# Effect of Mexican Hirsutella citriformis (Hypocreales: Ophiocordycipitaceae) Strains on Diaphorina citri (Hemiptera: Liviidae) and the Predators Chrysoperla rufilabris (Neuroptera: Chrysopidae) and Hippodamia convergens (Coleoptera: Coccinellidae)

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Source: Florida Entomologist, 99(3) : 509-515

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.099.0325

# Effect of Mexican *Hirsutella citriformis* (Hypocreales: Ophiocordycipitaceae) strains on *Diaphorina citri* (Hemiptera: Liviidae) and the predators *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) and *Hippodamia convergens* (Coleoptera: Coccinellidae)

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**Abstract**

Due to its role in the transmission of *Candidatus* Liberibacter asiaticus, a pathogen associated with huanglongbing, a catastrophic disease of citrus in the world, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) has become a very dangerous invasive pest. To contribute to increasing alternatives for its management, we evaluated against *D. citri* adults the pathogenicity of conidia and blastospores of 8 Mexican strains of the entomopathogenic fungus *Hirsutella citriformis* Speare (Hypocreales: Ophiocordycipitaceae). Furthermore, we conducted tests with non-target insects that included the predators *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) and *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae). Experiments in the laboratory included the application of the fungus by contact and through liquid sprays. Strains were collected from citrus areas of Mexico. They were grown in PDAY media and applied at $1 \times 10^6$ conidia per mL. All bioassays were conducted under controlled conditions at 26 ± 1 °C, a 16:8 h L:D photoperiod, and 76 ± 4% RH and maintained during 26 d after inoculation. Tests with *D. citri* were performed 3 times at different dates. Mean mortality by *H. citriformis* strains on *D. citri* adults ranged from 82 to 92%; INIFAP-Hir-1 strain produced the highest rate. Sprayed conidia produced 69% mortality. Use of sprayed blastospores caused 32 to 49 % mortality. LC50 obtained for INIFAP-Hir-1 strain was $3.4 \times 10^6$ conidia per mL. IB-Hir-1, IB-Hir-2, INIFAP-Hir-1, and INIFAP-Hir-2 strains inoculated by contact on *H. convergens* adults caused 9 to 11% mortality; in larvae of *C. rufilabris*, mortality ranged from 19 to 25%. In both tests, there were no statistical differences when compared with the untreated control. Unlike *D. citri*, all dead predator specimens showed absence of *H. citriformis* mycosis. The results suggest potential for the integration of this fungus in the management of *D. citri*. Data obtained from the predators could support safe use of this biological control agent.

Key Words: huanglongbing; Asian citrus psyllid; biological control; non-target insect

**Resumen**

Debido a su función en la transmisión de *Candidatus* Liberibacter asiaticus, un patógeno asociado al Huanglongbing, una enfermedad catastrófica de los cítricos en el mundo, *Diaphorina citri* Kuwayama (Hemiptera: Liividae) se ha convertido en una plaga invasiva muy peligrosa. Para contribuir a incrementar las alternativas para su manejo, evaluamos la patogenicidad de conidias y blastosporas de 8 cepas mexicanas del hongo entomopatógeno *Hirsutella citriformis* Speare (Hypocreales: Ophiocordycipitaceae) contra adultos de *D. citri*; además realizamos pruebas contra insectos no blanco, los depredadores *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) y *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae). Los experimentos en laboratorio incluyeron aplicación del hongo por contacto y mediante aspersiones. Las cepas fueron colectadas en diferentes áreas citrícolas de México, cultivadas en medio PDAY y asperjadas a $1 \times 10^6$ conidia/mL; todos los bioensayos fueron conducidos bajo condiciones controladas a 26 ± 1 °C, 16:8 L:O, 76 ± 4% HR y mantenidos durante 26 días después de la inoculación; las pruebas contra *D. citri* fueron realizadas 3 veces en diferentes fechas. La mortalidad causada por *H. citriformis* contra adultos de *D. citri* fue 82-92%; la cepa INIFAP-Hir-1 registró el mayor porcentaje. Las conidias asperjadas produjeron 69% de mortalidad; mientras que el uso de blastosporas causó 32-49%. La $CL_{50}$ obtenida para la cepa INIFAP- Hir-1 fue $3.4 \times 10^6$ conidias/mL. Las cepas IB-Hir-1, IB-Hir-2, INIFAP-Hir-1 e INIFAP-Hir-2 inoculadas por contacto contra adultos de *H. convergens* causaron 9-11% de mortalidad; en larvas de *C. rufilabris* fue de 19-25%; en ambas pruebas no existieron diferencias estadísticas con respecto al testigo. A diferencia de *D. citri*, todos los especímenes de depredadores muertos mostraron ausencia de micosis por *H. citriformis.* Los resultados sugieren potencial para la integración de este hongo en el manejo de *D. citri*; datos obtenidos de los depredadores podrían respaldar el uso seguro de este agente de control biológico.

