OPTIMIZATION OF PATHOGENICITY TESTS FOR SELECTION OF NATIVE ISOLATES OF ENTOMOPATHOGENIC FUNGI ISOLATED FROM CITRUS-GROWING AREAS OF MÉXICO ON ADULTS OF *DIAPHORINA CITRI* KUWAYAMA (HEMIPTERA: LIVIIDAE)

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ABSTRACT

Huanglongbing (HLB), considered one of the most lethal diseases of citrus worldwide, has reached the main areas of Mexican lime (*Citrus latifolia* Tanaka) fruit production on the Pacific coast of México. Growers have initiated intensive use of insecticides in order to control populations of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), the vector of the pathogen, 'Candidatus Liberibacter asiaticus' associated with huanglongbing. Presently, costs of insecticides and the side effects of their use are major concerns, because they could impair the management strategy against the vector; and thus, ecologically and economically viable alternatives to conventional insecticides are required in the short term. Therefore the goal of this study was to evaluate the pathogenicity of 27 native isolates and 3 strains of entomopathogenic fungi and determine their potential as biological control agents of *D. citri* by using 2 different bioassay methods. Bioassays were performed under laboratory conditions (26 ± 2 °C, 60 ± 5% RH and 16:8 h L:D) by exposing adult insects to a concentration of 1×10^8 conidia per milliliter using 2 different application methods, i.e., spraying the spores onto the citrus seedlings and spraying the spores directly onto the adult psyllids. The results showed that by direct spraying the adults, HIB-24 (*B. bassiana*) and HIB-32 (*I. fumosorosea*) isolates showed the highest mortality (60.66%). Regarding spraying of the seedlings, HIB-19 (*I. fumosorosea*) showed the highest percentage of mortality (62.02%). The results from this study demonstrate potential for using entomopathogenic fungi in the management of *D. citri* in México.

Key Words: *Diaphorina citri*, entomopathogenic fungi, biological control

RESUMEN

Huanglonbing (HLB), es considerado una de las más letales enfermedades de los cítricos alrededor del mundo, y ha alcanzado las principales áreas de producción de limón Mexicano (*Citrus latifolia* Tanaka) en la costa del pacifico de México. Los productores han iniciado el uso de insecticidas para controlar las poblaciones del psílido asiático de los cítricos, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), el vector del patógeno 'Candidatus Liberibacter asiaticus' asociado con el HLB. Actualmente los costos de los insecticidas y los efectos secundarios de su uso son las principales preocupaciones, ya que podrían perjudicar la estrategia de manejo contra el vector; y por lo tanto, alternativas ecológicas y económicamente viables a los insecticidas convencionales serian necesarias a corto plazo. Por tanto, el objetivo de este estudio fue evaluar la patogenicidad de 27 aislados nativos y 3 cepas de hongos entomopatógenos para determinar su potencial como agentes de control biológico sobre *Diaphorina citri* usando 2 diferentes métodos de bioensayo. Los bioensayos fueron realizados bajo condiciones de laboratorio (26 ± 2 °C, 60 ± 5% H.R y 16:8 h L:O) mediante la exposición de insectos adultos a una concentración de 1×10^8 conidios por mililitro utilizando 2 diferentes métodos de aplicación, es decir, por asperjado de esporas en las plántulas de cítricos y por asperjado directo a los psílidos adultos. Los resultados mostraron que para el asperjado directo a los adultos los aislados HIB-24 (*B. bassiana*) y HIB-32 (*I. fumosorosea*) mostraron el mayor porcentaje de mortalidad (60.66%). Respecto al asperjado de plántulas el aislado HIB-19 (*I. fumosorosea*) mostró el mayor porcentaje de mortalidad (62.02%). Los resultados de este estudio demuestran el potencial para el uso de hongos entomopatógenos en el manejo de *D. citri* en México.

