Mortality Evaluation of Armyworm (*Spodoptera frugiperda* J. E. Smith) by using *Metarhizium anisopliae In vitro*

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The biological control use to reduce pest insect populations is an alternative to the use of synthetic insecticides technique. The use of entomopathogenic fungi and microbial control has some advantages over the use of chemical insecticides, which emphasizes the high specificity of the host and low environmental contamination. However, the production of biological control agents such as *Metarhizium anisopliae*, presents constraints on the optimal concentration of conidium, the potential well may have unknown native strains. It was evaluated in this research, the mortality of fall armyworm (*Spodoptera frugiperda* J. E. Smith) in laboratory conditions by using concentrations of conidium from a native strain and a commercial strain of *M. anisopliae*. Different suspensions of conidium were conducted where $53x10^4$, $9x10^4$ were obtained and with $5x10^4$ / mL of native strain of *M. anisopliae* and $36x10^4$, $7x10^4$, and $4x10^4$ con/mL of commercial strain of *Metarhizium*. The concentration of $53x10^4$ con/mL of the native strain, reported a mortality of 72.5% and the lowest was obtained from the strain of *Metarhizium* commercial in concentration of $4x10^4$ con/mL, equivalent to 32.5%. The highest death rate for both strains occurred at 72 hrs. post-infection with 11 larval on average of three concentrations of native *Metarhizium* and 6 commercial strains larval. These results demonstrate the feasibility of using *M. anisopliae* for biological control armyworm (*S. frugiperda*) *in vitro*.

Key words: Biological control, Native strain, Conidium concentration and Mortality rate.

At present, food production faces the challenge of maintaining a high level of quality, considering aspects of food safety and production systems fairer income for producers[1]. In Mexico there are several common and important pests that cause damage in agricultural crops, among the most important are: the armyworm, the fruit fly, the boll weevil and the apple tree, spider mites, midges white, leafhoppers or aphids[2].

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The fall armyworm (*S. frugiperda*) can cause a reduction in corn production, ranging from 20% to the total loss of the crop, attacking at early stages of plant development and even in times of flowering[3]. In Mexico it is in all regions where maize is grown, but the damage is more severe in the tropics and subtropics, is considered the most damaging and voracious pest for the crop[4]. This insect makes scratches on the tender parts of the leaves, which then appear as small translucent areas and once the larval reach their development, start eating foliage preferably in the bud; hence its name[5].

In the last years the use of toxic substances in Mexico has increased pesticide use

and increases annually by 10%, so it is necessary to modify these agricultural practices to reduce the risk to the health of producers and consumers[6]. However, its overuse has caused resistance, plus its accumulation in the environment affects the flora and benefical wildlife[7]. In order to minimize these negative consequences, it is proposed to decrease the use of conventional pesticides and develop new strategies for an Integrated Pest Management (IPM), mainly through biological control; this being a method of controlling pests more rational and respectful with the environment[8]. The use of beneficial organisms as a sustainable alternative insect control is recommended within an integrated pest management, as it has no harmful effects on the environment, human health and animals[7].

Particularly, fungi have been one of the best alternatives for pest control in recent years; also exhibit a broad host spectrum[9]. Entomopathogenic fungi are a group of microorganisms with over 700 species within 90 genera that can infect insects[10,11].

The diseases caused by fungus in insects commonly; reduce populations significantly, demonstrating that bio-insecticides may be a viable option for solving the problems of insect pests in agriculture[2,12]. Among the fungi used as biological insecticides are included *Beauveria bassiana* and *Metarhizium anisopliae*[13,14]. *M. anisopliae* attacks more than 200 species of insects and mites of various genres, in orders Orthoptera, Hemiptera, Lepidoptera, Dermaptera, Hymenoptera, Coleoptera, among others[15]. The present study aimed to identify and characterize the native strain of *Metarhizium* CP-MA1, in addition to evaluating their pathogenic effect on in vitro armyworm.