Palabras Clave: huanglongbing; psílido asiático de los cítricos; control biológico; insectos no blanco

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*Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is one of the most important invasive pests in the citrus industry of the world; this qualification has been assigned mainly by its role as a vector of *Candidatus* Liberibacter, a bacterium associated with huanglongbing (Bové 2006; Yang et al. 2006). Nowadays, huanglongbing is considered as one of the most devastating diseases that plague the crop worldwide; its management has been achieved basically through the elimination of infected plants, use of certified plant material, and vector control (Bové 2006; Gottwald et al. 2007; National Research Council 2010; da Graça et al. 2015). Presently, effective eradication methods against the rapidly dispersing vector are unavailable (Bové 2006). Management of *D. citri* has been constrained to the use of chemical control (Yang et al. 2006; Belasque et al. 2010; Stansly et al. 2013; Qureshi et al. 2014). However, intensive use of pesticides has began to produce collateral effects that are noticed in resistance selection to insecticides in the vector (Yang et al. 2006; Tiwari et al. 2011), resurgence of controlled key pests, and outbreaks of secondary pests (Yang et al. 2006; Monzó et al. 2012).

Vector management needs to be addressed toward the integration of methods that allow a reduction in the use of insecticides as well as in insect population density and huanglongbing infection and dispersion rate (Yang et al. 2006; Stansly et al. 2013). Some beneficial insects (Aubert 1990; Hoy & Nguyen 2001; Michaud 2004; López-Arroyo et al. 2009; Qureshi & Stansly 2009; Cortez-Mondaca et al. 2010) and entomopathogens have been considered as biological control agents of *D. citri* (Subandiyah et al. 2000; Yang et al. 2006; Meyer et al. 2007; Avery et al. 2009). Easy integration of fungi with pesticides has motivated the exploration of diverse fungal species, e.g., *Beauveria bassiana* (Bals.-Criv) Vuill. (Hypocreales: Cordycipitaceae) (Padulla & Alves 2009; Gandarilla-Pacheco et al. 2013; Casique-Valdes et al. 2015), *Hirsutella citriformis* Speare (Hypocreales: Ophiocordycipitaceae) (Subandiyah et al. 2000; Meyer et al. 2007; Casique-Valdes et al. 2011; Hall et al. 2012; Romero-Rangel et al. 2012; Cortez-Madrigal et al. 2014; Orduño-Cruz et al. 2015; Perez-Gonzalez 2015a,b), *Isaria fumosorosea* Wize (= *Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae) (Subandiyah et al. 2000; Meyer et al. 2007; Hoy et al. 2010; Gandarilla-Pacheco et al. 2013), and *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (= *Verticillium lecanii*) (Hypocreales: Cordycipitaceae) (Rivero-Aragon & Grillo-Ravelo 2000).

*Hirsutella citriformis* was found producing remarkable natural epizootics on *D. citri* in the field (Aubert 1987; Subandiyah et al. 2000; López-Arroyo et al. 2009). Due to its characteristic sporulation in the insect (Hall et al. 2012), visual detection of attacked specimens in the trees frequently suggest a potential high value of the fungus for *D. citri* biological control. However, Hall et al. (2012) indicated an estimated contribution of *H. citriformis* in the reduction of vector populations by 4%. Hence, it is necessary to expand the exploration for highly pathogenic *H. citriformis* strains that allows re-evaluating the role that this fungus species may have for biological control of *D. citri*. Variability in pathogenicity among strains of *H. citriformis* may occur (Orduño-Cruz et al. 2015; Pérez-González et al. 2015a), as it was reported for other species of entomopathogenic fungi, even in those tested against *D. citri* (Mena et al. 2003; Hoy et al 2010). Thus, this factor may allow identifying more aggressive strains with the potential to cause increased mortality in *D. citri* while it proves to be harmless to other non-target insects.

The objective of this study was to evaluate 8 native strains of *H. citriformis*, obtained from diverse areas of the Mexican citriculture, against *D. citri* adults, and to test non-target effects in the predators *Chrysoperla rufilabris* (Burmeister) (Neuroptera: Chrysopidae) and *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), beneficial insects that attack *D. citri* in areas where the fungus has been found in Mexico (Cortez-Mondaca et al. 2010).

## Materials and Methods

### FUNGAL STRAINS

Eight *H. citriformis* strains obtained from 8 citrus-producing areas of Mexico were used (Table 1). Six strains were deposited in the National Institute of Forestry, Agriculture, and Livestock (INIFAP) and 2 in the Biotechnology Institute at the Biological Sciences Department, Nuevo León Autonomous University (IB-UANL). Strains were grown in Petri dishes containing potato dextrose agar medium (PDA) with 0.5% yeast extract (PDAY); they were incubated for 6 to 7 wk at 25 ± 1 °C until conidia production.

