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Alphitobius diaperinus, bioassays, biological control, entomopathogenic fungi.

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ABSTRACT

The pathogenicity of insect-pathogenic hyphomycetous isolates to the lesser mealworm *Alphitobius diaperinus* (Panzer) was tested in this study. Thirty isolates of *Beauveria bassiana* (Balsamo) Vuillemin, *Paecilomyces amoenoroseus* (Hennings), *P. fumosoroseus* (Wize) Brown & Smith, *P. lilacinus* (Thom) Samson, *P. tenuipes* (Petch) Samson, *Metarhizium anisopliae* (Metschnikoff) Sorokin, and *M. anisopliae var. acridum* were initially screened by sprinkling dry conidia onto adult *A. diaperinus* or by allowing adults to walk on Petri dishes with sporulating fungal isolates. The two most virulent isolates, CNPSo-Ma352 (*M. anisopliae*) and CNPSo-Ma356 (*M. anisopliae*), killed 30% and 26.7% of the dry-conidia treated adults, respectively. These two isolates were selected for LD_{50} bioassays. LD_{50} of CNPSo-Ma352 was 4.5×10^4 conidia per larva, and 2.1×10^5 conidia per adult, and for strain CNPSo-Ma356, LD_{50} was 2.2×10^4 conidia per larva and 1.3×10^5 conidia per adult. Larvae were 5-6 times more susceptible than adults. A larger number of conidia required to cause 50% mortality in insect evaluates, suggesting the reduced susceptibility of *A. diaperinus* to entomopathogenic fungi. Nevertheless, these and other strains of fungus offer an alternative method for controlling of lesser mealworm in poultry houses when associated to integrated management.

INTRODUCTION

The lesser mealworm or little beetle, *Alphitobius diaperinus* (Panzer) is the most important poultry-house pest in Brazil, where it successfully breeds, feeds, and develops in the mixture of warm litter and poultry droppings. Environmental conditions in the poultry house (25-30 °C, humidity between 50% and 70%) allows beetles to complete one life cycle during the chicken growing cycle (approximately 50 days) (Salin *et al.*, 2000; Chernaki & Almeida, 2001). Beetles (adults and larvae) feed on feed residues, feces, and dead or dying birds (Pfeiffer & Axtell, 1980). They are considered to be important vectors of pathogens, such as *Salmonella typhimurium* (Loeffler), *Escherichia coli* Lignieris, avian leukosis virus, and turkey enterovirus and rotavirus (McAllister *et al.*, 1994; 1995). Additionally, due to high beetle population levels, adult insects may become local nuisances during downtime, when adults often leave the poultry house and enter nearby residences. The control of *A. diaperinus* is important for poultry health and production, as well as for reducing the nuisance they cause. It is difficult, however, to obtain good control because chemical insecticides are not effective, and repeated applications may cause the development of resistance. In addition, this may cause chemical insecticide residue problems. Therefore, other alternatives must be developed to control this important pest.

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Beetles have natural enemies and pathogens, including mites (Steinkraus & Cross, 1993), protozoans (Apuya *et al.*, 1994), nematodes (Alves *et al.*, 2005), and entomopathogenic fungi (Steinkraus *et al.*, 1991; Crawford *et al.*, 1998; Geden *et al.*, 1998). Natural epizootics of *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) are found in lesser mealworm populations in poultry farms (Steinkraus *et al.*, 1991; Castrillo & Brooks, 1998). Apparently, however, natural occurrence of entomopathogenic fungi is limited (Steenberg & Jespersen, 1997). Yet, larvae of the lesser mealworm are susceptible to the infection of strains of most hyphomycete species: *Beauveria bassiana*, *B. brongniartii* (Saccardo) Peth, *Paecilomyces farinosus* (Dicks) Brown and Smith, *P. fumosoroseus* (Wise) Brown & Smith, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Verticillium lecanii* (Zimm.) Viégas, *Acremonium* sp. Link ex Fries and *Fusarium* sp. Link ex Gray (Steinkraus *et al.*, 1991; Crawford *et al.*, 1998; Geden *et al.*, 1998). *M. anisopliae* was found associated with litter in poultry houses in Brazil (Alves *et al.*, 2004), and, according to Geden & Steinkraus (2003),this fungus presents the potential to control these beetles.

