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Abstract

In the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), exposure to α-copaene, a botanically derived male attractant, and ginger root oil (GRO), *Zingiber officinale* (Roscoe), which contains α-copaene, increased the mating success of wild males, and GRO enhanced mating competitiveness of mass-reared males from a bisexual, mass-reared strain. The present study extends this research by examining the effects of GRO exposure on the mating success of mass-reared males from a genetic sexing strain based on a temperature sensitive lethal (*tsl*) mutation. Such strains are currently used for nearly all sterile insect technique (SIT) programs for this insect. In addition, potential negative effects of GRO exposure on male survival and female remating propensity were investigated. Following exposure to GRO, males from the *tsl* mass-reared strain showed enhanced mating performance against wild-like males from two recently established colonies. Against wild-like males from a Guatemala strain, the proportion of matings obtained by males from the *tsl* mass-reared strain increased from 16% per replicate for non-exposed (control) individuals to 30% for GRO-exposed (treated) individuals. Against wild-like males from a Madeira strain, the proportion of matings obtained by treated, *tsl* mass-reared males was 39% per replicate compared to only 16% for control, *tsl* mass-reared males. Survivorship was similar between GRO-exposed and non-exposed males from the *tsl* strain, and females mated initially to treated or control *tsl* mass-reared males displayed similar remating propensity. The application of pre-release, GRO-exposure to males in the SIT against medfly is discussed.

Key Words: *Ceratitis capitata*, sterile insect technique, ginger root oil, mating behavior

Resumen

Los machos silvestres de la mosca mediterránea *Ceratitis capitata* (Wiedemann) expuestos a a-copaene, un atrayente del macho derivado botanicamente y el aceite de la raiz de jengibre (GRO), *Zingiber officinale* (Roscoe), lo cual contiene a-copaene, aumentó el éxito copulatorio de los machos silvestres, y GRO mejoró la habilidad de machos criados en masa de un variedad sexual genética para competir en el apareamiento. Este estudio extiende los efectos de la exposición a GRO sobre el éxito copulatorio de machos criados masivamente de una variedad genética sexual basados sobre una mutación letal sensible de la temperatura (*tsl*). Actualmente, se usa tales variedades en casi todos los programas de técnica de insecto estéril (TIS) para este insecto. Además, se investigaron los efectos negativos potenciales de la exposición a GRO sobre la sobrevivencia de los machos y la propensidad de las hembras para aparearse de nuevo. Después de exponerlos a GRO, los machos de la variedad *tsl* criados en masa mostraron un mayor capacidad para aparearse contra los machos del tipo-silvestre de dos colonias recién establecidas. Contra los machos del tipo-silvestre de la variedad de Guatemala, la proporción de los apareamientos obtenidos de los machos de la variedad *tsl* criados en masa aumentó de 16% por réplica por individuos no expuestos (control) a 30% por los individuos expuestos a GRO (tratado). Contra los machos del tipo-silvestre de la variedad de Madeira, la proporción de los apareamientos obtenidos por los machos *tsl* criados en masa tratados fué 39% por réplica comparado a solo 16% en el control del los machos *tsl* criados en masa. La sobrevivencia de los machos de la variedad *tsl* criados fué similar entre los expuestos a GRO y no expuestos, y las hembras que se aparearon inicialmente con los machos *tsl* tratados o el control machos tsl criados en masa muestraron una propensidad similar para aparearse de nuevo. Se discute la aplicación de exponer los machos a GRO en TIS, antes de liberarlos contra la mosca mediterranean.

The Sterile Insect Technique (SIT) is an environmentally friendly approach for suppressing or eradicating insect pests and is widely used in integrated programs against tephritid fruit fly pests, particularly the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Hendrichs et al. 1995). The technique involves mass production, sterilization (using irradiation), 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 population of the wild population. Because the success of the SIT depends on the ability of mass-reared, sterile males to obtain copulations with wild females, it is essential that the mass-rearing protocol itself does not produce males with diminished mating competitiveness (Calkins 1984).

