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Behavioral Assay on Asian Citrus Psyllid Attraction to Orange Jasmine

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Abstract The Asian citrus psyllid (ACP) is an important pest because it transmits a bacterium putatively responsible for huanglongbing, a devastating citrus disease. Research on ACP chemical ecology is of interest with respect to identifying attractants and repellents for managing the psyllid. We report on an assay for investigating ACP attraction to the foliar odor of one of its host plants, orange jasmine (*Murraya exotica* L.). Flush shoots from this plant were placed into 25 dram vials covered with a paper wrapper, a lid with a small entrance hole was snapped onto each vial, the vials were placed into a small cage, adult ACP were released into the cage, and the location of the adults was determined 24 h later. A positive response required an adult to find and enter a vial. When single males or females were released, they were attracted into vials with flush 64 % of the time. At release rates of 25, 50 or 100 adults with 30 repetitions for each rate, relatively large mean percentages of adults (ca. 83 %) positively responded to

Highlights • A behavioral assay was investigated for assessing adult Asian citrus psyllid (ACP) attraction to flush shoots of orange jasmine (*Murraya exotica*), a plant genotype highly attractive to ACP.
• Flush shoots were placed into 25 dram vials, a lid with a small hole was snapped onto each vial, the vials were placed in a cage, and adult ACP were released into the cage - a positive response required adults to find and enter a vial within 24 h.
• When single adults were released, they were found inside vials 64 % of the time. When groups of 25 to 100 adults were released, 83 % were found in vials.
• When vial lids were treated with an insect repellent, only 8 % of adults were found in vials at the end of the assay period.

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flush shoots. ACP can escape from the assay vial and will do so if flush is not present. Working with ACP in empty vials, fluoropolymer resin applied to the inside of vials reduced escapes but did not eliminate them. An entrapment funnel for reducing escapes was tested, but it reduced ACP response rates. A good method of preventing escapes remains to be developed. When two vials of flush were placed in the assay cage and one was treated with a candidate repellent, only 8 % of adults settled in the vial with the repellent.

Keywords *Diaphorina citri* · huanglongbing · citrus greening · behavior assay

Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psylloidea), is an invasive insect pest of citrus in the United States, first found during 1998 in Florida (Halbert and Manjunath 2004). ACP is an important pest of citrus because it transmits bacteria putatively responsible for a serious disease of citrus known as huanglongbing (= citrus greening disease) (Bové 2006). Huanglongbing (HLB) attributed to the bacterium "*Candidatus* Liberibacter asiaticus" (CLas) was first found in south Florida during 2005 and subsequently has spread throughout the state's citrus growing regions (Gottwald 2010). Citrus trees affected by HLB severely decline in health and productivity (Bové 2006; Gottwald 2010). HLB has no cure, and the disease has thus put the Florida citrus industry in serious jeopardy. Meanwhile, ACP and huanglongbing are spreading to other areas in the Americas (Halbert et al. 2010; Hall et al. 2013).

Grower, regulatory and research personnel have keen interest in attractants and repellents for monitoring and managing ACP. ACP has been shown to be attracted to young flush shoots of its host plants, which is not surprising because ACP reproductive biology is dependent on young flush (Husain and Nath 1927). Plant volatiles may be principally involved in attracting ACP to flush, and in fact several chemical volatiles associated with ACP host plants have been shown to attract ACP (Patt and Sétamou 2010). While research on insect attractants and repellents can be conducted in the field, it is often most productive to initiate such research in a laboratory under controlled conditions. Insect responses to different chemical volatiles can be assessed using glass olfactometers with filtered/humidified air delivery systems. Wind tunnels can also be useful for investigating insect responses to volatiles. However, insect species vary in their behavioral responses under different conditions and therefore some laboratory procedures for investigating attractants or repellents may be better or worse than others. Some assays restrict the number of individual insects tested at a time. Some insect species may require flight space to respond to a volatile while others may need to walk. Gauging whether a response is positive may sometimes be somewhat arbitrary, e.g., an insect must walk past a certain point in an olfactometer in a certain amount of time. Moderate to large percentages of insects may show no or inconsistent responses using some assay procedures, thus requiring additional repetitions to complete a response dataset.

Behavioral studies on ACP responses to chemical volatiles have largely been conducted using traditional glass olfactometers with charcoal-filtered, humidified air.

