Application of Orange Oil to Pre-Release Holding Boxes Increases the Mating Success of Sterile Males of the Mediterranean Fruit Fly in Field Cage Trials (Diptera: Tephritidae)

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Abstract. Previous research showed that exposure to the aroma of orange oil (*Citrus sinensis* L.) increased the mating success of male Mediterranean fruit flies (medfly), *Ceratitis capitata* (Wiedemann). This work, however, involved the exposure of small groups of males (n = 25) in small containers (volume 400 ml). In implementing the Sterile Insect Technique (SIT), several programs use plastic adult rearing containers (PARC boxes, 0.48 by 0.60 by 0.33 m) to hold newly emerged males before release (≈ 36,000 males per box). The objective of this study was to determine whether the application of orange oil to individual PARC boxes increases the mating competitiveness of sterile *C. capitata* males. Orange oil was applied to paper placed on the screened opening on the top of PARC boxes. Two doses (0.25 and 1.0 ml) were tested, and the paper was either covered by a Petri dish lid (to reduce volatilization) or was left uncovered. Using field cages, we ran mating trials in which oil-exposed (treated) or non-exposed (control) sterile males competed against males from a recently established (from wild flies) colony for females from the same colony. In all trials, the wild-derived males obtained significantly more matings than the sterile males. In those trials involving uncovered, oil-laden paper, there was no difference in mating success between treated and control sterile males. However, when the paper was covered, the treated males obtained significantly more matings than the control males at both doses. These results are compared with similar, previously conducted experiments involving ginger root oil, and the potential use of orange oil in medfly SIT is discussed.

Key words: Sterile Insect Technique, *Ceratitis capitata*, mating success, orange oil

Introduction

The Sterile Insect Technique (SIT) is an environmentally benign approach for suppressing or eradicating insect pests and is widely used in integrated programs against tephritid fruit flies, particularly the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Hendrichs et al. 2002, Klassen 2005). The technique involves mass production, irradiation (sterilization), and release of males of the target species into the environment. Matings between sterile males and wild females yield infertile eggs, which reduces the reproductive potential of the wild population. Thus, the success of the SIT depends, to a large degree, on the ability of released, sterile males to attract and obtain matings with wild females. This factor is especially important for species, such as *C. capitata*, characterized by 'complex' mating behavior (Lance and McInnis 2005), in which males produce multiple sexual signals using various modalities (visual, acoustic, and olfactory), and females display a high degree of mate selection based apparently on male courtship performance (Whittier et al. 1992, 1994). Unfortunately, the mass-rearing procedures inherent to the SIT often lead to a reduction in the mating competitiveness of released medfly males, and sterile males typically have low

mating success relative to wild males (Rossler 1975, Lance et al. 2000). Thus, an important challenge for the SIT is the development of simple and inexpensive means to enhance the mating performance of released, sterile males. One potentially productive approach involves the pre-release exposure of males to particular chemical attractants. In particular, a series of experiments (Shelly et al. 2005 and references therein) have demonstrated that exposure to the aroma of ginger root oil (*Zingiber officinale* Roscoe), containing the known male attractant α-copaene (Flath et al. 1994a, b, Nishida et al. 2000), greatly enhances the mating success of sterile males of *C. capitata*. While initial studies involved exposure to small groups of males (25 individuals) held in small containers (400 ml), recent data showed that applications of 0.0625 - 1.0 ml of ginger root oil to the screen-covered opening of a larger storage boxes used in several SIT programs (Plastic Adult Rearing Containers, or PARC boxes; 0.48 by 0.60 by 0.33 m holding ≈ 36,000 males) significantly increased the mating competitiveness of the sterile males (Shelly et al. 2004a).

The goal of the present study was to determine whether the application of orange oil to PARC boxes similarly increases the mating success of sterile males of *C. capitata*. Earlier research (Papadopoulos et al. 2001, Shelly et al. 2004b, Shelly 2005) demonstrated that male mating success was increased following exposure to the peel of oranges (*Citrus sinensis* L). In addition, tests involving small groups of wild males (n = 250 showed that the aroma of commercially available orange oil boosted mating success to the same degree as ginger root oil (Shelly et al. 2004b, Papadopoulos et al. 2006). In light of these previous findings, we anticipated that orange oil also would be effective when applied to the large number of sterile males held in PARC boxes.

