San Andrés Aura, V.; Ayala Mingol, I.; Abad Payá, MDC.; Primo Millo, J.; Casteñera, P.; Moya Sanz, MDP. (2014). Laboratory evaluation of the compatibility of a new attractant contaminant device ciontaining Metarhizium anisopliae with Ceratitis capitata sterile males. Biological Control. 72:54-61. doi:10.1016/j.biocontrol.2014.02.007.

Laboratory evaluation of the compatibility of a new attractant contaminant device containing *Metarhizium anisopliae* with *Ceratitis capitata* sterile males

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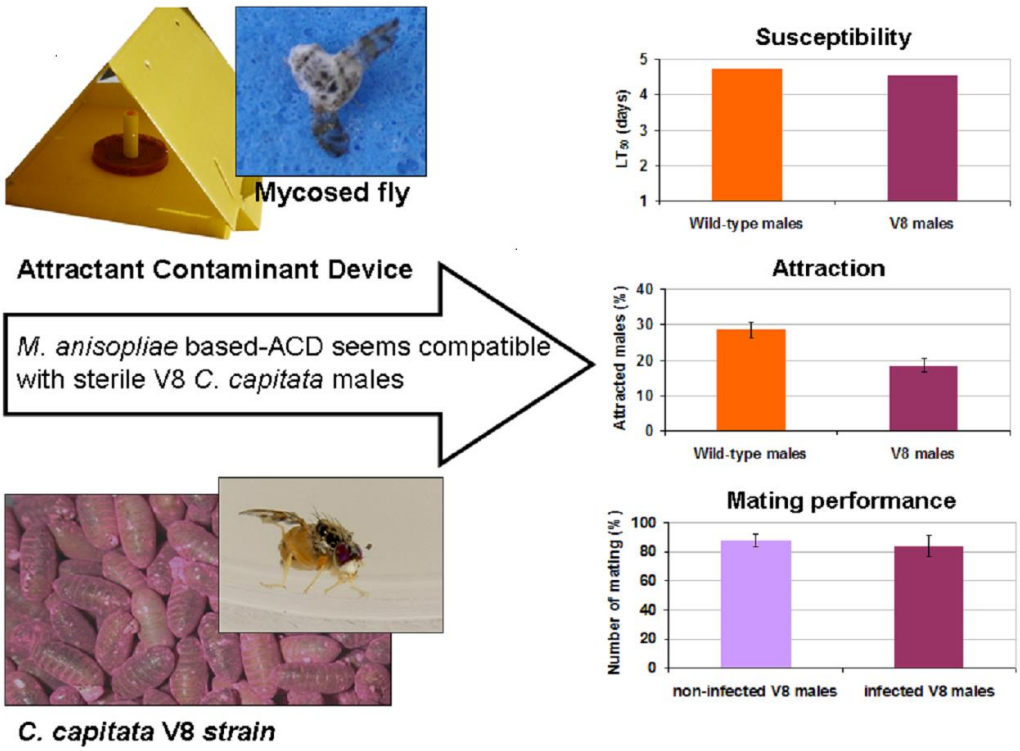
HIGHLIGHTS

- Sterile and wild-type *C. capitata* males showed similar susceptibility to *M. anisopliae*
- Sterile males were less attracted to infective dishes than wild-type males
- Under laboratory conditions, *M. anisopliae* did not impair mating performance of sterile males
- Infective device seems to be compatible with *C. capitata* Sterile Insect Technique under laboratory conditions

GRAPHICAL ABSTRACT



ABSTRACT

Laboratory experiments were conducted to evaluate the compatibility of using the entomopathogenic fungus *Metarhizium anisopliae*, to be dispensed in a new attractant contaminant device (ACD), jointly with sterilized *Ceratitis capitata* males, as an integrated approach to control this major pest. The exposure of sterile Vienna 8 (V8) strain and wild type (WT) males to the contaminating part (infective dish) of the ACD showed similar susceptibility levels to the fungal strain (LT_{50} value of 4.52 and 4.72 days, respectively). Sterile V8 males were significantly less attracted to the infective dish (18.4%) than WT males (28.5%).

As the success of Sterile Insect Technique (SIT) heavily relies on the mating success of sterile males in the field, mating performance of infected males was assessed. Around 85% of the females were mated, independently of the male strain and treatment (fungus–treated or untreated males) indicating that mating performance was unaffected by the fungus under laboratory conditions. Females showed a greater tendency to remate if previously mated to fungus-treated males, either V8 or WT.

Our data suggest that this *M. anisopliae* based-ACD does not impair the performance of *C. capitata* sterile males and, therefore, it could be used combined with area wide SIT-based programs, providing that these results are validated in field conditions. The implications of this combined strategy to control *C. capitata* are discussed.

Keywords: integrated control; entomopathogenic fungus; mating behavior; Medfly; Tephritidae.

1. Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most widespread and damaging fruit pests worldwide, being recorded in more than 400 host plant species (Liquido et al., 1991; Aluja and Mangan, 2008). In Spain, it is considered a major pest of citrus due to direct yield losses and to quarantine restrictions. Control strategies have been mainly based on field monitoring and aerial and terrestrial treatments with organophosphate insecticides, especially malathion, mixed with protein baits (San Andrés et al., 2007a). However, their continued use resulted in side effects, such as the field-evolved resistance of medfly to malathion in Spanish citrus areas (Magaña et al., 2007; 2008). Recently, emphasis has been placed on implementing safer environmental measures to control adult medfly (Urbaneja et al., 2009).

