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3 **Development of an attract-and-infect system to control *Rhynchophorus ferrugineus***  
4 **with the entomopathogenic fungus *Beauveria bassiana***

5

6 **Dembilio Ó<sup>1¥</sup>, Pilar Moya,<sup>2¥</sup> Sandra Vacas,<sup>2</sup> Lola Ortega-García<sup>3</sup>, Enrique**  
7 **Quesada-Moraga,<sup>3</sup> Josep A. Jaques,<sup>1†</sup> Vicente Navarro-Llopis<sup>2\*</sup>**

8

9 <sup>1</sup> Universitat Jaume I (UJI). Unitat Associada d'Entomologia Agrícola UJI-IVIA  
10 (Institut Valencià d'Investigacions Agràries), Departament de Ciències Agràries i del  
11 Medi Natural, Campus del Riu Sec. 12071. Castelló de la Plana (Spain)

12 <sup>2</sup> Universitat Politècnica de València. Instituto Agroforestal del Mediterráneo. Camino  
13 de Vera s/n. Edificio 6C. 46022. Valencia (Spain)

14 <sup>3</sup> Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba.  
15 Campus de Rabanales. Edificio C4 Celestino Mutis. 14071 Cordoba, Spain.

16 <sup>¥</sup> Both authors contributed equally to this study and should be considered as first author.

17 \* Correspondence to: V Navarro-Llopis, Universitat Politècnica de Valencia. Instituto  
18 Agroforestal del Mediterráneo. Camino de Vera s/n. Edificio 6C. 46022. Valencia  
19 (Spain). E-mail: vinallo@ceqa.upv.es

20 <sup>†</sup>Formerly Josep A Jacas

21

22 **Abstract**

23 BACKGROUND: A new *Beauveria bassiana*-based Attract and Infect Device (AID) to  
24 control *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) was developed.  
25 The virulence and persistence of the fungal formulation used in the AID was evaluated  
26 in laboratory. Semi-field and field trials were carried out to validate results and evaluate  
27 the efficacy of the devices.

28 RESULTS: In laboratory conditions, an  $LT_{50}$  of 4.33 days was obtained when adults (7-  
29 10 day-old) were exposed to the Inoculation Tunnel (IT) containing  $1 \times 10^{10}$  conidia/g  
30 in an oil-based fungal formulation. This formulation maintained conidia viability at 50  
31 % for up to 2 months. Moreover, when adults were exposed to 2.5-month field aged ITs  
32 mortality still reached 50% 40 days after exposition. In addition, no differences were  
33 observed between ITs aged in early-spring or those aged in summer suggesting that  
34 fungal formulation is not strongly affected by environmental factors in Mediterranean  
35 basin conditions. Semi-field assays showed that the device allowed an easy transit of  
36 weevils through the IT, which effectively became attracted and infected. Using one AID  
37 per ha in 4-ha plot field trials a reduction of more than 50% on the percentage of  
38 infested sentinel palms was obtained compared to plots treated with mass trapping, also  
39 installed at 1 trap per ha.

40 CONCLUSIONS: Based on the reported results in efficacy and persistence of this new  
41 AID in the field and on its potential in reducing *R. ferrugineus* populations and palm  
42 infestation, this device showed potential as a tool for the management of *R. ferrugineus*.

43

44 **Keywords:** red palm weevil; Curculionidae; entomopathogenic fungi; infective device

45

## 46 **1 INTRODUCTION**

47 In the last 10 years, the red palm weevil *Rhynchophorus ferrugineus* Olivier  
48 (Coleoptera: Curculionidae) has become the most destructive pest of palms in the world,  
49 particularly in the Mediterranean basin.<sup>1-2</sup> In this region the Canary Islands Date palm,  
50 *Phoenix canariensis* Hort ex Chabaud, is widely used as ornamental, whereas the date  
51 palm, *P. dactylifera*, is mostly grown for its fruit in the southern countries of this basin.<sup>3</sup>  
52 This weevil, native to south Asia and Melanesia, previously colonized most of  
53 southwestern Asia, the Arabian Peninsula in the mid-1980s, and Middle East and Egypt  
54 at the beginning of 1990s.<sup>4</sup> Later on, it was detected in other regions including the  
55 Canary Islands, the Caribbean, and southern China.<sup>1</sup> This pest is multivoltine and  
56 depending on climatic conditions, it can have from one single generation per year (i.e,  
57 Northern Mediterranean basin countries) to several overlapping generations in warmer  
58 climates.<sup>5</sup>

59 Control methods against *R. ferrugineus* are based on regular preventive treatments  
60 because early detection is not easy because of the hidden habits of most of its life  
61 cycle.<sup>6</sup> Pesticides, such as imidacloprid or chlorpyrifos, or entomopathogenic  
62 nematodes, are usually applied by spraying on the crown using different devices.<sup>7-8</sup>  
63 However, as the effect does not last for more than 1.5-2 months, at least 5-7 treatments  
64 per year may be required.<sup>8-9</sup> Systemic insecticides (mainly neonicotinoids and  
65 avermectins) can be applied by stipe injection. Although the efficacy of this technique  
66 has improved by use of low-pressure injectors,<sup>2</sup> the number of applications required is  
67 still high.

