Entomologia
Experimentalis et Applicata

DOI: 10.1111/eea.12171

Combination of the fungus *Beauveria bassiana* and pheromone in an attract-and-kill strategy against the banana weevil, *Cosmopolites sordidus*

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Accepted: 17 January 2014

Key words: microbial control, mycoinsecticides, entomopathogenic fungi, formulation, Cosmolure, delivery system, fat pellet, hydrogenated oil, shelf life, Coleoptera, Curculionidae

Abstract

An attract-and-kill approach based on pellets from soybean or palm stearin fats blended with the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. sensu lato and the aggregation pheromone sordidin (Cosmolure®) was tested against the banana weevil, *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae). The viability of *B. bassiana* conidia, blended with hydrogenated oil and exposed for up to 150 min to heating at 50 °C, was not affected and the aggregation pheromone did not undergo any decomposition. Conidial viability in pellets decreased by 50% after an average of 15.1 and 9.1 days at 25 and 40 °C, respectively, when packaged in polypropylene bags. Active packaging (hermetic bag + O_2/moisture-absorbing sachet) increased the shelf lives almost 10 and 6 times at 25 and 40 °C, respectively. In olfactometer bioassays, fat pellets amended with pheromone (sordidin, 1% wt/vol) were highly attractive to *C. sordidus* adults for up to 15 days, after which the pheromone release rate had decreased by about 90% and pellets were no longer attractive. Pellets with pheromone and conidia were as attractive to *C. sordidus* as banana rhizomes, and considerably more attractive than pieces of pseudostem. In no-choice experiments conducted in boxes, survival of insects exposed to fungus-impregnated pellets was affected by fat type (soybean fat vs. palm stearin) and bioassay temperature (25 vs. 30 °C), with results favoring soybean fat pellets at the higher temperature (96.9% of mortality after 18 days and ST_{50} of 7.7 days). However, mortality levels were low (21.7% for soybean fat pellets) or very low (1–5% for palm stearin pellets) in choice experiments carried out at 25 °C when fungus-impregnated pellets were applied before or after exposure of pseudostem residues to insects, respectively. The potential of this delivery system to manage *C. sordidus* populations and other insect pests (including those with cryptic habits) is discussed.

Introduction

Several species of entomopathogenic fungi (EF) have been studied as microbial control agents of invertebrates, and some of these species developed as commercial mycoinsecticides are already available worldwide (Li et al., 2010). Inundative applications of mycoinsecticides have targeted insects distributed in over 40 taxonomic families, many of them aerial pests such as whiteflies, aphids, and thrips (Faria & Wraight, 2007). According to these authors, 11 technical-grade active ingredients or formulation types have been identified, the majority being sprayable preparations. As fungal infection occurs by contact between the infective propagules and the insect cuticle, susceptible species that reside in difficult-to-reach sites, hidden in plant tissues or underground, are not usually exposed to conventional spraying.

Field performance of mycoinsecticides can be considerably improved with appropriate formulations, leading to enhanced persistence and insecticidal activity (Burges, 1998; Lacey et al., 2001; Jackson et al., 2010). In addition,

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the development of mechanisms that lead to greater exposure of insects to EF is critical for the successful control strategies of cryptic species. Feeding attractants have been tested in association with EF to control cryptic social insects, such as peanut oil-baited alginate pellets targeting the red imported fire ant, *Solenopsis invicta* (Bextine & Thorvilson, 2002) and cellulose baits for termite control (Wang & Powell, 2004). Another possibility would be the use of mass trapping and attract-and-kill devices, which rely on attraction of insects to a lure, in combination with a large-capacity trap or an insecticide-impregnated target, as recently reviewed by Witzgall et al. (2010). In this regard, semiochemicals have been explored under field conditions in association with EF, including pheromone-baited pseudostem traps for the banana weevil, *Cosmopolites sordidus* (Tinzaara et al., 2004, 2005), and devices baited with pheromone and co-attractants for attraction and contamination of sap beetles with fungal propagules (Dowd & Vega, 2003). In general, the use of mass trapping or attract-and-kill devices is expensive or labor intensive, and adoption in larger agricultural fields is not always economically feasible or practical (Vega et al., 1995; Tinzaara et al., 2004). Hidalgo et al. (1998) proposed the control of a grain storage pest based on solid fat pellets made of hydrogenated rapeseed oil amended with EF conidia. Subsequently, the same principle was tested to control another stored product insect, this time with the incorporation of an aggregation pheromone (Smith et al., 1999). Despite the simplicity of this attract-and-kill system, mortality of *Prostephanus truncatus* (Horn) adults in laboratory trials exposed to pellets for 24 h was 96–100% within 6 days.

Fat pellets are a simple and relatively inexpensive formulation and delivery system that, if successful, could be adopted even in low-value crops to control pests, including those with cryptic lifestyles. In the present study, we tested the attractiveness and efficiency of the fat pellet approach to control agricultural pests using adult banana weevil and a virulent isolate of *Beauveria bassiana* (Bals.) Vuill. sensu lato as our model. The aggregation pheromone sordidin, identified and synthesized in the 1990s (Beauhaire et al., 1995), was used in the formulation to attract the insects.

Materials and methods

Capture and laboratory maintenance of *Cosmopolites sordidus* adults

Pseudostem traps were installed in 2010 in a commercial banana plantation in Quixeré (05°09′S, 38°00′W), Ceará State, Northeastern Brazil. Traps were inspected every 10 days and groups of 200–250 captured adults were kept for a 40-day period in 1-l plastic containers, half-filled with sterilized vermiculite and pseudostem pieces (replaced weekly) and capped with a screened lid. Dead insects found in the containers were incubated in wet chamber (Petri dishes lined at the bottom with moistened filter paper) for disease confirmation, to determine the natural background infection by *B. bassiana* in the field population. The healthy insects submitted to quarantine were used in laboratory bioassays.