### EXPERIMENTAL INSECTS

Adults of *D. citri* were collected with a buccal plastic aspirator from shoots of Valencia orange trees from a 3-yr-old commercial orchard. Collected insects were transported to the laboratory and those that appeared healthy were selected for the bioassays. They were anesthetized using a cotton ball impregnated with 80 µL chloroform before being subjected to the treatments.

Adults of *H. convergens* were collected in the field and transported to the laboratory, where they were maintained in separate cages to eliminate those that were parasitized or damaged. For feeding, they received frozen eggs of *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae) and a source of water mixed with honey. Larvae of *C. rufilabris* were the 3rd generation of field-collected females maintained in the laboratory with water and a volumetric mixture of yeast, sugar, honey, and powdered milk used as artificial diet. Larvae were fed with *S. cerealella* frozen eggs. All the insects were maintained under laboratory conditions at 26 ± 1 °C, 60 ± 4% RH, and a 16:8 h L:D photoperiod.

### BIOASSAYS AGAINST *D. CITRI* ADULTS

#### Test of Conidia Inoculated by Contact

Anesthetized *D. citri* adults were transferred to Petri dishes containing sporulating cultures of the 8 strains, where they remained for

**Table 1.** *Hirsutella citriformis* monoconidial strains isolated from *Diaphorina citri* obtained from 8 citrus-growing areas of Mexico.

| Strain | Locality | Date of collection | Host plant |
|---|---|---|---|
| INIFAP-Hir-1 | Huimanguillo, Tabasco | Aug 2008 | *Citrus sinensis* (L.) Osbeck |
| INIFAP-Hir-2 | Mocochá, Yucatán | Sep 2011 | *Citrus tangerina* Tanaka |
| INIFAP-Hir-4 | Xolol, San Luis Potosí | Oct 2009 | *Citrus sinensis* (L.) Osbeck |
| INIFAP-Hir-5 | Nuevo Israel, Quintana Roo | Jan 2010 | *Murraya paniculata* (L.) Jack |
| INIFAP-Hir-6 | Edzná, Campeche | Sep 2011 | *Murraya paniculata* (L.) Jack |
| INIFAP-Hir-7 | Tapachula, Chiapas | Dec 2011 | *Murraya paniculata* (L.) Jack |
| IB-Hir-1 | Tlapacoyan, Veracruz | Sep 2011 | *Citrus latifolia* Tan. |
| IB-Hir-2 | Tecomán, Colima | Sep 2011 | *Citrus latifolia* Tan. |

1 to 2 min; insects for the untreated control were transferred to sterile PDAY for the same time. Afterwards, 15 insects from each treatment were allocated to a plastic container (6 cm height × 8 cm top diameter × 5 cm bottom diameter) that contained a Valencia orange tender leaf for feeding. The leaf was placed on a 2 cm thick sponge layer located on the bottom of the plastic container; to maintain leaf turgidness and high relative humidity, the sponge was saturated with sterile water. Each treatment had 7 replications. We used a completely randomized experimental design. For the total experiment, there were 63 plastic containers with 15 *D. citri* adults per container. All of them were placed in an environmental chamber at 26 ± 1 °C, 76 ± 4% RH, a 16:8 h L:D photoperiod, and maintained for 26 d. Every 2 or 3 d, the leaf was replaced, sterile water was added to the sponge, and the dead insects were removed and counted. The whole experiment was performed 3 times at different dates. All dead insects were placed in a humidity chamber to verify mycelial growth from the evaluated fungal strains.

### Test of Conidia Inoculated by Spraying

To perform the sprayed-conidia bioassay, INIFAP-Hir-1 and IB-Hir-2 strains were selected from the previous test. The conidial inoculum was obtained by adding 10 mL sterile water to each *H. citriformis* strain cultured in Petri dishes that showed abundant sporulation. Conidia were suspended by scraping lightly the medium with a Drigalski spatula. This suspension was mixed for 3 min; then, 1% w/v acacia gum (Desarrollo de Especialidades Químicas, S. A. de C. V. Monterrey, Mexico) was added to serve as adherent. Finally, the concentration was adjusted at $1 \times 10^6$ conidia per mL. The conidia suspension was prepared 1 h before the bioassay. For the untreated control, the solution contained only sterile water and 1% acacia gum. Treatments were sprayed to 15 healthy and anesthetized insects per replication that were placed on absorbent paper in a plastic tray. The spray of the conidia suspension was made with a commercial manual sprayer (15 mL volume; 0.3 mm nozzle diameter) at approximately 15 cm distance from the insects; conidia were sprayed for 3 consecutive times for each array of insects. Afterwards, we followed the same procedure as in the previous test.