68 Some alternatives to chemical control are the use of entomopathogenic nematodes<sup>7</sup> or  
69 fungi. Several strains of *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium*  
70 *anisopliae* (Metchn.) Sorokin (Hypocreales: Clavicipitaceae) have been isolated from

71 wild *R. ferrugineus* populations.<sup>10-14</sup> These entomopathogenic fungi have been tested  
72 against *R. ferrugineus* by direct injection,<sup>15</sup> application of fungal spores to the crown or  
73 stipe by spray or painting,<sup>11</sup> release of *R. ferrugineus* adults contaminated with spores<sup>15-</sup>  
74 <sup>16</sup> or by a combination of these techniques with, i.e., mass trapping.<sup>17</sup> The use of attract  
75 and infect devices could be, probably, the most efficient way to spread the inoculum of  
76 the fungus by horizontal transmission to other individuals, including those in already  
77 infested palms. Several authors have tried to develop this kind of devices against *R.*  
78 *ferrugineus*.<sup>18-19</sup> However, none of them has demonstrated their efficacy in field trials  
79 yet. The main objective of this study has been (1) to develop an effective  
80 autoinoculation system and (2) to evaluate the efficacy of this attract and infect device  
81 (AID) in field conditions.

82

## 83 **2 MATERIALS AND METHODS**

### 84 **2.1 Entomopathogenic fungus**

85 The *B. bassiana* strain used in the experiment was isolated from an infected pupa  
86 originally collected in a date palm grove near the town of Catral, Spain, and belongs to  
87 the fungal collection of the Departamento de Ciencias y Recursos Agrícolas y  
88 Forestales of the University of Córdoba (Spain) with the reference code EABb 07/06-  
89 Rf.<sup>11</sup> This strain was deposited with accession No. CECT- 20752 on May 13, 2009,  
90 following the Budapest Treaty, in the Spanish Collection of Culture Types (CECT) at  
91 the University of Valencia (Spain).

### 92 **2.2 Stock colonies**

93 Adult weevils collected in the province of Valencia in traps baited with ferrugineol (the  
94 male *R. ferrugineus* aggregation pheromone) dispensers and plant kairomones (ethyl

95 acetate and pieces of palm fronds) were used in some of our assays (see below) and also  
96 to start our stock colonies. These colonies were established in 2007 and have been  
97 periodically supplemented with the introduction of additional wild specimens. Adult  
98 weevils were reared in a controlled environment cabinet at  $25 \pm 1$  °C,  $75 \pm 5\%$  R.H. and  
99 a 16 h light photoperiod in perspex cages ( $30 \times 30 \times 45$  cm depth) with a density of  
100 100-120 weevils per cage.<sup>20</sup>

### 101 **2.3 Experimental insects**

102 Both laboratory-reared and wild specimens were used in our assays. Seven to ten day-  
103 old laboratory-reared adult *R. ferrugineus* were used in the laboratory assays to assess  
104 both the virulence of the fungus and its capacity to be horizontally transmitted to  
105 healthy adults when formulated as when used in the AID. The same type of insects was  
106 used in semi-field experiments. On the other hand, wild adults were used to assess the  
107 performance of the inoculation tunnels (IT) after several field ageing times because not  
108 enough laboratory-reared insects were available. In this case, trap-collected adults were  
109 maintained in our insectary and periodically examined during two to three weeks before  
110 the onset of the assay to discard weak, presumably unhealthy specimens.

### 111 **2.4 Plant material**

112 For both field and semi-field assays, 5-year old potted *P. canariensis* palms obtained  
113 from an officially inspected nursery, and therefore considered as *R. ferrugineus*-free,  
114 were used. The stipe of these palms was 0.35 to 0.55 m high and 0.30 to 0.40 m wide. In  
115 the semi-field assays, these plants were kept inside a double mesh security enclosure  
116 containing 24 independent cages ( $4 \times 3 \times 3$  m), under natural light and temperature  
117 conditions, and watered twice per week. In field-assays, palms were watered only once  
118 per week.

## 119 **2.5 Attract-and-Infect Device (AID)**

120 The commercially available black pyramidal trap Picusan® (Fig. 1A),<sup>21</sup> supplied by  
121 Sansan Prodesing SL (Valencia, Spain), was conveniently modified and used in our  
122 assays. This trap consisted of three parts: 1) a cylindrical base (25 cm in diameter, 6 cm  
123 height); 2) a rough (1 mm between grooves) black pyramid with a 66% slope and a  
124 funnel inserted onto the upper side; and 3) a green cover on the top leaving a 4-cm  
125 opening between the upper side of the pyramid and the top. This cover had a small  
126 basket inserted in its center where a 1-g ferrugineol dispenser (Pherosan RF, Sansan  
127 Prodesing SL) was set. The main modification of the standard trap consisted of an L-  
128 shaped pipeline (2.5 cm in diameter) connected to the funnel and to the base of the  
129 pyramid (Fig. 1B) to allow insects to freely enter and leave the trap. A removable lid  
130 opening outwards protected the exit hole on the pyramid. The lower part of the L-  
131 shaped pipeline was transformed into an inoculation tunnel containing the fungal  
132 formulation.

