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Localized Autoinoculation and Dissemination of *Isaria fumosorosea* for Control of the Asian Citrus Psyllid in South Texas

Patrick J. Moran[1*], Joseph M. Patt[2], H. Enrique Cabanillas[3], John L. Adamczyk[4], Mark A. Jackson[5], Christopher A. Dunlap[5], Wayne B. Hunter[2], and Pasco B. Avery[6]

[1]*Exotic and Invasive Weeds Research Unit, Western Regional Research Center, USDA-ARS, 800 Buchanan Street,, Albany, CA 94701*
[2]*Subtropical Insect Research Unit, U.S. Horticultural Research Laboratory, USDA-ARS, 2001 South Rock Road, Ft. Pierce, Florida, 34945, USA*
[3]*Honey Bee Research Unit, USDA-ARS, 2413 E Hwy 83, Weslaco, TX 78596*
[4]*Thad Cochran Southern Horticultural Laboratory, USDA-ARS, 810 Highway 26, West, Poplarville, MS 39470*
[5]*Crop Bioprotection Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, 1815 North University Street, Peoria, Illinois, 61604, USA*
[6]*Indian River Research and Education Center, University of Florida Institute of Food and Agricultural Sciences, 2199 South Rock Road, Fort Pierce, Florida, 34945, USA*

*Corresponding author: Email: Patrick.Moran@ars.usda.gov

ABSTRACT

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, vectors the causal organism of citrus greening disease. Control approaches involving entomopathogenic fungi may be useful on ornamental and abandoned citrus and other rutaceous hosts, which could act as vector and pathogen reservoirs. A Texas isolate of the fungus *Isaria fumosorosea* (*Ifr*) caused 95% mortality in applications to *D. citri*. In laboratory bioassays at 27 °C, 70% of psyllids died within one week when exposed to yellow cards that had been sprayed with liquid blastospore suspension. A potentially more practical approach of coating cards with filtered dry powder containing blastospores and emulsified wax adhesive did not cause significant mortality, but efficacy was 35% higher when the adhesive contained a blend of stimulatory citrus volatiles than on cards with no volatiles. Under greenhouse conditions (25 to 37°C), yellow cards with *Ifr* as either dried liquid suspension or powder were hung in cages containing psyllids. Mortality was low (< 40%) in all tests, but the inclusion of citrus volatiles increased mortality by 23%. Psyllids caged with blastospore powder-coated cards for one week did not transfer inoculum horizontally to other adults or vertically to offspring. However, when psyllids were exposed to these cards for 24 hours in a Petri dish and then released into cages containing unexposed psyllids, 25% of those psyllids died within three weeks. The results provide insight into chemical stimuli and physical fungal exposure conditions associated with an autodissemination device to aid in control of *D. citri.*

Additional Index Words: autodissemination, citrus volatiles, *Diaphorina citri*, entomopathogen, fungus

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U.S. citrus production involves over 600,000 ha and yields $5.3 billion annually (NASS, 2009). The subtropical Lower Rio Grande Valley (LRGV) of southern Texas contains 10,000 ha of production groves, and annual grower value exceeds $50 million (Sauls, 2008). Grapefruit (*Citrus* × *paradisi* Mcfadyen) and seedless orange (*C. sinensis* L.) are the predominant commercial plantings. Numerous arthropods cause damage in Texas, including mites, mealybugs, whiteflies, aphids, armored and soft scales, and the Mexican fruit fly *Anastrapha lutens* (Leow) (Diptera: Tephritidae) (Cartwright and Browning, 2005a,b,c; Cartwright et al., 2005). However, the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), first detected in the LRGV in 2001 (da Graca et al., 2008) is the most serious emerging threat to Texas citrus. Nymphs feed upon and damage new flush foliage on nursery plants and young trees (Hall and Albrigo, 2007; Rogers et al., 2010) but the potential for incurable damage to both young and

mature trees exists because *D. citri* can vector '*Candidatus* Liberibacter asiaticus' and '*Candidatus* Liberibacter americanus', presumed causal agents of huanglongbing (HLB) or citrus greening disease (Bové, 2006; Pelz-Stelinski et al., 2010). Citrus greening was first detected in the LRGV in January 2012 (da Graca et al., 2008; Texas Agrilife Extension, 2012) and has been found in southeastern Louisiana (Hummel and Ferrin, 2010), Belize (Manjunath et al., 2010) and the Yucatán region of México. The disease has killed thousands of trees in Florida since 2005 (FDACS, 2008; Tiwari et al., 2010).

