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Observations on the entomopathogenic fungus *Hirsutella citriformis* attacking adult *Diaphorina citri* (Hemiptera: Psyllidae) in a managed citrus grove

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Abstract A two-year field study was conducted in an orange grove in the United States (Florida) to characterize the phenology of the entomopathogen *Hirsutella citriformis* Speare infecting adults of the Asian citrus psyllid, *Diaphorina citri* Kuwayama. On the average, 23% of adults observed on mature leaves were killed by *H. citriformis*. These dead psyllids were characterized as being mummified and covered to various extents by synnemata produced by the fungus. Mummified cadavers were most abundant on citrus leaves during the fall and winter months, with the maximal percentage of mummified psyllids sometimes exceeding 75% of the total number of adults observed. Mummified cadavers were nearly absent each spring, presumably because relative humidity levels were suboptimal for the fungus at this time. Based on dispersion analyses, a monitoring plan for mummified cadavers would best include multiple samples in individual trees as well as multiple tree samples throughout a grove. Mummified cadavers with synnemata, which serve as point sources for new infections of the fungus in psyllids, were observed to remain on leaves for a mean of 68 days (one cadaver remained on a leaf for 168 days). Rainfall was positively correlated with the number of days mummies remained on leaves while mean daily air temperature was negatively correlated. Mummified cadavers were abundant in the summer during 2006 but not during 2007. This may have been a density-dependent consequence of low psyllid host populations in the grove in 2007. Alternatively, combination sprays of oil and copper applied during 2007 may have suppressed the fungus. This latter possibility prompted a laboratory investigation into the toxicity to *H. citriformis* of six chemicals commonly used in citrus. Copper hydroxide, petroleum oil, and elemental sulfur at maximum label rates each significantly reduced the infectivity of a laboratory culture of *H. citriformis* while copper sulfate pentahydrate, aluminum tris and alpha-keto/humic acids did not.

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This finding indicates that citrus growers interested in capitalizing on *H. citriformis* as a biological control agent of *D. citri* should avoid applying high rates of copper hydroxide, oil or sulfur.

Keywords Biological control · Asian citrus psyllid · Citrus greening disease · Huanglongbing · Microbial control · Fungal pathogens

Introduction

The Asian citrus psyllid [*Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)] is an important pest of citrus in North America, primarily because it vectors the bacterium '*Candidatus* Liberibacter asiaticus' (Bové 2006). This and other species of '*Ca.* Liberibacter' are phloem-limited bacteria thought to be responsible for huanglongbing disease (HLB) (also known as citrus greening or yellow shoot disease) (Halbert and Manjunath 2004; Hung et al. 2004). HLB is regarded as one of the world's most serious diseases of citrus (Bové 2006; Gottwald 2010). Citrus trees infected with HLB typically have a reduced lifespan, exhibit a decline in productivity and can produce misshapen, sour-tasting fruit.

A fully integrated pest management program for *D. citri* and HLB would include the combined use of chemical, cultural and biological control practices (Hall and Gottwald 2011). With respect to biological control, multiple arthropod predators and parasitoids are known to attack the psyllid worldwide (Hall 2008). In addition, an array of entomopathogenic fungi have been reported to infect *D. citri* including *Hirsutella citriformis* Speare (Rivero-Aragon and Grillo-Ravelo 2000; Subandiyah et al. 2000; Étienne et al. 2001; Meyer et al. 2007), *Isaria fumosorosea* Wize (=*Paecilomyces fumosoroseus*) (Samson 1974; Subandiyah et al. 2000; Meyer et al. 2008), Lecanicillium (=*Verticillium*) *lecanii* Zimm. (Rivero-Aragon and Grillo-Ravelo 2000; Xie et al. 1988), *Beauveria bassiana* (Bals.) Vuill. (Rivero-Aragon and Grillo-Ravelo 2000), and *Cladosporium* sp. nr. *oxysporum* Berk. & Desm. (Aubert 1987). *Hirsutella citriformis* and *I. fumosorosea* have been observed infecting *D. citri* in the United States (Meyer et al. 2007, 2008; Hall et al. 2008), and information has been published on the identification and detection of these entomopathogens in *D. citri* (Meyer et al. 2007, 2008; Avery et al. 2009). However, little is known regarding the phenology of these fungal pathogens or their impact on field populations of *D. citri*.

Presented here are results of a study on the phenology of *H. citriformis* in an orange grove in the United States (Florida) during 2006 and 2007. We investigated the seasonal dynamics of the *H. citriformis*–*D. citri* interaction, assessed the morbidity rate due to *H. citriformis* infection, and obtained information on the dispersion of *H. citriformis* within the grove. Results of the field study prompted laboratory evaluations of the effect of various chemicals commonly used in citrus on the infectivity of *H. citriformis*. Together, this research provides the first investigation into the field dynamics of an entomopathogen of *D. citri* in the US, and advances understanding of how to incorporate the fungus into existing HLB management practices.

