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1 **Laboratory evaluation of the compatibility of a new attractant contaminant device**

2 **containing *Metarhizium anisopliae* with *Ceratitis capitata* sterile males**

3

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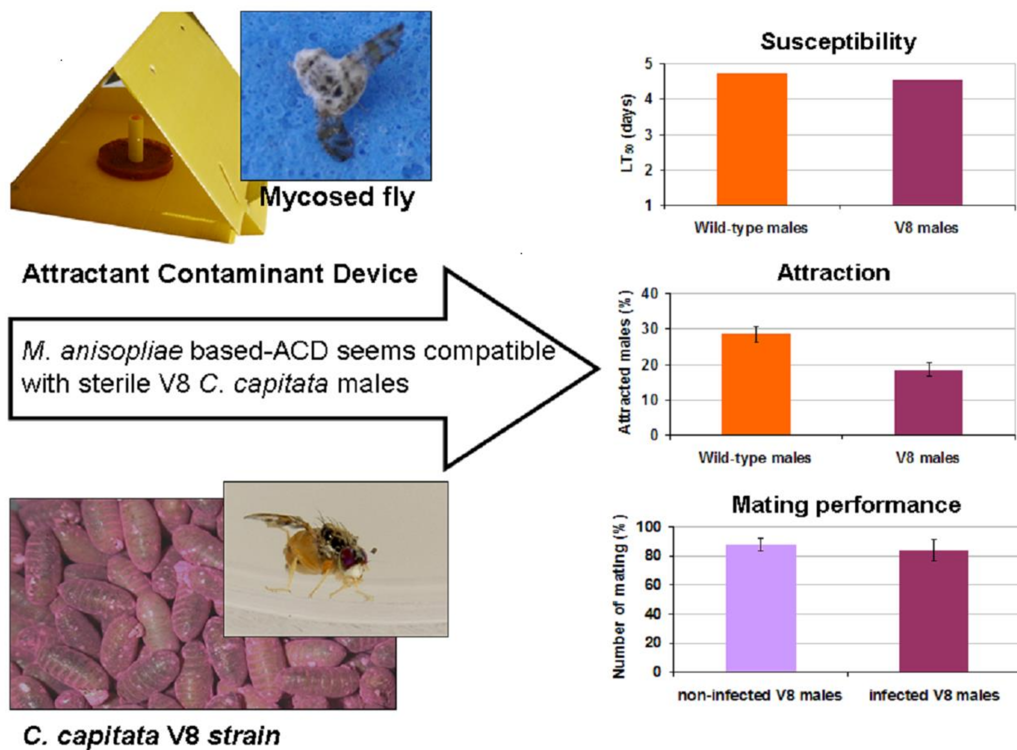
HIGHLIGHTS

- 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



34 **ABSTRACT**

35 Laboratory experiments were conducted to evaluate the compatibility of using the

36 entomopathogenic fungus *Metarhizium anisopliae*, to be dispensed in a new attractant

37 contaminant device (ACD), jointly with sterilized *Ceratitis capitata* males, as an integrated

38 approach to control this major pest. The exposure of sterile Vienna 8 (V8) strain and wild

39 type (WT) males to the contaminating part (infective dish) of the ACD showed similar

40 susceptibility levels to the fungal strain (LT_{50} value of 4.52 and 4.72 days, respectively).

41 Sterile V8 males were significantly less attracted to the infective dish (18.4%) than WT males

42 (28.5%).

43 As the success of Sterile Insect Technique (SIT) heavily relies on the mating success of

44 sterile males in the field, mating performance of infected males was assessed. Around 85% of

45 the females were mated, independently of the male strain and treatment (fungus–treated or

46 untreated males) indicating that mating performance was unaffected by the fungus under

47 laboratory conditions. Females showed a greater tendency to remate if previously mated to

48 fungus-treated males, either V8 or WT.