### 133 *2.5.1 Fungal formulation*

134 The fungal formulation used in the AID was made according to Primo-Yúfera et al.<sup>22</sup>  
135 with some modifications. Briefly, the *B. bassiana* strain was cultured in Petri dishes  
136 containing potato dextrose agar (PDA) medium (Difco, BD, Madrid, Spain)  
137 supplemented with yeast extract (1%) (Difco, BD, Madrid, Spain) at 26 °C in dark  
138 conditions. Previously to each experiment, viable germinating conidia were counted  
139 after 24 h of incubation at 26 °C in PDA.<sup>23</sup> In all cases, germination of conidia was over  
140 96%. Conidia from 18-20-day-old cultures were suspended in mineral oil and removed  
141 from each dish with a 10-mL pipette. Suspensions from four Petri dishes were  
142 combined in a sterile Falcon tube (50 mL), sonicated during 2 min and filtered through  
143 four layers of cheesecloth to obtain pure conidia. After centrifugation (3000 rpm, 3 min;

144 Rotina 46, Hettich, Germany), oil exceeding 10 mL was removed. Conidia  
145 concentration, estimated using a haemocytometer (Improved Neubauer chamber), was  
146 adjusted to obtain  $2 \times 10^{10}$  conidia in a final volume of 4 mL. Then, 2 g of a clay carrier  
147 were added and manually stirred to complete homogenization.

#### 148 2.5.2 Inoculation tunnels (ITs)

149 The formulation above was spread on a piece of black corrugated plastic (PVC) tube  
150 (100 mm long  $\times$  25 mm diameter), which constituted the contaminant component of the  
151 AID. In all cases, the final fungal concentration in the inoculation tunnel (IT) was  $1 \times$   
152  $10^7$  conidia  $\text{mm}^{-2}$  ( $2 \times 10^{10}$  conidia per IT). The control tunnel was prepared as  
153 described for the ITs but conidia were previously sterilized. In this case, fungal conidia  
154 were harvested in dry conditions by scraping the surface of the culture plate and the  
155 amount (by weight) corresponding to  $2 \times 10^{10}$  conidia was moist heat sterilized ( $121^\circ \text{C}$   
156 for 30 min) in an autoclave (Presoclave 15, JP Selecta, Barcelona, Spain). Afterwards,  
157 sterile conidia were poured in a 50 mL-Falcon tube in which mineral oil (up to reach 4  
158 mL) and clay carrier (2 g) were added to complete the formulation of the inactivated-  
159 fungus, control tunnel.

## 160 2.6 Laboratory bioassays

### 161 2.6.1 Infectivity of *B. bassiana* in the IT

162 This assay was performed using AIDs without the ferrugineol dispenser. The AID was  
163 placed inside a plexiglass cage ( $40 \times 30 \times 40$  cm) to easily recover the contaminated  
164 insects. Sixteen 7-15 day-old *R. ferrugineus* adults, 8 males and 8 females, from the  
165 stock colony were forced to cross the IT by introducing them through the upper part of  
166 the L-shaped pipeline. To ensure that weevils were not able to step back and get out  
167 through the upper part of the trap, or to avoid re-entry of trap-leaving insects, the upper

168 opening was partially closed once the insects had been introduced into the AID.  
169 Twenty-four hours later insects having crossed the IT once (i.e., those in the cage) were  
170 recovered. These insects were individually introduced into small-aerated plastic cages  
171 (11.0 × 4.5 × 7.5 cm) with a non-treated partner of the opposite sex and left undisturbed  
172 during 24 h to assess horizontal transmission. Then, couples were separated and each  
173 insect was introduced in a new clean cage where they were fed an apple slice and moist  
174 paper (replaced as needed). Survival was assessed daily for 10-12 days in the case of  
175 insects contaminated in the IT, or up to 30 days for those exposed to horizontal  
176 transmission. To confirm mycosis, each dead insect was individually surface-sterilized  
177 by immersion during 1 min in a 0.3% sodium hypochlorite solution (x 2 times). Then, it  
178 was rinsed using sterile distilled water (x 2 times; 1 min each) and individually  
179 incubated in a wet dark chamber at 26°C for 20 days. Mycosis was assumed when the  
180 sporulated mycelia of the fungus was observed growing from the cadaver. Lethal time  
181 50 (LT<sub>50</sub>), the time required to kill 50 % of the insects, was estimated according to San  
182 Andrés et al.<sup>24</sup> and used as an estimation of fungal virulence. Five assays, each  
183 consisting of two replicated ITs and a control tunnel, were carried out.

184 Additionally, two couples per assay (a total of 10 couples), treated as above were used  
185 to determine the per capita rate of propagule pick up by either direct exposition to the IT  
186 or by horizontal transmission. Thus, conidia picked up by each insect were recovered by  
187 three successive washes of dichlorometane (5 mL each) which were combined in a glass  
188 tube and concentrated up to 5 mL under gentle nitrogen stream. Conidia concentration  
189 was estimated as described in section 2.5.1.

