Use of a Solid Formulation of Beauveria bassiana for Biocontrol of the Red Palm Weevil (Rhynchophorus ferrugineus) (Coleoptera: Dryophthoridae) Under Field Conditions in SE Spain *

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USE OF A SOLID FORMULATION OF *BEAUVERIA BASSIANA* FOR BIOCONTROL OF THE RED PALM WEEVIL (*RHYNCHOPHORUS FERRUGINEUS*) (COLEOPTERA: DRYOPHTHORIDAE) UNDER FIELD CONDITIONS IN SE SPAIN*

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Abstract

We describe the effect of a *Beauveria bassiana* solid formulation on *Rhynchophorus ferrugineus* infesting naturally canary palms in SE Spain in the field. The formulation included a highly pathogenic strain of *B. bassiana* derived from *R. ferrugineus*. The formulation was applied 3 times in 2009 in 2 sites (Catral and El Hondo), at 3-month intervals. *Beauveria bassiana* caused 70-85% *R. ferrugineus* mortality. *Beauveria bassiana* solid formulation with high RPW pathogenicity and persistence, could be applied as a preventive as well as curative treatment for RPW control. Our *B. bassiana* formulation can be a significant component of an IPM strategy for RPW control.

Key Words: RPW, entomopathogenic fungi, biological control, mycoinsecticide, *Phoenix canariensis*

Resumen

En este artículo se describe el efecto de un formulado sólido a base de *Beauveria bassiana* contra *Rhynchophorus ferrugineus* infestando de forma natural palmeras canarias en campo (SE España). El formulado incluye un aislado obtenido de *R. ferrugineus* altamente patogénico contra la plaga. El formulado se aplicó tres veces en dos localidades (Catral y El Hondo) en 2009, con un intervalo de tres meses entre las aplicaciones. *Beauveria bassiana* causó un 70-85% de mortalidad en *R. ferrugineus*. Este formulado seco con alta patogenicidad sobre *R. ferruginues* y elevada persistencia en campo, podría utilizarse como medida tanto preventiva como curativa para su control. Nuestro formulado sólido y seco a base de *B. bassiana* podría aplicarse en programas de manejo integrado de *R. ferrugineus*.

Translation provided by authors.

The red palm weevil (RPW) *Rhynchophorus ferrugineus* is a devastating palm pest that has caused large economic losses in palm farming (Murphy & Briscoe 1999; Faleiro 2006). This beetle can affect a wide range of palms (Barranco et al. 2000) 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.). This pest is at present widely distributed in Oceania, Asia, Africa and Europe and was found in Curaçao and Marruecos, in 2008, and USA, in 2010 (EPPO 2006a, 2007a, 2007b, 2009, 2010). RPW was introduced in Spain mainland in 1995 (Barranco et al. 1996a; 1996b) and then spread to all palm growing areas in the Mediterranean and the Canary Islands.

Although this pest has produced significant economic losses, there are no effective measures for early monitoring and control of RPW yet. Several methods have been used for early detection of RPW such as physical methods for sound detection of feeding larvae (Al-Manie & Alkanhal 2004; Soroker et al. 2004; Faleiro 2006; Mankin et al. 2008; Pinhas et al. 2008; Ilyas et al. 2009; Gutiérrez et al. 2010; Siriwardena et al. 2010). These methods could allow early detection of RPW before visual symptoms appear, but they are still under experimentation. The late detection of the presence of the weevil because of its endophytic larval development reduces the efficacy of insect control using standard practices such as chemical insecticides.

Entomopathogenic fungi are important regulators of insect populations under natural conditions (Bidochka et al. 2000). Mitosporic fungi such as *Beauveria bassiana* have been used for the biological control of insect species and of coleopterans in particular (Tanada & Kaya 1993). Entomopathogenic fungi are being put forward as biological control agents in Integrated Pest Management (IPM) programs to control current *R. ferrugineus* outbreaks (Murphy & Briscoe 1999; Faleiro 2006). This strategy includes: epidemiological measures, mass trapping, chemical treatments and biological control (Murphy & Briscoe 1999; Faleiro 2006; Gindin et al. 2006; El-Shufty et al. 2007). Sewify et al. (2009) successfully reduced the incidence of *R. ferrugineus* under field conditions in Egypt using a native strain of *B. bassiana* isolated from a *R. ferrugineus* cadaver.

Most deuteromycetes are facultative pathogens which can live saprophytically in the soil (Bidochka et al. 2000). In a previous study (Asensio et al. 2003) we found *B. bassiana* as the most abundant entomopathogen in soils from SE Spain where palm groves are widely grown. Consequently, we have isolated *B. bassiana* from naturally infested RPW in palm groves in E and SE Spain (Güerri-Agulló et al. 2010).

We have already studied the mode of infection of *R. ferrugineus* larvae and adults by strains of *B. bassiana* derived from *R. ferrugineus* using dry conidia and conidia suspensions using SEM (Güerri-Agulló et al. 2010).