49 Our data suggest that this *M. anisopliae* based-ACD does not impair the performance of *C.*

50 *capitata* sterile males and, therefore, it could be used combined with area wide SIT-based

51 programs, providing that these results are validated in field conditions. The implications of

52 this combined strategy to control *C. capitata* are discussed.

53 *Keywords:* integrated control; entomopathogenic fungus; mating behavior; Medfly;

54 Tephritidae.

55 **1. Introduction**

56 The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera:

57 Tephritidae), is one of the most widespread and damaging fruit pests worldwide, being

58 recorded in more than 400 host plant species (Liquido et al., 1991; Aluja and Mangan, 2008).

59 In Spain, it is considered a major pest of citrus due to direct yield losses and to quarantine

60 restrictions. Control strategies have been mainly based on field monitoring and aerial and

61 terrestrial treatments with organophosphate insecticides, especially malathion, mixed with

62 protein baits (San Andrés et al., 2007a). However, their continued use resulted in side effects,

63 such as the field-evolved resistance of medfly to malathion in Spanish citrus areas (Magaña et

64 al., 2007; 2008). Recently, emphasis has been placed on implementing safer environmental

65 measures to control adult medfly (Urbaneja et al., 2009).

66 Area-wide SIT programs have been proved successful in the suppression,

67 containment, prevention or eradication of fruit flies (Dyck et al., 2005; Vreysen et al., 2007),

68 having been implemented in more than 150,000 ha in the Valencian region (Spain). Other

69 strategies used in the Mediterranean area are the chemosterilization by lufenuron (Navarro-

70 Llopis et al., 2004; 2007; 2010) or the use of bait stations (Mangan and Moreno, 2007;

71 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.,

91 2013) has also been considered. Accordingly, the combination of long lasting *M. anisopliae*
92 based-attractant contaminant devices with an area wide-SIT program to control *C. capitata*
93 could be a particularly valuable environment-friendly method for medfly management.
94 However, to our knowledge, there is no data on the effects of *M. anisopliae* against *C.*
95 *capitata* sterile strains, including the Vienna 8 (V8) strain. Laboratory and field studies are
96 needed to assess the potential effects of this combined strategy on the performance of *C.*
97 *capitata* sterile males, since a higher susceptibility of sterile males over wild ones to this
98 entomopathogenic fungus could risk the compatibility of both methods.

99 The goal of this work was to assess, under laboratory conditions, the feasibility of
100 using *M. anisopliae* with *C. capitata* V8 sterile males, which are currently used in the
101 ongoing SIT program in Spain and elsewhere. A first step of our work was to test the level of
102 fungal virulence against medfly sterile males, as well as the level of attraction of sterile males
103 to infective dishes of the attractant contaminant device. A second step consisted in assessing
104 mating performance of infected sterile males and the remating frequency of females
105 previously mated with infected sterile males.
106

107 **2. Material and methods**

108

109 *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 x 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

186 Eppendorf tube and vortexed during 1 minute. The suspension, containing dislodged conidia,

187 was transferred to a sterile graduate polypropylene vial (10 ml). For each fly, this process was

188 repeated twice and successive suspensions were added to the same polypropylene vial. Then,

189 the vial was centrifuged (300 rpm during 3 min.) in a Rotina 46 Centrifuge (Hettich,

190 Germany) and 1.0 ml exceeding supernatant was removed. Finally, the pellet was

191 resuspended with a gently shake and conidial concentration was estimated using a Neubauer

192 camera.

