Research Article

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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 were evaluated in the laboratory. Semi-field and field trials were carried out to validate the results and establish the potential of this device as a control tool.

RESULTS: In laboratory conditions, a 50% lethal time (LT_{50}) of 4.33 days was obtained when adults (7–10 days old) were exposed to the inoculation tunnel (IT) containing 1×10^{10} conidia g^{-1} in an oil-based fungal formulation. This formulation maintained conidium 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 exposure. In addition, no differences were observed between ITs aged in early spring and those aged in summer, suggesting that the 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 were effectively attracted and infected. Using the AIDs in 4-ha plot field trials, a reduction of >50% in the percentage of infested sentinel palms was obtained.

CONCLUSION: Based on the results obtained in terms of the efficacy and persistence of this new AID in the field and its potential in reducing *R. ferrugineus* populations and palm infestation, this device could become a key 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, causing particularly severe damage in the Mediterranean Basin.[1,2] In this region, the Canary Islands Date palm, *Phoenix canariensis* Hort ex Chabaud, is widely used as an ornamental plant, whereas the date palm, *Phoenix dactylifera*, is mostly grown for its fruit in the southern countries of the Mediterranean Basin.[3] The weevil *R. ferrugineus*, native to southern Asia and Melanesia, colonized most of southwestern Asia in the early 1980s, the Arabian Peninsula in the mid-1980s, and the Middle East and Egypt at the beginning of the 1990s.[4] Later, 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 (in 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 as a consequence of it remaining hidden for 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 five to seven treatments per year may be required.[8,9] Systemic insecticides (mainly neonicotinoids and

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avermectins) can be applied by stipe injection. Although the efficacy of this technique has been 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 a combination of these techniques with, for example, mass trapping.[17] The use of attract-and-infect devices (AIDs) is 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 researchers have tried to develop this type of device for use against *R. ferrugineus*.[18,19] However, their efficacy in field trials has not yet been demonstrated. The main objectives of this study were (1) to develop an effective autoinoculation system and (2) to evaluate the efficacy of this 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 13 May 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 °C and 75 ± 5% relative humidity (RH) with 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 *R. ferrugineus* adults were used in the laboratory assays to assess both the virulence of the fungus and its capacity to be horizontally transmitted to healthy adults in the formulation used in the AID. The same type of insect was used in semi-field experiments. However, wild adults were used to assess the performance of the inoculation tunnels (ITs) after several field ageing times because insufficient laboratory-reared insects were available. In this case, trap-collected adults were maintained in our insectary and periodically examined for 2 to 3 weeks before the start 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 The attract-and-infect device (AID)

The commercially available black pyramidal trap Picusan® (Fig. 1A),[21] supplied by Sansan Prodesing SL (Valencia, Spain), was appropriately modified and used in our assays. This trap consisted of three parts: (i) a cylindrical base (25 cm in diameter and 6 cm in height); (ii) a rough (1 mm between grooves) black pyramid with a 66% slope and an upper entry with a funnel inside and (iii) a green cover on the top leaving a 4-cm opening between the top of the pyramid and the cover. 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 IT 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) at 26 °C in dark conditions. Before 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 >96%. Conidia from 18–20-day-old cultures were suspended in mineral oil [Paraffinic White Oil (0.84–0.87 kg L^{-1} density at 15 °C) from Texaco, White Plains, NY, USA] and removed from each dish with a 10-mL pipette. Suspensions from four Petri dishes were combined in a sterile Falcon tube (50 mL), Corning Life Science, Corning, NY, USA, sonicated for 2 min and filtered through four layers of cheesecloth to obtain pure conidia. After centrifugation (3000 rpm for 3 min, 1509 g; Rotina 46; Hettich, Kirchlengern, Germany), oil exceeding 10 mL was removed. The concentration of conidia, estimated using a hemocytometer (Improved Neubauer chamber, Agar Scientific Ltd, Essex, UK), 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 described above was spread on a piece of black corrugated plastic [polyvinyl chloride (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 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