For practical use, biocontrol agents must be developed for mass production (Rombach et al. 1988). Most common substrates for fungal entomopathogens include primary products from agriculture (Alves & Pereira 1989; Moore & Prior 1993; Zimmermann 1993). Some simple and cheap methods for production of biocontrol fungi have been developed using agricultural waste products (Steinmetz & Schönbeck 1994; Leite et al. 2005; Kassa et al. 2008; Sahayaraj et al. 2008; Machado et al. 2010; Kim et al. 2010).

Our group has investigated the use of agricultural by-products for the production of entomopathogenic fungi (Lopez-Llorca & Carbonell 1998). We have developed palm by-products for the production of *B. bassiana* (Asensio et al. 2007). Based on this previous work, we have developed and tested in bioassays and semifield conditions a solid mycoinsecticide based on *B. bassiana* for biological control of RPW (Güerri-Agulló et al. 2011). In this paper we describe the effect of a *B. bassiana* formulation on RPW in naturally infested palms (*Phoenix canariensis*) at 2 field sites in SE Spain. The formulation was applied 3 times during 2009 at 3-month intervals. We also analyze the efficacy of the *B. bassiana* formulation in the same field sites one year after the last application.

MATERIALS AND METHODS

Field Trial Descriptions

Selection of Palms Groves Naturally Infested with RPW for Trials.

Two palm groves in SE Spain with a regular plantation pattern (1.5-2 m between palms) containing 875 *Phoenix canariensis* Hort palms in total were selected for the experiments in 2008. Palm groves included naturally RPW infested and non-infested palms. RPW infestation in these palm groves had been identified by TRAGSA (Spain). The palm groves were selected with the approval of Generalitat Valenciana and the consent of the owners for field application of a *Beauveria bassiana* formulation. Infested individuals were marked at the start of the experiments. *Beauveria bassiana* treated palms were georeferenced using GPS (Series GeoXM, Trimble GeoExplorer) and were plotted to indicate their position in each experimental site.

Catral Palm Grove

In the municipality of Catral (SE Spain) a rural plot (2 lots, A: ca. 30 m × 88 m and B: ca. 26 m × 110 m, Fig. 1a) with young (1.5 m from bottom to start of the crown) *P. canariensis* were selected for experiments. Lot A had suffered a severe RPW infestation prior to our experiments (Mr. J. J. Lopez-Calatayud, TRAGSA, personal comunication). Therefore, many palms were cut and the plantation frame was broken (Fig. 1a, lot A). In this plot a total of 240 (lot A: 89; lot B: 155) palms were selected for experiments. Of these 17 palms were infested by RPW (lot A:12; lot B: 5). In lot A (Fig. 1a) all palms (89) were treated with *B. bassiana*. Lot B, which was left untreated, served as a control.

El Hondo Palm Grove

In the municipality of Elche (SE Spain) a rural plot with 3 lots (Fig. 1b; lot A: ca. 22 m × 254 m, lot B: ca. 28 m × 253 m and lot C: ca. 33 m × 85 m;) with young (1 m from bottom to start of the crown) *P. canariensis* were selected for experiments. Lot C had suffered a severe RPW infestation prior to our experiments (Mr. J. J. López-Calatayud, personal comunication). In this plot a total of 631 palms were selected for experiments; and of these 22 palms were infested by RPW.

Lots A and B included 2 palms each naturally infested by RPW. Lot C included 18 palms naturally infested by RPW. The rest of palms had no visual symptoms of RPW infestation at the starting of the experiments. In lots A and B (Fig. 1b) of each 2 adjacent palms one was *B. bassiana* treated and the other left untreated throughout the lots. In lot C all RPW infested palms were treated with *B. bassiana* and 10 non-infested palms were also treated (Fig. 1b).

Application of *B. bassiana* Formulation to Palms in the Field

Beauveria bassiana isolate 203 used in experiments was obtained from naturally infected RPW adults in SE Spain (Güerri-Agulló et al. 2010).