Area-wide SIT programs have been proved successful in the suppression, containment, prevention or eradication of fruit flies (Dyck et al., 2005; Vreysen et al., 2007), having been implemented in more than 150,000 ha in the Valencian region (Spain). Other strategies used in the Mediterranean area are the chemosterilization by lufenuron (Navarro-Llopis et al., 2004; 2007; 2010) or the use of bait stations (Mangan and Moreno, 2007; Navarro-Llopis et al., 2013). Among all environmentally-friendly approaches, the biological

72 control with entomopathogenic fungi have demonstrated to hold a great potential for adult
73 flies due to their way of infecting the host (Ekesi et al., 2007; Charnley and Collins, 2007),
74 and it has proved to be efficient for the control of *C. capitata* and for a wide range of fruit fly
75 pests (De la Rosa et al., 2002; Ekesi et al., 2002; Dimbi et al., 2003; Konstantopoulou and
76 Mazomenos, 2005; Quesada-Moraga et al., 2006; 2008).
77 Different strategies regarding the dissemination of the fungi and the contamination of
78 fruit flies have been considered, including cover sprays (Ortu et al., 2009; Daniel and Wyss,
79 2010) or fungus contamination devices (Primo-Yúfera et al., 2002; Ekesi et al., 2007).
80 Fungus contamination devices rely on the attraction of the insects to baited stations where
81 they are contaminated with the pathogen (Dimbi et al., 2003; Maniania et al., 2006). This
82 entails a reduction of the amount of inoculum released to the field and a high specificity by
83 decreasing the possibility of infecting non-target species. The effectiveness of this type of
84 systems for controlling several tephritids were preliminary assayed under field conditions and
85 promising results were reported (Moya, 2003; Ekesi et al., 2007). A subsequent 3-year field
86 trial revealed that an attractant contaminant device (ACD) containing *Metarhizium*
87 *anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) was able to reduce the
88 population levels of *C. capitata* more efficiently than conventional chemical treatments
89 (Navarro-Llopis et al., *submitted*). The integration of fungal pathogen into SIT, using sterile
90 males as vectors of entomopathogens (Toledo et al., 2007; Ekesi et al., 2007; Flores et al.,

2013) has also been considered. Accordingly, the combination of long lasting *M. anisopliae* based-attractant contaminant devices with an area wide-SIT program to control *C. capitata* could be a particularly valuable environment-friendly method for medfly management. However, to our knowledge, there is no data on the effects of *M. anisopliae* against *C. capitata* sterile strains, including the Vienna 8 (V8) strain. Laboratory and field studies are needed to assess the potential effects of this combined strategy on the performance of *C. capitata* sterile males, since a higher susceptibility of sterile males over wild ones to this entomopathogenic fungus could risk the compatibility of both methods.

The goal of this work was to assess, under laboratory conditions, the feasibility of using *M. anisopliae* with *C. capitata* V8 sterile males, which are currently used in the ongoing SIT program in Spain and elsewhere. A first step of our work was to test the level of fungal virulence against medfly sterile males, as well as the level of attraction of sterile males to infective dishes of the attractant contaminant device. A second step consisted in assessing mating performance of infected sterile males and the remating frequency of females previously mated with infected sterile males.

2. Material and methods

2.1. Insect material

110 *Ceratitis capitata* adults were obtained from a laboratory colony maintained at the
111 Centro de Ecología Química Agrícola (Valencia, Spain) since 1995. This colony, hereafter
112 named WT strain (wild type strain), is annually out crossed with wild population coming
113 from Valencia orchard infested fruits, to prevent the loss of sexual performance (Joachim-
114 Bravo et al., 2009)
115 Sterile males of the V8 strain were obtained from the mass rearing facility in
116 "Caudete de las Fuentes" (Valencia, Spain). V8 strain contains the *tsl* (temperature sensitive
117 lethal) mutation that allows the elimination of females at the egg stage. Males used in the
118 current study were dyed and irradiated as pupae two days before emergence under hypoxia at
119 95 Grays (gamma irradiation), as the proteolytic activity of medfly adults is not affected at
120 this dose (San Andrés et al., 2007a).
121 In all the experiments, the WT flies were separated by sex within 24 h after emerging
122 to ensure the use of unmated insects. Males and females were then placed separately in
123 ventilated cages (20 x 20 x 20 cm) with an access hole (12 cm-diameter) in a lateral side
124 covered with a fine muslin sleeve and four ventilation holes (5 cm-diameter) in the top side
125 also covered with muslin fabric. Adult flies were maintained on a 4:1 volumetric mixture of
126 sugar: yeast hydrolysate (Sigma, Madrid, Spain) and water until they were sexually mature
127 (4-5 day-old) to simulate field conditions, where different sources of protein are available.
128 Similarly, V8 males were maintained in the same conditions up to 4-5 day-old, but provided

