Evaluation of the Entomopathogenic Fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the Red Palm Weevil *Rhynchophorus ferrugineus*

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The red palm weevil (RPW, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) is one of the most severe pests of various palm species, including date palms. While examining the susceptibility of RPW to two entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana,* strains of the former were found to be more virulent than those of the latter, achieving 100% larval mortality within 6–7 days. The most virulent strains of *M. anisopliae* were then tested on RPW eggs and adults. Incubation in a substrate treated with *M. anisopliae* spores increased egg mortality and reduced their hatchability. The total percentage mortality of eggs and hatched larvae was 80–82%, compared with 34% in the controls. RPW adults were challenged with two types of fungal formulation: dry powder and aqueous suspension. Cumulative adult mortality of 100% was achieved in 2–3 weeks for the dry rice-based formulation and in 4–5 weeks for the spore suspension. As a result of decreased longevity, treated females had a shorter oviposition period and three times lower fertility than the controls. Possible strategies for fungus application are discussed in the light of the high susceptibility of eggs and larvae to fungal infection.
KEY WORDS: *Rhynchophorus ferrugineus*; RPW; entomopathogenic fungi; *Metarhizium anisopliae; Beauveria bassiana;* date palms.

INTRODUCTION

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), is one of the most severe pests of various palm species, including date palms (8). The weevils develop within the tree trunk, destroying its vascular system and eventually causing the collapse and death of the tree. The pest is widely distributed in Oceania, Asia, Africa and Europe. The RPW causes severe damage to coconuts in Southeast Asia (8). It appeared in the Middle East in the 1980s and has heavily damaged date production by destroying many thousands of date palms (16). Infestation was first reported in Israel and Jordan in 1999 (13).

It is commonly accepted that RPW adults are attracted to dying and damaged parts of palm trees, but this does not preclude attacks to undamaged palms (16). Females oviposit in the splitting bark (1), at the base of young leaves, or in wounds on the leaves and trunks (5). Eggs are laid close to the surface and the hole is cemented over. The hatched grubs tunnel into and feed on the surrounding tissue, thereby destroying it. Pupation occurs inside a cocoon. In young trees, cocoons are found at the base of the palm trunk, near or below

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the soil surface. Usually all life stages, including adults, are present within the same palm tree (5).

With the exception of adults, the life cycle of RPW is mostly concealed within the tree trunk, so detection of infestation and treatment of infested trees are problematic. Current recommended methods for RPW management involve monitoring and mass-trapping of adults with pheromone lures, cultural control, and chemical treatments (1,24). It is common knowledge that intensive chemical treatment leads to the development of resistance, and therefore alternative methods should be considered. Recently, natural parasites and pathogens of RPW have been extensively studied to evaluate their potential as biological control agents (20,21,23). Entomopathogenic fungi are seldom isolated from *Rhynchophorus* spp.: of 95 isolates of various microorganisms that were found in dead RPWs and in their natural habitats, only three isolates were fungi (21). *Metarhizium anisopliae* (Metsch.) Sorokin was isolated from *R. bilineatus* in New Guinea, after treatment of young palms against the scarabaeid *Scapanes australis* with a formulation based on *M. anisopliae* spores (17). In Iran, *M. anisopliae* was isolated from adults and *Beauveria bassiana* (Bals.) Vuill. from pupae (7). In addition, a fungus that was identified as *Beauveria* sp. was associated with some cocoons of *R. ferrugineus* and infected the adults that emerged in an artificial rearing (22).

Unlike other insect pathogens, fungi infect the host by contact, penetrating the insect cuticle. The host can be infected both by direct treatment and by transmission of inoculum from treated insects or cadavers to untreated insects or to subsequent developmental stages *via* the new generation of spores (14,18). These unique characters make entomopathogenic fungi especially important for the control of concealed insects. In the case of *Rhynchophorus* spp., most of the life cycle passes within the tree trunk, making the pest inaccessible to direct-contact treatment. Adults are the only exposed stage and can be infected upon emergence. The questions remain of whether the treated adult will effectively convey the infection onwards and, if so, how and where it can best be treated.

