

Field Trial of Aqueous and Emulsion Preparations of Entomopathogenic Fungi Against the Asian Citrus Psyllid (Hemiptera: Liviidae) in a Lime Orchard in Mexico¹

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Liberibacter-caused Huanglongbing disease vectored by the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), has spread to most citrus-growing areas worldwide. In Mexico, research efforts to manage the disease and to slow its spread have intensified since it was detected there in 2009. Integration of microbial control with other management practices should be investigated for *D. citri* and the disease (Meyer et al. 2008, J. Invertebr. Pathol. 99: 96–102). To that end, we assessed the impact of applications of entomopathogenic fungi on *D. citri* nymphs in a single test conducted in a commercial Persian lime (*Citrus latifolia* Tanaka) orchard in Veracruz, Mexico, in 2011.

Three fungi were evaluated. *Beauveria* and *Metarhizium* isolates were isolated from soil with the insect-bait method using *Tenebrio molitor* L. larvae (Sánchez-Peña et al. 2011, J. Insect Sci. [BioOne Paper No. 1, 10 pg.]) in the citrus-growing region in Nuevo Leon, Mexico. *Isaria fumosorosea* Wize was isolated from *D. citri* collected in Gomez Farias, Tamaulipas (Casique-Valdez and Sanchez-Peña 2010, 58th Annual Meeting Southwestern Branch, Entomological Society of America, Cancun, Mexico). One isolate each of *Beauveria* sp. (B6C), *I. fumosorosea* (IF8B19), and *Metarhizium* sp. (M11) were selected for field tests based on (a) activity against *D. citri* nymphs in the laboratory and (b) stable and uniform production of conidia on autoclaved rice grains (Casique-Valdez 2010, MS Thesis, Universidad Autónoma Agraria Antonio

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Narro, Saltillo, Coahuila, Mexico). Isolates were deposited in the collections of S.R.S.-P. and M.J.B.

The *Beauveria* B6C isolate was morphologically determined to *Beauveria bassiana* (Balsamo) Vuillemin as per Humber (2011, Entomopathogenic fungal identification, APS-ESA, USDA-ARS, Ithaca, NY). Sequencing data and multiple-alignment analyses showed that the *I. fumosorosea* IF8B19 isolate possessed a 99% identity to and maximum scores with the partial sequence of the 28S ribosomal RNA gene from *I. fumosorosea* isolated in Florida (GenBank: EF429304.1; Meyer et al. 2008). The *Metarhizium* M11 isolate, with the double-digested restriction fragment-length polymorphism banding patterns and the 99.2% identity with the EF-1 α gene sequence (GenBank HM748309.1), was most closely related to the strain identified as *Metarhizium brunneum* Petch "alternate" (Wyrebeek et al. 2011, Microbiol. 157: 2904–2911).

Fungal preparations used in the field test were produced on autoclaved rice–water mixtures in 1-L polyethylene bags (30 $\times$ 40 cm) using methods adapted from Aquino et al. (1977, Bol. Tec. CODECAP 5: 7–11) and Grace and Jaronski (2005, Solid Substrate Fermentation Workshop, USDA-ARS, Northern Plains Agricultural Research Laboratory, Sidney, MT). Individual bags containing 110 g of rice and 100 ml of water were inoculated with 10 ml of 4-d-old liquid cultures of the respective fungi plus 1 mg/ml of penicillin (Bristol-Myers Squibb, Mexico). Bags were incubated for 15 d at room temperature. Conidia were harvested by rubbing the fungus–rice mixture in antiaphid mesh sacks (20 $\times$ 30 cm). The resulting conidial powders were refrigerated less than 2 mo at 4°C until used. Percentage of germination of each preparation was determined on potato dextrose agar plates on which conidia were spread. The surface of each plate was examined microscopically 48 h later. Conidia with germ tubes longer than the conidial diameter were considered germinated. Viability exceeded 80% for each fungus.

The study site was a Persian lime orchard in Martínez de la Torre, state of Veracruz, Mexico (20°02'30.79" N, 97°05'55.41" W) where the climate is tropical (annual average temperature 23.4°C, precipitation 1,840 mm) (Domínguez-Guadarrama and Lepe-Romano 2011, Tlapacoyan, Veracruz. Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México). Treatments were applied on 13 and 14 April 2011 at 5 p.m. The orchard had not been sprayed with pesticides for at least 12 mo. Weather data were recorded using EL-USB-1 Data Loggers (Lascar Electronics, Erie, PA) placed 150 m from the orchard.