The spread of citrus greening in the Americas is tied to dispersal and feeding by *D. citri* (Gottwald, 2010). Control with nsecticides is most effective during emergence of spring flush foliage (Hall and Albrigo, 2007; Sétamou et al., 2008; Rogers et al., 2010), which is required for oviposition, although adults are abundant in summer and are targeted throughout the year (Hall et al., 2008; Pluke et al., 2008; Sétamou et al., 2008; Flores et al., 2009; Qureshi et al., 2009). Control of nymphs is important, as they become the most efficient adult vectors of HLB (Inoue et al., 2009; Pelz-Stelinski et al., 2010). The use of sublethal doses of antifeedants to reduce impacts on beneficials (Boina et al., 2009, 2011), off-season insecticide applications to kill overwintering adults (Qureshi and Stansly, 2010), interplantation with guava (Zaka et al., 2010) and removal or treatment of abandoned groves (Tiwari et al., 2010) may improve *D. citri* control. The parasitoid *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) was released for biological control in Florida (Skelley and Hoy, 2004) with low to moderate impact (Qureshi et al., 2009) and is present in Texas (De León and Sétamou, 2010). Numerous generalist predators consume *D. citri* (Qureshi and Stansly, 2009). Chemical and biological control tactics, in commercial groves in the LRGV may be insufficient due to the presence of widespread, diverse ornamental and residential ('dooryard') citrus trees/shrubs and other rutaceous hosts. For example, orange jasmine/jessamine (*Murraya paniculata* (L.) Jack), a popular ornamental, is suitable for oviposition (Halbert and Manjunath, 2004), due in part to attractive foliar volatiles (Patt and Sétamou, 2010) in combination with visual stimuli (Wenninger et al., 2009a). Orange jasmine can host asymptomatic infections of *'C.* L. asiaticus' and '*C.* L. americanus' (Lopes et al., 2005; Manjunath et al., 2008; Damsteegt et al., 2010).

Fungal entomopathogens have shown great promise for control of insect pests (Hajek and Leger, 1994; Goettel et al., 2000; Hesketh et al., 2010), with over 100 products commercialized (de Faria and Wraight, 2007). Because they do not need to be ingested, fungi may be the only entomopathogens suitable for control of piercing-sucking hemipteran pests, such as armored scales, aphids, whiteflies and psyllids, and many fungal species are known (Evans and Prior, 1990; Goettel et al., 2000; McCoy et al., 2000; Wraight et al., 2000; de Faria and Wraight, 2007; Zimmermann, 2008). At least six entomopathogenic fungal genera infect *D. citri* (Hall, 2008; Meyer et al., 2008), including *Hirsutella citriformis* Speare (Meyer et al., 2007) and *Isaria fumosorosea* (*Ifr*)Wize (Meyer et al., 2008) that occur naturally in Florida. Three *Ifr* isolates, including two commercial strains, *PFR*97 WDG® (Certis Inc., Columbia, MD, USA) and *Ifr* 9901 (NoFly™ WP) (Natural Products, Houston, TX, USA), and a south Texas isolate (*Ifr* 3581) are pathogenic against *D. citri* (Meyer et al., 2008; Avery et al., 2009, 2011; Hunter et al., 2011) and are available as blastospore formulations (Jackson et al., 2003).

The local strain *Ifr* 3581 could be an important component of an integrated pest management system for *D. citri* in commercial, residential, and abandoned citrus in the LRGV of Texas. The use of *Isaria* spp. is compatible with biological control by parasitoids and predators (Avery et al., 2008; Zimmermann, 2008) and fungal dispersal could be enhanced by antifeedant insecticides (Boina et al., 2009). However, large-scale application (to 10^{14} propagules ha^{-1}) under the adverse environmental conditions (high temperature and solar radiation and low humidity) predominant in south Texas is not practical (Vidal and Fargues, 2007; Jackson et al., 2010; Jaronski, 2010). Natural dissemination of fungal propagules may occur via movement of a target insect, its natural enemies, and habitat associates such as honeybees (Baverstock et al., 2009, 2010). Autodissemination devices containing blastospores or other propagules and attractants may further facilitate disease outbreaks in *D. citri*, as these devices have been used successfully against a number of coleopteran, dipteran, and lepidopteran pests (Renn et al., 1999; Dowd and Vega, 2003; Jan-Scholte et al., 2004; Maniania et al., 2006; Vega et al., 2007; Migiro et al., 2010) and a stinkbug (Hemiptera: Pentatomidae) (Tsutsumi et al., 2003), but little is known about their utility for control of homopterous Hemiptera such as psyllids. *D. citri* adults can be attracted by artificial visual and olfactory stimuli (Hall et al., 2007, 2008; Sétamou et al., 2008; Hall, 2009; Wenninger et al., 2009a; Patt and Sétamou, 2010; Patt et al., 2011) and adult psyllids can auto-inoculate and disseminate propagules to leaves after alighting on fungal-coated yellow tags (Avery et al., 2009). The objective of this study was to evaluate the efficacy of *Ifr*3581 exposed to *D. citri* on attractant cards in laboratory tests, and in cages in greenhouses under south Texas conditions, to guide development of an autodissemination device.