Materials and Methods

Study insects. Mass-reared males were from a temperature sensitive lethal (*tsl*) strain (Vienna-7/Tol-99) produced by the California Department of Food and Agriculture Hawaii Fruit Fly Rearing Facility, Waimanalo, Oahu. This strain possesses a sex-linked mutation, such that treating eggs with high temperature kills female zygotes, thereby allowing production of males exclusively (Franz et al. 1996). Male pupae were dyed (fluorescent pink) and irradiated 2 d before eclosion in air at 150 Gy of gamma irradiation from a ^{137}Cs source. After irradiation, pupae were placed in six paper bags (100 ml pupae/bag; 1 ml contains ≈ 60 pupae), which, in turn, were placed in individual PARC boxes held at 22-26 °C, 50 – 90% RH, and a 12:12 (L:D) photoperiod of artificial and natural light. Under these conditions, most adult emergence occurred 2 d after pupal placement, and emerging *tsl* males were fed a sugar-agar gel placed on the screened opening on top of the box.

Because wild flies were not available in sufficiently large numbers, we used flies from a recently established colony (8-9 generations removed from the wild; hereafter referred to as REC flies) started with > 500 adults reared from coffee fruits collected on Kauai. The colony was housed in a screen cage and provided with a sugar (sucrose) and protein (yeast hydrolysate) mixture (3:1 by weight), water, and an oviposition substrate (perforated plastic vials containing small sponges soaked in lemon juice). Eggs were placed on standard larval diet (Tanaka et al. 1969) in plastic containers over vermiculite for pupation. Adults used in the mating trials were separated by sex within 24 h of eclosion, well before reaching sexual maturity at 5 – 7 d of age (T.E.S., unpublished data) and kept in screen-covered buckets (5-liter volume; 100-125 flies per bucket) with ample food (sugar-protein mixture) and water.

Exposure to orange oil. Orange oil (Oil Orange Valencia C.P. FCC), which contains trace amounts of α-copaene (S. Young, personal communication), was obtained from Citrus and Allied Essences Ltd. (Lake Success, NY). For all tests, the oil was applied to the PARC box for a 24-h period starting 3 d after the day of peak adult emergence, i.e., expo-

sure was terminated when the *tsl* males were 4 d old. We did not investigate the effects of applying the oil at the time of pupal placement, because such 'pupal-adult' exposure to ginger root oil had a smaller effect on male mating performance (Shelly et al. 2004a). Two doses of orange oil (0.25 and 1.0 ml) were tested. Using a pipette, we applied the oil to 10-cm squares of blotter paper and placed the paper on the screened opening on the top of the PARC box. In addition to the 2 doses, we also presented the oil-impregnated blotter paper in 2 ways, either uncovered or covered with a Petri dish lid. In our laboratory environment, uncovered blotter paper dried out within a 24-h period, and, as shown below, appeared to have no effect on male mating success. By placing the lid over the blotter paper, we sought to reduce volatilization of the oil away from the box and potentially increase its effect on male behavior. For each PARC box set up with orange oil, we also set up a PARC box that received the same quantity of pupae but no oil, thus yielding non-exposed (control) *tsl* males. To mimic SIT programs, we stacked an empty PARC box on top of all treated and control boxes. In all instances, boxes receiving the orange oil treatment were kept in a separate room from those not receiving the treatment to prevent inadvertent exposure of control *tsl* males. REC males were not exposed to orange oil in any test.

Immediately following exposure, we removed one paper bag from the PARC box, quickly transferred it to screen cages, and gently shook it to disperse the males. Sterile males were stored in plastic buckets with ample food (sugar-agar gel) for use the following day (i.e., the majority of *tsl* males were 5 d old when tested), except in one test in which *tsl* males, after exposure to 1 ml of orange oil with the Petri dish lid covering the blotter paper, were held 3 d before testing to examine the effect of oil exposure over a slightly longer interval. Control *tsl* males were removed from the PARC box on the same day and handled in the same manner as the treated males. Following removal from the PARC boxes, control and treated *tsl* males were stored in separate rooms.

Mating trials. Mating trials were conducted at the USDA-ARS-PBARC laboratory in Honolulu. Groups of 75 REC males (7 – 11 d old), 75 REC females (8 – 13 d old), and either 75 oil-exposed *tsl* males or 75 non-exposed *tsl* males were released between 0800-0830 hrs (males were released 15 min before females) in nylon mesh field cages (3 m diameter, 2.5 m high) that contained two artificial trees (each 2 m tall with approximately 450 leaves resembling those of *Ficus benjamina* L.). Artificial trees were used, because they provided a chemically neutral substrate on which the flies displayed the entire complement of natural activities. The cages were monitored over a 3-h period, mating pairs were collected in vials, and the males were identified under a UV light (pink dye = *tsl* male, undyed = REC male). On most test days, we set up 4 field cages, 2 with treated *tsl* males and 2 with control *tsl* males. The *tsl* males from a given PARC box were used in only one field cage, thus *tsl* males from four different PARC boxes were typically used on a given test day. Air temperature ranged between 26-31 °C during the trials.