LD_{50} is often used to determine the concentration of the pathogen or of the compound will kill 50% of the population in question. Thus, LD_{50} provides a useful value to compare results from different laboratories, hosts, and entomopathogenic strains or species. The aim of this study was to assess the susceptibility of *A. diaperinus* to the entomopathogenic fungi: *Beauveria bassiana*, *Paecilomyces amonoeroseus*, *P. tenuipes*, *P. lilacinus*, *P. fumosoroseus*, *Metarhizium anisopliae* and *M. anisopliae* var. *acridum,* and to determine the lethal doses (LD_{50}) of the most pathogenic isolates.

MATERIAL AND METHODS

Alphitobius diaperinus

Larvae and adult lesser mealworms were collected from poultry litter in a broiler farm in the municipalities of Cascavel and Curitiba, state of Paraná, southern Brazil. Colonies were kept in plastic trays (37 x 26 x 13 cm) with poultry litter and rabbit feed, at 24 ± 2 °C, in continuous darkness, and 50 - 70% relative humidity.

Tests were conducted using randomly selected adults of approximately the same size (sex unknown), and 1.0 to 1.3-cm long.

Source of fungal strains

Thirty isolates of the fungi *Beauveria bassiana* (CNPSo-Bb11, CNPSo-Bb13, CNPSo-Bb14, CNPSo-Bb15, CNPSo-Bb18, CNPSo-Bb40, CNPSo-Bb70, CNPSo-Bb71, CNPSo Bb357 and LPB), *Metarhizium. anisopliae* (CNPSo-Ma3, CNPSo-Ma4, CNPSo-Ma5, CNPSo-Ma6, CNPSo-Ma12, CNPSo-Ma69, CNPSo-Ma98, CNPSo- Ma356, CNPSo- Ma352), *M. anisopliae* var. *acridum* (CNPSo- Ma358) and *Paecilomyces amonoeroseus* (CNPSo-Pam73, CNPSo-Pam112), *P. tenuipes* (CNPSo-Pae41, CNPSo-Pae92), *P. lilacinus* (NRRL, NRRL-895, NRRL-6594), *P. fumosoroseus* (CNPSo-Pf79 CNPSo-Pf81 CNPSo-Pf83) were maintained in the collection of entomopathogenic fungi cultures at Embrapa Soja (Sosa-Gómez & Silva, 2002) and in the Laboratory of Biotechnological Processes of the Federal University of Paraná. In these collections, isolates are stored at –20 °C in silica gel with nonfat dry milk, according to Smith & Onion (1983). These isolates were screened for virulence against adult *A. diaperinus* in the laboratory. Each isolate was plated on spell out (PDA) medium (Alves *et al.*, 1998) at 26° ± 2 °C, in continuous darkness, and 60 - 65% relative humidity for 7 - 10 days prior to the use in the bioassay.

Selection and virulence of fungi isolates

Dry conidia from 1-2 week-old colonies were sprinkled onto the surface of the adult beetles. For the hydrophilic isolates CNPSo-Bb11, CNPSo-Bb13, LPB, CNPSo-Ma4, CNPSo-Ma5, CNPSo-Ma358, and NRRL-895, with low spore formation, insects were placed directly on the fungal colonies for 60 sec. Fifteen insects were used for each isolate, and replicated twice (total of 30 adults per isolate), including the control group. After inoculation (or no treatment, in the case of controls), insects were maintained in sealed (with fine textured cloth) 500-mL plastic containers with rabbit feed and slightly moistened sawdust, and kept in a climate chamber at 27 °C in constant darkness for 20 days. Dead insects were daily removed, and placed on Petri dishes with moistened cotton and filter paper, at room temperature, until signs of fungal infection appeared, or for 7 days, whichever came first. For counting, the 30 isolates were prepared by aerosol conidia suspension on slides with a PDA culture film, and incubated in the dark for 20 hours, at 26°-28 °C. The two most virulent isolates were chosen for further bioassays (LD_{50}).

LD_{50} estimation

The two isolates chosen for further bioassays were plated on Petri dishes with spell-out medium (PDA) or in adult beetles, and were incubated at 26 °C, 60-65%

relative humidity, and complete darkness. When isolates were sporulating, conidia were collected by scraping dry conidia from the surface of the culture plate. Dry conidia were then placed in test tubes with sterile distilled water containing 0.01% Tween 80. Conidial suspensions were then filtered, homogenized, and adjusted to the correct concentrations with the use of a hemacytometer.

