EVALUATION OF THE PATHOGENICITY OF MULTIPLE ISOLATES OF *BEAUVERIA BASSIANA* (HYPOCREALES: CLAVICIPITACEAE) ON *RHYNCHOPHORUS FERRUGINEUS* (COLEOPTERA: DRYOPHTHORIDAE) FOR THE ASSESSMENT OF A SOLID FORMULATION UNDER SIMULATED FIELD CONDITIONS

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Abstract

A solid state formulation of *Beauveria bassiana* (Balsamo) Vuillemin has been developed for biological control of the Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier, 1790). Two kinds of bioassays (dry conidia and dipping) using 10 isolates from several coleopterans in Mediterranean environments, identified 2 RPW derived isolates (193 and 203) as most pathogenic to RPW larvae and adults (zero survival within first 4-5 d for dry conidia, and 14 and 23 d for dipping bioassays). Isolate 203 ($5.1 \times 10^8 \pm 1.9 \times 10^8$ conidia g^{-1}) was formulated with fragmented date seed into solid granules and tested in palms infested with RPW under semi-field conditions in Feb, Apr/May and Jun of both 2007 and 2008. *Beauveria bassiana* significantly reduced RPW adult survival with respect to controls in May 2007 and in the Apr/Jun 2008 experiments. Total RPW adult mortality was achieved within 30 days for all *B. bassiana* treatments, and was associated with increasing numbers of insects with signs of mycosis in 2008 experiments. *Beauveria bassiana* formulation reduced RPW multiplication in artificially infested palms compared to controls, and a positive correlation between numbers of larvae and time post-infestation was recorded. The suppression of RPW adult populations by *B. bassiana* persisted for at least 3 months under semi-field conditions. The *Beauveria bassiana* solid formulation, which induces great adult mortality and persistence in the field, could be applied as a preventive as well as a curative treatment for the integrated management of RPW.

Key Words: entomopathogenic fungi, mycoinsecticide, *Phoenix canariensis*, solid state formulation, Red Palm Weevil

Resumen

En el presente estudio, se describe un procedimiento para el desarrollo de una formulación en estado sólido basada en *Beauveria bassiana* (Balsamo) Vuillemin para el control biológico del picudo rojo, *Rhynchophorus ferrugineus* (Olivier, 1790). Al realizar dos tipos de bioensayos (conidios secos e inmersión directa) utilizando diez aislados de diversos coleópteros en ambientes Mediterráneos, se identificaron dos aislados derivados del picudo rojo (193 y 203) como los más patogénicos para los estadios larvarios y adultos del insecto (supervivencia nula en los primeros 4-5 días y 14 y 23 días para bioensayos con conidios secos e inmersión directa respectivamente). El aislado 203 de *B. bassiana* ($5.1 \times 10^8 \pm 1.9 \times 10^8$ conidios g^{-1}) se formuló en sólido utilizando dátiles fragmentados y se ensayó en palmeras infestadas con el picudo rojo bajo condiciones de semi-campo en los meses de Febrero, Abril/Mayo y Junio de 2007 y 2008. *Beauveria bassiana* redujo significativamente la supervivencia de los insectos adultos con respecto a los controles en los experimentos de Mayo de 2007 y Abril/Junio

2008. Se alcanzó la mortalidad completa de los adultos del picudo rojo en 30 días para todos los tratamientos con *B. bassiana* y lo que se correlacionó con el incremento del número de insectos con signos de micosis para los ensayos de 2008. El formulado de *B. bassiana* redujo la multiplicación del insecto en palmeras infestadas artificialmente con respecto a los controles, registrándose una correlación positiva entre el número de larvas y el tiempo de infestación posterior. La capacidad del formulado de *B. bassiana* para suprimir las poblaciones de insectos adultos tuvo una persistencia de al menos tres meses bajo condiciones de semicampo. El formulado sólido de *B. bassiana*, que ha generado una alta mortalidad en adultos así como una elevada persistencia, podría utilizarse tanto en tratamientos preventivos como curativos en el manejo integrado del picudo rojo.

Palabras Clave: Hongos entomopatógenos, micoinsecticidas, *Phoenix canariensis*, formulación sólida, Picudo rojo de las palmeras

The red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae) is a devastating palm pest that has caused large economic losses in palm farming (Murphy & Briscoe 1999; Faleiro 2006). This weevil is a problem in a wide range of palms (Barranco et al. 2000; Llácer et al. 2012) including economically important species such as the date palm (*Phoenix dactylifera* L.), Canary Islands date palm (*P. canariensis* Hort), coconut (*Cocos nucifera* L.) and African oil palm (*Elaeis guineensis* Jacq.). RPW was introduced into mainland Spain in 1995 (Barranco et al. 1996a, 1996b), and then spread to all palm growing areas in the Mediterranean and recently to the Canary Islands.

Early detection of RPW is difficult under field conditions (EPPO 2007; Mankin et al. 2008; Pinhas et al. 2008). Mass trapping is useful for monitoring the pest but does not provide sufficient control in the field. Cryptic larval development reduces the efficacy of external chemical insecticide treatments against larvae. Furthermore, the multivoltine nature of RPW (Wattanapongsiri 1966) makes it difficult to manage this insect. Several researchers have demonstrated successful control of multivoltine coleopterans by combining microbial and chemical insecticides (Shapiro-Ilan et al. 2011). Populations of *Agrilus planipennis* (Coleoptera: Buprestidae), an invasive Asian beetle recently discovered in North America, are controlled through auto-contamination trapping systems, which involve *Beauveria bassiana* (Balsamo) Vuillemin plus Z-3-hexenol (Lyons et al. 2012). The most successful strategy for management of RPW has been found to be a combination of mass trapping, chemical treatments and biological control (Murphy & Briscoe 1999; Faleiro 2006; Gindin et al. 2006; El-Sufty et al. 2007).

Entomopathogenic fungi are important regulators of insect populations under natural conditions (Bidochka et al. 2000). Mitosporic fungi such as *B. bassiana* have been used for biological control of insect species and of coleopterans in particular (Tanada & Kaya 1993). *Beauveria bassiana* is recognized as a species complex comprising genetically diverse lineages (Uma Devi et al. 2006; Fernandes et al. 2009). The genetic variability among isolates can be expressed as differences in virulence (Kryukov et al. 2010) and in resistance to adverse environmental factors such as extreme temperature (Fernandes et al. 2008) and UV-radiation (Fernandes et al. 2007). In a previous study (Asensio et al. 2003) we determined that *B. bassiana* is the most abundant entomopathogen in soils from SE Spain, where palm groves are common. Subsequently, we isolated *B. bassiana* from RPW naturally infested in palm groves in E and SE Spain (Güerri-Agulló et al. 2010). In the present study, we describe a procedure to develop a mycoinsecticide based on *B. bassiana* for biological control of RPW. The most pathogenic *B. bassiana* isolate in 2 bioassays against RPW was formulated in solid state using fragmented date seed. The formulation was tested under semi-field conditions in palms infested with RPW. The efficacy of the *B. bassiana* formulation on RPW survival and reproduction were assessed. In addition, the long-term efficacy of the formulation was also evaluated.

