EVALUATIONS OF A NOVEL ISOLATE OF *ISARIA FUMOSOROSEA* FOR CONTROL OF THE ASIAN CITRUS PSYLLID, *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE)

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Abstract

A fungal pathogen that killed adult *Diaphorina citri* Kuwayama (Asian citrus psyllid) in Florida citrus groves was isolated, characterized molecularly and morphologically and identified as a novel isolate of *Isaria fumosorosea* (*Ifr*) (= *Paecilomyces fumosoroseus*) from the Asian citrus psyllid (*Ifr* AsCP), but no concentration-mortality or time-response data were obtained. When adult psyllids were sprayed with spores at 28°C, time response (LT_{50}) values of 111 and 102.5 h at spore concentrations of 1×10^7 and 1×10^8 spores/mL, respectively, were obtained. The LT_{99} was 167.4 and 174.6 h, respectively, for the 2 spore concentrations. After 192 h, the LC_{50} value was 6.8×10^5 spores/mL and the LC_{99} was 2.2×10^8 spores/mL. Ten serial passages of *Ifr* AsCP were carried out on malt extract agar (MEA) and dilute Sabouraud dextrose agar and yeast (SDY) media. The pathogenicity to adult psyllids did not decline, but spore yield declined on the SDY medium and *Ifr* AsCP consistently produced more spores on SDY than on MEA media. *Ifr* AsCP was highly pathogenic to the psyllid when healthy adults were exposed to spores from psyllid cadavers stored at -74°C. Two pilot field trials were conducted in Florida citrus groves to assess methods for confirming infection; *Ifr* AsCP spores infected immature psyllids when applied at a rate of 1×10^7 spores/mL, but monitoring for infected nymphs required special handling methods.

Key Words: *Diaphorina citri*, *Isaria fumosorosea (= Paecilomyces fumosoroseus)*, concentration-mortality data, timed-response data, field evaluation methods, stability

Resumen

Un hongo patógeno que mató adultos de *Diaphorina citri* Kuwayama (sílido asiático de los cítricos) en huertos de cítricos en la Florida fue aislado, molecularmente y morfologicamente caracterizado e identificado como un aislado novedoso de *Isaria fumosorosea* (*Ifr*) (= *Paecilomyces fumosoroseus*) del sílido asiático de los cítricos (*Ifr* AsCP), pero no se obtuvo datos de concentracion-mortalidad o tiempo-letal. Cuando los sílidos adultos fueron rociados con esporas a los 28°C, los valores obtenidos del tiempo letal (TL_{50}) fueron 111 y 102.5 horas a la concentracion de esporas de 1×10^7 y 1×10^8 esporas/mL, respectivamente. El TL_{99} fue 167.4 y 174.6 horas, respectivamente, para las dos concentraciones de esporas. Después de 192 horas, el valor CL_{50} fue 6.8×10^5 esporas/mL y la CL_{99} fue 2.2×10^8 esporas/mL. Se hicierion diez pasos seriales de *Ifr* AsCP sobre un extracto de malto y agar (MEA) y un medio de agar de dextrosa Sabouraud diluido y levadura (SDL). La patogenicidad de los adultos sílidos no disminuiron, pero el rendimiento de esporas sobre el medio SDL y el *Ifr* AsCP consistentemente produjó mas esporas sobre SDL que sobre el medio MEA. El *Ifr* AsCP fue altamente patogenico a los sílidos cuando los adultos sanos fueron expuestos a las esporas que provienen de los cadáveres de sílidos almacenados a los -74°C. Se realizaron dos pruebas piloto en huertos de citricos de la Florida para evaluar los métodos para confirmar infecciones; las esporas de *Ifr* AsCP infectaron los inmaduros de los sílidos cuando fue aplicado a la tasa de 1×10^7 esporas/mL, pero para el monitoreo de ninfas infectadas se requiere métodos especiales de manejo.

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, is a vector of citrus greening disease, which is the most serious disease of citrus. Citrus greening disease, caused by the bacterium *Candidatus* Liberibacter asiacticus, renders fruit unusable and ultimately kills the trees (da Graca 1991; Garnier et al. 2000; Halbert et al. 2000; Halbert 2005). Establishment of citrus greening disease

in Florida was confirmed in 2005 and the disease is widely distributed (Halbert 2005; Bouffard 2006; http://www.doacs.state.fl.us/pi/chrp/greening/maps/cgsit_map.pdf).