129 only a sugar diet and water, to simulate the standard prerelease diet mostly used in SIT
130 programs worldwide (FAO/IAEA, 2007).
131 Environmental conditions were 25 ± 1 ºC, 65 ± 5% RH and a photoperiod of 16:8 h
132 (L:D) in an environmental chamber.
133
134 *2.2. Fungal strain*
135 *Metarhizium anisopliae* CECT 20768 is a fungal strain deposited in the "Colección
136 Española de Cultivos Tipo" (CECT). Currently, it is the active ingredient in the infective dish
137 (contaminating part) of the ACD designed for controlling *C. capitata* (Primo-Yúfera et al.,
138 2002; Navarro-Llopis et al., *submmited*).
139 The fungus was cultured in potato dextrose agar (PDA) Petri dishes. The conidia from
140 7-8 day-old PDA cultures were harvested by adding mineral oil (10 ml). Conidial
141 concentration was estimated using a haemocytometer (Improved Neubauer chamber) and
142 adjusted to obtain the appropriated conidia dose to be used in the infective dish. When
143 aqueous suspensions of the fungus were used, conidia were harvested with a solution of
144 Tween 80 (0.05%) in distilled water.
145 Conidial viability of the fungus was determined 24 h before each bioassay according
146 to Castillo et al. (2000). In all cases, the percentage of germination was higher than 97.0%.
147

148 *2.3. Infective dish*

149 Infective dishes were prepared according to Primo-Yúfera et al. (2002) and Moya
150 (2003) with some modifications to be adapted to laboratory conditions. Briefly, each infective
151 dish consisted of the bottom of a 5-cm-diameter Petri dish. A carboximethylcellulose-based
152 semi-solid gel was used as adherent material to support the adsorbent material and to
153 maintain a suitable microenvironment for conidial persistence. The adsorbent material, which
154 is based on a sepiolite granular formulation of trimedlure (TML) (Corma et al., 2000),
155 contained the *M. anisopliae* conidia suspended in mineral oil. This material was uniformly
156 spread over the adherent material to achieve a dose of about 3.1×10^8 conidia per dish.

157

158 *2.4. Susceptibility of C. capitata V8 sterile and WT males to M. anisopliae by contact*
159 *application on the infective dish*

160 Approximately six hundred V8 pupae were placed in ventilated methacrylate cages
161 (20 x 20 x 20 cm) and adults were allowed to emerge. The pathogenicity of *M. anisopliae*
162 was tested against 4-5 day-old V8 males by exposing them to the infective dishes (10 dishes/
163 bioassay).

164 Ten slightly cold anesthetized V8 males were ventrally deposited on the infective dish
165 and maintained for 30 s. Then, they were transferred to a ventilated plastic cylinder (16 cm
166 high, 13 cm diameter) and provided water *ad libitum* and a sugar: yeast hydrolysate 4:1

167 (wt:wt) diet because in post-release conditions, males might be able to find carbohydrates and
168 proteins natural sources (Hendrichs and Hendrichs, 1990). Control V8 males were equally
169 exposed to fungus-free plates.

170 For comparative purposes, susceptibility of 4-5 day-old WT males to the infective
171 dishes was also evaluated. Flies (10 per plate) were subjected to both fungal and control
172 treatments using the same infective or control dishes as indicated above.

173 For each bioassay, ten replicates per treatment (fungal and control treatments) and fly
174 strain (V8 and WT strains) were performed and a total number of two bioassays were carried
175 out. The experimental arena was checked for 10 days and dead males daily recorded and
176 removed. After surface sterilization using 3% sodium hypochlorite solution, cadavers were
177 incubated on PDA at 28 °C for 10 days to confirm mycosis which was assumed when the
178 sporulated mycelia of the fungus was superficially observed (Selman et al., 1997).

179 Virulence levels were established through the lethal time 50 (LT_{50}) values which is
180 the time required (expressed as days) to kill 50% of the flies.

181 The amount of inoculum gathered by each fly was estimated according to Dimbi et al.
182 (2003) with some modifications. Two additional V8 and 2 WT males were exposed to each
183 one of the 10 infective plates. Just after the exposure period (30 s.), they were individually
184 transferred to sterile 1.5 ml Eppendorf tubes and frozen (-50 °C) until assayed. Later, an
185 aliquot (1.0 ml) of a 0.5% Tween 80 solution in sterile distilled water was added to each

Eppendorf tube and vortexed during 1 minute. The suspension, containing dislodged conidia, was transferred to a sterile graduate polypropylene vial (10 ml). For each fly, this process was repeated twice and successive suspensions were added to the same polypropylene vial. Then, the vial was centrifuged (300 rpm during 3 min.) in a Rotina 46 Centrifuge (Hettich, Germany) and 1.0 ml exceeding supernatant was removed. Finally, the pellet was resuspended with a gently shake and conidial concentration was estimated using a Neubauer camera.

2.5. Effect of the infective dish on the attraction of C. capitata V8 sterile males

The infective dish superficially contained a small quantity (20 mg/g emitter) of TML, which is an appropriate amount for assessing *C. capitata* attractiveness to the infective dish under laboratory conditions. An attraction bioassay to evaluate the behavior of WT and V8 males was performed. The V8 sterile male were exposed to Ginger Root Oil (GRO) aromatherapy to improve sterile males mating competitiveness (Shelly et al. 2007), as presently used in most of the ongoing SIT programs worldwide, whereas WT males were not subjected to any previous treatment before the experiment to simulate field conditions. Thus, 24 h before the assay, five hundred 4 day-old V8 sterile males were exposed to volatiles emitted by a piece of cotton containing 100 µl of GRO (Lluch Essence S.L., Spain), during 3 h, in a ventilated methacrylate cage (30 x 40 x 30 cm). The next day, and 1 h before starting