Conidia were formulated either as aqueous (0.025% Tween 20) (Sánchez-Peña and Thorvilson 1995, J. Invertebr. Pathol. 65: 248–252) or as emulsifiable suspensions in agricultural mineral oil (Purespray® 22E, PetroCanada, Mississauga, ON, Canada) at 0.5% in water. Suspensions were blended for 30 s at maximum speed in an Osterizer® blender. Formulations were adjusted to 8 to 10 L in water at a concentration of 1.0×10^8 conidia/ml except for *I. fumosorosea*, which was at 5.6×10^7 spores/ml because it produced less conidia than the other two fungi under the conditions described. Suspensions were applied immediately after preparation (Table 1).

Each treatment was replicated 8 to 10 times using randomly assigned trees. Trees were 1 to 2.5 m tall, planted 8 m apart within rows on 6-m centers. A completely randomized design was conducted with a factorial arrangement of 3 $\times$ 2 (fungi $\times$ number of applications $\times$ formulation type). Control preparations of oil

Table 1. Fungal treatments applied to foliage in a lime orchard against *Diaphorina citri* nymphs.

Treatment	Formulation*	Conidia/ml	No. of Applications**
<i>B. bassiana</i>	T20	1×10^8	1
<i>B. bassiana</i>	T20	1×10^8	2
<i>B. bassiana</i>	ME	1×10^8	1
<i>B. bassiana</i>	ME	1×10^8	2
<i>I. fumosorosea</i>	T20	5.6×10^7	1
<i>I. fumosorosea</i>	T20	5.6×10^7	2
<i>I. fumosorosea</i>	ME	5.6×10^7	1
<i>I. fumosorosea</i>	ME	5.6×10^7	2
<i>M. brunneum</i>	T20	1×10^8	1
<i>M. brunneum</i>	T20	1×10^8	2
<i>M. brunneum</i>	ME	1×10^8	1
<i>M. brunneum</i>	ME	1×10^8	2
Control (oil 0.5%)	ME		1
Control (oil 0.5%)	ME		2

* T20 = conidial suspension in Tween 20 at 0.025% in water. ME = conidial emulsion of agricultural mineral oil (Purespray foliar 22E) at 0.5% in water.

** With 2 applications, the second applications were made after an interval of 24 h.

emulsions (0.05%) without conidia were also applied, and absolute controls were not treated with conidia. Individual trees receiving two applications were treated on an interval of 24 h. (The rationale of two applications was to infect nymphs emerging from eggs or those that molted soon after the first application and thus lacked conidial deposits on the cuticle.) Fungi were applied using a gasoline-powered backpack sprayer (Stihl SR-420®, 2.6 kW power, displacement 56.5 cm³) (Andreas Stihl, Dieburg, Germany) at a medium setting (force) with a number 0.5 nozzle delivering approximately 100 L/ha (approximately 1 L per tree).

Samples were collected either 72 h after the first application or 48 h after the second application on 16 April 2011 between 6 and 8 p.m. A sample unit was a plant shoot, and two samples (7 to 10 shoots each) were randomly collected per replicate (tree). Each sample was placed in a zip-lock plastic bag (16.5 × 14.9 cm) for transport to the laboratory. Prior to processing in the laboratory (2–3 d later), one of the two bags collected per tree was kept at room temperature (25°C–30°C) while the other was placed in a cooler with ice, then refrigerated (4°C) to prevent sample loss due to possible growth of fungal saprophytes. In the laboratory, nymphs were retrieved from bags and transferred to petri dishes lined with moistened filter paper and maintained at room temperature for 72 h. Percentage of infection of nymphs per tree was determined by counting nymphs with fungal sporulation on the cuticle

Table 2. Mean percentage of infection of *Diaphorina citri* nymphs for each fungal species combined across formulations, number of applications, and incubation conditions. Within-column values (means $\pm$ SE) followed by the same letter are not significantly different (Tukey, $P < 0.05$).

Fungus	No. of Replications	% Infection
<i>Beauveria bassiana</i>	35	63.34 $\pm$ 4.55 a
<i>Isaria fumosorosea</i>	32	47.96 $\pm$ 4.21 ab
<i>Metarhizium brunneum</i>	32	35.81 $\pm$ 4.48 b
Control (mineral oil 0.5%)	17	1.18 $\pm$ 0.742 c

using a stereomicroscope. In the case of *B. bassiana*, nymphs were also considered infected if they had a reddish-to-pink coloration, often without mycelial development on the cuticle, as observed in infected whiteflies (*Trialeurodes vaporariorum* (Westwood): Hemiptera: Aleyrodidae) (Sánchez-Peña and Vazquez-Jaime 1996, Pp. 23–27, Memorias XIX Congreso, Sociedad Mexicana de Control Biológico, Culiacan, Sinaloa, Mexico; Wraight et al. 1998, J. Invertebr. Pathol. 71: 217–226).