Biological material

The strain used in this investigation is the CP-MA1 of *Metarhizium* spp, comes from the Municipality of Tetela de Ocampo, Puebla-Mexico; are deposited and maintained in potato dextrose agar culture (PDA) at Institute of Genetic Resources Center for Agroecology Sciences of BUAP. The strain identity was confirmed by amplification of the region ITS-1 5.8r and ITS-4. In the MEGA 4.0 program, phylogeny was constructed with *Neighboard Joining* algorithm with 1000 *bootstrap* replicates, whereas the nucleotide substitution model Kimura 2-p[16].

In the study was included as a reference strain of *Metarhizium* commercially available in the city of Puebla-Mexico in the company "Agrobionsa of Mexico" where the Meta-BLAS product was purchased. Thereafter activation of the mycelium in petri dishes of 9 cm diameter, using PDA culture medium and incubated at ambient temperature was performed.

Identification of Metarhizium spp

Genomic DNA extraction. From PDA plates with spore cultures 72 hrs., of incubation, a scraping was conducted for DNA extraction kit ZR Fungal / Bacterial DNA MiniPrep (ZIMO Research D6005). The DNA was run on an agarose gel 1%, in a horizontal electrophoresis chamber (BIO-RAD) at 70 volts for 60 min using a power source EC105. Genomic DNA extractions were stored at -20.

ITS amplification. PCR reactions were performed in a final volume of 50 L, using 1 ul of genomic DNA, 5 uL of 10X buffer, 1.5 uL of magnesium chloride 50 mM (1.5 mM final), 1 ul 10 mM dNTPs (0.2 mM), 1 uL of each initiators; oligonucleotides used outside the ITS-1 region (5'TCCGTAGGTGAACCTGCGG3') and ITS-4 (5'TCCTCCGCTTATTGATATGC3')[16] plus 5 mM (final 0.1 mM) and 0.4 mL of the enzyme Taq DNA polymerase 5 U / µL and completing the volume to 50 µL with sterile milliQ water. Subsequently amplification 1 cycle of 3 min at 94°C and 35 cycles of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C was performed. Finally there was a final extension step of 1 min at 72°C and held at 4°C. And the products obtained were purified using the DNA Purification Kit Wizard®.

Sequencing. Amplified products were concentrated and transferred to a 0.2 mL eppendorf tube with approximately 20 µL of the final product at a concentration of 50 ng. The sample was sent for sequencing in both directions to Center for Biomolecular Detection Autonomous University of Puebla (CBD-BUAP). The sequence obtained for each genomic region of each fungus was compared *in silico* on their homology to those existing in the public data base present in the BLAST website of the National Center of Biotechnology Information (NCBI) of the USA (http://www.ncbi.nlm.nih.gov/BLAST/)[17].

Growth rate and development rate

The growth rate and the development rate of *M. anisopliae* strains were determined in petri

dishes (4.5 cm in diameter) in culture medium (PDA), incubated at room temperature for eight days, the growth rate was measured every 24 hrs., until the completion of the total colonization of the strains, the macroscopic morphology of the colonies in texture, density, and color aerial mycelium were recorded. Growth rate and development rate was determined using the following formula: TD = VCF-VCI / No. days[18].

Preparation of suspensions and viability of conidia

Preparations of strains were taken in culture medium (PDA) with conidia Monosporic isolated of *M. anisopliae* (a commercial and other native) of 10 days of development, was subsequently added 10 mL of sterile distilled water (SDW), for suspend and remove spores. After 15 min recovery was performed and was brought to 10 mL in a graduated cylinder, thereby the mother suspension was prepared; of which it took a 1 mL and deposited in test tubes with 9 mL of ADW, being thus prepared the 10-1 dilution; the process being 1 ml of this dilution (10-1) to tubes with 9 mL of ADE, until a dilution of 10 to 10^{19} was repeated. For the test of mortality in the present investigation, the dilutions 10-1 10-5 10-10 for both strains of *M. anisopliae* were taken. From the dilutions performed previously, the concentration of conidia was determined by taking a sample of 60µL with a micropipette and visualizing in Neubauer chamber (Marienfeld, Germany) and a compound microscope (Leica Inc., USA) was observed under a microscope at 10x and 40x. The process of counting of conidia[19], was performed three times with the following formula: Total Spores = No. of spores / 8 x10^4.