Wenninger et al. (2009) and Patt and Sétamou (2010) studied ACP responses to host plant volatiles associated with flush shoots using a glass Y-tube olfactometer. Mann et al. (2011) used a glass T-maze olfactometer to assess individual adult female behavioral responses to garlic and wild onion and reported that sulfur volatiles associated with these plant species repelled ACP. Onagbola et al. (2011) reported that ACP responses to citrus host plant volatiles were inhibited by guava leaf volatiles in experiments with glass Y-tube and four-arm olfactometers. Zaka et al. (2010) used a Y-tube olfactometer to assess ACP responses to guava volatiles, but they also used a cage assay in which adult ACP were released into a cage and given a choice to settle on citrus and guava leaves in flasks of water. Ruan et al. (2015) used a cage study with small potted plants to study ACP attraction and settling behavior.

The objective of research presented here was to assess effectiveness of a relatively inexpensive, simple assay for investigating ACP responses to volatiles associated with young flush shoots of orange jasmine [*Murraya exotica* L. (= *Murraya paniculata* auct. non.)], a plant species known to be highly attractive to ACP (Patt and Sétamou 2010; Hall et al. 2013). The basic assay procedures are as follows. A young flush shoot is placed into a large plastic vial; a lid with a small entrance hole is snapped onto the vial; one to several such vials is/are placed together onto the floor of a small cage; adults are released into the cage; and the location of adults in the cage is determined 24 h later.

The vial assay described here is conceptually similar to containerized traps that release a volatile attractant, permit entry by the target insect, and then confine it. Examples include the McPhail trap for use with tephritid fruit flies (Steyskal 1977; Shelly et al. 2014) and pail traps for use with hematophagous arthropods (Muirhead-Thomson 1991; Burkett-Cadena et al. 2015; Springer et al. 2015). The design and arrangement of the vials necessitates that the test insect both fly and crawl to locate the source of test odor, thus it measures behavioral response in three dimensions rather than two dimensions as in a Y-tube. The assay also provides insects with more time to acclimate to the test area than does a Y-tube. Placing the odor source in a vial largely eliminates the influence of visual cues which are present in other types of settling assays in which an array of sprigs or leaf disks is offered to the test insect. Reported here are the results of several experiments conducted to assess adult ACP attraction to orange jasmine flush using these assay procedures. Of primary interest was how many ACP would find their way into assay vials and settle on a shoot (positive response) and the consistency in assay results.

Materials and Methods

ACP adults for these studies were obtained from a colony established in 2000 at the USDA-ARS U.S. Horticultural Research Laboratory (Fort Pierce, FL). The psyllids were originally collected from citrus in the field and subsequently reared in an air-conditioned greenhouse in cages containing orange jasmine until March 2010, when *Citrus macrophylla* Wester was substituted as the rearing plant. The colony is maintained using procedures similar to those described by Skelley and Hoy (2004), with no infusion of wild types. The colony is confirmed quarterly by qPRC (Li et al. 2006) to be free of CLas. ACP for the study were reared on *C. macrophylla*, but the adults were transferred to orange jasmine plants for a 3- to 6-day period prior to the assay as a

possible measure to enhance their recognition of this host plant. On Fridays, adult ACP (~7 days old) from *C. macrophylla* were placed into a cage with three orange jasmine plants containing new young flush. Adults from this cage of orange jasmine were subjected to the assay the following week on Monday, Tuesday, Wednesday, and Thursday, thus they ranged from about 10 to 13 days old.

The large plastic vials used for the assay were 25 dram clear-plastic tubes measuring 39 × 85 mm I.D. (diameter x height) (tube #8925, BioQuip Products, Inc., Gardenia, CA) with white snap-on plastic lids (Fig. 1a). A cork borer was used to cut a small hole through the plastic lid to serve as an entranceway into the vial (the hole was off-centered to help hide a vial's contents) (Fig. 1b). Prior to running the assay, the vials and lids were soaked in hot soapy (Alconox detergent, Alconox, Inc., White Plains, NY) water for several hours, rinsed with distilled water, and allowed to air dry.

