Acoustic Assessment of *Beauveria bassiana* (Hypocreales: Clavicipitaceae) Effects on *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae) Larval Activity and Mortality

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ABSTRACT *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae) is an economically important pest of palm trees in the subtropics. *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Clavicipitaceae), has been shown to be pathogenic against *R. ferrugineus* in laboratory and field studies. However, because they remain inside the trunks until adulthood, the slowing of feeding and increases in mortality of internally feeding *R. ferrugineus* larvae over time after *B. bassiana* treatment has not been established. To explore the potential of acoustic methods to assess treatment effects, sound impulses produced by untreated, 10^4-, and 10^6-conidia ml^{-1} *B. bassiana*-treated larvae in palms were recorded for 23 d, after which the palms were dissected and the larvae examined. Analyses were performed to identify trains of impulses with characteristic patterns (bursts) produced frequently by moving and feeding larvae but only rarely (3–8% of the larval rate) by interfering background noise or tree vibrations. The rates of bursts, the counts of larval impulses per burst, and the rates of impulses in bursts decreased significantly over time in both *B. bassiana* treatments but not in the control. This supports a hypothesis that larvae had briefer movement and feeding bouts as they became weaker after infection, which reduced the counts of larval impulses per burst, the rates of bursts, and the rates of impulses in bursts. There is considerable potential for use of acoustic methods as tools for nondestructive assessment of effects of biological control treatments against internally feeding insect pests.

KEY WORDS detection, entomopathogenic fungi, biological control

Rhynchophorus ferrugineus (Olivier) (Coleoptera: Dryophthoridae), the red palm weevil, causes significant damage to a wide range of palm species worldwide. In Spain, *R. ferrugineus* is an important pest of date [*Phoenix dactylifera* L. (Arecales: Arecaceae) and canary palms (*P. canariensis* Chabaud; Ferry et al. 2002, EPPO 2008). Adults can be monitored and trapped with pheromone–food attractant baits, but the larvae feed hidden inside the trunks, making it difficult to detect and control (Fiaboe et al. 2011).

In Spain and other Mediterranean countries where infestation is prevalent in urban areas, there is a strong emphasis on the development of integrated pest management strategies based on chemical treatments, pheromone traps (Faleiro and Chellapan 1999), and biological control such as entomopathogenic fungi (Shah and Pell 2003, Gindin et al. 2006, Dembilio et al. 2010, Güerri-Agulló et al. 2011). The most commonly used control treatments are insecticides such as Diazinon, Imidacloprid, and Phosmet (Abbas et al. 2010). However, heavy use of chemical treatments causes environmental damage and harms nontarget organisms, and also leads to the development of insecticide resistance. Pheromone traps are excellent monitoring devices but capture only adults, leaving the more harmful larvae to destroy the trunk and emerge later. Moreover, the potential for adults to be attracted to palm trees located near pheromone traps (Roda et al. 2011) and the capability of adults to escape from dry or nearly dry traps (Fiaboe et al. 2011) suggests that pheromone traps may be more useful monitoring or control tools in commercial palm areas than in zones with highly valuable historic palms like Elche-Alicante, Spain. Consequently, there remains interest in development of additional alternatives such as entomopathogenic fungi for *R. ferrugineus* management.

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) is an entomopathogenic fungus that has been shown to cause *R. ferrugineus* mortality

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in the laboratory (Gindin et al. 2006, Güerri-Agulló et al. 2010, Ricaño et al. 2013). A solid formulation of *B. bassiana* isolate Bb 203 dusted around the crowns, as well as the upper stems and petioles of palm trees, was demonstrated to reduce *R. ferrugineus* populations in southeastern Spain palm groves (Güerri-Agulló et al. 2011). Some of the field treatment effects could be assessed by observations of visible differences in damage in treated and untreated palms, but the effects on larvae hidden inside the palms could not be observed directly.

Exploring the potential of other methods to assess *B. bassiana* treatment effects in field environments, we hypothesized that comparisons of the temporal and spectral patterns of sounds produced by larvae inside trees might reveal significant differences between signals produced by fungus-treated and untreated larvae. Previous laboratory studies reported that fungus-treated larvae often produce weaker movements or eat for shorter intervals than untreated larvae (Ekesi 2001, Nussenbaum and Lecuona 2012). In addition, several previous studies, including Hussein et al. (2010), Gutiérrez et al. (2010), and Rach et al. (2013), have demonstrated that *R. ferrugineus* larvae can be detected acoustically. Temporal and spectral pattern analyses of sounds produced by insect larvae in trees have been applied to distinguish such sounds from background noise in laboratory and field environments (Mankin et al. 2008a,b, 2011). Large insect larvae moving and feeding in trees produce trains (groups) of brief, broadband sound impulses separated by intervals <25 ms that can be identified by specialized software. Trains with characteristic patterns that contain >6 and <200 impulses (designated as bursts) are known to be reliable indicators that insects are present within the sensor detection range (Mankin et al. 2008a). We hypothesized that *B. bassiana* treatment effects on larvae might be reflected in a slower rate of bursts or a smaller count of larval impulses per burst. In addition, treatment effects might be reflected in a smaller count of "burst impulses" per recording, where burst impulse refers to impulses occurring only within and not outside a burst. To consider whether sublethal and lethal effects might be identifiable, we conducted experiments with 30-d-old larvae at two different treatment levels, about two and four orders of magnitude below levels known to cause 100% mortality in previous studies (Dembilio et al. 2010, Ricaño et al. 2013).

