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Pathogenicity of the Hypocreales Fungi *Beauveria bassiana* and *Metarhizium anisopliae* against Insect Pests of Tomato

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Abstract. Silverleaf whitefly, *Bemisia tabaci* Gennadius (Aleyrodidae), potato/ tomato psyllid, *Bactericera cockerelli* Sulc. (Triozidae), and western flower thrips, *Frankliniella occidentalis* (Pergande) (Thripidae), are insect pests of economic importance in tomato (*Solanum lycopersicum* L.) and other solanaceous vegetables in México. Soil is the main reservoir for many entomopathogenic fungi known as biological control agents important for IPM, but only a few strains obtained from soil have been used against insect pests. In this study, the biological activity of two native isolates of entomopathogenic fungi (from soil at Chihuahua, México), was evaluated against immatures of the three insect pests in a laboratory. *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) (Bb-CIAD1) isolate was more virulent than the *Metarhizium anisopliae* (Metschnikoff) Socorin (Hypocreales: Clavicipitaceae) (Ma-CIAD1) isolate. Mortality based on mycosis varied significantly 8 days after inoculation. The number of immature insects infected by fungi was correlated with spore concentration. *Beauveria bassiana* (Bb-CIAD1) and *M. anisopliae* (Ma-CIAD1) had LT_{50} values of 5.6-6.4 and 5.3-6.5 days, respectively. The results indicated that psyllids, thrips, and whiteflies were susceptible to the native fungal isolates with potential as microbial control agents. Based on the results, we suggest the native isolates of entomopathogenic fungi from soil could be used as biological control agents of many other insect pests.

Resumen. La mosquita blanca, *Bemisia tabaci* Gennadius (Aleyrodidae), el psílido de la papa y tomate, *Bactericera cockerelli* Sulc. (Triozidae), y el thrips de las flores, *Frankliniella occidentalis* (Pergande) (Thripidae), son plagas de importancia económica del tomate (*Solanum lycopersicum* L.) y otras solanáceas en México. El suelo es el principal reservorio de muchos hongos entomopatógenos, conocidos

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como agentes de control biológico importantes para el MIP, pero solo algunas cepas obtenidas del suelo han sido utilizadas contra plagas insectiles. En este estudio, se evaluó, bajo condiciones de laboratorio, la actividad biológica de dos aislados nativos de hongos entomopatógenos (de suelo en Chihuahua, México), contra inmaduros de los tres insectos plaga. El aislado de *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) (Bb-CIAD1), fue más virulento que el aislado de *Metarhizium anisopliae* (Metschnikoff) Socorin (Hypocreales: Clavicipitaceae) (Ma-CIAD1). La mortalidad basada en la micosis, varió significativamente a los 8 días después de la inoculación. El número de insectos inmaduros infectados por hongos, fue correlacionado con la concentración de esporas. *Beauveria bassiana* (Bb-CIAD1) y *M. anisopliae* (Ma-CIAD1), tuvieron un LT_{50} de 5.6-6.4 y 5.3-6.5 d, respectivamente. Los resultados, indican que los psílidos, thrips y las mosquitas blancas, fueron susceptibles a estos aislados nativos de hongos entomopatógenos con potencial como agentes de control microbial. Basado en los resultados, sugerimos que los aislados nativos de hongos entomopatógenos obtenidos del suelo, pueden ser usados como agentes de control biológico de muchos otros insectos plaga.

Introduction

Silverleaf whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), potato/tomato psyllid, *Bactericera cockerelli* Sulc. (Hemiptera: Triozidae), and western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), are the main insect pests of economic importance in tomato (*Solanum lycopersicum* L.) and other solanaceous crops in México (Brown and Nelson 1988, Munyaneza et al. 2007). The pests cause millions of dollars in damage directly by feeding and indirectly by transmitting bacteria and viruses (Moritz et al. 2004, Munyaneza 2010, Butler and Trumble 2012). Insecticides are commonly used to control the insects. However, insecticides kill natural enemies and over time can induce development of resistance to insecticide (Jensen 2000, Liu and Trumble 2005). An alternative to use of insecticide is entomopathogenic fungi (Hypocreales) known for their pathogenicity to a wide range of hosts, including psyllids, thrips, and whiteflies and considered efficient biological control agents for IPM (Ansari et al. 2007; Lacey et al. 2008, 2009; Rios-Velasco et al. 2011; Skinner et al. 2012). Entomopathogenic fungal strains tested have been isolated directly from insect hosts (Sun and Liu 2008, Zhu and Kim 2011). The soil is the main natural reservoir for native entomopathogenic fungi (Bidochka et al. 1998, Klingen and Haukeland 2006, Meyling and Eilenberg 2006, Sun and Liu 2008), but only a few strains isolated from soil have been tested against insect pests (Sánchez-Peña et al. 2007). The objective of this study was to evaluate the biological activity of *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Metschnikoff) (Hypocreales: Clavicipitaceae) from soil against immature *B. tabaci, B. cockerelli*, and *F. occidentalis* under laboratory conditions.

