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Beauveria Bassiana Applied to Broiler Chicken Houses as Biocontrol of Alphitobius Diaperinus Panzer (Coleoptera: Tenebrionidae), an Avian Pathogens Vector

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ABSTRACT

Beauveria bassiana is naturally found in broiler chicken houses, and has been shown to be active against the lesser mealworm, *Alphitobius diaperinus*. The effectiveness of soil application of *B. bassiana* in emulsifiable oil-type formulation (Unioeste 4 isolate) to control the lesser mealworm in commercial poultry house was evaluated. The fungus was applied to the dirt floor of poultry house at 4.2×10^9 conidia/m^2 (treated aviary) and the insect population was assessed before and 96, 146 and 216 days after application (DAA). In the control aviary, no treatment was performed to control those insects. Molecular techniques were used to confirm the presence of the fungus in dead *A. diaperinus*. Significant treatment effects were observed, with 56% and 73% of insect population reduction on 96 and 146 DAA, respectively. However, on 216 DDA, insect population resumed to values close to those initially observed. In the control aviary, the population grew steadily, reaching almost 110% the initial population on 96 DAA, and close to 200% on 216 DDA. The results demonstrate the potential of the applied control strategy, even with a single application of the fungus; however, reapplications may be required after 3-6 months for more effective control.

INTRODUCTION

The lesser mealworm *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) is a ubiquitous and significant insect pest in commercial poultry house litter. The young chicks ingest larval and adult of the lesser mealworms, which negatively affects their performance and behavior, and causes gastrointestinal lesions (Despins & Axtel, 1995; Japp, 2008). The insect plays a potential role as vector of viruses, protozoa (*Eimeria*), and bacteria (*Campylobacter* and *Clostridium*) (Goodwin & Waltman, 1996; Skov *et al.*, 2004). The beetle is also the intermediate host for the nematode *Hadjelia truncata* (Alborzi & Rahbar, 2012) and it is a vector of *Salmonella* (Segabinazi *et al.*, 2005; Vittori *et al.*, 2007; Hazeleger *et al.*, 2008; Roche *et al.*, 2009; Chernaki-Leffer *et al.*, 2010).

The control of *A. diaperinus* is based on poultry house management practices and chemical insecticides, mainly pyrethroids, which are applied to the internal surfaces of the facilities and poultry litter (Bellaver *et al.*, 2003; Lambkin *et al.* 2012). However, despite the initial population reduction, these insecticides have proven to be ineffective (Uemura *et al.*, 2008; Santos *et al.*, 2009).

Alternative control measures under study include insecticidal plants, inert powders (hydrated lime and diatomaceous earth) and entomopathogenic nematodes (Watson *et al.*, 2003; Marcomini *et*

al., 2009; Alves *et al.*, 2012; Lambkin *et al.*, 2012). There are several reports on natural occurrence of entomopathogenic fungi infecting *A. diaperinus* in aviaries in Brazil and the United States, and on the selection of isolates against this pest, specially *Beauveria bassiana* (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) (Steinkraus *et al.*, 1991; Geden *et al.*, 1998; Alves *et al.*, 2004, 2005; Rohde *et al.*, 2006; Chernaki-Leffer *et al.*, 2007b; Santoro *et al.*, 2008; Oliveira *et al.*, 2011).

Despite the high mortality observed in laboratory, the use of *B. bassiana* is limited by product formulation, the physicochemical characteristics of the surface treated (cardboard tube traps, soil or litter), and application methods (Geden *et al.*, 1998; Alexandre *et al.*, 2006; Alves *et al.*, 2008; Gazoni *et al.*, 2011). In addition, poultry litter is rich in ammonia, which has strong fungistatic action, affecting the germination of different fungal species (Bacon, 1986). Previous studies showed that application of a *B. bassiana* emulsifiable oil formulation to poultry litter caused only 40% mortality of lesser mealworm adults (Crawford *et al.*, 1998). The use of *B. bassiana* for lesser mealworm control in poultry houses has been discouraged (Geden *et al.*, 1998). However, under laboratory conditions, a *B. bassiana* isolate formulated in a dry powder was more efficient when applied on poultry house soil than when applied on poultry litter (Alves *et al.*, 2008). Soil application is also recommended for the chemical control of the lesser mealworm due to its efficacy and safety (Uemura *et al.*, 2008; Santos *et al.*, 2009).