Materials and Methods

Entomopathogenic Fungi. The *B. bassiana* strain used in the experiment, Bb 203, was isolated from naturally infected *R. ferrugineus* adults in southeast Spain (Daimés, Elche; CBS 121097; Güerri-Agulló et al. 2010) and is maintained in the fungal collection of the Glen Biotech and Department of Plant Pathology, University of Alicante. The fungus was kept in darkness at 4°C on corn meal agar (CMA; BBL Sparks, MD). A solid formulation of *B. bassiana* was prepared according to Güerri-Agulló et al. (2010). Conidial suspensions were obtained by shaking 2 g of 15-d-old solid formulation in 20 ml of 0.2% Tween 80. Conidial concentration was determined using a Neubauer hemocytometer and subsequently adjusted to 10^8 conidia ml^{-1}. A series dilution was made to 10^6 conidia ml^{-1} (10^6 treatment) and 10^4 conidia ml^{-1} (10^4 treatment). Sterile distilled water containing 0.2% Tween 80 was used as control.

Palms. Assays were performed on 5-yr-old potted *P. canariensis* obtained from an officially inspected nursery (Elche, Alicante, Spain) and certified free of *R. ferrugineus* infestation. They were maintained in a greenhouse on the University of Alicante campus. Palms were watered twice weekly and kept inside independent cylindrical, wire cloth cages to avoid accidental additional infestation. A 6 by 60-mm screw (PZ3, Standers, Leroy Merlin, Lezennes, France) was inserted near the base of the palm for use as a signal waveguide. The palm then was prepared for artificial infestation by drilling two 30-mm-diameter by 6-cm holes into the palm at opposite ends of a diameter. Three test palms used as no larva controls were prepared for artificial infestation but were not inoculated with larvae.

Insects. Adult *R. ferrugineus* were collected in Elche using bucket traps baited with 4-methy-5-nonanol and 4-methyl-5 nonanone (Kaakeh et al. 2001). Insects were maintained in the laboratory in an incubator at 25 ± 0.5°C in darkness. Plastic boxes (40 by 30 by 21 cm) were set with a folded piece of moistened filter paper containing thin green apple slices that were replaced three times per week. Adults from the stock colony were sexed by visual inspection of their snouts (Prabhu and Patil 2009). Adults were bred in pairs in individual 100-ml specimen bottles (Deltalab, Barcelona, Spain) using green apple as both food and oviposition substrate. To maintain the humidity of rearing containers, 20 by 5 cm filter paper (Refe 1510, Filtros Anoia S. A., Barcelona, Spain) was wet with distilled water and placed into the specimen bottle.

After 2 d, eggs were collected from both apple and paper. The eggs were placed in a sterile 9-cm-diameter plastic Petri dish with an artificial diet substrate (Alarcón et al. 2002). Egg hatching was recorded daily for up to 6 d. Emerged larvae were individually transferred to 20-ml coulter tubes containing 10 ml of artificial diet, and after 15 d, they were transferred into specimen bottles containing 50 ml of artificial diet, which was replaced every 15 d.

Larvae were inoculated using a dipping technique. With the larval head held by hand, the abdomen was dipped into the *B. bassiana* conidial suspension and then placed into a coulter tube and transferred into a hole previously drilled into a test palm. The hole was plugged with the coulter tube to prevent larval escape.

Larvae from the 30-d age group were randomly selected into groups of 16 for controls, which were placed in pairs into 8 palms. Twenty larvae exposed to the 10^4 treatment were placed in pairs into 10 palms, and 10 exposed to the 10^6 treatment were placed into 5 palms.

The palms were dissected 25 d after artificial infestation to check the status of larvae and palm damage.

Visual symptoms of infestation, insect status (alive, dead, or absent) and stage (larva, pupa, or adult), and damage (tunnel-distance and number of petioles invaded by larvae per palm) were recorded.

Acoustic Recordings. Beginning on the first day after infestation, 180-s recordings of acoustic signals from each test palm were made between 1000 and 1900 hours on 14 different days over a 23-d period. A sensor–preamplifier module (model SP-1 L, Acoustic Emission Consulting [AEC] Inc., Sacramento, CA) was connected by a magnetic attachment to the signal waveguide screw at the base of each palm. The signals were fed from the sensor module through an amplifier (AED-2010, AEC Inc. Sacramento, CA) to a digital audio recorder (model HD-P2, Tascam, Montebello, CA) at a 44.1-kHz digitization rate. The recording procedures were similar to those described by Dosunmu et al. (2014).