Until citrus greening disease was discovered in Florida, control of Asian citrus psyllid relied heavily on native predators (Michaud 2004) and parasitoids introduced in a classical biological control program (Hoy et al. 1999, 2001; Hoy & Nguyen 2000; Skelley & Hoy 2004). The predators are most effective when nymphal psyllid densities are high and the parasitoids cause high rates of mortality late in the growing season (Hoy et al. unpublished; Qureshi et al. 2009). The occurrence of fungal pathogens attacking *D. citri* in Florida has been noted (Halbert & Manjunath 2004; Michaud 2004; Meyer 2007; Meyer et al. 2007, 2008), but detailed information on the distribution, phenology, or impact of these fungi on the population dynamics of *D. citri* is not yet available.

Methods for managing citrus greening disease are being developed and include monitoring, removing symptomatic trees, replanting groves with certified disease-free nursery stock, development of resistant trees, and suppression of *D. citri* populations with pesticides (Browning et al. 2006; Stansly & Rogers 2006; Rogers & Stelinski 2009). Petroleum oil and synthetic organic insecticides have been used to suppress *D. citri* populations in Florida (Rae et al. 1997; Browning et al. 2006; Srinivasan et al. 2008; Cocco & Hoy 2008) but, now that greening has been confirmed in Florida, growers are applying sprays more frequently in an attempt to reduce the likelihood of disease transmission (Padrick 2006). Unfortunately, many synthetic organic pesticides can cause secondary outbreaks of pests that are under substantial biological control (McCoy 1985; Browning 1990; Hoy 2000). As a result, control tactics that would suppress the Asian citrus psyllid and still allow natural enemies to survive are needed to sustain Florida's citrus industry.

During Sep 2005, a fungal pathogen was found killing adult *D. citri* in Florida citrus groves (Meyer et al. 2008). Based upon morphological and molecular methods, a novel strain was identified as *Isaria fumosorosea* (*Ifr*) (previously known as *Paecilomyces fumosoroseus* or *Pfr*) and designated as *Ifr* from the Asian citrus psyllid or *Ifr* AsCP. *Ifr* AsCP is distinct from (Meyer et al. 2008), but related to, another isolate (*Pfr* 97) that was originally collected from scales in Apopka, Florida and commercialized as a microbial insecticide (Vidal et al. 1998). *Ifr* AsCP also is related to a strain isolated from *D. citri* in Indonesia (Subandiyah et al. 2000). Because *Ifr* AsCP was found attacking *D. citri* in citrus groves in Florida, we considered that it might be uniquely adapted to citrus grove environmental conditions and to the Asian citrus psyllid.

The objectives of this study were to develop concentration-mortality and time-response data, to resolve how stable this isolate is after undergoing serial transfers on 2 different media because attenuation of Hyphomycetes after serial transfers on media has been shown to alter virulence and host specificity (Vandenberg & Cantone 2004), and to determine if frozen infected cadavers could infect live psyllids. In addition, we evaluated methods for assessing infection status of psyllids treated with *Ifr* AsCP in 2 pilot field trials.

MATERIALS AND METHODS

Colony of *D. citri*

A greenhouse colony of *D. citri* was established by collecting 10 adult females with orange abdomens from citrus during Mar 2006. The colony was maintained with methods modified from Skelley and Hoy (2004) in a greenhouse at 20-32°C under a 16L:8D photoperiod. Adult *D. citri* were allowed to oviposit on tender new growth (flush) produced by small potted citrus trees held in mesh cages (61 × 61 × 61 cm). Upon emergence, adult *D. citri* were collected to initiate another generation or used for evaluation of conidial (spore) pathogenicity in the serial transfer test or for concentration-mortality tests.