The concentrations used were from 2.8×10^3 conidia/mL to 1.8×10^5 conidia/mL. Each adult or larva was inoculated by pipetting 2 µl of the relevant concentration of the isolates CNPSo-Ma352 or CNPSo-Ma356 (by monocanal micropipette) onto the ventral side of the insect. Control insects received the same volume of sterile water (0.01 % Tween 80). Inoculated insects were then placed in 500 mL plastic containers as described above.

Each treatment comprised four replicates, with 640 adults and 520 larvae tested for each isolate. Dead insects were daily removed, and individually placed on Petri dishes with moistened cotton and filter paper, at room temperature, until signs of fungal infection appeared, or for 7 days, whichever came first. Samples of conidia from the dead animals were treated and counted as described above.

Statistical analysis

Probit analysis was used to determine LD_{50}, based on cumulative mortality per treatment (LeOra Software, 1987). Slopes and y-intercepts of the mortality regressions of the two treatments were compared ($\alpha = 0.05$).

RESULTS

Entomopathogenic fungus selection

All isolates were viable. CNPSo-Ma5 had the lowest germination rate (72%), while all other isolates presented germination rates of 85% or higher. The two most virulent fungi were isolate CNPSo-Ma356, which killed 30% of the adults and all insects killed by this isolate developed mycosis, and isolate CNPSo-Ma352, which killed 26.7% of the adults, and 75% of the insects killed by this isolate developed mycosis. These two isolates were therefore used in further bioassays. All other fungal isolates caused much lower or no mortality (Table 1).

Virulence determination

The two isolates, CNPSo-Ma352 and CNPSo-Ma356, presented similar ($p > 0.05$) virulence for adults and for larvae, but adults were much less susceptible than larvae. LD_{50} of CNPSo-Ma352 for larvae was 4.5×10^4 (n = 168; slope= 2.52 ± 0.89; CL = 2.8×10^4 to 9.6×10^4) and LD_{50} of CNPSo-Ma356 was 2.2×10^4 (n = 161; slope = 2.47 ± 0.68; CL = 1.3×10^4 to 3.8×10^4). LD_{50} of CNPSo-Ma352 for adults was 21.2×10^4 (n = 280; slope = 0.87 ± 0.26; CL = 9.2×10^4 to 2.36×10^6)

Table 1 - Assay for virulence of pathogenic fungus. Number of deaths and percentage of deaths with evidence of fungus in 30 trials of *Alphitobius diaperinus* adults inoculated with different isolates of *Beauveria bassiana, Paecilomyces* spp and *Metarhizium* spp dry conidia.

	Isolates[a]	Total Deaths/ % Death with evidence of fungus			Isolates[a]	
Beauveria bassiana	CNPSo-Bb11	1	-	*Paecilomyces* spp	*P.amoenoroseus*	CNPSo-Pam73
	CNPSo-Bb13	2	-			CNPSo-Pam112
	CNPSo-Bb14	2	50,0		*P.fumosoroseus*	CNPSo-Pf79
	CNPSo-Bb15	6	-			CNPSo-Pf81
	CNPSo-Bb18	2	-			CNPSo-Pf83
	CNPSo-Bb40	0	-		*P. tenuipes*	CNPSo-Pae41
	CNPSo-Bb70	1	-			CNPSo-Pae92
	CNPSo-Bb71	0	-		*P. lilacinus*	NRRL[b]
	CNPSo-Bb357	2	-			NRRL-895[b]
	LPB[b]	0	-			NRRL-6594[b]
Control group		0	0			

[a] Sosa-Gòmez & Silva (2002). [b] Laboratory of Biotechnological Processes of the Federal University of Paraná. [c] As nearly all mortality was apparently due to fungus, these two strains were chosen for LD_{50} studies.

and LD_{50} of CNPSo-Ma356 was 13.0 x 10^4 (n = 281; slope = 0.96 ± 0.27; CL = 5.8 x 10^4 to 1.4 x 10^6) (Figure 1). When LD_{50} for larvae and adults were compared in both isolates, larvae were 4.7 times (CNPSo-Ma352) and 5.9 times (CNPSo-Ma356) more susceptible than adults.