#### 190 *2.6.2 Field persistence of fungal activity in the IT*

191 Conidia viability in the ITs was evaluated from the moment when AIDs were set in the  
192 field until their removal in (1) Valencia (39°29'02.4"N; 0°20'25.1"W; outdoor

193 conditions) from 3 February to 24 April, and (2) Sagunt (39°39'51"N; 0°17'31"W) from  
194 14 April to 17 June (spring ITs) and from 24 June to 12 August (summer ITs), in 2014.  
195 Although already replaced in 17 June from the field trial, several spring ITs were  
196 maintained in the field until 12 August for a longer evaluation period. Every two weeks,  
197 a small amount (20-30 mg) of infective material from the IT was taken to the laboratory.  
198 The sample was weighted and 1 mL of mineral oil (the same oil as when preparing the  
199 infective material) was added. The sample was then stirred in a vortex (2 min) and  
200 sonicated (2 min). The suspension was allowed to precipitate the inorganic material and  
201 the oil was transferred to another vial. The remaining solid was washed again with 1 mL  
202 of mineral oil as before and added to the previous oil sample. From this suspension, 10-  
203 fold serially diluted oil suspensions were prepared to obtain the colony forming units  
204 (cfu) per mg of infective material. Fifty  $\mu$ L of each suspension were inoculated in a  
205 Petri dish containing *B. bassiana* CTC selective medium, consisting of PDAY [potato  
206 dextrose agar (Difco; BD) supplemented with 1 g L<sup>-1</sup> yeast extract (Difco; BD) + 0.5 g  
207 L<sup>-1</sup> chloramphenicol (Sigma-Aldrich, Madrid, Spain) + 0.001 g L<sup>-1</sup> thiabendazole  
208 (Sigma) + 0.25 g L<sup>-1</sup> cycloheximide (Sigma).<sup>25</sup>

209 The number of cfu obtained when the IT were assembled was considered as 100%  
210 viability and subsequent recordings were referred to this result. For each ageing time,  
211 three ITs were analyzed.

## 212 **2.7 Semi-field assay**

213 A semi-field field trial was carried out in a greenhouse with 6 independent meshed  
214 cages (4 × 3 × 3 meters). An AID was placed in the center of the cage and immediately  
215 after, three *R. ferrugineus* adult males and nine females were released. Three cages were  
216 provided with an infective AID (treated cages), whereas three additional cages had an  
217 AID with an inactivated fungal formulation (see 1.6 section) (untreated cages). A cotton

218 bud coated with fluorescein (Sigma-Aldrich, Madrid, Spain) was placed at the exit of  
219 each tunnel to mark the insects going through the AID. Forty-eight hours after trap  
220 placement, three palms with a crown of 0.35 to 0.55 m high and 0.30 to 0.40 m wide  
221 were introduced into each cage and left there with the weevils for 3 additional days.  
222 After this period, weevils were recovered, counted and inspected with a black light  
223 source to check how many of them had walked through the tunnel.

224 Palms exposed to the weevils were left in the greenhouse for 2 additional months to  
225 allow immature development. After this period, the palms were thoroughly dissected  
226 and the numbers of larvae, pupae and adults were counted. Twenty larvae from each  
227 cage were maintained in a dark wet chamber to record the number of individuals  
228 showing signs of infection.

## 229 **2.8 Field assays**

230 The field assay consisted of seven replicates, three of them in the province of Valencia  
231 (two in the municipality of Montcada (39°35'20"N; 0°23'55"W) and one in Sagunt  
232 (39°39'51"N; 0°17'31"W)), one in Córdoba (37°55'13"N; 4°43'30"W) and the remaining  
233 three in the island of Ibiza (the municipalities of Sant Carles (39°01'46"N; 1°30'29"E),  
234 Santa Eulària del Riu (38°58'52"N; 1°26'16"E) and Sant Antoni 38°59'16"N;  
235 1°20'23"E). Each trial consisted of two 4-ha plots. One of these paired plots was  
236 supplied with 4 AID set at the corners of a 100 × 100 m square (infective plot with 1  
237 AID ha<sup>-1</sup>). The other plot received 4 standard Picusan® traps set also at the corners of a  
238 100 × 100 m square (mass trapping plot with 1 trap ha<sup>-1</sup>). Trap density was chosen  
239 according to conventional mass trapping protocols employed by the Valencian  
240 Community local government. Infective and mass trapping plots of each trial were  
241 separated at least 200 m. Both plots had in the center a standard Picusan® trap, baited  
242 with a 1 g ferrugineol dispenser (Pherosan RF, Sansan Prodesing SL, Náquera, Spain)

243 and a DDVP strip (Biagro SL, Valencia, Spain) and were used to monitor *R. ferrugineus*  
244 populations in each plot. Dry traps baited with ferrugineol instead of traps containing  
245 pheromone, water and molasses were used in order to evaluate infection rate in the  
246 captured adults. Although traps baited with water and molasses are more attractive to  
247 weevils, infection rate evaluation would not have been feasible in soaked adults.

248 Both standard Picusan® traps and AID were placed in field on 14 April 2014 and trials  
249 ended four months later. Inoculation tunnels in AID were replaced once on 24 June, and  
250 ferrugineol dispensers were not replaced during the assay. Weevils captured in all the  
251 central standard Picusan® traps and the four traps of the mass trapping plots were  
252 counted weekly. Moreover, the weevils of the central traps were taken to the laboratory  
253 to ascertain whether they had been infested by *B. bassiana*. Thus, they were processed  
254 as described above in order to confirm mycosis (see 2.6.1 section). In addition, four  
255 palms were set around the central trap of the infective and mass trapping plots as  
256 sentinel plants in the assays carried out in Montcada, Sagunt and Córdoba. These palms  
257 remained in place for the four months that the trial lasted and were watered weekly. On  
258 12 August they were thoroughly dissected to assess *R. ferrugineus* attack and the  
259 number of larvae, pupae and adults were counted. Three inoculation tunnels of each  
260 area were also taken to the laboratory at the end of the trials to assess conidia viability  
261 and insecticidal activity against wild *R. ferrugineus* as above. Insecticidal activity was  
262 measured in 10 adults per tunnel.