Fresh, young jasmine flush shoots for the assay were excised from greenhouse plants, completely submerged in a beaker containing water, returned to a lab and processed immediately. We used flush shoots with two leaves, each with at least some young leaflets appropriate for oviposition (Fig. 1a). A 0.5 ml micro-centrifuge tube (catalog no. 1615–5500, Seal-Rite natural, USA Scientific, Inc., Orlando, FL) measuring 3.8 cm long (with the cap removed) was used to hold a flush shoot inside the assay

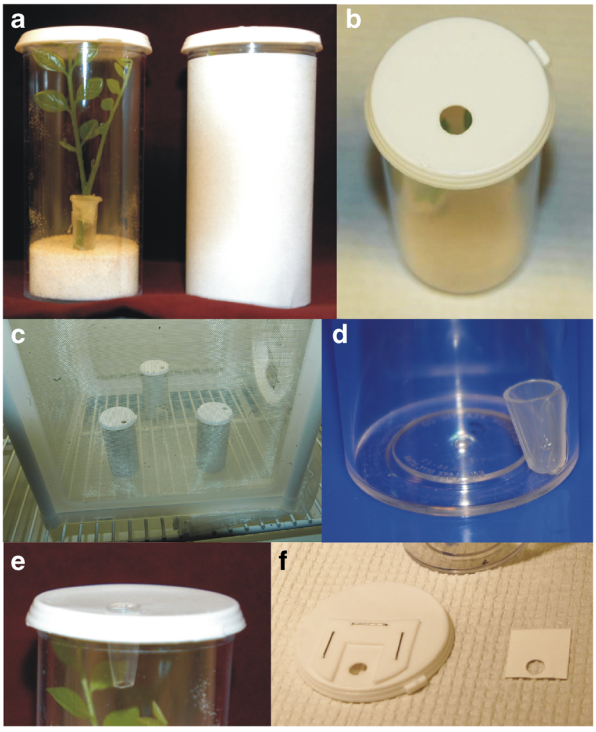

Fig. 1 Behavioral assay on Asian citrus psyllid attraction to orange jasmine. **a** To the *left*, an assay vial with cover removed showing orange jasmine flush shoot in centrifuge tube with water supported by sand, and to the *right* an assay vial with paper cover. **b** 7 mm entranceway through snap-on lid into an assay vial. **c** Three assay vials in the assay cage. **d** Plastic support alternative to sand. **e** Entrapment funnel with rim. **f** Assay vial lid with holder for blotter paper, and to the *right* a piece of blotter paper prepared for a repellency test

vial (Fig. 1a). Tap water was placed into the centrifuge tube, and the opening was sealed with Parafilm 'M' laboratory film (American National Can, Chicago, IL). The film was punctured, creating a small hole into the centrifuge tube, and then the cut end of a flush shoot was slipped through this small hole into the water. As an indicator of flush amount, the exposed length of each flush shoot leaf was recorded.

Twenty-five g of sand (Sakrete Natural Play Sand– Arena Fine, Bonsal American, Charlotte N.C.) was placed down into the bottom of the vial, providing a depth of about 1.5 cm (Fig. 1a). The sand had been cleaned and sterilized by sifting it with a #20 metal sieve, catching the sifted sand in a plastic tub; the tub with sand was flooded with tap water, the sand was stirred in the water and the water was decanted – this was repeated until the water decanted was clear; the sand in the tub was similarly rinsed an additional 6 times with distilled water, and finally placed in an aluminum pan in a drying oven for 2 days at 200 °C. After sand was added to the assay vial, the bottom end of the flush shoot's centrifuge tube was pressed down into the sand with the tube in a vertical position. The assay vial's lid was then snapped into place. A piece of light grey paper (plain printer paper, laser-jet printed with a Microsoft PowerPoint background - white, background 1, darker 15 %) was wrapped around and secured with tape (Scotch Magic Invisible Tape, 3M, St. Paul, MN) to the outside of the vial to hide the contents.

Depending on the experiment, two or three such vials with shoots were placed together onto the floor of a cubic cage (30 × 30 ×30 cm, Bugdorm-1 insect cage, DP1000, Megaview Science Co., Ltd., Taichung, Taiwan) (Fig. 1c). The assay cage was screened on three sides (24×24 mesh/6.45 cm^2 on two side panels, 32×32 mesh/6.45 cm^2 on the back panel) while the other three sides were made of a solid translucent material. A stocking net sleeve on one solid panel allowed access into the cage. When two vials were used, they were placed onto the cage floor about 16 cm apart, each about 3 cm from the center of the closest wall. For three vials, they were placed onto the cage floor positioned ~14 cm from each other similar to the points of a regular triangle. The cage was placed into an environmental chamber set at 28 °C, 14 h daily illumination, ambient relative humidity. Adult ACP for the assay were aspirated into a small glass tube, which was then placed onto the floor at the exact center of the cage. The glass tube lid was removed to allow the adults to exit, and their location in the cage was determined 24 h later.