The potential of entomopathogenic fungi as biocontrol agents against several weevil species has been evaluated (2,11,12). Passive mechanical transmission of fungi within insect populations has been observed for various entomopathogenic fungi, *e.g. B. bassiana, M. anisopliae* and *Paecilomyces fumosoroseus* (6,14,18). In these studies adults were often the most suitable or even the only possible stage for treatment. Evidence for successful application of fungi *via* adults was obtained from experiments on the pinhole borer, *Platypus* spp.: adults that were contaminated with *B. bassiana* or *M. anisopliae* spores transferred the fungal infection to larvae, which resulted in 50–100% larval mortality (9).

The aim of the present study was to test the susceptibility of the various RPW stages to the entomopathogenic fungi *M. anisopliae* and *B. bassiana*, in order to develop formulations and application strategies suitable for future use in biological control.

MATERIALS AND METHODS

Insect rearing A RPW colony was established in the laboratory on sugarcane as both food and oviposition substrate, following Rahalkar *et al.* (19). Adults were set to mate and oviposit in groups of at least five pairs placed on a substrate of moist sugar cane sawdust or on sugar cane logs. From the first larval stage to adult emergence, the RPWs were reared individually at 27–29°C. For egg harvesting, the adults of both sexes were kept on sugarcane sawdust. Eggs were collected every 2 days.

Preparation of fungi The virulence of seven *M. anisopliae* and three *B. bassiana* isolates to *R. ferrugineus* larvae was tested in the first screening. The fungal isolates used in this study (listed in Table 1) were obtained from private collections or were isolated from soil or from various insects in countries with hot climates. The isolates found most virulent to larvae were used for egg and adult bioassays. Fungi were grown for 2 weeks at 25°C on Sabouraud dextrose agar (Difco). Spores were harvested by washing the dishes with an aqueous solution of 0.01% Triton X-100; subsequently the spore suspension was filtered through several layers of cheesecloth to remove mycelium. Spore concentration was determined with a hemacytometer and adjusted to 5×10^7 spores ml^{-1} for egg bioassay, to 2×10^7 spores ml^{-1} for larval bioassay, and to 10^8 spores ml^{-1} for adult bioassay. In order to obtain a dry spore formulation, *M. anisopliae* Ru isolate was grown on a solid rice-based medium for 3 weeks and dried at 32^{o}C. The procedure was as follows: Rice washed in cold running water was mixed with water and sunflower oil (500 ml of water and 20 ml of oil per kg of rice). Before sterilization in an autoclave, rice was heated until water had been absorbed. The autoclaved rice was inoculated with ∼25 ml spore suspension (10^6 spores ml^{-1}). Inoculated rice was incubated at 28°C for 2–3 weeks until sporulation. Subsequently, bags were opened and rice with spores was dried in a ventilated chamber at 30–32°C for 2–3 days.

BIOASSAY PROCEDURES

Selection of most pathogenic isolates Isolates were selected using larvae in plastic boxes (35 × 20 × 30 mm) containing 2.5 g of moist sugar cane sawdust. Groups of five or six larvae were located in 9-cm petri dishes lined with filter paper, sprayed with 2 ml of spore suspension containing 2×10^7 spores ml^{-1} and transferred to boxes (one larva per box) 5–10 min later. The control group was treated with an aqueous solution of 0.01% Triton X-100. The boxes were incubated at 27°C in darkness for 7–14 days. The larvae used for the bioassay weighed 50–190 mg. The bioassay was repeated twice and the results were averaged.

Eggs exposure The two most virulent isolates of *M. anisopliae,* Ru and MA, were evaluated in this bioassay. Eggs for bioassay (1–4 days old) were obtained from egg-laying cages. The eggs were placed singly in plastic boxes each containing 2.5 g sugar cane sawdust pretreated as follows: the sawdust was sprayed with fungal spore suspension (5×10^7 spores ml^{-1} in 0.01% Triton X-100) or an aqueous solution of 0.01% Triton X-100 as control (both at a rate of 0.2 ml per gram of sawdust) and well mixed. The boxes with eggs were incubated for 10 days at 27°C in darkness. Egg hatching and mortality of emerging larvae were monitored during this period. The weights of the larvae that survived the treatment with *M. anisopliae* and of those in the control groups were examined 10 days after inoculation. The egg bioassay was repeated three times with three different batches of eggs (10–24 eggs per treatment of each test group).