Origin and culturing of the fungal strain

The strain CG1013 of *B. bassiana*, known to be virulent to *C. sordidus* (Lopes et al., 2011), was originally isolated from an infected *C. sordidus* adult collected from a banana plantation (15°43′S, 47°54′W) in Brasilia-DF (Brazil) and is deposited at the Invertebrate Fungal Collection at Embrapa Genetic Resources and Biotechnology. Conidia were inoculated on Potato Dextrose Agar medium (PDA; Difco Laboratories, Detroit, MI, USA) and incubated for 10–12 days at 25 ± 0.5 °C and L12: D12 photophase. Wet conidia were scraped with a spatula and immediately used on pellet preparation or dried over silica gel for 4 days at 25 ± 2 °C before pellet preparation. Previous studies have shown that moisture content of samples kept over silica gel is ca. 3.8% (Xavier-Santos et al., 2011), whereas those of wet conidia scraped from Petri dishes is usually >98%. Conidial viability was above 94% for both dry and wet conidia by the time they were used.

Pellet preparation

We started the study with four different hydrogenated oils, referred to as fats in this study: organic refined palm, palm kernel, and palm stearin fats (Grupo Agropalma, Belém, Brazil), and soybean fat (Bunge Alimentos, Gaspar, Brazil). Pellets were prepared by placing the fat in a water bath at 50 ± 1 °C until completely melted. Seven hundred microliters of any one fat were pipetted into each transparent gelatin capsule 20 × 8 mm (size 00; Catalent Pharma Solutions, Sorocaba, Brazil) and cooled at room temperature (10–15 min at 18 ± 2 °C). Pellets were stored at −20 °C and, at the time of use, were released from gelatin capsules by dipping them into ice water for 15 min and then keeping them at room temperature. The solid fat pellets were obtained without deformation or mass loss. In order to select fat pellets that would not lose excessive mass at higher temperatures, pre-weighed pellet types (779.8 ± 4.02 mg) were placed on a 3-cm diameter filter paper disk (198.4 ± 0.70 mg) and this was set on an absorbent paper in a Petri dish. Petri dishes were held at 28, 33, or 38 ± 0.5 °C for 24 h. The pellet-filter paper disk set was weighed at the end of this period to determine the mass loss of each pellet under the selected temperatures. Four replicates were prepared for each treatment combination (fat material × temperature).

Conidial viability and aggregation pheromone formulation

Palm stearin (PF) and soybean fat (SF) were amended with the commercial pheromone Cosmolure® (ChemTica International, Costa Rica) at 1% (wt/vol) and/or *B. bassiana* conidia while still liquid. Cosmolure is a male-produced aggregation pheromone composed of four diastereoisomers of sordidin (Jayaraman et al., 1997) comprising 0.226% (wt/wt) of the commercial product. Around 20 mg of wet or dry conidia was mixed with 6 g of the two different fats in a water bath at 50 ± 1 °C. The mixture was stirred using a stainless steel spatula for 60 s and fats were transferred into gelatin capsules after 15, 60, 90, 120, and 150 min of water bath exposure, as previously described. After solidification, each pellet was removed from its capsule and placed into a glass tube with 5 ml of deodorized kerosene (Sigma-Aldrich, St. Louis, MO, USA), and then stirred in a vortex until completely dissolved. A five-fold dilution with kerosene was performed before viability assessments. Conidial suspensions were finally homogenized for 30 s in a vortex and 10 µl was poured into Petri dishes containing PDA medium. Inoculated Petri dishes were sealed with parafilm after the kerosene had been spread over the agar, and then incubated at 25 ± 0.5 °C in darkness for 22 h. Each treatment (fat type vs. conidia moisture content vs. time of exposure) was replicated three times, and a total of 300 conidia in different microscopic fields at 400× magnification were scored in each replicate. Conidia were considered germinated when germ tube length was at least as long as the diameter of the ungerminated conidia. This experiment was performed twice.

Conidial viability in pellets while in storage

Cosmulure (1% wt/vol) was added to the hot melted PF and SF with dry conidia, prior to preparing the pellets, and pellets without the pheromone were also prepared as a control treatment to detect any harmful effect of Cosmolure against conidia. Pellets of both fats prepared with conidia, either with or without Cosmolure, were placed in gelatin capsules, stored either in bags (8–9.5 × 12–13 cm), either 100% polypropylene (code P200-2.1.8; Brassaco Embalagens, Brasilia, Brazil) or aluminized material in order to avoid gas and moisture exchange (High Gas Barrier for RP System; Mitsubishi Gas Chemical, Tokyo, Japan). One O_2-absorbing sachet (SoftPost 300 cc; SoftPost do Brasil, São Paulo, Brazil) and one H_2O-absorbing sachet (CaçaUmidade 50 g; SoftPost do Brasil) were enclosed in each hermetic bag before heat sealing, whereas polypropylene bags were heat sealed without the addition of sachets. Packages remained at 25 ± 0.5 °C for 1 week (equilibrium period) and were then stored at 25 or 40 ± 0.5 °C for up to 160 days. Each bag with just one pellet represented a replicate and three bags were prepared for each treatment (fat type vs. package type vs. temperature) and for each assessment date, totaling 18 bags per treatment. Conidial viability was determined at six different storage periods, varying according to tested temperature and packaging type, using the methodology already described. All experiments were performed twice with aluminized and polypropylene bags.