Percentage infection data (Tables 2–5) were arcsine-transformed and analyzed for normality using the Anderson–Darling and Shapiro–Wilk tests, and for homogeneity of variances using Levene’s test. Percentages of infected nymphs were then subjected to a generalized linear model with independent measurements (GLM procedure, SPSS 2003, Version 12.0. SPSS Inc. Chicago, IL), and means were separated with Tukey’s honestly significant difference (Sokal and Rohlf 1995, Biometry, 3rd ed., Freeman, New York). A confirmatory nonparametric analysis of variance (Kruskal–Wallis test; Lowry 2013, <http://www.vassarstats.net/textbook>) was performed in addition to the GLM analysis on the infection data (see Tables 4, 5) considering fungus as main factor for overall significant differences among fungal species.

The monthly mean temperature for April 2011 was 28.4°C, mean monthly relative humidity (RH) was 70.9%, and precipitation was 10 mm. During the test (13–16 April), the mean temperature was 30.1°C, mean RH was 71.36% (range 32%–

Table 3. Mean percentage of infection of *Diaphorina citri* nymphs for incubation conditions (either 4°C or 25°C–30°C) combined across fungal species, number of applications, and formulations. Within-column values (means $\pm$ SE) followed by the same letter are not significantly different (Tukey, $P < 0.05$).

Incubation Temperature	No. of Replicates	% Infection
25°C–30°C	64	46.09 $\pm$ 3.62 a
4°C	58	33.91 $\pm$ 1.32 b

Table 4. Mean percentage of infection of *Diaphorina citri* nymphs for each fungal species, formulation type, and number of applications (1 or 2) (combined across incubation conditions of samples). Within-column values (means $\pm$ SE) followed by the same letter are not significantly different (Tukey, $P > 0.05$).

Fungus	Formulation*	No. of Applications	No. of Replicates	% Infection
<i>B. bassiana</i>	ME	1	10	54.2 $\pm$ 8.17 ab
<i>B. bassiana</i>	ME	2	8	83.5 $\pm$ 6.57 a
<i>B. bassiana</i>	T20	1	9	67.00 $\pm$ 5.71 ab
<i>B. bassiana</i>	T20	2	8	50.50 $\pm$ 12.25 ab
<i>I. fumosorosea</i>	ME	1	7	53.85 $\pm$ 10.34 ab
<i>I. fumosorosea</i>	ME	2	8	38.25 $\pm$ 8.11 bc
<i>I. fumosorosea</i>	T20	1	7	49.28 $\pm$ 11.69 ab
<i>I. fumosorosea</i>	T20	2	10	50.70 $\pm$ 5.37 ab
<i>M. brunneum</i>	ME	1	6	38.16 $\pm$ 12.12 bc
<i>M. brunneum</i>	ME	2	9	31.11 $\pm$ 7.45 bcd
<i>M. brunneum</i>	T20	1	8	37.75 $\pm$ 8.44 bc
<i>M. brunneum</i>	T20	2	9	37.22 $\pm$ 10.02 bc
Control (oil .5%)	ME	1	8	0.00 $\pm$ 0.00 d
Control (oil .5%)	ME	2	9	2.11 $\pm$ 1.26 d

* T20 = conidial suspension in Tween 20 at 0.025% in water. ME = conidial emulsion of agricultural mineral oil (Purespray foliar 22E) at 0.5% in water.

95%), and a negligible amount of rain fell 16 April; however, there was an increase in RH on that day. The daily maximum RH level (93%–95%) occurred for a maximum period of about 1 h in all days of the test.

Percentages of infection in nymphs were normally distributed (Shapiro–Wilk $W = 0.9832$, $P = 0.2386$, Anderson–Darling $A = 0.4112$, $P = 0.3354$). Levene's test also showed homogeneity of variances when individual factors (fungus species, formulation, number of applications) were considered: fungus species ($F_{4,92} = 0.9348$, $P = 0.396$), formulation ($F_{1,92} = 0.584$, $P = 0.447$), and number of applications ($F_{1,92} = 0.6367$, $P = 0.427$). Residuals also were normally distributed when plotting the fitted values against the residuals and inspecting the normal quantile plot.