Cultivation of *Ifr* AsCP

Mycosed *D. citri* that had been exposed to field-collected cadavers were obtained from Jason Meyer. Inoculated insects were held in a sterile 50-mL centrifuge tube (USA Scientific, Ocala, FL) containing a single mature sour orange leaf (*Citrus aurantium* L.) and a water-soaked cotton ball to maintain RH at approximately 100%. These cultures were held in a growth chamber at 28 ± 1°C under a 16L:8D photoperiod. Spores from the infected insects were used to inoculate 15 × 1.5-cm petri plates (Fisherbrand, Fisher Scientific, Suwanee, GA) containing dilute SDY media (7.5 g Sabouraud dextrose agar, 5 g yeast extract, and 15 g agar/L of water) and maintained in the growth chamber under the same conditions. Spores from these *in vitro* cultures were harvested 23 d after inoculation by scraping fungal hyphae and spores off the plates with a sterilized spatula and suspending them in 20 mL of autoclaved deionized water. The suspension was filtered through Miracloth (Calbiochem, EMD Biosciences Inc., La Jolla, CA) and spore concentrations were determined with a hemocytometer. New SDY plates were inoculated with 0.5 mL of a 1×10^7/mL spore suspension. In a similar manner, *Ifr* AsCP was maintained on Malt Extract Agar (MEA) plates (45 g of malt extract agar/L water, MP Biomedi-

cals, Inc., Solon, OH). Plates were sealed with parafilm (Pechiney Plastic Packaging, Menasha, WI) and held in a growth chamber at 28 ± 1°C under a 16L:8D photoperiod for 23 d.

Serial Transfer of *Ifr* AsCP on MEA or SDY Media

Every 23 d, spores were harvested from *Ifr* AsCP grown on SDY and MEA plates and used to inoculate 2 plates each with 0.5 mL of spore suspension at 1×10^7 spores/mL. The pathogenicity of these spores was assessed in newly emerged adult psyllids from the greenhouse colony. Ten adult psyllids (4- to 5-d old) were aspirated from the colony into a 50-mL centrifuge tube, chilled for 10 min on ice and then placed in a 15 × 1.5-cm sterile petri dish and sprayed with 2 mL of spore suspension (1×10^7 spores/mL) that had been harvested from 2 MEA or 2 SDY plates. Psyllids were treated with autoclaved deionized water as a control. Treated psyllids were then individually transferred to 50-mL centrifuge tubes along with a mature sour orange leaf. A cotton ball moistened with tap water and squeezed to remove excess water was placed in the lid. A single Kimwipe (Kimberly-Clark Corp, Roswell, GA) was used to cover the cotton before fastening the lid. The tubes were incubated at 28 ± 1°C under 16L:8D photoperiod. Psyllids were observed for mortality every 24 h starting 4 d after treatment because Meyer et al. (2008) had shown that mortality did not occur until at least 6 d after exposure to 1×10^7 spores/mL under these conditions.

Mean (standard deviation = SD) percentage germination for transfers 8, 9, and 10 on SDY media was determined by spreading a thin layer of SDY medium on a plain microscope slide (3 × 1 × 1 mm) (Fisher Brand, Fisher Scientific, Pittsburgh, PA). The slide containing the medium was placed into a chamber with 100% RH overnight and then smeared with 100 µL of spore suspension diluted to 1,000 spores/100 µL. The microscope slides were held under 100% RH overnight at 24-25°C and 100 spores were scored for germination. A total of 5 slides were scored after each transfer and the mean percentage germination was calculated.

Storage of Infected Psyllid Cadavers

Different methods have been used to store fungal isolates, including storing the fungus on agar slants under mineral oil, storing the fungus along with the growing medium under water or mineral oil, freezing at very low temperatures, or storing the fungus on growing medium at room temperature (Dhingra & Sinclair 1995). These methods require an additional step prior to culturing or use of the fungus, which may lead to contamination due to handling or improper sterilization techniques, as well as additional time before the fungus can be used or tested.

To determine if *Ifr* AsCP could be stored at -74°C on psyllid cadavers, which would allow us to maintain *Ifr* AsCP on psyllids without the need to continuously transfer it to new hosts, cadavers were obtained by touching live adults to an infected psyllid with abundant spores and the newly infected psyllids were held in 50-mL centrifuge tubes with a citrus leaf for food at 28 ± 1°C under a 16L:8D photoperiod for 72 h, after which 100% mortality was observed. After an additional week, the cadavers were evaluated to confirm that they had spores and the tubes then were placed into sealed plastic bags in a -74°C freezer. Every 2 weeks, 2 cadavers were randomly chosen and thawed to room temperature. Ten adult psyllids that were 4- to 5-d old were collected from the greenhouse colony in a 50-mL centrifuge vial and chilled for 10 min on ice. Two psyllid cadavers were placed in the tube along with 10 chilled psyllids. The vial was tapped several times on a tabletop to ensure the chilled psyllids encountered the cadaver and then the psyllids were held individually in a new 50-mL tube, as described previously. A control also was included, where chilled psyllids were touched with water. The vials were held in a growth chamber at 28 ± 1°C under 16L:8D photoperiod. This procedure was followed 5 times every 2 weeks and data were recorded as the number of dead psyllids 24, 48, and 72 h after exposure to the cadavers.