Materials and Methods

Immature *B. tabaci* (3rd and 4th nymphal instars), *F. occidentalis* (1st and 2nd larval instars), and *B. cockerelli* (4th and 5th nymphal instars) were collected from tomato plants in two greenhouses at Cuauhtémoc, Chihuahua, México

(Campo 2A, N 28° 27' 16"-W 106° 55' 54", 2,067 m above sea level and Venezuela N 28° 25' 36" -W 106° 53' 54", 2,047 m above sea level) and transported to the Centro de Investigación en Alimentación y Desarrollo, A. C., Campus Cuauhtémoc, Chihuahua. *Bemisia tabaci* and *B. cockerelli* were treated using techniques described by Wilkey (1962) and Hodges and Evans (2005), and *F. occidentalis* specimens were prepared according to Johansen-Naime and Mojica-Guzmán (1997) for slide mounting and identification by their micro- and macroscopic characteristics (Mound et al. 1989, Burckhardt and Lauterer 1997, Hodges and Evans 2005). Dr. Jhonathan Cambero-Campos (Área Biológico Agropecuaria y Pesquera, Universidad Autónoma de Nayarit, Tepic Nayarit, México) confirmed the identities.

Mealworm, *Tenebrio molit*or L., bait was used to isolate entomopathogenic fungi from soil samples from the La Concepción apple (*Malus domestica* Borkh.) orchard (N 28° 23.9' -W 107° 1.6', 2,194 m above sea level) at Cuauhtémoc, Chihuahua, México. Mealworm larvae from a colony maintained at 25 ± 2°C were used as bait. Each soil sample was baited with 50 larvae in a rectangular plastic food container, closed with a perforated lid, and incubated in the dark for 1 week at 28°C and 90 ± 5% relative humidity. Dead larvae were transferred individually with a piece of cotton to Petri dishes, sealed with Parafilm, and incubated at 28°C. The cadavers were periodically inspected for the presence of external fungal growth.

Entomopathogenic fungi were purified, maintained, and propagated on potato dextrose agar (PDA) and identified according to micro- and macroscopic characteristics. Mycelia (hyphae) from cultures were mounted with lactophenol-blue on slides and observed with the aid of a Carl Zeiss microscope (Jena, Germany) (Barnett and Hunter 1972, Humber 1997, Dugan 2006). *B bassiana* (Bb-CIAD1) and *M. anisopliae* (Ma-CIAD1) for molecular identification were grown on potato dextrose agar medium covered with sterile cellophane for 7 days at 28°C. Mycelia were removed and macerated in a porcelain mortar. A buffer (Tris-HCl, NaCl, EDTA, SDS; 200 mM, pH = 8, 250 mM, 25 mM, 0.05%, respectively) was added for DNA extraction according to the protocol described by Raeder and Broda (1989). Total DNA was used to amplify the internal transcribed spacer (ITS) of the 18s rDNA, using the universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAACAAGG-3') and ITS4 (5'-TCCTCCGCTT ATTGATATGC-3'), where the fragment might be approximately 710 bp (White et al. 1990). The PCR products were verified in 1.0% agarose gel through electrophoresis and detected by staining with orange G. PCR conditions and reactions were: initial denaturalization at 94ºC for 5 minutes, followed by 30 amplification cycles of denaturalization at 94°C for 30 seconds, 30 seconds at 55ºC for annealing, and 45 seconds at 72ºC for extension. After the cycles were completed, the samples were incubated for an additional 10 minutes at 72ºC. PCR products were purified using purification kit DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), and sequenced by an ABI sequencer (Applied Biosystems) by the Macrogen Company (Rockville, MD), according to the method described by Sanger et al. (1977). Sequences were compared with the NCBI databases using the BLAST algorithm (Altschul et al. 1990).