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

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). Sterile conidia were then poured into a 50-mL Falcon tube to which mineral oil (up to a final volume of 4 mL) and clay carrier (2 g) were added to complete the formulation of the inactivated fungus for the 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 ($40 \times 30 \times 40$ cm) to easily recover the contaminated insects. Sixteen 7–15-day-old *R. ferrugineus* adults, eight males and eight 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, and to avoid re-entry of insects leaving the trap, the upper opening was partially closed once the insects had been introduced into the AID. Twenty-four hours later, insects that had passed through the IT once (i.e. those in the cage) were recovered. These insects were individually introduced into small aerated plastic cages ($11.0 \times 4.5 \times 7.5$ cm) with a non-treated partner of the opposite sex and left undisturbed for 24 h to assess horizontal transmission. Then, couples were separated and each insect was introduced to 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 for up to 30 days for those exposed to horizontal transmission. To confirm mycosis, each dead insect was individually surface-sterilized by immersion for 1 min in a 0.3% sodium hypochlorite solution (two times). Then, it was rinsed using sterile distilled water (two times for 1 min each) and individually incubated in a wet dark chamber at 26 °C for 20 days. Mycosis was assumed when the sporulated mycelia of the fungus were observed growing from the cadaver. The 50% lethal time (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 including 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 exposure to the IT or 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 a gentle nitrogen stream. The concentration of conidia was estimated as described in Section 2.5.1.

2.6.2 Field persistence of fungal activity in the IT

Conidial viability in the ITs was evaluated from the moment the AIDs were set up 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 most of the ITs used in the spring field trial were removed on 17 June, some of them, were maintained in the field until 12 August for a longer evaluation period. Every 2 weeks, a small amount (20–30 mg) of infective material from the IT was taken to the laboratory. The sample was weighed and 1 ml of mineral oil (the same oil as used 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 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 was inoculated in a Petri dish containing *B. bassiana* CTC selective medium, consisting of potato dextrose agar (Difco, BD) supplemented with 1 g L^{-1} yeast extract (Difco, BD) (PDAY), 0.5 g L^{-1} chloramphenicol (Sigma-Aldrich, Madrid, Spain), 0.001 g L^{-1} thiabendazole (Sigma-Aldrich, Madrid, Spain) and 0.25 g L^{-1} cycloheximide (Sigma-Aldrich, Madrid, Spain).[25]

The number of cfu obtained when the ITs were assembled was considered as 100% viability and this was used as the reference value for subsequent measurements. 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 six independent meshed cages ($4 \times 3 \times 3$ m). An AID was placed in the center of the cage and then three *R. ferrugineus* adult males and nine adult females were immediately released. Three cages were provided with an infective AID (treated cages), whereas three additional cages had an AID with an inactivated fungal formulation (see Section 2.5.2) (untreated cages). A cotton bud coated with fluorescein (Sigma-Aldrich) 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 on the island of Ibiza [in 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 four AIDs set at the corners of a 100 × 100 m square (infective plot with 1 AID ha^{-1}). The other plot had four standard Picusan® traps also set at the corners of a 100 × 100 m square (mass-trapping plot with 1 trap ha^{-1}). The infective and mass-trapping plots of each trial were separated by 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 AIDs were placed in the field on 14 April 2014 and trials ended 4 months later. ITs in the AIDs 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 from 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 Section 2.6.1). 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 4 months that the trial lasted and were watered weekly. On 12 August they were thoroughly dissected to assess *R. ferrugineus* attack and the numbers of larvae, pupae and adults were counted. Three ITs from each area were also taken to the laboratory at the end of the trials to assess conidial 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 SPSS v16.0.1 for Windows (SPSS Inc., IBM, Armonk NY, USA). Mortality data for insects exposed to the AID in the laboratory were further used to calculate the average survival times (ASTs) in days using Kaplan–Meier survival analysis.[27]

For the semi-field trial, an analysis of variance (ANOVA) followed by a least significant difference (LSD) test ($P < 0.05$) was conducted for the total number of insects captured in each treatment. Differences in the percentage of adults captured showing fungal outgrowth in the field trials were analyzed using a *t*-test. In this case, data were first 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. As for the semi-field trials, the number of *R. ferrugineus* per palm was analyzed using ANOVA and an LSD test at $P < 0.05$.