Soil is the natural ecosystem of *B. bassiana*, and poultry houses provide appropriate temperature and moisture conditions for the fungus. In addition, the fungus can remain in the soil in dead insects, infecting the following generations of lesser mealworms, which use this environment to pupate (Leger, 2008; Garrido-Jurado *et al.*, 2011).

A field study in laying hen houses resulted in limited action just 15 days after treatment with aqueous and granular formulations of *B. bassiana* (Geden & Steinkraus, 2003). However, no studies for biological control of the lesser mealworm in broiler houses are known.

Thus, the laboratory and field studies reported here were conducted to assess management of lesser mealworm in broiler houses using *B. bassiana* applied as an emulsifiable oil formulation to the soil floor.

MATERIALS AND METHODS

Fungal isolates

The virulent fungal isolate of *B. bassiana* used was 'Unioeste 4' (Rhode *et al.*, 2006), derived from the entomopathogenic fungi collection of the Agricultural Biotechnology Laboratory, UNIOESTE, Brazil. This fungus was cultured on rice in polypropylene bags. After growth, conidia were collected and formulated in 1% emulsifiable oil (oil dispersion – OD; Faria & Wraight, 2007) at concentration of 7×10^8 conidia/mL, with a viability of 90%.

Testing the toxicity of the fungal isolate in the laboratory

Lesser mealworm adults were collected from several commercial broiler houses and maintained in the laboratory in plastic containers (50 × 30cm – diameter × depth) with broiler litter and feed. Forced contact bioassays were conducted in the laboratory by immersing groups of 20 adults in 1 mL of the fungus formulation diluted in distilled water (concentration of 1.7×10^8 conidia/mL), and agitated for 10 seconds. In addition, a conidial suspension in Tween 80 solution (0.01%) was prepared (1.7×10^8 conidia/mL), and controls were prepared as emulsifiable oil (1%) and Tween 80 (0.01%) solutions in distilled water without conidia. Five groups of 20 beetles were used for each treatment and for the controls.

After immersion treatment, insects groups were transferred to Petri dishes lined with filter paper pre-moistened with distilled water and commercial poultry feed. Insects were kept in plastic containers with moistened polyurethane foam under controlled conditions (26 ± 2° C and 14-hour photophase), and evaluated daily for a period of ten days. The dead insects were removed and immersed in 70% alcohol and distilled water solution, and individually transferred to a humid chamber to allow conidial development. Conidiogenesis on dead insects was used as confirmation of confirm fungus-induced mortality. Percent mortality was submitted to analysis of variance (ANOVA) and means were compared by Tukey's test ($p<0.05$), using SAS statistical analysis software (SAS, 2003). The experiment was repeated three times to corroborate results.

Efficacy of the formulation in the field

Field experiments were undertaken in two commercial poultry houses in Cafelândia, State of Paraná, Brazil (24° 20' 54" S; 53° 17' 58" W). Each

house was 100m long and 12m wide with compacted earth floors, and held 17,000 broiler chickens. At the start of the trial, after broiler harvesting, the inside walls and earth floor of each house were washed and treated with a glutaraldehyde-based disinfectant solution (1L of the product/1000L of water per poultry house). Three days after cleaning procedure, the earth floor of poultry houses (Aviary 1) was treated with *B. bassiana* OD formulation containing 1×10^{12} conidia/L (5L of formulation in 1000L of water). *B. bassiana* treatment was done through the pressure-washing machine used to sanitize the chicken house. The fungus material was added to the tank in the pressure-washing machine, and sprayed on the soil floor of the poultry house under pressure (50 psi) at the rate of 4.2×10^9 conidia/m^2. Following application of the *B. bassiana*, the aviary was left empty with the curtains lowered for 48 hours, after which the curtains were lifted for ventilation and fresh poultry litter was placed on the dry floor. Similar cleaning procedures were performed in aviary 2 but no treatment for insect control was applied (control treatment).

