El Colegio de la Frontera Sur

Patogenicidad de *Beauveria bassiana* sobre estados inmaduros de *Diaphorina citri* (Hemiptera: Liviidae), bajo condiciones de laboratorio y jaula de campo.

TESIS
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Dedicatorias

A Dios

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Índice

I. Introducción

Los cítricos constituyen un producto agrícola básico en México, siendo fuente de empleo y de ingresos en zonas rurales. México es considerado el quinto productor de cítricos en el mundo, con 566,083 hectáreas (FAO 2015). En Chiapas se tienen establecidas 3,489 hectáreas, de las cuales 2,340 hectáreas corresponden al cultivo de naranja y 1,149 a limón. La mayor parte de los municipios con cítricos en el Estado son de traspatio, pero pueden encontrarse huertos comerciales en algunas regiones de la entidad, tales como La Trinitaria, Benemérito de las Américas, La Libertad, Marqués de Comillas, entre otros. El Estado de Chiapas no sobresale en superficie sembrada de cítricos, sin embargo no deja de ser importante por la vulnerabilidad de la llegada de plagas de importancia económica que afectan a estos cultivos (SIAP 2010).

Existen varias plagas que disminuyen el rendimiento y/o calidad de los cítricos, sin embargo, uno de los problemas más importantes a los que se está enfrentando la citricultura actual, es la presencia del psílido asiático de los cítricos, *Diaphorina citri* (Nava et al. 2010). Esta plaga inició la invasión al país por el Estado de Campeche durante 2002 (Mellin-Rosas 2011), posteriormente fue detectado en los meses de Julio y Agosto de 2003 en las regiones citrícolas de los estados de Tamaulipas y Nuevo León (Coronado y Ruiz 2004), y finalmente se dispersó a los 23 estados citrícolas del país (Torres- Pacheco et al. 2013).

Este insecto causa un daño directo al extraer la savia de las hojas, peciolos y brotes tiernos, y en infestaciones fuertes provoca deformación de los mismos así como la caída de follaje y flores (Rogers y Stansly 2006). Sin embargo, es mucho más relevante el daño que causa como vector, ya que transmite la enfermedad conocida como Huanglongbing (HLB), enverdecimiento, dragón

amarillo o greening ocasionada por una bacteria restringida al floema denominada *Candidatus liberibacter* spp. (Bové 2006), enfermedad devastadora de los cítricos, reportada en México en Yucatán, Quintana Roo, Nayarit y Jalisco desde el año 2009, (Trujillo-Arriaga 2009), un año después se reportó en Campeche, Colima, Sinaloa y Michoacán, y en el 2011 ya estaba presente en Baja California Sur, Chiapas, Hidalgo, San Luis Potosí y Veracruz (Gandarilla-Pacheco et al. 2011).

Son muchas las acciones que se han implementado para tratar de controlar y disminuir la presencia del vector. Dentro de estas se encuentran el control químico, biológico y cultural (Qureshi y Stansly 2007). El control biológico podría ser utilizado como una buena estrategia para la reducción de las poblaciones de *D. citri*, ya que su uso ayudaría a minimizar el impacto ambiental provocado por el uso irracional de productos químicos (Qureshi y Stansly 2009). En este sentido los hongos entomopatógenos constituyen uno de los grupos de mayor importancia en el control de insectos y han sido utilizados como alternativas para el control de diversas plagas de importancia económica (Goettel et al. 2000). Debido a que la gran mayoría de insectos son susceptibles a las enfermedades causadas por estos microorganismos, y dado su mecanismo de invasión, los convierte en excelentes agentes de control ya que actúan como insecticidas de contacto (Téllez-Jurado et al. 2009).

Existen varias especies de hongos entomopatógenos que se han utilizado para controlar las ninfas y adultos de *D. citri*, y que pueden ser útiles en un manejo integrado de la plaga. Dentro de estos se encuentran: *Isaria fumosorosea* Wize (*Paecilomyces fumosoroseús*) (Gandarilla-Pacheco et al. 2013; Lezama-Gutiérrez et al. 2012; Avery et al. 2009; Samson 1974; Stauderman et al. 2012; Subandiyah et al. 2000), *Hirsutella citriformis* Speare (Dwiastuti y Kurniawati 2007; Étienne et

al. 2001; Pérez-González et al. 2015; Rivero-Aragón y Grillo-Ravelo 2000), *Beauveria bassiana* (Bals.) Vuill. (Ferreira-Pinto et al. 2012; Lezama-Gutiérrez et al. 2012; Yang et al. 2006), entre otros. A pesar de los esfuerzos realizados para controlar las poblaciones del insecto, el potencial de los hongos para el control del psílido sigue siendo escaso y los resultados muy heterogéneos, además de que los trabajos se han enfocado solo a las ninfas y adultos, dejando de lado los huevecillos. Por lo anterior, el objetivo del presente trabajo fue evaluar la patogenicidad del hongo *B. bassiana* sobre los estados inmaduros de *D. citri*, en condiciones de laboratorio y jaula de campo.

II. Capitulo

Pathogenicity of *Beauveria bassiana* on *Diaphorina citri* under laboratory conditions.

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ARTICLE

Pathogenicity of *Beauveria bassiana* on *Diaphorina citri* under laboratory conditions

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ABSTRACT

Diaphorina citri (Kuwayama) is the major phytosanitary problem of citrus cultivation in Mexico and the worldwide. It is the vector of the bacteria responsible for Huanglongbing, a devastating disease of citrus crops. The first actions implemented for control of *D. citri* were application insecticides, but low environmental impact alternatives are being sought. This study was conducted to evaluate the pathogenicity of *Beauveria bassiana* (Balsamo) strains and a commercial product on immature stages of *D. citri* in the laboratory. The bioassays showed that a concentration of $1x10^8$ conidia/mL of the four strains and the *B. bassiana* commercial product (Bb-Rhy, Bb-Dc, Bb-18, Botanigard and Bb-Hy) caused 58, 56, 45, 43 and 21 % mortality on nymph stages, respectively. The best lethal times were found for Bb-Rhy and Bb-Dc with 8.5 and 9.5 days. When the nymph stages were grouped in a bioassay using only the Bb-Rhy strain, I-III nymph mortality was statistically different from that of IV-V nymphs, which were more susceptible. The lethal concentrations of the most pathogenic strains on nymph IV-V instars was $8.34x10^6$ for strain Bb-Dc, while for strain Bb-Rhy it was $1x10^7$ conidia/mL. None of the evaluated strains nor the commercial product had any effect on *D. citri* eggs; 95 % of the eggs hatched and the insects continued development to adulthood.