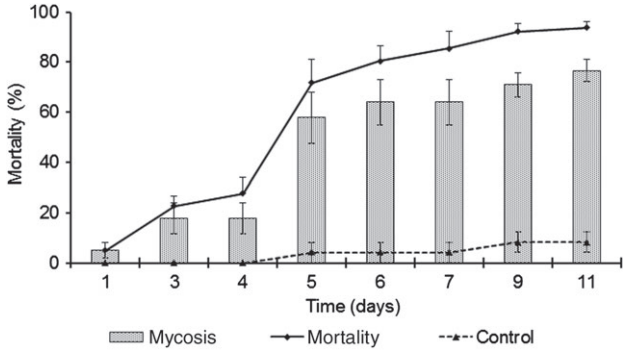

Figure 2. Mortality of insects ($n = 16$) directly exposed to the inoculation tunnel (IT) in the laboratory. Values shown are mean and standard error (SE). Solid lines depict the mean (± SE) percentage of dead insects in fungal and control treatments. Bars correspond to mean (± SE) percentage of weevils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

3 RESULTS

3.1 Laboratory bioassays

Mortality of insects exposed to the AID in the laboratory reached 72 and 92% 5 and 9 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 4 days after treatment. However, mortality data were only recorded when insects had definitely died. The estimated LT_{50} and AST were 4.33 days [95% fiducial limits: 3.90 and 4.80 days; slope ± standard error (SE): 5.980 ± 0.283; $\chi^2 = 276.3$; df = 18; $P \leq 0.001$] and 6.21 days, respectively. The mean (±SE) 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 value almost halved ($1.02 \pm 0.39 \times 10^7$ conidia) when the tunnel had been passed through 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 mean (±SE) 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 2 months later, all the palms in both control and treatment cages were infested. However, the mean (±SE) number of *R. ferrugineus* found in palms exposed to an AID was 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 mean (±SE) infection rate of the individuals in the cages treated with AID was 4-fold that 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

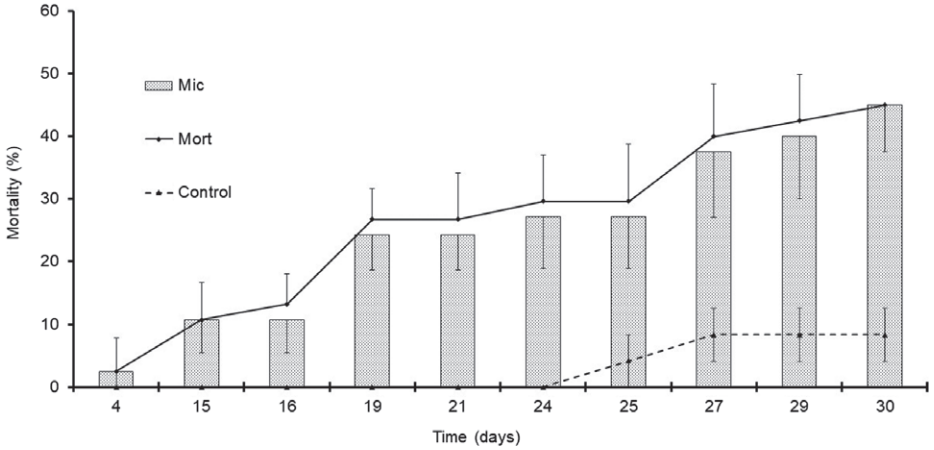

Figure 3. Mortality of insects that have been contaminated by horizontal transmission after being paired with insects directly exposed to the inoculation tunnel (IT) in the laboratory. Values shown are mean and standard error (SE). Solid lines depict the mean (± SE) percentage of dead insects in fungal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

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

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

[a] Percentages of fungal outgrowth at the same location followed by the same letter did not significantly differ in the χ^2 test.
[b] Total corresponding to the two trials set up at Montcada and one at Sagunt (province of Valencia).
[c] Total corresponding to the three trials set up on the island of Ibiza.

showed fungal outgrowth are 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 (data not shown).