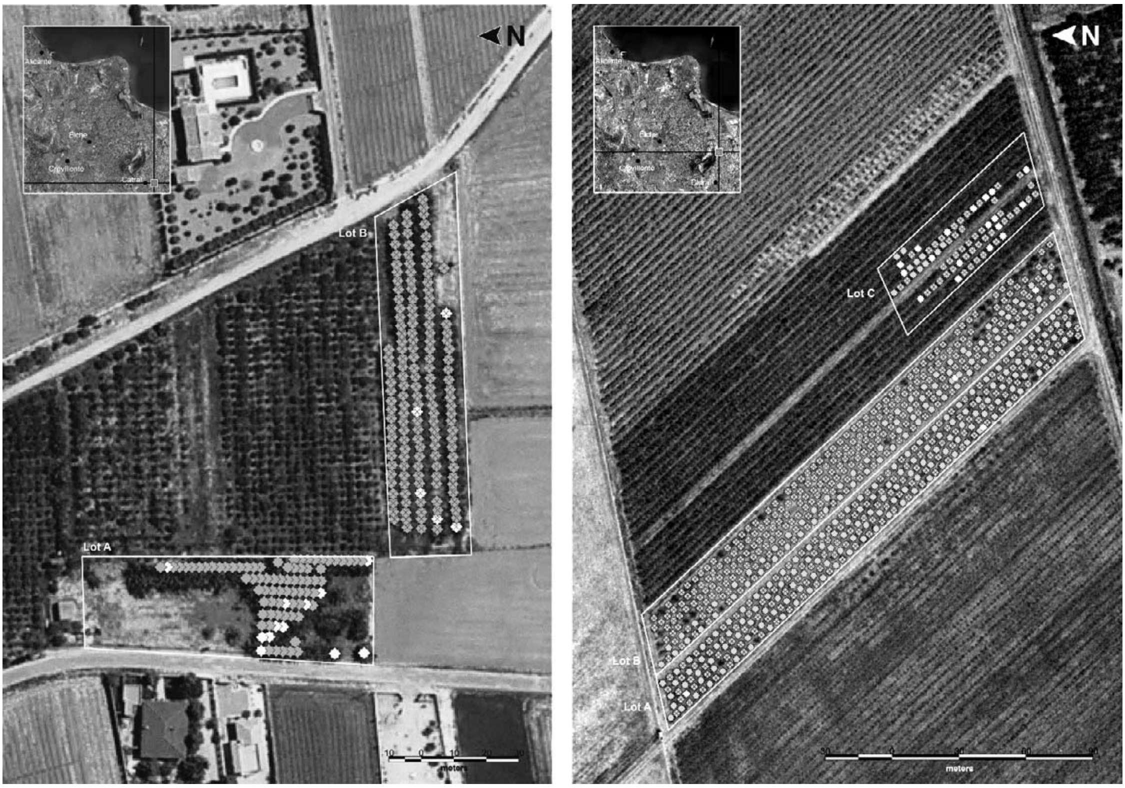

Fig. 1. *Beauveria bassiana* application to naturally infested canary palms (*Phoenix canariensis*) with *Rhynchophorus ferrugineus* in two palm groves in SE Spain. (a) Catral palm grove (stipe height 1.5-2 m). (b) El Hondo Palms grove (stipe height 1-1.5 m). Palms with X were left untreated. Marked palms in white were RPW infested. Palms marked in green displayed no RPW symptoms. A version of this figure in color is available in the supplementary figures at http://www.fcla.edu/FlaEnt/fe944.htm#InfoLink1.

This isolate was formulated as a solid in ca. 5mm granules as in Asensio et al. (2008) with further modifications. Selected *P. canariensis* palms were treated with ca. 500 g of the *B. bassiana* formulation per palm. *Beauveria bassiana* formulation was dusted around the palm crown to cover the spaces between the stem and pecioles using a 2 m long pole. Control palms were left untreated. The *B. bassiana* formulation was applied 3 times throughout the year (starting March 2009) at approximately 3-month intervals.

Assessment of *B. bassiana* Application on RPW and RPW Infestation in Palms

Three months after the starting of experiments the effect of *B. bassiana* application on RPW survival was assessed. Nine months later, RPW infestation in palms was also assessed. RPW survival was monitored in selected palms by pulping them. The upper part of leaves of each selected palm was removed and the remains (petioles, palm crown and stem) dissected using a chain saw until no signs of RPW were found. RPW infested material was carefully searched for insects (larvae, pupae and adults) and their numbers scored. Each insect was scored as dead or alive and whenever present insects with signs of mycoses were also scored. Palm infestation by RPW was scored with 2 levels. Level 1 included palms without visual symptoms of RPW damage (Fig. 2a). Level 2 included palms with large numbers of leaves asymmetrically placed in the crown (Fig. 2f-2g).

For the Catral palm grove 3 months after the *B. bassiana* application 6 RPW infested palms were pulped. Of these, 2 were controls (untreated) and the rest were *B. bassiana* treated palms. Nine months later 11 palms were also pulped. Of these, 2 palms were controls and RPW infested. Of the remaining 9 *B. bassiana* treated palms, 8 were RPW infested.

For the El Hondo palm grove 3 months after the *B. bassiana* application, 9 palms were pulped. Of these, 3 were controls (untreated) and RPW infested. Of the remaining 6 *B. bassiana* treated palms only 1 was non-infested. Nine months later 18 palms were also pulped.

