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DIAPHORINA CITRI (HEMIPTERA: PSYLLIDAE) INFECTION AND DISSEMINATION OF THE ENTOMOPATHOGENIC FUNGUS *ISARIA FUMOSOROSEA* (HYPOCREALES: CORDYCIPITACEAE) UNDER LABORATORY CONDITIONS

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ABSTRACT

The infectivity and horizontal transfer of *Isaria fumosorosea* Wize among *Diaphorina citri* Kuwayama was measured using a detached leaf bioassay in which blastospores were sprayed on citrus leaf sections or yellow plastic tags (artificial attractant surface). Four leaf sections or three leaf sections and one yellow tag were placed together in a Petri dish chamber. One to four of the leaf sections or the yellow tag was sprayed with *I. fumosorosea* ($1.2 - 1.7 \times 10^3$ blastospores/mm^2). After treatments dried, a single adult psyllid was released into each chamber. Mortality due to *I. fumosorosea* for the adult psyllid was observed 4.9 ± 0.21- 6.1 ± 0.37 d following exposure to the pathogen. The rate of colonization by *I. fumosorosea* on adults in chambers with untreated leaf sections and one treated yellow tag was as effective in inducing mortality as in chambers with one treated leaf section at 8 days post application. Under high humidity, *I. fumosorosea* blastospores readily produced hyphae on the surface of leaves, which was useful for determining if adults were responsible for transmission of the fungus. In chambers with a single treated leaf section, adults came into contact with blastospores and moved these around to the non-treated leaves. The same phenomenon, of psyllid infection and subsequent spreading of the fungus to non-treated leaves, was observed when psyllids were placed into chambers with a treated yellow tag. The use of *I. fumosorosea* inoculated yellow tags has potential as a psyllid dissemination technique for managing pest populations.

Key Words: autodissemination; blastospores; *Diaphorina citri*; fungal development index; *Isaria fumosorosea;* Huanglongbing

RESUMEN

La infectividad y transferencia horizontal de *Isaria fumosorosea* Wize entre *Diaphorina citri* Kuwayama fue medida usando un bioensayo de una hoja despegada en el cual blastosporas fueron rociadas sobre secciones de hojas de cítricos o sobre etiquetas plásticas de color amarillo (superficie de atrayente artificial). Cuatro secciones de hojas o tres secciones de hojas y una etiqueta amarilla fueron puestas juntas en una cámara de plato Petri. Una de las cuatro secciones de hojas o la etiqueta amarilla fueron rociados con *I. fumosorosea* ($1.2 - 1.7 \times 10^3$ blastosporas/mm^2). Después de que se seco los tratamientos, un solo adulto de psílido fue liberado dentro de cada cámara. La mortalidad debido a *I. fumosorosea* para el adulto del psilido fue 4.9 ± 0.21- 6.1 ± 0.37 días después de exponerlo al patógeno. La tasa de colonización de *I. fumosorosea* sobre los adultos en las cameras con secciones de hojas no tratadas y con una etiqueta amarilla tratada fue tan efectiva en inducir mortalidad como en las cámaras con una sección de hoja tratada a los 8 días después de la aplicación. Bajo condiciones de alta humedad, las blastosporas de *I. fumosorosea* fácilmente producieron hifae sobre la superficie de las hojas, que fue útil para determinar si los adultos psílidos fueron responsables para la transmisión del hongo. En las cámaras con una sola sección de una hoja tratada, los adultos se pusieron en contacto con las blastosporas y los removieron a las hojas no tratadas. El mismo fenómeno, de la infección del psílido y el espacir el hongo a hojas no tratadas fue observado cuando los psílidos fueron puestos en cámaras con una etiqueta amarilla tratada. El uso de etiquetas amarillas inoculadas con *I. fumosorosea* tiene un potencial como una técnica para diseminar los psílidos para el manejo de poblaciones plagas.

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), was first discovered in Florida in 1998 and has since dispersed rapidly throughout the state (Halbert & Manjunath 2004). The insect has a narrow host range consisting of plants in the family Rutaceae, including citrus and citrus relatives such as orange jasmine, *Murraya paniculata* (L.) Jack (Tsai et al. 2000). *Diaphornia citri* is a vector of the phloem-limited bacterium *Candidatus* Liberibacter asiaticus, which is always associated with citrus- huanglongbing (HLB), commonly referred to as 'citrus greening disease' (Hung et al. 2004; Manjunath et al. 2007). HLB is one of the most serious plant diseases in citrus on a worldwide scale (Bové 2006) and has been reported in Florida (Tsai et al. 2000; FDACS 2009).

