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

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Abstract

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

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

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

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

1 INTRODUCTION

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

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

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

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

2 MATERIALS AND METHODS

2.1 Entomopathogenic fungus

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

2.2 Stock colonies

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

acetate and pieces of palm fronds) were used in some of our assays (see below) and also to start our stock colonies. These colonies were established in 2007 and have been periodically supplemented with the introduction of additional wild specimens. Adult weevils were reared in a controlled environment cabinet at 25 ± 1 ºC, 75 ± 5% R.H. and a 16 h light photoperiod in perspex cages (30 × 30 × 45 cm depth) with a density of 100-120 weevils per cage.[20]

2.3 Experimental insects

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

2.4 Plant material

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

2.5 Attract-and-Infect Device (AID)

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

2.5.1 Fungal formulation

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

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

2.5.2 Inoculation tunnels (ITs)

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

2.6 Laboratory bioassays

2.6.1 Infectivity of B. bassiana in the IT

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

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

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

2.6.2 Field persistence of fungal activity in the IT

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

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

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

2.7 Semi-field assay

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

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

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

2.8 Field assays

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

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

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

2.9 Statistical Analysis

Mortality data in virulence experiments were corrected using Abbott´s formula when necessary.[26] The median lethal time (LT_{50}) value was estimated by probit analysis using the SPSS v16.0.1 for Windows (SPSS Inc., 2008). Mortality data of insects exposed to

the AID in the laboratory were further used to calculate the Average Survival Times (AST) in days using the Kaplan–Meier survival analysis.[27]

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

3 RESULTS

3.1 Laboratory bioassays

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

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

3.2 Semi-field assays

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

3.3 Field assays

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

When infestation of sentinel palms was assessed at the end of the assay, 37.5% of the palms placed in the infective plots were infested, whereas this percentage increased to 81.3 % of the palms placed in the mass trapping plots (Table 2). Indeed, the mean number of weevils developing per palm in the mass trapping plots was more than 3-fold 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^{-1} 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.[21]

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 ± 1.46% and 30.62 ± 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 ± 1.48% and 81.21 ± 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 ± 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,[28-29] leafminer[30] and tsetse flies,[31]
348 coleopterans, such as palm weevils,[19] and lepidopterans.[32] Our work describes an AID
349 for controlling *R. ferrugineus*. According to Vega et al.,[33] 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.[34] 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.[35]

356 Entomopathogenic conidia are, in many cases, very sensitive to weather conditions,[36]
357 which is a key point of the system's efficacy together with the horizontal transmission
358 of the pathogen.[31] 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.[11] tested the virulence of this strain against

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

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

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

If we consider the initial load of an IT (2.0×10^{10} conidia) and that of a weevil crossing it (6.7×10^{7} conidia), the maximum number of insects that could be effectively infected in each AID could reach 900, which is about 30 times higher than weekly captures in similar infested areas.[21] This is obviously a simplification, as the continuous 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^{-1} of the new AID was
420 reduced by more than 50% compared to mass trapping plots also installing 1 trap ha^{-1}.
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

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

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

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

*Percentage fungal outgrowth at the same location followed with the same letter did not significantly differ in the χ^2 test

[1]Total corresponding to the two trials set at Montcada and one at Sagunt (province of Valencia)

[2]Total corresponding to the three trials set at the Island of Ibiza

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

Location	Treatment			
	Mass Trapping		AIDs	
	Weevils/palm (Mean ± SE)	Infested palms (%)	Weevils/palm (Mean ± SE)	Infested palms (%)
Sagunt	21.5 ± 11.28	100	7.25 ± 3.57	75
Montcada A	19.5 ± 6. 26	100	13.5 ± 9.43	50
Montcada B	26.75 ± 9.44	100	0	0
Córdoba	0.25 ± 0.25	25	0	0
Mean*	17.0 ± 6.7a	81.3± 21.7a	5.2 ± 3.8b	31.25 ± 18.6b

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

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^{b}	4.06 ± 2.06^{a}
Pupa	2.44 ± 0.77^{a}	0.88 ± 0.43^{a}
Adults	0.56 ± 0.25^{a}	0.19 ± 0.14^{a}

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

FIGURE CAPTIONS

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

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

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

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

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

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