MATERIALS AND METHODS

Plants, insects, and pathogen. Orange jasmine (*Murraya paniculata* (L.) Jack, cv. 'Lakeview'), Valencia sweet orange (*Citrus* x *sinensis* (L.) Osbeck , and sour orange (*Citrus* × *aurantium* L.) were obtained as seedlings from a local nursery (Valley Garden Center, Weslaco, TX, USA) or propagated from seed, and maintained in a greenhouse at 25 to 40° C under ambient lighting. Asian citrus psyllids were field-collected in 2008 and maintained on orange jasmine in cages (0.4 m width × 0.8 m depth × 0.7 m height) made of plastic PVC pipe and white muslin (0.4 mm mesh) with a zippered closure, in either a growth chamber at 27°C, 60% RH and 14:10 L:D photoperiod, or in a greenhouse between 25 and 35 °C with ambient light and RH. Adults (100) were transferred to new cages monthly. New adults were observed within three weeks (Skelley and Hoy, 2004; Nava et al., 2010). Adults of mixed ages, sex, and abdominal color (blue/green, orange, or brown) were used in bioassays. Attraction to the visual and (in some cases) olfactory cues on *Ifr* dessiminator cards may have varied with all of these parameters (Wenninger et al., 2009a,b). Adults were provided with healthy foliage or seedlings during lab or greenhouse bioassays, respectively.

The fungus, *Isaria fumosorosea* Wize (Ascomycota: Order Hypocreales, Family Cordycipitaceae) (formerly known as *Paecilomyces fumosoroseus*) was isolated from the sweetpotato whitefly *Bemisia tabaci* biotype B (Gennadius) on cabbage in McAllen, Hidalgo County, Texas in 1992 (Wraight et al., 1998) and is preserved in the USDA-Agricultural Research Service Entomopathogenic Fungi Collection (ARSEF) as *Ifr* 3581 (henceforth *Ifr*). Molecular evidence indicates that isolates of *Ifr* from whiteflies represent a different species (Zimmermann, 2008). Blastospore powders of *Ifr* were produced on basal liquid media with 80 g l^{-1} glucose and 13.2 g l^{-1} amino acid mix added (Murashige and Skoog (MS) medium) as described in Jackson et al. (1997, 2003). Blastospores were separated from the fermentation broth by adding diatomaceous earth (Hyflo® super cel, World Minerals, Inc., Santa Barbara, CA) or ground cotton burrs (< 250 µm, provided by Gregory Holt, USDA/ ARS, Lubbock, TX) as filter aids at a ratio of 1 g filter aid per 2×10^{10} blastospores. The resultant filter cake was air-dried to less than 4% moisture, yielding 1×10^{9} to 10×10^{9} blastospores g^{-1} diatomaceous earth or cotton burr powder. For liquid applications, blastospore powder was suspended (20 mg ml^{-1}) in sterile water and agitated with a stir bar for 30 min. After 1 h settling time, blastospore suspension was collected and concentration measured with a hemacytometer (Bright-Line, Reichert, Buffalo, NY, USA) at 400× magnification.

Fungal pathogenicity and viability. To confirm the pathogenicity of *Ifr* 3581, groups of 10 to 20 adult *D. citri* (22 groups total across five pooled bioassays) were cold-(4°C) anesthetized for 30 min and sprayed at a dose of 2×10^{7} to 5×10^{7} blastospores ml^{-1} using a Potter Precision laboratory spray tower (Burkard Scientific, Uxbridge , UK) and 1 ml suspension per replicate group. Controls were sprayed with water. Psyllids were released into 15-cm diameter Petri dishes containing one orange jasmine leaf inserted into a sponge containing half-strength Hoagland's solution, which kept leaves turgid for one week. Psyllids were maintained at 27°C, 60 to 70% RH in growth chambers on a 14:10 L:D cycle. Mortality was assessed after 7 d. Mycosis was confirmed by placing dead psyllids on top of a piece of Parafilm® (Pecheney Packaging, Chicago, IL, USA) in a sterile Petri dish lined with moist filter paper and cadavers were examined for mycelial growth on the film 3 to 4 d later. In an additional test, blastospores were sprayed onto four orange jasmine plants hosting robust colonies of *D. citri* nymphs and adults.

Blastospore viability was evaluated using a previously described germination assay (Jackson et al 1997). Briefly, ~50 mg blastospore powder was suspended in 50 ml potato dextrose broth and incubated at 28°C and 300 rpm in a rotary shaker incubator. Following 6 h incubation, 100 blastospores were observed microscopically for germ tube formation. The average (± SE) germination rate was 87.4 ± 2.3% (M. Jackson, unpublished data). To confirm blastospore viability prior to each experiment, 9-cm Petri plates (three per test) containing full-strength Sabouraud dextrose agar medium with yeast (SDAY, 65 g L^{-1} SDA and 10g L^{-1} yeast extract) (Becton-Dickinson, Sparks, MD, USA) were coated with 1 ml suspension applied with a sterile spreader. Plates were incubated in the dark at 25°C for 18 h. Spore germination was determined by adding three drops of lactophenol-cotton blue and three cover slips per plate and examining 100 spores under each slip at 400× for germination using the criterion of Goettel et al. (2000).