193

194 *2.5. Effect of the infective dish on the attraction of C. capitata V8 sterile males*

195 The infective dish superficially contained a small quantity (20 mg/g emitter) of TML,

196 which is an appropriate amount for assessing *C. capitata* attractiveness to the infective dish

197 under laboratory conditions. An attraction bioassay to evaluate the behavior of WT and V8

198 males was performed. The V8 sterile male were exposed to Ginger Root Oil (GRO)

199 aromatherapy to improve sterile males mating competitiveness (Shelly et al. 2007), as

200 presently used in most of the ongoing SIT programs worldwide, whereas WT males were not

201 subjected to any previous treatment before the experiment to simulate field conditions. Thus,

202 24 h before the assay, five hundred 4 day-old V8 sterile males were exposed to volatiles

203 emitted by a piece of cotton containing 100 μl of GRO (Lluch Essence S.L., Spain), during 3

204 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 x 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 x 10^7 conidia/ml (1 x 10^5 conidia/fly) (data not shown). Based on this finding, a

220 slightly lesser dose (5 x 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

224 methacrylate cages (20 x 20 x 20 cm) and provided with sugar: yeast hydrolysate 4:1 (wt:wt)

225 diet and water until they reached sexual maturity. Sterile males were obtained and kept in a

226 sugar diet plus water, as described previously.

227 Five day-old sterile males were inoculated with one microliter of a conidial aqueous

228 suspension of 5 x 10^7 conidia/ml (5 x 10^4 conidia/fly) on the ventral surface of the abdomen

229 of the adults previously anesthetized with ice. Fungus untreated insects were inoculated with

230 the same volume of a sterile aqueous solution of 0.05% Tween 80. Twenty-four hours before

231 the fungal treatment, V8 males were exposed for 3 h to 100 µl of GRO in a ventilated

232 methacrylate cage (30 x 40 x 30 cm). Three replicates were performed for each treatment. For

233 each replicate 14 V8 males (fungus treated or untreated) were used. As the goal of the

234 experiment was to evaluate the effect of the ongoing process of infection on the capability of

235 the sterile males to mate and transfer sperm, and taking into account that generally

236 germination of *M. anisopliae* conidia takes place within the first 20 h after contact

237 (Zimmermann, 2007), a latent period of 48 h was established. Thus, from the second day of

238 treatment, 7 virgin WT females (male: female ratio, 2:1) were introduced in the mating

239 scenario (ventilated methacrylate cage of 20 x 20 x 20 cm) and allowed to mate for 24 h.

240 Then, females were removed and stored in 70% ethanol at 4 ºC. The process was daily

241 repeated along 6 days. The number of new virgin females introduced each day changed

242 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 $MgCl_2$, 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

262 cage (30 x 40 x 30 cm). Three replicates by treatment and medfly strain were performed.

263 Forty-eight h later, and always starting the experiment at 10 am, 2h later of “light on” in daily

264 photophase, each group was transferred to a new ventilated methacrylate mating cage of 30 x

265 40 x 30 cm (Gavriel et al., 2009) and allowed to settle for 30 min. Then, 50 WT virgin

266 females were introduced into the mating arena. In each scenario, mating pairs were checked

267 continuously during 3 h. Copulating pairs were collected into glass vials (15 ml volume) 5

268 min after the mating initiation and copula duration was recorded. To ensure that mated

269 females had been inseminated, only females with copula duration longer than 100 min were

270 selected for the remating tests (Taylor and Yuval, 1999). After copula completion, mated

271 females were transferred to a new methacrylate cage (30 x 40 x 30 cm) and provided with the

272 standard sugar and yeast hydrolysate (4:1; wt:wt) diet and water. Non-mated females and

273 males were discarded.

274 *Remating procedure*. Remating tests were performed following the same procedure

275 described, and as a continuation of the first mating procedure. Thus, mated females from each

276 group were allowed to remate with healthy virgin WT males. The remating test was

277 performed two days after their first copula when a maximum level of *C. capitata* female

278 remating has been reported (Gavriel et al., 2009; Kraaijeveld et al., 2005). This process was

279 carried out over three consecutive days using the remaining non-remated females from each

280 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).