Unfortunately, the mass-rearing procedures inherent to the SIT do often lead to a reduction in the mating competitiveness and viability of released *C. capitata* males, particularly in long-established strains (Shelly et al. 1994; Lance et al. 2000). The deterioration of strains results from a combination of factors, including genetic drift with its concomitant loss of genetic variability and intense artificial selection imposed by the laboratory environment (Leppla & Ozaki 1991). Aside from changing strains frequently, there is currently no effective way to avoid this decrease in quality. The now widespread use of genetic sexing strains to produce only males for release in medfly SIT programs (Robinson et al. 1999) has led to the development of filter rearing systems to maintain stability of the strains (Caceres & Fisher 2000). This low stress, low population size system, as well as providing a more efficient means for strain replacement, also provides a more natural environment for the flies and hence helps maintain some of the important behavioral components of fly quality.

This filter system notwithstanding, however, a constant and important challenge for the SIT is the development of simple and inexpensive means to enhance the performance of released, sterile *C. capitata* males in the wild. Recent research (Shelly 2001; Shelly & McInnis 2001) in Hawaii reveals that exposure of males to particular attractants, especially those containing the compound α-copaene, provides a strong advantage in mating competition over non-exposed males. For example, in one series of trials, Shelly and McInnis (2001) recorded the mating frequency of sterile, mass-reared males (from a standard bisexual strain) and wild males competing for wild females. In the absence of chemical exposure, mass-reared males obtained only 26% of all matings. However, following exposure to the odor of ginger root oil (GRO), which contains α-copaene, the mating frequencies were reversed, and mass-reared males accounted for 75% of all matings.

The primary objective of the present study was to determine whether exposure to GRO similarly enhanced the mating competitiveness of males from a genetic sexing strain based on a temperature sensitive lethal (*tsl*) mutation. This is a crucial undertaking as sexing strains based on the *tsl* mutation are being used in nearly all SIT programs for the medfly. As described below, males

from this strain were tested against males from two laboratory colonies established recently with wild flies from Guatemala and Madeira, respectively. In addition, we examined potential effects of GRO exposure on male survival and remating propensity of females.

MATERIALS AND METHODS

Study Animals

Laboratory flies were from a *tsl* strain designated Vienna-7-2000 Mix that had been mass-reared at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria, for approximately 1 year (12 generations) prior to this study. The strain was originally constructed through interpopulational crosses, involving wild flies from 7 different populations worldwide. Like other *tsl* strains, Vienna-7-2000 Mix possesses a sex-linked mutation, such that treating eggs with high temperature kills all female zygotes, thereby allowing production of males exclusively (Franz et al. 1996). The rearing and release of only males provides economic and biological benefits, and consequently *tsl* genetic sexing strains are replacing bisexual strains in mass-rearing facilities worldwide (Hendrichs et al. 1995). Males used in the present study were irradiated as pupae 1 day prior to eclosion at 100 Gy using a cobalt 60 gamma irradiator, and following irradiation pupae were at dusted with a pink fluorescent dye for identification. Larvae of the *tsl* mass-reared strain were reared on a wheat bran diet, and adults were fed a mixture of sugar and protein (yeast hydrolysate) (3:1 by volume).

Wild-like flies were from 2 recently colonized strains. One was started using flies reared from coffee berries (*Coffea arabica* L.) collected in Guatemala, and the other was initiated using flies reared from oranges (*Citrus sinensis* (L.)) collected in Madeira. In both cases, larval development occurred *in situ*, pupation occurred in sawdust, and pupae were shipped by air to the Seibersdorf facility. Both colonies were started with 500-1000 adults, and papayas were supplied for oviposition and subsequent larval development. Adults were fed the same protein-sugar mixture as the *tsl* mass-reared males. When used in the present study, the Guatemala- and Madeira-derived flies were 4 and 2 generations removed from the wild, respectively. Adults of the wild-like strains were separated by sex within 1-2 d of emergence, well before attaining sexual maturity at 7-9 days.