Key words: *D. citri*, eggs, nymphs, *B. bassiana*, pathogenicity

INTRODUCTION

Diaphorina citri Kuwayama (Hemiptera: Liividae) is the main phytosanitary problem of citrus production at the worldwide. It causes direct damage to the plant when it feeds on sap from leaves, petioles and shoots. However, its economic importance lies in the fact that it is the vector of the bacteria that causes Huanglongbing (HLB) (Miranda et al. 2008), a devastating disease of citruses (Torres-Pacheco et al. 2013). Presence of the vector was first reported in the Caribbean and the United States in 1998. By 2001, it had reached Rio Grande, Texas. In Mexico, this insect was initially recorded in Tamaulipas and Nuevo León in the North (Sosa-Armenta et al. 2012), and in the southern states of Campeche and Quintana Roo (Ortega-Arenas et al. 2013). According to Torres-Pacheco et al. (2013), the vector is now present in the 23 citrus-producing states of the country, including Chiapas.

HLB affects only plants of the Rutaceae family and is caused by the bacteria *Candidatus Liberibacter* spp. The first report of this disease was in India in the 18th century; one century later, damage was reported in China. It is widely distributed in Asia, Africa and America. In Mexico, its presence was reported in the states of Yucatán, Jalisco and Nayarit in 2009 and in Sinaloa and Colima in 2010 (López-Collado and López-Arroyo 2013). In Chiapas, it was first reported in Benemérito de las Americas (SIAP 2010). SAGARPA (2013) reported that, besides this municipality, the disease had also been found in La Trinitaria, La Libertad and Marqués de Comillas.

Alemán et al. (2007) reported that chemical control had been implemented with systemic and contact insecticides such as Imidacloprid, Tiametowam, Dimetoato and Lambda-cihalotrina, among others. Biological control with parasitoids (*Tamarixia radiata)*, predators (*Chrysoperla externa, Zelus renardii*) and entomopathogenic fungi (*Hirsutella citriformis*, *Isaria fumosorosea,*

62 *Metarhizium anisopliae* and *Beauveria bassiana*) is an alternative that has low environmental
63 impact (Fernández and Miranda 2005).
64 Entomopathogenic fungi are one of the most important groups for biological insect control and
65 have been used as an alternative in the control of economically important pests. Because most
66 insects are susceptible to diseases caused by these microorganisms and, given their unique
67 mechanism of invasion, these fungi have become excellent control agents acting as contact
68 insecticides (Téllez-Jurado et al. 2009).
69 Ferreira-Pinto et al. (2012) found that the lethal concentration (LC_{50}) of *B. bassiana* on *D. citri*
70 nymphs was $0.4X10^7$ conidia/mL. Moreover, Gandarilla-Pacheco et al. (2013) evaluated the
71 potential of *B. bassiana, I. fumosorosea, H. citriformis* and *M. anisopliae* against *D. citri* nymphs
72 and adults. They observed that *B. bassiana* isolates produced 100 % mortality in nymphs and 80
73 % in adults. Cruz-Cruz et al. (2013) evaluated pathogenicity of *M. anisopliae, H. citriformis* and
74 three strains of *B. bassiana* against *D. citri* adults and found that the *B. bassiana* strain was the
75 most pathogenic.
76 The objective of this study was to evaluate the pathogenicity of *B. bassiana* fungi on immature
77 stages of *D. citri* under laboratory conditions to evaluate the potential of this fungus in the
78 biological control against the Asian citrus psyllid.
79 **MATERIALS AND METHODS**
80 The study was conducted in the biological control laboratories of El Colegio de la Frontera Sur
81 (ECOSUR) located in the city of Tapachula, Chiapas. Here, *D. citri* was reared and *B. bassiana*
82 strains were reactivated for the bioassays to determine pathogenicity, median lethal time (LT_{50})
83 and median lethal concentration 50 (LC_{50}) of the different strains.

D. citri rearing

D. citri adults (females and males) were collected in Colonia La Hermosa Provincia, municipality of Tapachula, Chiapas. The insects were transported in 50 mL plastic centrifuge tubes to ECOSUR facilities where they were released in a nursery with 180 *Murraya paniculata* (L) Jack (Sapindales: Rutaceae) plants covered with organza fabric. The plants were pruned one week before releasing the adults to stimulate shoot growth and thus encourage oviposition and development.

Seeding the fungus in inclined tubes

In the laminar flow bell, silica gel crystals, where different *B. bassiana* strains are preserved, were placed in test tubes containing Agar Dextrose Sabouraud (ADS) culture medium. The tubes were sealed with Parafilm and incubated at 27 ± 2°C and 75±2 % relative humidity for 30 days. This procedure was done for each of the strains.

Reactivation of the _B. bassiana_ strains

The four strains used and the commercial product (Table 1) were reactivated using 50 adult *Anastrepha ludens* flies per strain. The flies were submerged for 30 seconds in a suspension of conidia, placed in 1 L plastic containers with food (hydrolyzed protein) and water, and incubated in the entomopathogenic fungus reproduction room at 27 ± 2°C and 75±2 % RH.

Dead flies were counted every 24 hours and placed in 9 cm sterilized Petri dishes together with a sterile slide and a cotton ball moistened with sterile distilled water and incubated at 27±2 ºC and 75±2 % RH for 10 days.

Once mycelia appeared on the flies, fungus conidia were extracted by the groove technique using a sterile bacteriological loop and placed in test tubes with ADS culture medium. The tubes were sealed with Parafilm and incubated at 27±2 °C with 75±2 % RH for 30 days.

Count and viability of *B. bassiana* strain conidia

The content of each tube was scraped with a bacteriological loop, and 5 mL sterile distilled water and 0.05 % Tween 80 were added; the solution was deposited in a sterile glass vial. Dilutions were done to facilitate conidia counting for each strain in a Neubauer chamber. The suspension in the tubes was shaken in a vortex for 2 min every time a dilution was made. For later bioassays, the concentration of each strain was adjusted to $1x10^8$ conidia/mL.

For the viability test, sterile slides were used. One drop of ADS medium was placed at each end where later a drop of conidial suspension was also placed and covered with a slide cover. These slides were placed in Petri dishes with a cotton ball moistened with sterile distilled water. The dishes were sealed with Parafilm and incubated for 24 hours at 27 ± 2 °C with 75±2 % RH. Three dishes were used for each strain. The proportion of viable conidia was determined by observation at 40X with a compound microscope. Germinated conidia were those that formed germ tubes. Viability was expressed as percentage of germination (Goettel and Inglis 1997). Viable strains were those in which germination was above 90 %.

Bioassays to determine pathogenicity on *D. citri* eggs

Thirty branches of *M. paniculata* 8 cm long with turgid shoots of good coloring and not longer than 5 cm infested with *D. citri* eggs were selected. The nymphs found were eliminated. Each of the four strains and the commercial product were adjusted to a concentration of $1x10^8$ conidia/mL and sprayed onto the shoots with eggs using plastic atomizers 3 cm in diameter by 9 cm long. One mL of fungal solution/shoot was applied, while the control received only sterile distilled water with 0.05% Tween 80. After applying the fungus, the *M. paniculata* branches were placed in flat-bottom vials that contained sterile distilled water and stoppered with a cotton ball. These tubes were placed in 1 L plastic containers, which were covered with organza fabric, and

incubated at 27 ± 2°C with 75±2 % RH. Emergence of nymphs in both treatments and the control was monitored.