Palabras Clave: *Diaphorina citri*, hongos entomopatógenos, control biológico

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is a major pest of citrus mainly because it is the vector of '*Candidatus* Liberibacter asiaticus', one of the 3 bacterial agents each of which is thought to be responsible for huanglongbing (HLB), also called citrus greening (Bové 2006). This disease is considered to be one of the most destructive of *Citrus* spp. (Sapindales: Rutaceae) in the world, by the severity of symptoms, the rapidity with which it spreads and by affecting all commercial species of citrus (da Graca 1991; Tsai & Liu 2000; Halbert & Manjunath 2004; National Research Council 2010). *Diaphorina citri*, first found in México in 2002 in the states of Campeche and Quintana Roo, has since been distributed to all citrus regions of the country (López-Arroyo et al. 2009). HLB was detected in July 2009 in Tizimín, Yucatán, also in 2009 in Quintana Roo, Nayarit and Jalisco, and in 2010 in Campeche, Colima, Sinaloa and Michoacán, and in 2011 in the Baja California Sur, Chiapas, Hidalgo and San Luis Potosí. Clearly HLB represents a serious problem for the citrus industry, because of the possibility of severe epidemics that would degrade this important economic fruit industry which annually produces 6.9 million tons of citrus in México (SENASICA 2012, www.senasica.gob.mx). Different strategies for managing *D. citri* have been based on various chemical, biological and cultural controls, which are necessary to achieve sustainable management of the pest and thus reduce the incidence of the disease (Qureshi & Stansly 2007). *Diaphorina citri* is subjected to various levels of biological control throughout its geographic distribution. The species complex of biological control agents attacking *D. citri* varies geographically. However, in many areas in Asia (where *D. citri* originated) as well as in many areas the psyllid has invaded, the complex of predators usually includes various species of lady beetles (Coleoptera: Coccinellidae); syrphid flies (Diptera: Syrphidae); lacewings (Neuroptera: Chrysopidae and Hemerobiidae); and spiders (Aranae) (Aubert 1987; Michaud 2002; González et al. 2003). There is relatively little known regarding the extent to which these predators reduce infestations of *D. citri*, but some are regarded as important biological control agents. Furthermore, the psyllid is attacked in Asia by 2 primary parasitoid species, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae). *Tamarixia radiata* has been regarded generally as the better of these 2 parasitoids against *D. citri*. Classical biological control projects have been conducted to establish these 2 parasitoids in a number of countries invaded by *D. citri* including Mauritius, Réunion Island, and the United States, specifically in Florida (Hall 2008). On the other hand the use of entomopathogenic fungi has emerged as an alternative to control psyllid populations (Hall 2008; Avery et al. 2009, 2011). A number of species of entomopathogenic fungi have been reported to infect *D. citri* worldwide including *Isaria fumosorosea* Wize (= *Paecilomyces fumosoroseus*) (Samson 1974; Subandiyah et al. 2000; Álvarez-González et al. 2003; Meyer et al. 2008; Avery et al. 2009, 2011), *Hirsutella citriformis* Speare (Rivero-Aragón & Grillo-Ravelo 2000; Subandiyah et al. 2000; Étienne et al. 2001; Álvarez-González et al. 2003; Meyer et al. 2007; Reyes-Rosas et al. 2009; Hall et al. 2012), *Lecanicillium lecanii* Zimm. (*Verticillium lecanii*) (Xie et al. 1988; Rivero-Aragón & Grillo-Ravelo 2000); *Beauveria bassiana* (Bals.) Vuill. (Rivero-Aragón & Grillo-Ravelo 2000; Álvarez-González et al. 2003; Yang et al. 2006); *Cladosporium* sp. nr. *oxysporum* Berk. & M.A. Curtis and *Capnodium citri* Berk. and Desm. (Aubert 1987). The fungus, *H. citriformis*, was reported to be common in Guadeloupe Islands during periods when humidity was greater than 80% (Étienne et al. 2001). In Florida, cadavers of adult *D. citri* killed by *H. citriformis* (Meyer et al. 2007) have been observed from mid-summer through winter, mainly in larger trees (Hall et al. 2008). The objective of this study was to determine the difference in the effectiveness of 2 bioassay methods in order to test the pathogenicity of native isolates of *B. bassiana*, *I. fumosorosea* and *M. brunneum* isolated from soils from citrus regions of México with potential for control of *Diaphorina citri*.