Analysis of variance on the effect of single and combined factors indicated that percentage of infection of *D. citri* nymphs was not affected either by type of formulation used ($F_{1,92} = 0.843$, $P = 0.433$) or by number of applications ($F_{1,92} = 0.014$, $P = 0.907$). The lack of significance for number of applications indicates that

Table 5. Mean percentage of infection of *Diaphorina citri* combined across fungus, incubation at 4°C or 25°C –30°C, formulation type, and number of applications. Within-column values (means ± SE) followed by the same letter are not significantly different (Tukey, *P* < 0.05).

Fungus	Formulation*	Applications	Incubation (°C)	Replicates	% Infection
<i>B. bassiana</i>	ME	1	25–30	5	61.8 ± 9.6 abcd
<i>B. bassiana</i>	ME	1	4	5	46.6 ± 13.3abcde
<i>B. bassiana</i>	ME	2	25–30	5	80.8 ± 10.1 ab
<i>B. bassiana</i>	ME	2	4	3	88.0 ± 6.42 a
<i>B. bassiana</i>	T20	1	25–30	5	67.8 ± 2.4 abc
<i>B. bassiana</i>	T20	1	4	4	66.0 ± 13.6 abc
<i>B. bassiana</i>	T20	2	25–30	4	66.0 ± 8.12 abc
<i>B. bassiana</i>	T20	2	4	4	35.0 ± 21.7 abcde
<i>I. fumosorosea</i>	ME	1	25–30	3	32.6 ± 7.1 abcde
<i>I. fumosorosea</i>	ME	1	4	4	69.7 ± 12.3b abc
<i>I. fumosorosea</i>	ME	2	25–30	5	31.0 ± 9.4 abcde
<i>I. fumosorosea</i>	ME	2	4	3	50.3 ± 14.1 abcde
<i>I. fumosorosea</i>	T20	1	25–30	4	67.5 ± 12.1 abc
<i>I. fumosorosea</i>	T20	1	4	3	25.0 ± 12.1 bcde
<i>I. fumosorosea</i>	T20	2	25–30	5	54.4 ± 6.4 abcde
<i>I. fumosorosea</i>	T20	2	4	5	47.0 ± 9.0 abcde
<i>M. brunneum</i>	ME	1	25–30	3	44.0 ± 10.4 abcde

Table 5. Continued.

Fungus	Formulation*	Applications	Incubation (°C)	Replicates	% Infection
<i>M. brunneum</i>	ME	1	4	3	32.3 ± 24.3 abcde
<i>M. brunneum</i>	ME	2	25–30	5	39.0 ± 8.8 abcde
<i>M. brunneum</i>	ME	2	4	4	21.2 ± 11.9 bcde
<i>M. brunneum</i>	T20	1	25–30	6	46.1 ± 8.1 abcde
<i>M. brunneum</i>	T20	1	4	2	12.5 ± 12.5 cde
<i>M. brunneum</i>	T20	2	25–30	4	54.7 ± 17.7 abcde
<i>M. brunneum</i>	T20	2	4	5	23.2 ± 7.8 bcde
Control (oil 0.5%)	ME	1	25–30	4	0 e
Control (oil 0.5%)	ME	1	4	4	0 e
Control (oil 0.5%)	ME	2	25–30	4	4.00 ± 2.61 e
Control (oil 0.5%)	ME	2	4	5	0.60 ± 0.60 e
Absolute control	—	—	25–30	4	0 e
Absolute control	—	—	4	3	0 e

* T20 = conidial suspension in Tween 20 at 0.025% in water. ME = conidial emulsion of agricultural mineral oil (Purespray foliar 22E) at 0.5% in water.