To evaluate the conidia germination the Marin and Bustillo methodology was followed, five points were placed with a suspension of 1x10^4 conidia / ml in Petri dishes (100 x 15 mm) with PDA. 100 conidia were observed and the number of germinated conidia of the five points was recorded and this value directly represents the germination rate in percentage of an experimental unit[20].

Preparation of the diet (*S. frugiperda* J. E. Smith)

An artificial diet was prepared with SOUTHLAND PRODUCTS INC (870-265-3747) that included a series of synthetic based on soy flour and wheat, preservatives and multivitamins substances[21]. The artificial diet was developed in based on a modification made by Dilcia in 1989[22]. To which, was performed 1 420.00 mL of diet in two stages. First 710 mL of sterile distilled water was measured in a test tube, then was placed in an aluminum pan and was put to boil for 5 minutes, immediately withdrew from the fire and was placed 150 g of SOUTHLAND diet and was dissolved with an electronic stirrer to homogenize the mixture. Subsequently, it was placed in a water bath with a micropipette and was added 5 mL of diet on transparent plastic cups and was allowed to solidify.

Inoculation of *M. anisopliae*

The larvae of armyworm (*S. frugiperda*) third urge used in this research come from a hatchery of the phytosanitary department of Colegio de Postgraduados, Campus Montecillo, State of Mexico.

The methodology treatments of inoculation consisted of placing 20 larvae in immersion in a petri dish, then the excess inoculum on larvae[23] was removed and placed in each plastic container with semi transparent cover 25 mL capacity with lid, which has a circular window in the center to facilitate gas exchange with 5 mL of diet[24] and incubated at 28°C. In the case of the control, sterile distilled water was used.

The design for this study was a randomized block, where three conidial concentrations (10-1, 10-5 to 10-10) with two strains of *Metarhizium* were compared. A total of 7 treatments were evaluated including control, each treatment had 40 replicates; thus having a total of 280 third instar larvae of armyworm. Larvae were evaluated every 24 hrs. starting from the third day after the experiment began.

The dead larvae were selected separately in trays and were observed until it was detected sporulation of *M. anisopliae*. During the monitoring period, was registered the number of live larvae and dead with sporulation due to the effect of the entomopathogenic fungus.

The mortality expressed in percent was determined by the following formula:

$$\% M = \frac{Lv}{Po} x100$$

In which:

% M = Percentage of larval mortality

Po = Initial Population

Lv = Dead larvae per treatment

The results obtained were subjected of an analysis of variance (Anova) and in to a test mean separation by Tukey ($p < 0.05$) using SPSS version 17 (Statistical Package for Social Sciences) to determine differences between treatments.

RESULTS AND DISCUSSION

Identification of fungus

The morphological identification of the CP-MA1 strain was performed in order to confirm the taxonomic classification of the genre indicated by Eberlein in 2010[25], for which were used the proposed keys by Barnett and Hunter in 1998[26]. The CP-MA1 strain showed white and cottony colonies, that started green yellow and became dark green olive and crusted areas with abundant aerial mycelium white and deep yellow reverse; also presents conidiophore with whorls 2-3 branches each, with dark olive green tones that clarified to the apex, conidiogenous cells 6.0-10.0 (8,1µ) x 2.0-2.5 (2,1µ), subhyaline to slightly green conidia, cylindrical to slightly ellipsoidal, 5.0-8.5 (6,6µ) x 2.0-3.0 (2,4µ).

Amplification and sequencing PCR-ITS

With the amplified region ITS-1 and ITS-4 a product of 522 base pairs (bp) was obtained (Figure 1), White in 1990, indicate that the size of the amplified product is approximately 650 bp for the genus *M. anisopliae*[16]. The PCR product, sequenced was compared with fungal sequences published through search program homology nucleotide-nucleotide BLAST. This showed an identity with the 522 nucleotide of 99% with *M. anisopliae var lepidiotum*. This sequence was included in the database of National Center for Biological Information (NCBI) with the access number YSGREUGG01R. The phylogenetic tree (Figure 2) was constructed with 9 access of *M. anisopliae* available in the database (NCBI), was condensed to values greater than 50, this means that the clades with values below 50 were eliminated and instead the emphasis is given to reliable clades with values above 50.