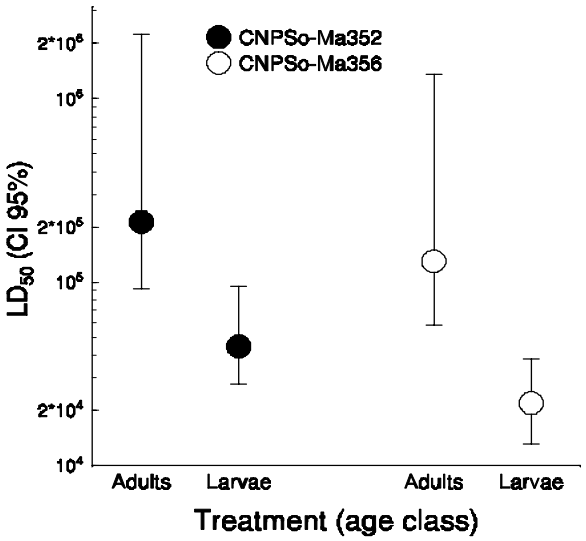

Figure 1 - A comparison of LD_{50} between MA352 and MA356 fungal strains, and adult and larval lesser mealworm beetles. Adults are less susceptible than larvae ($p < 0.05$), and strains present similar virulence. Lethal Dose 50% axis is shown on a logarithmic scale, as LD_{50} presents logarithmic distribution.

DISCUSSION

Many authors have stressed that fungal susceptibility is reasonably well-known for beetles, and in general, when beetles are exposed to large concentrations of conidia (10^7 conidia/mL), mortality can be very high (up to 98%, Steinkraus *et al.*, 1991). *A. diaperinus* larvae and pupae are more susceptible than adults to a variety of isolates of *B. bassiana*, *B. brongniartii*, *P. farinosus*, *P. fumosoroseus*, *M. anisopliae*, *Verticillium lecanii*, *Acremonium* sp., and *Fusarium* sp. (Steenberg & Jespersen, 1997). It was shown that *B. bassiana* isolates under poultry-house conditions can cause high mortality rates in *A. diaperinus* (Crawford *et al.*, 1998). Recently, Alexandre *et al.* (2006) evaluated the effect ofpoultry litter (new and used) by submerging insects in a fungal suspension (10^8 conidia/mL), and observed that *B. bassiana* isolates were more sensitive to high temperature than *M. anisopliae* because conidia viability, vegetative growth, and virulence were negatively affected.

CNPSo-Ma12, CNPSo-Ma352, and CNPSo-Ma356 (*M. anisopliae*) isolates are more virulent to adult *A. diaperinus* as compared to *Beauveria* spp. and *Paecilomyces* spp. isolates (also see Perez *et al.*, 1999). Adults are usually less susceptible (Steenberg & Jespersen, 1996 and 1997; Geden *et al.*, 1998). Also, last-instar larvae in this study were five (CNPSo-Ma352) to six (CNPSo-Ma356) times more susceptible than adults to *M. anisopliae.*

Average LD_{50} doses were 2.2 x 10^4 and 4.5 x 10^4 conidia per larva, and 13.0 x 10^4 and 21.2 x 10^4 conidia per adult (CNPSo-Ma356 and CNPSo-Ma352, respectively). These doses are quite high as compared to other studies. For example, LD_{50} of *Metarhizium* spp. in *Locusta migratoria migratorioides* is from 60 to 300 conidia per grasshopper (Nowierski *et al.*, 1996). Therefore, the use of fungi to control the lesser mealworm in poultry houses in Brazil will require further field studies to determine the cost-benefit ratio of each strategy and the virulence of the fungus (or conversely, susceptibility of the host).

Although a larger number of conidia is required to cause 50% mortality of the evaluated insect, this research study provides data that indicates that these and other fungus strains offer an alternative method for controlling the lesser mealworm in poultry houses. We recommend the controlled and studied application of fungi to poultry houses with significant infestations, perhaps simultaneously with studies on the impact of chemical as well as botanical or desiccants pesticides (diatomaceous earth) and growth regulators on fungal efficiency. Also, we believe that research studies on the best way to apply fungi to pests is an area that may yield important results in terms of cost reduction (for instance, Arends traps impregnated with conidia, according to Geden & Steinkraus (2003).

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