Concentration-Mortality and Time-Response Data

Five different concentrations of *Ifr* AsCP (1×10^4 to 10^8 spores/mL), plus a water control, were used to conduct a concentration-mortality test at 28 ± 1°C under a 16L:8D photoperiod. The bioassay was repeated 5 times at 7-10-d intervals. *Ifr* AsCP spores were scraped off 2 SDY plates that had been inoculated 23 d earlier. A series of plates was inoculated at different times so that each replicate was conducted with spores from plates that were 23 d old. The spore suspension was vortexed in autoclaved deionized water for 1 min and then filtered. Spores were counted and the required concentrations were obtained by serial dilutions. Twenty adult psyllids that were 4- to 5-d old (both sexes) were used for each concentration in each replicate. Psyllids were collected in 50-mL centrifuge tubes and chilled for 10 min on ice and then placed in a petri dish. Psyllids were sprayed with 2 mL of each spore concentration. Then the psyllids were individually transferred to a sterile 50-mL centrifuge tube and maintained as described previously. Psyllids were observed for mortality every 24 h until 10 d after treatment. Data were analyzed by probit analysis in PoloPlus (Le Ora Software 2002-2003).

Evaluation of Infection with *Ifr* AsCP During Pilot Field Trials

SDY plates (n = 20) that had been inoculated 24 d previously with *Ifr* AsCP were used to prepare spore suspensions for field application. The spore suspension was prepared as described above. The spore suspension (concentrated) was stored overnight at 4°C in sterile 50-mL centrifuge tubes and transported to the field in an ice chest containing ice packs. The spore suspension was diluted to 1×10^7 spores/mL (2.7 L) with autoclaved deionized water immediately before spraying trees at the Citrus Research and Education Center (CREC) near Lake Alfred, Florida.

At the CREC, 3 'Hamlin' orange trees, ca. 2-3 m in height and with flush less than 4 cm in length were sprayed with 1 L of autoclaved deionized water/tree as controls. Subsequently, 3 trees were sprayed with 900 mL of 1×10^7 spores/mL/tree. Before spraying the trees, a pretreatment sample of 5 shoots was collected randomly from each tree, and each shoot was placed in a separate clear plastic bag (25.4 × 30.4 cm polyethylene bag, Fisher Scientific, Suwanee, GA) secured with twist ties. The sprays were applied with a battery-operated sprayer (Scorpion sprayer, AgSouth, LLC, Union City, TN). An hour after treatment, a post-treatment sample of 5 shoots per tree was collected from both the *Ifr* AsCP- and water-treated trees and placed into clear plastic bags secured with twist ties. The bags were stored in an ice chest with ice packs for the trip to Gainesville, Florida.

Immediately after returning to Gainesville, the numbers of psyllid nymphs, adults and/or eggs were recorded and shoot length measured. Insects also were examined for evidence of infection with *Ifr* AsCP. The shoots were replaced into the bags with 2 moist paper towels to maintain a high RH and stored in a growth chamber at 23 ± 1°C under a 16L:8D photoperiod. After 5 d, the number of nymphs and adults that were dead or alive was recorded. Dead nymphs and/or adults recovered from plastic bags were placed on 9.5 × 1.5 cm petri plates containing crystal violet dodine medium (Chase et al. 1986) to observe if any fungal growth occurred. Dead nymphs were evaluated for visible signs of *Ifr* AsCP.

Foliage samples were randomly collected weekly twice more after the spray and scored as described above. Data were analyzed by ANOVA (SAS 1999) and means were separated with Fisher's least significant difference (LSD) at a 5% level of significance. Weather data were obtained from the weather station at the CREC (http://fawn.ifas.ufl.edu/scripts/reportrequest.asp), and mean daily temperature, RH, and rainfall were obtained.