Materials and Methods

Plants

Twenty cm stem diam (about 2-3 yr-old) *Phoenix canariensis* Hort. (Arecales: Arecaceae) grown in 30-cm diam pots were used for experiments. Pots contained pit compost and were watered and fertilized as required. Nine and 12 *P. canariensis* palms were treated with *B. bassiana* formulation in 2007-2008, respectively. The same numbers were used as controls (non treated).

Insects

Rhynchophorus ferrugineus larvae used in this study were reared in a SANYO MLR-351 incubator at 25 ± 0.5 °C in complete darkness. Adults were bred in pairs using sugarcane (*Saccharum officinarum* L.; Poales: Poaceae) as food and oviposition substrate. Eggs were collected from the sugarcane to sterile 9-cm diam plastic Petri dishes with an artificial food substrate (Alarcon et al. 2002). Emerged larvae were individually transferred to 20 mL blood cell counter polystyrene vials with a 30 mL cap (VWR® International, Barcelona, Spain)

containing 15 mL of artificial diet. Fifteen days later, larvae (instar 4) were transferred to new vials with fresh food diet. After 30 days, larvae were then transferred to 100 mL plastic containers with 30 mL of artificial diet; diet was replaced every 15 days for 3 months. Larvae were then transferred into 150 mL plastic containers with 15 g of palm and sisal fibre mixture or sisal and Spanish broom fibre mixture as a pupation substrate. Adults emerged 1 month later and were either used for experiments or kept for RPW breeding.

Beauveria bassiana Isolates and Inocculum Preparation for Pathogenicity Bioassays

Ten *B. bassiana* isolates from several Mediterranean environments (Table 1) were screened for RPW pathogenicity (Table 1). All fungi had been kept in the Phytopathology Laboratory collection (University of Alicante, Spain) at 4 °C in the dark on corn meal agar (CMA, BBL Sparks, Maryland, USA). Conidial suspensions (3×10^7 conidia/mL) of each *B. bassiana* isolate (Table 1) in 0.05% Tween 20 were used for dipping experiments (see below).

Dry conidia from 24-day-old CMA cultures of *B. bassiana* isolates grown at 25 °C in complete darkness were used to prepare a solid formulation in 250 mL flasks containing 11 g of fragmented (c.a. 5 mm diam) date seed as substrate. Fragmented date seeds were saturated in water and sterilized before inoculation with 1 mL of a *B. bassiana* conidia suspension (1×10^6 conidia mL^{-1}) (Asensio-Berbegal et al. 2008). The fragmented date seed with *B. bassiana* was shaken every 2 days after the fifth day of inoculation. A 15 day-old solid state formulation was used for assays with the RPW.

Evaluation of *B. bassiana* Isolates Against RPW Adults in a Dipping Bioassay

Adults collected in the field were inoculated with *B. bassiana* isolates as follows: Fifteen insects were dipped independently (1 insect per container) into 60-mL polypropylene containers (VWR® International, Barcelona, Spain) each containing 20 mL of conidia suspensions of either dose shown above of a specific *B. bassiana* isolate (Table 1) for 3 s. Control insects were dipped in 0.05% Tween 20 only. After inoculation, insects were placed in moist chambers at 25 °C for 12 h. Insects were then transferred to sterile Petri dishes with moist sterile filter-paper disks. Since RPW is very sensitive to desiccation, the filter paper was kept moist regularly with sterile distilled water. Petri dishes were incubated at 25 °C in the dark and insect survival was recorded daily for 35 days. Dead insects were surface sterilized with 1% NaClO for 1 min, rinsed 3 times with sterile distilled water for 1 min and placed on sterile filter paper in a moist chamber to determine the cause of death. These bioassays were carried out twice. A Kaplan-Meier test was applied to the insect survival data to screen the virulence of *B. bassiana* isolates.

Evaluation of *B. bassiana* Isolates against RPW Larvae and Adults Using CMA Cultures

Beauveria bassiana isolates were also screened for pathogenicity to RPW in laboratory bioassays using CMA cultures. RPW larvae (7-10 and 30 day old) and adults (up to 1 month after emergence from pupae) were independently inoculated with a specific *B. bassiana* isolate as follows: 5 insects were placed independently (1 insect per dish) in 9

TABLE 1. *BEAUVERIA BASSIANA* ISOLATES USED IN BIOASSAYS AGAINST DIFFERENT LIFE STAGES OF *RHYNCHOPHORUS FERRUGINEUS*.

Isolate no.	Host or Origin	Place of isolation/Source
23	Soil (*Galleria mellonella* bait)	Xaló River, Llíber (Alicante, SE Spain)/(Asensio et al., 2003)
33	*Thaumathopoea pytiocampa* (Lepidoptera)	Valencia (E Spain)
53	*Rhizotrogus chevrolati* (Coleoptera)	Setúbal (Portugal)/(Lopez-Llorca, 1992)
118	*Haplothrips tritici* (Thysanoptera)	Murcia (SE Spain) /(Lopez-Llorca, 1993)
119	*Langia* sp. (Coleoptera)	Orihuela (Alicante, SE Spain), CBS 121098
193	*R. ferrugineus* (Coleoptera)	Olocau (Valencia, E Spain)/ (Güerri-Agulló et al. 2010), CBS 121096
203	*R. ferrugineus* (Coleoptera)	Daimés (Elche, SE Spain))/ (Güerri-Agulló et al. 2010), CBS 121097
205	*Hypothenemus hampe* (Coleoptera)	ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, USA)
206	Soil (*Galleria mellonella* bait)	Palmeral, Universidad Miguel Hernández (Elche, SE Spain)/This report
207	*R. ferrugineus* (Coleoptera)	Almería (S Spain)/This report

cm Petri dishes each containing a 24-day old CMA colony of a specific *B. bassiana* isolate. Isolates 33, 193, 203, 206 were tested against 7-10 day larvae and adults. Isolates 23, 33, 53, 118, 119, 193, 203, 205 and 206 were tested against 30 day old larva). The insects were exposed to the isolates for 15 min with occasional shaking.

Three replicates of 5 insects each were tested for each isolate. Controls consisted of insects placed in Petri dishes containing CMA only. The spore (conidial) dose per insect was evaluated in RPW adults by washing each *B. bassiana*-inoculated insect in 20 mL of 0.02% Tween 20 in sterile distilled water after vortexing for 5 min. Three insects per isolate (53 and 203) were processed. The experiment was repeated twice. Conidia in washing suspensions were counted using a haemocytometer. After inoculation insects were placed in moist chambers the same as for the dipping bioassay protocol. Pathogenicity of *B. bassiana* isolates was determined as their lethal infection capacity. These bioassays were carried out twice (each 1 with 3 replicates).