318

319 *3.2. Effect of the infective dish on the attraction of C. capitata V8 sterile males*

320 Results from the attraction bioassay in laboratory cages are shown in Table 2. The

321 percentage of attraction was significantly different among infective and non-infective dishes

322 as well as between fly strains ($F_{3, 28}$ = 29.159, $P < 0.001$). Both V8 and WT males showed a

323 significantly higher attraction to the infective dishes than to the control ones. In addition, WT

324 males were significantly more attracted than V8 males to either infective or non-infective

325 dishes.

326

327 *3.3. Effect of M. anisopliae on the sexual performance of C. capitata V8 sterile males*

328 *3.3.1. Sperm transference detection*

329 The application of CcYsp marker (Fig. 1) indicated that treated V8 males developing

330 the fungal infection during two days before being exposed to virgin females were able to

331 qualitatively transfer sperm similarly to untreated V8 males. Percentages of mating higher

332 than 90% were observed in most cases, including those of the sixth day, when the treated V8

333 males were already showing mortality values of about 50% (Table 3). GLM-U disclosed no

334 significant differences respect to the capability of mate and transfer sperm between the two

335 treatments ($F_{1, 35}$ = 0.185, P = 0.671) and time ($F_{5, 35}$ = 0.480, P = 0.788) and no interaction

336 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

375 **4. Discussion**

376 There is an increasing need to develop new safer environmental measures to control 377 *C. capitata*. In this context, we have investigated the compatibility of a new *M. anisopliae*-378 based attractant-contaminant device with the sterile males used in SIT programs. This 379 approach involves a new notion of a combined strategy leading to a reduction of the wild 380 population density by using *M. anisopliae*-based ACDs, as it has been reported for *C.* 381 *capitata* in citrus orchards (Navarro-Llopis et al., *submitted*), and to a significant 382 improvement in the efficiency of SIT programs (Klassen, 2005). This combined strategy 383 would be particularly important in those regions with high population density, as usually 384 occurs in the Spanish citrus growing area. However, to be compatible with SIT, it will be 385 necessary that sterile males will not be affected in their performance.

386 We found that the exposition of *C. capitata* V8 sterile males to the infective dish 387 entailed a higher susceptibility to *M. anisopliae* than when aqueous conidial suspensions 388 were used to provoke the infection (sperm transference assay). This effect can be related to 389 the oil-fungus formulation contained in the infective dish, since it has been reported that oil 390 formulations enhance pathogens efficacy (Prior et al., 1988; Barson et al., 1994) accelerating 391 the mycosis process on the host in comparison to aqueous formulations. According to Prior et 392 al. (1988) oil prevents conidia from drying, increases their adhesion ability to the insect 393 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

432 experimental conditions that exposure to fungal inoculation does not adversely affect the

433 mating performance of *C. capitata* up to the day when mortality due to inoculation begins.

434 Similarly, Novelo-Rincon et al. (2009) reported, in field-cages experiments, no significant

435 differences in sexual performance of *Anastrepha ludens* sterile males when used as vector or

436 not of *Beauveria bassiana* conidia. Yet, open field experiments are needed to validate these

437 data.

438 Remating frequency in *C. capitata* is a complex issue and remating rates in wild

439 females are variable depending on the environmental conditions and strains (Mossinson and

440 Yuval, 2003, Vera et al., 2003; Kraaijeveld et al., 2005; Gavriel et al., 2009). We have found

441 that females showed higher tendency to remate if previously mated to *M. anisopliae*-treated

442 males, either V8 or WT, though the percentages of remating remained similar in all

443 treatments and within the range of values reported by different authors (Mossinson and

444 Yuval, 2003; Gavriel et al., 2009). In addition, the remating rate under laboratory conditions

445 might not reflect the situation in the field, since it could be related to the density of flies

446 released in laboratory cages and the cage conditions could also stimulate either indoors or

447 outdoors remating (Vera et al, 2003). Moreover, the significant minor attraction of V8 sterile

448 male to the infective dish might suggest that a smaller proportion of them in relation to WT

449 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

469 integration of these two friendly strategies, the SIT and the fungus attractant-contaminant

470 device against the Mediterranean fruit fly, *C. capitata*. Nevertheless, it will be necessary to

471 ascertain whether similar results are obtained under field conditions.