Each treatment was replicated five times. The effect of the different *B. bassiana* strains on *D. citri* eggs was observed every 72 hours for a period of 13 days. A completely randomized design was used, in which the experimental units were the *M. paniculata* branches infested with *D. citri* eggs, and the treatments were the four fungal strains, the commercial product and the control.

Bioassays to determine pathogenicity and LT_{50} on first to fifth instar nymphs

To conduct this bioassay, the methodology described above was used, with the difference that, in this case, the first to fifth instar nymphs were counted on the selected shoots. Each treatment was replicated five times and the dead nymphs were counted and placed in humid chambers to stimulate sporulation. The number of nymphs affected by mycosis was also counted.

Mortality of the five *D. citri* nymph instars caused by the different *B. bassiana* strains was determined every 24 hours for 13 days. A completely randomized design was used in which the experimental units were the *M. paniculata* branches infested by nymphs of first to fifth instar, and the treatments were the four strains, the commercial product and the control.

Pathogenicity and LT_{50} bioassays on the two groups of nymphs, instars I-III and IV-V, using the Bb-Rhy strain

Because the results obtained in the previous bioassay showed that the first to third instar nymphs were little affected by the different fungal treatments, another bioassay was conducted using the most aggressive strain, Bb-Rhy, and separating the nymphs into two groups: one group of first to third instar and the other group of fourth and fifth instar. The same procedure was followed as that used previously.

Bioassays to determine LC_{50}

For these bioassays, 28 *M. paniculata* branches 8 cm long that had turgid shoots with good coloring and up to 5 cm long infested with nymphs of only fourth and fifth instar (25 nymphs per shoot) were used. This was because the previous results showed that the first to third instar nymphs were not susceptible to the fungus. The strains used were the most pathogenic and had the shortest lethal time in the previous bioassays (Bb-Dc and Bb-Rhy).

The procedure used was that of the previous bioassays, with the variant that, in this case, the following six concentrations were prepared: $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, and $1x10^9$ conidia/mL for each strain. The control was distilled water with 0.05% Tween 80.

Using the same method as in the previous bioassay, strains Bb-Dc and Bb-Rhy and the control were sprayed on the nymphs and incubated at 27 ± 2°C and 75±2% RH. Mortality of *D. citri* nymphs was determined every 24 hours for a period of 13 days. The dead insects were counted and placed in humid chambers to stimulate sporulation, and the number of nymphs affected by the fungi were counted.

A completely randomized experimental design was used in which the treatments were the six concentrations plus the control. Four replicates of *M. paniculata* shoots infested by fourth and fifth instar nymphs were done.

Data analysis

The results on pathogenicity obtained were analyzed with a Probit analysis using R software version 3.0.2, in which the survival curves of the *D. citri* nymph instars subjected to five *B. bassiana* strains were obtained.

LC_{50} was estimated using a Probit model of R software version 3.0.2, with which the log-probit mortality line of the *D. citri* nymph instars at different concentrations of *B. bassiana* strains was obtained. Data on mortality were corrected using the formula of Schneider-Orelli (Zar 1999).

RESULTS

Effect on eggs

The results obtained in the bioassay show that the four strains and the commercial product of *B. bassiana* evaluated (Bb-18, Bb-Dc, Bb-Rhy, Bb-Hy and Botanigard) at a concentration of $1x10^8$ conidia/mL had no negative effect on *D. citri* eggs: 95 % of the eggs hatched and the insects continued their development normally (Figure 1). The same percentage was observed in the control. None of the eggs that did not hatch, of either the treatments or the control, exhibited mycosis.

Pathogenicity and LT_{50} of *B. bassiana* on first to fifth instar nymphs.

The highest percentages of corrected mortality in the pathogenicity tests at a concentration of $1x10^8$ conidia/mL were those by the strains Bb-Rhy (58.11%) isolated from the soybean weevil *Rhyssomatus nigerrimus*, Bb-Dc (55.99%) isolated from *D. citri* and Bb-18 (45.61%) isolated from a lepidopteran larva (Figure 2). The treatments that did not achieve 50 % corrected mortality in *D. citri* nymphs were Bb-Hy (21.22%) isolated from the coffee borer *Hypothenemus hampei* and the commercial strain Botanigard (43.64%). The highest percentages of mycosis also occurred with treatments Bb-Rhy (69.51%) and Bb-Dc (58.27%) (Table 2). The dead nymphs in the control did not develop mycosis, demonstrating the action of the fungus on the different nymph stages of the insect (Table 2). The dead nymphs in the treatments turned red and lost volume; these effects were not observed in the control nymphs.

Survival analysis showed that there were significant differences among treatments (X^2=21.94, d.f.=5, P=0.0005). With the Bonferroni correction, statistical differences were also found (Bonferroni=0.0033). However, with the interactions, treatments 3 and 4 were different from the rest, but were statistically equal to each other (P=0.3219), indicating that these two strains were the most pathogenic for the *D. citri* nymph instars (Figure 3).

Median lethal times of the of the strains using a concentration of $1x10^8$ conidia/mL were 8.5 days (with intervals of 8.5 and 9.5 days) and 9.5 days (with intervals of 9.5 and 10.5 days) for the Bb-Rhy and Bb-Dc strains, respectively. The effect of the fungus on nymphs decreased after 8 days; that is, after this time, the fungus loss effectiveness against the nymphs (Figure 3).

The pathogenicity tests showed that nymph mortality was different among the five instars of *D. citri* (Table 3). The most susceptible to infection caused by the fungus were fourth and fifth instars (X^2=276.8; d.f.=4; P=0).

Pathogenicity and LT_{50} of the Bb-Rhy strain used against I-III and IV-V instar nymphs.

The highest percentage of corrected mortality in the pathogenicity tests using the Bb-Rhy strain against the two groups of *Diaphorina citri* nymphs was found in the group of fourth and fifth instar (IV-V) nymphs, with 69.4 %. In contrast, mortality in the group of first to third (I-III) instar nymphs was only 18.98 % (Table 4).

The statistical analysis and Bonferroni correction, performed on the data of this bioassay showed that effects of the treatments were statistically different (X^2=128.1272, P=$2.2e^{-16}$) (Bonferroni= 0.0083). The Tukey test showed that Bb-Rhy treatment of the IV-V group was statistically different from the rest, and that the Bb-Rhy I-III was similar to the control (Table 5).

The survival curve of the different treatments reveals that survival of nymphs in treatment Bb-Rhy (I-III) was greater than 50 % (Figure 4), and therefore, it was not possible to calculate LT_{50}. The Bb-Rhy (IV-V) treatment had low survival; lethal time was 5.5 days with intervals of 3.5 and 6.5 days.

The percentages of mycosis for the Bb-Rhy treatments were 45 % for Bb-Rhy (I-III) and 68. % for Bb-Rhy (IV-V). The nymphs of the control did not develop mycosis. It should be mentioned that the control nymphs continued their development into adulthood. Likewise, the nymphs in the fungus application treatments that did not die also reached adulthood.