Evaluation of Pathogenicity of *B. bassiana* Isolates in RPW using Solid State Formulations

Bioassays for pathogenicity were also performed in Petri dishes containing 2.75 g of the solid state formulation (see above) for *B. bassiana* isolates nos. 33, 193 and 203 ($5.1 \times 10^8 \pm 1.9 \times 10^8$ conidia g^{-1}). *Beauveria bassiana* isolates sporulated within the same range (15 days) in the solid formula (e.g. $1.5 \times 10^8 \pm 9.1 \times 10^7$ conidia g^{-1} for isolate 119). All pathogenicity bioassays (with 3 replicates each) were performed twice.

Evaluation of Pathogenicity of a *Beauveria bassiana* Solid State Formulation on RPW in Simulated Field Conditions

Pathogenicity of *B. bassiana* (isolate 203) on RPW adults in palms (3 per treatment and sampling date) was tested in *P. canariensis* palms held in pots treated with 50 g/palm of the *B. bassiana* formulation, which was dusted into the space between stem and petiole insertion. Each palm was enclosed in a cylindrical metallic net (5 × 5 mm mesh size). Three days later, 5 RPW adults (3 females + 2 males), collected from naturally infested palms in the field were placed on the top of each palm and the net enclosure with a 30 cm diam circular top was closed and sealed with packaging tape (Fig. 1). Controls were prepared similarly but the solid formulation was not inoculated with the fungus. Each treatment (*B. bassiana* inoculations and controls) was replicated 3 times per sampling occasion. Palms were irrigated as required and were placed in a protected roofed enclosure cage where temperature was recorded but not controlled (semi-field conditions). Three experiments were conducted in Feb, May and Jun 2007 and Feb, Apr and Jun 2008 to represent a wide array of environmental conditions. Mean temperatures recorded for the experiments

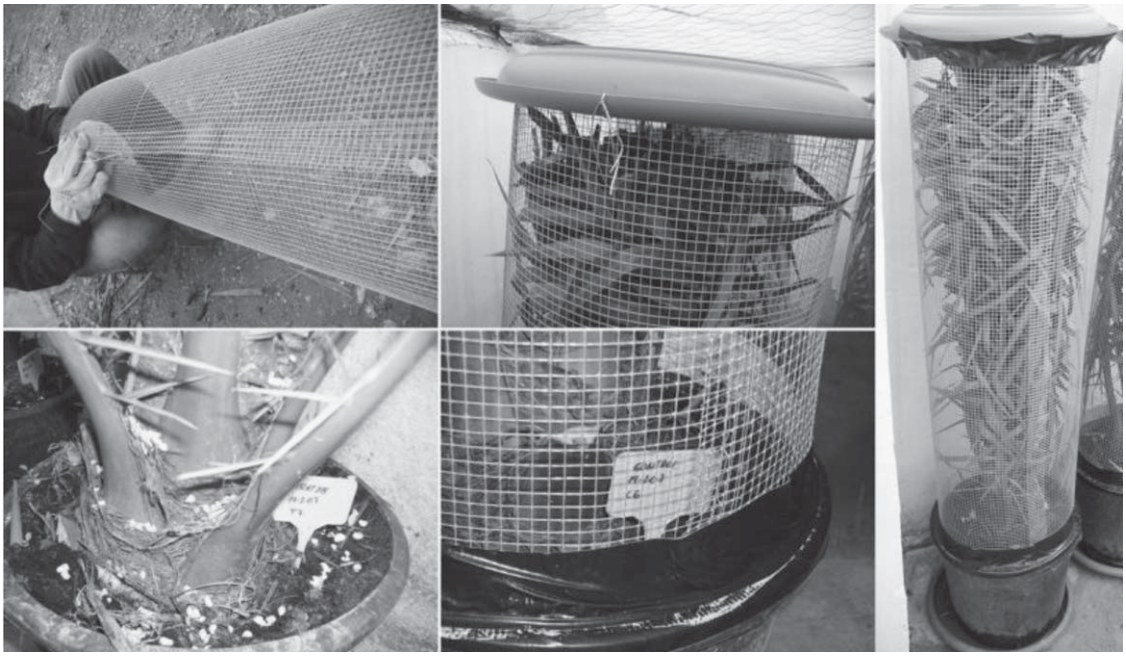

Fig. 1. System for evaluation of the pathogenicity of a *Beauveria bassiana* solid state formulation against the red palm weevils infesting *Phoenix canariensis* in a simulated field condition.

were 15.0 ± 1.3 °C (Feb 2007) and 15.5 ± 1.3 °C (Feb 2008), 19.6 ± 2 °C (May 2007) and 18.9 ± 1.4 °C (Apr 2008), 25.1 ± 2.3 °C (Jun 2007) and 26.4 ± 1.3 °C (Jun 2008). For each experiment, 3 palms per treatment were destructively sampled 7, 14 and 30 days after insect placement (2007 experiments) or 7, 14, 24 and 30 days (2008 experiments). These palms were carefully examined for insects in petiole insertions, roots and potting mixture. When required, palms were pulped to find insects. Insect survival per palm and numbers of dead RPW adults with signs of *B. bassiana* were recorded. RPW adults were then removed and, when possible, the palms were enclosed again in their cylindrical metallic nets and kept under semi-field conditions for 60 d. Palms were then checked for RPW larvae and their numbers were scored. Long-term efficacy of *B. bassiana* formulations also was assessed. Palms were treated in 2008 (Feb and Apr) with either *B. bassiana* or non-inoculated formulation (controls). After 3 months, palms were infested with RPW adults and, 24 d later, insect survival and fungus infection were also scored as described above.

Statistical Analyses

Statistical analyses were performed using R version 2.10.1 (R Development Core Team 2009). Data were checked for normality using the Shapiro-Wilk test, and Levene's test was used to study homogeneity of variance across groups. Data following a normal distribution were compared using 1-way ANOVA. Non-normal data were compared using Kruskal-Wallis (K-W) rank sum test. Pearson's correlation coefficient was used to explore a possible relationship between numbers of RPW larvae and time post-infestation. To determine the survival rate of the insects with respect to assessed strains, mortality curves were plotted and estimation of significant differences in survival was analyzed by the Kaplan-Meier test ($P < 0.05$) (logrank method, Wilcoxon) using SPSS 19 statistical software (IBM SPSS statistics, 2010).