A second pilot field trial was conducted with spores that were 'mass' produced by Dr. Jarrod Leland (USDA-ARS) and shipped to Gainesville, FL in a dry state in vacuum-sealed plastic bags. *Ifr* AsCP was grown in a liquid culture for 3 d, and 50 mL of inoculum was added to 1 kg of barley along with 400 mL of sterile water. The barley was kept in a low-oxygen bag at 60% RH, with the bag manipulated daily to prevent clumping. Spores were harvested after 10 d and stored at 2-4°C until the trial was conducted. The day before the spray was applied, the dry *Ifr* AsCP spores were suspended in autoclaved deionized water and vortexed for 1 min. The suspension was then filtered through a double layer of autoclaved Miracloth (Calbiochem, EMD Biosciences, Inc. La Jolla, CA) and spores were counted with a hemocytometer. The spore suspension (concentrated) was stored overnight at 4°C in sterile 50-mL conical centrifuge tubes and transported to field in an ice chest containing ice packs. The spore suspension was diluted to 1×10^7 spores/mL with autoclaved deionized water in the field just before spraying.

The 3-y-old block of trees sprayed in this pilot field trial was located at the Florida Citrus Foundation research grove, in Orange County, near Winter Garden, Florida and consisted of a Minneola cross (LB89), planted 3.8 m apart in the row with rows 6.1 m apart. The 20 trees were ca. 1.2-1.5 m tall, and the 2 treatments were randomly chosen by coin toss. Trees initially contained flush less than 4 cm long with eggs and small psyllid nymphs. Ten trees were treated first with 500 mL of autoclaved deionized water and then 10 trees were treated with 500 mL of 1×10^7 spores/mL/tree with a battery-operated sprayer (Scorpion sprayer, AgSouth, LLC, Union City, TN).

Sampling and analysis of field samples was conducted as described above, except that each shoot was placed into a separate zip-lock plastic bag (Great Value Zip Close bags 17.8 × 20.3 cm, Walmart Stores Inc., Bentonville, AR) with the goal of maintaining higher RH and preventing loss of psyllid nymphs from the bag. In addition, three dead nymphs per shoot or per bag that appeared to be infected with fungus were placed on a petri dish (6.0 × 1.5 cm, Fisher's Scientific, Suwannee, GA) containing SDY media and the identity of the fungus was subsequently confirmed by the polymerase chain reaction (PCR) (Meyer 2007; Meyer et al. 2008). Foliage samples were collected for 4 weeks after the sprays and scored as described above. Data were analyzed by ANOVA and means were separated with Fisher's least significant difference (LSD) at 5% level of significance. Weather data were obtained from the weather station on the site and mean daily temperature, RH, and rainfall were obtained.

RESULTS AND DISCUSSION

Serial Transfer of *Ifr* AsCP on MEA and SDY Media

Ifr AsCP produced on SDY medium produced significantly more spores than on MEA (Fig. 1). When spore production was evaluated over time, there was no significant difference in spore production on MEA over the 10 transfers, with spore production ranging from 6.1×10^7 to 7.14×10^7 per 10-cm plate (Fig. 1). Spore production on SDY medium was more variable, and appeared to decline over the 10 transfers. Whether this was due to genetic changes in the *Ifr* AsCP being transferred or due to a decline in the quality of the media used was not determined. Media was prepared fresh for each transfer, but the dry media was stored at room temperature for >10 months.

Viability of spores was maintained when *Ifr* AsCP was grown on SDY media. Mean (SD) percentage germination for transfers 8, 9, and 10 were 97 (2.7), 96 (3.9), and 96 (3.5), respectively. Serial transfer for only 10 generations might not be sufficient to show reduced rates of germination, but the production of spores declined for *Ifr* AsCP grown on the SDY medium.

The colony phenotypes of *Ifr* AsCP on the 2 media differed consistently throughout the 10 transfers. Mycelia and spores on the SDY medium appeared dense and grayish in color. When the plates were turned over, there was intense yellow pigmentation. By contrast, the mycelia and spores on the MEA plates were less dense, lighter in color, and no pigmentation was seen on the underside of the cultures.

