 spores per mL and 3.15 d, respectively. These results match partially with those reported by Han et al (2014) who reported that the isolates *Metarhizium anisopliae* FT83 showed 100% cumulative mortality to control second instar larvae of *S. exigua* 3 d after treatment at 1×10^7 conidia per mL, and *I. fumosorosea* (formerly *Paecilomyces fumosoroseus*) FG340 caused 100% mortality 6 d after treatment at 1×10^4 conidia per mL. In a recent study, 14 fungal entomopathogenic strains were field-collected and isolated from soil samples and infected *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) larvae in Guanajuato, Mexico. The LC_{50} on neonate larvae was estimated at 2.8×10^5, 16×10^5, 26×10^5, and 36×10^5 conidia per mL for strains Ma41, Bb9, Ma22, and Mr8, respectively (Cruz-Avalos et al. 2019). In another work, *M. anisopliae* isolate Ma002 killed 87.5 and 81.25% of *S. exigua* larvae under laboratory and greenhouse conditions (Freed et al. 2012). This selective advantage of *M. anisopliae* seems to be given by the fact that Kershaw et al. (1999) described that all the isolates of *Metarhizium*, whether pathogenic or non-pathogenic, evoked melanization of the cuticle, and the growth of the fungus in the hemolymph was associated with the virulence toward the lepidopterous insect pests. The more virulent isolates tended to grow more profusely than the less virulent isolates. On the other hand, sublethal effects on *S. exigua* have been documented widely in the case of chemical insecticides (Rodríguez-Enríquez et al. 2010; Wang et al. 2013), whereas in the case of entomopathogenic fungi, the reports still are considered scarce because generally when fungal bioassays are carried out, only the percentages of mortality and the time of exposure is taken into account, and little importance has been given to the post-treatment effects. Additionally, in this work, individuals who did not succumb to infection by the different fungi evaluated were monitored to document their effects on the *S. exigua* cycle. The obtained results showed that the interruption of the metamorphosis was not influenced by the concentration of spores used, but it is concerning for the species of the fungus used since the highest percentage of interruption of the metamorphosis was caused by the GHA strain of *B. bassiana*, whereas the Ma strain of *M. anisopliae* induced the slightest interruption of the biological cycle. For example, in a study with *Helicoverpa zea*, Leckie et al. (2008) evaluated the effects on larval growth, development, and mortality of different rates of dried, ground mycelia and water-soluble metabolites from a fermentation broth culture of different isolates of *B. bassiana* incorporated into a synthetic diet and fed to neonate *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae). Development was delayed, weights of larvae were lower, and mortality was high for larvae fed with the highest rates (1.0 and 5.0%, w/v) of mycelia incorporated diet compared to control. Borges et al. (2010) reported that the beauvericin, a secondary metabolite initially extracted from mycelia of *B. bassiana*, also has been isolated from different species of *Fusarium* Link (Hypocreales: Nectriaceae) and *Paecilomyces* Samson (Eurotiales: Trichocomaceae) and can alter molting, metamorphosis, and fertility. However, these differences between species seem to be due to the presence of toxic secondary metabolites that are synthesized when the fungus has penetrated the exoskeleton (Téllez-Jurado et al. 2009) and has reached hemocoel as part of the virulence factors during pathogenesis, so they are considered to have insecticidal properties (Vey et al. 2001), as well as other metabolites with anti-

Table 5. Metamorphosis interrupted of *Spodoptera exigua* treated with isolates of native species of entomopathogenic fungi under laboratory conditions (26 °C, 65 ± 5% RH, 14:10 h [L:D] photoperiod).

	Metamorphosis interrupted (CI 95%)*			
	Concentration (conidia per mL)			
Key	1×10^5	1×10^6	1×10^7	1×10^8
HEB-1	14 (13.97–14.03)	19 (18.96–19.04)	9 (8.97–9.03)	22 (21.96–22.04)
GHA	23 (22.96–23.05)	19 (18.96–19.04)	20 (19.96–20.04)	22 (21.96–22.04)
HIB-12	9 (8.97–9.03)	17 (16.96–17.04)	10 (9.97–10.03)	11 (10.97–11.03)
Ma	10 (9.97–10.03)	2 (1.99–2.01)	6 (5.98–6.02)	8 (7.97–8.03)
Mean ± SE	14 ± 3.18	14.25 ± 4.10	11.25 ± 3.03	15.75 ± 3.66

*Metamorphosis interrupted is reported in percentage (%).
No mortality was recorded in controls (Tween 80 control, untreated control).

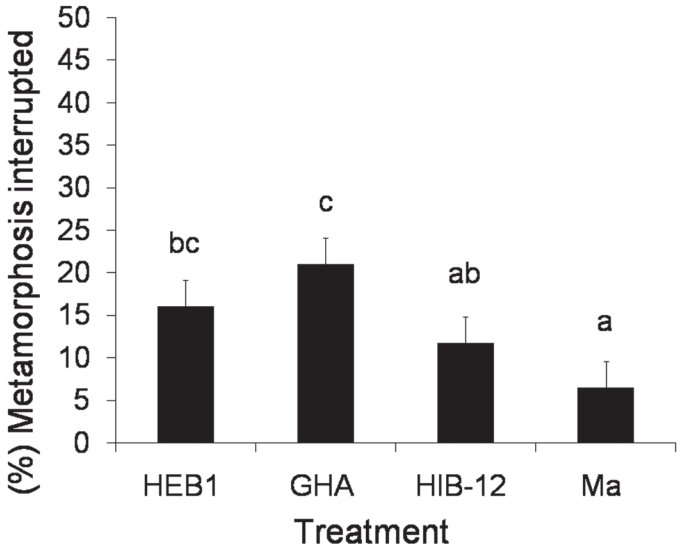

Fig. 2. Interruption of the metamorphosis of *Spodoptera exigua* caused by isolates (HEB1, HIB-12) and collection strains (GHA, Ma) of entomopathogenic fungi under laboratory conditions (26 °C, 65 ± 5% RH, 14:10 h [L:D] photoperiod). Lines in the bars indicate the standard error.