## 263 **2.9 Statistical Analysis**

264 Mortality data in virulence experiments were corrected using Abbott's formula when  
265 necessary.<sup>26</sup> The median lethal time (LT<sub>50</sub>) value was estimated by probit analysis using  
266 the SPSS v16.0.1 for Windows (SPSS Inc., 2008). Mortality data of insects exposed to

267 the AID in the laboratory were further used to calculate the Average Survival Times  
268 (AST) in days using the Kaplan–Meier survival analysis.<sup>27</sup>

269 For the semi-field trial, an ANOVA followed by LSD test ( $P < 0.05$ ) was conducted  
270 with the total number of insects captured in each treatment. Differences in percentage of  
271 adults captured showing fungal outgrowth in the field trials were analyzed using a  $t$ -test.  
272 In this case, data were previously transformed ( $\arcsin(\sqrt{x+1})$ ) to meet the  
273 assumptions of ANOVA. Differences in palm infestation in the field assays were  
274 assessed using a Chi square-test. Same as for semi-field trials, the number of *R.*  
275 *ferrugineus* per palm was analyzed using ANOVA and LSD test at  $P < 0.05$ .

276

## 277 **3 RESULTS**

### 278 **3.1 Laboratory bioassays**

279 Mortality of insects exposed to the AID in the laboratory reached 72 and 92 % five and  
280 nine days after treatment, respectively (Fig. 2). Remarkably, most of these insects did  
281 not move and only reacted if gently touched with a small brush as soon as four days  
282 after treatment. However, mortality data were only recorded when insects definitively  
283 died. The estimated  $LT_{50}$  and AST were 4.33 days (95% fiducial limits: 3.90 and 4.80  
284 days; slope  $\pm$  standard error:  $5.980 \pm 0.283$ ;  $X^2 = 276.3$ ,  $df = 18$ ;  $P \leq 0.001$ ) and 6.21  
285 days, respectively. The average conidial load picked up by a single adult weevil when  
286 leaving a freshly-made IT was  $2.23 \pm 0.46 \times 10^7$  conidia. This amount almost halved  
287 ( $1.02 \pm 0.39 \times 10^7$  conidia) when the tunnel had already been crossed by 23 individuals.  
288 Interestingly, evidence of horizontal transmission was observed starting 15 days after  
289 pairing with inoculated insects and mortality reached 45 % on day 30 (Fig. 3). The  
290 conidial load of these insects was estimated at  $2.16 \pm 0.51 \times 10^6$  conidia, which is about

291 10-fold lower than what was observed when insects were directly exposed to the fresh  
292 IT.

### 293 **3.2 Semi-field assays**

294 More than 88% of the adult weevils recovered in the cages were marked with  
295 fluorescein, and this is indicative that most of them had passed through the ITs. When  
296 palms were dissected two months later, all the palms in both control and treatment cages  
297 were infested. However, the number of *R. ferrugineus* found in palms exposed to an  
298 AID were significantly lower than in control palms ( $32.3 \pm 3.7$  and  $51.0 \pm 2.4$ ,  
299 respectively;  $F = 16.78$ ;  $df = 1, 16$ ;  $P < 0.001$ ). Furthermore, the infection rate of the  
300 individuals in the cages treated with AID was 4-fold than in the control ( $28.3 \pm 3.9$  and  
301  $7.1 \pm 2.1$ , respectively;  $F = 35.84$ ;  $df = 1, 16$ ;  $P < 0.001$ ).

### 302 **3.3 Field assays**

303 The total number of weevils captured in the center of infective and mass trapping plots  
304 and the percentage of these insects that showed fungal outgrowth is shown in Table 1.  
305 No differences in fungal outgrowth were detected between insects captured in plots  
306 treated with mass trapping and plots treated with AIDs. In addition, some weevils  
307 captured in traps located outside the trial areas (500 to 3000 m away) were evaluated for  
308 fungal outgrowth and their rate of infection was significantly lower than what was  
309 observed in the trial areas (same authors, unpublished results).

310 When infestation of sentinel palms was assessed at the end of the assay, 37.5% of the  
311 palms placed in the infective plots were infested, whereas this percentage increased to  
312 81.3 % of the palms placed in the mass trapping plots (Table 2). Indeed, the mean  
313 number of weevils developing per palm in the mass trapping plots was more than 3-fold  
314 the number found in palms in the infective plots (17.0 versus 5.2, respectively, Table 3).