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). Therefore, mass trapping at a density of 1 trap ha^{-1} was not enough to control palm infestation in 4-ha plots. However, the same density of infective traps resulted in a 46% reduction of infested palms. Efficacy of both mass-trapping and attract-and-infect techniques might be improved by using water and co-attractants as described in previous research.[21]

3.4 Laboratory evaluation of both fungal formulation persistence and infective activity of ITs used in the field

The viability of the fungal formulation in the ITs evaluated 63 and 82 days after field exposure under outdoor conditions prior to field assays (3 February to 24 April 2014) (Fig. 4) was 70 and 45%, respectively. Fungal viability measured in parallel with field assays in Sagunt (Fig. 5A) remained >50%, 67 days after the start of the test. Although most of these tunnels were replaced by newly made ITs on that date, some of them were allowed to further age in the

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

	Treatment			
	Mass trapping		AIDs	
Location	Weevils per palm (mean ± SE)	Infested palms (%)	Weevils per 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 ± SE[a]	17.0 ± 6.7a	81.3 ± 21.7a	5.2 ± 3.8b	31.25 ± 18.6b

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

Table 3. Mean (± SE) number of *R. ferrugineus* weevil stages[a] found in sentinel palms depending on treatment (Sagunt, Montcada and Córdoba trials combined)

Weevil stage	Mass-trapping plot	Infective plot
Larva	14.00 ± 3.27b	4.06 ± 2.06a
Pupa	2.44 ± 0.77a	0.88 ± 0.43a
Adult	0.56 ± 0.25a	0.19 ± 0.14a

[a] For each weevil stage, values followed by a different letter in the same line were significantly different in a paired data Student's *t*-test (larva, $t = 2.71$; $P = 0.016$; pupa, $t = 2.04$; $P = 0.059$; adult, $t = 1.46$; $P = 0.164$).

field for a longer evaluation period (white bars in Fig. 5A). Viability decreased to almost 30 and 12% after 3 and 4 months of aging, respectively. Interestingly, the viability decrease observed was similar for both the initial and replaced ITs up to 50 days of aging, even though they had been exposed to different environmental conditions: spring (Fig. 5A) and summer (Fig. 5B). At the end of both periods, three ITs per plot were taken to the laboratory to evaluate both their infective activity and their fungal viability. The spring ITs (aged in the field for 2.5 months) caused 50% mortality in approximately 45 days (Fig. 6A). Mortality in the ITs from the Ibiza trials was higher than in those from Valencia (63.3 versus 35.6% at day 34, respectively). However, mortality in the Ibiza trial remained the same until the end of the assay. At that time (2.5 months of ageing), mean (±SE) fungal viability was 35.26 ± 1.46% and 30.62 ± 1.28% for the Ibiza and Valencia trials, respectively. At the end of the second period (Fig. 6B), mortality was slightly higher than at the end of the first period. Mean mortality was about 60% by day 40. However, the ITs from both locations showed a more homogenous response (86.83 ± 1.48% and 81.21 ± 3.06% mean (±SE) fungal viability, respectively). The mean (±SE) viability of the ITs that were allowed to remain in the field until the first week of September in Ibiza (ageing period of 70 days) was 43.32 ± 0.49%.

4 DISCUSSION

The use of entomopathogenic fungi in attract-and-infect traps has been developed for several pests including dipterans, such as fruit flies,[28,29] leafminers[30] and tsetse flies,[31] coleopterans, such as palm weevils,[19] and lepidopterans.[32] Our work describes an AID for controlling *R. ferrugineus*. According to Vega *et al.*,[33] insects are attracted to the infective source in the device, become infected, leave the source and then disseminate the pathogen to other members of the target population. Similar attract-and-infect traps used against Triatominae have demonstrated a high efficacy in

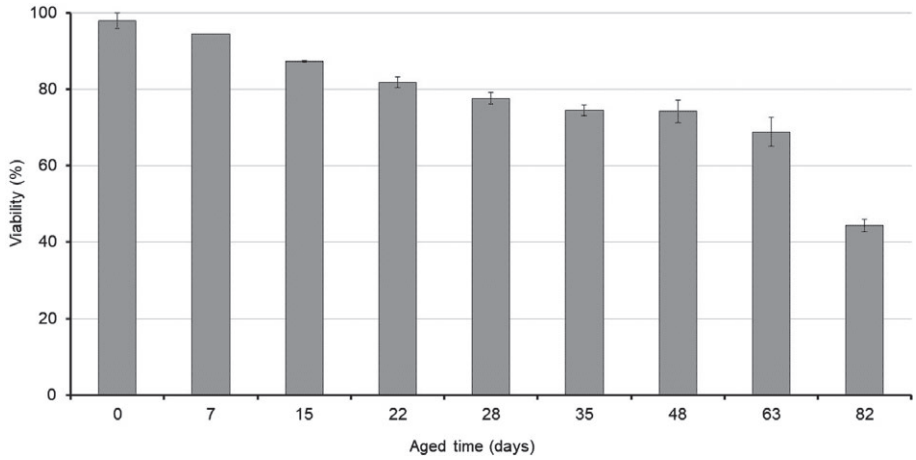

Figure 4. Mean (± SE) persistence of the fungal formulation in the inoculation tunnel (IT) which had been aged in the field from 3 February to 24 April.