Disseminators and fungal application. Squares (25 cm^2) cut from yellow non-sticky cardboard cards (Pherocon® AM, Trécé, Adair, OK) were used as disseminators. In most bioassays, cards were folded every 1 cm to create edges to exploit the tendency of *D. citri* adults to move along edges of leaves (Yasuda et al., 2005). Two methods were used to coat cards with spores. In 2009 bioassays, liquid *Ifr* 3581 blastospore suspension (1 ml) was applied in the Potter spray tower to cards, producing a deposition rate of 320 to 450 spores mm^{-2}. Cards were allowed to dry

for 30 minutes at 27 °C, 60% to 70% RH and then immediately used in bioassays. In 2010 bioassays, cards were first coated with 1 ml of an emulsified wax material that acted as an adhesive (Splat®, a (proprietary mixture of food-grade ingredients) (ISCA Technologies, Riverside, CA, USA) and that had been colored bright yellow by the manufacturer. *Ifr* 3581 powder (0.5 g) was then applied via gentle shaking onto the card surface. Based on a 96% germination rate (see Results) and an average of 5×10^9 blastospores g^{-1} powder, the exposure concentration was 9.6×10^5 viable blastospores mm^{-2}.

Laboratory bioassays and effect of citrus volatiles. Suspension- or powder-coated cards were placed individually in 15-cm diameter Petri dishes with groups of 15 to 30 adult *D. citri* and an orange jasmine leaf with nutrient solution. Mortality was determined 7 d after release. In some 2010 tests involving blastospore powder, yellow Splat® was scented with a 'generic citrus scent' (Patt et al., 2011) attractive to *D. citri* (Patt and Sétamou, 2010; Patt et al. 2011) and reflective of important South Texas hosts of *D. citri*, that contained a mixture of the following terpenes: 100µl linalyl acetate (Sigma-Aldrich Fine Chemicals, SAFC, St Louis, MO, USA); 90 µl β-ocimene (SAFC); 80 µl (r) -(+)-Limonene (SAFC); 80 µl linalool (SAFC); 30 µl β-myrcene (SAFC); 20 µl β-caryophyllene (SAFC); 20µl citronellal (Aldrich Chemistry, St Louis, MO, USA); 10 µl geranial (SAFC); 10 µl sabinene (Berjé, Bloomfield, NJ, USA); 1 µl geranyl acetate (Aldrich Chemistry); and 1 µl (Z) 3-hexen-1-ol acetate (SAFC). This scent mixture was added at a rate of 1 µl ml^{-1} Splat®, and ~ 1 ml of scented (or unscented) Splat® was used to coat one side of each 25 cm^2 card.

Greenhouse cage bioassays and effect of citrus volatiles. In four 2009 bioassays, cards coated with dried liquid blastospore suspension (no Splat®) were hung using monofilament fishing line vertically from the top of metal cube cages (30 cm l, w, h) screened with 0.8 mm plastic mesh and containing either one orange jasmine seedlng (one bioassay without citrus volatiles) or one sour orange seedling (one bioassay without volatiles and two with volatiles). In two of the four 2009 bioassays, cards were baited with 1µl generic citrus scent on a cotton plug inserted into a rubber septum stapled to the top of the card (across all four tests, n = 7 cages with volatiles and *Ifr*; n = 7 cages with volatiles without fungus; n = 6 cages with no volatiles and *Ifr,* and n = 6 cages with no fungus). Each cage received 50 adult psyllids. Cages were maintained in a greenhouse under conditions similar to those used for plant maintenance.

In two 2010 cage bioassays, cards with Splat® adhesive and no citrus volatiles, with or without *Ifr* (n = 8 and 4, respectively), were suspended vertically with one sweet or sour orange seedling and 30–60 adult *D. citri* per cage. In an additional 2010 test, adhesive with citrus volatiles was used on cards either with (n = 12) or without (n = 4) *Ifr*.

In 2009 and 2010 cage tests, mortality was examined after 7 d. Greenhouse temperature and relative humidity were recorded hourly with a HOBO H8 meter (Onset, Bourne, MA, USA) between 25 August and 13 September 2009. For comparison, field conditions were monitored for a similar period inside the canopy of an orange tree in a citrus grove.

Horizontal and vertical transfer of *Ifr* from cards. In 2010, the ability of yellow Splat® cards with or without citrus volatiles to act as autodisseminators of *Ifr* blastospores was examined. In the first transfer bioassay, 20 psyllids were placed for 6 h in a microfuge tube containing 0.5 g blastospores+diatomaceous earth as a positive inoculation control, and then released into cages (n = 8) without cards containing one orange jasmine and one sweet orange seedling. Cages to assess autoinoculation followed by horizontal trasnfer (n = 8) contained seedlings, 20 psyllids and one Splat®- and *Ifr*-coated yellow card with no citrus volatiles, while 8 other cages contained a card with Splat® only (negative inoculation control). After one wk, cards were removed, and all three cage types received 40 additional psyllids and were maintained for two wk. Dead adults were counted and collected weekly. Percent cumulative mortality after three wk, divided by the total number of psyllids released, represented a measure of horizontal transfer because the percentage (33%) that consisted of directly-exposed psyllids was known. The number of nymphs and new adults produced in the cage was determined by counting total live adults and nymphs and subtracting adults originally released in cages. The three week time frame was sufficient to yield a new adult generation on orange jasmine (Skelley and Hoy, 2004; Nava et al., 2010). Reproduction per surviving parent was determined. An assumption was made that all dead adults were from the parental cohort of 60 total psyllids.