LC_{50} of the two *B. bassiana* strains (Bb-Dc and Bb-Rhy) on IV and V instar nymphs

LC_{50} of the strain Bb-Dc

The median lethal concentration for strain Bb-Dc, one of the most pathogenic, was $8.34x10^6$ conidia/mL, with intervals of $1.63x10^6$ and $1.41x10^7$ (Figure 5). The percentage of mortality increased as the dosage increased, achieving a corrected mortality of 91.81 % for the highest concentration ($1x10^9$) and 12.6 % for the lowest concentration ($1x10^4$). The nymphs that did not die from effect of the fungus continued their development to adulthood. For the control, mortality was 2.30 % (Figure 6); the nymphs of this treatment that did not die continued their development to adulthood.

The percentages of mycosis were higher with the highest concentrations. For the concentration $1x10^9$, the percentage was 93.39 %, and for the concentration $1x10^4$, the percentage of mycosis was 23.08 % (Table 6). Because the nymphs of the control that died did not develop mycosis, it is evident that nymph mortality in the rest of the treatments was caused by the fungus.

LC_{50} of the strain Bb-Rhy

The median lethal concentration for the strain Bb-Rhy was $1x10^7$ conidia/mL, with intervals of $1.62x10^6$ and $1.81x10^7$ (Figure 7). Mortality increased with increased dosages. With the highest dosage ($1x10^9$), a 74.24 % corrected mortality was achieved and for the lowest concentration ($1x10^4$) it was 23.94% (Table 7). The nymphs that did not die from fungal infection continued their development to adulthood. Mortality in the control was 7.69 %; the nymphs that did not die continued their development into adulthood.

The percentages of mycosis were higher with the highest concentrations. With the concentration of $1x10^9$ conidia/mL it was 79.59%, and with the concentration of $1x10^4$ the percentage was de 23.52% (Table 7). The control nymphs that died did not develop mycosis, demonstrating that death of nymphs in the rest of the treatments was by effect of the fungus.

DISCUSSION

This paper is the first report on the little impact caused by *Beauveria bassiana* on the eggs of *Diaphorina citri*. The results obtained show that the four strains and the commercial product of *B. bassiana* evaluated have no negative effect on *D. citri* eggs; 95 % hatched and continued normal development into the nymph stage. The only reports we found were the resistance of white fly eggs to certain pathogenic fungi. Gindin et al. (2000), reported that *Bemisia argentifolii* eggs were immune to infection by *Verticillium lecanii,* but when the eggs were close to hatching, fungus hyphae can infect the following stage. This did not occur in our study: the nymphs that hatched reached adulthood, and therefore, it can be stated that *B. bassiana* did not affect the psyllid during this development stage.

Moreover, Abdel-Baky et al. (1998) found that *Cladosporium* spp. fungus caused low percentages of mortality in *Bemisia* spp. eggs, 14 to 28%. Mohammad and Deghairi (2008) also found that *B. bassiana* had little impact on *B. tabaci* eggs, causing average mortality of 4.49%. Both reported that the phenomenon is due to the structure of the chorion, which acts as a strong barrier against the invasion of fungal spores. Because the fungus germ tube requires time to germinate and penetrate, it is probable that this time is longer than that of embryo development. There may also be antifungal compounds on the eggs that inhibit conidial germination (Meeks et al. 2000). Therefore, it is important to continue evaluating other strains and other pathogenic fungi against the eggs of this psyllid to determine whether any might be pathogenic.

Regarding the *D. citri* nymph instars, the strains Bb-Rhy and Bb-Dc were more pathogenic at a concentration of $1x10^8$ conidia/mL. These strains achieved 58.11 and 55.99 % mortality, respectively: Padulla and Alves (2009) obtained 72 % mortality for second to fourth instar with a strain of *B. bassiana* at a concentration of $5x10^7$ conidia/mL. Torres-Acosta and et al. (2011), who evaluated *B. bassiana* with different bioassay techniques and two methods of inoculating

first to third instar nymphs, obtained 73 % mortality with a concentration of $1x10^8$ conidia/mL. Lezama-Gutiérrez et al. (2012) used a concentration of $2x10^{13}$ conidia/mL of the strains *Metarhizium anisopliae, Cordyceps bassiana* and *Isaria fumosorosea* and obtained percentages of mortality that oscillated between 35 and 60 %. It is recommendable to continue evaluating under field conditions the strains that were the most pathogenic in our study to determine their potential for control of *D. citri* nymphs. As we have seen, *D. citri* nymphs are susceptible to *B. bassiana*. However, percentages of mortality varied. The variability may be due to origin of the isolate (different host, different geographic location). In addition, percentage of mortality was not proportional to the percentage of mycosis; in the case of the strain Bb-18, a high percentage of mortality was obtained, but the percentage of mycosis was low. In contrast, the commercial strain, which caused a high percentage of mycosis, caused low mortality. This phenomenon may be related to virulence and production of secondary metabolites that intervene in the ability of the pathogen to cause the disease (Padulla and Alves 2009), as well as to the intrinsic conditions of the pathogen, natural susceptibility or resistance of the insect host, among other important factors (Alves 1998).

The absence of mycosis in the treatments might also be attributed to the presence of the fungus *Cladosporim* spp., which was observed in several dead nymphs. Although in its natural form it can be phytopathogenic or saprophyte (Pasco et al. 2004), this fungus has been mentioned in the literature as a growth inhibitor of pathogenic fungi (Mellin-Rosas et al. 2011). Gandarilla-Pacheco et al. (2013) state that high mortality caused by pathogenic fungi is important since it assures a large decrease in the pest population. However, percentage of mortality caused by mycosis is also important because it increases the probability that the pathogen disseminates throughout the field. For this reason, an isolate that causes high rates of mortality and is capable of colonizing and causing mycosis in high percentages in the infected dead insect a much more

296 effective pathogen, than one that causes high mortality but whose sporulation on the host is low
297 from the perspective of control. In our study, the two strains that were most pathogenic for the *D.*
298 *citri* nymphs also had high percentages of mycosis and thus are feasible for control of the psyllid.
299 The results of our study demonstrate that the pathogenicity of the strains was statistically
300 different for different nymph stages of the psyllid. The highest mortality, and therefore the
301 highest susceptibility, was recorded in the fourth and fifth instars. Malekan et al. (2012) reported
302 similar results when they compared the effect of *B. bassiana* and *Lecanicillium muscarium* and
303 their combination with the insecticide imidacloprid on different stages of *Trialeurodes*
304 *vaporariorum* nymphs. They found that the percentage of mortality and the levels of infection
305 caused by the fungus were higher in the third and fourth instars than in the first and second instar
306 nymphs, indicating that the later stages were highly susceptible to fungus treatment. Poprawski et
307 al. (2000) also mention that third instar nymphs of *T. vaporariorum* were more susceptible than
308 the first when inoculated with the fungi *Paecelomyces fumosoroseus* and *B. bassiana.*
309 In contrast, there are studies that mention that first nymph instars, are more susceptible to the
310 action of certain pathogens, such as the case of *Bemisia tabaci* with *L. muscarium* in tomatoes
311 and verbena. It was observed that the second instar of the white fly was more susceptible to
312 infection caused by the fungus (Cuthbertson et al. 2005). Gindin et al. (2000) indicates high
313 pathogenicity of *Verticillium lecanni* to all developmental stages of *B. argentifolii* except eggs.
314 Siongers and Coosemans (2003) found that the product Botanigard caused higher mortality in *T.*
315 *vaporariorum* first instar nymphs.
316 There are also reports that indicate that the effectiveness of fungi is not affected by the different
317 nymph instars. Lacey et al. (2009) assessed the insecticidal activity of isolates of *B. bassiana, M.*
318 *anisopliae,* and *I. fumosorosea* on adults and first and third stage nymphs of the potato psyllid