Attractiveness of fat pellets to banana weevil adults

A two-choice olfactometer was used to test the attractiveness or repellency of PF or SF pellets to *C. sordidus* adults. The olfactometer was constructed in an acrylic block with a central hole [Y shaped, 2.5 cm internal diameter (i.d.)]. The trunk of the apparatus measured 20 cm and each arm measured 18 cm and was separated by an angle of 80°. The acrylic block was covered (above and below) with glass. Charcoal-filtered and humidified air was passed through each arm at a constant flow of 800 ml/min. Fat pellets removed from gelatin capsules were introduced in glass chambers (25 ml) located close to the air entrance in one of the arms, and connected to the olfactometer by silicone tubes. The olfactometer was positioned horizontally in an environmentally controlled room (26.0 ± 1.0 °C) and illuminated from above by red incandescent lamps (40 W) as the only source of light. For each bioassay, a single unsexed adult was introduced at the base of the Y-tube and its behavior was monitored for 10 min. The initial choice (the arm of the olfactometer into which the insect entered first for at least 5 cm and remained for at least 20 s) and residence time in each area (percentage of entire observation time) were scored. To avoid any bias in the insect responses, the position of the treatments was inverted every 2–3 bioassays, at which time the entire apparatus was cleaned with fragrance-free liquid soap, rinsed thoroughly with water, and dried in convector ovens (160 °C for the glass material and 60 °C for the acrylic box). The effect of fat pellets (with and without pheromone/conidia) and plant parts (pseudostem or rhizome) was studied and, for each treatment, a total of 20–25 insects were tested. Each adult weevil was used only once and not re-used in subsequent olfactometer bioassays. All bioassays were performed between 10:00 and 16:00 hours.

Attractiveness of fat pellets over time

In another set of bioassays, formulation attractiveness to *C. sordidus* adults was tested over time. Fat pellets (PF and SF) with conidia and pheromone were placed in open glass plates (9 cm in diameter) and were held at 25 ± 0.5 °C for 1, 5, or 15 days. After each time interval, bioassays (n = 20–25 insects per formulation type) were performed

to compare the attractiveness of these pellets with a control treatment (pheromone-free pellets kept under the same conditions).

Release rate of sordidin from fat pellet formulations

To study the release rates of the sordidin incorporated into fat pellets (PF and SF), air entrainment was performed. Volatiles were collected by introducing each pellet (n = 5 for each formulation type) in a 10-ml glass syringe. An air flow of 1 l/min was drawn sequentially through a bed of 4–12 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), the glass container and through one trap (glass tube of 10 × 0.5 cm i.d.) containing Super Q (100 mg each; Alltech Associates, Deerfield, IL, USA) using a negative pressure air flow. Volatiles collection was performed at different time intervals (1, 4, 10, 15, and 19 days at 25 °C) for each formulation containing Cosmolure. At each time interval, the pellets were aerated for 24 h and the trapped volatiles were eluted with n-hexane (ca. 500 µl); the eluates were stored at −20 °C until needed for further use. For quantitative analyses, the volatile collections were concentrated under a gentle stream of nitrogen to a volume of 100 µl, and analyzed using a Shimadzu 17A gas chromatograph equipped with a DB–5 column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA) and a flame ionization detector. The oven temperature program was 50 °C/2 min, and then increased at the rate of 15 °C/min to 250 °C. Data were collected with Autosystem Software in ASCII format and processed using Excel (Microsoft, Redmond, WA, USA). Injections were made in splitless mode. For sordidin quantification, an internal standard of 10 µl of dodecanein hexane (0.1 mg ml^{-1}) was added to each sample, after which samples were concentrated under a gentle stream of nitrogen to a volume of 100 µl. The amount of each compound identified was estimated by comparing the area of the internal standard to the area for sordidin. The response factor for dodecane and sordidin was assumed to be 1.0.

Insecticidal activity of fat pellets against banana weevil adults

No-choice experiment. The efficacy of *B. bassiana*-formulated PF and SF pellets, with pheromone (1% wt/vol) and with or without dry conidia (0.5 g/10 g of fat, about 2×10^9 conidia g^{-1} of fat), was evaluated. The experiment was conducted in 250-ml plastic boxes (11 × 11 × 3.5 cm) with moistened filter papers on the bottom and 16 banana weevil adults per box, previously washed in distilled water to remove plant residues. Following removal from a gelatin capsule, one pellet was immediately placed in the center of a box. The box was sealed with a plastic lid and incubated at either 25 ± 0.5 or 30 ± 0.5 °C for 24 h. Following the exposure period, insects were transferred into 150-ml plastic containers with a screened lid and half-filled with sterilized vermiculite and pieces of pseudostem as a food source. The containers remained in a chamber at 25 ± 0.5 °C, 90 ± 5% r.h. and darkness throughout the experimental period. Insect mortality was assessed every 3 days until the 18th day and cadavers were transferred to a wet chamber for disease confirmation. Each treatment (fat type vs. temperature) was performed with four replicates (plastic boxes), totaling 64 insects per treatment. All experiments were performed three times on different dates.

Choice experiments. The experiment was carried out in plastic trays (50 × 30 × 15 cm) half-filled with moistened sterilized vermiculite (ca. 200 ml water per 3.2 l vermiculite) and pieces of pseudostem (ca. 50 g in each corner of the tray). A thin vaseline layer was applied on the upper border of the tray to prevent insect escape. Thirty banana weevil adults were released into each tray 24 h before one fat pellet was placed into the center of the tray on top of vermiculite. Trays were maintained in a dark room at 24 ± 0.5 °C and 86 ± 5% r.h. Six days after pellet application, insects were transferred into plastic containers and kept as previously described. Insect mortality was assessed at the time of insect transfer and every 3 days, for 24 days. Cadavers were transferred to a wet chamber for disease confirmation. Each treatment was performed with four replicates (trays), totaling 120 insects per treatment. The experiment was repeated twice; however, in the second experiment the food source (pseudostem pieces) was offered only 24 h after pellet application.