205 the assay, groups of fifty aromatherapy-treated V8 males were introduced in 30 x 30 x 30 cm
206 cages, the lateral and top sides of which were made of wire mesh to avoid saturation effects
207 Insects were allowed to settle during 1 h. Then, a 5 cm infective dish (3.1×10^8 conidia) was
208 placed in the bottom of the cage and only water was provided. Attraction was recorded every
209 10 min during 3 h. The percentage of attraction was obtained considering the average number
210 of males alighting the dish along the fixed attractant period respect to the total number of flies
211 in the cage. Controls were simultaneously performed in the same conditions exposing them to
212 fungus-free dishes. Two bioassays with 5 replicates per treatment and fly strain were
213 performed.
214
215 *2.6. Effect of M. anisopliae on the sexual performance of C. capitata V8 sterile males*
216 *2.6.1. Sperm transfer detection*
217 Previous assays allowed us to obtain a LT_{50} = 6.29 days when 4-day-old sterile V8
218 males were treated by topical application with 1µl of a Tween 80 (0.05%) suspension
219 containing 1×10^7 conidia/ml (1×10^5 conidia/fly) (data not shown). Based on this finding, a
220 slightly lesser dose (5×10^4 conidia/fly) was assayed to obtain an adequate period of
221 observation according to our experimental design.
222 Newly emerged (<24 h) WT flies were first separated by sex to ensure that only
223 unmated females would be used. Females were maintained separately in ventilated

methacrylate cages (20 x 20 x 20 cm) and provided with sugar: yeast hydrolysate 4:1 (wt:wt) diet and water until they reached sexual maturity. Sterile males were obtained and kept in a sugar diet plus water, as described previously.

Five day-old sterile males were inoculated with one microliter of a conidial aqueous suspension of 5 x 10^7 conidia/ml (5 x 10^4 conidia/fly) on the ventral surface of the abdomen of the adults previously anesthetized with ice. Fungus untreated insects were inoculated with the same volume of a sterile aqueous solution of 0.05% Tween 80. Twenty-four hours before the fungal treatment, V8 males were exposed for 3 h to 100 µl of GRO in a ventilated methacrylate cage (30 x 40 x 30 cm). Three replicates were performed for each treatment. For each replicate 14 V8 males (fungus treated or untreated) were used. As the goal of the experiment was to evaluate the effect of the ongoing process of infection on the capability of the sterile males to mate and transfer sperm, and taking into account that generally germination of *M. anisopliae* conidia takes place within the first 20 h after contact (Zimmermann, 2007), a latent period of 48 h was established. Thus, from the second day of treatment, 7 virgin WT females (male: female ratio, 2:1) were introduced in the mating scenario (ventilated methacrylate cage of 20 x 20 x 20 cm) and allowed to mate for 24 h. Then, females were removed and stored in 70% ethanol at 4 ºC. The process was daily repeated along 6 days. The number of new virgin females introduced each day changed according the number of surviving males in order to maintain the male: female (2:1) ratio

243 described above. Sperm presence on female spermathecae was analyzed with the molecular
244 marker CcYsp according to San Andrés et al. (2007b). PCR was performed in a thermocycler
245 Mastercycler Eppendorf Gradient S using the following conditions: 300 nM dNTPs, 1 X Taq
246 buffer, 2mM MgCl2, 0.75 U of Taq polymerase (Biotools), 10 pmol of each primer, and 10
247 ng of total DNA. Amplification conditions were: one denaturation cycle at 94 ºC for 5 min,
248 30 cycles at 94 ºC for 40 s, 55 ºC for 40 s, 72 ºC for 1 min, followed by a final extension
249 cycle at 72 ºC for 4 min. PCR products were run in a 2% agarose D-1 low electroendosmosis
250 (EEO) gel (Pronadisa, Sumilab S.L., Madrid, Spain).
251
252 *2.6.2. Effect of M. anisopliae on the mating of C. capitata V8 sterile males.*
253 To examine the potential effect of the fungal infection (2 days post fungus
254 inoculation) on the V8 male sexual performance and on their corresponding mated females,
255 virgin WT females were allowed to copulate with sterile or WT, treated or untreated with *M.*
256 *anisopliae*, males.
257 *First mating procedure*. WT females and males and V8 males, all of them virgin and
258 sexually mature (5 day-old) were obtained as previously described.
259 Fifty V8 males and 50 WT males were subjected to fungal treatment by contact on the
260 infective dishes (10 flies/dish) as described above. The corresponding controls were made
261 using fungus-free dishes. Each group of fifty flies was placed in a ventilated methacrylate

cage (30 x 40 x 30 cm). Three replicates by treatment and medfly strain were performed. Forty-eight h later, and always starting the experiment at 10 am, 2h later of "light on" in daily photophase, each group was transferred to a new ventilated methacrylate mating cage of 30 x 40 x 30 cm (Gavriel et al., 2009) and allowed to settle for 30 min. Then, 50 WT virgin females were introduced into the mating arena. In each scenario, mating pairs were checked continuously during 3 h. Copulating pairs were collected into glass vials (15 ml volume) 5 min after the mating initiation and copula duration was recorded. To ensure that mated females had been inseminated, only females with copula duration longer than 100 min were selected for the remating tests (Taylor and Yuval, 1999). After copula completion, mated females were transferred to a new methacrylate cage (30 x 40 x 30 cm) and provided with the standard sugar and yeast hydrolysate (4:1; wt:wt) diet and water. Non-mated females and males were discarded.