Growth rate and development rate

The radial growth of the mycelium ranged from 0.27 to 0.21 cm / day (Table 1). The values observed in this study are similar to those found

Table 1. Macroscopic characteristics of *M. anisopliae* strains on PDA medium

Culture Medium	Macroscopic characteristics of the colonies of strains of *M. anisopliae*						
	Key	Texture	Density	Aerial Mycelium	Color	Radial growth rate (cm / day)	Rate of development (mm / day)
PDA	CP-MA1	Cottony	Abundant	Abundante	Olive green / dark	0.27	3.3
	Commercial strain	Woolly	Abundant	Regular	Green / Yellow	0.21	2.8

Table 2. Microscopic characteristics of *M. anisopliae* strains on PDA medium

Treatment	Macroscopic characteristics of the colonies of strains of *M. anisopliae*				
	Key	Concentrations of conidium x10^4 con/mL*		% Spore viability *	
10/1	CP-MA1	53	a	89	a
	Commercial strain	36	b	76	b
10/5	CP-MA1	9	c	68	b
	Commercial strain	7	d	54	c
10/10	CP-MA1	5	de	33	d
	Commercial strain	4	e	17	e

* Different letters in the same column indicate significant differences with multiple range Tukey Kramer test (± = 0.05)

by Skrobek in 2001, who evaluated the mycelial growth of *M. anisopliae var. anisopliae* on SDA at 26°C, recorded growth rate of 0.33 to 0.36 cm / day[26]. Meanwhile, Rachappa in 2007, reported values of mycelial growth of 0.38 to 0.39 cm / day in isolates of *M. anisopliae*, which is slightly higher than those found in the present study[27].

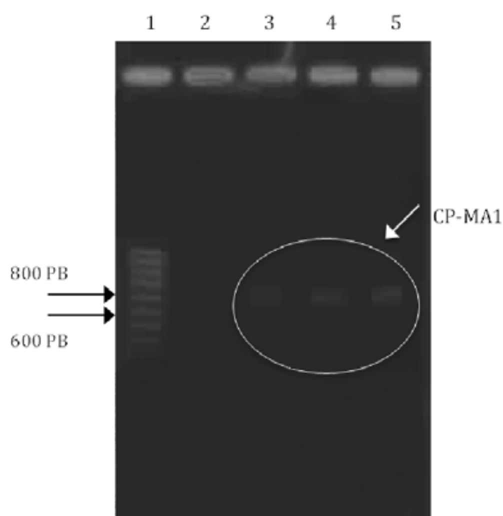

Fig. 1. Lane 1: molecular weight marker 1 kb (Promega, USA), Lane 2: negative control, lane 3, 4 and 5: amplification of internal transcribed spacer (ITS) of the ribosomal DNA, with the combined ITS1 and ITS4 primers (approximate size of the band: 520-550 bp) of the CP-MA1 x triplicate.

Preparation of suspensions and viability of conidia

By counting the conidia in Neubauer chamber, the concentration of conidia for each of the concentrations used was determined. The highest concentration of conidia was obtained in 1/10 with $53x10^4$ con/mL of *Metarhizium* native and $36x10^4$ con/mL with commercial *Metarhizium*. The lowest concentrations were obtained with 5/10 native *Metarhizium*, 5/10 commercial *Metarhizium*, native *Metarhizium* 10/10 and commercial *Metarhizium* 10/10; with $9x10^4$, $7x10^4$, $5x10^4$ and $4x10^4$ con/mL respectively. No significant differences were observed in concentrations of commercial *Metarhizium* 10/10, native *Metarhizium* 10/10 and the concentration 5/10 commercial *Metarhizium*. Instead, concentrations of 5/10 native *Metarhizium*, *Metarhizium* commercial 1/10 and *Metarhizium* native 1/10, showed significant difference. The ranges percentages of viability are very heterogeneous among strains of *M. anisopliae*, the difference between the highest was 72% and the lowest was 13% viability (Table 2).