MATERIALS AND METHODS

Colony of *Diaphorina citri*

For the establishment of a colony of *D. citri*, adults were collected from shoots or leaves of mature sour orange, *Citrus aurantium* (L.), and trifoliate orange, *Poncirus trifoliata* (L.), which were established in germination beds. Around 200 insects (females and males) were collected and released in ventilated cages of 2 different sizes, i.e., 48 × 48 × 60 cm and 60 × 65 × 160 cm. These were kept in a room with controlled conditions at 26 °C and 16:8 h L:D. Each cage contained 2 'Volkameriana' lemon trees (*Citrus volkameriana* V. Ten. & Pasq.), which had tender vegetative shoots "flush" for adults to feed on and reproduce. When the seedlings were matured, the insects were collected for introducing them into cages with new flushing plants.

Activation of Entomopathogenic Fungi

Native isolates of entomopathogenic fungi were obtained from citrus growing areas of México (Table 1). For this study we selected 27

isolates and 3 strains (GHA; *Beauveria bassiana*, Pfr-612; *Isaria fumosorosea* (formerly *Paecilomyces fumosoroseus*) and Met; *Metarhizium brunneum* (formerly *M. anisopliae*) from the collection of the Institute of Biotechnology, FCB-UANL, stored cryogenically (10% glycerol at -80 °C). The strains were thawed at room temperature, inoculated on potato dextrose agar (PDA) and incubated at 28 °C for 14 to 21 days (Table 1).

Preparation of Conidial Suspensions

After each strain had been incubated for 14 days, 10 mL of Tween® 80 solution at 0.1 % (v/v) was added and then the surface of each plate was scraped with a spreader to obtain a concentrated suspension of conidia. A Neubauer chamber was used to determine the concentration of these suspensions, and each suspension was adjusted to a concentration to 1×10^8 spores/mL. The spore suspensions were used immediately for bioassays.

Spore Viability

Spore viability was determined at the time of treatment by spraying 1 mL aliquots of spore suspension onto PDA plates (10 cm diam Petri dish) and incubating at 25 °C for 18 h. Spore germination was halted by placing 3 separate drops of lactophenol cotton blue and 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 X magnifications with a compound microscope, and determining the proportion of spores that possessed a distinct germ tube, as defined by germ tube lengths that were 2 times the diam of the spore (Goettel & Inglis 1997). All experiments were repeated at least 3 times for each entomopathogenic isolate and strain under the same conditions.

Bioassay Chambers

To test the pathogenicity of the entomopathogenic fungi used in this study, we used a series of special chambers. Each of these chambers consisted of a 2 L plastic container with a tight lid. Each one these plastic containers had a window in its side 9 cm wide × 22.5 cm long covered with mesh to prevent the psyllids from escaping (Fig. 1).

TABLE 1. ENTOMOPATHOGENIC FUNGI ISOLATED FROM CULTIVATED SOILS OF CITRUS ORCHARDS IN DIFFERENT STATES OF MÉXICO.