Mating Tests

We performed 3 experiments that measured the influence of GRO on the mating competitiveness of *tsl* mass-reared males. First, wild-like males from

the Guatemala colony competed against GRO-exposed (treated) or non-exposed (control) *tsl* mass-reared males for wild-like females from the Guatemala line. Second, wild-like males from the Madeira colony competed against treated or control *tsl* mass-reared males for wild-like females from the Madeira line. Wild-like males were not exposed to GRO in either of these experiments. Third, treated, *tsl* mass-reared males competed against control, *tsl* mass-reared males for wild-like females from the Guatemala line.

To expose *tsl* mass-reared males, we applied 20 μl of GRO to a small piece of filter paper using a microcapillary pipette. The paper was then placed on the bottom of a transparent, plastic drinking cup (400 ml volume), 25 males were placed in the cup using an aspirator, and the cup was covered with nylon screening. Exposure commenced at 1100 h and continued until 1400 h. As reported previously (Shelly 2001), GRO acted as an arrestant, and males were generally quiescent. Males did not aggregate near the filter paper and were not observed touching it. Following exposure, treated males were removed from the exposure cups, placed in holding containers, and moved to an adjacent room. The exposure procedure was conducted in a room isolated from any other flies to prevent inadvertent exposure of control males. For all tests, treated males were used the day after exposure to GRO.

Mating tests were conducted during July-August, 2001, in 2 field-cages (2.5 m high; 3 m diameter) enclosed in a greenhouse (6 by 4.8 by 3 m high) at the Seibersdorf facility. Sections of the ceiling and sides of the greenhouse were open, allowing free movement of air. During the tests, temperature generally ranged from 21-29°C, and relative humidity ranged from 45-80%. Each cage contained 3 potted orange trees whose collective canopy reached the top of the tent. In experiments 1 and 2, 100 *tsl* mass-reared males (control or treated) and 100 wild-like males and females from the same strain were used. In experiment 3, 100 treated and 100 control *tsl* mass-reared males and 100 wild-like females from the Guatemala colony were used. When tested, *tsl* mass-reared males were 6-7 days old, and wild-like flies were 9-12 days old.

Males were released at 0815 h, and females were released 20 min later. Mating pairs were collected over the next 4 h. At the end of a test, all flies were removed from the field cages; new flies were used for all tests. For experiments 1 and 2, males were identified as *tsl* mass-reared or wild-like by squashing the head of each male and examining the everted ptilinum under ultraviolet light for the presence/absence of pink dye. For experiment 3 (where only *tsl* mass-reared males were used), males were marked 2 days before testing by cooling them for several minutes and placing a dot of enamel paint on the thorax. This

procedure had no obvious adverse effects, and males resumed normal activities within minutes of handling. For a given trial, we marked either control or treated males, alternating the marked group between successive trials.

Female Remating

The remating frequency of wild-like females from the Guatemala strain was compared among individuals mated first to 1) wild-like males from the Guatemala line, 2) *tsl* mass-reared males exposed to GRO, and 3) non-exposed, *tsl* mass-reared males. To obtain initial matings, 40-60 females (9-11 d old) were placed in transparent, plastic cages (41 by 31 by 31 cm) with an equal number of males from a given strain or treatment (*tsl* mass-reared males: 6-9 d old; wild-like males: 9-15 d old). Flies were placed in the cages at 0900 h, and mating pairs were collected for the following 5 h. Copulation was allowed to continue without interruption, but copulation durations were not recorded. Following break-up, females were placed in new cages containing food and water. Groups of mated females contained 14-36 individuals (x = 22 females), and each group constituted a replicate. Three days later, males from the Guatemala colony (9-14 d old) were placed with the once-mated females (in 1:1 sex ratio), and rematings were recorded over the following 5 h. Cages were held at 21-25°C and were illuminated by fluorescent lights on a 10:14 h light:dark cycle.

Male Survivorship

To determine whether exposure to GRO affected male survivorship, we placed groups of 20 treated or control males from the *tsl* mass-reared strain in small plastic containers (18 by 11 by 9 cm with one end covered by nylon screening) with ample food and water and counted survivors 5 d later. All males were 3 d old at the start of the experiment. Treated males were exposed to GRO (following the above protocol) from 0800-1100 h and then transferred directly to the test containers. Control males were placed in the containers at the same time but were simply transferred from holding cages. The males were held under the same temperature and light regime noted above.