Remating procedure. Remating tests were performed following the same procedure described, and as a continuation of the first mating procedure. Thus, mated females from each group were allowed to remate with healthy virgin WT males. The remating test was performed two days after their first copula when a maximum level of *C. capitata* female remating has been reported (Gavriel et al., 2009; Kraaijeveld et al., 2005). This process was carried out over three consecutive days using the remaining non-remated females from each previous day. The number of healthy virgin WT males (5 day-old) introduced each day in the

281 remating arena (mating methacrylate cage of 30 x 40 x 30 cm) was the corresponding number
282 to reach the male: female (1:1) proportion. Copulating pairs were collected continuously for 3
283 h and re-copula duration recorded as described above. As for the first mate, females staying
284 in copula for over 100 min were considered remated.

285 To evaluate horizontal transmission of the fungus during the first-mating process,
286 mortality of WT females used in the mating/remating bioassays was recorded throughout the
287 experiment and cadavers were handled, as previously described, to confirm mycosis.

288

289 *2.7. Statistical analysis*

290 Mortality data in pathogenicity experiments was corrected using Abbott's formula.
291 The median lethal time (LT_{50}) values were estimated by probit analysis. Parallelism test of
292 probit regression lines was conducted using chi-square goodness-of-fit tests. Relative median
293 potency and its 95% confidence interval were calculated because the slopes were not
294 significantly different. The number of matings, rematings and their duration and female
295 mortality were subjected to analysis of variance (ANOVA) followed by means separation
296 using the Tukey's test ($P \leq 0.05$).

297 Attraction data from the attractant bioassay in laboratory cages were analyzed by one-
298 way ANOVA followed by Tukey´s test ($P \leq 0.05$).

299 To establish differences in the number of mating along the time through the sperm
300 transfer detection bioassay, a generalized lineal model univariate (GLM-U) procedure
301 followed by a Tukey´s test ($P \leq 0.05$).
302 When necessary, data were transformed (logarithmic or angular transformation) in
303 order to comply with analyses assumptions. In all cases the lack of significant differences
304 among variances was verified with the Levene´s statistic ($P > 0.05$). All analyses were
305 performed using the SPSS v.16.0.1 for Windows (SPSS Inc., 2008).
306
307 **3. Results**
308
309 *3.1. Susceptibility of C. capitata V8 sterile and WT males to M. anisopliae by contact*
310 *application on the infective plate*
311 No significant differences were found between the amount of inoculum gathered by
312 the WT and V8 males ($F_{1, 38} = 1.13$, $P = 0.294$) (Table 1), which allowed us to compare
313 susceptibility results between medflies strains. Based on the estimated LT_{50} values, V8 and
314 WT males showed a similar level of susceptibility (4.52 and 4.72 days, respectively). No
315 difference in susceptibility between both *C. capitata* strains was observed according to the
316 results of the parallelism between probit mortality lines and relative potency of WT respect to
317 V8 (Table 1).

3.2. Effect of the infective dish on the attraction of C. capitata V8 sterile males

Results from the attraction bioassay in laboratory cages are shown in Table 2. The percentage of attraction was significantly different among infective and non-infective dishes as well as between fly strains ($F_{3, 28}$ = 29.159, P < 0.001). Both V8 and WT males showed a significantly higher attraction to the infective dishes than to the control ones. In addition, WT males were significantly more attracted than V8 males to either infective or non-infective dishes.

3.3. Effect of M. anisopliae on the sexual performance of C. capitata V8 sterile males

3.3.1. Sperm transference detection

The application of CcYsp marker (Fig. 1) indicated that treated V8 males developing the fungal infection during two days before being exposed to virgin females were able to qualitatively transfer sperm similarly to untreated V8 males. Percentages of mating higher than 90% were observed in most cases, including those of the sixth day, when the treated V8 males were already showing mortality values of about 50% (Table 3). GLM-U disclosed no significant differences respect to the capability of mate and transfer sperm between the two treatments ($F_{1, 35}$ = 0.185, P = 0.671) and time ($F_{5, 35}$ = 0.480, P = 0.788) and no interaction was observed ($F_{3, 35}$ = 1.043, P = 0.415).

337

338 *3.3.2. Effect of M. anisopliae on the mating of C. capitata V8 sterile males*

339 Results from the mating experiments are shown in Table 4. The percentage of matings
340 (about 85.0%) was not significantly affected either by the treatments or the fly strain ($F_{3, 8}$ =
341 0.208, P = 0.888). However, the mating duration was affected, though only statistically
342 significant differences were found between treated- and untreated WT males ($F_{3, 8}$ = 5.633, P
343 = 0.023), the former showing the longest period of copulation (193.3 min). No significant
344 effect of the fungus was observed on the mating duration between virgin WT females and
345 virgin V8 males, either treated or untreated with *M. anisopliae*. Untreated WT males showed
346 the shorter mating duration (160.6 min) with no significant differences from those showed by
347 the V8 males, independently if treated or not.

348 Two days after its first copulation, each female was allowed to copulate again with
349 sexually mature healthy WT males. Table 5 shows the percentages of remated females during
350 the following three days of the experiment. Females that mated first to treated males (either
351 WT or V8) tended to remate more than females mated first to untreated males although this
352 effect was more evident between females mated first to treated and untreated WT males.