Direct feeding by *D. citri* nymphs is primarily on new citrus growth or "flush" (Hall & Albrigo 2007) which can result in distorted, reduced growth of new leaf tissue. Probing by the adult psyllid while searching for the best feeding area on a leaf can transmit HLB. Infected citrus trees may only live 5-8 years and produce irregular shaped, bitter, unmarketable fruit (Halbert & Manjunath 2004; Bové 2006). Considering the seriousness of the disease and its -vector, controlling psyllid populations by the use of chemical insecticides, removing confirmed diseased trees and planting disease-free nursery stock are recommended as management strategies for this pathosystem (Childers & Rogers 2005; Brlansky et al. 2006; Rogers et al. 2006). The present paradigm of an intensive insecticidal control program is economically unsustainable for the grower and will likely interfere with biological control programs in Florida citrus (Michaud & Grant 2003; Michaud & Olsen 2004; Hoy 2005; Stansly & Qureshi 2008). Thus, an integrated pest management (IPM) strategy is needed to minimize the use of chemical insecticides and to develop sustainable alternatives for managing psyllid populations.

The entomopathogenic fungus, *Isaria fumosorosea* (*Ifr)* Wize (= *Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae), was recently isolated from mycosed *D. citri* collected from the underside of foliage on orange trees in Polk County, Florida (28°06'295" N, 81°42'895" W) (Meyer et al. 2008). Presently, 2 *Ifr* strains are available for research as blastospore formulations in the U.S.A., *PFR* 97 20% WDG® (Certis, Columbia, MD, USA) and *Ifr* 3581 from the USDA/ARS, NCAUR, Peoria, IL, USA (Jackson et al. 1997). *Ifr* has several characteristics that favor its further evaluation for controlling *D. citri*; it is native to Florida, can infect a wide range of citrus pests, and is compatible with non-target arthropods (Sterk et al. 1995a, b; Avery 2002; Avery et al. 2008).

Growing concerns about the negative effects of chemical insecticides on workers, food supply, and the environment make microbial control of arthropod pests of tree fruit crops an attractive alternative (Puterka 1999; Subandiyah et al. 2000; Slininger et al. 2003; Dolinski & Lacey 2007; Lacey & Shapiro-Ilan 2008). The most common fungal pathogen application technique, spraying trees with conidial suspensions, can become cost prohibitive for multiple treatments of groves. Therefore, development of a low-cost autodissemination technique for entomopathogenic fungi where the insect can spread the fungus via horizontal transmission to conspecifics (e.g., during mating) is warranted. Similar autodissemination techniques for controlling pests have been evaluated in other systems (Maniania 2002; Dowd & Vega 2003; Tsutsumi et al. 2003; Scholte et al. 2004; Maniania et al. 2006).

Adult psyllids are attracted to yellow sticky cards in the field (Hall & Albrigo 2007; Hall et al. 2007, 2008; Hall 2009); therefore, it was hypothesized that yellow tags (non-sticky artificial attractant) sprayed with *Ifr* blastospores could potentially be used to horizontally spread the fungus by acquisition and dissemination to other leaves and psyllids in the field. The objectives were (1) to compare the efficacy of yellow tags and leaves sprayed with *Ifr* blastospores for infecting and colonizing the psyllid and (2) to assess the horizontal transfer of blastospores by the movement of the adult psyllids under laboratory conditions. A Fungal Development Index (FDI), similar to that of Avery et al. (2004), was designed to assess the effect and development of *Ifr* dosages on the post-lethal period of infected adult psyllids.

Materials and Methods

Source of Insects

The USDA-ARS laboratory colony of *D. citri* was established during early 2000 at the U.S. Horticultural Research Laboratory, Fort Pierce, FL. Originally collected from citrus, the psyllids have been continuously reared on orange jasmine, *Murraya paniculata* (L.) Jack housed in Plexiglas ($0.6 \times 0.6 \times 0.6$ m) or BugDorm-2 cages (MegaView Science Education Services Co., Ltd., Taichung, Taiwan). Original colony has not had field collected psyllids added since establishment.

Citrus Leaves

Duncan grapefruit (*Citrus paradisi* Macf.) seedlings were grown in Premier Pro-mix General Purpose Growing Medium from seed in size C10 "Cone-tainers"™ (Stuewe & Sons, Inc., Corvalis, OR) for approximately 6 months. Detached leaves of similar age and size were washed with water and placed in a fume hood to air dry.

Fungal Blastospore Preparation, Deposition, and Viability

A fungal, dessication-tolerant, blastospore-diatomaceous earth formulation of *Ifr* ARSEF strain 3581, supplied as a powder in vacuum packed 10-g bags was produced and stabilized as previously described (Jackson et al. 2003) and stored at 4°C. The blastospore suspension was prepared by mixing 2 g of the powder in 100 mL of sterile distilled water, stirring the suspension with a magnetic bar for 30 min and then allowing the diatomaceous earth to settle from the suspension for an additional 30 min. The suspension (50 mL) was then pipetted to a Nalgene® aerosol sprayer (Nalge Nunc International, Rochester, NY). Two aliquots were taken prior to spraying from the suspension and the concentration of *Ifr* blastospores/mL was determined with a hemacytometer.

To determine the deposition of *Ifr* blastospores/mm^2, 12 plastic microscope cover slips (Fisherbrand® 22 × 22 mm, Fisher Scientific, Pittsburgh, PA) were placed randomly on paper among the leaf sections and yellow plastic tags and sprayed simultaneously and in an identical fashion. The cover slips were allowed to dry for 30 min in a fume hood, then placed upside down on a glass microscope slide in a 50-µL drop of acid fuschin stain. Blastospore density was assessed with a compound light microscope (400X) and a 10 mm reticle grid (Hunt Optic and Imaging, Pittsburg, PA).