315 Therefore, mass trapping at a density of 1 trap ha<sup>-1</sup> was not enough to control palm  
316 infestation in 4 ha-plots. However, the same density of infective traps resulted in a 46%  
317 reduction of infested palms. Efficacy of both mass trapping and attract and infect  
318 techniques might be improved by using water and co-attractants as described in  
319 previous research.<sup>21</sup>

### 320 **3.4 Laboratory evaluation of both fungal formulation persistence and infective** 321 **activity of field-used ITs**

322 The viability of the fungal formulation in the IT evaluated under outdoor conditions  
323 prior to field assays (3 February to 24 April 2014) (Fig. 4) was 70 and 45% 63 and 82  
324 days after field exposure, respectively. Fungal viability measured in parallel with field  
325 assays in Sagunt (Fig. 5A) remained over 50%, 67 days after the start of the test.  
326 Although most of these tunnels were replaced by newly-made ITs at that date, some of  
327 them were allowed to further age in the field for a longer evaluation period (white bars  
328 in Fig. 5A). Viability decreased to almost 30 and 12% after 3 and 4 months of aging,  
329 respectively. Interestingly, the viability decrease observed was similar for both the  
330 initial and replaced ITs up to 50 days of aging, even though they had been exposed to  
331 different environmental conditions: spring (Fig. 5A) and summer (Fig. 5B). At the end  
332 of both periods, three ITs per plot were taken to the laboratory to evaluate both their  
333 infective activity and fungal viability. The spring ITs (aged in the field for 2.5 months)  
334 caused 50% mortality in approximately 45 days (Fig. 6A). Mortality in the ITs from the  
335 Ibiza trials was higher than in those from Valencia (63.3 versus 35.6% at day 34).  
336 However, mortality in the Ibiza trial remained the same until the end of the assay. At  
337 that time (2.5 months ageing), fungal viability was  $35.26 \pm 1.46\%$  and  $30.62 \pm 1.28\%$   
338 for Ibiza and Valencia trials, respectively. At the end of the second period (Fig. 6B),  
339 mortality was slightly higher than in the first one. Mean mortality was about 60% by

340 day 40. However, the ITs from both locations showed a more homogenous response  
341 ( $86.83 \pm 1.48\%$  and  $81.21 \pm 3.06\%$  fungal viability, respectively). The mean viability  
342 of the ITs, which were allowed to remain in the field until the first week of September  
343 in Ibiza (ageing period of 70 days) was  $43.32 \pm 0.49\%$ .

344

#### 345 **4 DISCUSSION**

346 The use of entomopathogenic fungi in attract and infect traps has been developed for  
347 several pests including dipterans, such as fruit flies,<sup>28-29</sup> leafminer<sup>30</sup> and tsetse flies,<sup>31</sup>  
348 coleopterans, such as palm weevils,<sup>19</sup> and lepidopterans.<sup>32</sup> Our work describes an AID  
349 for controlling *R. ferrugineus*. According to Vega et al.,<sup>33</sup> insects are attracted to the  
350 infective source in the device, become infected, leave the source and then disseminate  
351 the pathogen to other members of the target population. Similar attract and infect traps  
352 used against Triatominae have demonstrated a high efficacy in reducing pest  
353 populations and a 52.4% population mortality.<sup>34</sup> When infective traps were applied in  
354 houses, infection rate was reduced up to 85%, and a significant reduction in fertility and  
355 fecundity of infected females was obtained.<sup>35</sup>

356 Entomopathogenic conidia are, in many cases, very sensitive to weather conditions,<sup>36</sup>  
357 which is a key point of the system's efficacy together with the horizontal transmission  
358 of the pathogen.<sup>31</sup> Therefore, the main objective of this research was to develop a device  
359 and a formulation protecting spores from adverse environmental conditions for as long  
360 as possible and which, at the same time, should be effective for weevil attraction and  
361 infection.

362 The fungal strain used in this study had previously shown promising activity results  
363 against *R. ferrugineus*. Dembilio et al.<sup>11</sup> tested the virulence of this strain against

364 laboratory-reared and field-collected adults by immersion in eight conidial aqueous  
365 suspensions ranging from  $5.16 \times 10^6$  to  $6.73 \times 10^9$  conidia mL<sup>-1</sup>, reporting that adults  
366 survived around 16 days on average when dose was  $5.16 \times 10^9$  conidia mL<sup>-1</sup> and  
367 mortality was null with doses below  $5.16 \times 10^8$  conidia mL<sup>-1</sup>. Following these reported  
368 conditions, we subsequently proved that a single weevil treated with the most effective  
369 dose ( $5.16 \times 10^9$  conidia mL<sup>-1</sup>) was able to acquire a fungal load of  $6.7 \pm 0.9 \times 10^7$   
370 conidia (unpublished results). Based on these results, the fungal dose required in the IT  
371 to ensure that crossing weevils acquire between 2 and  $6 \times 10^7$  conidia was  $1.0 \times 10^{10}$   
372 conidia g<sup>-1</sup> of solid carrier ( $2 \times 10^{10}$  conidia IT<sup>-1</sup>). was established to be used in our  
373 studies because 1) the maximum amount of free oil that could be used to suspend about  
374  $6.0 \times 10^7$  conidia without provoking oil-toxicity effects when applied on a *R.*  
375 *ferrugineus* adult was 6 µl and, 2) from an economic point of view, it was a relatively  
376 high but commercially feasible concentration to be used. According to the present work,  
377 the oil-based formulation of the fungus used in ITs showed an LT<sub>50</sub> of 4.33 days with an  
378 average load of  $2.2 \times 10^7$  conidia per weevil, which is 3-fold less conidia than the  
379 amount gained by a weevil being immersed in a conidial aqueous suspension of  $5 \times 10^9$   
380 conidia mL<sup>-1</sup>. These results suggest that the fungal oil-based formulation enhances  
381 virulence, as a 3-fold lower fungal load reduced the time required to kill insects by  
382 approximately 4 times compared to Dembilio et al.<sup>11</sup> The enhancement of fungal  
383 virulence with oil formulations had been previously reported.<sup>36-37</sup> This is attributed to an  
384 increase of conidia adhesiveness to the insect cuticle and an interference with its  
385 defensive nature resulting in an acceleration of the fungal outgrowth process in the host  
386 compared to aqueous formulations. Furthermore, oil prevents conidia from drying and  
387 helps increase the fungal agent's persistence.<sup>38</sup>