To avoid high levels of ventilation fan and other background noises present in the greenhouse, the palm in each recording was moved temporarily to a nearby storage cabin and then returned to the greenhouse. In addition, the signals were monitored with headphones as they were being collected to avoid faulty readings or periods of loud background noise. Nevertheless, because the cabin was not fully soundproofed, periods of vehicular noise (car, train, helicopter, etc.), wind, and bird calls were detected on different occasions, and signal processing was conducted to discriminate such background noises from larval sound impulses, as described in the next section. To assist in these analyses, background sounds were recorded from each test palm on the day its waveguide screw was inserted and before its petioles were drilled for larval placement.

Signal Processing. Insect movement and feeding sounds typically are produced as trains (groups) of 3–30-ms impulses with similar, distinctive spectral patterns (Mankin et al. 2011). Mean spectra (profiles) of such sounds can be used to help distinguish insect sounds from background noises. However, because the spectra of insect-produced sounds are modified as the signals travel through tree structures (Mankin et al. 2008a,b), the larval sound impulses in this study could not be distinguished from background noises reliably by their spectra alone without also considering the temporal pattern of the impulses. Therefore, we conducted a two-stage spectral and temporal pattern assessment process developed in Mankin et al. (2008b) to provide a more reliable indicator whether signals detected in the recordings were larval sounds or incidental background noise.

In the first stage of the assessment procedure, recordings were prescreened using the Raven 1.3 software sound analysis program (Charif et al. 2008) to survey the different types of signals that had been recorded and locate intervals that contained groups of sound impulses without confounding background noise (Mankin et al. 2011). Signals in each recording were examined whenever their amplitudes exceeded an amplitude threshold (Mankin et al. 2008b) set just above the background noise. Signals that were not composed of groups of brief impulses were discarded from further analysis.

Four types of sound impulses with distinctive spectral features were identified during the prescreening of multiple recordings collected over the duration of the experiment. Mean spectral averages (profiles) of the four frequently occurring types of larval sound impulses were constructed using the DAVIS insect signal analysis program (Mankin et al. 2008b), as described in the Results section. Next, the sound impulses in each recording were least-squares matched by DAVIS against each of the four profiles and were assigned to the profile type of best fit. Impulses that failed to match within a total least-squares difference of 40 dB between 1 and 15 kHz were discarded as a background noise (Mankin et al. 2008b).

In the final stage of analysis, DAVIS classified impulse trains containing >6 and <200 profile-matching impulses, as larval bursts in each recording, based on the high likelihood that they were produced by larvae and not by extraneous background sounds (Mankin et al. 2008a,b; Dosunmu et al. 2014; Herrick and Mankin 2012). The burst type was assigned as the type of which the plurality of impulses in the burst had been classified. The times and types of each burst and the count of impulses in each burst (burst impulses) were saved in a spreadsheet for statistical analyses. Multiple bursts of several different types were observed in recordings from most of the infested palms. An overall rate of bursts was calculated by summing the numbers of bursts of each type and dividing by the recording duration. Mean counts of larval impulses per burst were calculated by dividing the total counts of impulses in bursts by the number of bursts in the recording. Overall rates of burst impulses were calculated by summing the numbers of impulses of each type in the recording and dividing by the recording duration. The overall rates of bursts, the counts of larval impulses per burst, and the overall rates of larval impulses occurring in bursts were tested as potential indicators of larval activity in subsequent statistical analyses. Two-tailed, paired *t*-tests were conducted, palm by palm, to compare mean rates of bursts, mean number larval impulses per burst, and mean rates of burst impulses at the end of the study, 22–23 d after treatment, against the mean rates 15 d previously, when the rates were near overall mean values.

Results

Larval Sound Impulse Characteristics. The larvae in each palm produced sound impulses with a broad range of amplitudes, spectral features, and temporal patterns. An example showing a typical range of signals is a 2-s section of recording from a control palm with untreated larvae (Fig. 1). The example has two impulse trains, one with 35 impulses and the other with 10.

Four profiles that characterized the spectra of the most commonly observed larval impulses were