### Test of Blastospores Inoculated by Spraying

We evaluated effects of blastospores from 5 *H. citriformis* strains (INIFAP-Hir-2, INIFAP-Hir-4, INIFAP-Hir-5, INIFAP-Hir-7, and IB-Hir-1) sprayed against *D. citri*. To obtain blastospores from these strains, they were grown on PDA in Petri dishes at 25 ± 1 °C for 2 wk. Thereafter, four 1 $cm^2$ mycelial colony pieces were extracted with a scalpel and used to inoculate potato dextrose broth (PDB) in 300 mL glass Erlenmeyer flasks that were incubated for 11 d at 25 °C on a shaker at 250 rpm. Produced blastospores were adjusted to $1 \times 10^6$ blastospores per mL as described for bioassays with conidia. Insects in the untreated control were sprayed with PDB sterile diluted medium. Thereupon, we follow the same procedure as in the spray of conidia that was described previously.

### Test to Determine Lethal Concentration of *H. citriformis* INIFAP-Hir-1 Strain

The *H. citriformis* strain that produced the highest mortality in the test of inoculation by conidia contact (INIFAP-Hir-1) was selected to determine its LC50 and LC90. For this bioassay, 6 concentrations of conidia ($1 \times 10^4$, $1 \times 10^5$, $0.5 \times 10^6$, $1 \times 10^6$, $1 \times 10^7$, and $1 \times 10^8$ conidia per mL) in sterile water were applied by spraying, using the same procedure described previously. Sterile water was used for the untreated control. Subsequent procedures were the same as described previously.

### BIOASSAY AGAINST INSECT PREDATORS

Strains INIFAP-Hir-1, INIFAP-Hir-2, IB-Hir-1, and IB-Hir-2 were used for bioassays against *H. convergens* adults and *C. rufilabris* 1st instar larvae. Both predator species were inoculated in the same way as *D. citri* in the infection by contact test. In the case of *H. convergens*, for each experimental replication there were cohorts of 10 specimens kept in a Petri dish. For *C. rufilabris,* we used a pair of larvae per Petri dish; to avoid cannibalism, we added in each cage a zig-zag folded paper strip (length: 10 cm; width: 1 cm) that acted as a barrier between the larvae. There were 5 replications per treatment for *H. convergens* and 8 replications per treatment for *C. rufilabris*. After inoculation, insects were maintained during 24 d for *H. convergens* and 30 d for *C. rufilabris* under the environmental conditions described before for *D. citri*. Every other day, we examined all the insects, removed dead specimens, changed the Petri dish and the paper strip, and provided the food indicated before. Retrieved dead insects were placed in a humidity chamber to promote or verify mycelial growth from the evaluated fungal strains.

### DATA ANALYSES

One-way ANOVA was performed to analyze mortality data from the bioassays. Before analysis, data were transformed by using arc sin ($\sin^{-1}\sqrt{x+1}$). Treatment means were compared using Tukey's test ($\alpha$ = 0.05) (SPSS 2008). We used a computerized program (US Applied and Environmental Health, 1989) to run probit analysis to determine LC50 and LC90 for the INIFAP-Hir-1strain; for this analysis, data were transformed by using arc sin ($\sin^{-1}\sqrt{x+1}$).

## Results

### VIRULENCE AGAINST *D. CITRI*

All *H. citriformis* strains were able to kill *D. citri* adults in the contact bioassays (Fig. 1; Table 2). Mortality was observed beginning 6 d post exposure to conidia in a range from 1.8 to 8.2%, with the highest mortality in insects exposed to strain INIFAP-Hir-1 and the lowest in

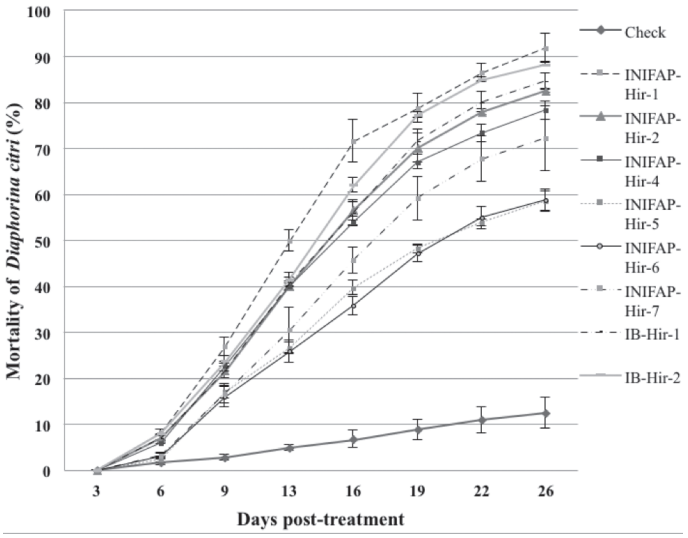


**Fig. 1**. Overall mean mortality of *Diaphorina citri* adults caused by conidia of 8 *Hirsutella citriformis* strains applied by contact (3 separate bioassays) under controlled conditions (26 ± 1 °C, 76 ± 4% RH, 16:8 h L:D photoperiod). Error bars represent the standard error (*n* = 7).