Adult treatment An initial experiment was designed to compare the effectiveness of two application methods: (*a*) spraying with spore suspension or (*b*) contact with dry spores obtained *in situ* on SDA medium. (*a*) Groups of eight adults were sprayed with 3 ml of spore suspension containing 1×10^8 spores ml^{-1} in petri dishes lined with filter paper and left there for 10 min. (*b*) Groups of 13–15 adults were put individually in petri dishes with 10-day-old fungal cultures for 1 min, in contact with spores. Control groups of eight or nine

adults were treated with an aqueous solution of 0.01% Triton X-100 in the spraying tests, or with talcum powder in the dry spores tests. Subsequently, each treated or control weevil was placed in an individual box with 50 g of moist sugar cane sawdust, and incubated at 28°C under a 12:12 L:D regime for 2–7 weeks. Fresh substrate was added to the boxes every week.

In order to evaluate the possibility of fungal transmission from surface-treated females to their progeny (eggs and larvae) during oviposition, 15 mated females were dusted individually with *M. anisopliae* spores in 5 g of a solid rice-based formulation. They were then transferred in groups of five to large plastic boxes (180×145×140 mm), provided with ten 3-cm-long logs of sugar cane as oviposition substrate, and incubated at 28°C under a 12:12 L:D regime until they died. The control group comprised 15 untreated females which were incubated under the same regime up to completion of oviposition. Twice a week the sugar cane logs were replaced with new ones, and the old ones with eggs were incubated at 27°C in darkness for 14 days. The logs were then cut up and the eggs or emerged larvae were counted. The latter were placed in individual boxes with moist sugar cane sawdust and incubated for an additional 2 weeks to compare their survival with that of the controls.

Analysis of results Results of the bioassay were recorded as mortality percentages in eggs, larvae and adults, as the hatching percentages of eggs, or as the weight of the surviving larvae. Student's t-test or one-way ANOVA was used to compare the effects of the experimental and control treatments on the eggs and larval mortality, and on larval weight. Prior to statistical analysis, data expressed as proportions were subjected to angular transformation. Alpha was 0.05. Data are presented as means ± SD. Statistical analyses were performed with the StatView for Power PC software, version 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA).

RESULTS

Selection of most virulent pathogenic isolates Seven isolates of *M. anosopliae* and three isolates of *B. bassiana* were subjected to an initial assay on larvae to select fungi with high pathogenicity against *R. ferrugineus*. The tested fungi differed in their pathogenicity to *R. ferrugineus* larvae: at 5 days post-inoculation (PI), mortality of treated larvae ranged from 0 to 20% for *B. bassiana* and from 40% to 100% for *M. anisopliae* (Table 1), and by 7 days PI the mortality of larvae treated with *M. anisopliae* isolates Ru, MA, M7 reached 100%. Mortality did not occur in the control group within any of the test durations. *M. anisopliae* strains were more virulent than those of *B. bassiana*, killing the treated larvae relatively quickly (LT_{50}: 3.5–5 days), in comparison with the *B. bassiana* strains that began to affect the larvae only after 5–6 days (LT_{50}: 5.8–6.5 days).

The first signs of penetration by both fungi were identical and appeared 2–3 days PI as large dark brown or black spots on the larval cuticle. One to 2 days later, the color of larvae killed by *B. bassiana* changed from sandy to dark pink. After development inside the larvae, both *M. anisopliae* and *B. bassiana* strains appeared to break through the cuticle and conidia emerged on the surfaces of the cadavers (Fig. 1a-c).