353 In all cases, the number of rematings was higher the first day of exposition. No
354 significant differences were observed among females mated first to treated and untreated V8
355 males or treated WT males, with remating percentages ranging from 13.2 to 18.9%. Only the

356 females first mated to untreated WT males showed a significantly lower remating rate ($F_{3, 8}$ =
357 12.88, P = 0.002). On the second day, the remating percentages ranged from 0.9 to 4.3%, but
358 no significant differences were found among treatments ($F_{3, 8}$ = 1.79, P = 0.225). The third
359 and last day of evaluation, more variable remating rates were obtained. Again females mated
360 first to treated V8 males showed a significantly higher remating percentage ($F_{3, 8}$ = 10.15, P =
361 0.004), although no significant difference was found with the females mated first to treated
362 WT males.

363 Similarly to the first-mating part of the experiment, remating duration was also
364 recorded. The average values of remating duration over the 3-days experiment for the females
365 first mated to WT males (treated or not) and V8 males (treated or not) when remated to
366 healthy WT males are shown in Table 5. Unlike first-mating experiments, no significant
367 differences on this remating parameter were found ($F_{3, 8}$ = 1.361; P = 0.378).

368 Finally, female mortality was recorded during the entire bioassay in order to assess
369 fungus horizontal transmission during the first mate. At the end of the experiment, average
370 percentage of female mortality coupled with treated or untreated WT and treated or untreated
371 V8 males were 14.39 ± 2.14, 9.86 ± 1.47, 8.26 ± 0.48 and 9.89 ± 2.14, respectively. No
372 significant differences were found among the treatments ($F_{3, 8}$ = 1.270; P = 0.148). None of
373 the dead females showed signs of mycosis.

374

4. Discussion

There is an increasing need to develop new safer environmental measures to control *C. capitata*. In this context, we have investigated the compatibility of a new *M. anisopliae*-based attractant-contaminant device with the sterile males used in SIT programs. This approach involves a new notion of a combined strategy leading to a reduction of the wild population density by using *M. anisopliae*-based ACDs, as it has been reported for *C. capitata* in citrus orchards (Navarro-Llopis et al., *submitted*), and to a significant improvement in the efficiency of SIT programs (Klassen, 2005). This combined strategy would be particularly important in those regions with high population density, as usually occurs in the Spanish citrus growing area. However, to be compatible with SIT, it will be necessary that sterile males will not be affected in their performance.

We found that the exposition of *C. capitata* V8 sterile males to the infective dish entailed a higher susceptibility to *M. anisopliae* than when aqueous conidial suspensions were used to provoke the infection (sperm transference assay). This effect can be related to the oil-fungus formulation contained in the infective dish, since it has been reported that oil formulations enhance pathogens efficacy (Prior et al., 1988; Barson et al., 1994) accelerating the mycosis process on the host in comparison to aqueous formulations. According to Prior et al. (1988) oil prevents conidia from drying, increases their adhesion ability to the insect cuticle and interferes with the defensive nature of the cuticle.

394 The exposure of *C. capitata* V8 sterile males to the infective dish showed a LT_{50}
395 value (4.5 days) similar to the one found for WT male (4.7 days), which suggests a lack of a
396 higher fungal virulence against V8 males. Moreover, our results suggest that even if V8
397 males are attracted to the ACD and become infected immediately after released, their
398 expected life-span would be in the range of the mean life-span of the V8 males under field
399 conditions, which vary from 3-5 days after their release (Paranhos et al. 2010). These
400 findings, although need to be tested in the field, seem to support the idea of a combined
401 strategy with SIT, since the use of this approach would be compatible with V8 males released
402 in the SIT program.
403 We found that males of the V8 and WT *C. capitata* strains showed a higher attraction
404 to infective dishes with conidia than to the controls without conidia. This increased
405 attractiveness could be attributed to the effect of male-attractant-semiochemicals emitted by
406 the fungus as recently stated by Roy *et al*. (2007). However, although there is evidence of
407 insect attraction to volatiles released from fungi, these are non-entomopathogenic fungi,
408 predominantly (Baverstock et al., 2010). Therefore, further studies are needed to clarify this
409 issue.
410 In addition, we also found that sterile V8 males were significantly less attracted to the
411 infective dishes than WT males, though the potential effect of the food supply and the GRO
412 exposure cannot be ruled out. This lower tendency shown by V8 males to approach and alight

413 on the infective dishes might suppose a sterile male advantage by reducing their
414 contamination rate in the field and would contribute to favor the SIT-ACD combined
415 strategy. Although, it must be considered that, in the field, other stimuli as the whole ACD
416 containing the long-lasting and large-distance attractants participate in the attraction and they
417 might alter the laboratory results, a recent work of Shelly and Edu (2009) reports that mass-
418 reared males of *C. capitata* from a *tsl* genetic sexing strain are less likely to be captured in the
419 field in trimedlure-baited traps than males from a recently established (wild-type) strain. This
420 report is in accordance with our laboratory results and supports our data in the field.
421 However, field studies with the ACD are required to ensure this specific aspect of the
422 attraction.