Materials and methods

Seasonal incidence of adult *Diaphorina citri* killed by *Hirsutella citriformis*

Field incidence of *H. citriformis* infecting adult *D. citri* was investigated weekly from January 2006 through April 2008 at a citrus grove (27°39′ 16.93″ N, −80°28′ 15.80″ W) in Indian River County near Vero Beach, Florida, USA. This grove (0.7 ha) contained 'Temple' orange [*Citrus reticulata* Blanco × *C. sinensis* (L.) Osbeck] trees (37 years old, ~3.4 m tall) on the rootstock 'Cleopatra mandarin' (*C. reshni* Hort. ex Tan.). The trees were subjected to an annual spray program primarily aimed at controlling the plant pathogenic fungus *Mycosphaerella citri* Whiteside (Browning et al. 1995), which causes an important defoliating disease commonly called 'greasy spot.' Over the course of the study, no hard insecticides were applied to the trees.

The grove was subjected to the following management practices during the study. Petroleum oil (spray oil 435) was applied at a low dose on 3 April 2006 (2 l in 1,170 l spray per ha) with a nutritional spray. Another petroleum oil (470) was applied with nutritional sprays at low rates on 17 April (8 l ha^{-1}) and 15 June 2007 (9 l ha^{-1}) (both sprays 1,560 l spray per ha). Moderate rates (all lower than maximum labeled rate) of petroleum oil (470) for greasy spot control were applied on 29 June (70 l in 5,500 l spray per ha), 27 July 2006 (28 l in 2,340 l spray per ha), 20 July (37 l in 1,560 l spray per ha), and 6 Sept 2007 (28 l in 2,340 l spray per ha). Foliar treatments of macronutrients and micronutrients were applied approximately monthly during the spring and summer and included nitrogen and KeyPlex 1000DP (KeyPlex, Winter Park, FL, USA). Kocide (35% copper hydroxide) was applied for greasy spot control at 2.5–3.7 kg ha^{-1} (lower than maximum labeled rate) with each foliar spray during 2007 except on 15 June 2007. No copper was applied during 2006. Liquid fertilizer (5–0–8 N–P–K) was applied to the soil beneath the trees from February through October during each year of the study. No pruning or hedging of trees was conducted in the grove.

To assess morbidity, counts of live adults and adults killed by *H. citriformis* were made weekly on 20 pairs of mature leaves (including the stem between the leaves) per tree in each of 30 trees randomly selected each week. The leaf pairs were arbitrarily chosen around the outer tree canopy 1–2 m above ground. Mummified psyllids containing characteristic synnemata (Fig. 1a) were considered to have been killed by *H. citriformis*. Mean numbers of live adults and mummified cadavers per pair of mature leaves were calculated weekly for each tree. A total of 118 weekly samplings were conducted throughout the experiment. Selected leaves with mummified cadavers observed during the study were tagged to assess the time duration that the cadavers remained on leaves. Each tagged leaf was examined once a week until the cadaver was no longer present.

Air temperature and relative humidity data during the two-year study were obtained hourly using a data logger (WatchDog Model 100-T/RH, Spectrum Technologies, Inc., Plainfield, IL, USA) placed inside the canopy close to the trunk of one tree in the grove. Hourly rainfall data were obtained 0.4 km from the study site with a rain gauge operated by the Florida Research Center for Agricultural Sustainability. The environmental variables were summarized for four time periods relative to a sample date: 7 or 60 consecutive days preceding a sample date (no temporal lag), or 7 or 14 consecutive days ending seven days before a sample date (seven days temporal lag). For each of the time periods, summary environmental variables for correlation analyses were calculated based on either averages [e.g., average relative humidity and average temperature] or summations

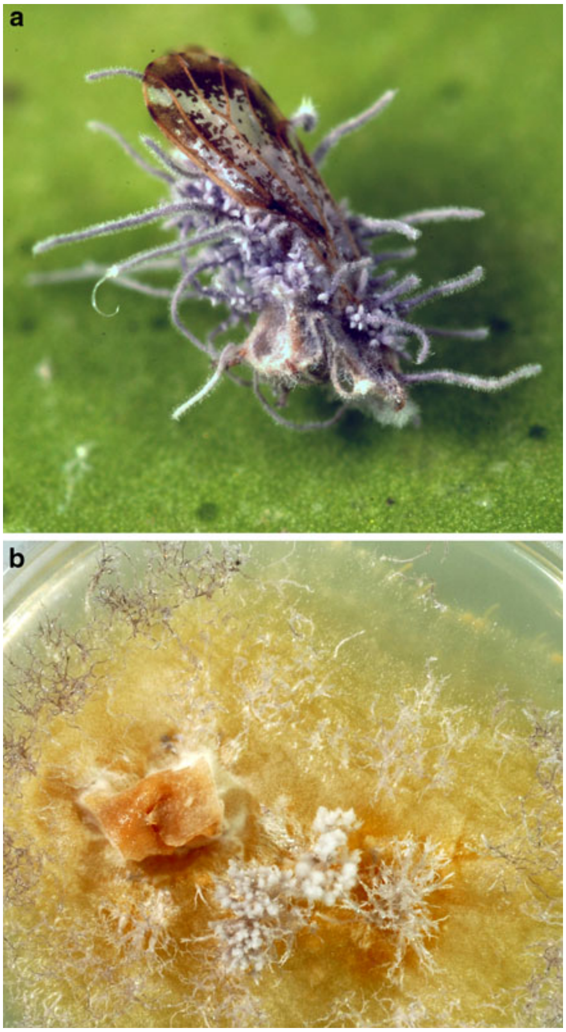

Fig. 1 **a** *Hirsutella citriformis* growing on a mummified adult *Diaphorina citri* (note the synnemata extending from the cadaver). **b** The same *H. citriformis* isolate growing on mycological media after approximately five weeks (note the production of well-developed, dark synnemata at the periphery of the culture)