319 *Bactericera cockerelli*. They concluded that in the case of nymphs, age did not affect
320 effectiveness.
321 It is not entirely known why the results regarding susceptibility of the nymphs to pathogenic
322 fungi vary. Malekan et al. (2010) mention that for the case of *T. vaporariorum*, the differences in
323 mortality are possibly due to nymph molting in the first stages shortly after fungus inoculation
324 and also to the permanence of the conidia on later nymph stages.
325 Regarding lethal times, in the pathogenicity tests in which five nymph stages of *D. citri* were
326 evaluated without considering which instar they belonged to, at a concentration of $1x10^8$
327 conidia/mL, LT_{50} was 8.5 days for the Bb-Rhy strain. However, in the following bioassay in
328 which the nymphs were classified by instar, the median lethal time was 5.5 days, which was
329 possible to calculate only for fourth and fifth instar nymphs since mortality of first, second and
330 third instar nymphs did not reach 50 %. This difference lies precisely in that IV and V instar
331 nymphs are more susceptible to *B. bassiana*. For this reason, when all the instars were evaluated
332 together, LT_{50} increased. The LT_{50} for IV and V instars in this study coincide with that reported
333 by Ferreira-Pinto et al. (2012), who found LT_{50} of 5.7 days at a concentration of $1x10^9$
334 conidia/mL *B. bassiana* on *D. citri* nymphs. Padulla and Alves (2009) found that for second to
335 fifth instar *D. citri* nymphs, TL_{50} was 4.71 days.
336 The median lethal concentration of the strains that were pathogenic was $8.34x10^6$ Bb-Dc
337 conidia/mL and $1x10^7$ Bb-Rhy conidia/mL, observation of both strains was six days. The study
338 conducted by Padulla and Alves (2009) found that the lethal concentration of the *B. bassiana*
339 strain used on second to fourth instar *D. citri* nymphs was $2.3x10^7$ conidia/mL. Ferreira-Pinto et
340 al. (2012) confirmed that the LC_{50} of *B. bassiana* on *D. citri* nymphs was $0.4x10^7$ conidia/mL
341 after ten days of evaluation. These concentrations are very similar and it can be concluded that
342 the strains evaluated are quite efficient compared with the fungus *Hirsutella citriformis* on *D.*

343 *citri* nymphs for which they obtained lethal concentrations of $2.26x10^{13}$ conidia/mL on third
344 instar nymphs and $1.31x10^{13}$ conidia/mL for fourth instar nymphs. Was not possible to calculate
345 CL_{50} for fifth instar nymphs (Dwiastuti and Kurniawati 2007).
346 Will be recommended to evaluate the effectiveness of these two strains under field conditions to
347 determine their potential as agents of biological control against *Diaphorina citri*.

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453 **Table 1** Description of the *B. bassiana* strains used in the bioassays of pathogenicity and median
454 lethal time (LT_{50}) on *D. citri* nymphs

Strain	**Place collection**	**Origin of the strain**
Bb-Dc	Tapachula	*Diaphorina citri*
Bb-Hy	Finca "Alianza"	*Hypothenemus hampei*
Bb-Rhy	"Tinajas", 2a. sección	*Rhyssomatus nigerrimus*
Bb-18	Papantla, Veracruz	Larvae-Lepidóptera (unidentified)
Botanigard (commercial)	Unknown	Unknown

455 **Table 2** Observed mortality, corrected mortality and *D. citri* first to fifth instar nymphs with
456 mycosis caused by four strains and a commercial product of *B. bassiana*

Treatments	Total nymphs	Mortality nymphs	Observed mortality(%)	Corrected Mortality (%) (Schneider-Orelli)	Mycosis	Mycosis (%)
Control	319	36	11.29	--------	0	0
Bb-18	257	133	51.75	45.61	4	3.01
Bb-Dc	228	139	60.96	55.99	81	58.27
Bb-Rhy	261	164	62.84	58.11	114	69.51
Bb-Hy	352	106	30.11	21.22	56	52.83
Botanigard	274	137	50.00	43.64	45	32.85

457 **Table 3** Mortality of the five *D. citri* nymph instars caused by four strains and a commercial
458 product of *B. bassiana*. The pathogenicity tests showed that nymph mortality was different
459 among the five *D. citri* nymph instars; the most susceptible to infection caused by the fungus
460 were the fourth and fifth (X^2=276.8; d.f.=4; P=0)

Treatments	**Total nymphs**	**Mortality nymphs**	**Mortality/nymphs**				
			I	**II**	**III**	**IV**	**V**
Control	319	36	0	1	6	16	13
Bb-18	257	133	5	13	30	40	45
Bb-Dc	228	139	1	17	33	36	52
Bb-Rhy	261	164	1	11	43	51	58
Bb-Hy	352	106	3	10	32	27	34
Botanigard	274	137	6	15	30	28	58

461 **Table 4** Observed mortality, corrected mortality and *D. citri* nymphs with mycosis grouped by
462 nymph instars (first-third; fourth-fifth) caused by the *B. bassiana* strain Bb-Rhy

Treatments	Total nymphs	Mortality nymphs	Observed mortality(%)	Corrected Mortality (%) (Schneider-Orelli)	Mycosis	Mycosis (%)
Control (I-III)	117	3	2.56	-------	0	0
Control (IV-V)	60	6	10.00	-------	0	0
Bb-Rhy (I-III)	95	20	21.05	18.98	9	45
Bb-Rhy (IV-V)	69	50	72.46	69.4	34	68

463 **Table 5** Means (SD) of the mortality caused by the Bb-Rhy strains on two groups of *D. citri*
464 nymphs, I-III and IV-V instars

Treatments	mean	sd	n	Comparisons
Bb-Rhy(IV-V)	0.73	0.07	5	a
Bb-Rhy(I-III)	0.16	0.12	5	b
Control(IV-V)	0.10	0.15	5	bc
Control(I-III)	0.02	0.03	5	c

465 Different letters mean statistical differences at $P < 0.05$

Table 6 Observed mortality, corrected mortality and mycosis in *D. citri* fourth and fifth instar nymphs caused by the *B. bassiana* strain Bb-Dc using six different concentrations (conidia/mL) and a control