Statistical analyses

Most experiments (shelf life, boxes, and trays) were arranged in a completely randomized design. Percent germination data from fat pellet preparation experiments were normalized by arcsine transformation and analyzed by two- or three-way analysis of variance (ANOVA). Means were compared by Tukey–Kramer HSD ($\alpha = 0.05$). Survival analysis with the Weibull function was used to estimate the time to 50% insect mortality (ST_{50}) for each fat pellet in the plastic box experiment. The same analysis was performed to estimate the shelf life (time for initial viability to drop 50% at a specific temperature) of conidia-formulated pellets in the packaging experiment. Log-Rank testing with 5% probability was applied for comparisons among survival curves. Insect mortality data from tray experiments and pellet storage experiment were analyzed by a generalized linear model (GLM) of mixed effects fit by a restricted maximum likelihood procedure,

attributing to the variable response a binomial distribution (logit). A two-way ANOVA was performed for insect mortality data and means compared by contrasts ($\alpha = 0.05$). The time for conidial viability in pellets to reach 50% of the original percentage germination (shelf life) during storage at 25 or 40 °C was estimated for different package systems and fat types. In olfactometer bioassays, insects that had not made a choice after 5 min were considered as non-responders, and they were not included in the statistical analyses. The choices made by the banana weevil adults in the olfactometer assays were analyzed by logistic regression and estimation of the probability of choosing one of the signals tested. The fitted model contained a factor for the side (left or right) on which volatiles were presented to control this variable. The hypothesis of no-preference (50% first choice to each volatile signal) was tested by means of a χ^2 Wald test. The time spent in each odor field (residence time) was analyzed by Wilcoxon's matched-pairs test (W). The dynamics of pheromone release rate was analyzed by a non-linear (negative exponential) regression model using time as independent variable and quantity of volatiles released in 24 h as the dependent variable. These tests were performed in R (R Development Core Team, 2006). ANOVAs and survival analyses were performed using the JMP statistical software package (JMP Software, 2007).

Results

Conidial viability following pellet preparation

The organic refined palm and palm kernel fats were solid below 25 °C, but started to melt at 28 °C and lost about 10–20% of their weight after a 24-h period, whereas weight loss at 38 °C was 39.7 ± 0.4 and 79.0 ± 0.2%, respectively; in addition, both changed to a clear liquid at 40 °C. Soybean fat was solid below 28 °C (without pellet deformation), and started to melt around 30 °C; mass loss was less than 20% at 33 °C and 25.5 ± 0.5% at 38 °C, and it liquefied at 42 °C. Palm stearin was solid below 34 °C, no loss weight was observed below 38 °C, and it became liquid at 50 °C. As the first two fats started to melt below 30 °C, we selected palm stearin and soybean fats for further studies.

The germination methodology using kerosene was not harmful to *B. bassiana* conidia. However, as has been observed with other oils, there was a slight delay in germination compared with unformulated conidia (data not shown). Viability readings for dry or wet conidia in water suspension were performed at 18 h, when >95% of living conidia had germ tube lengths longer than the diameter of ungerminated conidia. When suspended in kerosene, similar levels of germination required at least 22 h. The three-way (fat type*exposure time*moisture content of conidia) and all two-way interactions were not significant, with all P-values ≥0.80. The main effect of fat type ($F_{1,100} = 0.016$, $P = 0.90$) and exposure time of conidia at high temperature in the water bath ($F_{4,100} = 0.531$, $P = 0.71$) were not significant, although there seems to be a trend of reduced germination with time (Table 1). Initial moisture content of conidia (wet vs. dry) had no significant impact on germination rates for either fat type ($F_{1,100} = 0.706$, $P = 0.40$) (Table 1).

Conidial viability in pellets while in storage

Viability of fat-formulated conidia decreased with storage period at both 25 and 40 °C, and active packaging clearly extended the shelf lives (Figure 1). As expected, the increase in storage temperature caused a faster decrease in conidial viability for both packaging systems (hermetic vs. polypropylene bags). In general, conidial shelf lives without active packaging (i.e., in polypropylene bags) averaged 15.1 days (14.2–15.7) at 25 °C and 9.1 days (8.6–9.5) at

Table 1 Mean (± SE) viability (%) of *Beauveria bassiana* conidia during fat pellet preparation at 50 ± 1 °C for various durations (15–150 min) in water bath (after 22 h of incubation on potato dextrose agar medium at 25 ± 0.5 °C and darkness)

Treatment[1]	n					
PF+Bb (dry)	6	86.8 ± 3.64Aa	84.1 ± 4.52Aa	85.0 ± 4.56Aa	83.3 ± 5.22Aa	84.2 ± 4.74Aa
PF+Bb (wet)	6	90.6 ± 3.34Aa	89.2 ± 4.03Aa	88.0 ± 3.72Aa	87.4 ± 4.72Aa	86.8 ± 4.53Aa
SF+Bb (dry)	6	89.1 ± 2.54Aa	84.7 ± 4.73Aa	83.0 ± 4.61Aa	83.1 ± 5.12Aa	81.6 ± 4.96Aa
SF+Bb (wet)	6	90.6 ± 3.34Aa	86.8 ± 4.97Aa	86.0 ± 5.81Aa	86.8 ± 4.88Aa	83.9 ± 5.26Aa

Conidia viability was not affected by fat pellet treatment or duration of exposure to 50 °C (ANOVA: P>0.05).

[1]Viability of dry and wet conidia before mixture in fats were 96.0 ± 0.9 and 97.5 ± 0.91%, respectively. PF, palm stearin fat; SF, soybean fat; Bb, *B. bassiana* strain CG1013.

Means followed by the same letter in a column (lowercase) or in a row (uppercase) did not differ significantly (Tukey–Kramer HSD: $P > 0.05$).