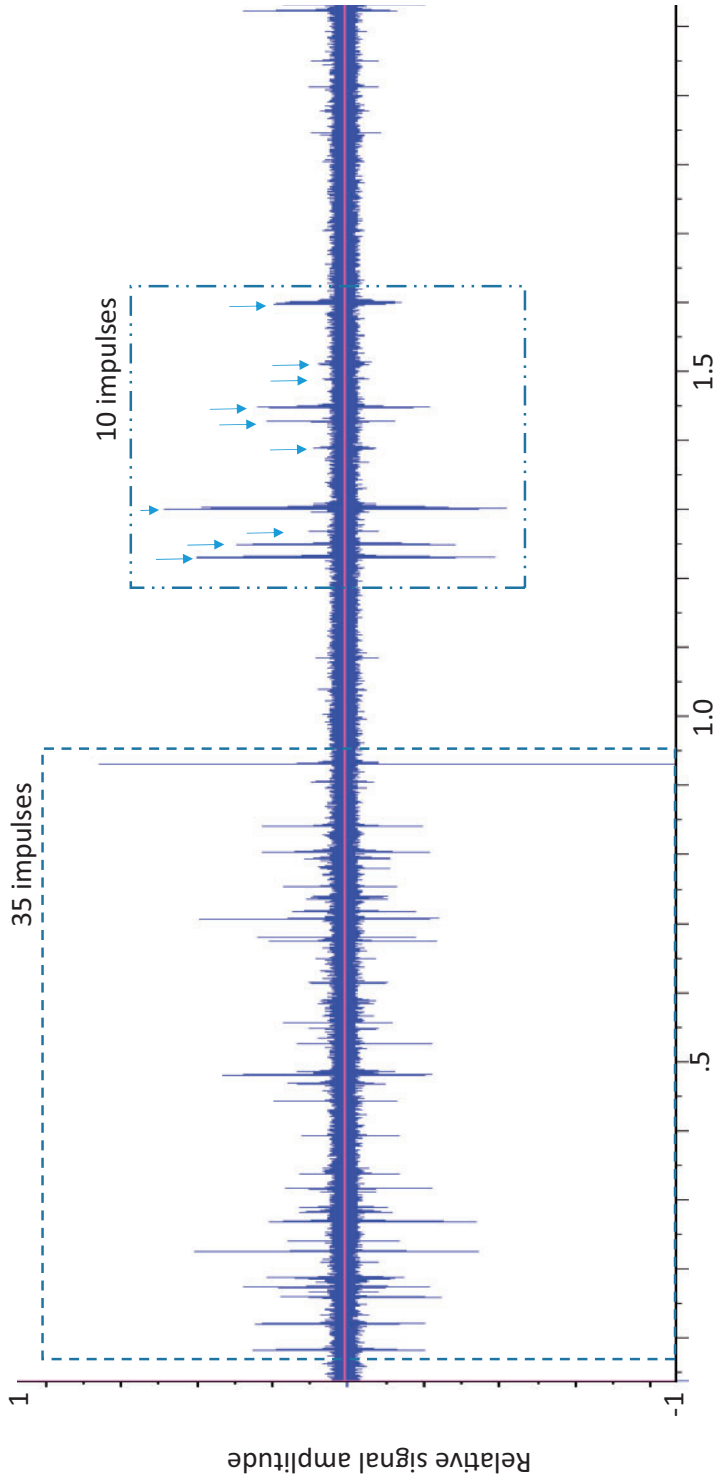

Fig. 1. Example of typical amplitudes and temporal patterns of impulses recorded from a control palm with untreated larvae. Two impulse trains separated by a quiet interval of 30 ms are indicated by boxes, one with 35 impulses (dashed line), and one with 10 impulses (dash-dot-dotted line). Impulses are marked with arrows in the second train (10 impulses).