Results

When the oil-laden paper was left uncovered, orange oil had no apparent effect on the mating success of *tsl* males at either dose. At both doses, REC males achieved significantly more matings than control or treated *tsl* males, which, in turn, did not differ significantly from one another (Table 1). With the paper uncovered, treated *tsl* males obtained, on average, 28% and 32% of the total matings per replicate following exposure to 0.25 or 1.0 ml of orange oil, respectively, proportions that were statistically indistinguishable from one another or from those observed for the corresponding control *tsl* males (Fig. 1).

At both doses, REC males also had a mating advantage over control and treated *tsl* males when the oil-laden paper was covered by a Petri dish lid (Table 1). However, in contrast to

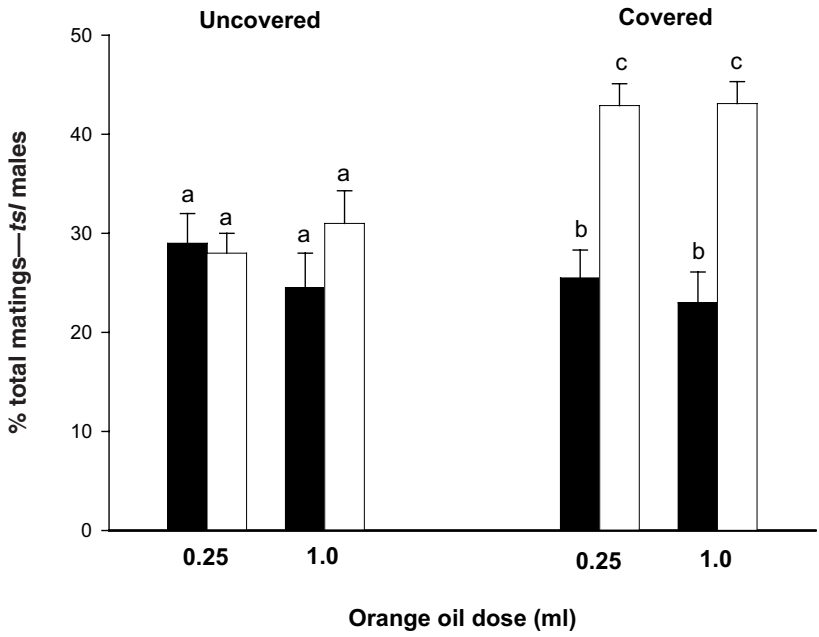

Figure 1. Relative mating success of non-exposed (control - solid bars) and orange oil-exposed (treated - open bars) *tsl* males in mating trials. Bar heights represent means (± 1 SE; n = 12 in all cases). Letters above bars refer to comparisons of means across doses for uncovered and covered paper, respectively; means indicated by different letters were significantly different (P = 0.05) using the Tukey test following ANOVA (with arcsine transformed values).

the preceding experiment, treated *tsl* males exposed to covered paper obtained significantly more matings than control *tsl* males at either dose. Among treated males, the number of matings obtained per replicate was similar for the 2 doses. As these results suggest, treated *tsl* males achieved, on average, a higher proportion of the total matings per replicate than control *tsl* males for both doses, and the mean relative mating success was similar for males exposed to 0.25 versus 1.0 ml (Fig. 1). Pooling data across the 2 doses, we found that, on average, *tsl* males exposed to covered, oil-laden paper accounted for a significantly higher proportion of the total matings per replicate than *tsl* males exposed to uncovered, oil-laden paper (43% versus 30%, respectively; $P < 0.001$, t-test using arcsine transformed data).

At the single dose tested (1 ml), trials involving *tsl* males held for the 3 d after exposure to covered blotter paper showed that, while REC males obtained significantly more matings than control *tsl* males, REC males and treated *tsl* males had similar mating success (Table 1). Consistent with this result, treated *tsl* males obtained a significantly greater number of matings than control *tsl* males.

Table 1. Number of matings obtained by REC and *tsl* males in mating trials.