[e.g., total precipitation (rain)]. Number of hours with relative humidity greater than or equal to 80% (RH80) or 90% (RH90) were also calculated for the correlation analyses. Phenology of *H. citriformis* in this managed grove was characterized by plotting over time mean numbers of live adults and mummified cadavers per leaf pair and percentages of adults observed to be killed by the fungus. Correlation analyses (Spearman rank coefficients) were conducted between the abundance of live adults, mummified adults, and proportion of mummified adults per sample date and the summary environmental variables. Correlations were also investigated between the number of days that mummies remained on leaves and data for total rainfall, mean daily air temperature and mean daily relative humidity. Each of these environmental variables was calculated for the time period during which a cadaver was first discovered (tagged) until the day before the cadaver was observed as absent from the sample site. All correlation analyses were conducting using PROC CORR (SAS Institute 2008) ($P = 0.05$).

Spatial distribution of *Hirsutella citriformis* based on mummified cadavers

Dispersion within the grove of live adults and adults killed by *H. citriformis* (within trees, among trees, and over all samples per sample date) was investigated using Taylor's power law (Taylor 1961; Harcourt 1965; Southwood 1978). According to this power law, the relationship between mean number of insects per sample and the associated variance can be used to characterize spatial dispersion using the following equation: variance $= a \times \text{mean}^b$, where a and b are constants. The a coefficient is largely a sampling factor while the b coefficient is an index of aggregation characteristic of the species (Southwood 1978). A b value of 1.0 indicates a random dispersion, values less than 1.0 indicate a uniform dispersion, and values greater than 1.0 indicate an aggregated dispersion. The coefficients were determined by a linear regression of log (variance) on log (mean), with the resulting intercept and slope equal to log (a) and b, respectively. For dispersion within trees, the simple mean and variance among counts per tree were calculated for the Taylor's analyses. For dispersion among trees, the mean count per tree was first calculated, and then the mean and variance in counts per tree per sampling date were calculated for Taylor's analyses. Overall dispersion was based on the mean and variance over all samples taken on each date. All regression analyses were conducted using PROC GLM (SAS Institute 2008). Dispersion of live adults and mummified cadavers across the grove also was investigated using color-filled, contour spectrum plots generated with SigmaPlot (Systat Software 2008) to summarize the three-dimensional data for the grove (row position, tree position, and cumulative numbers of live adults or mummified cadavers per tree).

Effect of chemicals used in citrus on infectivity of *Hirsutella citriformis*

The toxicity of six chemicals commonly used in citrus (Table 1) to *H. citriformis* was assessed using a laboratory culture of the fungus (USDA Agricultural Research Service Collection of Entomopathogenic Fungi, ARSEF 11665). The culture was derived from mummified cadavers of adult *D. citri* collected on citrus in Collier County, FL, USA (Fig. 1a). Synnemata from these cadavers were removed and propagated on plates of mycological media (10 g glucose, 5 g sucrose, 5 g yeast extract, 0.5 g peptone and 15 g agar per liter of water). Inoculated plates sealed with Parafilm (American National Can, Menasha, WI, USA) were incubated for 3–4 weeks at 26°C, 14 h daily illumination. Synnemal formation was stimulated by removing the Parafilm and incubating the plates for an additional 7–10 days (Fig. 1b). Subsequently, the culture was continually propagated by transferring synnemata to new media every 5–6 weeks.

This in vitro culture was used to evaluate the effect of each of the six chemicals on infectivity of *H. citriformis*. This was accomplished by spraying cultures of the fungus with each chemical. The cultures were about five weeks old with an abundance of synnemata when they were sprayed. Each chemical was mixed in water at a concentration equivalent to the chemical's highest labeled rate for citrus as applied in 935 l of spray per ha. The commercially formulated products were diluted in 100 ml of tap water to achieve the desired concentration. Small subsamples (a 7–10 mm^2) of the fungus culture were removed from Petri dish cultures and placed in a Petri dish of water agar (a 7–10 mm^2 of agar had been removed from the center of the dish and a subsample was slipped into this space). Each Petri dish with a subsample of fungus was misted thoroughly with a chemical (approximately 0.19 ml of diluted product per 9.6 cm^2) and allowed to dry for 24 h (1 h with Petri dish lids off followed by 23 h with dish lids on). A 180-ml Nalgene aerosol spray bottle (#2430-0200, Fisher Scientific, Pittsburgh, PA, USA) was used to apply the chemicals. Eight subsamples of the in vitro culture were treated with each chemical, and infectivity of the subsamples to healthy adult psyllids was assessed. These healthy psyllids were obtained from a colony maintained by USDA (Hall et al. 2010).