Mortality Assessment of Armyworm (*S. frugiperda* J. E. Smith) in vitro

The sporulation of *M. anisopliae* on larvae was performed 5 to 6 days after his death. Initially they were fluffy white colonies, characteristic state of mycelial growth (Fig. 3). The emergence of the fungus was performed in different ways, such as mouthparts and abdomen. Fig. 3a shows that the emergence of the fungus was carried

Table 3. Assessment of mortality armyworm (*S. frugiperda*) in vitro

Treatment	Key	Dead larvae	% M*		Lethal time Death in larvae 72 hrs.	96 hrs.	120 hrs.	144 . hrs	Pupae infected	Adult emergence *	
Control Group	Water	0	0	e	0	0	0	0	0	40	d
10/1	CP-MA1	29	72.5	a	14	9	5	1	11	6	a
	Commercial Strain	21	52.5	b	9	7	3	2	19	11	b
10/5	CP-MA1	22	55.0	b	11	6	3	2	18	12	b
	Commercial Strain	17	42.5	c	6	5	4	2	23	17	c
10/10	CP-MA1	19	47.5	b	9	5	4	1	21	13	b
	Commercial Strain	13	32.5	d	5	4	2	2	27	18	c

* Different letters in the same column indicate significant differences with multiple range Tukey Kramer test (± = 0.05).

out in the bottom of the head, mouthparts colonizing and invading the body of the larva by producing enzymes; meanwhile in Fig. 3b, are observed two colonies of *M. anisopliae* in different areas, the first is located on the side of the thorax and the second in the abdomen; likewise in Fig. 3c, is observed sporulation of the fungus in the lower abdomen and finally seen in Fig. 3e, is observed fungal sporulation with green coloration, typical of this genre[28]. It was also observed the presence of *M. anisopliae* in adults (Fig. 3f), since the infection can occur if the spores get into the cells before they were capped, killing the host[29].

Mortality of armyworm larvae, using native *Metarhizium* occurred at 72 hrs. where the highest mortality rate for each of the treatments (TL_{50}) was recorded. Regarding the mortality rate caused by the commercial strain of *Metarhizium* occurred between 72-96 hrs. where the highest rate of mortality was recorded for each of the treatments. Regarding the mortality rate caused by the commercial strain of *Metarhizium* occurred between 72-96 hrs. where the highest rate of mortality was recorded for each of the treatments (TL_{50}). The concentration of 1:10 ($53x10^4$ con/mL) caused higher mortality in 29 larvae, equivalent to 72.5% of the total population corresponding to this treatment; having significant differences with other concentrations, subsequently native *Metarhizium* 5/10 ($9x10^4$con/mL) with a total of 22 larvae (55%), immediately commercial *Metarhizium* 1/10 ($36x10^4$ con/mL) with 21 larvae (52.5%), then native *Metarhizium* 10/10 ($5x10^4$ con/mL) with 19 larvae (47.5%), commercial *Metarhizium* 5/10 ($7x10^4$con/mL) with 17 larvae (42.5%), and finally commercial *Metarhizium* 10/10 ($4x10^4$ con/mL) with

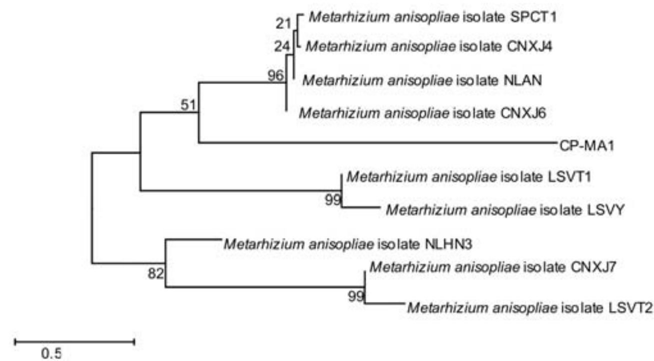

Fig. 2. Phylogenetic tree of the CP-MA1 generated by the Neighbor-Joining method with 1000 replicates condensate (values > 50) based on rDNA sequences of the ITS region with 9 *M. anisopliae* access the database (NCBI).