Results

Evaluation of RPW Adult Survival Rates after Dipping in *B. bassiana* Isolates

RPW adults dipped in *B. bassiana* 203 showed the lowest survival rates from 10 d after inoculation (DAI) onwards (Fig. 2). This resulted in zero survival by 15 DAI. All insects treated with *B. bassiana* isolates 33 and 193 displayed zero survival between 20 and 30 DAI. The other isolates caused zero survival between 34 and 37 DAI. Control (un-treated) insects displayed 80% survival 40 days after treatment.

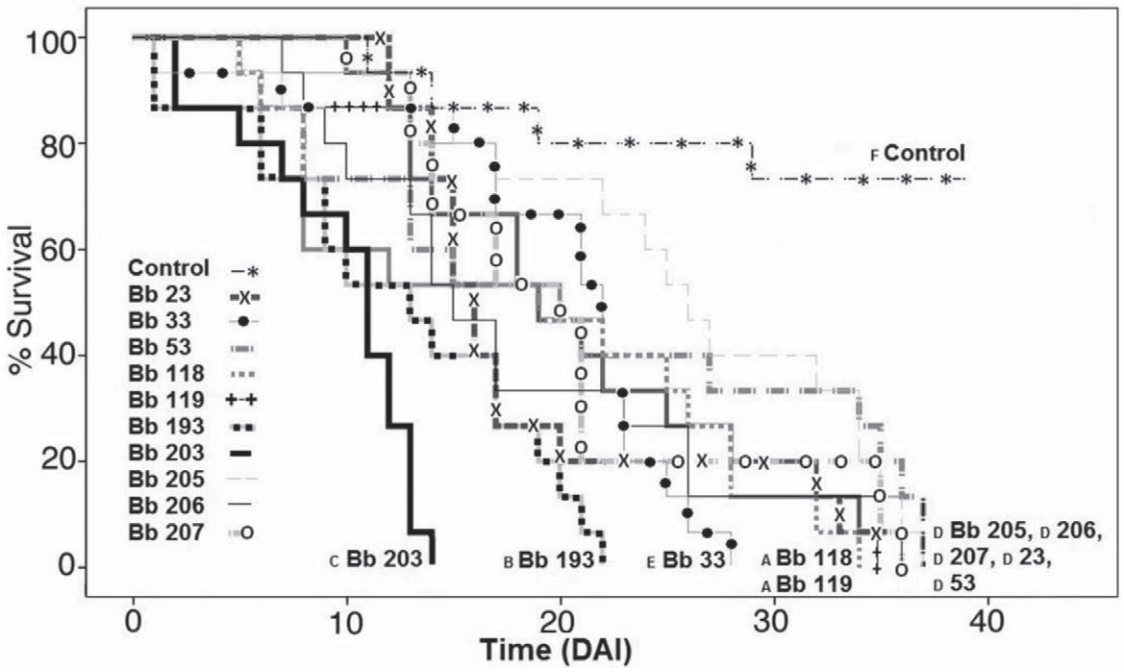

Fig. 2. Evaluation of RPW survival after exposure to dipping treatments of *B. bassiana* isolates (10^7 conidia/mL). Comparisons among treatments were performed using the logrank test ($P < 0.05$) applied to the Kaplan-Meir estimator of the survival function. Different capital letters represent significant differences. Time was measured in days after inoculation (DAI).

Sporulation of *B. bassiana* Isolates on CMA and Conidia Acquisition by RPW Adults

The sporulation of *B. bassiana* isolates grown on CMA used in this study is shown in Fig. 3. All isolates except for 193 had the same order of magnitude of 10^5 conidia cm^{-2} (range of 5.44×10^4 - 9.53×10^3) (Fig. 3a), varying from 1.55×10^5 (isolate 23) to about 6×10^5 conidia cm^{-2} (isolate 53). Because insects were exposed to a CMA culture of the same age, sporulation per culture was determined as well (Fig. 3b). *Beauveria bassiana* cultures contained approximately 10^7 conidia/culture except for isolates 23 and 193, which had about 10^6 conidia/culture. When inoculated with CMA cultures, RPW adults received approximately the same dose (about 10^6 conidia/insect) from the isolate used (range of 2.27×10^6 - 5.02×10^5 conidia/insect for isolate 53 and 5.45×10^6 - 1.59×10^6 conidia/insect for isolate 203, respectively).

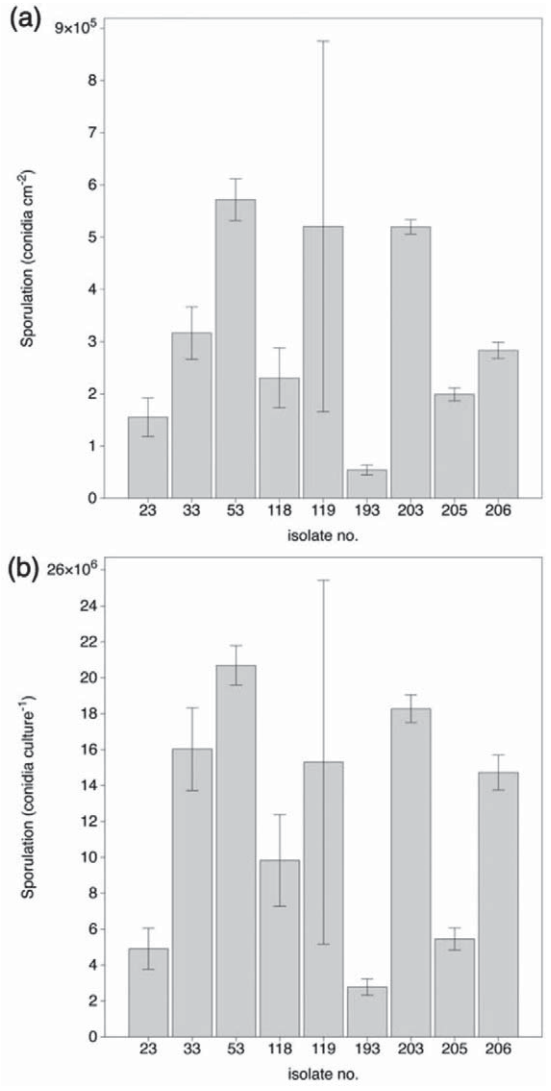

Fig. 3. Sporulation of *B. bassiana* isolates used for pathogenicity bioassays agaist RPW. a) Density of conidia (as conidia cm^{-2}) of *B. bassiana* cultures on corn meal agar (CMA). b) Number of conidia per CMA 24-day culture of each isolate.