In a second 2010 transfer test, groups of 30 psyllids were exposed to *Ifr*3581 blastospore powder on cards in Petri dishes for 24 h to facilitate autoinoculation in a simulated enclosed autodissemination device, followed by release into cages without cards (n = 4), or, to simulate open field autoinoculation and horizontal transfer , were released into greenhouse cages with one Splat® plus blastospore-coated card with (n = 4) or without (n = 8) citrus scent, or containing a card with Splat® only (n=4) (negative inoculation control). After one wk, cards were removed (where applicable) and an additional 45 psyllids were released per cage. Mortality and reproduction were assessed after two additional wk as above.

Statistical analysis**.** Data for assays that used similar procedures were combined. To account for

variance in control mortality, percent mortally arising from direct *Ifr* spray application, exposure to *Ifr*-coated cards (either liquid or powder method) in Petri dishes, and exposure to cards in cages are reported as control-corrected percentages using Abbott's formula with 95% confidence intervals, calculated as in Rosenheim and Hoy (1989). Intervals overlapping zero indicate non-significant mortality. To compare fungus-associated mortality with non-fungus controls, percentage mortality data were ranked and subjected to two-factor analysis of variance using SAS PROC GLIMMIX (SAS Version 9.1.3) (SAS, 2004) with citrus volatile and fungal application main effects plus an interaction factor. Least-square means of ranks were compared with Tukey correction and $\alpha = 0.05$. In tests of horizontal and vertical transfer, a similar ranked data approach was used in one-factor ANOVAs to examine the effects of *Ifr* exposure method on cumulative mortality and reproduction per surviving parent. Untransformed counts of nymphs and adults produced in cages met normality and equality of variance requirements and were compared without ranking.

RESULTS

Assessment of fungal inoculum. Dry blastospore preparations of *Ifr* 3581 contained (mean ± SE) $4.6 \pm 0.7 \times 10^9$ blastospores g^{-1}. Liquid fungal suspensions, used to test pathogenicity by direct spray contact and in 2009 dissemination tests, contained $3.1 \pm 0.4 \times 10^7$ blastospores ml^{-1}. Spore germination after 18 hours was 96% ± 1.5%. In five pooled bioassays, direct spray contact with blastospores led to control-corrected adult *D. citri* mortality (mean ± lower and upper 95% confidence intervals) of 96.1% ± 5.5% after 7 d. All 50 adult psyllids from a representative test showed white fungal growth consistent with mycosis within that time frame (Fig. 1). Mycosis was observed in both nymphs and adults collected as cadavers from psyllid-infested orange jasmine plants that had been sprayed one week earlier with blastospores (Fig. 1).

Ifr efficacy in laboratory bioassays and effect of citrus volatiles. In five 2009 tests using liquid fungal suspension-coated cards in a total of 17 Petri dishes and 15 psyllids per dish, control-corrected mortality was 70.5 ± 29.3%. In 2010 tests, when blastospores were applied as powders containing diatomaceous earth or cotton burr attached with Splat® yellow adhesive, there was no difference in mortality between diatomaceous earth and cotton burr filter aids (one-way ANOVA on ranked data, $F = 1.2$, df = 1,16, $P = 0.29$, data not shown). Mortality of Asian citrus psyllids exposed in Petri dishes to either *Ifr* blastospore powder