The viability of blastospores were assessed with 2 potato dextrose agar plates sprayed at a rate of 6.0×10^7 blastospores/mL. After the plates had been incubated for 12 h at 25 ± 1.0°C, 100% RH, the percent viability was determined by viewing a total of 200 blastospores. Blastospores were considered to have germinated if a germ tube had formed. This procedure was repeated for each repetition of the experiment, and the mean percent viability was 85 ± 8.3%.

Bioassay Petri Dish Chambers

Petri dishes (100 mm × 15 mm) were lined with filter paper and moistened with 800 µL sterile distilled water. To prepare the leaf sections, each leaf was cut 2.5 cm from the tip across the midrib. The adaxial side of leaves of similar size and top side of yellow plastic tags (Xpress Tags, Brooklyn, NY), were cut to mimic the shape and surface area (range: 101-125 mm^2) of the leaf section. These sections were sprayed until runoff with a Nalgene® aerosol sprayer held at approximately a 45° angle. The spray was either sterile distilled water (DW) or an *Ifr* blastospore suspension (6.0×10^7 blastospores/mL) in sterile water. Sprayed leaves and yellow tags were air dried for 30 min.

Bioassay leaf section treatments inside the Petri dish consisted of 4 sections total, with 1, 2, 3 or 4 leaf section(s) sprayed with *Ifr*. The yellow tag treatments consisted of 3 leaf sections (sprayed with water) and 1 yellow tag (sprayed with either *Ifr* or water) placed on moistened filter paper. Leaf section treatment combinations were arranged in the following ratios of fungus (blastospores) to distilled water (*Ifr* to DW): 0:4 (control), 1:3, 2:2, 3:1, 4:0, and yellow tag treatment combinations 1:3 and 0:4 (control). Treatment combinations were oriented in a cross pattern with the leaf section or yellow tag tip pointed toward the center of the dish prior to introducing an adult psyllid inside the Petri dish.

A single (<1 week old) adult psyllid (sex not identified) was allowed to walk on the inside of the Petri dish lid. The lid was then turned over, placed over the bottom of the dish and the adult psyllid was allowed free movement. Each dish chamber was sealed with Parafilm® and transferred to a Precision 818® low temperature fluorescent illuminated incubator (Precision, Winchester, VA, USA). All treatments were maintained at 25 ± 1.0°C under a photoperiod of 16:8 (L:D) at approximately 100% RH for 14 d and observed on a daily basis. There were 8 replicate dish chambers for each treatment and the experiment was repeated 4 times.

Determining *Ifr* Acquisition and Horizontal Transfer by the Psyllid

Leaf sections and yellow tags were treated and arranged inside the dish chambers as described above for all treatments. Two groups of treatments (8 replicates/treatment) were compared, one with the psyllid present, the other without the psyllid present. The group without the presence of an adult psyllid served as a control for assessing spread of the blastospores within dish chambers in the absence of a psyllid. Leaf section treatment combinations were arranged in the following ratios of fungus (blastospores) to distilled water (*Ifr* to DW): 0:4 and 1:3. The yellow tag treatments were conducted as previously described.

After a pilot study, fungal hyphae from spores transferred by the psyllid were first observed to grow on the leaf surface under high relative humidity conditions (Avery, unpublished data; Fig. 1). Therefore, this new finding was used to evaluate the transfer of fungal spores among leaves in the dish chamber. Untreated leaf sections inside the dish chambers were monitored for the presence of *Ifr* hyphae growing on the whole leaf with a dissecting binocular microscope (40X). Data obtained from replicated experiments after 14 d were used as criteria for determining acquisition and horizontal transfer by the adult psyllid. In cases where the insect died and mycosed on an untreated leaf section, the leaf was recorded as contaminated by *Ifr* and horizontally transferred by the adult psyllid.

Fungal Development Index (FDI) Assessment

The degree of fungal development of *Ifr* on psyllid adults was assessed by a Fungal Development Index (FDI; see Table 3 for summary) modified from Avery et al. (2004). The FDI was used as a measure for estimating establishment speed or infection rate of hosts in each treatment. All assays were rated daily until sporulation of *Ifr* was observed (FDI value 3.0) on the insect host. Each adult was assessed under a dissecting binocular microscope (40X), and the FDI value for the stage of fungal development observed was recorded. The FDI was used to assess the fungal growth of blastospores after infection of the adults until colonization at 25 ± 1.0°C and 100% RH.

The FDI values of 0.0-0.5, which represented the beginning of the growth phase and initial germination of the blastospore, were not assessed. An FDI value of 1.0 was assumed once the insect died; however, this value was not recorded until confirmation of *Ifr* fungal hyphae was first noticed extending from any part of the body or wings. Once the fungus protruded through the exoskeleton of the host insect (FDI values 1.5-2.0), the insect would not recover from the infection. Conidiogenesis was represented by FDI values 2.5-3.0. Each adult was scored for 8 d according to the FDI as a replicate and results were expressed as a daily mean value for all adult psyllids in each treatment.