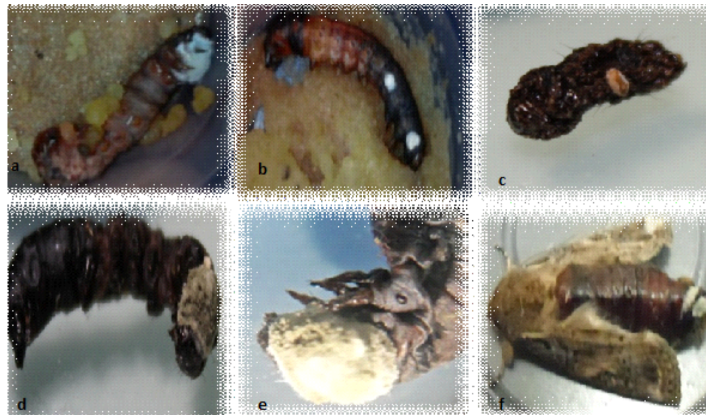

Fig. 3. Colonization of native *Metarhizium* on armyworm larvae (*S. frugiperda* J. E. Smith); **a, b** and **c**: colonization of *M. anisopliae* in growth state, over the body of larva; **d**: fungal sporulation with radial growth and greenish coloration; e: *M. anisopliae* colonies observed in a microscope (40X resolution), and f: emergence and colonization of the *M. anisopliae* against adults emerged in armyworm.

13 larvae (32.5%) (Table 3).

Laboratory studies carried out by Lezama in 1996 have shown that *S. frugiperda* is susceptible to *M. anisopliae* in biological egg state and larval state, with mortality rates of 100% and values TL_{50} with 2.5 2.9 1.3 days in egg and larva to 3.1 days, on a concentration of 1x10^8 conidia per mL, similar to those obtained in this investigation[30]. Also Nájera in 2005, report that the native strain of *M. anisopliae* original from the state of Jalisco-Mexico, caused an 80% mortality within 30 days of starting the experiment on larvae of *Phyllophaga* spp., at a concentration of 2x10^8 to/g[31]. This last fact is related with mortality results obtained using the native strain of *Metarhizium* 1/10 on a concentration of 53x10^4 con/mL. Concentrations of commercial *Metarhizium* strain, except the 1/10 concentration (36x10^4 con/mL), showed reduced virulence compared to the native strain, since virulence is determined by factors that depend on their genetic information, thereby influencing the production of enzymes and toxins for infection[32,33].

Larvae that moved to the pupae state

Of each of the applied dose, the concentration where the largest numbers of pupae were obtained was commercial *Metarhizium* 10/10 (4x10^4 con/mL) with 27 pupae, followed by concentration of commercial *Metarhizium* 5/10 (7x10^4 con/mL) with 23 pupae. However, be noted that the native strain of *Metarhizium* in concentration 1/10 (53x10^4 con/mL) only 11 represented pupae, that have significant differences with other treatments (Table 3).

Adult emergence

The concentration where adult emergence that reached lowest point of the armyworm, was native *Metarhizium* 1/10 with 6 adults, equivalent to 15% of the total population of this treatment (Table 3). Meanwhile, concentrations where there was more adult emergence was commercial *Metarhizium* 5/10, and commercial *Metarhizium* 10/10; 17 (42.5%) and 18 (45%) larvae respectively. These data relate to those reported by Angel-Sahagún in 2005, who evaluated the sensitivity of eggs, pupae and adult *Haematobia irritans* isolates three entomopathogenic fungi *B. bassiana, M. anisopliae* and *P. fumosoroserus* used at a concentration (con/mL) of 1x10^6 for eggs, 1x10^8 for pupae and the two for adults, finding that all stages are susceptible to the action of entomopathogenic fungi with reduced hatching from 3.8 to 6.3% to 72% hatching of the control treatment, mortality between 50 and 71.3% in pupae and 90% in adults[34]. In general, it was shown that the highest concentration of native *Metrahizium* 1/10 (53x10^4 con/mL), was a better control in adult emergence of *S. frugiderda*.

CONCLUSIONS

Most conidia production occurred in the native strain of *M. anisopliae* with 53x10^4 con/mL in culture medium PDA, representing increased production of reproductive structures compared to the commercial strain. Concentration of 10/1 *M. anisopliae* strain native had higher mortality rate 72.5% at 72 hrs. post-infection as compared to the commercial strain 10/10 with 32.5% was the lowest result obtained in the present research. These results demonstrate the feasibility of using *M. anisopliae* for biological control armyworm (*S. frugiperda* J. E. Smith).

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