Treatments	Total nymphs	Mortality nymphs	Observed mortality(%)	Mortality (%) (Schneider-Orelli)	Mycosis	Mycosis (%)
Control	87	2	2.30	-------	0	0
$1x10^4$	89	13	14.61	12.6	3	23.08
$1x10^5$	86	26	30.23	28.59	10	38.46
$1x10^6$	82	33	40.24	38.83	15	45.45
$1x10^7$	83	37	44.58	43.28	20	54.05
$1x10^8$	95	57	60	59.06	41	71.93
$1x10^9$	100	92	92	91.81	85	92.39

Table 7 Observed mortality, corrected mortality and micosis in *D. citri* fourth and fifth instar nymphs caused by the *B. bassiana* strain Bb-Rhy using six different concentrations (conidia/mL) and a control

Treatments	Total nymphs	Mortality nymphs	Observed mortality(%)	Mortality (%) (Schneider-Orelli)	Mycosis	Mycosis (%)
Control	91	7	7.69	-------	0	0
$1x10^4$	71	17	23.94	17.60	4	23.52
$1x10^5$	71	22	30.99	25.24	7	31.81
$1x10^6$	63	26	41.27	36.38	9	34.61
$1x10^7$	82	38	46.34	41.87	24	63.15
$1x10^8$	64	44	68.75	66.15	32	72.72
$1x10^9$	66	49	74.24	72.09	39	79.59

Fig. 1 Effect of four strains and one commercial product of *B. bassiana* on *D. citri* eggs using the following treatments: T1: Control, T2: Bb-18, T3: Bb-Dc, T4: Bb-Rhy, T5: Bb-Hy and T6: Botanigard (commercial product).

Fig. 1 Observed mortality and corrected mortality of *D. citri* first to fifth instar nymphs caused by four strains and one commercial product of *B. bassiana*. The treatments were T1: Control, T2: Bb-18, T3: Bb-Dc, T4: Bb-Rhy, T5: Bb-Hy and T6: Botanigard

Fig. 3 Survival curve of *D. citri* first to fifth instar nymphs caused by four strains and a commercial product of *B. bassiana*. T1: Control, T2: Bb-18, T3: Bb-Dc, T4: Bb-Rhy, T5: Bb-Hy and T6: Botanigard

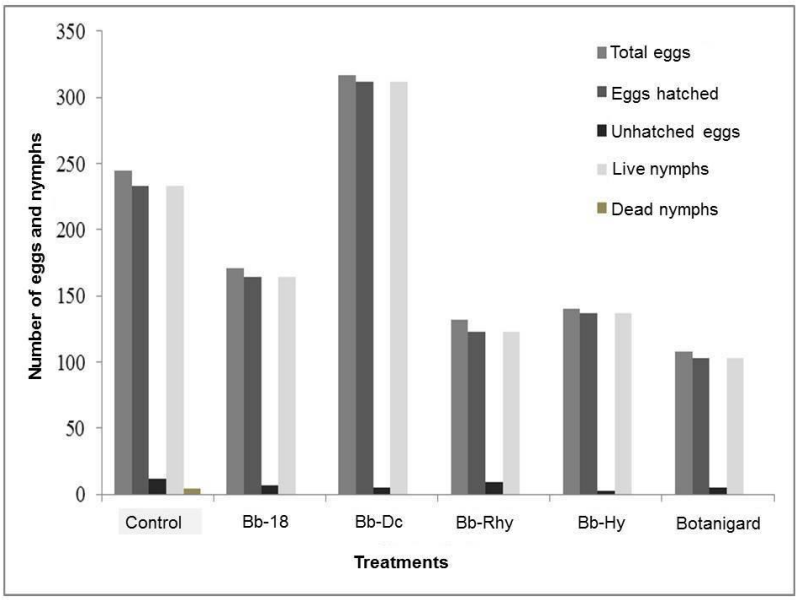
Fig. 4 Survival curve of *D. citri* nymphs grouped into first to third instar and fourth to fifth instar and LT_{50} of the strain Bb-Rhy. T1: Control (I-III), T2: Control (IV-V), T3: Bb-Rhy (I-III), and T4: Bb-Rhy (IV-V)

Fig. 5 Log-probit mortality line of *D. citri* IV and V instar nymphs caused by the de *B. bassiana* strain Bb-Dc.

Fig. 6 Observed and corrected mortality of *D. citri* IV and V instar nymphs caused by the *B. bassiana* strain Bb-Dc in concentrations of $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$ and $1x10^9$

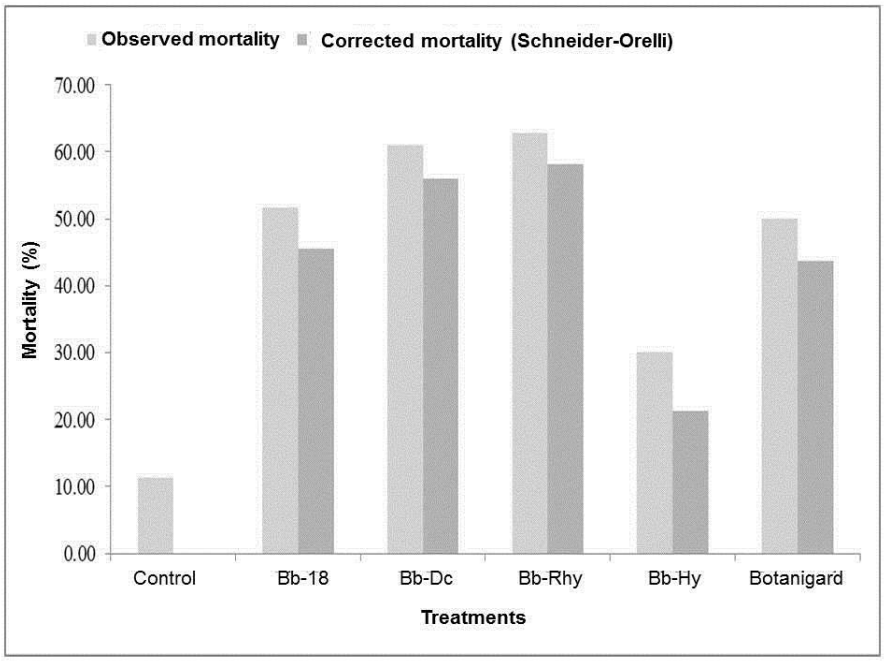
Fig. 7 Log-probit mortality line of *D. citri* IV and V instar nymphs caused by the de *B. bassiana* strain Bb-Rhy in concentrations of 1x104, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$ y $1x10^9$.