Lesser mealworm was sampled according to Godinho & Alves (2009). For sampling purposes, each house was longitudinally divided in two sections (left and right), and each longitudinal section was then divided into three cross sections, establishing to establishing 36 sampling spots in total. Litter samples (30cm wide x 30cm deep) were collected by the wall, under the feeder, and by the pillar in each section, as shown in Figure 1.

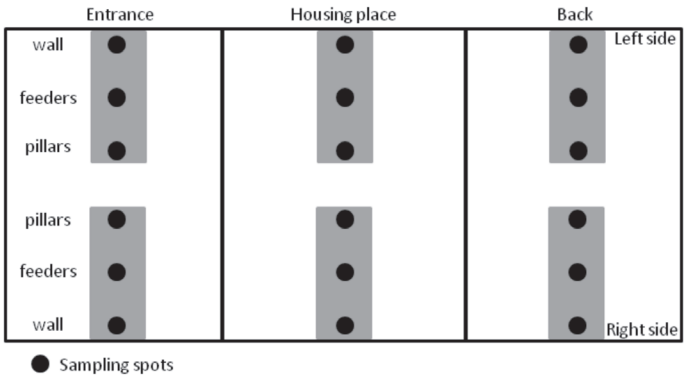

Figure 1 – Poultry house sampling spots (black circles) location, in three transversal sections (gray), along poultry house length.

The collected material was kept at -10 °C for 48 hours, after which lesser mealworm adults were counted. Insect sampling was always done during the last week of the flock and then repeated over three subsequent flocks (96, 146, and 216 days after fungus application).

The initial assessment of the insect population before the treatment represented the total insect population in each poultry house (100%). In the subsequent assessments in each flock, percentual variations were calculated in relation to this initial population. The efficiency of the control strategy for the lesser mealworm was calculated as reductions of the insect population initially observed (Godinho & Alves, 2009).

Molecular characterization

Over the course of the field trials, a large amount of dead beetles, with signs of fungus infection, was found outside the treated poultry house (Aviary 1). This was atypical and was observed over the time of the trial exclusively in the perimeter of Aviary 1. Samples of these insects were collected and fungus was isolated on selective culture media (Alves *et al.*, 1998). Molecular characterization was performed in order to identify the fungus and determine if its origin matched the one used for the treatment of the broiler house.

Two isolates obtained from samples collected in the Aviary 1 and the isolate Unioeste 4 were multiplied in Petri dishes containing Spore Production Media - ME (Alves et al., 1998) and kept at 26 ± 2 °C and 14-hour photophase for seven days. Conidia were harvest by scrapping culture media surface and were suspended in 0.01% Tween 80 solution by vortex agitation. A suspension with 1×10^8 conidia/mL of each isolate was inoculated in Erlenmeyer flasks containing 150 mL of no-agar ME. Cultures were incubated for 72 hours at 120 rpm and 26 ± 1 °C. The mycelium was collected from the liquid cultures, filtered using a Buchner funnel, and washed with sterile distilled water before being stored at -20 °C prior to DNA extraction. Genomic DNA was extracted according to the methodology modified by Raeder & Broda (1985).