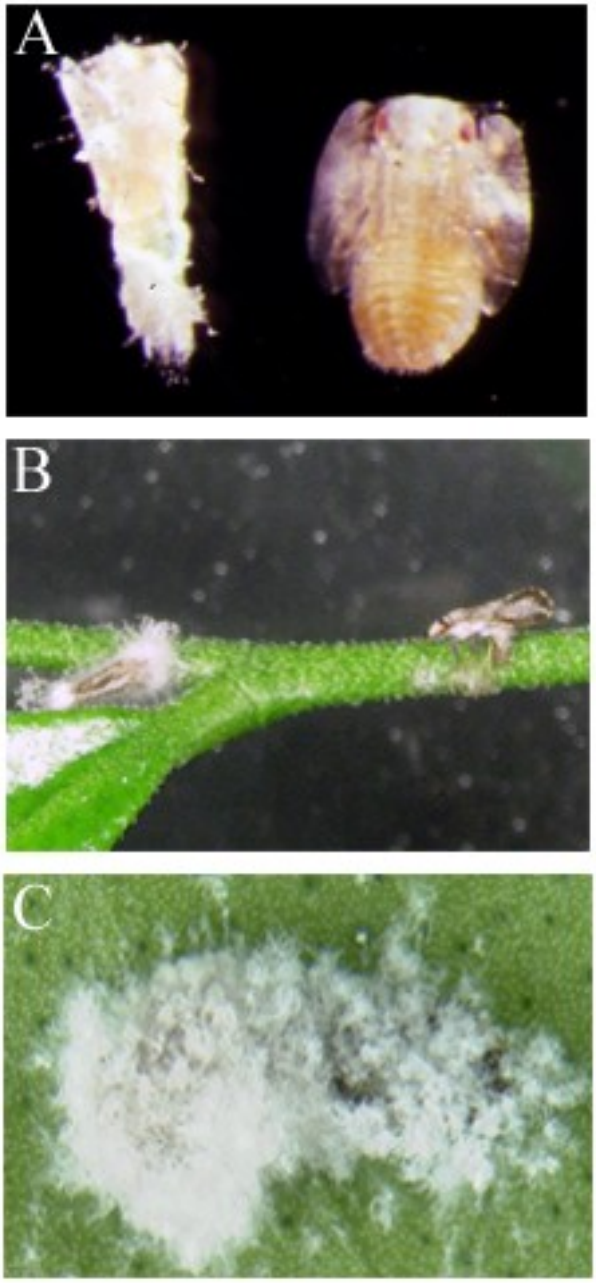

Fig. 1. Mycosis caused by application of a south Texas isolate of *Isaria fumosorosea* (*Ifr* 3581) on the Asian citrus psyllid *Diaphorina citri*; A, Mycosed nymphs photographed seven days after application of liquid blastospore suspension to nymphs feeding on orange jasmine foliage; B, Mycosed dead (left) and live (right) adult psyllids on orange jasmine seven days after blastospore application; C, Adult killed and mycosed as a result of autodissemination. Photos B and C show hyphal growth of *Ifr* 3581 on leaf blade and petiolar surfaces. Photo A by E. Cabanillas, photo B by J.P. Ramos (USDA-ARS, Weslaco, TX), photo C by P. Avery.

type did not differ from controls (no fungus) (Table 1), as confidence intervals for psyllids exposed to *Ifr* included zero. However, the use of citrus volatiles increased mortality by 35% compared to cards with fungus and adhesive but no volatiles, and the effect of volatiles was significant (Table 1).

Ifr efficacy in greenhouse bioassays and effect of citrus volatiles. Greenhouse conditions mirrored summer field conditions in south Texas, with daily average temperatures of (mean ± SE) 29.5 ± 0.3°C in the greenhouse and 29.0°C in the field, respectively. Greenhouse high temperatures reached 37.0 °C (± 0.6° C) and relative humidity (RH) declined to 52% (± 1.5%) during the day. Nighttime (2100–0800 h) minimum daily temperature averaged 25.6 ± 0.6°C, and relative humidity ranged from 90–92%. In 2009 tests, control-corrected mortality in cages in which psyllids were exposed to dried liquid blastospores for one week was always less than 40%, but was 23% higher, with non-overlapping confidence intervals, in cages containing *Ifr* cards to which citrus volatiles had been attached, than in cages with *Ifr*-coated cards without volatiles (Table 1). The effects of both fungal treatment and citrus volatile effects were significant (Table 1).

In 2010 greenhouse tests involving cards coated with *Ifr*3581 powder, the effect of diatomaceous earth vs. cotton burr material was not significant (F = 2.05, df = 1,18, P = 0.17, data not shown). As in 2010 laboratory tests, *Ifr* exposed to psyllids as powder on cards in greenhouse cages had no efficacy with or without citrus volatiles, and neither the effects of fungal treatment or citrus volatile treatment were significant (Table 1).

Horizontal and vertical transfer of *If* from cards. In the bioassay involving 20 psyllids rolled in fungal powder for 6 h as the positive inoculation control, a ratio of 0.33 indicated 100% mortality of psyllids exposed to fungus. Mortality (among 60 total per cage) after three weeks was at least 3-fold below 0.33 in the positive control cages, and in cages in which 20 psyllids were exposed to *Ifr* powder- coated cards without

Table 1: Mortality of Asian citrus psyllids in laboratory bioassays with *I. fumosorosea* (*Ifr* 3581) blastospore powder and emulsified wax adhesive on yellow cards, in greenhouse cage bioassays involving cards sprayed with liquid *Ifr* suspension with citrus scent mixture attached to some cards, or in greenhouse cages involving cards with *Ifr*3581 blastospores and adhesive, in some cases containing citrus scent mixture.

Fungal Treatment	Citrus Volatiles	Petri Plate tests spore powder[a]	Cage tests spore suspension[a]	Cage tests spore powder[a]
Fungus[b]	Yes	40.3 ± 42.8 % (8)	36.7 ± 13.5% (7)	4.8% ± 13.4% (12)
Fungus[b]	No	5.0 ± 16.6 % (10)	13.8 ± 5.6% (6)	0.7% ± 8.1% (8)
Control[c]	Yes	20.0 ± 31.2 % (4)	21.7 ± 16.0% (7)	4.2% ± 13.3% (4)
Control[c]	No	5.0 ± 12.4 % (3)	2.7 ± 5.1% (6)	5.0% ± 8.0% (4)
		ANOVA[d]		
Fungal Treatment	(F; df; *P*)	2.74; 1,21; 0.113	22.1; 1,22; 0.0001	1.68; 1,24; 0.208
Citrus Volatile Treatment	(F; df; *P*)	5.92; 1,21; 0.024	28.1; 1,22; <0.0001	0.09; 1,24; 0.765
Interaction	(F;df; *P*)	0.05; 1,21; 0.830	0.27; 1,22; 0.611	0.68; 1,24; 0.419

[a]Sample size of Petri plates (each containing 15 adult psyllids) or cages (each containing 50 adult psyllids in spore suspension tests or 30 to 60 psyllids in spore powder tests) in parentheses.
[b]Values calculated using Abbott's formula, modified to correct for variance in control mortality (Rosenheim and Hoy, 1989), ± 95% confidence intervals.
[c]Values calculated as mean ± 95% confidence intervals.
[d]ANOVAs comparing ranks for the four treatment categories, α = 0.05.