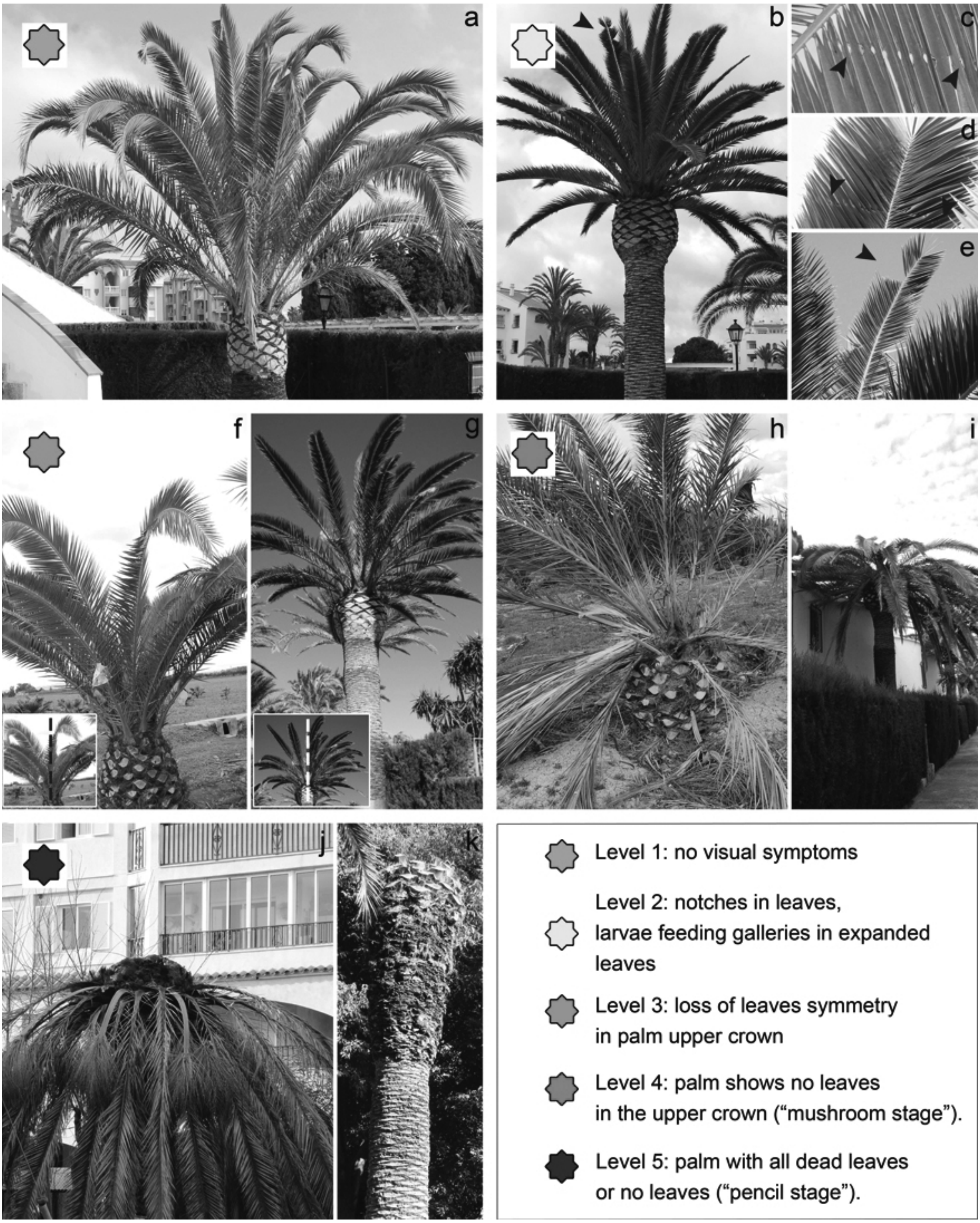

Fig. 2. Visual chart of RPW palm infestation based on empirical observations in this study. Chart shows (1 = no symptoms, 5 = dead palm). (a) Level 1: no symptoms. (b - e) Level 2: notches in leaves (b, e; arrowheads); larvae feeding galleries (c, d; arrowheads) in expanded leaves. (f - g) Level 3: loss of leaf symmetry in palm upper crown. (h - i) Level 4: palm shows no leaves in the upper crown ("mushroom stage"). (j - k) Level 5: palm with all dead leaves or no leaves ("pencil stage"). A version of this figure in color is available in the supplementary figures at http://www.fcla.edu/FlaEnt/fe944.htm#InfoLink1.

Of these 5 were controls and RPW infested. Of the remaining 13 *B. bassiana* treated palms, all were RPW infested. A summary of palm RPW infestations and *B. bassiana* treatments are given in Table 1. Palms without RPW were not counted for data collection.

Assessment of Long-Term Effect of *B. bassiana* Application on RPW Infestation in Palms

In view that a two-level scale of RPW infestation was insufficient for long-term assessment of *B. bassiana* treatments, a finer scale (with 5 levels) was empirically established in this study (Fig. 2). Twelve months after the last *B. bassiana* application palm infestation by RPW was scored with 5 levels (Fig. 2). Level 1 included palms without visual symptoms of RPW damage (Fig. 2a). Level 2 included palms with early RPW infestation symptoms mainly in the leaves, such as holes or notches in folioles and leaves, or missing folioles (Figs. 2b-2e). Even tracks caused by insect feeding could sometimes be detected. Level 3 included palms with large numbers of leaves asymmetrically placed in the crown (Figs. 2f, 2g). Level 4 included palms in an advanced stage of RPW infestation, with mostly flat or bent down leaves in the crown (Figs. 2h, 2i). Level 5 included dead palms without living leaves (Figs. 2j, 2k). All palms in Catral and El Hondo plots were georeferenced (Series GeoXM, Trimble GeoExplorer, gvSIG and IGN-España) and visually scored using the 1-5 scale previously described.