Evaluation of RPW Survival Rates after Exposure to *B. bassiana* Isolates in CMA Colonies or Solid State Formulations

The survival of RPW larvae and adults exposed to *B. bassiana* isolates either grown on CMA or in solid state formulations was compared (Fig. 4). When 7-10 day RPW larvae (Fig. 4a) were tested, survival decreased rapidly (zero survival for insects at 4 DAI compared to the control). Isolates 203 and 193 displayed statistically significant differences in survival rates ($P < 0.023$) from those of the other isolates. When 30-day RPW larvae were tested, significant differences in pathogenicity of *B. bassiana* isolates were found (Fig. 4c). Isolates 53 and 119 had the lowest pathogenicity (zero survival for insects approximately 16 and 9 DAI, respectively), and isolates 23, 33, 118, 205 and 206 showed a higher pathogenicity (zero survival between 6 and 9 DAI). Isolates 193 and 203 had the highest and similar pathogenicity towards RPW larvae (zero survival at 4-5 DAI) compared to the other isolates.

The isolates were tested on RPW adults (Fig. 4e). Isolates 33 and 206 were also included for comparative purposes. Again, isolates 193 and 203 were the most pathogenic, and their survival functions were significantly different from those of other isolates (zero survival at 11 and 12 days, respectively; $P < 0.032$). The solid granular formulations of these isolates were also tested on RPW. In these experiments, isolate 33 was also included. Pathogenicity of the fungi formulated on solid granules on RPW did not differ from what was found using CMA cultures as inocculum (Figs. 4b, 4d and 4f).

Evaluation of Solid State Formulation of *B. bassiana* on RPW in Artificially Infested Potted Palms

Similar effects of *B. bassiana* treatments on RPW were found in both potted palm experiments except for Feb 2007, where insect survival (especially for controls) was lower than in 2008 (Figs. 5a & 5c). For all *B. bassiana* treatments, insect survival decreased with time (c.a. 100%) at 7 days after palm infestation (DAPI) with RPW adults to values close to zero at 25 DAPI. Null survival of RPW adults was recorded in 2007 and 2008 experiments by 30 DAPI in treated palms, except for Jun 2007 where this value was missing (Figs. 5a and 5c). *Beauve-*

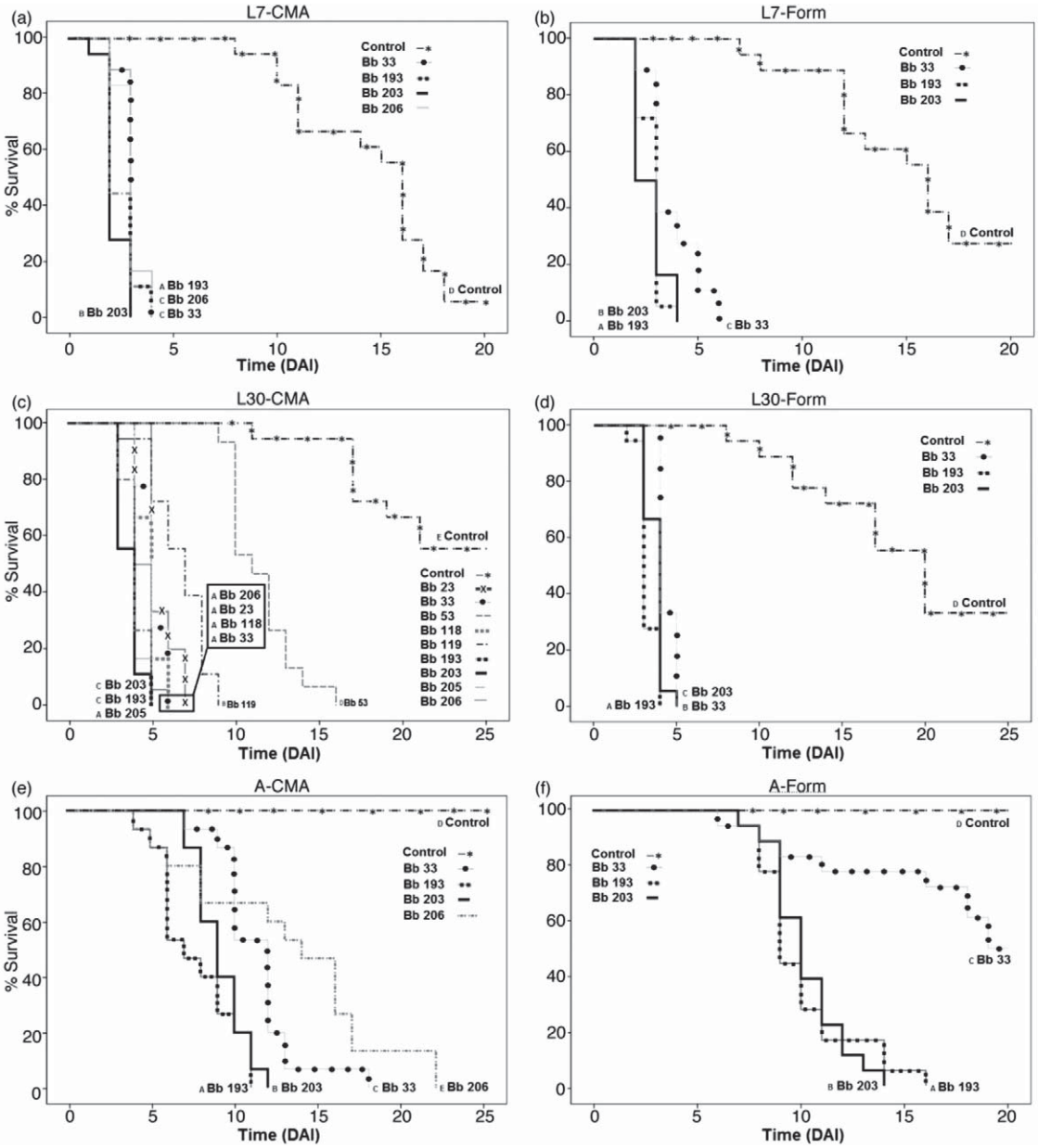

Fig. 4. Evaluation of pathogenicity of *B. bassiana* isolates on RPW in laboratory bioassays using dry conidia. Comparison between treatments were performed using logrank test ($P < 0.05$) applied to the Kaplan-Meier estimator of the survival function. 4a), b) RPW 7-10-day larvae (L7); 4c), d) 30-day larvae (L30); 4e), f) adults. In a, c and e, RPW was inoculated with corn meal agar (CMA) cultures of *B. bassiana* isolates. In b, d and f, RPW was inoculated with *B. bassiana* solid state formulations (Form). Controls were RPW mock inoculated (non-inoculated formulation or CMA plate). Different capital letters represent statistically significant differences. Time was measured in days after inoculation (DAI).

ria bassiana treatments showed a significantly lower survival than controls 14-30 DAPI and 14-24-30 DAPI for 2007 and 2008 experiments, respectively ($P < 0.05$). No differences in survival were found in 2007 and 2008 experiments, except for 14 DAPI in 2008 ($P < 0.05$). Dead insects in *B. bassiana* treatments often showed signs of fungus infection (Fig. 6). Percentage of insects with signs of infection increased with time after palm infestation in *B. bassiana* treatments (Figs. 5b, 5d and 6). Differences were found between insects with signs of *B. bassiana* infection