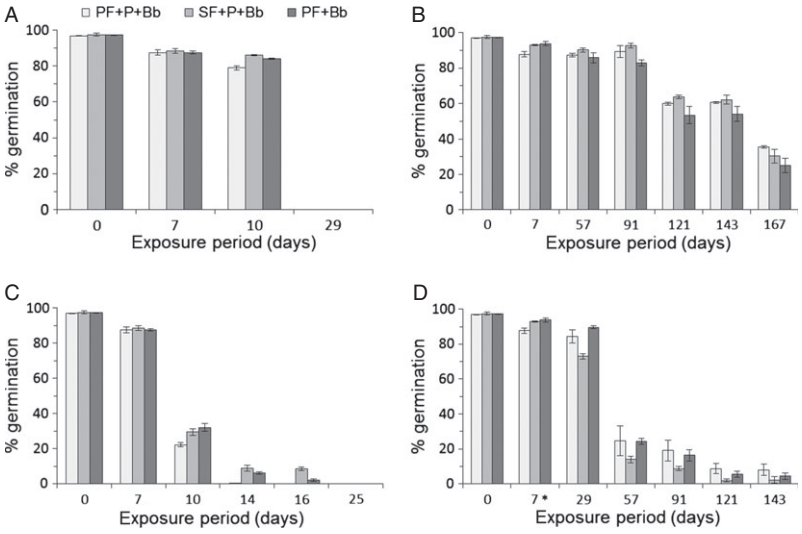

Figure 1 Mean (± SE) viability (%) of *Beauveria bassiana* conidia following fat pellet storage at (A, B) 25 ± 1 °C and (C, D) 40 ± 1 °C, in (A, C) polypropylene and (B, D) hermetic bags. *Bags were kept at 25 °C for 7 days ('Equilibrium period') before incubation at 40 ± 1 °C. PF, palm stearin fat; SF, soybean fat; P, aggregation pheromone sordidin; Bb, *B. bassiana* isolate CG1013.

40 °C. Active packaging (hermetic bag + sachet) increased the shelf lives of conidia almost 10 times at 25 °C and around six times at 40 °C, with averages of 146.3 (134.5–154.7) and 51.2 days (42.4–56.5), respectively. Conidial shelf lives for PF and SF pellets with pheromone and PF pellets without pheromone were similar at both tested temperatures, or had marginal differences under the same temperature and packaging regime.

Attractiveness of fat pellets to banana weevil adults

Cosmopolites sordidus males and females were equally responsive to pellets with pheromones and, therefore, unsexed adults were used for further studies. For PF+pheromone (PF+P), initial choice probability was 0.84 (95% IC = 0.95–0.59) for females and 0.94 (0.99–0.54) for males (females: $\chi^2 = 7.1$, d.f. = 1, P = 0.008; males: $\chi^2 = 7.2$, d.f. = 1, P = 0.007) and residence time was higher than in the PF (without pheromone) in both sexes (females: PF+P = 258.4 ± 38.65 s, PF = 70.1 ± 31.12 s; W = −128.0, P = 0.008; males: PF+P = 336.6 ± 42.8 s, PF = 29.4 ± 15.02 s; W = −143.0, P<0.001). Based on these results, all other bioassays with adults were performed regardless of gender.

PF and SF pellets without pheromone did not repel adults and no preference was observed between fat types (Figure 2). The addition of pheromone attracted significantly more adults when compared to fats without pheromone until the 15th day in PF pellets; on the other hand, SF pellets maintained attractiveness only in the first two periods (1 and 5 days) (Figure 3). Conidia formulated in fats with pheromone did not modify pellet attraction (Figure 4). SF pellets with pheromone were preferable to pseudostem pieces, however, rhizome pieces showed attractiveness similar to those of PF and SF pellets (Figure 4). In general, insects remained in the same olfactometer area after the initial choice. However, adult

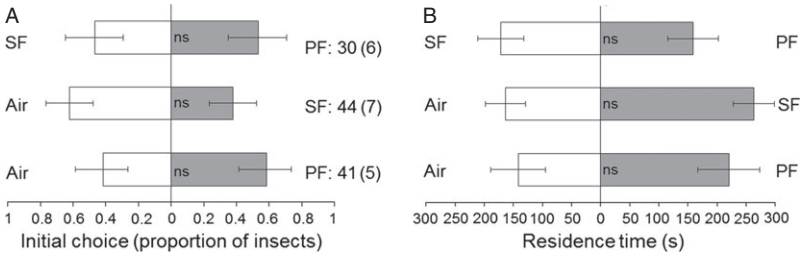

Figure 2 (A) Initial choice (mean ± 95% CI) and (B) residence time (mean ± SE) of *Cosmopolites sordidus* adults when stimulated with two fats or air (no pheromone and conidia used). SF, soybean fat; PF, palm stearin fat. Numbers to the right in (A) indicate the total repetitions used for statistical analyses of each combination and numbers in parentheses indicate the number of insects evaluated that did not respond to the treatments within 5 min. ns, non-significant differences.

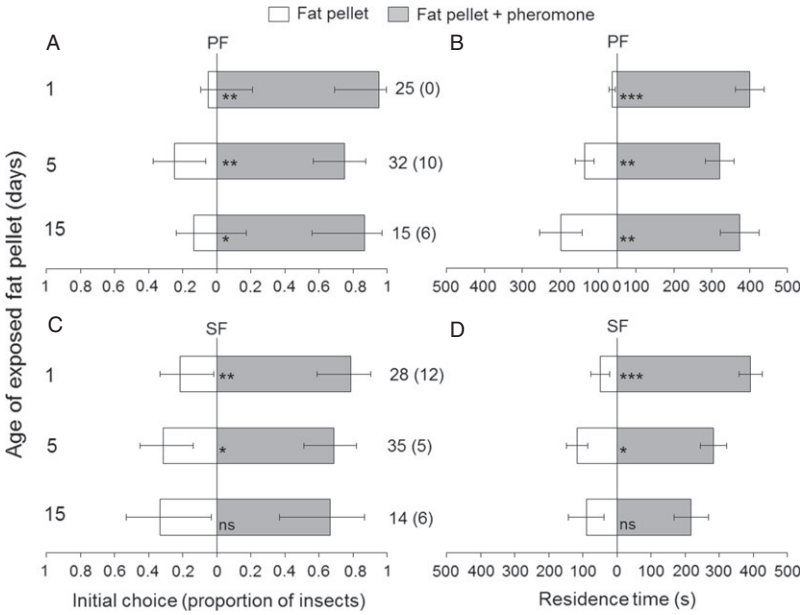

Figure 3 (A, C) Initial choice (mean ± 95% CI) and (B, D) residence time (mean ± SE) of *Cosmopolites sordidus* adults when stimulated with two fats with or without pheromone or the fats at different exposure times (1, 5, and 15 days) after the capsule removal. SF, soybean fat; PF, palm stearin fat. Numbers to the right in (A) and (C) indicate the total repetitions used for statistical analyses of each combination and numbers in parentheses indicate the number of insects evaluated that did not respond to the treatments (did not make choices after 5 min of experiment). *0.05>P>0.01, **0.01>P>0.001, and ***P<0.001; ns, non-significant differences.

residence time tended to reduce for older pellets (exposure time of 15 days) and also compared with rhizome, especially for SF pellets.