Statistical Analyses

The Mann-Whitney test (test statistic T) was used to compare 1) the number of matings obtained by competing male types in a given experiment and 2) the number of surviving control and GRO-exposed males. Variation in remating among females mated to different male types was assessed using the Kruskal-Wallis test (test statistic H).

RESULTS

Mating Tests

In experiment 1, wild-like males from the Guatemala line obtained significantly more matings per replicate than the control or treated males from the *tsl* mass-reared strain (Table 1). Although wild-like males were superior competitors in both instances, GRO exposure nonetheless improved the performance of the *tsl* mass-reared males. In a given replicate, treated, *tsl* mass-reared males achieved significantly more matings ($T = 109.0$; $P < 0.05$) and a significantly higher proportion of matings (30% versus 16%, respectively; $T = 114.0$; $P < 0.05$) than control males. There was no difference in the number of matings obtained by wild-like Guatemala males ($T = 92.5$; $P > 0.05$) or in the total number of matings ($T = 88.0$; $P > 0.05$) between trials involving control versus treated *tsl* mass-reared males.

GRO exposure had a more marked effect on the mating frequency of *tsl* mass-reared males in experiment 2. Wild-like males from the Madeira colony accounted for significantly more matings per replicate than control, *tsl* mass-reared males, but no difference in mating frequency was detected between wild-like Madeira males and treated, *tsl* mass-reared males (Table 1). In a given replicate, treated, *tsl* mass-reared males obtained significantly more matings ($T = 38.5$; $P < 0.05$) and a significantly higher proportion of matings (39% versus 16%, respectively; $T = 40.0$; $P < 0.01$) than control, *tsl* mass-reared males. There was no difference in the number of matings obtained by wild-like Madeira males ($T = 32.5$; $P > 0.05$) or in the total number of matings ($T = 33.0$; $P > 0.05$) between trials involving control versus treated males.

In experiment 3, treated, *tsl* mass-reared males obtained significantly more matings per replicate than control, *tsl* mass-reared males (Table 1). Over all replicates, treated males accounted for 75% (100/134) of all matings observed.

Female Remating

The incidence of female remating varied independently of the strain and GRO treatment of the initial mate. The average proportion of wild-like, Guatemala females remating per replicate was 11% (range: 4-16%) for individuals first mated to wild-like, Guatemala males, 9% (range: 5-17%) for individuals first mated to control, *tsl* mass-reared males, and 10% (range: 0-26%) for individuals mated first to treated, *tsl* mass-reared males ($H = 0.2$; $df = 2$; $P > 0.05$; 5 replicates were run for all 3 mating combinations).

Male Survivorship

GRO exposure had no apparent effect on survivorship of males from the *tsl* mass-reared strain. On average, only 0.8 control males (range: 0-2) and 1.0 treated males (range: 0-3) died over the 5-day test period ($T = 215.5$; $P > 0.05$).

DISCUSSION

The present findings show that exposure to GRO increased the mating success of males from a *tsl* mass-reared strain in competition with wild-like males from 2 different source populations. Following GRO exposure, the mating frequency of *tsl* mass-reared males increased approximately 1.9-fold (from 16% to 30% in a given replicate) and 2.4-fold (from 16% to 39% in a given replicate) in tests involving wild-like flies from Guatemala and Madeira, respectively. Although substantial, these increases were actually smaller than that observed for a mass-reared, bisexual strain in Hawaii, where the mating frequency of

TABLE 1. RESULTS OF 3 MEDFLY MATING EXPERIMENTS. IN EXPERIMENTS 1 AND 2, WILD-LIKE MALES COMPETED AGAINST CONTROL (A) OR TREATED (B) MASS-REARED MALES, AND IN EXPERIMENT 3 CONTROL AND TREATED MASS-REARED MALES COMPETED AGAINST ONE ANOTHER. VALUES FOR MATINGS REPRESENT MEAN NUMBER PER REPLICATE; VALUES IN PARENTHESES ARE RANGES. SIGNIFICANCE LEVELS OF T VALUES (MANN-WHITNEY TEST) ARE: ***$P < 0.001$; **$P < 0.01$; NS NOT SIGNIFICANT).