Entranceway Diameter

Using these assay procedures, we conducted an experiment to compare ACP attraction into vials with three different size entrance holes: diameters of 6, 7 or 10 mm (cork borer #3, #4 or #7, respectively). One of the three vials in the cage was assigned each size. Four different release rates of ACP were studied: 1, 25, 50 and 100 adults. ACP adults were released into the cage and 24 h later the number of adults in each of the three vials was determined. For releases of multiple adults, the number of ACP for each release was counted as they were aspirated from rearing cages and again after each assay repetition. There were 30 repetitions of each release rate. For releases of single ACP, there were 30 repetitions of each gender.

Two environmental chambers (I-30BLL, Percival Scientific, Inc., Perry, IA) were utilized for the experiment, allowing two repetitions of each release rate to be studied at a time. The chambers were fitted with GE EcoLux F20T12-CW-

ECO light bulbs, two mounted on the ceiling of each chamber and two additional bulbs mounted 22 cm below the ceiling; the top of the assay cage was 2 cm below the lower bulbs. Temperature and relative humidity in each chamber was monitored with a WatchDog data logger (Spectrum Technologies, Inc., Aurora, IL).

The experiment was conducted between February and July 2014, usually two repetitions on each of four successive days a week. Each day a different release rate was studied, thus two repetitions of each release rate were usually completed within five successive days. For each release rate, the position of each of the three assay vials in the cage was systematically rotated among repetitions.

Analyses of variance were conducted using PROC ANOVA or PROC GLM (SAS Institute 2010), and mean comparisons among treatments were investigated using Tukey's HSD test. Percentage data were arcsine-transformed (Gomez and Gomez 1984). Data associated with releases of 25, 50 or 100 adults were analyzed together while data from releases of single adults were analyzed separately. All statistical tests were conducted at the $P=0.05$ level of significance.

Plastic Receptacle Alternative to Sand

As an alternative to sand for holding a flush shoot's micro-centrifuge tube, we took a second centrifuge tube and cut the bottom end from the top about 1.5 cm above the bottom tip. The upper half with cap was discarded and the bottom end was used as a receptacle for a centrifuge tube containing flush. We used hot glue to secure the bottom half against the inside wall of the assay vial with the bottom tip touching the floor of the assay vial (Fig. 1d). An experiment was then conducted to compare ACP attraction into vials containing flush shoots supported by sand versus into vials with shoots supported by the plastic receptacle. The experiment was identical in almost all respects to the one conducted on entranceway sizes with the following notable exceptions. Only two vials were placed into the assay cage, one with a flush shoot supported by sand and one with a shoot supported by a plastic receptacle. A release rate of 50 adults and entranceway diameter of 6 mm were used, and the assay was repeated 30 times using two environmental chambers. The experiment was initiated during mid-May and concluded by mid-June. Arcsine-transformed percentages of adults ending up in vials with and without sand were compared using a *t*-test (PROC TTEST, SAS Institute 2010).

Attraction into Vials with and Without a Cover Hiding Contents

An experiment was conducted to compare ACP attraction and settling behavior in assay vials with and without the light-grey paper covering. The basic procedures used followed those of the experiment on entranceway sizes with the following notable exceptions. Only two vials were placed into the assay cage, one with a paper covering and one without a cover. A release rate of 50 adults and entranceway diameter of 6 mm were used, and the assay was repeated 30 times. The experiment was conducted during March and was expedited by using four environmental chambers. Arcsine-transformed percentages of adults ending up in vials with and without covers were compared using a *t*-test (PROC TTEST, SAS Institute 2010).

Preventing Escape from the Assay Vial

It might often be desirable to ensure that once an adult enters a vial that it cannot exit. We therefore conducted two experiments, one in which the inside wall of a vial was treated with fluoropolymer resin (PTFE-30), known to prevent some arthropods from climbing out of containers (Porter et al. 2013), and one in which adults had to walk through an entrapment funnel attached to the inside of a lid's entranceway.