Pathogenicity of entomopathogenic fungi to *R. ferrugineus* eggs The pathogenicity of the two most virulent isolates of *M. anisopliae*, selected in the initial screening on larvae, was tested against *R. ferrugineus* eggs. Both isolates (Ru and MA) killed the eggs quickly, without preliminary colonization on the egg surface. The characteristic symptoms, *e.g.*

TABLE 1. Origin of fungal isolates and their pathogenicity to *Rhynchophorus ferrugineus* larvae

Species	Isolate	Original source	Country	% Mortality[z] on day 5
Beauveria bassiana	Nb	Soil	Israel	20
	Kl	Soil	Israel	0
	FF	Unidentified Coleoptera	Ethiopia	20
Metarhizium anisopliae	Ru	*Amblyomma variegatum*	Kenya	80
	MA	Soil	Ethiopia	100
	M7	Soil	Israel	80
	I30	Unknown	Kenya	80
	29	*Pachnoda interrupta*	Ethiopia	40
	EE	Unknown	Ethiopia	40
	108	Unidentified Scarabaeidae	Germany	80

[z]Bioassay was repeated two times (5–6 larvae per isolate) and results were calculated as average of two replicates.

TABLE 2. Pathogenicity of *Metarhizium anisopliae* toward eggs and larvae emerged from eggs developing in treated substrate

Test[z] no.	Isolate	Number of exposed eggs	Egg mortality (%)	Egg hatch (%)	Mortality of emerged larvae[y]	Survival of eggs and larvae (%)
I	Ru	15	80	20	1/3	13.3
	MA	15	80	20	2/3	6.6
	Control	15	40	60	1/9	53.3
II	Ru	23	43.5	56.5	5/13	34.8
	MA	23	47.8	52.2	4/12	34.8
	Control	24	16.7	83.3	1/20	79.2
III	Ru	10	80	20	1/2	10
	MA	10	70	30	1/3	20
	Control	10	30	70	0	70

[z]A different batch of eggs was used for each test.
[y]Number of dead larvae out of total larvae emerged.

loss of turgor, dullness and darkening of the eggs, appeared 2–3 days after treatment; subsequently the eggs were destroyed and disappeared in the substrate. The mortality of eggs located in sugar cane sawdust containing fungal spores was 43.5–80%, compared with only 16.7–30% in control eggs of the same age group (Table 2). Some of the emerged larvae were infected with the strain to which they were treated as eggs, and died in a few days. In total, the averaged mortalities of treated eggs and hatched larvae, calculated from three tests, were 81.6% $\pm$13.5% for *M. anisopliae* Ru, 79.8% $\pm$ 13.7% for *M. anisopliae* MA, and 33.7% $\pm$ 13.2% for control. This treatment effect was significant (ANOVA, F=10.15, P=0.012). Moreover, the weights of the larvae that survived the treatment with *M. anisopliae* were significantly lower than those of the controls (22 $\pm$ 10 and 33 $\pm$ 12 mg for Ru and MA isolates, respectively, and 61 $\pm$ 8 mg for the control (ANOVA, F=32.8, $P<0.0001$).

Pathogenicity of entomopathogenic fungi to *R. ferrugineus* adults The bioassays on adults were conducted with the *M. anisopliae* isolate Ru, which proved to be one of the most virulent to the larvae. Results of the first experiment indicated that the mortality of *R. ferrugineus* adults differed according to the fungus application method. The mortality of adult weevils was recorded 1 week after contact with dry spores from grown culture and