Experiment	Replicates	Male strain	GRO?	Matings/Replicate	T
1A	9	Guatemala	no	39.4 (21-63)	126.0***
		Vienna-7 Mix	no	8.3 (2-20)	
1B	9	Guatemala	no	34.8 (19-53)	122.0***
		Vienna-7 Mix	yes	15.1 (11-27)	
2A	5	Madeira	no	32.0 (22-41)	40.5**
		Vienna-7 Mix	no	6.4 (1-11)	
2B	5	Madeira	no	27.6 (16-33)	35.5 NS
		Vienna-7 Mix	yes	19.2 (10-29)	
3	5	Vienna-7 Mix	no	6.8 (3-10)	40.0**
		Vienna-7 Mix	yes	20.0 (18-22)	

mass-reared males increased approximately 3-fold (from 26% to 75% in a given replicate) after GRO exposure (Shelly & McInnis 2001). It appears, therefore, that although GRO exposure may consistently elevate male mating success, the magnitude of this increase may vary among different combinations of mass-reared and wild flies. The mechanism by which GRO exposure affects male mating success is unknown, although it does not appear to reflect a simple elevation of male signaling activity (Shelly & McInnis 2001).

Existing data reveal considerable variation in the mating success of *tsl* mass-reared males (without the introduction of GRO). On the one hand, Hendrichs et al. (1996) conducted mating tests in field cage and reported that males of *tsl* mass-reared strain Vienna-42 obtained 39% of all matings in competition with wild males. In addition, males of *tsl* mass-reared strains have effectively reduced wild medfly populations in Guatemala (Rendon et al. 1996), Tunisia (Cayol & Zarai 1999), and Israel (Rossler et al. 2000). On the other hand, the data reported herein reveal low mating competitiveness of males from the Vienna-7 Mix 200 strain against wild-like males from two different source populations. Similarly, in field cage trials conducted in Guatemala, Lance et al. (2000) reported that *tsl* mass-reared males (from Vienna-42 and Toliman-*tsl* strains combined) accounted for only 18% of all matings in competition with wild males.

Importantly, the present study also demonstrates that exposure to GRO did not adversely affect male survival. Among mass-reared males, treated individuals displayed the same level of survivorship as control males under laboratory conditions. Subsequent tests conducted in field-cages in Hawaii have similarly detected no difference in the survival level between GRO-exposed and non-exposed males from a mass-reared, bisexual strain (T.E.S., unpublished data). Although open field testing would provide more robust findings, the existing data at least indicate that males from the *tsl* mass-reared strain do not suffer any immediate and massive die-off as a consequence of GRO exposure.

The present study also indicated that females mated initially to GRO-exposed, *tsl* mass-reared males displayed a similar tendency to remate as females first mated to non-exposed, *tsl* mass-reared males. Thus, treated and control males do not appear to differ in their ability to induce a female refractory period following mating. In addition, there was no difference in female remating propensity between individuals first mated to irradiated, mass-reared versus wild-like males. This latter finding differs from earlier reports. Working exclusively with mass-reared flies, Katiyar and Ramirez (1970) and Bloem et al. (1993) found that females initially mated with non-irradiated males were more likely to remate than females initially mated to irradiated males. In both of these studies, females were given multiple opportunities for remating over several weeks, and consequently the present finding may have reflected the single remating opportunity given only 3 d after the initial mating.

Based on the present results, it appears that pre-release exposure of males to GRO has the potential to increase the effectiveness of *tsl* genetic sexing strains in the SIT. Earlier work (Shelly & McInnis 2001) on a bisexual strain demonstrated that a male mating advantage was evident even when males were unable to contact the GRO source and when GRO was presented to immature males. These findings further indicate that it may be relatively easy, in terms of both logistics and cost, to incorporate a treatment with GRO into the current adult male handling procedures in operational programs.

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