With data obtained, as previously described, RPW infestation maps using GIS tools were constructed. Graphs indicating RPW incidence as well as level of RPW infestation using percentage of infestation were also calculated 12 months after last *B. bassiana* applications.

Statistical Analysis

Statistical analyses of the data 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 one-way ANOVA and Tukey's Honest Significant Difference method, or Student's t-test for analyzing differences among groups. Non-normal data were compared using Kruskal-Wallis (K-W) rank sum test and U-Mann-Whitney test with corrections for multiple testing.

TABLE 1. EFFECT OF *BEAUVERIA BASSIANA* ON RPW STAGES IN NATURAL INFESTED PALMS IN TWO PLOTS IN SE SPAIN.

Catral plot		RPW mortality		Dead RPW with *B. bassiana*	El Hondo plot		RPW mortality		Dead RPW with *B. bassiana*
	Stage No.	no.	%	(%)		Stage No.	no.	%	(%)
Control*					Control*				
	larvae 93	0	0	—		larvae 36	1	3	0
	pupae 4	3	75	33		pupae 6	1	24	0
	adults 21	3	14	67		adults 7	2	30	10
*B. bassiana**					*B. bassiana**				
	larvae 3	3	100	90		larvae 1	1	100	0
	pupae 7	7	100	83		pupae 3	3	100	100
	adults 26	24	94	53		adults 4	4	100	42
Control**					Control**		8	23	21
	larvae 78	1	1	0		larvae 35	22	83	21
	pupae 4	2	38	50		pupae 26	20	42	70
	adults 3	1	20	100		adults 46	8	23	21
*B. bassiana***					*B. bassiana***				
	larvae 1	1	100	50		larvae 2	1	42	17
	pupae 11	8	75	67		pupae 1	1	100	75
	adults 17	14	81	64		adults 5	5	100	56

No. = total RPW; no. = dead RPW

**B. bassiana* treated palms and untreated (control) palms scored three months after the first application.

***B. bassiana* treated palms and untreated (control) palms scored 12 months after the first application.

RESULTS AND DISCUSSION

Effect of *B. bassiana* on RPW in Naturally Infested Palms in the Field

Three months after first field application of *B. bassiana* formulation in Catral palm grove, 70% of RPW population within *B. bassiana* treated palms was dead. Twelve months after first field application, RPW mortality was ca. 85% (Fig. 3a). On the contrary untreated (control) palms had a much lower RPW mortality (Fig. 3a, ca. 5%). Palms with one *B. bassiana* treatment showed ca. 50% of dead insects with *B. bassiana* signs (Fig. 3b). After 3 treatments most dead insects showed signs of *B. bassiana* infection (ca. 70%, Fig. 3b). Dead insects from untreated palms also showed signs of *B. bassiana* infection (ca. 40-50%, Fig. 3b). Since RPW adults survive for a few days after acquisition of fungal inoculum in *B. bassiana* treated palms (Güerri-Aguló et al. 2011b), they may act as vectors of the fungus. They would then transmit *B. bassiana* infection to RPW in untreated palms. This could explain the high number of dead insects with *B. bassiana* signs in these palms (Fig. 3b). Moreover, non-motile RPW (pupae) were found with *B. bassiana* signs close to dead adults with the same signs in untreated palms.