The primers used in preliminary tests were based on those tested by Oliveira *et al.* (2011). Based on analyses of the RAPD profiles, three primers (Operon Technologies®) were selected based on consistent band patterns (OPB-10; OPQ-05; and OPZ-19). PCR reaction mixture was prepared for a final volume of 25 µL [2,5 µL PCR buffer (200 mM Tris-HCl, pH 8.8, 500 mM KCl concentrated 10x); 1.5 µL $MgCl_2$ (50 mM); 2.5 µL dNTP (2.5 mM); 1 µL of each primer (100 µM); 0.2 µL *Taq* DNA polymerase (5 U/ µL) and 1 µL DNA (20 ng)]. The amplification reactions were carried out

using a thermal cycler (MG PTC-200) scheduled for an initial denaturation at 94 °C for 5 min; followed by 39 cycles at 92 °C for 1 min, 35 °C for 1 min and 30 sec, and 72 °C for 2 min; and one cycle at 72 °C for 5 min. Subsequently, the samples were analyzed in 1.4% agarose gel (100 V), using a 1 Kb Plus DNA Ladder marker (Life Technologies®) as molecular weight pattern . Gels were stained with 10 mg/mL of ethidium bromide and photo-documented (L Pix Loccus Biotechnology).

The data obtained were analyzed using the Bionumerics software (Applied Mathematics, Kortrijk, Belgium, version 2.0). For grouping, the unweighted pair-group method was used with arithmetic mean algorithm (UPGMA) and Jaccard coefficient at a tolerance level of 3%.

Sequencing of rDNA-ITS region for isolate confirmation

Amplification and sequencing of rDNA-ITS region were determined using the forward and reverse primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC), respectively (White *et al.*, 1990). The amplification reactions were prepared for a final volume of 25 µL, containing 2.5 µL PCR buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl concentrated 10x); 1.2 µL $MgCl_2$ (50 mM); 5.0 µL dNTP (1.5 mM); 1 µL of each primer (50 mM); 0.2 µL *Taq* DNA polymerase (5 U/µL); and 20 ng genomic DNA, and performed in a thermal cycler (MG PTC-200). The amplification consisted of an initial denaturation at 95 °C/3 min, followed by 35 cycles at 94 °C/1 min, 57 °C/1 min, 72 °C/1 min, and a cycle at 72 °C/3 min. Next, the PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega) and the sequencing was performed with the BigDye® Terminator v 3.1 Cycle Sequencing kit on an ABI 3730 DNA Analyzer. The sequences were organized into contigs using the programs Phred (Ewing *et al.*, 1998), Phrap (www.phrap.org), and Consed (Gordon *et al.*, 1998), and compared with sequences in the GenBank database.

RESULTS AND DISCUSSION

Efficacy in the laboratory

Both total mortality and fungus-confirmed mortality varied from 82 to 92% for the aqueous suspension and oil-dispersion preparation, respectively. Mortality was significantly different from the water and oil controls (F=419.9; CV= 12.6; $p<0.01$) (Table 1).

Table 1 – Total and fungus-confirmed mortality of *Alphitobius diaperinus* adults after exposure to 1.7×10^8 *Beauveria bassiana* (Unioeste 4) conidia/mL aqueous suspension and oil dispersible formulation, over 10-day evaluation period (26 ± 2 ºC; RH: 60 ± 10%; photophase: 14 hours).

Treatment	Mortality (%)	
	Total	Confirmed
Control water	1.0 ± 1.0 B	0.0 ± 0.0 B
Oil Control	3.0 ± 3.0 B	0.0 ± 0.0 B
Unioeste 4 AS	89.0 ± 2.5 A	82.0 ± 4.1 A
Unioeste 4 OD	92.0 ± 3.4 A	88.0 ± 2.6 A
C.V. (%) = 12.6		

Means (± SEM) followed by the same letter in the column are not different by Tukey's test ($p<0.05$). AS = aqueous suspension; OD = oil dispersible

The oil control treatment had quick and ephemeral effect on insects, causing decreased walking activity in most insects and cessation of movement. However, this effect was temporary, and the assessment after 24 hours showed that the insects were in apparently normal conditions after treatment. Similar decrease in activity was observed in the insects treated with oil dispersible fungus application.