Pheromone release rate from fat pellets

Pheromone release rates after gelatin capsule removal were very similar for PF and SF pellets during a 20-day period at 25 ± 0.5 °C. A typical negative exponential curve was observed for both fats and no significant pellet deformation occurred (Figure 5).

Insecticidal activity of fat pellets against banana weevil adults—non-choice experiment

An indigenous *C. sordidus*-derived *B. bassiana* strain was isolated from a single adult during the quarantine period (mean level of natural infection of 0.03%), and insects that survived quarantine were used in bioassays. Significant differences in survival curves among distinct fat pellets in box bioassay were observed under 25 °C (χ^2 = 498.64, d.f. = 4, P<0.001) and 30 °C (χ^2 = 771.54, d.f. = 4, P<0.001). Control mortality was less than 3% and none of the dead control adults showed signs of the disease, confirming the almost absence of background fungal infection. Pellets without *B. bassiana* conidia caused less than 6% of adult mortality at 25 °C after an 18-day period. SF+P pellets without conidia caused 26% insect mortality after the same period at 30 °C and ST_{50} was not different from observed to PF+P pellets impregnated with the fungus at the same temperature (Table 2). However, 36% of the insects exposed to PF+P pellets with conidia succumbed to infection at 25 °C. SF pellets with pheromone and *B. bassiana* conidia (SF+P+Bb) were more effective against *C. sordidus* adults, causing mortalities of 71 and 96% at 25 and 30 °C, respectively.

Insecticidal activity of fat pellets against banana weevil adults—choice experiments

No differences in weevil mortality between fat types (PF+P and SF+P), with or without conidia, were observed in the choice bioassay in which food and shelter (pseudostem pieces) were offered before pellet application (Table 3, Exp. I). Mortality did not increase during the evaluation period for any treatments. There was also no interaction between pellet treatments and exposure time (χ^2 = 10.05, d.f. = 24, P = 0.99). Although there was no difference in mortality between treatments, all dead insects in SF+P pellets with conidia were successfully infected and showed signs of the disease. When pseudostem pieces were offered only 24 h after pellet application (second tray bioassay), insect contact with pellets and infection were higher (χ^2 = 288.53, d.f. = 4, P<0.001). In this case, differences between SF+PF with conidia and other treatments were observed from 9th day, reaching 21.7% of mortality at day 24 (Table 3, Exp. II). Mortality increased during the time for SF+P fat with conidia (χ^2 = 61.48, d.f. = 6, P<0.001); however, no interaction between pellet treatments and exposure time was observed (χ^2 = 11.62, d.f. = 24, P = 0.98).

Discussion

Solid fat impregnated with *B. bassiana* conidia and pheromone is a biodegradable, device-free formulation/delivery system with potential to control a range of mobile insect

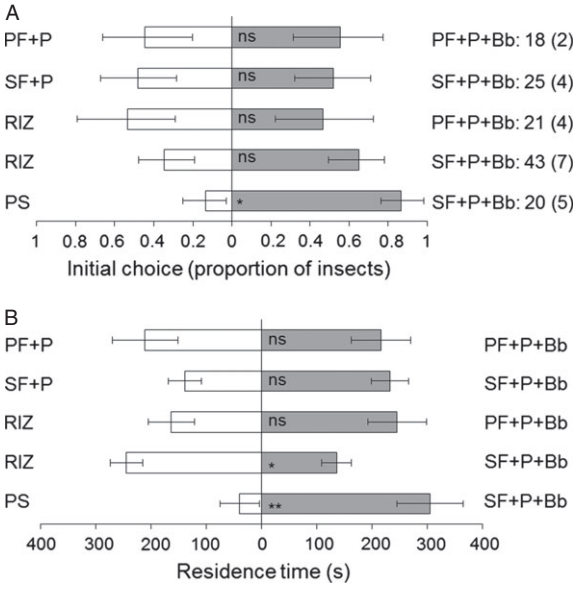

Figure 4 (A) Initial choice (mean ± CI 95%) and (B) residence time (mean ± SE) of *Cosmopolites sordidus* adults when stimulated with two fats with pheromone and *Beauveria bassiana* conidia or the fats with pheromone or banana rhizome. PF+P, palm stearin fat + pheromone; SF+P, soybean fat + pheromone; RIZ, banana rhizome; PS, banana pseudostem; PF+P+Bb, PF + pheromone + *B. bassiana* conidia; and SF+P+Bb, SF + pheromone + *B. bassiana* conidia. Numbers to the right in (A) indicate the total repetitions used for statistical analyses of each combination and numbers in parentheses indicate the number of insects evaluated that did not respond to the treatments (did not make choices after 5 min of experiment). *0.05>P>0.01, **0.01>P>0.001; ns, non-significant differences.