feedant activity in insects (Quesada-Moraga et al. 2006). The action of the secondary metabolites produced by the entomopathogenic fungi on the hosts is quite varied. They can participate in the solubilization of the cuticle of the insect or the inhibition of metabolic and physiological processes, preventing its development and causing its death (Borges et al. 2010). Therefore, it is considered important in subsequent studies that the participation of these compounds in the mechanism of action of entomopathogenic fungi to evaluate their effect on the post-treatment life cycle is elucidated.

The results obtained show that *M. anisopliae* was the most effective for the control of larval stages of S. *exigua*, which opens new paradigms for formulation and application studies in the field. In addition, the results in the interruption of the insect cycle should continue to be studied as a possible additional mechanism of population control of this lepidopteran.

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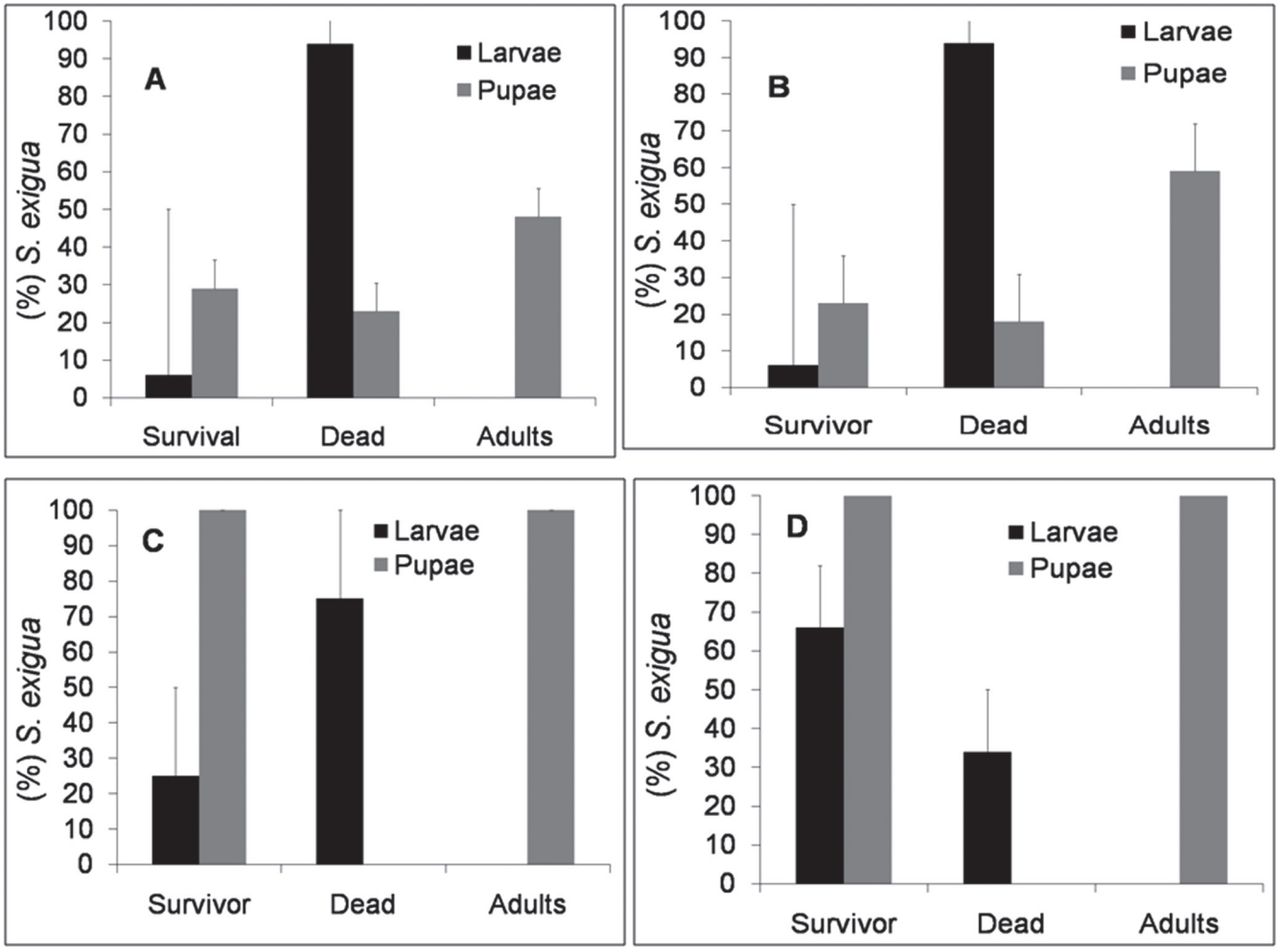

Fig. 3. Changes in the metamorphosis of *Spodoptera exigua* caused by isolates (HEB1, HIB-12) and collection strains (GHA, Ma) of entomopathogenic fungi under laboratory conditions (26 °C, 65 ± 5% RH, 14:10 h [L:D] photoperiod). (A) HEB1 (*Beauveria bassiana*); (B) GHA (*Beauveria bassiana*); (C) HIB-12 (*Metharizium anisopliae*); (D) Ma (*Metharizium anisopliae*). Lines in the bars indicate the standard error.

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