The mortality observed with *B. bassiana* treatments were similar previous studies with the same insect (Rohde *et al.*, 2006; Rezende *et al.*, 2009). However, the expected higher mortality using the oil-dispersible formulation did not reach significance levels. This type of preparation is recommended to improve the performance of fungi, particularly to increase fungus resistance to high temperatures (Alexandre *et al.*, 2006), which were limiting for the action of the fungus during the study. Despite of the similarity in the mortalities observed in the lab bioassay, the OD formulation was used in the field experiment due to other previously reported advantages (Daoust & Roberts, 1983; Starthers *et al.*, 1993; McClatchie *et al.*, 1994; Batista Filho *et al.*, 1998).

Efficacy of the formulation in the field

In pretreatment evaluations, more than 1000 adult *A. diaperinus* individuals were collected from each poultry house (Table 2). The adult beetle population in aviary 1 was significantly reduced by fungus treatment to 44% of the initial population. The treatment with *B. bassiana* provided long-term control of the insects, lasting over two production cycles or 150 days. However, seven months after the application, the number of adult beetles in the fungus-treated poultry house (Aviary 1) increased to values higher, but not significantly different from the original population. In contrast, in the control poultry house (Aviary 2), the

pest population remained high during the next two production cycles. Furthermore, at the end of the three production cycles (seven months), *A. diaperinus* population in the control poultry house doubled in relation to the original infestations levels.

Table 2 – Means (±EPM) of *Alphitobius diaperinus* adults in commercial poultry houses untreated (Control) and treated with 1 × 10^6 conidia/mL *Beauveria bassiana* (Unioeste 4) oil dispersion formulation applied on the soil.

Evaluation	Treated Aviary (1)	Control Aviary (2)
Pretreatment	1360.7 ± 354.1 A (100)	1114.9 ± 211.4 A (100)
1st (96 DAA*)	606.4 ± 141.7 B (44)	1221.1 ± 349.9 A^{ns} (109)
2nd (146 DAA)	372.1 ± 86.3 B (27)	1146.3 ± 321.4 A^{ns} (102)
3rd (216 DAA)	1515.4 ± 315.4 A^{ns} (111)	2195.4 ± 733.5 A (196)

*DAA = Days after application. Mean ± SEM of individuals at every assessment and respective percentage of infestation, between parentheses, in relation to pretreatment population. Means followed by the same letter in the same row do not differ by Mann--Whitney's test ($p<0.05$) (Comparison between aviaries); NS = not different from the insect population before treatment by Wilcoxon's test ($p<0.05$) (Comparison of every assessment with the pretreatment population in the same aviary).

The results suggest that poultry house management and replacement of the poultry litter did not provide protection against *A. diaperinus* since no decrease in the insect population was observed in the control aviary. Also we did not observe any effect of the poultry litter replacement in the preventing increases in the A. diaperinus population as observed previously (Lambkin et al., 2007). The use of fungus application on the soil and the placement of new litter provided significant protection against this pest population, as previously demonstrated with the application of chemical insecticides (Chernaki-Leffer *et al*., 2007a; Uemura *et al*., 2008).

The lack of insect control, as demonstrated in the control aviary, along with cryptic habits of insects (Axtell & Arends, 1990), allowed the population to reach high levels of more than 2000 individuals/sample in the third production cycle, corresponding to 196% of the original population.

Persistent activity is a recognizable advantage of biological control and the use of entomopathogenic fungi (Alves, 1998). The fungus treatment of the soil maintained the *A. diaperinus* population at low levels for two production cycles in contrast with conventional chemical treatments, which cause transitory reduction of the population, have a short residual effect, and gradually allows pest levels to increase when poultry litter is reused during the subsequent production cycles (Pinto *et al.*, 2005; Chernaki-Leffer *et al.*, 2007a; Uemura *et al.*, 2008; Santos *et al.*, 2009).

The protection period provided by the fungus treatment corresponds to the minimum interval adopted by the integrator company for poultry litter replacement or chemical insecticide application.