The *B. bassiana* CIAD-1 and *M. anisopliae* CIAD-1 isolates were propagated in rice (*Oryza sativa* L.) grain and incubated in plastic trays (25x18.5x7 cm). Spores were suspended using aseptic conditions into a 0.05% (v/v) Tween 80 sterile distilled water solution (pH 6.0) at 0°C. Spore viability was evaluated 1 day before the bioassay, by spreading 10 µl of spore suspension onto potato dextrose agar in Petri dishes. After 24 hours of incubation at 26 ± 1°C, the percentage of spores that

germinated was determined; a spore was considered viable if the germ tube was twice the length of the spore. Spore concentrations were estimated by using a hemacytometer (Neubauer Improved, Marienfield, Germany) and adjusted for each isolate (Figs. 1, 2).

All suspensions were shaken continually during the bioassay treatment period. The bioassays evaluated fungal virulence against immature *B. tabaci*, *B. cockerelli*, and *F. occidentalis* feeding on cherry tomato leaves (only those that had 25 specimens of each insect species were considered) dipped in a spore suspension (1.8×10^3 to 6.1×10^7 spores per milliliter for *B. bassiana* and 2.8×10^3 to 9.5×10^7 spores per milliliter for *M. anisopliae*) for 10 seconds (Lewis 1997), dried on brown paper at room temperature (25 ± 2°C), placed on moist filter paper in Petri dishes, sealed with Parafilm, and stored at 26 ± 1°C, a photoperiod of 14:12 light:dark hours, and >90% relative humidity (Rios-Velasco et al. 2011) in an environmental chamber (Model 6LM, Precision Scientific; Winchester, EUA). Check insects were handled similarly and treated with only 0.05% (v/v) Tween 80 water solution. Treated tomato leaves were sectioned into 4-5 cm^2 pieces. The number of treated insects that died was recorded every 24 hours for 8 days after inoculation, and fungal infection was confirmed by the presence of mycelium and conidia on the insect cuticle (Fig. 1) observed with the aid of a stereomicroscope (Leica GZ6, Barrington, New Jersey, EUA). The experiments were done in triplicate with each insect species in a randomized complete block design with 11 treatments (concentrations) and a check for each fungal isolate and insect species, where each treatment was a fungal concentration (i.e., a total of 75 immatures of each insect species per concentration was tested, with a total of 825 specimens for each isolate).

The percentage of insects that died was corrected using Abbott's (1925) formula, normalized using arcsine transformation, and analyzed using the Statistical Analysis System (SAS 2002) for balanced analysis of variance (ANOVA). Means were separated by Tukey's test ($P < 0.05$). To compare virulence among fungal species, PROBIT analysis was used to estimate the median lethal concentration compared based on the ratio test analysis and the median lethal time. A *t*-test rather than overlap test for LC_{95}% was used.

Results and Discussion

The insects were identified as *B. tabaci*, *B. cockerelli*, and *F. occidentalis* (Mound et al. 1989, Burckhardt and Lauterer 1997, Hodges and Evans 2005). Morphological characteristics confirmed the identity of the fungi *B. bassiana* and *M. anisopliae* (Barnett and Hunter 1972, Humber 1997, Dugan 2006). Sequencing data showed that isolates Bb-CIAD1 and Ma-CIAD1, identified initially as *B. bassiana* and *M. anisopliae*, showed 99-100% identity and maximum scores in both isolates with GenBank sequence (*B. bassiana* isolate 2773 18s ribosomal RNA gene partial sequence KC755391.1, *M. anisopliae*, isolate CNYN4 18s ribosomal RNA gene partial sequence FJ545306.1 and strain ARSEF4587 18s ribosomal RNA gene partial sequence AY646386.1) obtained from the BLAST algorithm NCBI database (Altschul et al. 1990).