Statistical Analysis

The mean number of days of adult psyllids survival post *Ifr* leaf section treatment compared with a yellow tag treatment were assessed by ANOVA ($\alpha = 0.05$) with mean separation by a Tukey's HSD test. In order to determine the percent transfer of *Ifr* blastospores to untreated leaf sections by adult psyllid movement compared to no psyllid present, data were arcsine-transformed and analyzed by ANOVA ($\alpha = 0.05$) with mean separation by a Tukey's HSD test. A Ryan-Einot-Gabriel-Welsh Multiple Range Test was used to analyze the effect of increasing the number of treated leaf sections on the development of *Ifr* on the adult psyllid (after initial mycosis until colonization; FDI value 3.0) and between the single treated leaf section compared to the yellow tag treatment using the FDI values. A regression analysis was used to determine if the infection rate of 1 treated yellow tag was as high or higher compared to a treated single leaf section against the psyllid over time. If results and trends per treatment were not significantly different between repetitions of the experiment based on an ANOVA ($\alpha = 0.05$), then the data were pooled and analyzed. All statistical tests were conducted by PROC GLM procedures of SAS (SAS Institute, Cary, NC, USA).

RESULTS

Efficacy of Treatments

All *Ifr* treatments were effective in inducing mortality in adult psyllids under the laboratory conditions tested. No significant differences in treatment results were observed ($F = 0.01$; $df = 3, 15$; $P = 0.100$) between repetitions of the experiment; therefore, the data over all repetitions were pooled and analyzed. The mean number of *Ifr* viable blastospores/mm^2 deposited on the leaf sections was 1,344 ± 149.7.

The number of days for the fungus to infect and induce mortality in an adult psyllid ranged from 4.9 to 6.1, and no mortality was observed in the control treatment (Table 1). Mortality rates of adults in chambers with an *Ifr*- treated yellow tag were not significantly different ($P > 0.05$) than mortality rates of adults in chambers with *Ifr*-treated leaves. The number of days adult psyllids survived in chambers with 3 *Ifr*- treated leaf sections was significantly shorter ($F = 5.60$; $df = 4, 155$; $P < 0.001$) compared to those treatments with fewer leaf sections treated. The days the psyllid survived in treatments with 3 or 4 leaf sections sprayed were similar ($P > 0.05$), 4.9 ± 0.21 and 5.2 ± 0.30, respectively.

Ifr Acquisition and Horizontal Transfer by the Psyllid

The acquisition and percent horizontal transfer of blastospores to untreated leaf surfaces (edge or center) is presented in Table 2. Psyllid movement within chambers did not affect the percent horizontal transfer of the blastospores to the edge of the untreated leaf sections in either the leaf section or yellow tag treatments ($F = 1.12$; $df = 2, 95$; $P = 0.348$). However, in both the leaf section and yellow tag treatments, the presence and

TABLE 1. MEAN TIME TO DEATH IN DAYS (± SEM) FOR ADULT PSYLLIDS AFTER RELEASE INTO PETRI DISH CHAMBERS[a] CONTAINING CITRUS LEAF SECTION (S) OR YELLOW TAGS SPRAYED WITH *ISARIA FUMOSOROSEA (IFR)*.

Treatment[a]	Time to death[b] (days)
1 Yellow tag sprayed	5.7 ± 0.23 ab
1 Leaf section sprayed	6.1 ± 0.37 b
2 Leaf sections sprayed	5.9 ± 0.30 b
3 Leaf sections sprayed	4.9 ± 0.21 a
4 Leaf sections sprayed	5.2 ± 0.30 ab

[a]Total number of leaf sections per Petri dish chamber was 4. The leaf sections and yellow tags were sprayed with blastospores of *Ifr* and allowed to dry before introducing a psyllid. The yellow tag replaced 1 leaf section. No mortality was observed for the untreated controls ($n = 32$/treatment).

[b]Mean survival values followed by different letters in a column are significantly different (Tukey's HSD test, $P < 0.001$).

TABLE 2. PERCENT HORIZONTAL TRANSFER (± SEM) OF BLASTOSPORES OF *ISARIA FUMOSOROSEA (IFR)* FROM TREATED TO THE UNTREATED LEAF SECTION EDGE OR CENTER BY ADULT PSYLLID MOVEMENT IN PETRI DISH CHAMBERS[a] HELD AT 25°C UNDER A 16-H PHOTOPHASE AFTER 14 D.

Treatment	Psyllid	% Horizontal transfer ± SEM[b]	
		Leaf edge	Leaf center
1 Leaf section sprayed	Absent	81.9 ± 6.4 a[c]	23.5 ± 5.8 a
1 Leaf section sprayed	Present	90.3 ± 4.3 a	84.8 ± 4.9 b
1 Yellow tag sprayed	Absent	73.6 ± 7.0 a	13.8 ± 4.0 a
1 Yellow tag sprayed	Present	90.4 ± 3.1 a	90.3 ± 3.7 b

[a]Total number of leaf sections per Petri dish chamber was 4. The leaf sections and yellow tags were sprayed with blastospores of *Ifr* and allowed to dry before introducing a psyllid. The yellow tag replaced 1 leaf section. No mortality for the control was observed (n = 24/treatment).