**Table 2.** Mortality of *Diaphorina citri* caused by conidia of 8 *Hirsutella citriformis* strains applied by contact (3 separate bioassays) under controlled conditions (26 ± 1 °C, 76 ± 4% RH, 16:8 h L:D photoperiod).

| | Mean mortality (% ) ± SE (*n* = 7) | | | Mycosis |
|---|---|---|---|---|
| Strain | Bioassay 1 | Bioassay 2 | Bioassay 3 | % ± 95% CI |
| Control | 7.08 ± 1.02e | 12.00 ± 0.89d | 18.50 ± 0.93c | 0.0 |
| INIFAP-Hir-1 | 98.33 ± 3.13a | 89.20 ± 5.89a | 88.07 ± 2.56a | 80.0 ± 29.6 |
| INIFAP-Hir-2 | 82.48 ± 3.46abc | 83.55 ± 3.43a | 81.70 ± 3.53a | 80.5 ± 29.3 |
| INIFAP-Hir-4 | 73.78 ± 6.90bc | 80.55 ± 5.12ab | 80.00 ± 3.93a | 78.3 ± 30.5 |
| INIFAP-Hir-5 | 64.88 ± 6.26cd | 52.12 ± 3.92c | 58.50 ± 3.19b | 48.7 ± 37.0 |
| INIFAP-Hir-6 | 50.78 ± 4.05d | 70.63 ± 3.14b | 43.00 ± 2.40b | 61.3 ± 36.1 |
| INIFAP-Hir-7 | 85.76 ± 2.23ab | 69.10 ± 2.21b | 30.31 ± 3.10b | 54.6 ± 36.9 |
| IB-Hir-1 | 79.73 ± 6.64bc | 89.20 ± 5.89a | 85.62 ± 4.06a | 73.0 ± 32.9 |
| IB-Hir-2 | 87.43 ± 3.87ab | 89.30 ± 2.17a | 87.68 ± 2.53a | 81.2 ± 28.9 |

Different letters within columns indicate significant differences (Tukey's test, α = 0.05). SE = Standard error. CI= Confidence interval.

untreated controls (Fig. 1). At 10 d after inoculation, we observed occurrence of the characteristic *H. citriformis* synnemata on *D. citri* cadavers. At 13 d after application, the evaluated fungal strains caused 25.8% (INIFAP-Hir-6) to 49.9 % mortality (INIFAP-Hir 1) in *D. citri* (Fig. 1). This trend of mortality continued among the treatments until the last record date, which was 26 d after exposure (Fig. 1), when overall mean mortality caused by strain INIFAP-Hir-1 in the 3 replicated bioassays ranged from 88.1 to 98.3%, followed by IB-Hir-2 (87.4–89.3%), whereas mean mortality in the untreated controls ranged from 7.1 to 18.5% (Fig. 1; Table 2). In the 3 bioassays, there were significant differences in overall mortality among treatments (bioassay 1: $F = 19.31$; df = 8,45; $P < 0.001$; bioassay 2: $F = 32.36$; df = 8,45; $P < 0.001$; bioassay 3: $F = 44.12$; df = 8,45; $P < 0.001$). Observed mycosis by *H. citriformis* ranged from 48.7% (INIFAP-Hir-5) to 81.2% (IB-Hir-2) (Table 2); dead specimens in the control did not show any sign of mycosis (Table 2).

When conidia were applied by spraying, INIFAP-Hir-1 and IB-Hir-2 strains caused 69% overall mortality in *D. citri* after 26 d; this value was significantly higher than that recorded in the untreated control ($F = 23.48$; df = 2,18; $P < 0.001$; Tukey's test, α = 0.05) (Fig. 2). In this test, we observed *H. citriformis* mycosis in dead specimens ranging from 20.5 to 62.7%.

In the test of *H. citriformis* blastospores in spray inoculation against *D. citri,* initial mortality was observed 6 d after the fungus application and ranged from 6.2% (INIFAP-Hir-2) to 13.9% (INIFAP-Hir-1), whereas mortality in the untreated control was 4.1% (Fig. 3). At 12 d post application, *H. citriformis* blastospores caused 20.4 to 38.0% mortality of *D. citri* (Fig. 3). After 26 d, the INIFAP-Hir-2 and INIFAP-Hir-5 strains sprayed as blastospores produced the highest overall mortality (48.7%) in *D. citri*; this value was statistically different from that recorded in the control (17.6%) ($F = 4.68$; df = 5,36; $P = 0.002$; Tukey's test, α = 0.05) (Fig. 3). Occurrence in humid chambers of mycosis by *H. citriformis* in *D. citri* dead specimens from the treatments with blastospore application was very low, approximately 10%; in the case of cadavers from the control, mycosis was null. Probit analysis showed that LC50 for strain INIFAP-Hir-1 was $3.40 \times 10^6$ conidia per mL, with 95% confidence interval 1.38 to 8.51 $\times 10^6$ conidia per mL. LC90 was $2.67 \times 10^9$ conidia per mL, with 95% confidence interval $2.18 \times 10^8$ to $2.44 \times 10^{11}$ conidia per mL. The regression equation obtained was $y = 4.31 + 0.44x$ ($\chi^2 = 7.0055$; $P = 0.05$).