Spore viability ranged from 98 to 100%. The fungal entomopathogens were very virulent against *B. tabaci*, *B. cockerelli*, and *F. occidentalis*, killing >80% with the greatest concentration (6.1×10^7 spores per milliliter of *B. bassiana* and 9.5×10^7 conidia per milliliter of *M. anisopliae*) (Figs. 1, 2). Numbers killed by *B. bassiana*

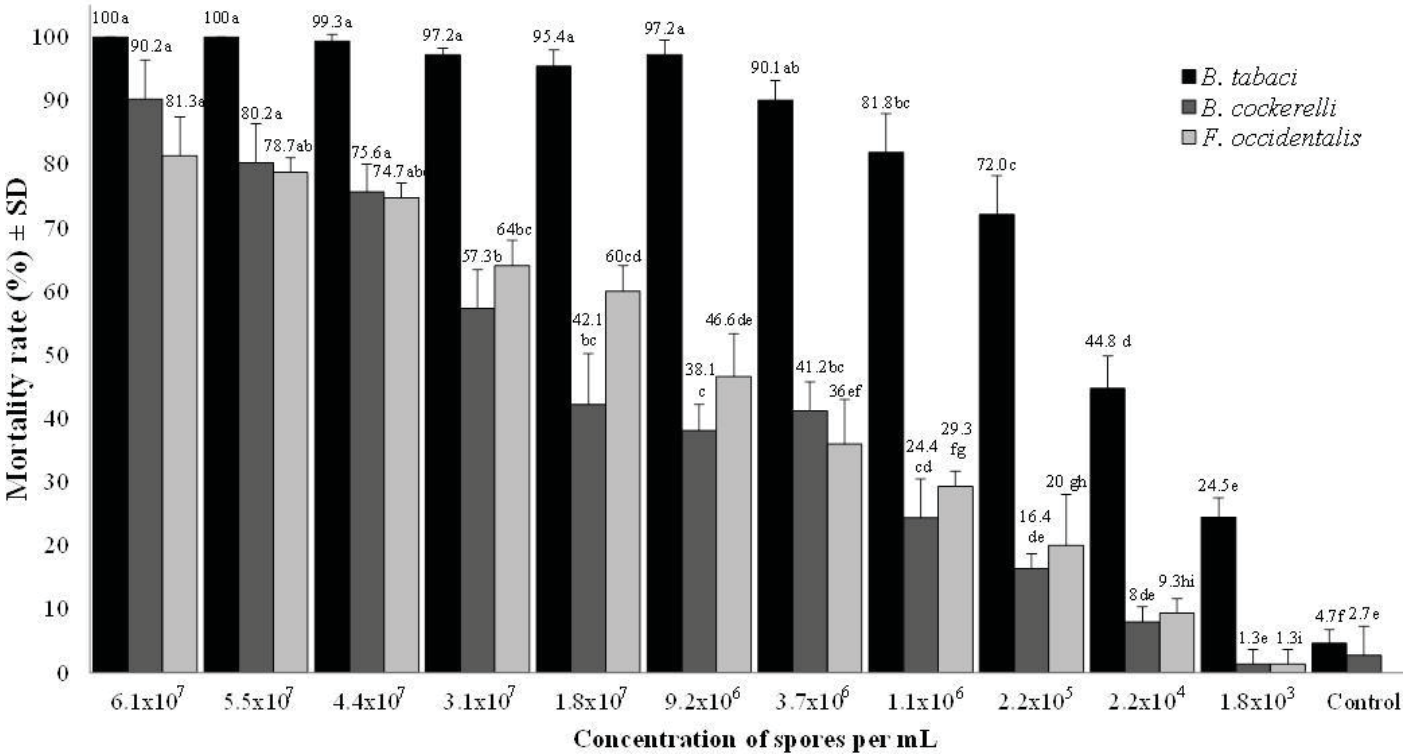

Fig. 1. Mean number ± SD of immatures of three insect pests of tomato dead by 8 days after treatment with *Beauveria bassiana* in México. Means followed by the same letter for a concentration are not statistically different (ANOVA) according to Tukey's test on arcsine transformed data ($P < 0.05$). Nontransformed means are presented. Specimens were submerged for 10 seconds into 50 ml of spore suspension.