Dose (ml)	Treatment[b]	Matings per replicate[c] REC males	*tsl* males
Uncovered[a]			
0.25	Control	28.7 (1.5)[A, e]	11.7 (1.2)[B, f]
	Treated	29.6 (1.8)[C, e]	11.5 (1.2)[D, f]
1.0	Control	31.5 (2.5)[E, e]	9.9 (1.4)[F, f]
	Treated	26.0 (1.3)[G, e]	12.2 (1.6)[H, f]
Covered			
0.25	Control	30.7 (2.2)[I, g]	10.5 (1.4)[J, j]
	Treated	29.5 (2.3)[K, g]	22.2 (1.9)[L, k]
1.0	Control	31.7 (2.4)[M, g]	9.8 (1.2)[N, j]
	Treated	27.8 (2.1)[P, g]	20.0 (0.9)[R, k]
1.0	Control[d]	27.5 (2.5)[S, g]	8.8 (1.6)[T, j]
	Treated[d]	21.8 (2.0)[U, h]	19.1 (1.8)[U, k]

[a]Uncovered – Petri dish was not placed over oil-laden, blotter paper on PARC box; Covered - Petri dish lid was placed over blotter paper.
[b]Control – *tsl* males not exposed to orange oil; Treated – *tsl* males exposed to orange oil.
[c]Values are means (± 1 SE; n = 12 in all cases). Upper case letters refer to within-row, pairwise comparisons (REC vs. *tsl* males); lower case letters refer to within-column comparisons (across dose and treatment category) for trials involving uncovered and covered blotter paper, respectively. For a given comparison, means followed by different letters were significantly different (P = 0.05) using the t-test (within-row) or the Tukey test following ANOVA (within-column). Assumptions of normality and equal variance were met in all cases.
[d]*tsl* males tested 3 d after exposure (as opposed to 1 d in all other tests).

Discussion

This study shows that orange oil, applied to PARC boxes on covered blotter paper, increases the mating competitiveness of sterile, *tsl* male medflies. This increase was not sufficiently large to overcome the mating advantage of REC males, but in absolute and relative terms, oil-exposed *tsl* males obtained significantly more matings than non-exposed *tsl* males. The 2 doses tested produced a similar increase in mating success, and, as was the case for ginger root oil (Shelly et al. 2004a), even smaller doses of orange oil may boost mating performance significantly. Even 3 d after exposure to covered paper (at 1.0 ml dose), treated *tsl* males displayed increased mating success relative to non-exposed males.

In contrast, when the oil-laden paper was left uncovered, there was no detectable effect of exposure on the mating success of *tsl* males. For both doses, uncovered blotter paper was completely dry following the 24 h exposure period, whereas a wet spot of orange oil was evident when the paper was covered by a Petri dish lid. Thus, while high volatility is obviously essential for aromatization, the volatilization rate and loss (away from the box) of (uncovered) orange oil may have been so high as to limit its impact on male behavior. While

a plausible explanation, recent experiments (T.E.S., unpubl. data) with ginger root oil reveal that the mating success of male medflies is increased following an exposure interval as short as 5 min, suggesting that, above some low threshold, the duration of exposure to ginger root oil is not a key determinant of male mating success. This implies, in turn, that the ineffectiveness of uncovered orange oil did not reflect high volatility alone but also some difference in its chemical constituency relative to ginger root oil, which rendered it effective only after relatively long exposure durations. In this regard, it is interesting to note that -copaene is present at a much higher concentration in ginger root oil than in orange oil (0.4% versus 'trace' [< 0.01%], respectively; S. Young, personal communication).

Even when a Petri dish lid was used to retard volatilization, the increase observed in mating success of *tsl* males was less pronounced than observed following exposure to ginger root oil. For example, after application of 1.0 ml of ginger root oil to PARC boxes, *tsl* males obtained approximately the same numbers of matings as REC males and accounted for an average of 53% of the total matings per replicate (Shelly et al. 2004a). In the present study, exposure to 1.0 ml of orange oil on covered paper boosted the mating success of treated males above that observed for control males. However, *tsl* males exposed in this manner were still competitively inferior to REC males and accounted for approximately 42% of the total matings per replicate.

Although apparently less effective, orange oil is much cheaper than ginger root oil. For example, for a purchase of 5 kg, orange oil costs approximately $9 per kg compared to $66 per kg for ginger root oil (prices for March 2006, Citrus and Allied Essences Ltd.). To potentially improve the efficacy of orange oil exposure and realize this cost savings, there is a need to develop a slow-release, delivery system that extends the time interval during which males are exposed to key volatiles. In addition, data collected using ginger root oil (T.E.S., unpubl. data) indicate that entire rooms (holding several hundred PARC boxes) can be aromatized, with *tsl* males gaining a mating advantage. Whether or not orange oil is likewise effective at this scale is unknown and merits testing.

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