[b]Mean percent horizontal transfer values were arcsine transformed before being analyzed. Untransformed values followed by different letters in a column are significantly different (Tukey's HSD test, $P < 0.001$).

[c]Mechanical transfer of blastospores from a treated leaf section or yellow tag to an untreated leaf edge occurred in all treatments.

movement of psyllids enhanced and had a significant positive effect on the acquisition and spread of the fungus to the central part of untreated leaf sections (F = 6.67; df = 2, 95; $P < 0.001$).

FDI Assessment of *Ifr*

Adult psyllids began succumbing to the fungus 2 d post release in all *Ifr* treatments. A 100-percent mortality of the adult psyllids occurred (FDI value 1.0) and all psyllids in treatments with 3-4 leaves sprayed had mycosed (FDI value 1.5) 5 d post release (Table 3). The fungi on the leaf section effectively infected and colonized the adult psyllid, as compared with the controls for the duration of the experiment under these growing conditions. The yellow tag treatment had a similar effect on the *Ifr* development as compared to the single leaf section treatment. *Ifr* developed on the adult psyllids exposed to the tag treatment at a similar rate compared with the sprayed leaf section treatments, except 5 d post application where 3-4 leaf section treatments showed a higher rate (F = 1.92; df = 3, 127; P = 0.0009) compared with the 1-2 leaf section treatments. The total percentage psyllid adults colonized (FDI value of 3.0: covered with mycelium and conidia) for all experiments after 8 d post release was 63 ± 8.7, 55 ± 9.1, 77 ± 7.6, 75 ± 7.8% for 1, 2, 3, 4 leaf section(s) treated and 73 ± 8.2% for the yellow tag treatments. The final percent mortality was 91 ± 5.2, 97 ± 3.2, 97 ± 3.2, 97 ± 3.1% and 100 ± 0.0%, respectively. Regression analyses between FDI value (Y) and days of exposure to *Ifr* treatment (X) were similar between the yellow tag treatment (Y = -0.9 + 0.44X; F = 347.0, $Pr > F = < 0.0001$, r^2 = 0.59, slope SEM = 0.023, 239 df) and single leaf section treatment (Y = -0.8 + 0.39X; F = 276.2, $Pr > F = < 0.0001$, r^2 = 0.52, slope SEM = 0.023, 239 df). These analyses indicated that *Ifr* blastospores sprayed on either a leaf or card, infected and developed on the adult psyllid at a similar rate over time. No natural mortality of the adult psyllids (controls) occurred until 8 d post release for either the 4 leaf sections (3.2 ± 3.2%) or 1 yellow tag plus 3 leaf sections (25.8 ± 8.0%) treated with water.

DISCUSSION

Assessment of *Ifr* Treatments

All *Ifr*-sprayed leaf section treatments resulted in a mortality of >95% of the adult psyllids under laboratory conditions after 8 d with 100% mortality on the yellow tag treatments during the same period. In addition, fungal development of *Ifr* on psyllids in the yellow tag treatment was similar to the single leaf section treatment, and comparable to the other leaf section treatments. This indicates that psyllids were attracted to the artificial yellow tag and then able to acquire and disseminate the blastospores to the surface of other untreated leaves. Some of the untreated leaf section edges may have become contaminated with the blastospores by mechanical transfer while in the Petri dishes.

Under these optimum growing conditions in the dish chambers, fungal hyphae were observed to grow on both the leaf (edge and center) and plastic tag surface. Lopez-Llorca et al. (1999) observed that *I. farinosa* first grew on the edges of the leaves and then colonized the palm leaf surface. This is the first report of *Ifr* producing hyphal extensions on either a leaf or an artificial surface (yellow tag) directly from *Ifr* blastospores (Fig. 1).

Moribund psyllids that were attached by mycelium to the filter paper, Petri dish or leaf section had succumbed to the fungal infection after they had walked around and contaminated the untreated leaf surfaces. Under high humidity (RH > 80%) some insects would mycose and form a sporulating cadaver cemented in a feeding position to any surface by hyphae growing from their

TABLE 3. FUNGAL DEVELOPMENT INDEX (FDI) VALUES OF MYCOSIS OBSERVED DAILY ON ADULT PSYLLIDS INFECTED WITH *ISARIA FUMOSOROSEA* (*IFR*) AFTER EXPOSURE TO SPRAYED CITRUS LEAF SECTION(S) OR A YELLOW TAG IN PETRI DISH CHAMBERS[a] HELD AT 25°C UNDER A 16-H PHOTOPHASE.