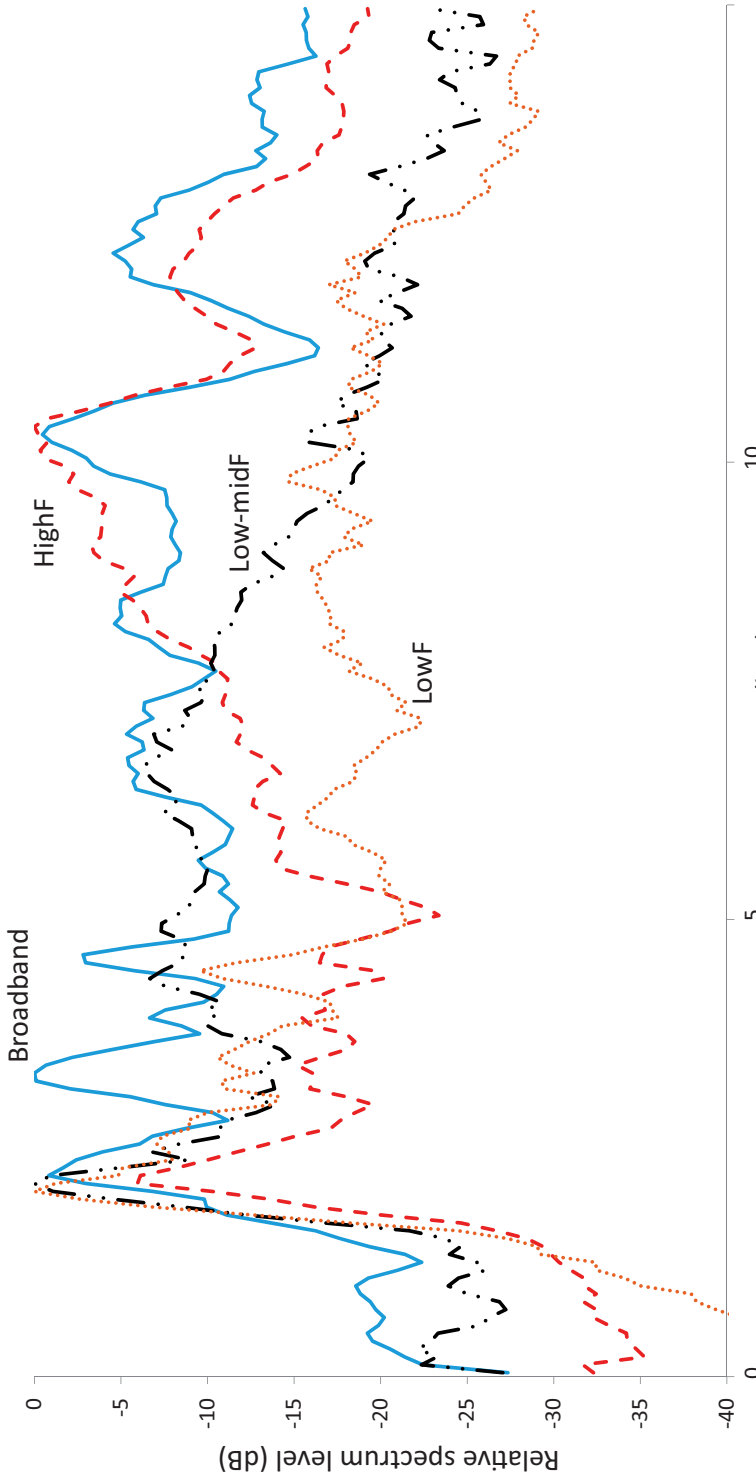

Fig. 2. Spectral profiles of four distinctive types of larval sound impulses detected in palms: Broadband (with energy over a broad range of frequencies), solid line; LowF (energy primarily near 1.9 kHz), dotted line; Low-midF (energy near 2.0 kHz and second broadband peak from 4.3–6.7 kHz), dash-dot-dotted line; and HighF (energy near 2.0 kHz and second broadband peak from 8–10.8 kHz), dashed line. Horizontal axis indicates frequency in kHz and vertical axis indicates relative spectrum amplitude in dB.

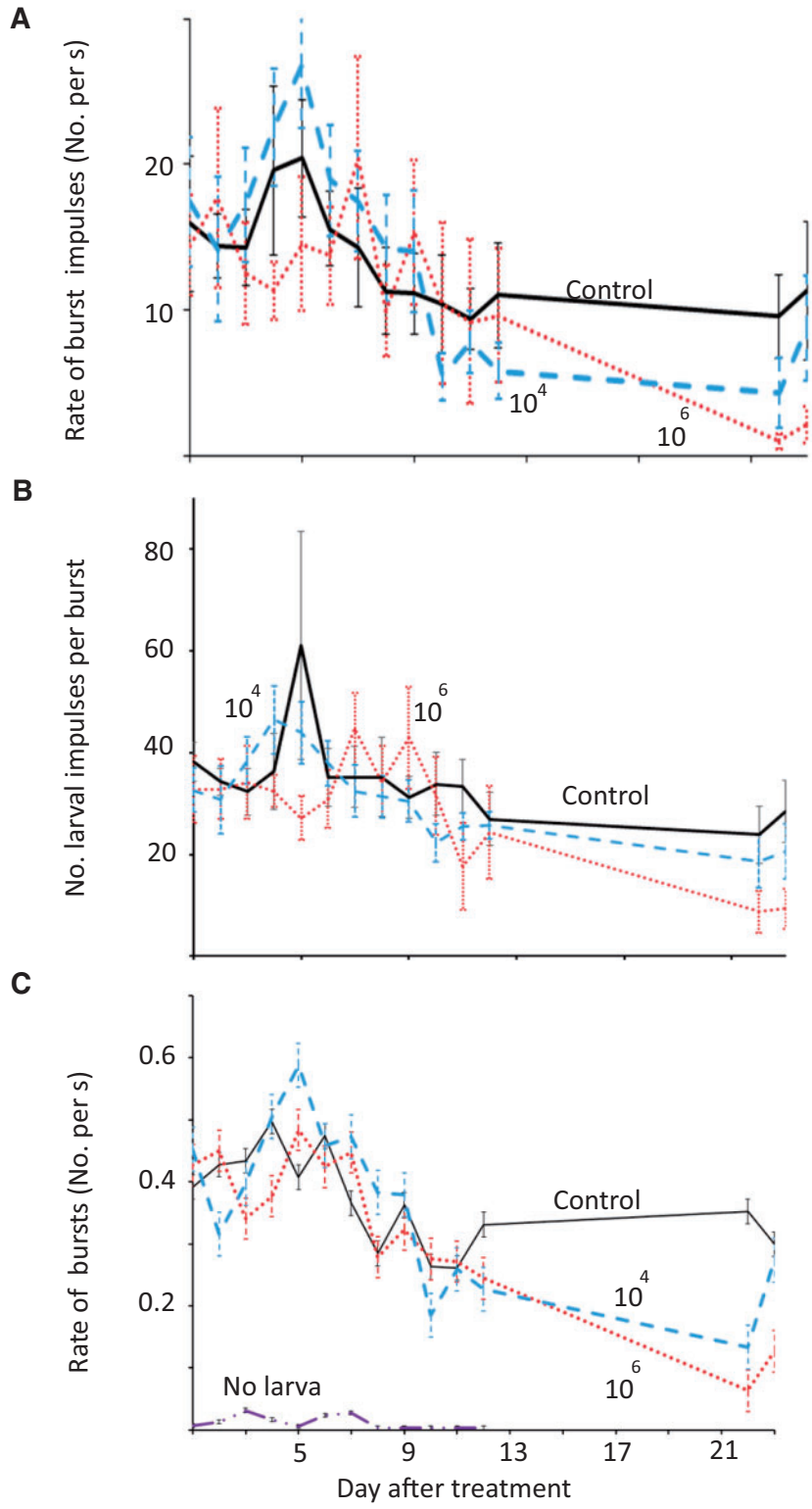

Fig. 3. Mean ± SEM of (A) rates of burst impulses, (B) number of larval impulses per burst, and (C) rates of bursts detected on different days after treatment with different doses of *B. bassiana*: control treatment, solid line; 10^4 treatment, dashed line; and 10^6 treatment, dotted line; no larva (background noise control), dash-dot-dotted line. SEs are shown by vertical bars, horizontal axis indicates day after treatment.