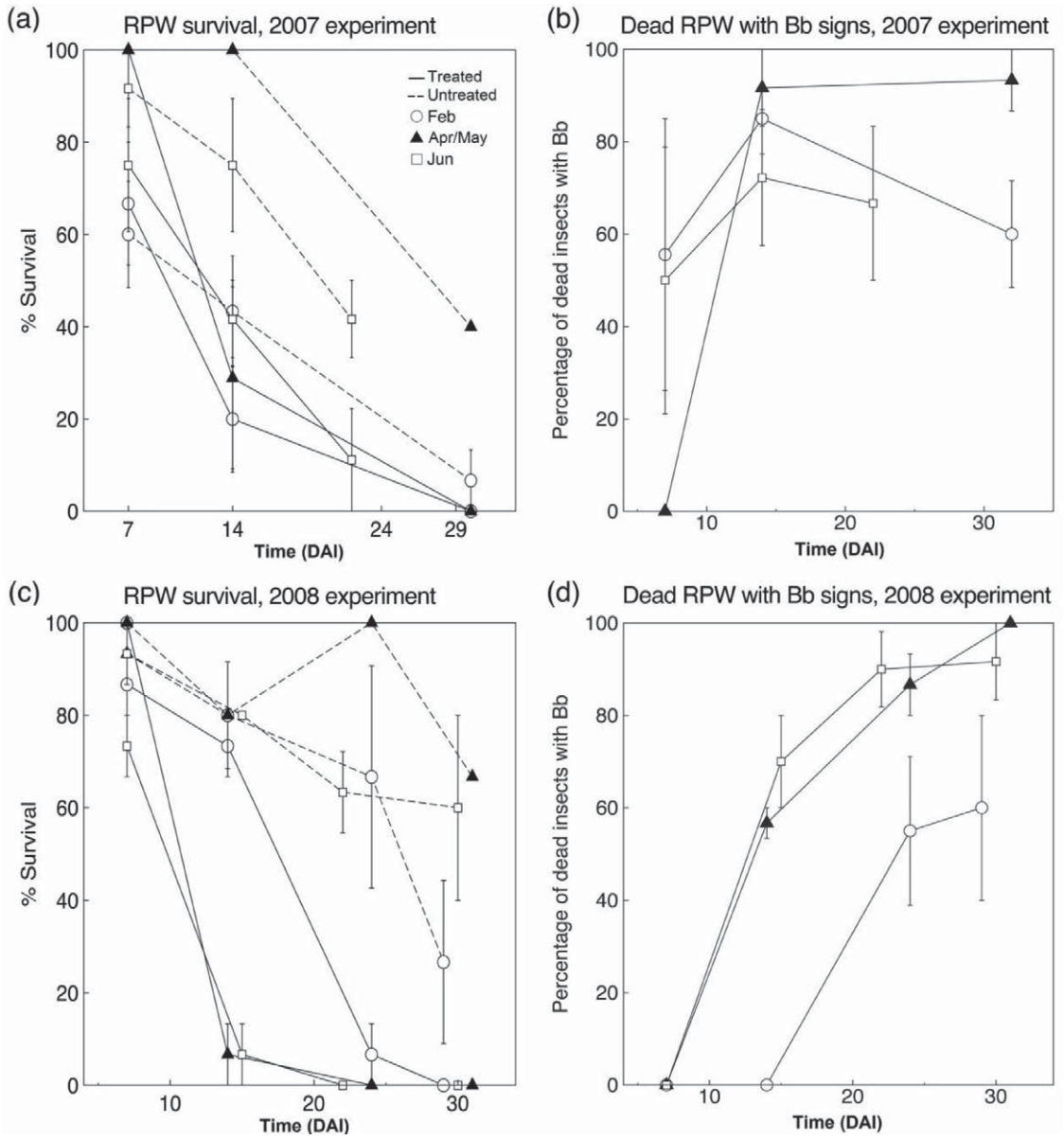

Fig. 5. Effect of *B. bassiana* on RPW survival in artificially infested palms under semi-field conditions. a) RPW survival, 2007 Experiment, b) Dead RPW with signs of *B. bassiana* infection, 2007 Experiment, c) RPW survival, 2008 Experiment, d) Dead RPW with signs of *B. bassiana* infection, 2008 Experiment. Solid line (*B. bassiana* treatments). Dashed line (controls). Feb (○), Apr/May (▲), Jun (□) experiments. Each value represents the mean of 3 replicates. Time was measured in days after inoculation (DAI).

and time post-infestation for May 2007 and Feb/Apr 2008 experiments ($P < 0.05$). No insects with signs of *B. bassiana* infection were found in control palms. In the Feb experiments (2007-2008) RPW adults with signs of *B. bassiana* infection were less frequently found at the end of the experiment (60%, Figs. 5b and 5d) than in Apr and Jun experiments (100% and 80% respectively, Figs. 5b and d), except for Jun 2007 where the value at 30 days was missing.

Dead insects were found on the surface of the potting mixture (Fig. 6a) and very often insects were also found buried in the substrate mixed with palm roots (Figs. 6b and 6d). Signs of *B.*

Fig. 6. Signs of *B. bassiana* infection and colonization of RPW in artificially infested palms under semi-field conditions. a) RPW infected adults with *B. bassiana* on the surface of the potting mixture. b) RPW infected adults with *B. bassiana* buried in the substrate mixed with palm roots. c) Restricted *B. bassiana* development from RPW antennae clubs (arrows). d) *B. bassiana* development from intersegmental regions (arrowhead) and body dismembration (circle). e and f) Extensive mycelium growth and sporulation of *B. bassiana* in RPW infected adults. f) Sporodochia *of B. bassiana* in RPW infected adults. Scale bar = 1 cm.

bassiana infection ranged from restricted mycelial development from RPW antennae clubs 7-14 DAI (Fig. 6c, arrows) to extensive mycelium growth and sporulation (24-30 DAI) (Figs. 6e and 6f) from inter-segmental regions. *Beauveria bassiana* conidiophore aggregates such as sporodochia were sometimes found on heavily infected insects (Fig. 6f).