	FDI values[c] days post release						
Treatment[b]	2	3	4	5	6	7	8
1 Yellow tag sprayed	0.1 ± 0.01 a	0.1 ± 0.07 a	0.3 ± 0.14 ab	1.0 ± 0.23 b	1.7 ± 0.23 ab	2.4 ± 0.15 a	2.8 ± 0.08 a
1 Leaf section sprayed	0.1 ± 0.07 a	0.1 ± 0.07 a	0.3 ± 0.11 ab	1.0 ± 0.21 b	1.6 ± 0.20 b	2.2 ± 0.19 a	2.4 ± 0.17 a
2 Leaf sections sprayed	0.1 ± 0.01 a	0.1 ± 0.08 a	0.4 ± 0.14 ab	1.1 ± 0.21 b	1.7 ± 0.21 ab	2.0 ± 0.21 a	2.5 ± 0.13 a
3 Leaf sections sprayed	0.1 ± 0.03 a	0.1 ± 0.04 a	0.6 ± 0.13 a	1.7 ± 0.19 a	2.2 ± 0.16 a	2.5 ± 0.17 a	2.7 ± 0.12 a
4 Leaf sections sprayed	0.0 ± 0.00 a	0.1 ± 0.08 a	0.6 ± 0.15 a	1.7 ± 0.19 a	2.2 ± 0.18 a	2.5 ± 0.16 a	2.7 ± 0.11 a
4 Leaf sections untreated	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 b	0.0 ± 0.00 c	0.0 ± 0.00 c	0.0 ± 0.00 b	0.1 ± 0.05 b[d]
1 Yellow tag untreated	0.0 ± 0.00 a	0.0 ± 0.00 a	0.0 ± 0.00 b	0.0 ± 0.00 c	0.0 ± 0.00 c	0.0 ± 0.00 b	0.1 ± 0.03 b[d]

[a]Total number of leaf sections per Petri dish chamber was 4. The leaf sections and yellow tags were sprayed with blastospores of *Ifr* and allowed to dry before introducing a psyllid. The yellow tag replaced 1 leaf section (n = 32/treatment).

[b]FDI values are as follows: 1.0 = insect is dead (whether infected with *Ifr* fungus or due to natural mortality); 1.5 = appearance of *Ifr* fungal hyphae protruded through the exoskeleton of the psyllid body; 2.0 = *Ifr* fungal hyphae protruded through head, thorax, wings of the psyllid body; 2.5 = *Ifr* fungal hyphae protruded through the exoskeleton as in 2.0, plus conidia are first formed anywhere on the psyllid body; 3.0 = *Ifr* fungus has colonized and formed conidia on all sections of the psyllid body.

[c]FDI values in a column followed by the same letter are not significantly different (REGW multiple range test, $P < 0.001$).

[d]Natural mortality (FDI = 1.0) 8 d post release for leaf section and yellow tag treatments were 3.2 ± 3.2% and 25.8 ± 8.0%. No mortality was observed due to *Ifr* infection.

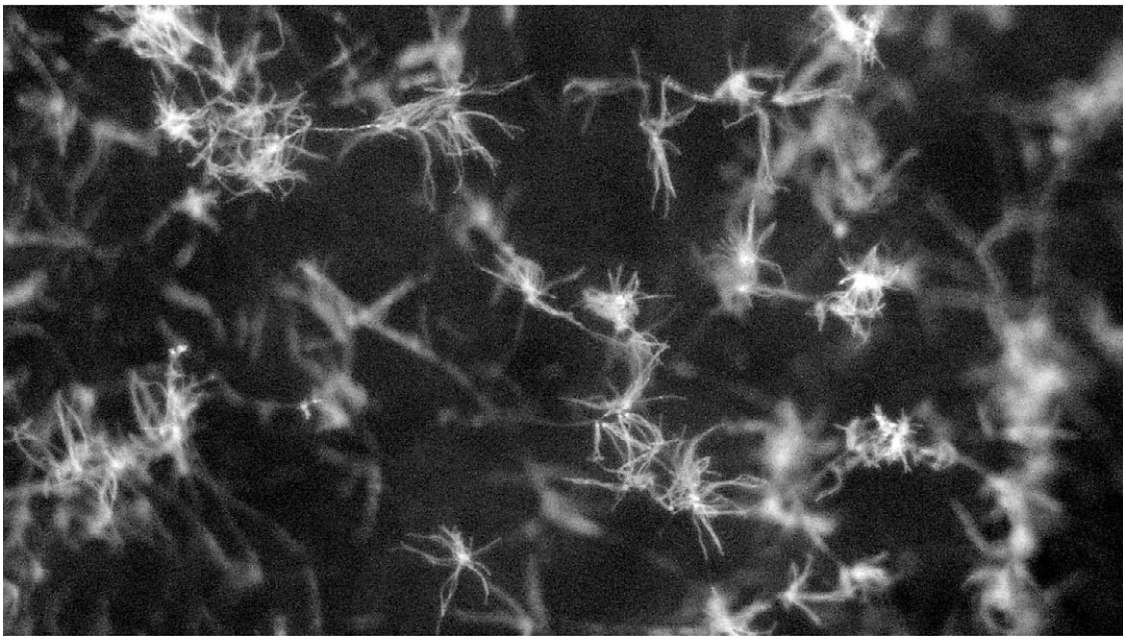

Fig. 1. *Isaria fumosorosea* hyphal growth on a citrus leaf surface exposed to high humidity conditions (40X).