Table 1 Chemicals screened for toxicity to *Hirsutella citriformis*

Trade name	Class	Active ingredient	Concentration (product/1 l water)[a]	Manufacturer
Aliette WDG	Phosphonate	Aluminum tris (80%)	6.0 g	Bayer CropScience
Citrus Soluble oil	Petroleum oil	Petroleum oil (99.3%)	100.0 mL	Loveland Products, Inc.
Kocide 101WP	Copper fungicide	Copper hydroxide (77%)	14.4 g	Griffin, L.L.C.
KeyPlex	Nutritional	Alpha-Keto acids (0.06%), humic acid (0.11%), and micro-nutrients (10.16%)	7.5 mL	KeyPlex
Magna-Bon	Bactericide	Copper sulfate pentahydrate (19.8%)	5.0 mL	Magna-Bon II, L.L.C.
Microfine sulfur	Elemental sulfur	Elemental sulfur (90%)	9.2 g	Diamond R. Fertilizer Co.

[a] Each chemical was mixed in water at a concentration equivalent to the chemical's highest labeled rate for citrus as applied in 935 l of spray per ha. Approximately 0.19 ml of product in water was applied per 9.6 cm^2

Healthy adult psyllids were aspirated into glass vials which were then placed on ice for 15–30 min to reduce their activity. Subsequently, six psyllids were picked up individually and rubbed against synnemata of each subsample of the fungus. After the psyllids were exposed to treated synnemata they were transferred to a test tube containing a live, detached leaf as described by Ammar and Hall (2011). The tubes were kept in an environmental chamber at 26°C and 14 h illumination per day. The psyllids were examined after two weeks to determine numbers of live insects (not infected), dead insects (without synnemata), and dead infected insects (synnemata present). As a positive control, a set of healthy psyllids was exposed to the in vitro culture treated with plain tap water. As a negative control, a set of healthy psyllids not exposed to the fungus were placed directly onto detached leaves.

Percentages of psyllids that died following exposure to the various treatments were analyzed as a completely randomized test with eight replications using PROC ANOVA (SAS Institute 2008), and mean separations among treatments were investigated using Fisher's LSD test ($P = 0.05$). Percentage data were arcsine-transformed prior to statistical analyses (Gomez and Gomez 1984).

Results

Seasonal incidence of adult *Diaphorina citri* killed by *Hirsutella citriformis*

Over the entire field survey, a weekly mean ± SE of 0.03 ± 0.01 live adult *D. citri* was observed per pair of mature leaves. A weekly mean of 0.05 ± 0.003 live adults was observed in 2006 compared to 0.02 ± 0.003 in 2007. Live adults were observed at an average rate of one per 33 samples (leaf pairs), frequently in the range of one per 15–25 samples, and occasionally more than one per ten samples. A peak equivalent to one live adult per 1.5 samples (three leaves) was observed during May 2006. The maximum number of live adults observed per sample (leaf pair) during the study was nine. With respect to incidence of *H. citriformis*, a weekly mean of 0.01 ± 0.0004 mummified cadavers was observed per pair of mature leaves. A weekly mean of 23.3 ± 1.1% of the adults observed were dead and mummified. The largest mean number of mummies per leaf pair observed per single sample date was 0.04 ± 0.01 (11 Oct 2006), equivalent to one mummy per 25 samples. The maximum number of mummified cadavers observed per sample during the study was four. No correlation was found between numbers of live adults and mummies ($r = -0.13$, $P = 0.17$, $N = 118$).

Mummified cadavers were most abundant during 2006 from summer through winter with a peak during early October (Fig. 2a). In contrast, during 2007 mummies were nearly absent during the summer but again were abundant during fall and winter (no single peak). Means of 0.02 ± 0.002 mummies per leaf pair and of 26.7 ± 2.4% adults mummified were observed during July–September 2006. During this same interval in 2007, means of only 0.0004 ± 0.0002 mummies per leaf pair and of only 2.4 ± 1.6% adults mummified were observed. Few or no mummies were observed on sample dates in May–June during either 2006 or 2007 (a mean of 0.002 ± 0.001 in 2006 and of 0.0002 ± 0.0002 in 2007), even though large numbers of psyllid hosts were available especially during May and June 2006 (Fig. 2b). Percentages of adults observed to be dead and mummified sometimes exceeded 75% during fall and winter, and 100% of adults observed per leaf pair were mummified on two sample dates during January and early March 2007.

A mean of 2.2 ± 0.3 cm cumulative rainfall occurred weekly during the study. Mean, maximum and minimum air temperatures averaged 22.7 ± 0.4, 29.5 ± 0.4, and 17.4 ± 0.5°C, and RH averaged 76.9 ± 0.1%. April and May were months during which RH was consistently the lowest, with daily averages ranging from 60 to 70% and an average of only 8.0 ± 0.8 h per day of RH $\geq$80%. Numbers of live adults on mature leaves were positively correlated with rain and air temperatures during the seven days prior to an assessment while numbers of mummies and proportions of adults mummified were negatively correlated with these variables (Table 2). Relative humidity variables (e.g., RH, RH90, RH80) during the seven days prior to an assessment were positively correlated with the number of mummified adults. RH90 over the 60 days prior to an assessment had the highest correlation with both number of mummies and proportion of adults mummified. When a temporal lag of seven days was used, the number of live adults could only be related to temperature while the proportion of mummified adults was negatively correlated with both precipitation and temperature.