388 The persistence of the attract and infect device capacities is crucial for the economic  
389 and technical feasibility of this method. These features would be hardly fulfilled if the  
390 device had to be serviced/replaced for less than one month. The device under study  
391 remained infective (over 50% of conidial viability) for 2 months, even during the driest  
392 and warmest seasons in the Mediterranean (summer). Even lower percentages of  
393 viability, as those corresponding to 2.5 ageing months (30-35%), have also been  
394 correlated in the laboratory with 50% mortality, 45 days after adult treatment. This  
395 residual efficacy is especially relevant if we keep in mind that the performance of  
396 treated insects was seriously impaired long before they died.

397 Previously developed devices maintain its infective capacity for at least 31 days in  
398 tsetse flies<sup>39</sup> and for almost 40 days against *Ceratitidis cosyra* (Walker).<sup>28</sup> An AID against  
399 *Ceratitidis capitata* (Wiedemann) using the same mesoporous technology employed  
400 herein has been recently reported.<sup>28</sup> This AID can remain active for almost 3 months in  
401 the field and only one replacement per year is needed to cover the whole season.  
402 Previous AIDs developed for *R. ferrugineus* control using conidia inoculated in rice lost  
403 fungal viability to around 40% after 4 weeks.<sup>18</sup> Contrarily, the new AID maintains  
404 conidial viability over 50% for at least 8 weeks. More recently, Hajjar and Ajlan<sup>19</sup> tested  
405 bucket traps covered with rough sackcloth soaked with a commercial oil-based  
406 formulation of *B. bassiana*. High infection rates of weevils and horizontal transmission  
407 occurred but only for 13 days.

408 If we consider the initial load of an IT ( $2.0 \times 10^{10}$  conidia) and that of a weevil  
409 crossing it ( $6.7 \times 10^7$  conidia), the maximum number of insects that could be effectively  
410 infected in each AID could reach 900, which is about 30 times higher than weekly  
411 captures in similar infested areas.<sup>21</sup> This is obviously a simplification, as the continuous  
412 reduction in infective material swept along by each crossing weevil, or progressively

413 reducing viability (approx. 50% of fungal viability after 2 months in the field) should be  
414 taken into account. Therefore, further studies are needed to provide real numbers of  
415 insects effectively infected by AID under real field conditions.

416 The results obtained in the present study show that the new AID is very effective at  
417 attracting and infecting weevils as more than 88% of the insects released in the semi-  
418 field assay passed through the IT and this resulted in 95% mortality. Moreover, the  
419 percentage of infested palms in field assays using 1 device ha<sup>-1</sup> of the new AID was  
420 reduced by more than 50% compared to mass trapping plots also installing 1 trap ha<sup>-1</sup>.  
421 Moreover, in some cases, 100% of the sentinel palms used remained uninfested in plots  
422 treated with AIDs. In the particular case of Córdoba, red palm weevil population level  
423 was lower than in Sagunt and Montcada and, consequently, damage in palms was lower.  
424 The only weevil found in this trial was in the mass trapping field and only one palm of  
425 the AID field showed symptoms of affectation but without any larvae, pupa or adult  
426 inside the palm.

427 Overall results can be taken as evidence of the potential of this method to reduce the  
428 impact of *R. ferrugineus*. Intriguingly, these values corresponded to the same fungal  
429 outgrowth rates when comparing insects captured in the central traps of infected versus  
430 mass-trapping plots. As our field assays were performed in 4-ha plots, cross-  
431 contamination between AID-treated plots and mass-trapping may have occurred and this  
432 may account for the lack of signification of the differences in infection rates recorded  
433 but explain differences in infestation of sentinel palms. Indeed, the autodissemination  
434 potential of strain EABb07/06-Rf, with male-to-female and female-to-male rates of  
435 transmission of 55% and 60% (Dembilio et al., 2010) points on that direction. Insects in  
436 the AID-plots may have received a full load of conidia, which would remain almost  
437 unchanged when infesting neighboring palms but significantly decrease when moving

438 to the mass-trapping plots. This is a hypothesis, though, that should be properly tested.  
439 Weevils retaining the full conidial load and being infected by the fungal strain are  
440 expected to have an overall 78% progeny reduction (Dembilio et al., 2010), which  
441 clearly accounts for the infestation reduction in the sentinel palms. As *R. ferrugineus* is  
442 able to easily move distances of over 100 m in a single flight<sup>40</sup> and over 1 km in a flight  
443 mill with some weevils flying distances exceeding 50 km in 24 h,<sup>41</sup> the separation  
444 between our plots (200 m) may have been insufficient to preclude this cross-  
445 contamination. Based on these recently reported results about *R. ferrugineus* flight  
446 capacities, optimal results from placing infective traps could be accomplished when  
447 applied to wide areas. Therefore, further studies should be carried out in large areas to  
448 test several infective trap densities as this would allow to ascertain the field efficacy of  
449 this technology and the potential to become an economically viable control method.