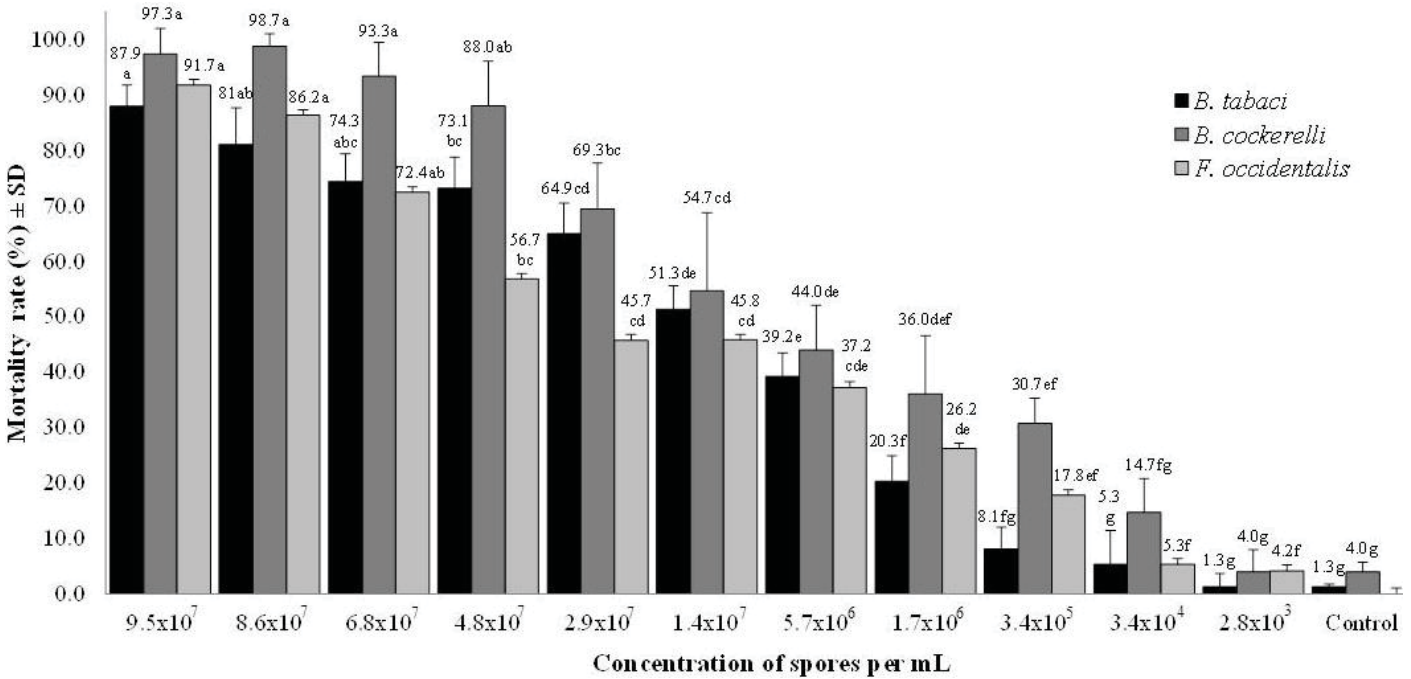

Fig. 2. Mean number ± SD of immature insects of three tomato pests dead by 8 days after treatment with *Metarhizium anisopliae* in México. Means followed by the same letter for a concentration are not statistically different (ANOVA) according to Tukey's test on arcsine transformed data ($P < 0.05$). Nontransformed means are presented. Specimens were submerged for 10 seconds into 50 ml of spore suspension.

differed significantly by insect (F = 286.52, df = 11, P < 0.0001 for *B. tabaci*; F = 72.84, df = 11, P < 0.0001 for *B. cockerelli*; and F = 98.66, df = 11, P < 0.0001 for *F. occidentalis*), as did that by *M. anisopliae* (F = 143.06, df = 11, P < 0.0001 for *B. tabaci*; F = 71.62, df = 11, P < 0.0001 for *B. cockerelli*; and F = 56.35, df = 11, P < 0.0001 for *F. occidentalis*). *B. bassiana* killed most immature *B. tabaci* (100%), while *M. anisopliae* killed most *B. cockerelli* (97%), achieving LT_{50} values of 5.6-6.4 for *B. bassiana* and 4.8-6.5 days for *M. anisopliae* in immature *B. tabaci*, *B. cockerelli*, and *F. occidentalis* (Table 1). Mycelia emerged on the cuticles of the immature insects 2-3 days after death, and most conidia were recorded on legs, wings, and thoraces of some adult cadavers.