Storage of Frozen Cadavers

After 10 weeks of storage at -74°C, *Ifr* AsCP spores on the psyllid cadavers retained a high degree of virulence. All adults that were tested died within 72 h after being exposed to the frozen cadavers. Thus, freezing for up to 10 weeks at -74°C
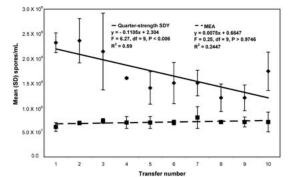
did not affect the infectivity of the cadavers and this method of storage has the advantage of reducing handling or processing of the fungus to separate it from mineral oil, water, or other storage media. This storage method should reduce the chance of attenuation of *Ifr* AsCP, because *Ifr* AsCP can be maintained on psyllid hosts continuously with little labor. When spores were isolated from the cadavers with a sterile needle and streaked on SDY plates, all single colonies had a phenotype typical of *Ifr* AsCP.

Concentration-Mortality and Time-Response Data

At concentrations of 1×10^7 and 1×10^8 spores/mL at 28°C under a 16L:8D photoperiod, the LT_{50} for *D. citri* adults was 111 and 102.5 h, respectively (Fig. 2, Table 1). No mortality was observed in the water controls. The LT_{99} was 167.4 and 174.6 h, respectively, for the 2 spore concentrations. The time-response lines for the 2 concentrations were not different.

A complete concentration-mortality line was calculated for adult psyllid mortality after 192 h (Table 2, Fig. 3). The LC_{50} value was 6.8×10^5 spores/mL and the LC_{99} was 2.2×10^8 spores/mL under these laboratory conditions.

Pilot Field Trials

During the first trial, the RH was approximately 42% on the day the sprays were applied, although RH increased later and averaged 60-70% (data not shown). Temperature averages ranged from approximately 12-15°C; only 2 rains occurred during the trial. The mean number of psyllid nymphs was not significantly different on the shoots prior to treatment with water or *Ifr*
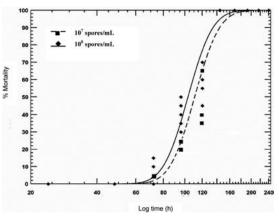

Fig. 1. Spore production of *Ifr* AsCP during 10 transfers on MEA and SDY media.

Fig. 2. Time-response curves for adults of *D. citri* to 2 concentrations of *Ifr* AsCP at 28 ± 1°C, 16L:8D photoperiod and ~100% RH. The lines were generated by Polo-Plus; the individual points indicate the actual mortality caused by the 5 replicates for each concentration.

TABLE 1. COMPARISON OF TIME TO DEATH (LT) OF ADULT *D. CITRI* FROM 2 CONCENTRATIONS OF *IFR* AsCP TESTED AT 28 ± 1°C, UNDER 16L:8D PHOTOPERIOD AND ~ 100% RH.

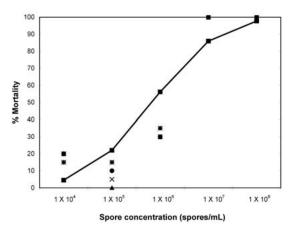|	Lethal Time (h) (95% fiducial limits) for	
	1×10^7 spores/mL	1×10^8 spores/mL
LT_{10}	88.5 (83.9 to 92.3)	76.5 (71.8 to 80.5)
LT_{50}	111.0 (107.8 to 114.2)	102.5 (99.0 to 106.0)
LT_{90}	139.2 (134.1 to 145.7)	137.5 (131.8 to 144.7)
LT_{95}	148.4 (142.2 to 156.8)	149.4 (142.2 to 158.9)
LT_{99}	167.4 (158.3 to 180.2)	174.6 (163.7 to 189.6)
Slope	13.0 ± 0.98	10.1 ± 0.7

Fig. 3. Concentration-mortality curve for adults treated with *Ifr* AsCP after 192 h at 28 ± 1°C, under a 16L:8D photoperiod and ~100% RH. The line was generated by Polo-Plus and the 5 means for each concentation are represented by the individual symbols, some of which are on top of each other.

AsCP ($F = 0.60$, $df = 1$, $P = 0.45$) or immediately after the sprays were applied (data not shown). The number of nymphs/shoot on the trees treated with water averaged 14.5 and 12.9 immediately before and after treatment, respectively, which was not significantly different ($F = 0.10$, $df = 1$, $P = 0.75$).