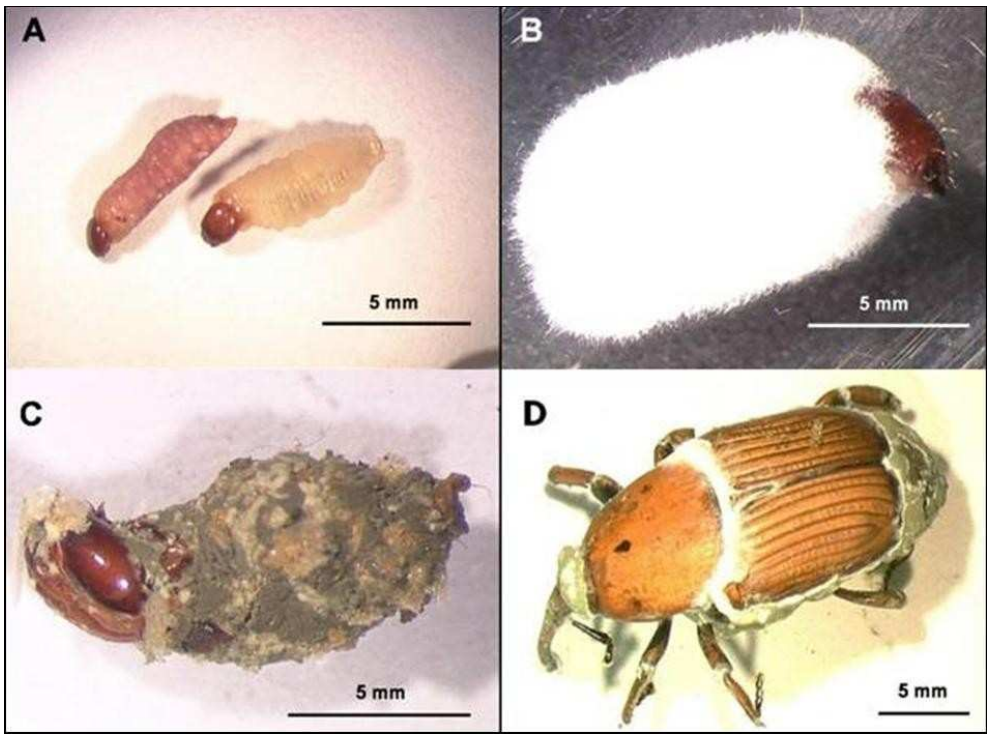

Fig. 1. Larvae and adults of *Rhynchophorus ferrugineus* infected with *Beauveria bassiana* and *Metarhizium anisopliae.* (A) Larvae killed by *B. bassiana* change color from pale-yellow to pink (here appear darker than control). Sporulation of *B. bassiana* (B) and *M. anisopliae* (C) on dead larvae. Sporulation of *M. anisopliae* on *R. ferrugineus* adult (D).

only 4 weeks after spraying with spore suspension. The maximum mortality of weevils reached 84.6% by 2 weeks after contact with dry spores, and 100% by 5 weeks after spraying. Mortality in control groups (aqueous Triton spray and talc dusting) was 12.5% and 22.2%, respectively.

Adults killed by the fungus did not change color, whereas dead adults in the control treatment darkened. After incubation of cadavers under moist conditions, fungi emerged on the dorsal and ventral surfaces of the weevil and formed conidiophores with conidia (Fig. 1d).

In order to evaluate the possibility of fungal infection transmission from the infected females to eggs and larvae within the oviposition tunnels, the females that had been treated with dry formulation of spores were allowed to oviposit in logs of sugar cane. Mortality of infected females reached approx. 60% by day 11 PI and 100% after 2–2.5 weeks PI. However, the oviposition was completed earlier, in 7 to 11 days (Fig. 2). For females in the control group, intensive egg laying continued for more than a month (Fig. 2). Mortality of most control females occurred after 1.5–2 months. The average reproductive period of infected females was 8.7 days, compared with more than 30 days for control females, and the total number of eggs laid by all infected females was 37 as compared with 115 in the control treatment. However, the average numbers of eggs laid by each female per day did