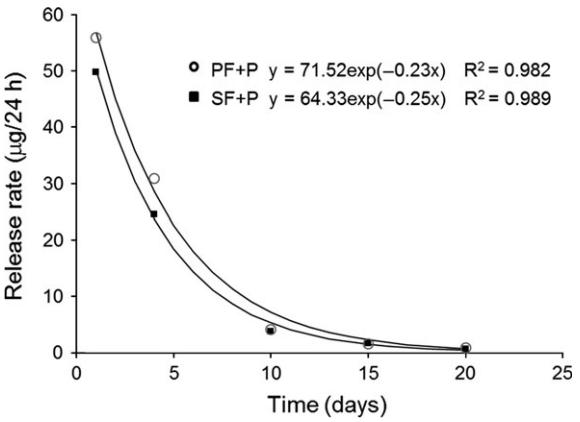

Figure 5 Pheromone release rate (µg/24 h) for fat pellets during a 20-day period at 25 ± 0.5 °C after gelatin capsule removal. PF+P, palm stearin fat + pheromone; SF+P, soybean fat + pheromone.

pests, including non-cryptic and cryptic species via an attract-and-kill strategy. Firstly, the lipophilic characteristic of conidia and pheromone enables homogeneous mixing with liquefied fats. Pellet preparation is quite simple and could be easily manufactured at large scale, and all materials are relatively inexpensive. This delivery system could be economically applied to the entire crop, establishing hundreds of infection sites. As pointed out by Dowd & Vega (2003), delivery strategies other than spraying avoid or minimize undesirable non-target effects of EF on beneficial arthropods and reduce the costs of application and equipment. Incorporation of the *C. sordidus* pheromone on fat pellets formulated with *B. bassiana* did not reduce pheromone attractiveness. In olfactometer studies, fat pellets impregnated with pheromone and *B. bassiana* were significantly attractive to *C. sordidus* adults and were at least as attractive as fresh parts of banana plants (rhizome and pseudostem). The manipulation of insect behavior using artificial devices to contaminate some individuals and promote auto-dissemination has been assessed for a number of insects and fungal species, such as the beetles *Carpophilus lugubris* Murray (Vega et al., 1995; Dowd & Vega, 2003), *Cylas formicarius* Fabricius (Yasuda, 1999), and *Ips typographus* (L.) (Kreutz et al., 2004). Traps combining pheromone and *B. bassiana* in order to promote subsequent fungal dissemination have been tested for the banana weevil under field conditions (Tinzaara et al., 2004, 2007). However, these elaborate traps have drawbacks such as the high cost for extensive agricultural areas and/or intensive labor related to trap set up in the field, limiting its adoption among farmers. Moreover, insects may be attracted to pheromone lures but can fail to enter the traps as previously suggested (Tinzaara et al., 2004).

The decline in pheromone release rate over time was similar for both types of fat pellets, yet olfactometer bioassays indicated that PF pellets but not SF pellets remained attractive to *C. sordidus* adults after 15 days exposure in the laboratory. This suggests that something in the soybean fat formulation may have interfered with attraction of *C. sordidus* to the pheromone. Potential hazardous effects of chemical compounds from semiochemical formulations on conidial activity, as suggested in other studies (Baverstock et al., 2010; Niassy et al., 2012), were not observed.

Shelf lives of conidia formulated in pellets were considerably shorter when packaged in polypropylene bags than when kept in hermetically sealed aluminized packages with O_2 and moisture-absorbing sachets. The advantages of this active packaging were recently shown (Faria et al., 2012) for pure *B. bassiana* conidia dried to very low water activity levels, with over 70% germination after 16 months at

Table 2 Mean (± SE) mortality (%) and survival time (ST_{50}) of *Cosmopolites sordidus* adults exposed to fat pellets impregnated with the pheromone Cosmolure (1%) with or without *Beauveria bassiana* conidia (2.5×10^9 conidia g^{-1}) in no-choice experiments

Temperature (°C)	Treatment[1]	n	% mortality[2]	ST_{50} (days)[3]	95% confidence interval
25	Control	192	1.6 ± 0.82e	>18.0	ND[4]
	PF+P	192	5.7 ± 2.10de	>18.0	ND
	PF+P+Bb	192	36.5 ± 7.33c	16.5	15.95–17.13
	SF+P	192	4.2 ± 1.77e	>18.0	ND
	SF+P+Bb	192	71.3 ± 8.23b	10.6	9.86–11.31
30	Control	192	2.6 ± 0.93e	>18.0	ND
	PF+P	192	5.2 ± 2.15e	>18.0	ND
	PF+P+Bb	192	26.1 ± 5.95cd	17.4	16.92–17.93
	SF+P	192	26.5 ± 5.59cd	17.6	17.10–18.18
	SF+P+Bb	192	96.9 ± 4.98a	7.7	7.28–8.20

[1]PF, palm stearin fat; SF, soybean fat; P, aggregation pheromone sordidin (Cosmolure®); Bb, *B. bassiana* strain CG1013.
[2]Means followed by the same letter are not significantly different (Tukey–Kramer HSD: P>0.05).
[3]Survival analysis with the Weibull function was used to estimate the time (days) to 50% mortality (ST_{50}) for each treatment.
[4]ND, not determined (calculation not possible because the horizontal line at 0.5 did not intersect a confidence interval).

40 °C and 63–65% germination after 3 months at 50 °C. In our treatment with active packaging, shelf lives of conidia formulated in pellets (with or without pheromone) were ca. 20 weeks at 25 °C and, therefore, similar to 16–22 weeks at 27 °C reported for fungus-impregnated pellets by Smith et al. (1999). Likewise, Smith and colleagues did not observe statistically significant differences between germination of conidia from pellets with or without pheromone.

There was a non-significant trend of declining viability with time of exposure to hot (50 °C) hydrogenated oil during pellet preparation, but viability was high after 15 min at 89.2% across all treatments, and even after 150 min was 84.1%. It is likely that a manufacturing process could reduce the time conidia were exposed to high temperatures. Although germination figures suggested little or no adverse effect on the conidia, they may have been weakened, with subsequent effects on shelf life, field persistence, and efficacy. Germination levels are an important indicator of conidial efficacy, but the robustness of germination is also important (Lopes et al., 2013). Dryer conidia are more resilient to the effects of heat (Faria et al., 2012), so further drying may also improve performance.