In both Feb experiments (2007 and 2008), which evaluated the effect of *B. bassiana* on insect survival, slight signs of RPW adult feeding were found (Fig. 7a). However, in both the controls and the *B. bassiana* treatments, no larvae and no signs of larval damage within palms were found (Fig. 7b). On the contrary, in the Apr/May and Jun experiments, eggs and larvae were found within palms (Figs. 7c-e). In both the Apr and Jun 2008 experiments, numbers of RPW larvae in palms treated with *B. bassiana* were lower than those in control palms (Table 2). In the Apr experiment, a positive correlation ($r < 1$) between numbers of RPW larvae and time post-infestation (DAI, Table 2) was found for control palms. RPW multiplication in Jun was higher than in Apr experiment for both control and *B. bassiana* treatment. In *B. bassiana* treatments we sometimes found feeding

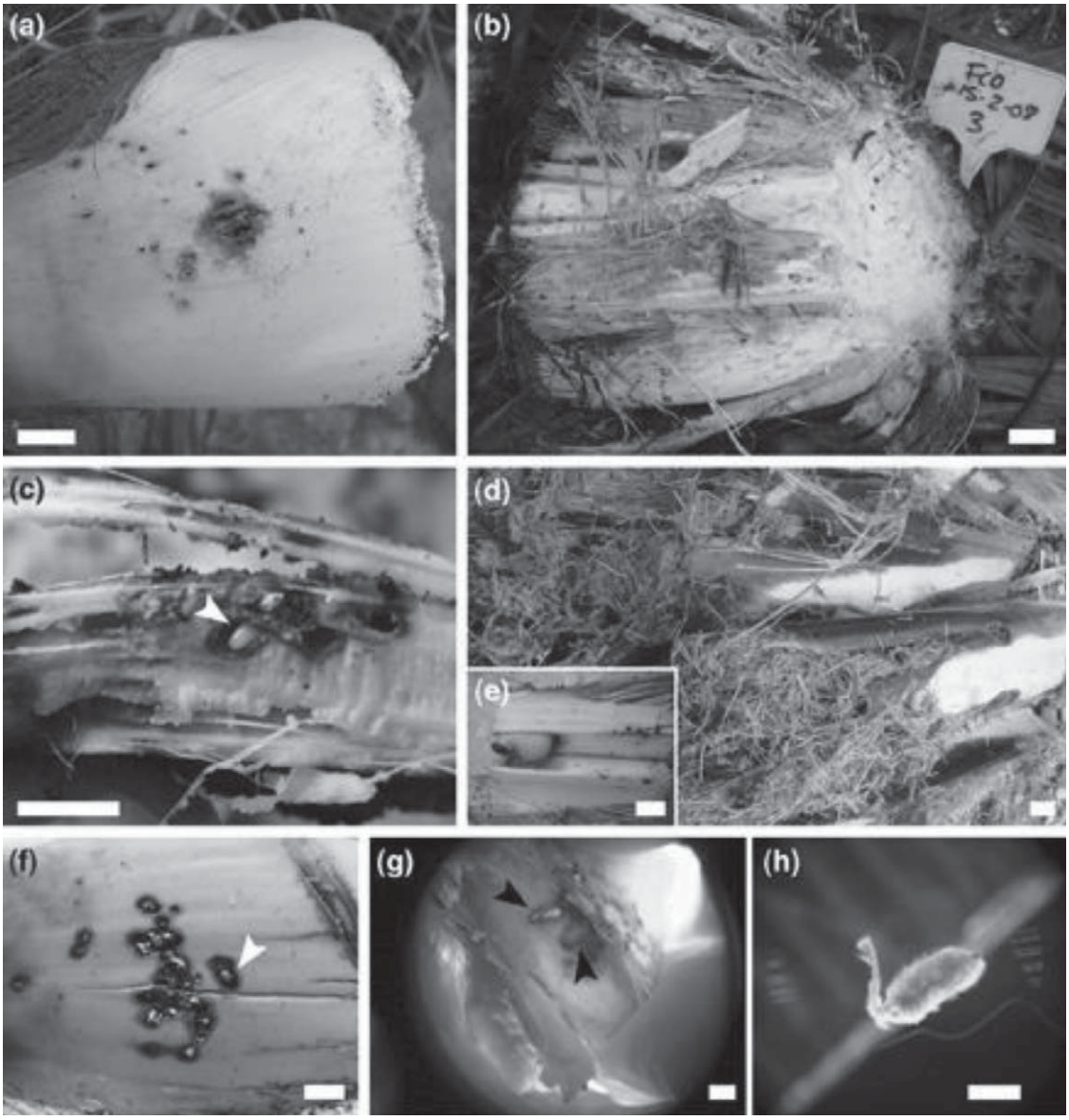

Fig. 7. RPW activity in artificially infested palms under semi-field conditions. a) RPW feeding galleries, Feb 2008 experiment (control). b) Dissection of palm from Feb 2008 experiment without signs of RPW larvae (control). c) RPW egg (arrowhead) on palm petiole Apr 2008 experiment (control). d) Palm with signs of RPW larval feeding Jun 2007 experiment (control). e) Detail of d with RPW larvae feeding on palm tissue. f) RPW feeding galleries colonized by *B. bassiana* (arrowhead). g) RPW infected egg (arrowhead) within palm tissue. h) Detail of Fig. g showing infected egg. Scale bars a-j = 1 cm; Scale bars g-h = 1 mm.

TABLE 2. EFFECT OF *BEAUVERIA BASSIANA* SOLID STATE FORMULATION ON RED PALM WEEVIL (RPW) MULTIPLICATION IN ARTIFICIALLY INFESTED PALMS UNDER SEMI-FIELD CONDITIONS.

		Beauveria bassiana		Control	
Experiment	Time (DAI)	N	RPW larvae / palm	N	RPW larvae/ palm
April 2008	7	3	4 ± 3	2	4 ± 1
	14	2	2 ± 1	3	0 ± 0
	24	3	1 ± 1	3	12 ± 2
	31	3	0.3 ± 0.3	2	24 ± 6
Pearson's correlation (r, p)		(-0.473, 0.142)		(0.85, 0.002)	
June 2008	7	3	14 ± 6	2	93 ± 8
	15	2	35 ± 13	2	76 ± 7
	22	2	17 ± 2	1	95
	30	1	41	0	nd
Pearson's correlation (r, p)		(0.434, 0.283)		(-0.128, 0.837)	

N = no. of palms tested; DAI = days after infestation with RPW adults; nd = no data.
Pearson's correlation coefficient (r) was calculated between numbers of RPW larvae and days post-infestation (DAI).

perforations colonized by the fungus (Fig. 7f). In these colonized perforations we also sometimes found *B. bassiana* infected RPW eggs (Figs. 7g and 7h).

Long-term Efficacy of *B. bassiana* Formulations

Beauveria bassiana formulation remained effective 3 months after application under semi-field conditions. RPW adult survival in Feb and Apr for *B. bassiana* long-term experiments were 0 ± 0% and 7 ± 12%, respectively. Survival of controls in Feb and Apr long-term experiments was 77 ± 30% and 38 ± 13%, respectively. Percentage of insects with external signs of infection in *B. bassiana* treatments was 40 ± 23% and 20 ± 20% for Feb and Apr experiments, respectively.