| Key isolate	Location of collection	Geographic location	Elevation (m asl)	Species
HIB-1	Guasave, Sinaloa	N 25°42'20" W 108°20'48"	50	*B. bassiana*
HIB-2	Ahome, Sinaloa	N 25°55'01" W 109°10'42"	10	*B. bassiana*
HIB-3	Ahome, Sinaloa	N 25°33'58" W 108°26'44"	10	*B. bassiana*
HIB-4	Ahome, Sinaloa	N 25°55'24" W 109°10'25"	10	*B. bassiana*
HIB-6	Guasave, Sinaloa	N 25°34'31" W 108°28'18"	50	*B. bassiana*
HIB-7	Ahome, Sinaloa	N 25°48'48" W 108°59'27"	10	*B. bassiana*
HIB-8	Tamazunchale, San Luis Potosí	N 21°17'15" W 98°43'38"	140	*B. bassiana*
HIB-10	Allende, Nuevo León	N 25°24'34" W 99°59'17"	460	*B. bassiana*
HIB-11	Hualahuises, Nuevo León	N 25°24'34" W 99°59'17"	400	*M. brunneum*
HIB-14	Hidalgo, Tamaulipas	N 24°14'52" W 99°26'11"	400	*B. bassiana*
HIB-15	Hidalgo, Tamaulipas	N 24°14'52" W 99°26'11"	400	*B. bassiana*
HIB-17	Ahome, Sinaloa	N 25°55'24" W 109°10'25"	10	*B. bassiana*
HIB-19	Linares, Nuevo León	N 25°09'13" W 99°51'10"	350	*I. fumosorosea*
HIB-20	Hualahuises, Nuevo León	N 24°53'03" W 99°38'55"	360	*I. fumosorosea*
HIB-21	Tikinmul, Campeche	N 19°46'20" W 90°12'10"	20	*I. fumosorosea*
HIB-22	Montemorelos, Nuevo León	N 25°10'03" W 99°57'11"	430	*I. fumosorosea*
HIB-23	Montemorelos, Nuevo León	N 25°10'03" W 99°51'10"	430	*I. fumosorosea*
HIB-24	Montemorelos, Nuevo León	N 25°19'09" W 99°54'07"	430	*B. bassiana*
HIB-25	Montemorelos, Nuevo León	N 25°10'03" W 99°57'11"	430	*B. bassiana*
HIB-26	Hampolol, Campeche	N 19°55'25" W 90°23'26"	10	*I. fumosorosea*
HIB-27	Hermosillo, Sonora	N 28°53'36" W 111°19'45"	210	*I. fumosorosea*
HIB-28	Montemorelos, Nuevo León	N 25°19'06" W 99°51'12"	430	*I. fumosorosea*
HIB-29	Montemorelos, Nuevo León	N 25°18'12" W 99°53'13"	430	*I. fumosorosea*
HIB-30	Montemorelos, Nuevo León	N 25°10'03" W 99°57'11"	430	*I. fumosorosea*
HIB-31	Chencolli, Campeche	N 19°48'48" W 90°16'25"	30	*I. fumosorosea*
HIB-32	Padilla, Tamaulipas	N 24°05'00" W 99°07'30"	153	*I. fumosorosea*
HIB-33	Pocyaxum, Campeche	N 19°40'30" W 90°20'30"	20	*I. fumosorosea*
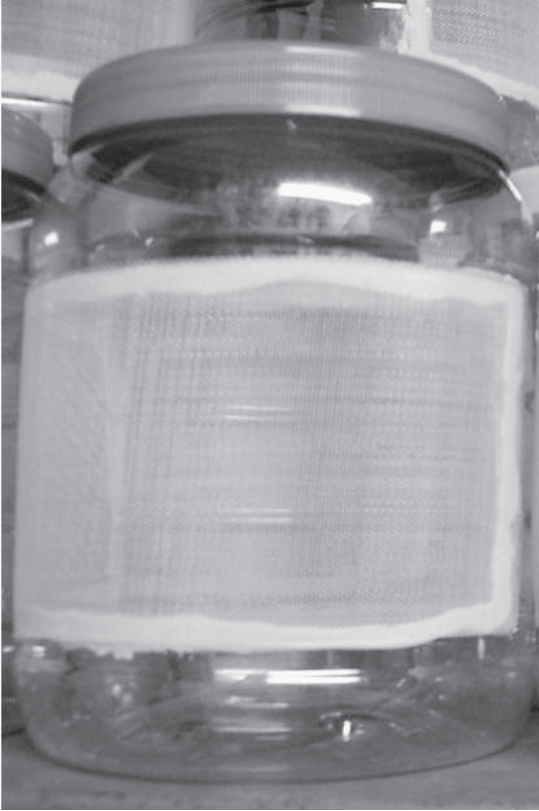
Fig. 1. Bioassay chamber used to test the pathogenicity of the entomopathogenic fungi. The chamber is a 2 L plastic container with a tight lid and a mesh-covered window in its side to prevent escape of the psyllids.