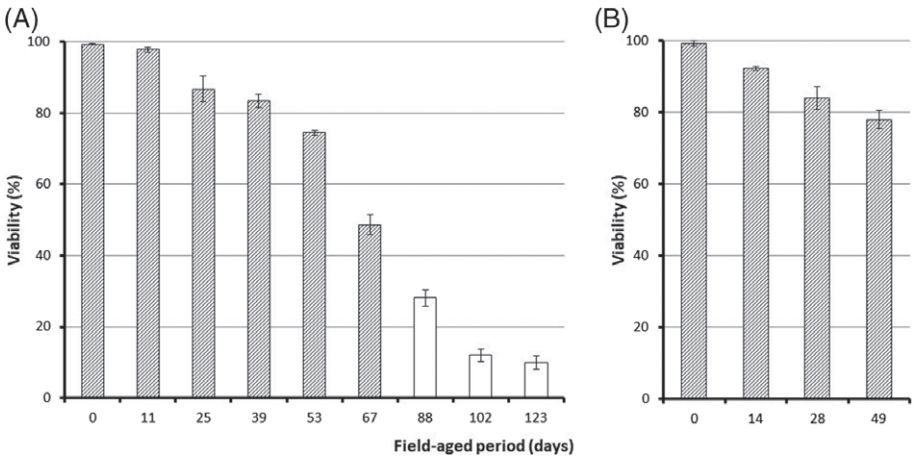

Figure 5. Fungal persistence in the attract-and-infect devices (AIDs) during the field trial conducted in Sagunto (Valencia, Spain) determined by periodically evaluating conidial viability in (A) inoculation tunnels placed in the field from 14 April to 17 June plus an extended ageing period (white bars) and (B) replacement inoculation tunnels placed in the field from 24 June to 12 August. Values of bars are mean (± SE) viability for four traps ($n = 4$).

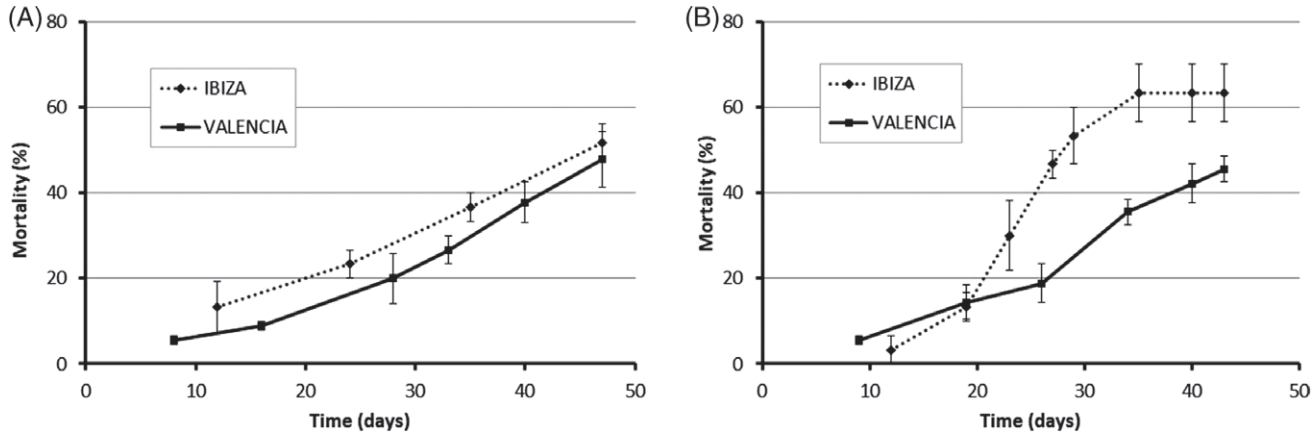

Figure 6. Laboratory evaluation of the infective activity (mean ± SE mortality) of three inoculation tunnels (ITs) (10 adults per tunnel) used in the field for (A) 2.5 months (from mid-April to late-June) and (B) 40 days (from the third week of June to the first week of August).