Table 2. Mortality after three weeks to Asian citrus psyllids exposed to *Ifr* 3581 in microfuge tubes for 6 h followed by release into cages for one week ('Tube'), blastospore-coated cards hung in cages for one week ('Card'), or not exposed ('None') plus other psyllids added after one week, and effect on reproduction.

Treatment	Percent psyllids dead after three weeks (mean ± 95% C.I.)	Total psyllid production (nymphs+adults) (mean ± 95% C.I.)	Production per surviving parent (mean ± 95% C.I.)
Tube	11.0 ± 7.0 [a]	102 ± 17 [a]	1.84 ± 1.12 a
Card	8.0 ± 2.0 [a]	102 ± 36 a	1.78 ± 1.59 a
None	7.0 ± 3.0 [a]	119 ± 22 a	1.98 ± 0.63 a
F; df; Pa	0.51; 2,21; 0.610	0.14; 2,20; 0.871	0.47; 2,21; 0.63

[a] Sample size = 8 cages for all treatments. Each cage had 20 psyllids for one week, and then 40 psyllids were added to cages. For percent mortality and production per parent, ANOVAs on ranked data. For total psyllid counts, ANOVA on untransformed data. Means with different letters are significantly different.

citrus scent for one week. Mortality in these *Ifr*-exposed groups was similar to that in cages containing no *Ifr*-exposed psyllids (Table 2), indicating a lack of autoinoculation precluding horizontal transfer to other psyllids after one week. Exposure did not affect psyllid progeny production (Table 2), indicating no adverse effects of *Ifr* exposure on females and no vertical transfer to their offspring.

In the test involving groups of 30 psyllids exposed to fungus-coated cards in a Petri dish for 24 h as thr the positive inoculation control, total mortality after three weeks (including 45 adults added after the first week) was 57%, significantly greater than in cages in which 30 psyllids were exposed to a card for one week with or without citrus scent, and then 45 more psyllids added (Table 3). A value of 40% (30/75) represented 100% mortality of directly-exposed psyllids, and so in the treatment involving a simulated enclosed autoinoculation device, approximately 25% of psyllids not exposed to blastospores died as a result of confinement with exposed psyllids, indicating limited horizontal transfer. Counts of new nymphs and adults did not vary in Tukey-adjusted mean comparisons (Table 3), although per capita reproduction per surviving parent among the the enclosed autoinoculation–exposed psyllids was 5-fold greater than production by psyllids exposed to *Ifr*-coated cards in cages , or not exposed to fungus. Neither of the *Ifr* exposure methods reduced live offspring production relative to negative control cages (Table 3), indicating no vertical transfer.

DISCUSSION

Ifr was efficacious in direct spray applications to *D. citri* adults, as in past studies of the same species or closely related fungi (Meyer et al., 2008; Avery et al., 2009, 2011). However, an autoinoculation method based on cards with a visual color stimulus, and in some cases chemical attractants, did not produce mortality levels sufficient to control field populations. The results do, however illustrate the roles of two factors that will be critical for autodissemination (Vega et al., 2007): The use of chemical and visual attractants; and 2, containment of fungal inoculum to both protect it from environmental extremes and to promote contact with the target pest.

About 75% of psyllids confined in Petri dishes containing a card coated with dried liquid *Ifr* blastospore suspension without citrus scent died within one week, demonstrating acquisition of inoculum by *D. citri* in dishes, albeit with lower efficacy than the level (100%) reported by Avery et al. (2009, 2011) in studies on blastospore-coated leaf sections or yellow tags. Application of blastospores as a liquid suspension, combined with the humid dish environment, likely stimulated germination on the surface of the cards (Jackson et al., 2010). Acquisition by psyllids may have thus been active, via contact with the surface of the card, or passive, via aerial dispersal of conidiospores inside dishes. Germination and conidia production can increase autodissemination device success (Migiro et al., 2010) but the yellow cards contained no

Table 3. Mortality after three weeks to Asian citrus psyllids exposed to *Ifr* 3581 on cards in Petri plates for 24 h followed by release into cages for one week ('Plate'), blastospore-coated cards without citrus scent hung in a cage for a week ('Card'), fungal cards with scent in a cage ('Card-S') or cards with no fungus ('None'), and to psyllids added to each cage after one week, and effect on reproduction.