A total of 42 individual mummified cadavers of *D. citri* were ultimately examined weekly to determine how long they remained on leaves. These included cadavers first observed on leaves during the months of July, August, October, November and December. The cadavers were observed to remain on leaves for a mean of 67.6 ± 6.9 days. The maximum observed duration of a mummified cadaver on a leaf was 168 days and the minimum was seven days. The number of days cadavers remained on leaves was positively correlated with rain ($r = 0.79$, $P = < 0.0001$), negatively correlated with mean daily air temperature ($r = -0.50$, $P = 0.0008$), and not correlated with mean daily RH ($r = -0.27$, $P = 0.09$) ($N = 42$ for each analysis).

Spatial distribution of *Hirsutella citriformis* based on mummified cadavers

Adult *D. citri* mummified by *H. citriformis* were observed at least once in every tree sampled during the study. Taylor's Power Law analyses were conducted on numbers of live and mummified adults excluding data from sample dates in which no adults were observed. Therefore, 115 and 81 of the sampling dates were included in the spatial analysis of live adult and mummified populations, respectively (Table 3). Comparisons (99% confidence intervals) of regression slopes (b) to a value of 1.0 indicated that, on any given sample date, live adults and mummified cadavers each were weakly aggregated within and among trees (Table 3). Over all samples on any given sample date, the dispersion of live adults was weakly aggregated while the dispersion of mummies was random. Contour plots revealed that live adults tended to be most prevalent in the southern half of the grove. Mummies were also most prevalent in this area (Fig. 3).

Effect of chemicals used in citrus on infectivity of *Hirsutella citriformis*

Copper sulfate pentahydrate (Magna-Bon), aluminum tris (Aliette) and alpha-keto/humic acids (KeyPlex) did not reduce the infectivity of *H. citriformis* based on percentages of psyllids that died after being exposed to fungus treated with these chemicals compared to fungus treated with plain water (Table 4). Copper hydroxide (Kocide), petroleum oil and sulfur were toxic to the fungus: an average of only 8% of adult *D. citri* died and produced synnemata when exposed to *H. citriformis* treated with any one of these three chemicals.

Discussion

Our study examined the phenology of *H. citriformis*, an entomopathogen of the invasive Asian citrus psyllid in Florida, USA. The significance of this vector with regard to HLB transmission in Florida, and throughout citrus growing regions of the world, supports research pursuits to improve understanding of its interactions with native and exotic natural enemies. This topic is of particular interest to growers intent on integrating biological control with other pest management tactics including both chemical and cultural approaches. The ability to elucidate the impact of biotic and abiotic factors on the biology of this host-entomopathogen interaction, as well as gaining information about how particular chemical