## PATHOGENICITY AGAINST PREDATORS

Overall mortality by *H. citriformis* in *H. convergens* adults ranged from 9% (INIFAP-Hir-1) to 11% (INIFAP-Hir-2) after 24 d of conidia application; these percentages did not differ significantly from that recorded in the untreated control ($F = 0.1623$; df = 4,20; $P = 0.953$) (Table 3). Dead specimens did not develop mycosis after being placed in a humidity chamber. In *C. rufilabris,* overall mortality by *H. citriformis* 30 d after application in larvae and recorded in emerged adults ranged

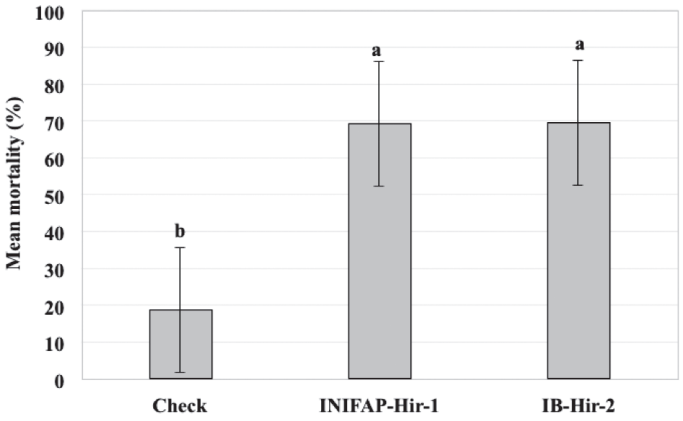


**Fig. 2.** Mean mortality of *Diaphorina citri* caused by conidia sprays of 2 *Hirsutella citriformis* strains under controlled conditions (26 ± 1 °C, 76 ± 4% RH, 16:8 h L:D photoperiod). Different letters indicate significant differences (Tukey's test, α = 0.05). Error bars represent the standard error (*n* = 7).

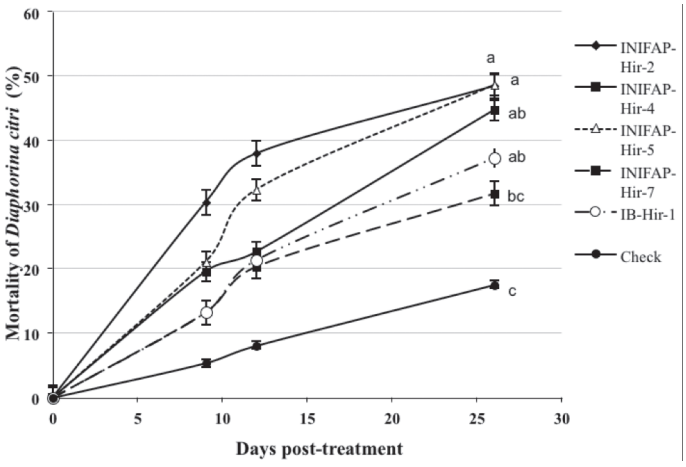


**Fig. 3.** Mean mortality of *Diaphorina citri* caused by blastospores of 5 *Hirsutella citriformis* strains under controlled conditions (26 ± 1 °C, 76 ± 4% RH, 16:8 h L:D photoperiod) during 26 d post inoculation. Different letters indicate significant differences (Tukey's test, α = 0.05). Error bars represent the standard error (*n* = 7).

**Table 3.** Overall mean mortality of the predators *Chrysoperla rufilabris* and *Hippodamia convergens* at 30 d and 24 d, respectively, after conidia of *Hirsutella citriformis* strains were applied by contact.

| | Mean mortality (% ) ± SE (*n*) | |
|---|---|---|
| Fungal Strain | *Chrysoperla rufilabris* | *Hippodamia convergens* |
| INIFAP-Hir-1 | 18.7 ± 0.5 (5) | 8.7 ± 0.3 (8) |
| INIFAP-Hir-2 | 25.0 ± 0.4 (5) | 11.2 ± 0.6 (8) |
| IB-Hir-1 | 18.7 ± 0.5 (5) | 10.7± 0.3 (8) |
| IB-Hir-2 | 20.0 ± 0.4 (5) | 10.6 ± 0.1 (8) |
| Control | 18.7 ± 0.5 (5) | 10.6 ± 0.6 (8) |

*Hippodamia convergens* received conidia as adults. *Chrysoperla rufilabris* received conidia as 1st instar larvae. SE = Standard error.

from 19% (INIFAP-Hir-1) to 25% (INIFAP-Hir-2); there were no statistical differences among treatments ($F$ = 0.577; df = 4,15; $P$ = 0.684) (Table 3). As in *H. convergens*, *C. rufilabris* dead specimens did not develop mycosis by *H. citriformis*.