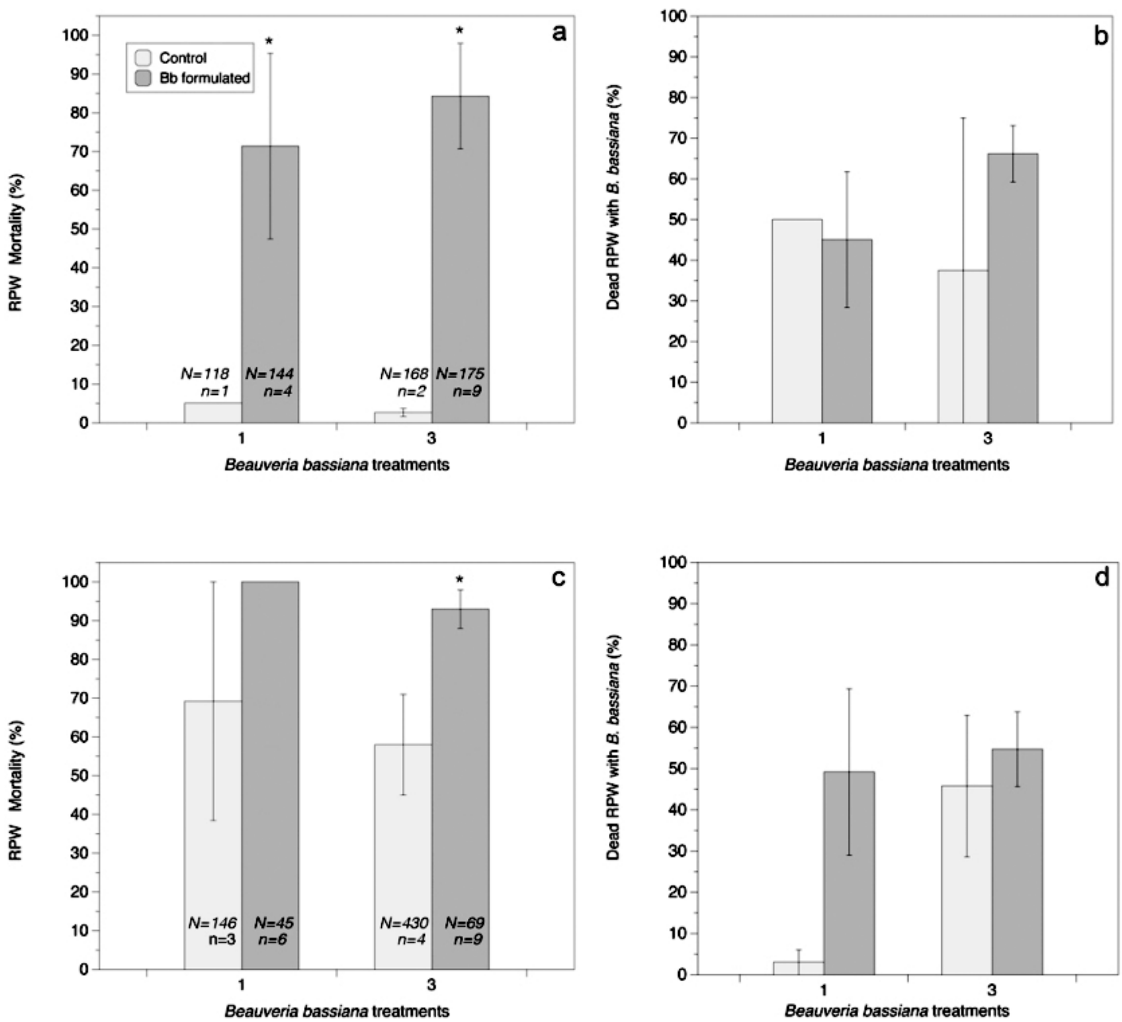

Fig. 3. Effect of *B. bassiana* on *Rhynchophorus ferrugineus* in naturally infested palms in two palm groves in SE Spain. Sites: Catral (a, b), El Hondo (c, d); n = no. palms assessed. N = sum of the insects present in palms assessed. *Significant Difference ($P = 0.05$). In each of the 4 panels, the bar graphs on the left present the data taken at 3 months post the first application, and the bar graphs on the right present the data taken at 12 months post the first application. Mortality rates are showed in (a) and (c). Dead insects with signs of *B. bassiana* are showed in (b) and (d).

In El Hondo palm grove a higher RPW mortality (90-100%; 55-65%) than in Catral was found for both treated and untreated palms (Fig. 3c), respectively. Three months after first field application of *B. bassiana* formulation in El Hondo palm grove, 100% of RPW population within *B. bassiana* treated palms was dead. Twelve months after the first field application, RPW mortality was ca. 95% (Fig. 3c). RPW mortality in untreated (control) palms was ca. 65% 3 months after the beginning of the experiment and ca. 55% 12 months after the beginning of the experiment.

Beauveria bassiana signs were found in dead RPW from both treated and untreated palms (Fig. 3d). RPW mycoses in untreated palms increased noticeably with time. This could be due to the increasing amount of inoculum for the repeated number of *B. bassiana* treatments.

In Catral, the RPW population was larger in untreated palms than in treated palms (Table 1). This was true for larvae in untreated palms (93-78) vs. treated ones (3-1) with just 1 or 3 *B. bassiana* treatments respectively (Table 1). The largest number of living adults was found in untreated palms, unlike treated ones (Table 1). RPW females lay more viable eggs than those infected with *B. bassiana* (Dembilio et al. 2010). This could explain the large numbers of larvae in untreated palms. Similar results were found in El Hondo palm grove (Table 1). The low number of larvae in treated palms could be due to the action of the entomopathogenic fungus.

Evolution of RPW infestation in *B. bassiana* treated palm groves.