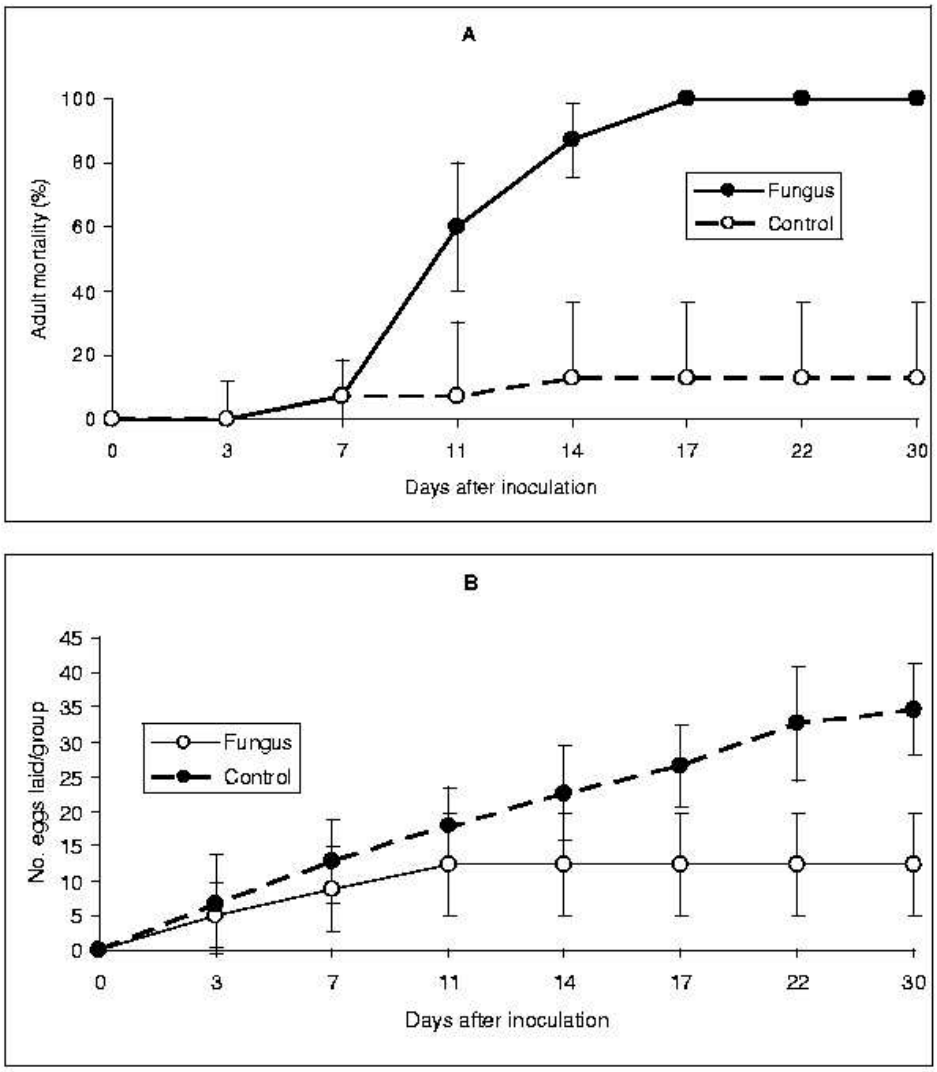

Fig. 2. The effect of *Metarhizium anisopliae* infection (strain Ru**,** rice-based formulation) on adult mortality (A) and oviposition (B). Bars indicate standard deviation of the mean.

not differ significantly: 0.7 ± 0.44 per treated and 1 ± 0.40 per control female, respectively. The weight of 3-week-old larvae from treated females was highly variable, ranging from 1.2 to 10.7 mg, and not significantly different from the control, which ranged from 1.2 to 35.0 mg.

DISCUSSION

The screening of fungi known to be pathogenic to RPW was aimed at evaluating the pathogenicity of *M. anisopliae* and *B. bassiana* to various development stages of RPW, and to develop a method for fungus implementation. The present investigation showed that the tested *M. anisopliae* and *B. bassiana* isolates infect the larvae and fully completed their life cycles by forming conidiophores with conidia on RPW cadavers. The red pigmentation of larvae killed by *B. bassiana* indicates the presence of a red pigment, oosporein, produced by *Beauveria* spp. (10,27).