tarsi. Similarly, Meyer et al. (2008) observed that moribund adult psyllids were lightly fastened to the leaf or to the side of a centrifuge tube by white mycelium of *Ifr*AsCP emerging from the tarsi. In addition, *Ifr* hyphae were observed to spread outwards from the cadavers and contaminate the surrounding leaf surface. On plant leaves, *Ifr* has been observed to colonize several millimeters across the leaf surface and infect aleyrodids (Wraight et al. 1998). Avery (2002) noted that the *Ifr* hyphae grew 21 mm across a simulated leaf surface to colonize other susceptible greenhouse whitefly pharate adults.

FDI Assessment

In all treatments, 83% of the adult psyllids were colonized and sporulating (FDI value: 2.5-3.0) by d 8. Infected insects (FDI value: 1.5) were alive and had fungal hyphae protruding from their leg joints immediately prior to mortality, similar to effects observed by Meyer et al. (2008). After psyllids succumbed to fungal infections, fungal development of *Ifr* progressed to an FDI value of 2.0 or higher the following day under continuous high (RH > 80%) humidity conditions.

Ifr infection rate on the adult psyllids was comparable to that recorded for the greenhouse whitefly maintained under similar laboratory conditions (Avery et al. 2004). All whitefly pharate adults were completely colonized (FDI value of 3.0) in 8 d following topical application and infection with *Ifr* blastospores under a 16 hr photophase and high relative humidity. Similarly, in sprayed leaf section treatments, over 97% of the adult psyllids were colonized in 8 d, while 100% of the psyllids were colonized in the yellow tag treatments. Overall, our data supported that a yellow card impregnated with blastospores is as effective in contaminating and killing the adult psyllid as spraying several leaf sections under laboratory conditions. However, the efficacy of *Ifr* for managing the psyllids by either spraying trees or by using yellow cards contaminated with fungal spores requires evaluation under field conditions. In addition, evaluation of the most suitable material for retaining the blastospores on cards in the field also warrants further investigation.

In autodissemination strategies, the ability of insects to acquire and horizontally transfer viable spores is vital to the effectiveness and ultimate success of a fungal biocontrol pest management program (Roy et al. 2001; Dowd & Vega 2003; Tsutsumi et al. 2003; Scholte et al. 2004; Maniania et al. 2006). The increase in the amount of viable inoculum on the leaf surface appears to positively correlate with the rate of acquisition and concomitant increase in mortality of the psyllid. For instance in Table 3 on d 5, as the concentration of *Ifr* inoculum increased among leaf sections per dish chamber from 1 leaf section to 3 leaf sections, the host infection and fungal development rate also increased 7 times. Bailey et al. (2007) found that when the *Microsphaeropsis ochracea* was increased in concentration per leaf surface, the host infection rate also increased. In contrast, Ugine et al. (2005) noted an inverse relationship between

acquisition rate (conidia acquired/total conidia applied) and residue concentration of *Beauveria bassiana* by western flower thrips. This size ratio concept is very important when designing an autodissemination system with entomopathogenic fungi and warrants further research.

A high incidence of *Ifr* hyphae being observed along the edges of some non-treated leaves without psyllids present during the experiments was noted (Table 2), which could be attributed at least partially to the edges of these non-treated leaves coming into accidental contact with the edge of a treated leaf. This scenario could be avoided in future studies by fixing the leaf sections to the filter paper. However, the significant increases in *Ifr* hyphae growing within the center of leaves and yellow tags was attributed to active dissemination by the adult psyllids. Regardless of whether the sprayed surface was an authentic leaf or an artificial attractant tag, psyllid movement caused significant contamination of unsprayed leaf section centers. Meyling et al. (2006) found that insects living in nettle plants could help spread and disperse *B. bassiana* from one site to another. In the field, the transfer of *Ifr* to leaves or flush where psyllids congregate could potentially lead to secondary infection. In a preliminary bottle cage experiment, it was observed that an entire psyllid population living on a citrus seedling became infected after several days exposure to a yellow tag sprayed with *Ifr* blastospores (Avery, unpublished data). Also, in a pilot field trial (Avery et al. 2009), 33-50% of psyllid eggs and 29-50% nymphs on citrus flush were found infected with *Ifr* 10-21 d post-spray, respectively. In addition, 100% (3/3) of the adult psyllids caught per yellow card were contaminated and infected with *Ifr* 28 d post-spray (Avery et al. 2009).

The autodissemination system using a yellow tag contaminated with *Ifr* blastospores has potential; however, there are many parameters that need to be investigated further in order to determine the efficacy of this strategy for managing psyllid populations in a citrus grove. The efficacy of a yellow tag contaminated with *Ifr* blastospores as a source for *D. citri* to spread the fungus to young citrus plants and other psyllids is presently being tested in cages (Moran et al. 2009); if results are promising then this autodissemination strategy will be evaluated in Texas door-yard citrus. However, persistence and viability of the *Ifr* blastospores on the yellow tag or leaf surface over time under field conditions will help determine the cost effectiveness of such a pest management strategy. To increase the persistence, viability and efficacy of the fungal blastospores, perhaps an adjuvant could be added. Dunlap et al. (2007) indicated that the speed of the *Ifr* blastospore germination was improved by adding keratin hydrolysate and the number of infective propagules was increased as well.