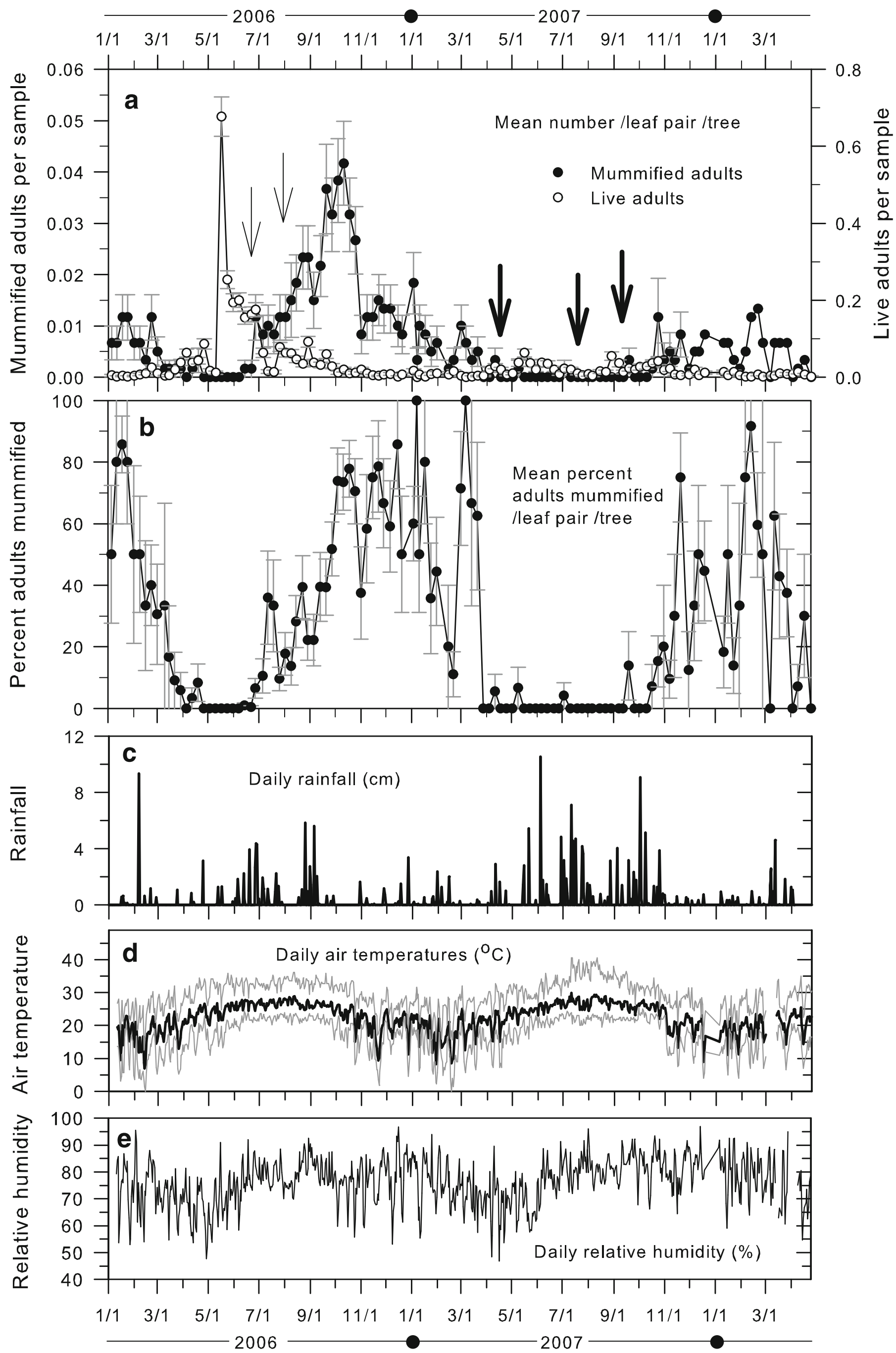

Fig. 2 Seasonal incidence of adult *Diaphorina citri* cadavers mummified by *Hirsutella citriformis* and environmental conditions, January 2006–April 2008. **a** Mean numbers of live adults and mummified cadavers per sample date (note the different *Y*-axis scales). The thin downward arrows indicate dates petroleum oils were applied at moderately high rates. The bold downward arrows indicate dates copper and petroleum oil were applied in combination each at moderately high rates. **b** Mean percentages of adult *D. citri* observed to be infected by *H. citriformis*. The error margins in panels **a** and **b** reflect SE among trees within each sample date. Information on rainfall, air temperature (mean, minimum, maximum) and humidity is presented in panels (**c**), (**d**) and (**e**), respectively

control regimens may influence this dynamic, are key elements we have begun to address here.

Populations of adult *D. citri* infesting mature citrus leaves are usually relatively small (Hall 2009). For example, a mean of 0.05 ± 0.01 live adults per pair of mature leaves was reported during 2005 and 2006 (Hall et al. 2008) in the same trees sampled in this report. Compared to a density of 0.05 adults per sample, densities of adults observed during the current study were similar during 2006 but lower during 2007. Yearly fluctuations in psyllid populations in trees not treated with insecticides may occur as a result of

Table 2 Spearman rank correlation analyses comparing counts of live, mummified, and proportion of mummified *Diaphorina citri* on mature citrus leaves for 118 sampling dates to various weather variables for listed time window lengths

Assessment variable	Weather variable[a]	No temporal lag		7 days temporal lag	
		7-day	60-day	7-day	14-day
Total number of live adults per sample date	Rain	0.27**	0.19	0.16	0.19*
	Temp	0.55***	0.37**	0.58***	0.54***
	RH	−0.01	−0.24*	−0.10	−0.19
	RH90	0.10	−0.28	0.01	−0.09
	RH80	0.07	−0.33*	−0.06	−0.14
Total number of mummified adults per sample date	Rain	−0.19*	−0.06	−0.23*	−0.19*
	Temp	−0.23*	0.08	−0.10	−0.07
	RH	0.23*	0.34**	0.23*	0.28**
	RH90	0.43**	0.82***	0.53***	0.71***
	RH80	0.42**	0.76***	0.46**	0.57***
Proportion of mummified adults per sample date	Rain	−0.31**	−0.22*	−0.33**	−0.33**
	Temp	−0.52***	−0.21*	−0.41***	−0.39***
	RH	0.13	0.33**	0.18	0.25*
	RH90	0.26	0.67***	0.38*	0.53**
	RH80	0.27	0.67***	0.39*	0.48**

Spearman correlations were significant at or below the 0.05(*), 0.005(**), or 0.0001(***) level of confidence

[a] Rain = total precipitation (cm), Temp = mean temperature (°C), RH = mean relative humidity (%) over the time period, RH90 = number of hours with relative humidity ≥90%, RH80 = number of hours with relative humidity ≥80%

Table 3 Results of Taylor's Power Law analyses on dispersion of live and mummified adult *Diaphorina citri* in a block of citrus trees

Adult psyllids	Variance source	*a*	*b*	99% Confidence interval for *b*[a]	r^2	*df*
Live	Within trees	1.32	1.10	1.07–1.12	0.94	960
	Among trees	0.17	1.21	1.13–1.29	0.93	115
	Overall	1.38	1.06	1.03–1.08	0.99	115
Mummified	Within trees	1.40	1.11	1.05–1.17	0.87	345
	Among trees	0.12	1.13	1.03–1.23	0.91	81
	Overall	1.22	1.03	0.96–1.09	0.96	81

[a] All *b* values were significantly greater than 1.0 (indicating aggregated dispersion) expect for the *b* associated with mummified adults over all samples per sample date (these cadavers were randomly dispersed)

differences in environmental conditions and particularly those that affect the relative amount of flush generated by trees over time, as *D. citri* oviposits exclusively on young flush and nymphs develop exclusively on flush (Husain and Nath 1927). Differences in flush abundance during spring might explain why the psyllid was more abundant during May and June in 2006 than in 2007, although we did not investigate flush abundance in this study. Reduced infestation levels during May and June 2007 were not attributed to applications of petroleum oil—oils alone are not currently recommended for managing *D. citri* in Florida citrus (Rogers et al. 2011) because they are less effective than conventional insecticides unless perhaps applied repetitively at high rates.