450

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596

597 **Table 1.** Total number of *R. ferrugineus* captured in the central  
 598 traps per location and percentage of weevils showing fungal  
 599 outgrowth

Location	Treatment	N	Fungal outgrowth (%)*
Sagunt and	Infective plot	35	45.2a
Montcada <sup>1</sup>	Mass trapping plot	34	65.6a
Ibiza <sup>2</sup>	Infective plot	152	69.6a
	Mass trapping plot	611	73.4a
Córdoba	Infective plot	8	25.0a
	Mass trapping plot	15	6.7a

600 \*Percentage fungal outgrowth at the same location followed  
 601 with the same letter did not significantly differ in the  $\chi^2$  test

602 <sup>1</sup>Total corresponding to the two trials set at Montcada and one at Sagunt (province of  
 603 Valencia)

604 <sup>2</sup>Total corresponding to the three trials set at the Island of Ibiza

605 **Table 2.** Damage assessment results in sentinel palms of the trials carried out  
 606 in Sagunt, Montcada and Córdoba.

Location	Treatment			
	Mass Trapping		AIDs	
	Weevils/palm (Mean $\pm$ SE)	Infested palms (%)	Weevils/palm (Mean $\pm$ SE)	Infested palms (%)
Sagunt	21.5 $\pm$ 11.28	100	7.25 $\pm$ 3.57	75
Montcada A	19.5 $\pm$ 6.26	100	13.5 $\pm$ 9.43	50
Montcada B	26.75 $\pm$ 9.44	100	0	0
Córdoba	0.25 $\pm$ 0.25	25	0	0
Mean*	17.0 $\pm$ 6.7a	81.3 $\pm$ 21.7a	5.2 $\pm$ 3.8b	31.25 $\pm$ 18.6b

607 \* Mean number of weevils per palm or percentage of affected palms followed by  
 608 a different letter were significantly different in the ANOVA test ( $F = 12.20$ ;  $df =$   
 609  $1,27$ ;  $P = 0.002$ ) and the  $\chi^2$  test ( $\chi^2 = 8.13$ ;  $P = 0,004$ ), respectively.

610

611 **Table 3.** Mean number of *R. ferrugineus* weevil stages\* found in sentinel palms  
612 depending on treatment (Sagunt, Montcada and Córdoba trials combined)

Weevil stage	Mass trapping plot	Infective plot
Larva	14.00 ± 3.27 <sup>b</sup>	4.06 ± 2.06 <sup>a</sup>
Pupa	2.44 ± 0.77 <sup>a</sup>	0.88 ± 0.43 <sup>a</sup>
Adults	0.56 ± 0.25 <sup>a</sup>	0.19 ± 0.14 <sup>a</sup>

613 \*For each weevil stage, values followed by a different letter in the same line were  
614 significantly different in a paired data *t*-student test (Larva  $t = 2.71$ ,  $P = 0.016$ ; Pupa  $t =$   
615  $2.04$ ,  $P = 0.059$ ; Adults  $t = 1.46$ ,  $P = 0.164$ )

616

617 FIGURE CAPTIONS

618 Fig. 1. (A) Picusan trap with exit hole; (B) bottom view of the infective trap design with  
619 inoculation tunnel; (C) trap sketch with components: (1) pheromone dispenser, (2) trap  
620 entrance with funnel, (3) infective tunnel, (4) exit hole.

621 Fig. 2. Mortality of insects (N=16) directly exposed to the inoculation tunnel (IT) in the  
622 laboratory. Values are shown as mean and standard error. Solid lines depict the mean ( $\pm$  se)  
623 percentage of dead insects in fungal and control treatments. Bars correspond to mean ( $\pm$  se)  
624 percentage of weevils showing mycosis signs.

625 Fig. 3. Mortality of insects which have been contaminated by horizontal transmission after  
626 being coupled with insects directly exposed to the inoculation tunnel (IT) in the laboratory.  
627 Values are shown as mean and standard error. Solid lines depict the mean ( $\pm$  se) percentage of  
628 dead insects in fungal and control treatments. Bars correspond to mean ( $\pm$  se) percentage of  
629 weevils showing mycosis signs.

630 Fig. 4. Mean ( $\pm$  se) persistence of the fungus formulation in the inoculation tunnel (IT) which  
631 has been aged in the field from 3 February to 24 April.

632 Fig. 5. Fungal persistence in the Attract and Infect Devices (AIDs) during the field trial  
633 conducted in Sagunto (Valencia, Spain) by periodically evaluating conidia viability in (A) initial  
634 placement of inoculation tunnels (from 14 April to 17 June + extended ageing period - white  
635 bars-) and (B) replaced inoculation tunnels (from 24 June to 12 August). Values of bars are  
636 means ( $\pm$  se) of viability in four traps (N=4).

637 Fig. 6. Laboratory evaluation of the infective activity (mean mortality  $\pm$  se) of 3 inoculation  
638 tunnels (ITs) (10 adults per tunnel) used in the field during (A) 2.5 months (from mid-April to  
639 late-June) and (B) 40 days (from the third week of June to the first week of August)

640