The macroscopic developments of the fungi were similar in the three insect host species (Fig. 3). No immatures in the check group were killed by fungal infection. Numbers killed within 8 days after treatment were used to estimate the LC_{50} and LT_{50} for *B. bassiana* against immature *B. tabaci*, *B. cockerelli*, and *F. occidentalis*, where the LC_{50} values were 6.3×10^7, 5.5×10^8, and 5.4×10^8 conidia per milliliter, respectively, with corresponding LT_{50} values of 5.6, 6.4, and 6.1 days (Table 1). LC_{50} and LT_{50} values estimated for *M. anisopliae* against *B. tabaci*, *B. cockerelli*, and *F. occidentalis* were 3.6×10^8, 3.5×10^8, and 5.3×10^8 conidia per milliliter, respectively, with corresponding LT_{50} values of 6.5, 4.8, and 5.3 days.

Studies using fungal isolates to obtain infective conidia from infected insects and cultured on PDA, rice, and wheat (*Triticum aestivum* L.), and commercial products confirmed the effectiveness and potential of entomopathogenic fungi *B. bassiana* and *M. anisopliae* as microbial control agents against psyllids, thrips, and whiteflies (Ansari et al. 2007, Ugine et al. 2007, Lacey et al. 2009, Skinner et al. 2012, Wang and Zheng 2012). However, few studies reported biological activity of entomopathogenic fungi isolated from soil (Ali-Shtayeh et al. 2002, Sánchez-Peña et al. 2007). Conidia that occur naturally in soil can kill many insect pests. Such exposure can cause horizontal transmission from an infected insect (as secondary inoculum source) to another, with several replications of *B. bassiana* and *M. anisopliae* that have cycles of approximately 5 days. Results of this study are within this range of lethal time.

Variations were observed in the number of insect hosts killed by the fungal isolates, with *B. bassiana* most virulent against immature psyllids, whiteflies, and thrips (71-86% mortality within 8 days), with LT_{50} values ranging from 3-4 days post-inoculation. Similar observations were reported by Mascarin et al. (2013a,b), who mentioned that isolates of the fungus were most virulent against *B. tabaci* nymphs with estimated LT_{50} of 3-6 days. Wraight et al. (1998) found 6.3×10^7 spores per milliliter of the fungus was required to kill 50% of nymphs, whereas Lacey et al. (2008) reported 3.6×10^8 spores per milliliter were required to kill the same number of nymphs. Shah and Pell (2003) and Mascarin et al. (2013a,b) observed that *B. tabaci* died within 3 to 14 days after treatment with *B. bassiana*. Sánchez-Peña et al. (2007) and Lacey et al. (2009) reported significant numbers of *B. cockerelli* killed by *B. bassiana* and *M. anisopliae* under laboratory and field conditions. Wang and Zheng (2012) reported 93.08% of *F. occidentalis* died by 6 days after treatment with *B. bassiana*, and Robb and Parrella (1991) found LT_{50} of 3.5 days. Susceptibility (mortality rate and LC_{50} and TL_{50s} parameters) of the insect was affected mainly by the fungal isolate, host species, and host development stage (Liu and Stansly 2000, Mascarin et al. 2013a,b). The experimental method, incubation time, environmental conditions, and chemical surfactants added to the conidial suspension were the same in their study.

Table 1. Median Lethal Concentration (LC_{50}) and Virulence (LT_{50}) of Two Entomopathogenic Fungi Isolates Applied to Immature Stages of *Bemisia tabaci*, *Bactericera cockerelli*, and *Frankliniella occidentalis* that are Tomato Pests In México

Entomopathogen	Pest insect	Number of insects treated	Lower limit	LC_{50} (95% fiducial limits)*	Upper limit	Slope (±SE)	Intercept (±SE)	χ^2	LT_{50} (95% CI) (days ±SE)
B. bassiana	*B. tabaci*	675	$2.0x10^7$	$6.3x10^7$	$3.2x10^8$	0.26±0.03	-2.03±0.16	13.5	5.6±0.28
	B. cockerelli	675	$2.0x10^8$	$5.5x10^8$	$3.2x10^9$	0.45±0.06	-3.93±0.41	16.5	6.4±0.68
	F. occidentalis	675	$2.7x10^8$	$5.4x10^8$	$1.5x10^9$	0.42±0.04	-3.70±0.31	2.5	6.1±0.63
M. anisopliae	*B. tabaci*	675	$2.3x10^8$	$3.6x10^8$	$6.5x10^8$	0.54±0.04	-4.65±0.30	3.7	6.5±0.52
	B. cockerelli	675	$1.9x10^8$	$3.5x10^8$	$7.5x10^8$	0.40±0.03	-3.44±0.24	9.6	4.8±0.24
	F. occidentalis	675	$3.2x10^8$	$5.3x10^8$	$1.1x10^9$	0.47±0.04	-4.13±0.27	8.3	5.3±0.19

*LC_{50} values were expressed as conidia per milliliter and estimated from three replicated bioassays. Twenty-five immature insects of each pest per each fungal concentration (eight units per concentration per fungus) were used; also 25 nontreated larvae (check) per replicate were used.