Mummified cadavers of adult *D. citri* killed by *H. citriformis* were most common during the fall and winter months during both study years, although they were generally less abundant during winter 2007/2008 than winter 2006/2007. Low incidence of mummies was observed during the months of April–June of each year, which we attributed to low levels of relative

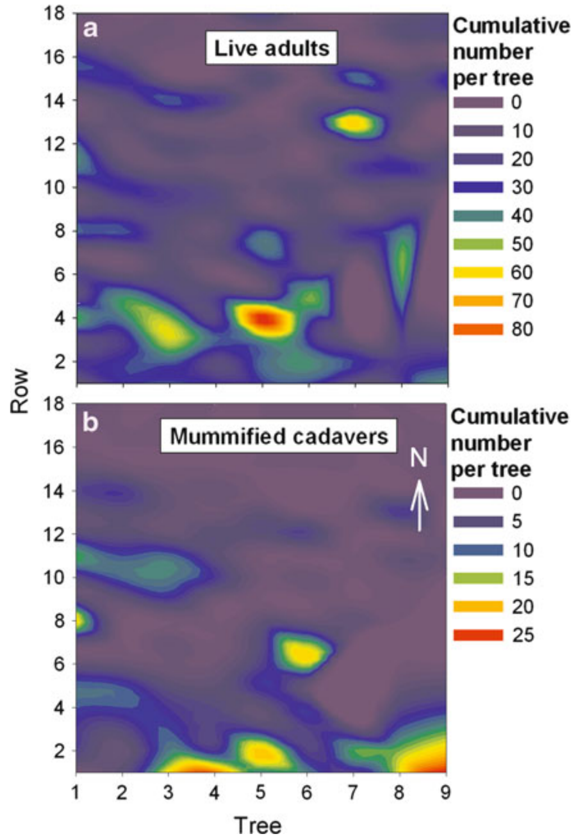

Fig. 3 Spatial distribution of live (**a**) and mummified cadavers (**b**) of adult *Diaphorina citri* across a 0.7 ha block of orange trees based on cumulative numbers observed per tree (January 2006–April 2008)

humidity. We found a positive correlation between relative humidity variables and total number or proportion of mummified adults whether humidity data were lagged by seven days or not. Étienne et al. (2001) reported that adult *D. citri* killed by *H. citriformis* were more common in Guadeloupe during periods when relative humidity was greater than 80%. Detailed studies on optimal relative humidity levels for this fungus remain to be conducted. During our study, a near absence of mummies between July and September 2007 could not be attributed to low relative humidity but most likely was a consequence of either (1) copper and oil applications that reduced the growth, infectivity and abundance of the fungus, or (2) a host density dependent response of the fungus to diminished psyllid populations in the grove. Higher amounts of rainfall occurred during July–September in 2007 than in 2006 and may have contributed to reduced numbers of diseased *D. citri* observed in 2007, as rainfall and occurrence of mummified adults were negatively correlated. Rainfall might inhibit movement of healthy psyllids in and around trees, reducing numbers that come into contact with conidia-bearing synnemata.

Adult psyllids exposed to mummified cadavers of *D. citri* generally contract the disease and die within 7–9 days, and synnemata first begin to appear seven -days later (Meyer et al. 2007). Some period of time elapses before the biology and behavior of a newly

Table 4 Effect of chemicals commonly used in citrus on infectivity of *Hirsutella citriformis* based on percentage mortality of adult *Diaphorina citri* exposed to fungus treated with different chemicals (consult Table 1 for chemical rates)

Hirsutella treated with the indicated chemical	Mean ± SE percentage mortality[a]	
	Adults killed[b]	Adults killed, mummies produced synnemata[c]
Alpha-Keto acids and humic acid	100.0 ± 0.0a	94.0 ± 2.9a
Aluminum tris	93.8 ± 4.4a	85.4 ± 5.8a
Copper sulfate pentahydrate	95.8 ± 2.7a	91.7 ± 4.5a
Water	100.0 ± 0.0a	95.8 ± 4.2a
Copper hydroxide	36.3 ± 14.0b	26.5 ± 12.1b
Petroleum oil	41.7 ± 15.4bc	19.3 ± 9.6bc
Elemental sulfur	18.8 ± 6.6cd	4.2 ± 4.2cd
Psyllids not exposed to *Hirsutella*	4.2 ± 2.7d	0.0 ± 0.0d

[a] Means in the same column followed by the same letter are not significantly different ($P = 0.05$), Fisher's LSD test. Analyses on arcsine-transformed data, raw data means presented