Field studies on lesser mealworm control using entomopathogenic fungi have been carried out in laying hen houses with applications of *B. bassiana* on the feces of the birds, achieving 60 to 90% reductions in pest populations and short persistence of 15 days (Perez *et al.*, 1999; Geden & Steinkraus, 2003). Despite the environmental condition differences between layer and broiler houses, the results of the present study suggest that changes in the application techniques or in formulations may improve the results of the use entomopathogenic fungi in layer houses. Long persistence and lack of risk to the poultry or human health (Haas-Costa *et al.*, 2010) further justify the use of *B. bassiana* for the control of *A. diaperinus* in poultry production.

Molecular characterization and sequencing for isolate confirmation

Using the three RAPD markers to analyze the three isolates of *B. bassiana* obtained from aviary 1 and the isolate Unioeste 4, bands were generated with sizes ranging from 500 bp to 2500 bp. The minimum and maximum numbers of bands observed were two (OPQ-05) and seven (OPB-10), respectively, and the profiles generated with significant bands were virtually identical (Figure 2).

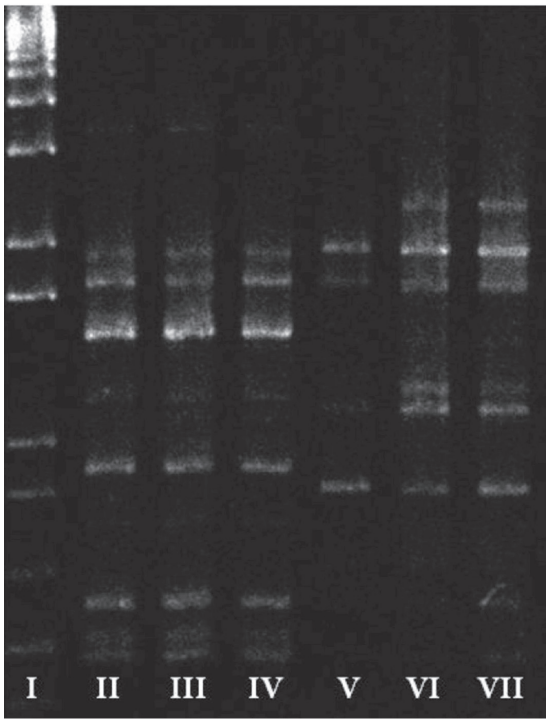

Figure 2 – RAPD profiles of Beauveria bassiana isolates: I) 1 Kb Plus marker; II) With OPB-10 primer Unioeste 4; III) Sample 1; IV) Sample 2; V) With OPZ-19 primer Unioeste 4; VI) Sample 1; VII) Sample 2.

The primers ITS1 and ITS4 amplified isolate fragments with approximately 540 bp. The sequences of the fragments showed high degree of identity with Unioeste 4 isolate data in the GenBank database (EF491629).

B. bassiana naturally occurs in commercial aviaries (Alves *et. al.*, 2005) and several studies on molecular characterization with isolates related to the lesser mealworm have been conducted (Castrillo & Brooks, 1998; Castrillo *et al.*, 1999; Santoro *et al.*, 2008; Oliveira *et al.*, 2011). No studies have reported on the re-isolation of fungi artificially produced and applied against *A. diaperinus* in field conditions. Our results confirmed the isolates obtained from Aviary 1 after used in the fungus application were the same as that applied in the treatment. Genetic confirmation that the applied isolate was the cause of fungal infections in the field eliminates natural infections as the potential cause of the mortality observed, and this was further corroborated by the fact that the dead *B. bassiana*-infected *A. diaperinus* were only found after the application of the fungus.

The treatment of the aviary with the entomopathogenic fungus *B. bassiana* (isolate Unioeste 4 in oil dispersion formulation), applied to the facilities and on the soil, is an efficient method to maintain the population of *A. diaperinus* under control for up to 150 days, with a single application fact. This was confirmed by genetic typing of the applied fungal isolate as the agent causing epizootic disease in the lesser mealworm population.

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