In Catral and El Hondo groves, the initial percentage of RPW infestation was around 10% (Fig. 4, Mar 2009). In Catral (Fig. 4a), the percentage of RPW infestation after 3 *B. bassiana* treatments increased to 40% (Oct 2009). This could be due to the large RPW infestation prior to our *B. bassiana* treatments. Besides in lot B (Fig. 1a), which was left untreated, RPW infestation rose from 3% at the start of the experiment to 45% 12 months after last *B. bassiana* treatment. This could have acted as a focus for RPW infestation of lot A (close to lot B). Twelve months after the last treatment with no further *B. bassiana* treatments (Oct 2010) RPW infestation dropped to 30% in lot A. Three zones with different levels of RPW infestation could be distinguished. In the inner zone, palms did not show visual symptoms of RPW infestation. The middle zone had palms with different degrees of RPW infestation and in the outer zone there were only few palm trees left (Fig. 5a). The persistence of the *B. bassiana* applications was detected by the extensive finding of *B. bassiana* infecting several RPW stages (Fig. 6a-c). Transmission of *B. bassiana* infection was detected since non-motile insects (pupae) in control palms were also found mycosed (Fig. 6b).

In El Hondo (Fig. 4b), the percentage of RPW infestation after 3 *B. bassiana* treatments dropped to 3% (Oct 2009). Twelve months later with no further *B. bassiana* treatments (Oct

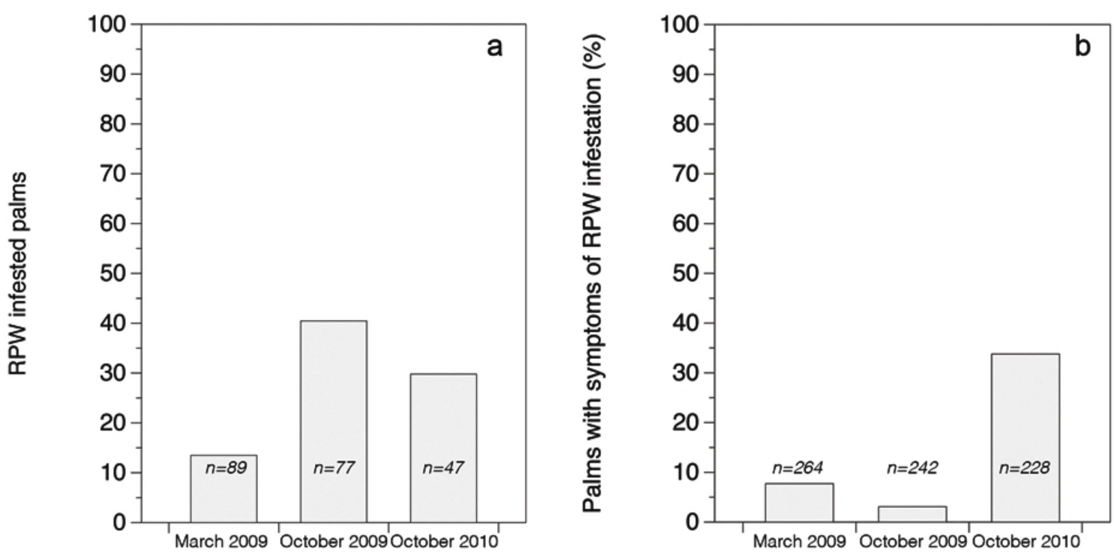

Fig. 4. Effect of *B. bassiana* treatment on RPW infestation in palms of two palm groves: (a) in Catral palm grove, (b) in El Hondo palm grove. RPW infestation before treatment (March 2009), after treatment (October 2009) and twelve months after the last treatment (October 2010); *n* = no. palms assessed.

Fig. 5. Long-term (12 months after last application) effect of *B. bassiana* treatment on spatial distribution of RPW infestation in 2 palm groves in SE Spain: (a) Catral and (b) El Hondo. RPW infestation was measured by visual symptom evaluation as in Fig. 2. A version of this figure in color is available in the supplementary figures at http://www.fcla.edu/FlaEnt/fe944.htm#InfoLink1.

2010) RPW infestation was 34% (Fig. 4b; Fig. 5b, rows: 1-9). RPW infestation was found to be dependent on the density of the previous *B. bassiana* treatments (Fig. 5b). Therefore, low infestations were found in treated (25%) and untreated (37%) palms within high-density *B. bassiana* treated areas (Fig. 5b, rows: 1-5) 12 mo after last *B. bassiana* application. In contrast at the same time, RPW infestation was high (62%) in untreated palms outside *B. bassiana* heavily treated areas (Fig. 5b, rows: 10-11). *Beauveria bassiana* infection may have been spread in the field by RPW adults. This could explain the presence of RPW (especially non-motile stages) infected with *B. bassiana* in untreated palms.

At least one application of *B. bassiana* reduced the RPW population in treated palms with respect to the control. Most of insects in treated palms were dead and presented signs of mycosis. Adding to that there were RPW larvae, pupae and adults with *B. bassiana* signs in untreated palms. We could consider that repeated *B. bassiana* field treatments could create natural epizootics in RPW populations. This would maintain a *B. bassiana* inoculum in palm groves, such as those in this study. Therefore subsequent *B. bassiana* treatments would increase this fungal inoculum and reduce the impact of RPW infestation.