Treatment	Percent psyllids dead after three weeks (mean ± 95% C.I.)	Total psyllid production (nymphs+adults) (mean ± SE)	Production per surviving parental adults (mean ±95% C.I.)
Plate	57.0 ± 21.0 a	98 ± 14 a	4.05 ± 3.15 a
Card	9.0 ± 4.0 b	44 ± 14 a	0.77 ± 0.58 b
Card-S	8.0 ± 9.0 b	87 ± 11 a	1.45 ± 0.47 ab
None	9.1 ± 6.0 ab	85 ± 13 a	1.44 ± 0.69 ab
F; df; Pa	5.33; 3,16; 0.010	3.34; 3,15; 0.048	8.60; 3,16; 0.001

[a] Sample size = 8 cages for fungal-coated cards without volatiles in cages ('Card'); n = 4 for all other treatments. Each cage had 30 psyllids for the first week and 45 psyllids were then added. For percent mortality and production per parent, ANOVAs on ranked data. For total psyllid counts, ANOVA on untransformed data. Means with different letters are significantly different.

substrate for fungal growth. In 2010 laboratory tests with blastospore powder, no significant mortality occurred, but presence of citrus scent in the adhesive Splat® material increased control-corrected mortality by 35%, a nonsignificant trend indicative of some acquisition of blastospores from cards by psyllids. Alighting events were observed in the first day after test initiation (data not shown). In a preliminary study, 40% of Asian citrus psyllids aquired blastospores after 16 h exposure to *Ifr* on yellow tags in a Petri dish (P. Avery – *unpublished data*). Determination of minimum alighting time required by *D. citri* for autoinoculation warrants further study, as exposure time influences efficacy (Ugine, 2005; Baverstock et al., 2010). Greenhouse conditions simulated south Texas field conditions under which an autodisseminator to control *D. citri* would have to function. Conditions were adverse during the day, as high temperatures exceeded typical tolerance levels for *Isaria* spp (Zimmermann, 2008). However, strains of *I. fumosorosea* and other entomopathogenic fungi vary in temperature tolerance based on geographic source (Zimmermann, 2008; Jackson et al., 2010) and another south Texas *Isaria* isolate from the sweetpotato whitefly, ARSEF 7028, tolerated incubation at 35°C for one week (Cabanillas and Jones, 2009). Moderate nighttime temperatures and RH ≥ 90% were favorable for spore germination. Mortality occurred among psyllids exposed in 2009 cage tests to dried liquid *Ifr* blastospore suspension, and mortality was enhanced, though still below practical levels, by citrus scent. However, in 2010 cage tests involving exposure to blastospore powder-coated cards for one week,*Ifr*-associated mortality either with or without citrus scent was indistinguishable from control cages, indicating that blastospore powder application did not lead to autoinoculation.

Despite low mortality in laboratory and greenhouse tests, the autodisseminator design involving powder application to cards was deemed more practical for field use than liquid application, based on both ease of fungal application and promotion of survival of the fungal inoculum on the moisture-retaining Splat® material , In two bioassays, no horizontal or vertical transfer occurred over three weeks from groups of 20–30 psyllids exposed to a blastospore powder coated-card in a cage for one week onto 40–45 additional adult psyllids added after one week, or to their progeny, consistent with the lack of autoinoculation of powder-coated card-exposed psyllids in prior cage tests. The failure of six hours of direct contact with fungal powder (containing diatomaceous earth) in a tube to lead to autoinoculation and death of the exposed cohort and horizontal transfer through premortem behavioral interactions such as mating or contact with a shared food substrate, or post-mortem fungal growth is consistent with prior work (Ugine et al., 2005; P. Avery, *unpublished data*) indicating that 16

to 24 h of exposure to fungus is needed.. In a separate test, 25% horizontal transfer did occur when psyllids were confined with *Ifr* powder on a card inside a humid Petri dish for 24 h prior to cage release as the positive control treatment. Containment of inoculum inside a high-humidity chamber may thus be a critical design feature for an autodisseminator targeting *D. citri*, as for other pests (Baverstock et al., 2010; Vega et al., 2007). However, confinement of *D. citri* in close proximity to *Ifr* for 24 h is not likely reflective of field encounters. Citrus scent, which was absent in this Petri dish exposure, might have increased card contact times and improved autoinoculation and dissemination. Horizontal transfer can occur via both behavioral interactions such as mating (Baverstock et al., 2010), or, as demonstrated for *D. citri* and other insects, through contact with hyphae germinated from blastospores previously deposited by live adults, or from mycosed cadavers (Avery et al., 2009, 2010). No vertical transfer occurred in our tests. Reproduction per surviving parent was actually highest for psyllids exposed to the 24-hr Petri dish positive inoculation control treatment, due possibly to mortality of some members of the parental cohort and increased availability of oviposition sites for females. Both visual and olfactory cue types are needed to elicit behavioral attraction of *D. citri* to host volatiles (Wenninger et al., 2009a). The results here demonstrate the need for improved autodisseminator designs to stimulate contact with inoculum via foraging, feeding, and/or mating behaviors, by illustrating the importance of chemical citrus scent stimuli, and of the method of exposure of psyllids to fungal propagules.

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