Conidia transferred from pellets to banana weevil adults produced some satisfactory levels of mortality at 25 °C (36.5% for palm stearin fat and 71.3% for soybean fat), and even higher at 30 °C in some cases (26.1% for palm stearin fat and 96.9% for soybean fat) and the fat probably assisted in sticking the conidia on the insect cuticle. In our experiments, mortality percentages for fungus-impregnated soybean pellets were better than for those based on palm stearin, likely because melting starts at 30 and 38 °C, respectively. As significant pellet deformation or melts did not occur for PF and SF pellets at tested temperatures, pheromone release was gradual and efficiently attracted the insect for a period of ca. 15 and 5 days, respectively. Therefore, temperature fluctuation after pellet application seems to be important with regard to the selection of fats to be used. High temperatures may cause complete pellet melt and a fast pheromone loss for fats with low degree of hydrogenation. On the other hand, low environmental temperatures would probably not allow a satisfactory conidia transfer to insect body for fats with high degree of hydrogenation. Therefore, the balance among fat type, hydrogenation degree, and exposure temperature could produce fat pellets with longer insecticidal activity and pheromone release. Other factors that may be of practical importance would be pellet size and insect targeted. Smaller pellets allow a greater surface area to volume ratio, requiring less product, but probably resulting in reduced persistency.

Horizontal transmission among *C. sordidus* adults infected with *B. bassiana* has been shown under laboratory conditions (Lopes et al., 2011). For social or gregarious insects, horizontal transmission of fungal diseases within the population could be greatly increased by contact with insects previously exposed to fat pellets impregnated with entomopathogenic conidia. This formulation/delivery strategy could also benefit from adoption of conservation biological control. For instance, although mulching is frequently reported as unfavorable to survival of hypocrealean entomopathogenic fungi (Meyling & Eilenberg, 2007), in cases in which it does not become a physical barrier to target pests, adoption of this farming practice is

Table 3 Mean (± SE) mortality (%) of *Cosmopolites sordidus* adults measured 6–24 days following 6 days of exposure to fat pellets impregnated with the pheromone Cosmolure (1%) with or without *Beauveria bassiana* conidia (2.5×10^9 conidia g^{-1}) in choice experiments at 25 ± 0.5 °C and 86.5 ± 0.5% r.h.

			% adult mortality after fat pellet exposure[1]						
Exp[2]	Treatment[3]	n	6 days	9 days	12 days	15 days	18 days	21 days	24 days
I	PF+P	120	0.8 ± 0.83 Aa	1.7 ± 0.96Aa	2.5 ± 0.83Aa	2.5 ± 0.83Aa	3.3 ± 1.36Aa	3.3 ± 1.36Aa	4.2 ± 0.83Aa
	PF+P+Bb	120	0.0 ± 0.0Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa
	SF+P	120	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	1.7 ± 0.96Aa	1.7 ± 0.96Aa	1.7 ± 0.96Aa
	SF+P+Bb	120	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.8 ± 0.83Aa	2.5 ± 1.60Aa	4.2 ± 1.60Aa	4.2 ± 1.60Aa	5.0 ± 1.67Aa
	Control	120	0.8 ± 0.83Aa	1.7 ± 1.67Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa
II	PF+P	120	0.0 ± 0.0Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa
	PF+P+Bb	120	0.0 ± 0.0Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa
	SF+P	120	0.0 ± 0.0Aa	0.0 ± 0.0Aa	0.0 ± 0.0Aa	1.7 ± 1.67Aa	1.7 ± 1.67Aa	1.7 ± 1.67Aa	1.7 ± 1.67Aa
	SF+P+Bb	120	0.0 ± 0.0Aa	10.0 ± 3.04Ab	16.7 ± 1.92ABb	18.3 ± 3.19ABb	21.7 ± 2.15Bb	21.7 ± 2.15Bb	21.7 ± 2.15Bb
	Control	120	0.8 ± 0.83Aa	0.8 ± 0.83Aa	0.8 ± 0.83Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa	3.3 ± 1.92Aa

[1]Means followed by the same letter in a column (lowercase) or in a row (uppercase) are not significantly different (Tukey–Kramer HSD: P>0.05).
[2]In experiment I, plant residues (pieces of pseudostem) were added to trays 24 h before pellet application, whereas in experiment II plant residues were added 24 h after pellet application.
[3]PF, palm stearin fat; SF, soybean fat; P, aggregation pheromone sordidin (Cosmolure®); Bb, *B. bassiana* strain CG1013; Control, no pellets.

likely to enhance persistence of conidia, and release of semiochemicals impregnated in fat pellets, due to protection against UV.

Despite the successful performance of SF pellets on banana weevil infection in no-choice experiments carried out in box at two temperatures (25 and 30 °C), mortality levels in choice bioassays were low or nil. Insects previously established on feeding sites (Exp. I) did not leave these sites to have contact with pellets. However, many insects seeking food or shelter were attracted by the pheromone and infected by the fungus when pseudostem residues were added 24 h after pellet application (Exp. II). This suggests that migrant *C. sordidus* adults looking for feeding or breeding sites would be more appropriate targets of the proposed attract-and-kill strategy, although confirmation under field conditions is required. Nevertheless, a wide range of insects could be suitable targets for this type of formulation/delivery system, including other weevil species and even social insects, which may pick up fat and conidia and return to their nests. So, the formulation shows some interesting potential, but requires targeting at particular pests.

Acknowledgments

We are thankful to A.L.M. Mesquita (EMBRAPA Tropical Agroindustry, Fortaleza, Brazil) for collecting adults of *C. sordidus* used in this study. We are also grateful to K.S. de Lima for help with the olfactometer bioassays, and M. Borges and M.C.B. Moraes (EMBRAPA Genetic Resources and Biotechnology) for reviewing the manuscript.

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