For the experiment with fluoropolymer resin, we applied 'INSECT-a-SLIP' (catalog no. 2871B, BioQuip Products, Inc., Rancho Dominguez, CA) to the inside surface of the assay vial. The resin was poured into an empty assay vial and the vial was rotated until the entire inner surface was coated. The vial was then turned upside down and placed on toweling to allow excess fluid to drain out, after which the vial was turned upright and allowed to dry overnight. The inside of the lid was treated in a similar manner. Adult ACP for the study were aspirated into glass tubes and held in a refrigerator until they were subdued enough to transfer them into assay vials. Fifteen adult ACP were placed directly into an empty assay vial treated with resin and 15 were placed in an empty vial not treated; lids with a 10 mm entranceway were snapped into place; the two vials were transferred into the assay cage; and the number of ACP remaining in each vial was determined 24 h later. Orange jasmine flush was omitted in an effort to discourage ACP from staying in the vials. We repeated the assay 30 times during July-August using four environmental chambers. Arcsine-transformed percentages of adults escaping vials with and without the resin were compared using a *t*-test (PROC TTEST, SAS Institute 2010).

For the experiment with the entrapment funnel, a small funnel was crafted from a 0.2 ml PCR tube (catalog no. 1402–4300, TempAssure natural with attached dome cap, USA Scientific, Inc., Orlando, FL) measuring 2 cm in length, ~6 mm outside diameter at the top just below a rim – the cap and bottom 0.4 cm of the tube were cut off and discarded, and the remaining 1.6 cm portion of the tube was slipped down through a vial's 6 mm entranceway. Because the PCR tube was tapered, the inside diameter of the funnel tapered from just under 6 mm at the top down to about 3.5 mm. Two configurations of the funnel were investigated, one in which the upper rim of the tube was retained and the funnel was pushed through a lid's entrance hole all the way down to the rim (Fig. 1e), and a second in which the rim was cut off and the funnel was pushed down into the hole until the upper edge was flush with the upper surface of the lid. Three vials containing orange jasmine flush were prepared following procedures previously described, one vial without a funnel, one with a funnel with a rim, and one with a funnel without a rim. Fifty ACP were introduced into the assay cage. The assay was repeated 30 times during January-February using two environmental chambers. Analyses of variance on arcsine-transformed percentages were conducted using PROC ANOVA (SAS Institute 2010), and mean comparisons among treatments were investigated using Tukey's HSD test.

Testing a Repellent

Of interest was if ACP attraction into vials containing orange jasmine flush could be reduced or prevented by adding a repellent. A candidate chemical (a proprietary repellent blend) based on its known repellency to other insect species was investigated.

Two vials containing orange jasmine flush were prepared following the assay procedures previously described, with entranceways measuring 6 mm in diameter. A 6 mm hole was punched near one edge of a 2 cm^2 piece of chromatography blotting paper (3MM CHR Whatman® Chromatography Paper, Whatman International Ltd., Maidstone, England). This piece of blotting paper was placed onto the assay vial's lid such that the paper's hole was aligned with the lid's hole. A U-shaped piece of plastic cut from the lid of an assay vial was stapled onto the lid as a frame for the blotting paper, under which the edges of the blotting paper were slipped anchoring it in place (Fig. 1f). Then 2.5 uL of the liquid repellent was applied to the blotting paper associated with one vial and allowed to air dry for around an hour, after which two assay vials containing orange jasmine flush were placed into the assay cage, one with and one without the repellent. Seventy ACP adults were released into the cage and the location of the adults was determine 24 h later. The assay was repeated 16 times during January using three environmental chambers. Arcsine-transformed percentages of adults ending up in vials with and without the candidate repellent were compared using a *t*-test (PROC TTEST, SAS Institute 2010).

Results and Discussion

Over all experiments, air temperatures averaged 28 °C and relative humidity averaged 33 %. Environmental conditions recorded during each experiment are detailed in Online Resource 1.

Entranceway Diameter

Releases of Single Adults Among the 30 releases of single females or males, at the end of the assay period 1 female and 3 male ACP were found dead inside the small glass tube or on the floor of the cage. These repetitions were excluded from analyses because the adults may have been injured during the collection process and unable to find a vial and make a choice before dying. Females were found in vials in 17 of 29 repetitions (58.6 %) and males were found in 19 of 27 (70.4 %). These percentages were not significantly different ($F_{1,25}$=0.66, P=0.42). The average positive response rate over all 56 releases of single adults was 64±7 %. Among adults that were found in one of the three vials at the end of the assay, there were no significant differences among the entranceway sizes with respect to which vial the adults settled in (Table 1).