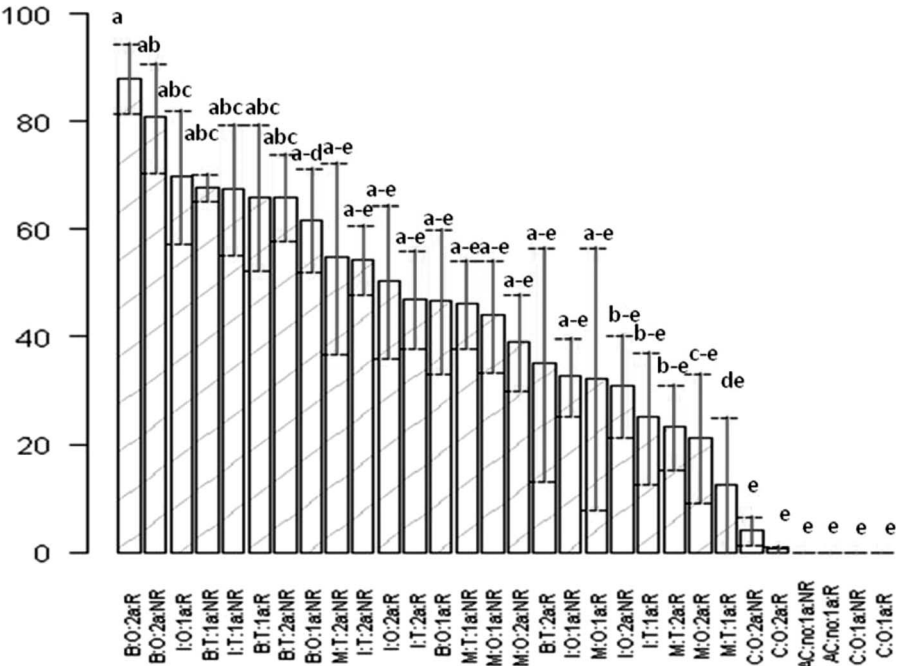


Fig. 1. Mean percentage of infection of *D. citri* nymphs combined for four factors: fungus (B = *B. bassiana*; I = *I. fumosorosea*; M = *M. brunneum*; C = control of 0.05% mineral oil in water; AC = absolute control), formulation type (O = mineral oil emulsion; T = Tween 20), number of applications on the field (1a = 1; 2a = 2), and incubation of samples (R = 4°C; NR = 25°C). Values of bars (means $\pm$ SE) followed by the same letter(s) do not differ significantly (Tukey, $P < 0.05$).

recruitment of nymphs after the first application was minimal, or that established infection levels were insensitive to the additional application.

Infection levels were significantly affected by fungal species ($F_{2,92} = 31.38$, $P < 0.001$). *Beauveria bassiana* treatments, as a whole, caused significantly higher infection levels than *M. brunneum* treatments (Tables 2, 4). The Kruskal–Wallis analysis confirmed the overall differences among fungal species, whether incubation of samples for 3 d either at 25°C or 4°C was or was not considered as factor ($H = 6.76$, $df = 2$, $P = 0.034$; $H = 8.41$, $df = 2$, $P = 0.0149$, respectively), confirming actual infection differences between the *B. bassiana* and *M. brunneum* isolates.

Infection levels by *I. fumosorosea* were intermediate between *B. bassiana* and *M. brunneum* (Tables 2, 4); however, the conidial concentration of *Isaria* was about 50% lower than that of the other two fungi (Table 1). Manner of incubation of the samples (i.e., 3 d at either 25°C or 4°C) significantly impacted observed infection levels (Table 3) ($F_{1,92} = 5.64$, $P = 0.019$) as did the interaction of formulation type and incubation temperature of the samples ($F_{1,92} = 7.72$, $P = 0.007$) and the

interaction of fungus, formulation type, and number of applications ($F = 5.69$; $df = 2$; $P = 0.005$) (Table 4).

The combination of all four factors (fungus, formulation, number of applications, temperature of incubation) was also significant ($F = 3.05$; $df = 2$; $P = 0.05$) (Table 5). The highest mean infection value (88%) was registered when *B. bassiana* was applied twice, formulated as emulsion in mineral oil, and collected samples were incubated at 4°C (Fig. 1). In general, application of *B. bassiana* and *I. fumosorosea* resulted in the highest percentages of infection (70%–88%) (Tables 2, 4, 5; Fig. 1).

The observation of dense fungal growth and production of conidia on exposed insects 5 to 6 d after field application (including 2–3 d in the field) indicated that it is likely that insects were infected in the field. The likelihood that nymphs were infected by residual spray in the plastic bags is remote, considering the relatively brief incubation time before full conidial production was observed (48 to 72 h in bags).

There are few reports of field evaluations of fungi for *D. citri* control (Grafton-Cardwell et al. 2014, Annu. Rev. Entomol. 58: 413–432). The results presented herein indicate that there might be significant potential for the use of entomopathogenic fungi in *D. citri* control in citrus. Further investigations should continue evaluating the effects of formulations, environmental aspects, and fungal load on inundative microbial control of insects in citrus orchards.

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