Fig. 8 Observed and corrected mortality of *D. citri* IV and V instar nymphs caused by the *B. bassiana* strain Bb-Rhy in concentrations of $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$ and $1x10^9$


350
300
250
200
150
100
50
0
Number of eggs and nymphs
Total eggs
Eggs hatched
Unhatched eggs
Live nymphs
Dead nymphs
Control
Bb-18
Bb-Dc
Bb-Rhy
Bb-Hy
Botanigard
Treatments

492

493 **Fig. 1**


Observed mortality
Corrected mortality (Schneider-Orelli)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Mortality (%)
Control
Bb-18
Bb-Dc
Bb-Rhy
Bb-Hy
Botanigard
Treatments

494

495 **Fig. 2**

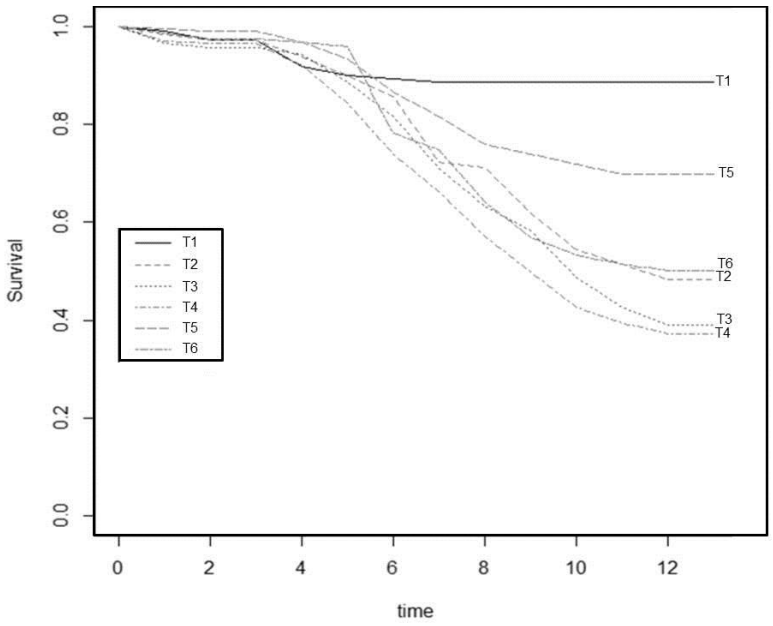
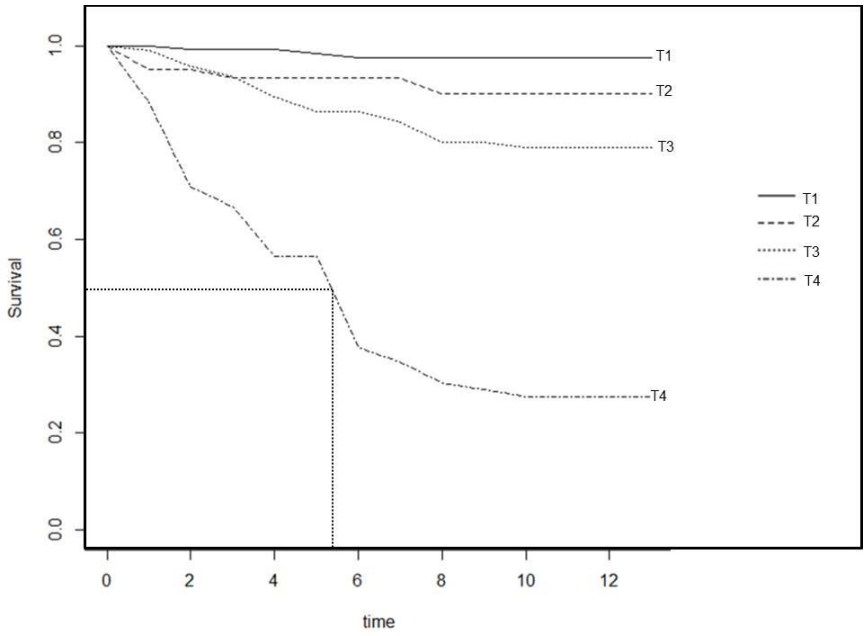
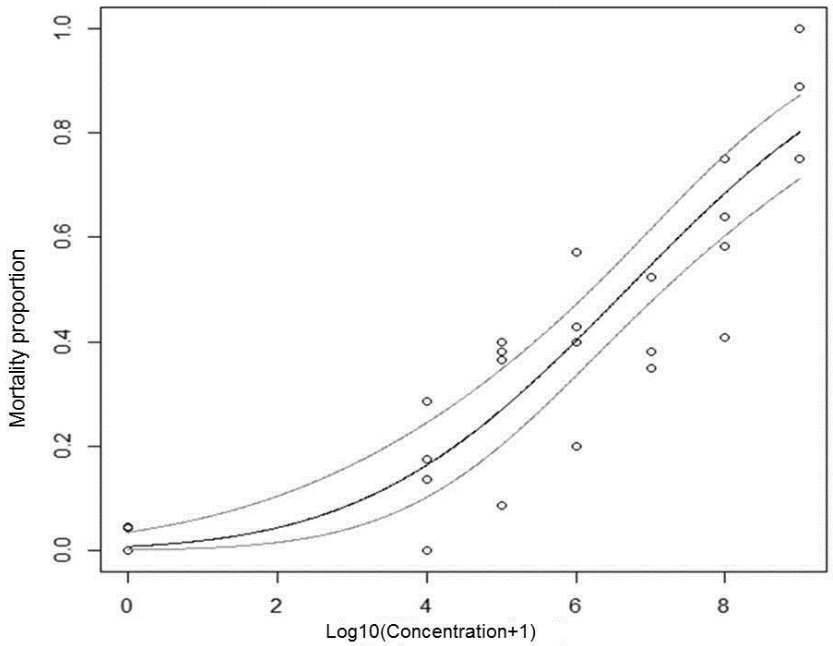

Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
time
T1
T2
T3
T4
T5
T6
T1
T5
T6
T2
T3
T4

Fig. 3



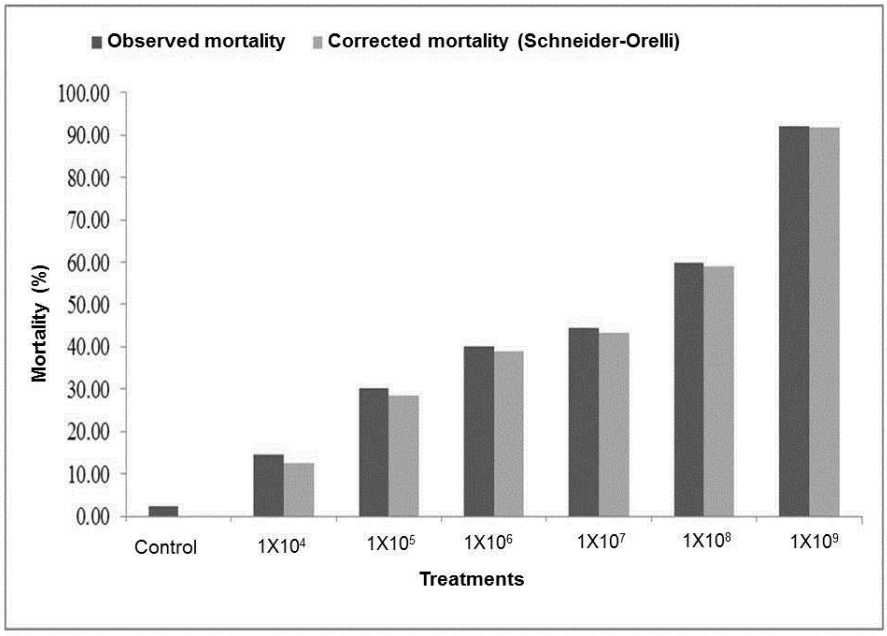
498

499 **Fig. 4**


Mortality proportion
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
Log10(Concentration+1)