RPW mortality results with our *B. bassiana* solid formulation were ca. 70-100% (after 1 application) and ca. 85-95% (after 3 applications). Our results differ from those of El-Sufty et al. (2007), who used a powder formulation of *B. bassiana* and obtained only 9% RPW mortality. These differences - among other reasons - could be due to the isolate used. As we have shown (Güerri-Agulló et al. 2011b) the selection of the isolate is a very important step for developing a mycoinsecticide.

There are only few examples of RPW biocontrol, especially in the field. There are, however, studies on weevil biocontrol with entomopathogenic fungi especially using liquid formulations. Ihara et al. (2009) applied *B. bassiana* liquid inoculum against chestnut weevil, *Curculio sikkimensis*, in pots with soil. They achieved a survival rate of 3% 6 months after application. *Beau-*

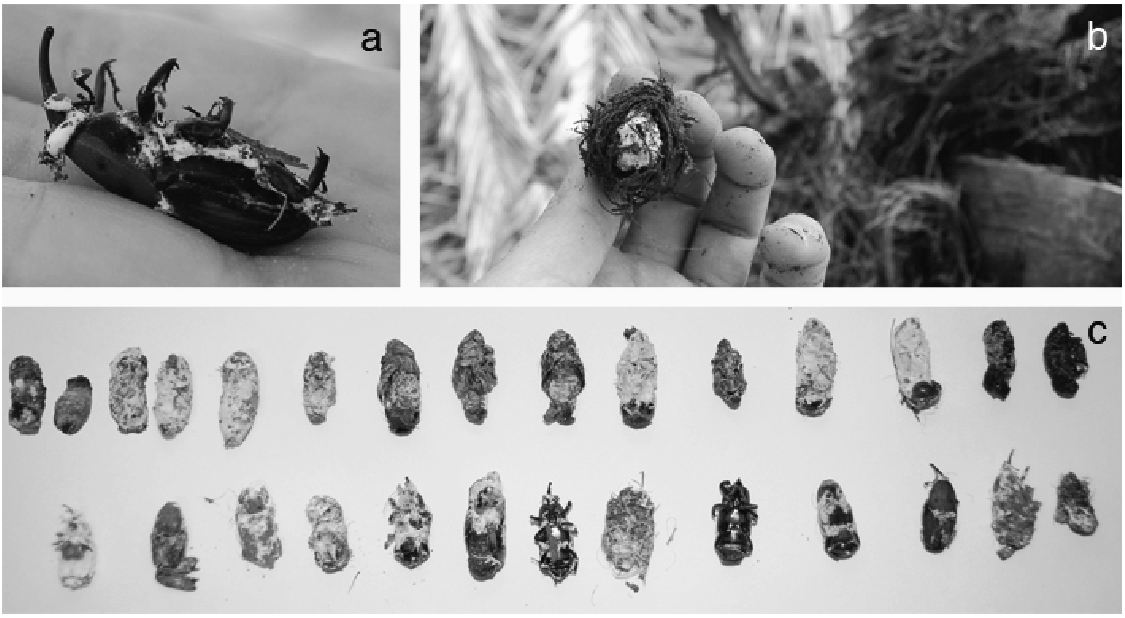

Fig. 6. Detection of *B. bassiana* induced RPW epizootics in Catral palm grove 12 months after last application. (a) RPW infected adult found in *B. bassiana* treated middle zone. (b) RPW infected pupa in untreated zone. (c) Numerous RPW individuals of different stages found infected in *B. bassiana* treated middle zone. For Catral zones see Fig. 5. A version of this figure in color is available in the supplementary figures at http://www.fcla.edu/FlaEnt/fe944.htm#InfoLink1.

veria bassiana (GHA isolate; Shapiro-Ilan et al. 2008) achieved ca. 80% mortality used against the pecan weevil, *Curculio caryae* (Horn), in the 15-d experimental period. Gemination percentage of the fungus was ca. 80% and increased until ca. 90% using UV protectors. Godonou et al. (2010) used two *B. bassiana* formulations for the management of the banana weevil, *Cosmopolites sordidu*s (Germar). They achieved ca. 75% mortality with both a powder formulation and an oil palm formulation, in a 30-d experimental period. RPW has a long life cycle within the palm. It is even possible that adults do not go outside palm during their life cycle. Therefore, biocontrol formulations must remain viable for at least 2-3 mo when applied to palms. We found our formulation to be viable for at least 3 months in both soil and palms (Asensio et al. 2008; Güerri-Agulló et al. 2011b).

We have shown in this study that our *B. bassiana* solid formulation causes RPW mortality, and reduces RPW populations and RPW palm infestation levels. We also presented preliminary evidence that *B. bassiana* induced RPW mortality is positively correlated with the number of palms treated in the field. Therefore *B. bassiana* could be implemented as a key component in an IPM program for RPW sustainable management in Mediterranean areas and similar palm growing conditions.

Acknowledgments

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