423 Entomopathogenic fungi are known to alter their host behavior to favor their own
424 development (Roy et al., 2007) and to reduce mating performance (Schaechter, 2000).
425 Therefore, for the integration of the *M. anisopliae*-based ACD and the SIT strategy, a key
426 point is to avoid impairing the sexual performance of V8 males after the fungus infection.
427 Our results revealed that the *M. anisopliae* strain used did not affect the capability of V8
428 males to qualitatively transfer sperm, even up to the time corresponding to about the LT_{50} for
429 the used experimental design (6-7 days). Moreover, the number of matings achieved by
430 fungal infected V8 males and their copula duration were also unaffected. These findings are
431 consistent with data reported by Dimbi et al. (2009) who demonstrated, using similar

experimental conditions that exposure to fungal inoculation does not adversely affect the mating performance of *C. capitata* up to the day when mortality due to inoculation begins. Similarly, Novelo-Rincon et al. (2009) reported, in field-cages experiments, no significant differences in sexual performance of *Anastrepha ludens* sterile males when used as vector or not of *Beauveria bassiana* conidia. Yet, open field experiments are needed to validate these data.

Remating frequency in *C. capitata* is a complex issue and remating rates in wild females are variable depending on the environmental conditions and strains (Mossinson and Yuval, 2003, Vera et al., 2003; Kraaijeveld et al., 2005; Gavriel et al., 2009). We have found that females showed higher tendency to remate if previously mated to *M. anisopliae*-treated males, either V8 or WT, though the percentages of remating remained similar in all treatments and within the range of values reported by different authors (Mossinson and Yuval, 2003; Gavriel et al., 2009). In addition, the remating rate under laboratory conditions might not reflect the situation in the field, since it could be related to the density of flies released in laboratory cages and the cage conditions could also stimulate either indoors or outdoors remating (Vera et al, 2003). Moreover, the significant minor attraction of V8 sterile male to the infective dish might suggest that a smaller proportion of them in relation to WT would become contaminated by *M. anisopliae.* This situation would contribute to counteract

450 the negative aspect of females increasing their remating frequency when mated to a *M.*
451 *anisopliae* infected V8 male.
452 Finally, female mortality has not been shown during the mating/remating bioassay
453 which suggests that fungus horizontal transmission was not achieved. Previous works using
454 the ACD in laboratory and field conditions (Navarro-Llopis et al., *submitted*) had already
455 demonstrated this lack of transmission which was attributed to the oil strongly adhering
456 conidia to the lipophilic fly cuticle hence avoiding its transfer by contact.
457 In summary, our data reveal that sterile V8 males presented a similar susceptibility to
458 *M. anisopliae* as WT males and that no adverse effects induced by fungal contamination were
459 detected at the level of sexual performance. Thus, based on these findings, the use of the *M.*
460 *anisopliae* based-attractant-contaminant device could be compatible with an area-wide SIT
461 program, but field experiments would be required before it can be applied in action programs.
462 The major advantages of using ACDs are those derived from its high effectiveness reducing
463 *C. capitata* in the field because, though no horizontal transmission is achieved, both *C.*
464 *capitata* males and females become contaminated due to the attractant properties of the
465 device (Navarro-Llopis et al., *submitted*). In addition, the device is highly selective delivering
466 the fungus, which increases notably its environmental safety. Moreover, highly persistent
467 field effectiveness (about three months) has been proved, which could contribute to its
468 economic feasibility in IPM programs. These findings may have practical implications for the

integration of these two friendly strategies, the SIT and the fungus attractant-contaminant device against the Mediterranean fruit fly, *C. capitata*. Nevertheless, it will be necessary to ascertain whether similar results are obtained under field conditions.

Acknowledgments

This work was partially supported by the "Comisión Interministerial de Ciencia y Tecnología" (CICYT, Spain) (Grant: AGL2006-13346-C02-02).

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TABLES CAPTIONS

Table 1. Time-mortality response of *C. capitata* males (V8 and WT) against *M. anisopliae* by contact application on the infective dish.

[a] Values (conidia/fly) represent the mean ± SE of conidia recovered and estimated using an hemocytometer. [b] Values in days. [c] Parallelism Test: $\chi^2 = 3.152$; $df = 1$; $P = 0.076$; common slope (±SE): 9.921 ± 0.213: Relative Potency of the response of WT males respect to V8 males (± 95% Fiducial Limits): 1.03 (0.01, 1.06)

Table 2. Percentage of attraction of *C. capitata* males (V8 and WT) exposed to infective *M. anisopliae* dishes under laboratory conditions.

[a] Values are mean ± SE over a 3 h period. [b] To simulate field conditions, only V8 males were subjected to a GRO-aromatherapy treatment 24 h before the experiment. Values followed by different letters are significantly different ($P < 0.05$).

Table 3. Sperm detection over time of *C. capitata* WT virgin females mated to *M. anisopliae*-treated or untreated *C. capitata* V8 males.

Ceratitis capitata V8 males were *M. anisopliae*-treated by topical application (1µl) using an aqueous suspension of 5.0×10^7 conidia/ml (5.0×10^4 conidia/fly), two days before exposition to the mating process. For each day, mating values are mean ± SE of percentage of females containing sperm in their spermathecae with respect to the number of females assayed that day, which was corrected as a function of the V8 males mortality to maintain the initial male: female (2:1) rate.

Table 4. Percentage of matings and their duration between *C. capitata* virgin WT females and virgin males (V8 or WT), either treated or untreated with *M. anisopliae*.

Ceratitis capitata WT and V8 males were fungus-treated by contact application on infective dishes two days before the mating process. Values (mean ± SE) followed by different letters within columns are significantly different ($P < 0.05$).