Bioassays

The treatments consisted of a high spore concentration (1×10^8 spores/mL), water plus Tween® 80 control, and an untreated control. To assess natural mortality, untreated adults (untreated control) and adults treated with 0.1% Tween® 80 (application control) were included in all experiments. Two methods were used for treating the *D. citri* psyllids, i.e., either the treatments were applied by 1) spraying the citrus seedlings previously infested, or 2) by directly spraying the psyllids.

For the first method, 'Cleopatra' mandarin (*Citrus reshni* Hort ex Tan.) seedlings growing on Sunshine Peat Moss® (Sun Gro® Horticulture, Vancouver, Canada) in styrofoam pots of 237 mL were inoculated. The pots were covered with brown paper to prevent contact between the insect and the ground (Fig. 2). Then the seedlings were placed for 72 h in reproduction cages of 48 × 48 × 60 cm. to induce a natural infestation. After this time, seedlings with at least 10 actively feeding adult psyllids were selected and placed individually into a bioassay chamber. Once the chamber was closed and sealed, the seedlings pre-
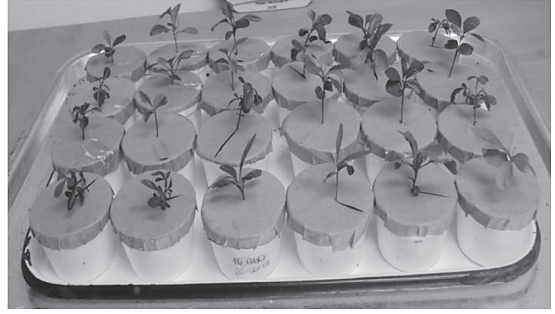

Fig. 2. Styrofoam pots covered with brown paper to prevent contact between the insect and the ground. A 'Cleopatra' mandarin seedling was grown on Sunshine Peat Moss® in each 237 mL pot. The seedlings infested by placing the pots into a cage with *Diaphorina citri*.

viously infested in the reproduction cages were sprayed through the window of mesh with 1 mL of conidial suspension using a Nalgene© aerosol sprayer (Fig. 3).

For the direct spray method the adult insects were collected in their resting positions from the reproduction chamber using an oral aspirator. Each group of 10 individuals was placed in a 250 mL plastic container. To facilitate the application of spore suspensions, the psyllid adults were kept in a refrigerator for 10 min at 7 °C, and

Fig. 3. 'Cleopatra' mandarin seedling placed into a bioassay chamber.

only those that survived the cold were used for the treatment. The treatments were applied by spraying 1 mL each of the suspensions of spores with a Nalgene© aerosol directly on the psyllid adults while they were in the container. After the psyllids had been sprayed, they were placed in a bioassay chamber that already contained a 'Cleopatra' mandarin seedling.

The sprayer used delivered 0.135 ± 0.04 mL, and therefore we standardized at 10 sprays per 10 adult insects and/or infested seedling. For the bioassay with the seedlings, the amount of suspension delivered was determined by placing a 10 cm diam Petri dish of that contained 1.5% water agar in one the bioassay chambers and the spraying 1 ml of suspension through the window. For the bioassay by the direct spray method against adult insects, the amount of suspension delivered was determined by spraying 1 mL of suspension directly on a 10 cm diam petri dish. Later, for each of these 2 methods were cut 5 agar blocks each 20 mm long × 20 mm wide × 3 mm high were cut out of the Petri dish. Finally each agar block was examined by a compound microscope at 400 x magnification.