After holding the immediate post-spray sample shoots for 5 d, no fungal infection was observed on any of the dead nymphs or adults, but this could have been due to the low RH in the field at the time of the spray application resulting in low infection rates, or to the low RH in the bags, which were secured with twist ties. All dead nymphs appeared to have starved and dried out, perhaps because the leaves had fallen off the tender young shoots so that adequate food was not available. In most samples, a large proportion (up to 100%) of nymphs and/or adults were missing from the bags, which had been closed with wire twist ties. We concluded that these small insects escaped through small openings where the bags were secured.

One week after the sprays were applied, there were significantly fewer psyllid nymphs ($F = 5.13$, $df = 1$, $P = 0.03$) on the foliated treated with *Ifr* AsCP (data not shown), but 2 weeks after sprays were applied, there were no differences in the

mean number of psyllid nymphs/shoot in the 2 treatments ($F = 0.39$, $df = 1$, $P = 0.54$). The number of nymphs was reduced in both, probably because the shoots had matured and predation had eliminated some of the nymphs.

Prior to spraying the trees, shoot lengths in the *Ifr* AsCP- and water-treated trees were significantly different ($F = 12.04$, $df = 1$, $P = 0.002$), with an average length of 3.5 cm in the trees to be treated with water and 2.2 cm in the trees to be treated with *Ifr* AsCP. However, immediately after the sprays were applied, samples were taken again and there were no significant differences ($F = 0.15$, $df = 1$, $P = 0.71$) in shoot length (2.2 and 2.1 cm, respectively), for the water- and *Ifr* AsCP-treated trees. One week after the sprays were applied, mean shoot length in the trees treated with *Ifr* AsCP averaged 7.9 cm, while the shoots in the water-treated trees averaged 4.6 cm, a significant difference ($F = 17.54$, $df = 1$, $P = 0.0004$). Two weeks after treatment, shoots from the *Ifr* AsCP-treated trees again were significantly longer than on the water-treated trees ($F = 7.78$, $df = 1$, $P = 0.01$). Thus, application of *Ifr* AsCP appeared to result in increased shoot lengths for 2 weeks after treatment, which could have been due to the reduced number of psyllids on the shoots. Unfortunately, because we could not confirm infection of nymphs with *Ifr* AsCP in the plastic bags, the reductions in the psyllid densities during the course of the experiment could be attributed to one or more factors, including the effects of *Ifr* AsCP, the production of adults that left the shoots, and predation on nymphs in both control and treatment trees by lady beetles, which were abundant in the block.

TABLE 2. CONCENTRATION-MORTALITY DATA AFTER 192 H FOR *IFR* AsCP APPLIED TO ADULTS OF *D. CITRI* AT 28 ± 1°C, UNDER 16L:8D PHOTOPERIOD AND ~100% RH.

	Lethal concentration (spores/mL) (95% fiducial limits)
LC_{10}	2.8×10^4 (5.8×10^3 to 7.5×10^4)
LC_{50}	6.8×10^5 (3.1×10^5 to 1.6×10^6)
LC_{95}	4.1×10^7 (1.2×10^7 to 3.3×10^8)
LC_{99}	2.2×10^8 (0.5×10^8 to 3.6×10^9)
Slope	0.92 ± 0.07

The data suggest the fungal spray reduced the number of psyllid nymphs, but the problems in containing the nymphs in the sample bags and confirming that the psyllids were infected with the fungus indicated that our evaluation methods were inadequate. It should be noted that *Ifr* AsCP-infected nymphs and adults were not observed in the block while sampling, perhaps because psyllids killed by this fungus do not adhere strongly to leaf surfaces (Meyer 2007; Meyer et al. 2008).

A second spray application was conducted on Aug 9, when the RH averaged ca. 67%. Over the course of this second trial, RH varied but averaged 70-80%. Five rains occurred during the trial and temperature averages ranged from 27-30°C. During this experiment, the shoot lengths of the trees in the 2 treatments did not differ significantly, except during week 3, when the shoots on the *Ifr* AsCP-treated trees were significantly longer ($F = 5.29$, $df = 1$, $P = 0.02$).