Release Rates of 25, 50 and 100 Adults Numbers of adults actually released were close to the target release rates of 25, 50 and 100: means±SEM of 25.8±0.3, 53.1±0.6 and 104.2±1.4 adults, respectively. The percentage of ACP found inside vials after the 24 h assay was 84±2, 79±2, and 85±1 % at release rates of 25, 50 and 100 adults per cage, respectively, with an overall mean of 83±1 %. There were no significant differences between the three percentages ($F_{2,58}$=2.1, P=0.13). These percentages exclude individuals found dead in the small glass tube or on the floor of the cage. However, only relatively low numbers of adults were found dead at any location 24 h after release and most dead adults were found outside of vials (Table 2). With respect to consistency of the assay, results of an inspection of the outcome of individual

Table 1 Attraction by single adult Asian citrus psyllids (ACP) to flush shoots of orange jasmine in vials with a large, medium or small entrance hole (10, 7, or 6 mm dia.)

Location of ACP at end of assay	Mean percent of repetitions ACP settled at the indicated location		
	Releases of single males (N=27)	Releases of single females (N=29)	Releases of single adults (over both genders, N=56)
Vial with 10 mm hole	22.2a	24.1a	23.2a
Vial with 7 mm hole	25.9a	13.8a	19.6a
Vial with 6 mm hole	22.2a	20.7a	21.4a
Not in a vial	29.6a	41.4a	35.7a

Means in the same column followed by the same letter are not significantly different, Tukey's HSD test. For males, $F_{3,78}$=0.1, P=0.94. For females, $F_{3,84}$=1.6, P=0.19. Over both genders, $F_{3,191}$=1.4, P=0.25. Analyses on arcsine-transformed percentages, raw means presented

repetitions of each release rate underscored the importance of running more than a few repetitions (Table 2). While an average of 20 to 30 % adults was found in each vial after 24 h, in some individual repetitions fewer than 5 % or more than 55 % adults were found in some vials regardless of the release rate or size of the entranceway.

A significant positive correlation was found between the number of ACP released and the number found in vials 24 h later, but increasing the number released from 25 to 100 did not increase the percentage ending up in vials (Table 3). Increasing the amount of flush in a vial increased numbers of ACP entering and settling in the vial, as there was a significant positive correlation between percentages of adults ending up in vials and the amount of flush in a vial based on the combined length of the flush shoot leaves. Although female ACP may produce a sex pheromone that attracts males (Wenninger et al. 2008), there was no correlation between numbers of females ending up in vials and either the total number or percentage of ACP settling in a vial. A significant, positive correlation was found between percentages of ACP in vials and the diameter of the entranceway. At a release rate of 25 ACP, significantly greater percentages of ACP were found in vials with 7 and 10 mm diameter entranceways (Table 2). At release rates of 50 or 100 per cage, larger percentages of ACP tended to be found in vials with 7 and 10 mm entrances but the differences among entrance sizes were not significant. An analysis of variance over all data with main effects entrance diameter, replication and release rate indicated that there were no significant differences among release rates in percentages of ACP settling in vials with different entrance diameters ($F_{2,325}$=0.05, P=0.94) but significantly greater percentages of ACP entered and settled in vials with the 10 mm diameter entrance ($F_{3,325}$=19.3, P=<0.0001, Table 4).

Plastic Receptacle Alternative to Sand

The mean percentage of ACP found inside vials after the 24 h assay was 74±2 % and, among the 30 repetitions, ranged from 49 to 86 %. Females constituted 48±2 % of the adults in vials with sand and 46±2 % of the adults in vials without sand. A mean of 51 ±3 % of the adults was found in vials with shoots supported by sand (range 20 to 79 %)

Table 2 Asian citrus psyllid (ACP) attraction to flush shoots of orange jasmine in vials with large, medium or small entrance holes (10, 7, or 6 mm dia.)