The yellow tag may only attract a few psyllids for dissemination of *Ifr* into the grove and timing of application will be crucial. In a field study where *D. citri* populations were monitored with yellow sticky traps, the mean number of adult *D. citri* per trap decreased significantly during periods of abundant new flush compared to trap captures immediately before and after new flush was present (Rogers, unpublished data). Therefore, the yellow tags will need to be hung prior to the emergence of the new preferred flush depending on the climatic conditions and phenology (usually before Mar and just prior to Aug) by the psyllids to be most effective. However, this autodissemination strategy could be augmented by the addition of an attractant in the future (El-Sayed et al. 2006; Suckling et al. 2007). Recently, Wenninger et al. (2008) provided behavioral evidence for a female-produced volatile sex pheromone for the adult psyllid. Perhaps this pheromone, once identified and synthesized could be added to the yellow tag to increase the effectiveness of attracting other adult psyllids and increase the dissemination of the *Ifr* into the grove, irrespective of the presence of flush.

In the field, the transfer of *Ifr* to leaves or flush where psyllids congregate could potentially lead to secondary infection producing sporulating cadavers and eventually under high humidity conditions an epizootic effect. In a preliminary caged laboratory experiment, it was observed that an entire psyllid population living on a citrus seedling became infected after 7 d of exposure to a yellow tag sprayed with *Ifr* blastospores (Avery, unpublished data) Also, in qualitative assays, Meyer (2007) recorded 100% mortality of adult *D. citri* that were exposed to *Pfr* AsCP on sporulating psyllid cadavers. The extent of the epizootic effect is dependent upon the density of the insects in the area where the sporulating cadavers are located (Furlong and Pell 2001; Avery 2002; Klinger et al. 2006).

Entomopathogenic fungi, which are efficient in killing soft bodied, sucking-piercing insects, are being investigated in different parts of the world as biocontrol agents for controlling the Asian citrus psyllid (Subandiyah et al. 2000; Pell 2008). However, currently there are no *Ifr* biopesticides registered for spraying and controlling the psyllid on fruit crops in the USA. Presently, Certis® in Maryland, USA, produces a blastospore formulation of *Ifr* (*Pfr*-97 20% WDG® that should become registered for use by citrus growers in 2010 (Dimock, personal communication).

Blastospores of different entomopathogenic fungi, including *Ifr* have been used extensively in pest management programs on a worldwide scale (Avery 2002). *Ifr* blastospores easily can be mass produced in a shake-flask liquid culture medium (Jackson et al. 1997, 2003; Lozano-Contreras et al. 2007) and only require 6-8 h to germinate (Vega et al. 1999). Considering that southern and

central Florida experiences high humidity, it seems that the environmental conditions are conducive for the use of this fungal biopesticide as part of an IPM program in managing all stages of the psyllid population.

Biopesticides can be used as an alternative in a spray program to break the cycle of harder chemicals and prevent the development of resistance (Moore 2008a). The use of *Ifr* is an environmentally friendly alternative that will have minimal effect on non-target beneficial arthropods present in the grove (Sterk et al. 1995a, b) and can be used with other strategies for sustainable pest management (Shah & Pell 2003). For instance, Étienne et al. (2001) reported *Tamarixia radiata*, a parasitoid of the Asian citrus psyllid established in the Guadeloupe Islands, has provided excellent control of the psyllid even in the presence of an entomopathogenic fungus of the psyllid, *Hirsutella citriformis*. Both *H. citriformis* and *Ifr* are types of native entomopathogenic fungi found in the Florida groves (Meyer et al. 2007, 2008), and should be compatible with *T. radiata* previously released for the control of the psyllid pest. However, the compatibility of *Ifr* with *T. radiata* for controlling the psyllids needs to be tested under field conditions. Lastly, biopesticides, such as *Ifr*, may be used effectively either alone or in rotation with traditional pesticides for added genetic resistance prevention (Er & Göçke 2004; Kantz 2007; Moore 2008b). However, which chemicals sprayed in the field are compatible with *Ifr* warrants further investigation.

These autodissemination laboratory studies are the first to evaluate the potential for using *Ifr* against the Asian citrus psyllid, whether sprayed on trees or on an artificial attractant surface. Based on the results, the use of *Ifr* for managing the citrus psyllid has demonstrated potential and warrants further testing under field conditions. Lastly, because the psyllid is attracted to the yellow color (Hall & Albrigo 2007; Hall et al. 2007; Hall et al. 2008; Hall 2009), the use of yellow cards impregnated with *Ifr* blastospores as part of an IPM strategy has potential for providing citrus growers with a cost-effective method for managing psyllids.

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