Incubation of Bioassays

Bioassay chambers were incubated in an environmental chamber at 26 ± 2 °C, 60 ± 5% RH and 16:18 h L:D. Insect mortality was evaluated 7 days after application for each treatment. The dead adults were counted and placed in a moist chamber to support sporulation. After spending 7 days in the moist chamber, the insects that displayed fungal development were counted as mycosed. All experiments were performed in triplicate and were repeated at least twice under the same conditions.

Statistical Analysis

Arcsine transformed mortality rates were corrected by the Schneider-Orelli formula (Ciba-Geigy 1981). Mortality rates were subjected to the Kolmogorov-Smirnov test to verify the normality of the data. Treatment data were analyzed by ANOVA (α = 0.01) with mean separation by the Scheffé *F*-test (α = 0.01). All statistical tests were conducted by IBM SPSS® v.19 Inc., New York, USA.

Results

Evaluation of Entomopathogenic Fungi by Spraying the Spores onto the Citrus Seedlings

Spore viability was 93 ± 3.7%; the mean number of conidia/mm² deposited on the agar blocks was 412 ± 62. The results showed that the mortality for *D. citri* ranged from 33.21% to 62.02%. Based on statistical analysis, it was determined that differences between treatments were highly significant (F = 10,364; df = 29, 59; P < 0.0001). The comparison of means between the entomopathogenic fungi evaluated showed that the isolate HIB-19 of *I. fumosorosea* sprayed onto the citrus seedlings induced the greatest highest mortality of *D. citri* adults, while isolate HIB-31 induced the least mortality (Table 2).

Evaluation of Isolates by Spraying the Spores Directly onto Adult Psyllids

Spore viability was 94 ± 3.86 %; the mean number of conidia/mm² deposited on the agar blocks was 433 ± 58. The results indicated that mortality for *D. citri* ranged from 34.44% to 60.66%. Differences between treatments were highly significant (F = 31.721, df = 29, 59 P < 0.0001), and comparison of means showed that isolates HIB-24 of *B. bassiana* and HIB-32 of *I. fumosorosea* sprayed directly onto *D. citri* adults induced the greatest mortality, while isolate HIB-22 of *I. fumosorosea* induced the least mortality (Table 2).

Discussion

Methods for the selection of microorganisms in the laboratory as control agents of insect pests involve a number of biological factors (e.g., greater percentage of effectiveness in a shorter time), and technological factors, (e.g., development characteristics of the microorganism). In the case of entomopathogenic fungi it is important that they present high sporulation rates, rapid growth, and retain their viability and infectivity (Goettel & Roberts 1992; Feng et al. 1994). Another important factor is the development of an effective test method to determine the true potential of an entomopathogenic fungus against a target insect (Posada & Vega 2005). Around the world, various laboratory methods have been developed to establish the effectiveness of different species of entomopathogens against *D. citri*, which focus on assessing insecticidal activity by measuring infectivity and virulence (Grifaldo-Alcántara et al. 2009). This study evaluated the pathogenicity of entomopathogenic fungi native to the Mexican citrus area using 2 different spray application techniques; however, percent mortality on psyllids by both methods was similar. This indicates that there was no difference between the two, which may be associated with factors related to entomopathogenic fungi and/or incubation conditions. For both methods we used plastic containers (PET) with a mesh in order to allow ventilation and prevent the passage of insects. In the market there is a wide variety of meshes with different pore sizes, which may be penetrable or impenetrable, but in general most of them are made of monofilament high density polyethylene

TABLE 2. MORTALITY OF *DIAPHORINA CITRI* TREATED WITH ISOLATES OF NATIVE SPECIES OF ENTOMOPATHOGENIC FUNGI BY 2 DIFFERENT METHODS OF APPLICATION UNDER LABORATORY CONDITIONS.