The mean number of psyllid nymphs on the trees prior to treatment and immediately after treatment were significantly different ($F = 4.81$, $df = 1$, $P = 0.04$; $F = 4.54$, $df = 1$, $P = 0.04$, respectively), with more psyllid nymphs on the trees treated with water. Reasons for the pre-treatment differences are unknown. One week after treatment, there were no differences in nymphal psyllid densities between the 2 treatments ($F = 0.01$, $df = 1$, $P = 0.90$), but during the following week, there were fewer ($F = 5.40$, $df = 1$, $P = 0.02$) psyllid nymphs/shoot in the *Ifr* AsCP-treated trees (mean = 0.08) compared to the water-treated trees (mean = 0.45). By the third week after sprays were applied, there were no significant differences in nymphal psyllid densities, and densities were very low (mean = 0.03 and 0 for the water- and *Ifr* AsCP-treated trees, respectively). Thus, it was difficult to determine if nymphal psyllid densities declined due to treatment with *Ifr* AsCP based on the counts.

However, a total of 437 nymphs from the trees treated with *Ifr* AsCP that were held in the zip-lock plastic bags in the laboratory to determine if they were infected with *Ifr* AsCP showed substantial levels of infection under laboratory conditions. At least 327 (75%) were visibly infected with a fungus that was morphologically similar to *Ifr* AsCP. To confirm that the fungus was *Ifr* AsCP, Meyer (2007) tested 9 randomly selected nymphs with *Ifr* AsCP-specific PCR primers and all were positive, indicating that *Ifr* AsCP had persisted in the plot for 3 weeks and had killed the majority of these psyllid nymphs. A total of 731 psyllid nymphs from the trees treated with water were held under the same conditions and 692 were recovered, with only 39 missing from the bags. Of the 692 nymphs recovered, 28 were dead but these were not infected with a fungus. Thus, the zip-lock plastic bags were well suited to containing the psyllid nymphs and the RH was sufficient that visible symptoms could be observed; fewer than 2% of the nymphs on shoots placed on the bags were missing when scored for infection. This suggests that the fungus did kill psyllid nymphs in the block, but that mortality from other natural enemies (especially predators such as coccinellids, which were abundant in the block) reduced psyllid densities in the water-treated trees.

CONCLUSIONS

The potential advantages of developing *Ifr* AsCP as a microbial pesticide could include the reduced use of synthetic organic pesticides in citrus, to which *D. citri* could become resistant. Use of this microbial pesticide might reduce disruption of numerous natural enemies in Florida's citrus through the use of synthetic pesticides, thus reducing the costs of additional control measures, although it remains to be determined whether this microbial product could disrupt the natural enemies, as well.

Potential negative aspects of *Ifr* AsCP as a microbial pesticide are likely to be its susceptibility to copper, which currently is being applied multiple times a year (up to 8) to control citrus canker (*Xanthomonas axonopodis* pv. *citri* (Hasse) Vaut.) (Timmer et al. 2006). However, if other selective canker control products are found, this hurdle might be overcome. Although other strains of *Ifr* (= *Pfr*) have been used successfully against other pests, the use of fungal microbial pesticides in IPM programs remains rare (Lacey et al. 2001; Montesinos 2003).

Alternatively, this pathogen may be an important naturally occurring mortality factor of *D. citri* in Florida's citrus groves, especially during periods of high RH, although the pathogen appears difficult to detect. Only a very few specimens of mycosed adult psyllids were discovered by Meyer (2007), and no infected nymphs were seen in his surveys. In these 2 pilot tests, no cadavers of nymphs or adults were seen in the field or on freshly collected foliage, perhaps because this pathogen does not cause infected psyllids to 'stick' to the leaf surface. Furthermore, we only saw mycosed nymphs when foliage was returned to the laboratory and held in tightly closed plastic bags for 3 d under high RH; evaluations on the day of collection using a dissecting microscope failed to indicate any psyllid nymphs were infected. Thus, unless someone is sampling and holding nymphs and adults under conditions designed specifically to detect this pathogen, they might not discover *Ifr* AsCP as a mortality factor in Florida's psyllid populations. Additional field studies should be conducted to resolve the role of this pathogen in regulating *D. citri* populations in Florida's citrus groves throughout the year.

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