Location of ACP at end of assay	Mean percent ACP (live + dead) at each location	Range in percent ACP (live + dead) at each location	Mean number live + dead ACP	Mean number dead ACP	Mean percent ACP female	Mean length (cm) of flush in vials
Release rate 25 adults						
Vial with 10 mm hole	28.8±2.7 a	8–54	7.4 ab	0.7 b	52.6 a	7.8 a
Vial with 7 mm hole	20.8±2.1 ab	4–46	5.4 b	0.5 b	49.9 ab	8.0 a
Vial with 6 mm hole	19.0±2.4 b	0–52	4.9 b	0.2 b	35.5 b	8.0 a
Not in a vial	31.3±2.5 a	4–59	8.1 a	3.3 a	52.0 a	–
Release rate 50 adults						
Vial with 10 mm hole	30.1±3.1 a	4–74	16.0 a	1.2 b	47.3 ab	8.2 a
Vial with 7 mm hole	28.0±2.4 a	7–56	11.2 a	0.9 b	42.3 b	8.2 a
Vial with 6 mm hole	21.2±2.8 a	4–72	11.2 a	0.5 b	46.0 ab	8.3 a
Not in a vial	20.7±1.6 a	6–38	11.2 a	3.1 a	56.5 a	–
Release rate 100 adults						
Vial with 10 mm hole	30.4±2.1 a	7–50	31.8 a	2.3 b	46.7 b	8.1 a
Vial with 7 mm hole	24.8±2.4 a	2–55	25.7 ab	1.6 b	46.6 b	7.7 a
Vial with 6 mm hole	22.1±1.8 a	8–45	23.1 b	1.8 b	49.1 ab	7.8 a
Not in a vial	22.7±1.3 a	6–35	23.6 ab	9.0 a	57.1 a	–

For each release rate, means followed by the same letter are not significantly different (P=0.05), Tukey's HSD test. Analyses on arcsine-transformed percentages, raw data means presented

Table 3 Results of correlation analyses (Pearson's coefficient) comparing numbers of Asian citrus psyllids (ACP) released into the cage and numbers and percentages of adults settling on flush in vials inside the cage. Analyses on data for release rates of 25, 50 and 100 per cage, includes dead ACP found on the floor of the cage at the end of the assay period

	Total number settled in vials	Percent settled in vials	Flush amount (cm)	Proportion of adults in vials that were female	Diameter of vial entrance hole
Total number released	0.71****	0.00 ns	−0.03 ns	0.04 ns	0.00 ns
Total number settled in vials	1.0	0.60****	0.11 ns	0.03 ns	0.18***
Percent settled in vials		1.0	0.20***	0.04 ns	0.28****
Flush amount (cm)			1.0	0.01 ns	0.00 ns
Proportion of adults in vials that were female				1.0	0.12*

ns not significant

*P=.05; **P=.01; ***P=.001; ****P=0.0001; N=270 for each analysis

and 49±3 % of the adults was found in vials with shoots supported by the plastic receptacle (range 21 to 80 %). There was no significant difference between these mean percentages (t=0.37, P=0.71, N=30).

Attraction into Vials with and Without a Cover Hiding Contents

The mean percentage of ACP found inside assay vials after the 24 h assay was 73±3 % and, among the 30 repetitions, ranged from 46 to 100 %. Females constituted 44±2 % of the adults in vials with covers and 46±3 % of the adults in vials without covers. Among ACP ending up in vials, a mean of 58±4 % of the adults was found in vials with covers (range 13 to 93 %) and 42±4 % of the adults was found in vials without covers (range 7 to 88 %); these mean percentages were significantly different (t=2.9, P=0.006, N=30). Lower percentages of ACP were attracted into and settled on flush in vials without covers even though it is plausible that adults would have been attracted to

Table 4 Asian citrus psyllid ACP) attraction to flush shoots of orange jasmine in vials with large, medium or small entrance holes (10, 7, or 5 mm dia.), analysis over all releases of 25, 50 and 100 adults

Location of ACP at end of assay	Mean percent ACP settled at the indicated location
Vial with 10 mm hole	32.8a
Vial with 7 mm hole	26.9b
Vial with 6 mm hole	22.8b
Not in a vial	17.5c

$F_{3,327}$=19.4, P=<0.0001. Means followed by the same letter are not significantly different, Tukey's HSD test. Analyses on arcsine-transformed percentages, raw data means presented

the light-green color of flush (visible to the human eye through the clear wall of the assay vial). Perhaps for some reason ACP were unable to see this green color, for example due to a glare. Another possibility is that adults may prefer to walk on paper than the plastic surface of the assay vial.

Preventing Escape from the Assay Vial

Nearly all (99±0.4 %) adult ACP had escaped the plain empty vial by the end of the assay period. Significantly fewer adults escaped from vials treated with resin (t=−11.3, P=<0.0001, N=30), but the percentage escaping was relatively large (62±3 %).

In assays with the entrapment funnels, a mean of 74±3 % of the adults ended up in vials. Among all adults released in the assay cage, a mean of 18±2 % ACP ended up in the vial with a rimmed funnel, 17±2 % ended up in the vial with a funnel without a rim, and 40±3 % ended up in the vial without an entrapment funnel. The differences between percentages of ACP ending up in vials with and without entrapment funnels were significant ($F_{3,87}$=16.3, P=<0.0001). Reductions in numbers of ACP entering vials with funnels may have been a consequence of the smaller entranceway created by the funnels, or perhaps the funnels reduced the amount of flush volatiles emitting from a vial.