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637 TABLES CAPTIONS

638 **Table 1**. Time-mortality response of *C. capitata* males (V8 and WT) against *M. anisopliae*

639 by contact application on the infective dish.

640

641 [a] Values (conidia/fly) represent the mean ± SE of conidia recovered and estimated using an

642 hemocytometer. [b] Values in days. [c] Parallelism Test: $\chi^2 = 3.152$; $df = 1$; $P = 0.076$; common

643 slope (±SE): 9.921 ± 0.213: Relative Potency of the response of WT males respect to V8

644 males (± 95% Fiducial Limits): 1.03 (0.01, 1.06)

645

646 **Table 2**. Percentage of attraction of *C. capitata* males (V8 and WT) exposed to infective *M.*

647 *anisopliae* dishes under laboratory conditions.

648

649 [a] Values are mean ± SE over a 3 h period. [b] To simulate field conditions, only V8 males were

650 subjected to a GRO-aromatherapy treatment 24 h before the experiment. Values followed by

651 different letters are significantly different ($P < 0.05$).

652

653 **Table 3**. Sperm detection over time of *C. capitata* WT virgin females mated to *M.*

654 *anisopliae*-treated or untreated *C. capitata* V8 males.

655

656 *Ceratitis capitata* V8 males were *M. anisopliae*-treated by topical application (1µl) using an aqueous

657 suspension of 5.0 x 10^7 conidia/ml (5.0 x 10^4 conidia/fly), two days before exposition to the mating

658 process. For each day, mating values are mean ± SE of percentage of females containing sperm in

659 their spermathecae with respect to the number of females assayed that day, which was corrected as a

660 function of the V8 males mortality to maintain the initial male: female (2:1) rate.

661 **Table 4**. Percentage of matings and their duration between *C. capitata* virgin WT females and
662 virgin males (V8 or WT), either treated or untreated with *M. anisopliae*.
663
664 *Ceratitis capitata* WT and V8 males were fungus-treated by contact application on infective dishes
665 two days before the mating process. Values (mean ± SE) followed by different letters within columns
666 are significantly different ($P < 0.05$).
667
668 **Table 5**. Percentage of rematings and their duration between WT females first-mated to *M.*
669 *anisopliae* treated or untreated *C. capitata* V8 or WT males and *C. capitata* WT males.
670
671 Females first-mated to *M. anisopliae* treated or untreated, *C. capitata* V8 or WT males, were allowed
672 to remate to healthy WT males two days after their first mate. For each day, values are percentages
673 (mean ± SE) of remated females respect to the total number of first-mated females in the mating cage.
674 Remating in Day 2 and Day 3 were performed with the remaining non-remated females from the
675 previous day. Percentage values followed by different letters within columns are significantly
676 different ($P < 0.05$).
677 Remating duration was obtained as the average value from all the rematings times recorded along the
678 3-days experiment.
679
680

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

682

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

695

696 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

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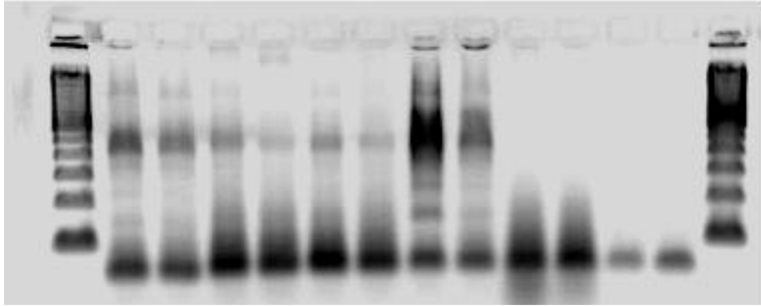
698

699 FIGURE CAPTIONS

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

707 Figure 1.



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