Table 1. Analysis of variance of effects of fungal treatment, palm, day, and the interaction between treatment and day on rates of bursts ($n = 359$ observations), number of impulses per burst ($n = 322$), and rates of burst impulses ($n = 338$)

Parameter	df	*F*	*P*
Bursts			
Treatment	3	34.12	<0.0001
Palm	22	2.48	0.0004
Day	13	5.16	0.0001
Treatment × Day	38	0.78	0.817
Impulses/burst:			
Treatment	2	9.66	<0.0001
Palm	20	5.09	<0.0001
Day	13	4.76	<0.0001
Treatment × Day	26	1.11	0.3364
Burst impulses:			
Treatment	3	10.67	<0.0001
Palm	22	3.39	<0.0001
Day	13	5.81	<0.0001
Treatment × Day	31	0.72	0.859

Table 2. Mean ± SEM change in rates of bursts 22 and 23 d after treatment compared with same palm 15 d previously

Treatment	Change in no. bursts s^{-1}	df	*t*	*P*
10^6	−0.268 ± 0.076	9	3.55	0.0062
10^4	−0.225 ± 0.060	19	3.75	0.0014
Control	0.0007 ± 0.053	15	−0.01	0.9898

constructed from mean spectra of groups of distinctive impulses collected on the first day of recording. A low frequency (LowF) profile contained a prominent low-frequency peak near 1.9 kHz (Fig. 2). The profile was constructed as a mean spectrum from 145 impulses collected in a palm infested with 10^4 treatment larvae. A low-midrange frequency profile contained a peak near 2.0 kHz and an additional peak at 6.7 kHz (Low-MidF in Fig. 2). The Low-Midf profile was constructed as a mean spectrum from 1,289 impulses collected in a palm containing untreated control larvae. A high-frequency profile contained a high-amplitude peak near 10.8 kHz (HighF in Fig. 2), and a broadband profile included numerous peaks from 2.0 to 12.5 kHz (Broadband in Fig. 2). The HighF profile was constructed as a mean spectrum from 66 impulses collected in a palm containing 10^4 treatment larvae. The Broadband profile was constructed as a mean spectrum of 994 impulses collected from a third palm with 10^4 treatment larvae.

The four profiles were applied by DAVIS matching analysis subroutines (Mankin et al. 2011) to classify each sound impulse in the experiment. Impulses that matched closely with a larval profile were classified as larval impulses, and trains containing >6 and <200 larval impulses were classified as bursts, used previously as indicators of the likelihood of insect presence (Mankin et al. 2008a,b).

Both trains marked in Figure 1 were classified as bursts according to the spectral and temporal pattern analyses of their impulses. Each train contained impulses matching more than one profile type. It should be noted that impulses occurring outside the bursts were discarded from analysis because the lack of a temporal pattern provided less certainty that they had been produced by larvae rather than background noise sources.

Table 3. Mean ± SEM change in nos. of larval impulses per burst 22 and 23 d after treatment compared with same palm 15 d previously

Treatment	Change in no. impulses or burst	df	*t*	*P*
10^6	−30.390 ± 4.982	9	6.10	0.0002
10^4	−12.212 ± 3.115	19	3.92	0.0009
Control	−9.031 ± 4.897	15	1.84	0.0850

Table 4. Mean ± SEM change in rates of burst impulses 22 and 23 d after treatment compared with same palm 15 d previously

Treatment	Change in no. impulses s^{-1}	df	*t*	*P*
10^6	−13.622 ± 4.156	9	3.28	0.0096
10^4	−9.331 ± 2.375	19	3.93	0.0009
Control	−2.364 ± 2.716	15	0.87	0.3977

Effects of *B. bassiana* Treatments on Larval Activity. The overall mean rates of bursts, mean counts of impulses per burst, and mean rates of burst impulses varied considerably among treatments over time after treatment (Fig. 3). The mean rate of bursts in the untreated control varied in a narrow range between 0.25 and 0.5 bursts s^{-1}, while the rates in both the 10^4 and 10^6 treatment decreased <0.3 bursts s^{-1} after the first week of testing (Fig. 3C). At least one burst was detected from each larva-infested palm on each day of testing, in conformance with previous findings that the occurrence of bursts in a recording is strongly correlated with the presence of active insects nearby the sensor.