Specimens were submerged for 10 seconds into 50 ml of a spore suspension.

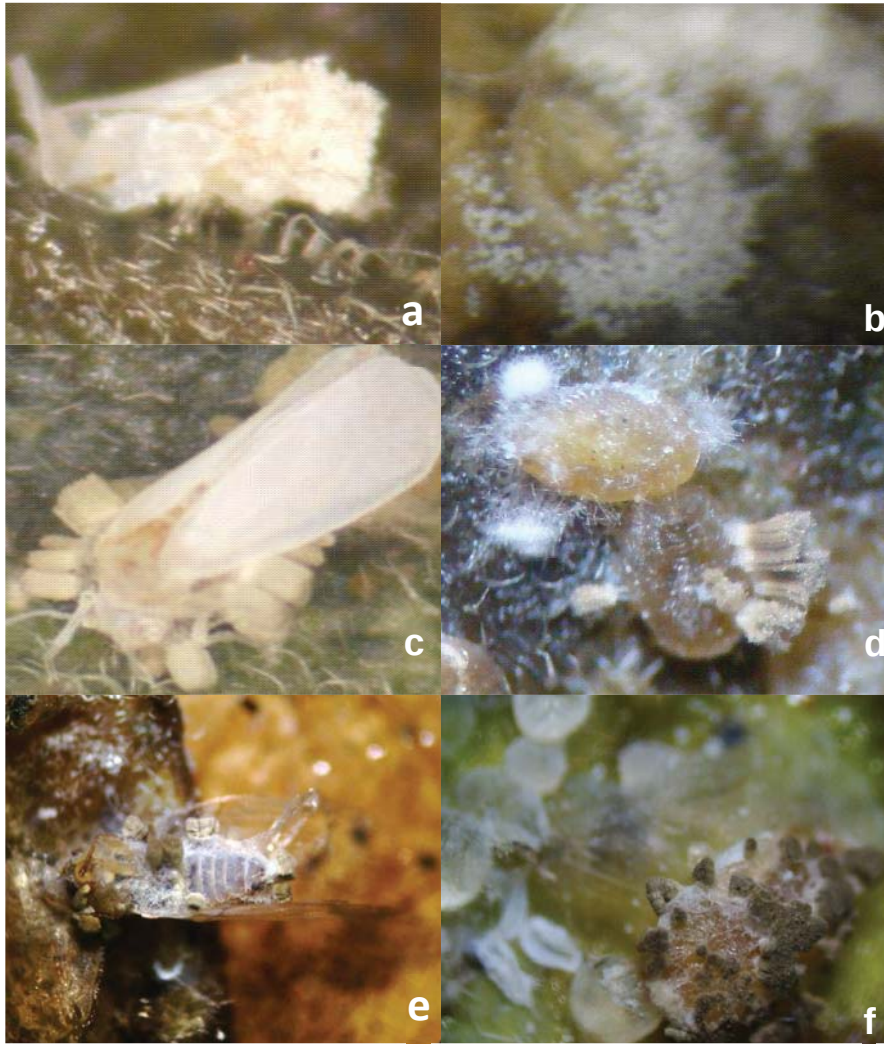

Fig. 3. Adult and nymph of *Bemisia tabaci* infected by, a-b: *Beauveria bassiana*; c-d: *Metarhizium anisopliae*; and e-f: *Bactericera cockerelli* adult and nymph, infected by *Metarhizium anisopliae*.

Results of the study indicated that *B. bassiana* and *M. anisopliae* from soil were pathogenic and very virulent against psyllids, thrips, and whiteflies. The isolates had potential for conidial production on rice grain and inoculum production on insects; hence, they are candidates for use in inundative biological control to manage the insect pests. A large sample of isolates of different species currently is

being used to identify effective formulation techniques and optimize efficacy and persistence of fungal isolates under greenhouse and field conditions.

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