## Discussion

In our study, all the tested Mexican *H. citriformis* strains showed potential as biological control agents of *D. citri* as they performed well in the bioassays using inoculation by conidia contact, by which they caused high mortality rates that were close to 90% (Table 2). Similar to the results of Meyer et al. (2007), we observed that mortality of *D. citri* specimens began 6 d after exposure to the fungus (Fig. 1); occurrence of characteristic *H. citriformis* synnemata on *D. citri* cadavers was observed 10 d after inoculation, which agreed with results by Casique-Valdés et al. (2011); however, the highest accumulated mortality (90%; Fig. 1; Table 2) in our study was recorded until 26 d after the application of the fungal strains, whereas Casique-Valdés et al. (2011) and Orduño-Cruz et al. (2015) reported 100% mortality just 6 d after inoculation, and Meyer et al. (2007) obtained the same rate at 9 d post inoculation. These differences in achieving the highest mortality could be due to genetic variability of the evaluated strains that could be associated with high pathogenicity as observed in many pathogens (see Mena et al. 2003; Hoy et al. 2010); hence, we were expecting some strain variability.

In addition, variations in bioassay procedures such as the use of different fungal conidia doses could explain the above differences; in Meyer et al. (2007) and Casique-Valdés et al.(2011), doses were unreported, and we realized based on the size of conidia (see Speare 1920; Perez-Gonzalez et al. 2015b) that Orduño-Cruz et al. (2015) could have overestimated the number of conidia attached to the insect and consequently used a very high dose of conidia, probably far beyond to the LC90 of 2.6 × $10^9$ conidia per mL estimated in our bioassay with sprayed conidia. Meyer et al. (2007) and Casique-Valdés et al. (2011) might have used a dose in these amounts. It is also possible that the first dead specimens observed in our trial were those that were infected with a greater number of conidia and that those that died later barely had received a lethal dose. Such differences in the number of conidia per insect could be due mainly to the inoculation method by not providing a homogeneous presence of conidia on the insect body.

Nonetheless, we emphasize that the observed mortality curves (Fig. 1) may resemble a possible pattern for infected *D. citri* by *H. citriformis* in nature. Because the *H. citriformis* conidia are characteristically sticky (Speare 1920; Meyer et al. 2007; Perez-Gonzalez et al. 2015b), they would not spread readily as seen in other entomopathogenic fungal genera such as *Beauveria* or *Metarhizium* (Mena et al. 2003; Padulla & Alves 2009). Thus, it is possible that *H. citriformis* would exhibit a behavioral manipulation of the host as it has been documented for *Ophiocordyceps unilateralis* (Tul. & C. Tul.) Petch (Hypocreales: Ophiocordycipitaceae) in ants (Andersen et al. 2009; Pontoppidan et al. 2009). *Hirsutella citriformis* could use of the host as a vehicle for dispersion. For instance, numerous healthy hosts could be infected with a lethal, sublethal, or innocuous concentration of conidia that eventually will kill the insect through time and on several host plants, possibly far away from the initial site of infection. This scenario would reflect a possible strategy or adaptation of the fungus to spread and survive as suggested by Casique-Valdés et al. (2011) and Hall et al. (2012).

Research on *H. citriformis* has been neglected, and there are many uncovered areas of study. From the stated above, an essential need is to demonstrate the mechanisms involved in the possible host manipulation, as well as to understand completely its role at the insect population level and, therefore, the settings and development of epidemics.

In the bioassays in which conidia were applied by spraying, mortality caused by the 8 strains was remarkably reduced (Fig. 2). Such low values when conidia in suspension were used for inoculation was also observed by Orduño-Cruz et al. (2015) in *H. citriformis.* A possible explanation for these results could be the damage or removal of the water-soluble mucilaginous envelope of conidia during the preparation of inoculum (Sánchez-Peña et al. 2011). These changes eventually could result in poor adherence to the cuticle of the host, as well as in low germination, decreased virulence, and consequently reduced host mortality as the one that we obtained in our tests (Fig. 2). We used acacia gum to overcome such factor; however, it was ineffective and it has prompted the need for further research in application techniques of the fungus that could support its successful use in the field.