Background Noise Trains. The no-larva treatment served as a control, and provided an estimate of the mean rate of occurrence of background noise trains misclassified as larval bursts by the signal analysis procedure. With uninoculated palms, bursts were detected at rates of 0.01667 ± 0.0064 bursts s^{-1} in recordings obtained over a 12-d period, slightly below the rate of 0.02 bursts s^{-1} suggested by Mankin et al. (2008a) as a threshold for low likelihood of infestation. As noted in Mankin et al. (2011), short broadband impulses can be produced by extraneous tapping or other percussive noises and by stress or mechanically induced acoustic emissions inside the tree. The 0.01667 s^{-1} mean rate of bursts in the no-larva control is ~3.3–6.7% of the 0.25 – 0.5 bursts s^{-1}mean rate of bursts in the untreated control, which can be used as an estimate of the fraction of background noise trains misclassified as larval bursts in this experiment.

An alternative estimate of the fraction of misclassified larval bursts can be obtained from the result that, in a random sample of five recordings, 49 of the 1,470 (3.3%) trains initially classified as bursts by temporal pattern analysis were discarded by the spectral pattern analysis because <50% of the impulses in those trains matched within a total least-squares difference of 40 dB of any larval profile. If the rate of noise impulses that matched one of the four profiles was the same order of magnitude as the rate of noise impulses that

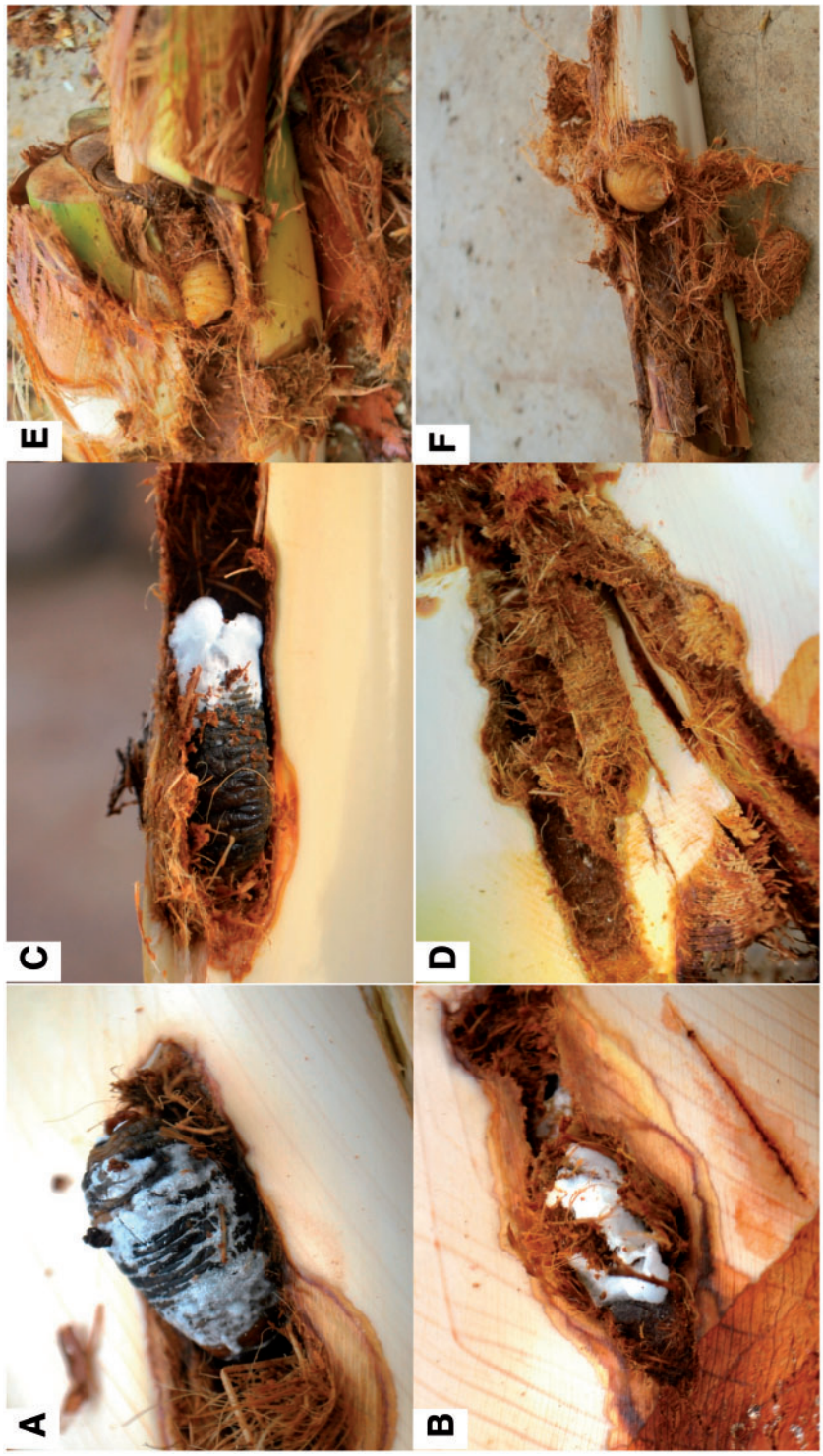

Fig. 4. Examples of mortality and hyphal growth observed for larvae exposed to 10^6 treatment (A–C), and tunneling damage observed with untreated control larvae (D–F).

failed to match a profile, ~3% the larval bursts plotted in Figure 3C potentially are misclassified background noise trains, and this estimate is the same order of magnitude as the fraction of misclassifications estimated from the no-larva control.