All of the screened *M. anisopliae* strains exhibited pathogenicity to all development stages of RPW, causing up to 80–100% mortality of larvae and adult weevils under laboratory conditions. When eggs were exposed to sawdust previously sprayed with *M. anisopliae* spores, the total survival of both the eggs and the emerged larvae was reduced by a factor of approximately two to three, relative to control. Moreover, larvae developing in sprayed sawdust with fungal spores had a significantly reduced weight in comparison with larvae which emerged in the control treatment. The phenomenon could result from lower food intake by infected insects and/or from the energetic cost of confrontation with the infection. There are a number of examples of reduction in food consumption in insects infected by pathogenic fungi (4,25,26). On the other hand, fungus contamination of the food source could repel larvae and thus reduce their food consumption, as was shown for the grasshopper *Stiphra robusta* exposed to leaves sprayed with *M. anisopliae* var. *acridum* (Vicentini, Ph.D. thesis, 1999; see 15). In any case, we can expect that larvae of lower weight may have lower fitness and thus a shorter life span.

The present study showed that, in addition to the direct effect on adult mortality, fungal treatment reduced female fertility. Females contaminated with the dry spore formulation had a shorter oviposition time than the controls (7–11 days compared with 30–45 days) and, moreover, the total number of eggs laid by infected females was three times less than that by control females. However, the daily numbers of eggs laid by each female, and the survival and weight of emerged larvae, did not differ significantly between treated and control insects. This may suggest that females did not transmit fungal spores to the oviposition tunnels. Thus, the main contribution of the fungus to RPW control was found to be through the premature death of the infected females.

Despite the observed susceptibility of all the development stages of the RPW to the entomopathogenic fungi under laboratory conditions, the practicability of achieving efficient control of RPW in the field seems problematic. The field efficacy of entomopathogenic fungi toward various pests depends on many factors, often related to the behavior of the insect host in its natural habitat. The soil is the natural habitat of fungi and, since the RPW pupae occasionally inhabit the soil, it is theoretically possible to infect them with fungal spores by soil treatment. However, pupation occurs inside a cocoon and young adults remain in the cocoon for 8–14 days, which gives rise to doubt about the feasibility of implementing fungi in soil.

The spraying of palms and of large areas between them to ensure contact between free-living adult RPW and fungal spores also presents a difficulty, because of the large fungal inoculum needed. The best strategy would be to treat only selected areas that are especially likely to attract adults. Adult weevils are usually cryptic, taking refuge between petioles and offshoot bases. They are highly attracted to wounds in palm trees, *e.g.* that are inflicted

during vegetative production practices that include the removal of offshoots (8). These areas are often the most attractive sites to females for oviposition and, therefore, may be the best candidates for treatment with a dry fungal formulation. The possibility of infecting the *Rhynchophorus* adults by this method was discovered by chance, after application of a rice-based formulation of *M. anisopliae* against the Scarabaeid *Scapanes australis* on young palms in New Guinea (17). The treatment of frond axils with this formulation caused infection not only to the target pest but also inflicted some incidental infection on *R. bilineatus.* The high mortality of adults to dry spores of a selected isolate indicates that there was proper contact between fungi and ovipositing females. Hydrophobicity of fungal spores could play a significant role in the success of the dry rice formulation. The attachment of a fungal spore to the cuticle surface is the initial and thus a crucial event in the establishment of mycosis (3). The spores of *M. anisopliae* possess an outer layer composed of interwoven fascicles of hydrophobic rodlets, which provided the adhesion to the insect cuticle due to non-specific hydrophobic forces (3). Thus, similar to the natural infection process, the application of dry spores could provide better adhesion relative to application of aquatic spore suspension.

Ideally, we would like to use artificially inoculated females as vectors of infection to their progeny *via* egg contamination during oviposition. However, in the present study we were unable to prove that such infection transfer occurred. We believe that the lack of success was due mainly to insufficient inoculum transferred by females to eggs. It is possible that this problem is connected with spore hydrophobicity. This character may interfere with fungus transfer from female hydrophobic surface to the egg under humid conditions during oviposition into the plant tissue. The mechanical transfer of fungal spores between the oviposition sites and even further into the oviposition tunnels would be possible, provided that a suitable fungus formulation could be developed. In light of the high susceptibility of eggs and larvae to fungal infection, we assume that in such a case the contamination of eggs and of the larval habitat would add to the effectiveness of this pest control method.

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