Testing a Repellent

A mean of 73±3 % of the adults ended up in the vial without the candidate repellent and 8±1 % ended up in the vial with the repellent (t=14.9, P=<0.0001, N=30). The results support that the assay can be useful for screening chemicals potentially repellent to ACP. We have not disclosed the identity of the repellent we studied due to reasons associated with an invention disclosure.

Assay Considerations

Relatively large percentages of ACP positively responded to odors associated with orange jasmine flush under conditions of the assay. The assay accommodated releases of up to 100 adults at a time and could have accommodated more. Greater numbers of adults ended up settled in vials with a 10 mm diameter entranceway at release rates of 25 to 100 adults, but there were no differences among the three sizes when single adults were released. Possibly increasing the number of adults released increases competition to enter a vial and this competition is accentuated by smaller entranceways. Although we have not carefully monitored adult activity during the assay period, we have observed adults to immediately begin exiting the glass release tube, some springing up away from the tube. Adults have been observed on the ceiling and sides of the assay cage. We assume adults explore the inside cage by walking and that, as they come into the vicinity of a vial's entranceway, they detect flush volatiles and are attracted into the vial. We also assume that the adults enter vials during the photophase based on observations by Wenninger and Hall (2007) that ACP activity is primarily diurnal.

Based on the results of the assays using 30 repetitions, one would expect single psyllids released into the cage to find and enter a vial ~64 % of the time when vials are baited with orange jasmine flush. Thirty repetitions may usually be adequate for

achieving this positive response rate assuming the error margin is acceptable (in our assays, a standard error of the mean of about 6 %), with no increase in the response rate at up to around 60 repetitions based on combined data across genders. Higher response rates by individual ACP might occur if vials are baited with a larger quantity of flush. Achieving a specific number of repetitions with single adults may require running additional repetitions if any adults die before entering a vial, which occurred 3 to 10 % of the time in our assays with 30 repetitions. When groups of 25, 50 or 100 adults were released into the assay cage, in each of 30 repetitions at least some adults were found in all three vials except in one repetition at a release rate of 25, in which adults were only found in two vials.

With respect to using sand to support a flush shoot, considerable efforts were deemed necessary to eliminate any possible odors associated with sand that might influence ACP behavior. Using the plastic receptacle for flush shoots instead of sand was easier, and percentages of adults ending up in vials with these plastic receptacles were not significantly different from percentages ending in vials supported by sand. However, there might be some advantages for using sand, e.g., studies on ACP responses to orange jasmine in vials under different humidity regimes, in which case different amounts of water might be added to the sand.

A number of parameters associated with the basic ACP attraction assay presented here remain to be explored. For example, an assay duration shorter than 24 h would expedite research on attractants and repellents. Twelve hours might be adequate based on research reported by Zaka et al. (2010) on a cage study assessing ACP attraction and settling behavior on citrus shoots with and without guava shoots – due to differences in assay protocols, a 12 h protocol using our assay would need to be assessed. Response rates of adults during the assay could vary according to their physiological state (age, the host plant they are reared on, if they have been starved, whether they are infected by CLas, and other factors). Changes in temperature, humidity, light intensity, and photoperiod could also affect response rates of ACP. Response rates might be different using a larger assay cage or larger assay vials. Preliminary research using vials with and without orange jasmine flush showed that at the end of the assay at least small numbers of adults were often found in the vial without flush, thus an attractant is not necessarily required for an adult to explore inside a vial. This raises the question, do some adults enter a vial and then exit from it? Adults entering a vial with an orange jasmine flush shoot are usually found feeding on the shoot at the end of the assay. Unless there were too many adults entering a vial and trying to colonize a shoot, there might be little reason to exit from a vial. If there was not a flush shoot or other host plant material in a vial, an adult might not stay even if the vial contained a strong chemical attractant. It would therefore be desirable to ensure that once an adult enters a vial that it cannot exit. Treating the inside surface of the assay vial with fluoropolymer resin reduced but did not prevent escapes. The entrapment funnel that we tested might have helped reduce numbers of ACP escaping from a vial, but we did not pursue it further because significantly fewer adults entered vials with the funnel. A good method to ensure adults cannot escape from a vial remains to be developed.

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