DISCUSSION

At present, *Rhynchophorus ferrugineus* is widespread in Europe, causing severe damage in native palm groves (EPPO 2008). Because of its nature, this is one of the most feared pests in the world for production and commerce of wide genera of palms, including *Phoenix dactylifera*. This has led to the use of biological agents including *B. bassiana* (Falerio 2006; Gindin et al. 2006; El-Sufty et al. 2007; Llácer et al. 2009; Güerri-Agulló et al. 2010; Llácer et al. 2013).

In this paper, we investigated the potential of *B. bassiana* application as a mycoinsecticide for biocontrol of the RPW. *Beauveria bassiana* isolates from diverse sources, including naturally infected RPW from Mediterranean habitats (close to RPW outbreaks), were evaluated (Asensio et al. 2003). This is the first approach based on multiple-isolate selection for developing a formulation to target RPW, since other studies reporting the effect of entomopathogenic fungi on RPW have just tested 1 isolate (El-Sufty et al. 2007; Dembilio et al. 2010). Because RPW larvae are very susceptible to *B. bassiana* infection, young 7-day larvae bred in the laboratory were not used as insect targets for isolate selection. Nevertheless, we found that isolates derived from 30-day RPW larvae (nos. 193 and 203), were the most pathogenic strains among all tested isolates. These isolates were also the most virulent on RPW adults.

Beauveria bassiana is a species-complex with large genetic variability among isolates (Uma Devi et al. 2006; Fernandes et al. 2009; Chen et al. 2010), and recent investigations have demonstrated that this fungus may cluster with other *Beauveria* species showing different genotypes and consequently, variations in their virulence (Johny et al. 2012). Through this experiment, we proposed a way to identify virulent isolates for the target insect. We conducted multiple infection bioassays using conidia suspensions (dipping) or dry conidia, which allowed us to identify *B. bassiana* isolate 203 (originated from RPW naturally infected in the field). We also showed that its virulence on RPW adults was lower than that on larvae, probably because of the weak structure and composition of the larval cuticle (Güerri-Agulló et al. 2010).

Previously, we had found that *B. bassiana* conidia had a low germination rate in water and consequently, they required an addition of nutrients for full germination (Palma-Guerrero et al. 2008). For *B. bassiana* isolate 119 in particular, only 14.7 ± 3.7% of conidia germinated in water (Palma-Guerrero, pers. com.). This effect may be found only when germination is estimated micro-

scopically, because full germination of the same conidial suspensions was found when they were placed on nutrient media. Since the insect cuticle does not provide nutrients to conidia of entomopathogenic fungi—but starves them and reduces germination (St. Leger et al. 1992)—dry inocula should be more efficient for host penetration and infection. In fact, dry *B. bassiana* conidia performed better than conidial suspensions at degrading RPW cuticle and developing abundant infection structures such as appressoria (Güerri-Agulló et al. 2010).

We also studied the effect on Lethal Time 60 of different concentrations of isolate 193 in 2 of the insect stages; 30-day-old larvae (stage 6) and adults. We noted that in order to kill the same percentage of the population, a lower concentration of conidia per larva was required than per adult (data not shown). In the same work, we evaluated the secondary inoculum that occurred after the death of the larvae and the emergence of the fungus, observing that there was a higher conidia concentration (Güerri-Agulló et al. 2011a).

In a previous assay, conidial suspensions were found less pathogenic to RPW than the same amount of dry conidia formulations in this study. The solid state formulation based on isolate 203 showed high effectiveness in controlling artificial RPW infestations in palms with zero adult survival. The differences in survival rates between the 2007 and 2008 experiments could be caused by differences in temperature, but also in the age and health conditions of the insects that were collected from field populations for both experiments. Insects used in the 2007 experiment were older than those in 2008, and this could have influenced the lower survival observed in the controls (especially in the Feb experiment). During the 2008 experiment, even with higher survival in the controls, insect survival declined faster when the *B. bassiana* formulation was applied to insects in Apr and Jun. Zero survival was delayed only by about 5 days in the Feb experiment with respect to Apr and Jun experiments.

RPW adult survival for *B. bassiana* long-term experiments set in Feb and Apr was similar to that found for experiments set in Apr and Jun (2008), where insects were added immediately after the application of the formulation. Therefore, the formulation can persist at least 3 months. In addition, insect survival decreased and signs of *B. bassiana* infection increased with time (DAI), pointing to mycosis as the cause for insect death.

In the 2008 experiment, the Feb application yielded significantly fewer insects with signs of *B. bassiana* infection than the Apr and Jun applications. In Feb, dead insects in *B. bassiana* treated palms only had post infection breakouts of the fungus, limited to zones with little sclerotised cuticle. On the contrary, after the later applications most insects were profusely colonised by the fungus, which sometimes sporulated massively. This may help fungus persistence and dispersion under field conditions.

It is important to mention that no RPW larvae were found within palms in Feb experiments. This implies that RPW females did not lay eggs in Feb. Consequently, this period was optimal for RPW control by *B. bassiana*. On the other hand, when *B. bassiana* was applied in Apr or Jun insect multiplication was significantly reduced and no correlation between time of adult presence in palms and insect multiplication was found for treated palms. This effect could be explained by RPW adult survival, but also, by *B. bassiana* reduction of RPW fertility and/or egg hatching (Dembilio et al. 2010).

It is known that in some Coleopteran species like *Hylobius abietis*, developmental stages such as larvae and pupa are highly susceptible to *Metarhizium* and *B. bassiana* infection which consequently produces death more rapidly than with adult weevils. This may be observed because their soft bodies pose less of a barrier to infection than the adult sclerotinised cuticle (Ansari & Butt 2012). In our assays when using 7-day-old larvae in the Jun experiment, this effect was especially evident.

Beauveria bassiana has cryptic habits in palm tissues (Gómez-Vidal et al. 2006), which could induce stress resistance proteins (Gómez-Vidal et al. 2009). It has been reported that after an endophytic phase inside banana plants, *Musa acuminata* (Colla), *B. bassiana* retains its morphology and pathogenicity against the banana weevil larvae, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae), and can offer protection against the damaging larvae feeding inside the rhizome (Akello et al. 2010). This could also explain the reduction in RPW larvae populations in palms treated with *B. bassiana*.

In previous assays we detected *B. bassiana* in the internal tissue of the palms, where fungus-infected eggs were also found. *Beauveria bassiana* could have reached palm tissues through feeding perforations, where females lay eggs. In addition, larvae are difficult to reach by the biocontrol agent, although it was noted that survival decreased to 30% in the field tests.

At the present time, we continue to test the solid state formulation of *B. bassiana* described in this paper in palm groves. The preliminary results indicate that the solid state formulation infects RPW adults under field conditions, but also pupae and larvae (Güerri-Agulló et al. 2011b). We believe that the solid state formulation described in this paper can be a significant component of an IPM strategy for RPW control.

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