Table 5. Percentage of rematings and their duration between WT females first-mated to *M. anisopliae* treated or untreated *C. capitata* V8 or WT males and *C. capitata* WT males.

Females first-mated to *M. anisopliae* treated or untreated, *C. capitata* V8 or WT males, were allowed to remate to healthy WT males two days after their first mate. For each day, values are percentages (mean ± SE) of remated females respect to the total number of first-mated females in the mating cage. Remating in Day 2 and Day 3 were performed with the remaining non-remated females from the previous day. Percentage values followed by different letters within columns are significantly different ($P < 0.05$).

Remating duration was obtained as the average value from all the rematings times recorded along the 3-days experiment.

681 Table 1.

	Dose (x 10^4)[a]	Slope ± SE	LT_{50} (95% CL)[b]	χ^2	*df*	*Sig*
WT males	2.4 ± 0.2	9.626 ± 0.331	4.7 (4.7 – 4.8)[c]	51.6	34	0.027
V8 males	2.1 ± 0.2	8.606 ± 0.327	4.5 (4.5 – 4.6)[c]	23.9	34	0.901

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683

684 Table 2.

Male strain + assayed dish	Attraction (%)[a]
V8[b] + non-infective dish	6.9 ± 1.1 a
V8[b] + infective dish	18.7 ± 2.0 b
WT + non-infective dish	19.6 ± 1.0 b
WT + infective dish	28.5 ± 2.2 c

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692 Table 3.

	♀ with sperm from V8 ♂ (%)		V8 ♂ mortality (%)	
Day	♀ x untreated V8♂	♀ x treated V8♂	Untreated V8♂	Treated V8♂
1	100.0 ± 0.0	95.2 ± 4.8	0	0
2	95.2 ± 4.8	100.0 ± 0.0	0	0
3	95.2 ± 4.8	94.4 ± 5.6	0	11.11± 2.8
4	81.0 ± 19.0	100.0 ± 0.0	5.6 ± 2.8	19.4 ± 2.8
5	100.0 ± 0.0	100.0 ± 0.0	5.6 ± 2.8	33.3 ± 4.8
6	100.0 ± 0.0	91.7 ± 8.3	13.89 ± 2.8	44.5 ± 2.8

693

694 Table 4.

Crosses	Number of matings (%)	Mating duration (min)
WT ♀ x untreated V8 ♂	88.0 ± 4.2 a	178.1 ± 1.8 ab
WT ♀ x treated V8 ♂	84.0 ± 7.2 a	172.7 ± 6.6 ab
WT ♀ x untreated WT ♂	82.0 ± 6.1 a	160.6 ± 6.8 a
WT ♀ x treated WT ♂	83.3 ± 4.7 a	193.3 ± 6.0 b

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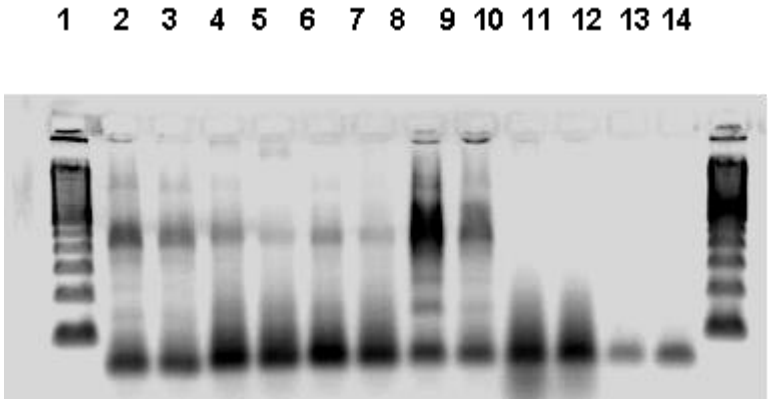
Table 5.

Remating crosses	Number of rematings (%)			Remating
♀(first-copulating ♂) x WT ♂	Day 1	Day 2	Day 3	duration (min)
♀(untreated V8 ♂) x WT ♂	13.2 ± 0.7 a	1.9 ± 1.9 a	2.2 ± 1.1 a	192.7 ± 8.0 a
♀(treated V8 ♂) x WT ♂	18.9 ± 1.5 a	3.4 ± 0.4 a	9.3 ± 0.4 b	179.7 ± 3.3 a
♀(untreated WT ♂) x WT ♂	5.5 ± 2.2 b	0.9 ± 0.9 a	3.3 ± 1.7 a	171.3 ± 10.5 a
♀(treated WT ♂) x WT ♂	17.0 ± 1.6 a	4.3 ± 0.7 a	6.6 ± 0.2 ab	195.3 ± 14.5 a

FIGURE CAPTIONS

Fig. 1. Sperm transference detection in *C. capitata* females mated to *M. anisopliae*–treated or untreatred *C. capitata* V8 sterile males by PCR analysis. Lanes 1 and 14 correspond to the molecular weight marker 100-bp ladder (Invitrogen). Lanes 2 to 9 correspond to CcYsp marker, which indicate sperm DNA of fungus-contaminated males (lanes 2, 4 and 6) or non-contaminated males (lanes 3, 5 and 7) on female spermathecae the days 2, 4 and 6 after treatment. Lanes 8 and 9 correspond to V8 DNA, lanes 10 and 11 to an unmated female and lanes 12 and 13 to negative control (no DNA template).

707 Figure 1.



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