[b] $F_{7,53} = 23.0$, $P = <0.0001$

[c] $F_{7,53} = 36.1$, $P = <0.0001$

infected adult are disrupted by the disease: we have occasionally observed mummies on yellow sticky traps (DGH personal observations), indicating that an infected adult psyllid may disperse prior to succumbing to the disease. Mummified cadavers adhered to citrus leaves serve as point sources of infection for healthy adult *D. citri*, and under laboratory conditions remain infectious for more than ten weeks (Meyer et al. 2007). Adults become infected when they come into contact with conidial-bearing synnemata associated with mummified cadavers. Healthy adults may come into contact with the cadavers accidentally, the chances of which are increased due to horizontal growth of synnemata extending from a cadaver. Mummified cadavers are often situated in a feeding position typically assumed by live adults, and healthy adults in search of a mate may be attracted to a cadaver by visual or chemical cues. Whether *H. citriformis* produces semiochemicals that attract healthy adults for disease dissemination purposes remains to be investigated. We observed that most mummified cadavers remained adhered to leaves in excess of two months. The duration decreased as mean daily air temperature increased but increased during rainy periods. Free moisture in the form of rain or dew on leaves apparently is favorable for the persistence of mummies on leaves. Our data did not show that relative humidity affected how long mummies remained on leaves. How the pathogen is maintained following periods when no mummified cadavers were observed on leaves is unclear—perhaps some were present but at too low of a density for detection under our sampling regimen.

Healthy adults and cadavers of *D. citri* killed by *H. citriformis* were mildly aggregated across the grove both within and among trees and were observed on both upper and lower leaf surfaces. The dispersion of adult *D. citri* in citrus had previously been shown to be aggregated (Costa et al. 2010; Sétamou et al. 2008). Throughout the two-year study, there was a prevalence of both live adults and mummified cadavers in the southern half of the grove. This was not unexpected assuming host-density dependent fungal dispersal, but it remains unknown why live adults were more common in this area of the grove.

On average over the two-year study, 23% of adults observed on mature leaves were killed by *H. citriformis*. This percentage was high enough to suggest that the fungus may have had significant negative impact on population levels of the psyllid. However, a better measure of the impact of the fungus might have included enumeration of all adults in a tree, notably adults on flush. Hall et al. (2008) reported nearly six times as many adults on flush shoots than on pairs of mature leaves. It is therefore probable that the actual percentage of adults killed by *H. citriformis* was lower than an average of 23%, possibly as low as 3–4% based on a sixfold difference in numbers of adults on flush versus mature leaves. In addition to not taking into account numbers of adults on flush, definite conclusions regarding levels of biological control of the psyllid afforded by the fungus could not be made from this study because numbers of mummified cadavers on mature leaves might not adequately reflect actual numbers of adults killed by the fungus—some infected adults end up mummified on tree limbs (Meyer et al. 2007), which we did not sample. Mummified cadavers have only been reported to form on mature citrus leaves and tree limbs. However, some adults might die as they feed on flush but do not remain as cadavers on flush due to the expansion process of developing leaves.

Evaluations of the toxicity of various chemicals used in citrus to *H. citriformis* were prompted by the marked difference in field-incidence of mummified cadavers during late summer between 2006 and 2007. We hypothesized that the fungus was more prevalent during late summer 2006 than 2007 because copper and oil were applied at moderately high rates in combination several times during summer 2007 and not during summer 2006. Other species of *Hirsutella* have been shown to be negatively impacted by pesticides to various extents including *H. brownorum* Minter et Brady; *H. kirchneri* (Rostrup) Minter, Brady et Hall; *H. necatrix* Minter, Brady et Hall; *H. nodulosa* (Petch); and *H. thompsonii* Fisher (Tkaczuk et al. 2004; Tkaczuk and Miętkiewski 2005; McCoy and Lye 1995). In laboratory studies, we found that petroleum oil, sulfur and copper hydroxide at their highest labeled rates were detrimental to *H. citriformis*. This suggested that such chemical control regimens may have a negative impact on biological control by the fungus in the field. Spray oils and other chemical treatments to citrus might negate biological control by reducing infectivity and growth of *H. citriformis* as well as the longevity of mummies on leaves. A number of pesticides including fungicides, insecticides and herbicides have been shown to negatively impact growth of various *Hirsutella* species (Tkaczuk et al. 2004; Tkaczuk and Miętkiewski 2005). Copper hydroxide was previously shown to be toxic to a *Hirsutella* species (*thompsonii*) (McCoy and Lye 1995). Not all pesticides may be toxic to *Hirsutella*, for example the miticide avermectin showed no detrimental effect to *H. thompsonii* (McCoy et al. 1982). Citrus growers interested in capitalizing on *H. citriformis* as a biological control agent of *D. citri* should consider avoiding or reducing the use of copper hydroxide, oil or sulfur, particularly at high rates. Although copper and oil may have suppressed the fungus in the grove during 2007, application rates of these chemicals were less than the rates we tested. Thus, it remains possible that the differences between 2006 and 2007 in summer abundance of mummified cadavers was a consequence of more live adults being present during 2006 than 2007. The outcomes of our study provide a basis for future research needed to further characterize the interaction between *D. citri* and *H. citriformis* on an increased scale and to explore the possibility of integrating this entomopathogen into sustainable management programs for psyllid and HLB control.

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