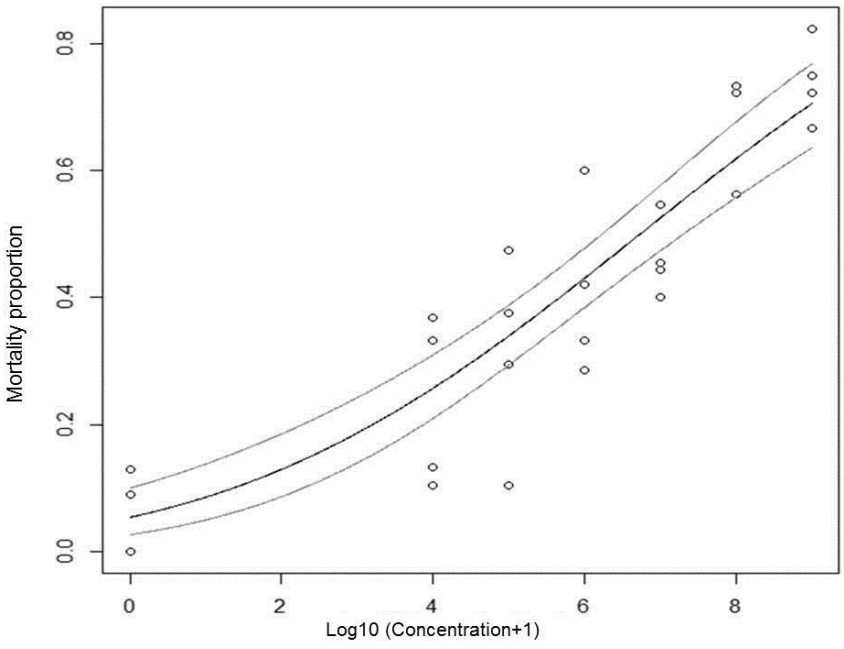
500

501 **Fig. 5**


Observed mortality
Corrected mortality (Schneider-Orelli)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Mortality (%)
Control
1X10⁴
1X10⁵
1X10⁶
1X10⁷
1X10⁸
1X10⁹
Treatments

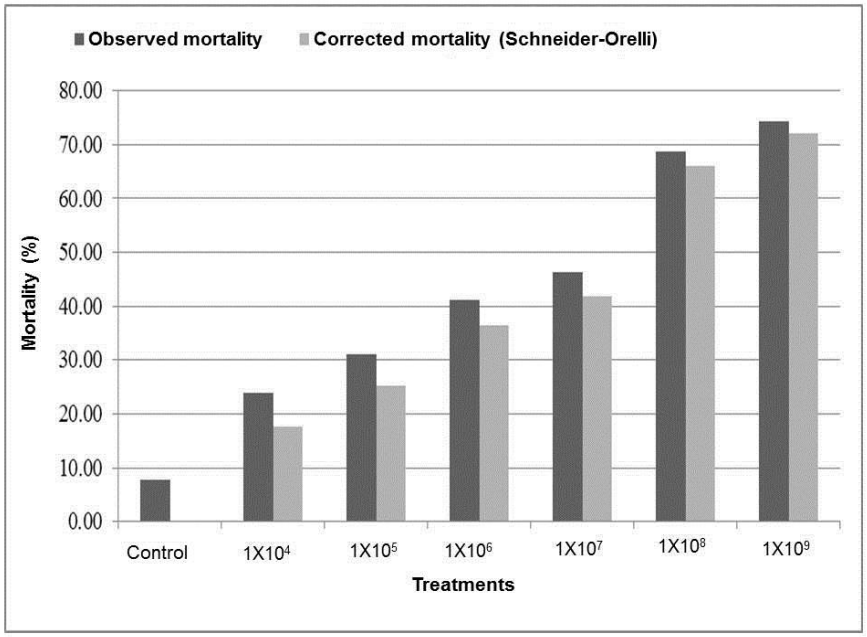
502

503 **Fig. 6**


Mortality proportion
0.0
0.2
0.4
0.6
0.8
0
2
4
6
8
Log10 (Concentration+1)

504

505 **Fig. 7**


Observed mortality
Corrected mortality (Schneider-Orelli)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Mortality (%)
Control
1X10⁴
1X10⁵
1X10⁶
1X10⁷
1X10⁸
1X10⁹
Treatments

506

507 **Fig. 8**

III. Conclusiones generales

Los bioensayos de patogenicidad de las cuatro cepas no comerciales y el producto comercial de *B. bassiana* a una concentración de $1x10^8$ conidios/mL, no tuvieron efecto sobre los huevecillos *de D. citri*, ya que el 95% de ellos eclosionaron y continuaron su desarrollo hasta el estado adulto. No existen reportes de trabajos realizados con huevecillos del psílido, por lo que se considera una aportación relevante.

Las cepas Bb-Rhy, Bb-Dc, Bb-18, Botanigard y Bb-Hy provocaron una mortalidad corregida de 58.11, 55.99, 45.61, 43.64 y 21.22 % respectivamente, en ninfas de primero a quinto instar obteniendo los menores tiempos letales las cepas Bb-Rhy y Bb-Dc con 8.5 y 9.5 días respectivamente.

Se encontró que los instares ninfales más susceptibles a *B. bassiana* fueron los estadios IV y V, ya que, cuando se agruparon en un bioensayo utilizando la cepa Bb-Rhy se observó que el tratamiento de las ninfas I-III fue diferente estadísticamente a las ninfas IV-V, siendo más susceptible éste último grupo, donde el tiempo letal fue de 5.5 días. Sin embargo la acción de los hongos en los diferentes estadíos de algunos insectos es discutida.

Las concentraciones letales de las dos cepas más patogénicas realizadas a los instares ninfales IV-V fue de $8.34x10^6$ para la cepa Bb-Dc y $1x10^7$ conidios/mL para la cepa Bb-Rhy. La cepa Bb-Dc fue evaluada en jaulas de campo utilizando una concentración de $1x10^8$ conidios/mL, donde se eligieron y enumeraron 36 plantas de *M. paniculata* contenidas en macetas con altas infestaciones de ninfas de *D. citri*. A 18 de las plantas se le aplicó la suspensión de la cepa Bb-Dc y el resto de

las plantas fueron utilizadas como testigo (solo se agregó agua con Tween al 0.05%). Las plantas fueron colocadas al azar. Inmediatamente después de la aplicación se tomaron al azar 2 plantas tratadas y 2 plantas no tratadas, dando inicio al conteo de las ninfas al tiempo cero. Diariamente se tomó el mismo número de plantas para determinar el total de ninfas existentes y los muertos por efecto del hongo, durante seis días de observación. En los resultados solo se obtuvo 20% de mortalidad en las ninfas al sexto día después de la evaluación. Se recomienda seguir realizando pruebas a nivel de jaula de campo, tomando en cuenta la duración de los bioensayos y el material biológico

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