		Application Method	
		Sprayed on seedlings	Sprayed directly on psyllids
Isolate	Species	Mortality (%)	
HIB-1	*B. bassiana*	37.46 abc	51.35 cdefg
HIB-2	*B. bassiana*	40.97 abc	43.28 abcde
HIB-3	*B. bassiana*	49.02 abc	47.29 abcdefg
HIB-4	*B. bassiana*	44.42 abc	51.35 cdefg
HIB-6	*B. bassiana*	43.28 abc	49.02 abcdefg
HIB-7	*B. bassiana*	38.64 abc	47.86 abcdefg
HIB-8	*B. bassiana*	41.55 abc	42.70 abcde
HIB-10	*B. bassiana*	49.60 abc	40.97 abcd
HIB-11	*M. brunneum*	37.46 abc	35.66 ab
HIB-14	*B. bassiana*	34.44 ab	35.06 ab
HIB-15	*B. bassiana*	49.07 abc	48.45 abcdefg
HIB-17	*B. bassiana*	50.76 abc	43.28 abcde
HIB-19	*I. fumosorosea*	62.02 c	43.28 abcde
HIB-20	*I. fumosorosea*	46.71 abc	45.00 abcdef
HIB-21	*I. fumosorosea*	38.05 abc	43.28 abcde
HIB-22	*I. fumosorosea*	58.05 abc	34.44 a
HIB-23	*I. fumosorosea*	44.69 abc	40.58 abcd
HIB-24	*B. bassiana*	51.94 abc	60.66 g
HIB-25	*B. bassiana*	41.55 abc	46.14 abcdefg
HIB-26	*I. fumosorosea*	56.78 abc	56.78 efg
HIB-27	*I. fumosorosea*	45.00 abc	42.70 abcde
HIB-28	*I. fumosorosea*	42.70 abc	50.76 cdefg
HIB-29	*I. fumosorosea*	50.18 abc	49.60 bcdefg
HIB-30	*I. fumosorosea*	48.47 abc	45.28 abcdef
HIB-31	*I. fumosorosea*	33.21 a	36.86 abc
HIB-32	*I. fumosorosea*	54.33 abc	60.66 g
HIB-33	*I. fumosorosea*	60.66 bc	54.33 defg
GHA	*B. bassiana*	46.16 abc	45.86 abcdef
Pfr-612	*I. fumosorosea*	52.53 abc	58.05 fg
Met	*M. brunneum*	45.00 abc	49.02 abcdefg
Mean±SD		46.49 ± 7.43	46.65 ± 7.00

Tween 80 control, mean ± SD: 22.6 4± 2.85. Untreated control, mean ± SD: 23.55 ± 2.64
Values in the same column followed with the same letter are not significantly different according to Scheffé *F*-test ($P \leq 0.01$).

containing additives such as UV light stabilizers and antioxidants.

The mesh used in the bioassay chambers was not impenetrable to spray droplets, but the sprayer used may have introduced variability. The pressure in this sprayer was manually created and its spray delivery cannot be considered to have been as uniform as that delivered by a spray tower that allows a fine spray for better coverage of microorganisms to provide a larger area of action for the conidia.

Variation in psyllid mortality exposed to different entomopathogenic fungal isolates or strains may be caused principally by factors such as light, temperature and humidity, which can independently or collectively affect fungi that oc-

cur naturally or that are introduced as biological control agents. These factors may also affect the growth and survival of fungi directly or indirectly through their host, and usually such effects differ between different species of fungi (Fargues et al. 1997). These factors, in turn, may affect the ability of entomopathogens to induce mortality in a particular host, and they can also be associated with differences in pathogenicity of the strains themselves, the inoculation technique and the concentration of spores (Moorhouse et al. 1994).

In the present study the conditions used for evaluation were designed for application to psyllids, and we consider these conditions to be appropriate for the main species of entomopathogenic fungi (Zimmermann 2007, 2008). Several

studies suggest that an entomopathogenic fungus tends to kill more rapidly at the temperature that is optimum for its growth (Moorhouse et al. 1994), and the speed of development of the mycelium and thus the evolution of the infection depend on the temperature (Ferron 1978). However, in general, it appears that the effect of temperature on mycosis has been less studied than its effect on hyphal extension (Thomas & Jenkins 1997).