Analysis of Variance. Based on the trends observed in Figure 3 for the effects of different treatments over the duration of the experiment, analysis of variance was conducted on the effects of treatment, time after treatment (day), and palm on burst rate, count of larval impulses per burst, and rate of burst impulses, considering also the interaction between treatment and time after treatment. Significant differences were found for treatment, palm, and time after treatment, but not for the interaction between treatment and time after treatment (Table 1).

Inspection of Figure 3 revealed no clear trends in the mean rates of bursts, the count of larval impulses per burst, and the mean rates of burst impulses in the 10^4 and 10^6 treatments during the first week after treatment, but decreases in these values appeared by the last two days of testing (days 22 and 23). Paired *t*-tests were conducted (Tables 2–4) to test the hypothesis that the three parameters had decreased at the end of testing from their values 15 d previously (days 7–8). In all of these comparisons, significant decreases were found for parameter values in the 10^4 and 10^6 treatments, but not in the untreated controls.

Effects of *B. bassiana* Treatments on Larval Survival. When the palms were dissected 25 d after treatment, the decreases in the numbers of surviving larvae generally reflected the decreases observed in burst rates in the different treatments. There was at least one surviving larva in each initially infested palm at day 25 and tunneling damage was observed in association with all surviving larvae (e.g., Fig. 4D–F). Five of the 10 larvae in palms with the 10^6 treatment had survived. The five that died exhibited hyphal growth and other visual signs of *B. bassiana* infection (e.g., Fig. 4A–C). Seventeen of the 20 larvae in palms with the 10^4 treatment survived to day 25. The three that died also exhibited hyphal growth. In addition, 2 of the 16 larvae in the untreated control died of unknown causes and 1 larva had pupated.

Discussion

The results suggest that the effects of different *B. bassiana* treatments against *R. ferrugineus* can be assessed successfully by three methods that compare different aspects of larval movement and feeding activity: the rate of bursts, the count of impulses per burst, and the rate of burst impulses, as seen in Figure 3 and Tables 2–4. The mean burst rates, counts of impulses per burst, and rates of burst impulses for the 10^6 and 10^4 treatments were significantly lower on the final two days of testing, days 22 and 23, than on days before any trends in burst rates had emerged, e.g., days 7 and 8 after treatment. In correlation with the reduction to 50% survival observed when the palms with 10^6 treatment larvae were dissected on day 25, the mean rate of bursts on days 22–23 decreased to 26% of the 0.363 bursts s^{-1} mean on days 7 and 8. Similarly, the 10^6 treatment mean count of impulses per burst decreased to 23% of the mean of 39 impulses per burst, and the mean rate of burst impulses decreased to 10% of the mean of 15.2 impulses s^{-1}. In correlation with the reduction to 85% survival observed when the palms with 10^4 treatment larvae were dissected, the mean rate of bursts on days 22 and 23 decreased to 47% of the days 7 and 8 mean of 0.428 bursts s^{-1}. Similarly, the 10^4 treatment mean count of impulses per burst decreased to 62% of the days 7 and 8 mean of the 32 impulses per burst, and the mean rate of burst impulses decreased to 41% of the days 7 and 8 mean of 15.8 impulses s^{-1}. Of the three different activity level measures, the rate of burst impulses on days 22 and 23 had the greatest fractional change from its mean value on days 7 and 8 because it factored in both effects of a decrease in burst rate and a decrease in the rate of impulses per burst.

The effects of *B. bassiana* treatments on activity and survival of hidden larvae correlate well with previous studies conducted outside of hidden environments (Fargues et al. 1994, Gindin et al. 2006; Nussenbaum and Lecuona et al. 2012). In such studies, live spores or conidia germinated when they contacted the insect cuticle. After germination, the fungus penetrated into the insect cuticle and grew within the body of its host which, once infected, reduced its feeding and movement activities. High doses of fungal treatments such as the 6.3×10^7 to 3.0×10^9 conidia ml^{-1} treatments in Dembilio et al. (2010) have been shown to cause 100% larval mortality in *R. ferrugineus* within 6–7 d. Malarvannan et al. (2010) found the least pupation (43.33%) in *Spodoptera litura* (F.) larvae treated with their highest dosages (2.4 by 10^7 conidia ml^{-1}) of *B. bassiana*.

Correlation was observed in this experiment between mean activity levels and subsequently observed fungal treatment effects even though the rate of sound production is variable and often decreases during periods of molting or pupation (Mankin et al. 2011). The ages of the larvae played a role here because the larvae were near their final stages of development but only one larva (an untreated control) had pupated by the end of the experiment.

Thus, there is considerable potential for use of acoustic methods as tools for nondestructive assessment of effects of biological control treatments against hidden insect pests under field conditions. The use of such methods could assist in optimization of doses and delivery methods for development of entomopathogenic fungal treatments as alternative pest control methods.

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