reducing pest populations and a 52.4% population mortality.[34] When infective traps were applied in houses, the Triatoma infestans mortality reached 83% and a significant reduction in the fertility and fecundity of infected females was obtained.[35]

Entomopathogenic conidia are, in many cases, very sensitive to weather conditions,[36] which is a key factor in the system's efficacy and in the horizontal transmission of the pathogen.[31] Therefore, the main objective of this research was to develop a device and a formulation that protected spores from adverse environmental conditions for as long as possible and that, at the same time, was effective for weevil attraction and infection.

The fungal strain used in this study had previously shown promising activity results against *R. ferrugineus*. Dembilio *et al.*[11] reported that laboratory-reared adults treated by immersion in a conidial aqueous suspension of 5.0×10^9 conidia mL^{-1} survived 17 days on average. Under the conditions reported by Dembilio *et al.*, we subsequently demonstrated that a single weevil was able to acquire a mean (±SE) fungal load of $6.7 \pm 0.9 \times 10^7$ conidia (unpublished data P. Moya and V. Navarro-Llopis), which is 3-fold more conidia than the amount acquired by a weevil passing through our ITs. In 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. These results suggest that the fungal formulation enhances virulence, as a 3-fold lower fungal load reduced the time required to kill insects by approximately 4 times compared with Dembilio *et al.*[11] Enhancement of fungal virulence with oil formulations had been previously reported.[36,37] This is attributed to an increase of adhesiveness of conidia to the insect cuticle and interference with its defensive properties, resulting in an acceleration of the fungal outgrowth process in the host compared with aqueous formulations. Furthermore, oil prevents conidia from drying and helps increase the fungal agent's persistence.[38]

The persistence of the infective capacity of the AID is crucial for the economic and technical feasibility of this method. This requirement would hardly be fulfilled if the device had to be serviced or replaced in less than 1 month. The device under study remained infective (> 50% conidial viability) for 2 months, even during the driest and warmest seasons in the Mediterranean (summer). Even lower percentages of viability, such 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 maintained their infective capacity for at least 31 days against 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 showed reduced fungal viability to around 40% after 4 weeks.[18] In contrast, the new AID maintains conidial viability at >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 passing through 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 passing weevil and progressively decreasing viability (approx. 50% fungal viability after 2 months in the field) should be taken into account. Therefore, further studies are needed to provide real numbers of insects effectively infected by the AID under field conditions.

The results obtained in the present study show that the new AID is very effective at attracting and infecting weevils, as >88% of the insects released in the semi-field assay passed through the IT and this resulted in 95% mortality. Moreover, the percentage of infested palms in field assays using the new AID was reduced by >50% and, in some cases, 100% of the sentinel palms used remained uninfested. In the particular case of Córdoba, the red palm weevil population level was lower than in Sagunt and Montcada and, consequently, damage in palms was lower. The only weevil found in this trial was in the mass-trapping field and only one palm of the AID field showed symptoms of infestation but without any larvae, pupae or adults inside the palm.

Overall, the results can be taken as evidence of the potential of this method to reduce the impact of *R. ferrugineus*. Intriguingly, the same fungal outgrowth rates were found when

comparing insects captured in the central traps of infective versus mass-trapping plots. As our field assays were performed in 4-ha plots, cross-contamination between AID-treated plots and mass-trapping plots may have occurred and this may account for the lack of significant differences in infection rates recorded but explain differences in infestation of sentinel palms. Indeed, the autodissemination potential of strain EABb07/06-Rf, with male-to-female and female-to-male rates of transmission of 55% and 60%, respectively, points in that direction.[11] Insects in the AID plots may have received a full load of conidia, which would remain almost 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 reduction in progeny of 78%,[11] which clearly accounts for the infestation reduction in the sentinel palms. As *R. ferrugineus* is able to easily move distances of >100 m in a single flight[40] and >1 km in a flight mill, with some weevils flying distances >50 km in 24 h,[41] the separation between our plots (200 m) may have been insufficient to preclude this cross-contamination. Based on the *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 one to ascertain the field efficacy of this technology.

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