Humidity is a factor in the development and spread of the infection because it facilitates fungal sporulation (Rath 2000), but humidity is not considered decisive for the penetration of the fungus and infection (Leucona et al. 2001). However, high humidity, close to saturation, is connected with infection periods due to an increase in the percentage of inoculum; and under these conditions auto multiplication of inoculum produces very high insect contamination, which strongly facilitates epizootic development of mycosis (Guglielmone et al. 1997). On the other hand when the humidity is very low, the effectiveness of entomopathogenic fungi can be very limited (Lo & Chapman 1998). In this study the relative humidity of the environmental chamber was approximately 60%, but the moisture levels within the bioassay chambers were not determined, and there may have been variations of moisture between them.

Furthermore different isolates of the same species of pathogen may behave differently with respect to pathogenicity. This behavior can be related to virulence and the production of secondary metabolites that influence the ability of the pathogen to cause disease (Padulla & Alves 2009). In addition to the intrinsic qualities of the pathogen, the susceptibility and/or the natural resistance of host insect itself, is another factor in the pathogenesis of an isolate (Alves 1998). In bioassays for the selection of strains, variations in pathogenicity has been observed, which may be a result of the genetic variability of the isolates with regard to virulence, specificity and host tolerance (Vestergaard et al. 1995; Alves 1998).

Subandiyah et al. (2000) conducted a test using 3 different concentrations of conidia of *I. fumosorosea*, and at 6 days they obtained up to 55.6% mortality when using 10^8 conidia/ mL. In another study Mellín-Rosas et al. (2009) evaluated 4 strains of entomopathogenic fungi at 1×10^7 conidia/mL with 10 days of incubation, and 2 of the *I. fumosorosea* strains induced mortalities of 62.71% and 77.38%. Stauderman et al. (2012) reported strains of *I. fumosorosea* in laboratory tests induced up to 100% mortality of adult psyllids at concentrations between 10^6 and 10^7 blastospores/mL after 12 days. These results demonstrate that the pathogenicity of fungal strains is highly variable, and that a single method of evaluation cannot be used as a universal measure of virulence (Grewal et al. 1994). Strains or isolates of the same fungal species may vary genetically with respect to its adaptation to a particular host; in addition host tolerance and geographical distribution may be factors in their pathogenesis (Vestergaard et al. 1995; Alves 1998; Coates et al. 2002).

The biological activity, i.e., toxicity, of several species of entomopathogenic fungi on insect pests has been demonstrated through various methods, which take into account the interaction of biotic and abiotic factors, such as temperature, relative humidity, origin of the fungus, age and species of the insect (Hernández-Díaz Ordaz et al. 2010). The results suggest that biologically active fungal species have the potential to be used as an element of mortality for management of these pests without the risk posed by different types of insecticides.

Imidacloprid and abamectin have been applied to suppress psyllid populations, and they require multiple applications (up to 6-8/yr). Such insecticides are expensive, disrupt the balance of natural enemies and may lead to the development of resistance by the psyllid (Srinivasan et al. 2008), while the biopesticides can be used as alternatives in a spray program to break the cycle of dependence on the harder chemicals and prevent the development of resistance (Moore 2008).

In México, biological control has been successfully applied against various pests (Rodríguez & Arredondo 2007), and in the case of citrus, entomopathogenic fungi are considered as one of the groups with potential for effective pest management. However the use of entomopathogens as biological control agents in an integrated pest management program should be linked to the study of the possible effects on non-target beneficial insects associated with the target pest. In the case of *D. citri* these beneficials include predators like *Olla v-nigrum* Mulsant, *Cycloneda sanguinea* (L.) (Coleoptera: Coccinellidae), *Chrysoperla spp.* (Neuroptera: Chrysopidae), and parasitoids such as *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), which are important natural mortality factors of *D. citri* populations (Quereshi & Stansly 2009). Also it is important to assess the effects on beneficial of chemicals commonly used in citrus production such as those reported by Hall et al (2012), who observed that high rates of copper hydroxide, oil or sulfur may affect the development of the entomopathogenic fungus, *H. citriformis*.

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