The LC50 and LC90 determined for the strain INIFAP-Hir-1 in our study (3.4 × $10^6$ and 2.6 × $10^9$ conidia per mL respectively), applied by spraying of conidia, are the first reported for *H. citriformis.* Hoy et al. (2010) determined for *I. fumosorosea* against *D. citri* LC50 and LC95 values of 6.8 × $10^5$ and 2.2 × $10^8$ conidia per mL, respectively. Although the LC values are relatively similar for both fungal species, *H. citriformis* due to its slow development would be more expensive to use in an extensive way. For example, the conidia of the *H. citriformis* strains used herein germinated after 48 to 72 h (O. P. G., unpublished data) and growth is reportedly slow (radial growth = 0.083 to 0.114 cm/d) (Meyer et al. 2007; Pérez-González et al. 2015a). For *I. fumosorosea*, Vu et al. (2009) indicated conidia germination within 12 to 24 h, and according to Cabanillas & Jones (2009), this fungus showed fast radial growth (0.33 cm/d); both traits were at least 3 times greater than in *H. citriformis.* With the estimated LC50 and LC90 for *H. citriformis* against *D. citri* as reference, its potential use for biological control of this insect requires investigation to facilitate the commercial production of the fungus and make it economically viable.

In the evaluation of 5 *H. citriformis* Mexican strains as blastospores, we found that they induced low mortality against *D. citri*. Similar results were obtained previously by Pérez-González et al. (2015a). In our bioassays, the average mortality ranged from 31 to 48% (Fig. 3), and mycosis occurred only in a small number of insects. A higher mortality in *D. citri* with the use of blastospores was reported by Orduño-Cruz et al. (2015); they observed 61 to 65% mortality caused by 2 strains of *H. citriformis* isolated from unidentified species of insects; mycosis data were unavailable. Low values of mortality and mycosis produced by blastospores recorded in this study may have been caused in part by toxins released during *H. citriformis* liquid-culture growth, as reported by Liu et al. (1995) and Rosas-Acevedo et al. (2003) for strains of *Hirsutella thompsonii* F. E. Fisher. Our mortality data suggest that the application of conidia for control of *D. citri* is preferable over the use of blastospores; however, it is important to continue determining conditions under which this fungus develops infective and virulent blastospores against *D. citri* as their use could be more practical and

they could be produced in high numbers in liquid culture in a short time (Avery et al. 2009; Romero-Rangel et al. 2012).

Tests of *H. citriformis* conidia inoculation by contact against *H. convergens* adults and *C. rufilabris* larvae produced low mortality without significant differences among treatments, including the untreated controls (Table 3). Data concerning *H. convergens* adults were similar to those reported by Gandarilla-Pacheco et al. (2012) in tests with *B. bassiana* and *I. fumosorosea*, in which they recorded 6 to 25% mortality. In contrast, the effect on *C. rufilabris* (18 to 25% mortality; Table 3) was lower than that reported by these authors for *Ceraeochrysa valida* (Banks) and *Eremochrysa punctinervis* (McLachlan) (Neuroptera: Chrysopidae) larvae, for which they recorded 50% mortality caused by a local strain of *I. fumosorosea* (Gandarilla-Pacheco et al. 2012).

Our study confirms the value of including tests for non-target effects. We here evaluated 4 *H. citriformis* strains under a hypothetical scenario of causing high mortality in diverse organisms different to our target. Hence, we would have the opportunity to select a possible fungal strain that produces no harmful effects for other organisms, thereby evading antagonistic interactions (Thomas & Lynch 2003) and allowing a relatively safe use for our evaluated agent for biological control. As there are many species of arthropods associated with citrus (Monzó et al. 2012), and as we tested the fungus in only 2 beneficial entomophagous insect species, it would be imprecise to state that *H. citriformis* appears to be a specific natural enemy for *D. citri*. Instead, we suggest further studies of this valuable natural enemy aimed to generate in the short term knowledge for its successful and safe use as a biological control agent. Plans should be made for its integration with other beneficial arthropods for a sustainable management of *D. citri* (Thomas & Lynch 2003), generating in this way alternatives to the present and intense use of pesticides against the vector of huanglongbing.

We conclude that the 8 Mexican fungal strains of *H. citriformis* tested against *D. citri* were pathogenic to the Asian citrus psyllid adults, with variability in performance associated with strain diversity. Among the strains, INIFAP-Hir-1 showed constantly favorable results. At the present time, use of conidia seems to be the best method for application of this fungus; the use of blastospores produced in liquid culture requires further investigation to determine optimal conditions to develop infective and virulent propagules against *D. citri.* Our data obtained from tests against the predators *C. rufilabris* and *H. convergens* could support the safe use of *H. citriformis* as a biological control agent of *D. citri.*

## Acknowledgments

We thank CONACYT (MEXICO) for the grant SAGARPA-CONACYT 2009-108591, and the PAICYT-UANL Program for partial support of this study. O. Pérez-González also thanks CONACYT for fellowship support. We are deeply grateful to Yesenia J. Salazar, Don Juan Herrera, Santos Díaz, Franco G. Robles, and numerous people at the INIFAP's General Terán Experimental Station for invaluable help in the collection of insects and maintenance of experiments.

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