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Endophytic and entomopathogenic strains of *Beauveria* sp to control the bovine tick *Rhipicephalus* (*Boophilus*) *microplus*

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ABSTRACT. Pathogenicity of strains of the entomopathogenic fungus *Beauveria bassiana* and endophytic strains of *Beauveria* sp against the bovine tick *Rhipicephalus* (*Boophilus*) *microplus* was tested in laboratory bioassays and under field conditions. Suspensions containing 10^5, 10^7 and 10^9 conidia/mL were prepared of each fungal strain for laboratory bioassays. The ticks were maintained at 28°C, 90 ± 5% relative humidity, and the following variables were evaluated: initial female weight, egg weight, hatching percentage, reproductive efficiency, and percentage control. For tests under field conditions, a *Beauveria* suspension containing 10^6 conidia/mL was sprayed on tick-

infested cows. After 72 h, the ticks were collected to estimate mortality under field conditions. Laboratory bioassays showed a mortality of 20 to 50% of the ticks seven days after inoculation with 10^7 *Beauveria* conidia/mL. Under field conditions 10^6 *Beauveria* conidia/mL induced 18-32% mortality. All *Beauveria* strains were effective in biological control of *R.* (*Boophilus*) *microplus* under laboratory and field test conditions. This is the first demonstration that endophytic fungi can be used for biological control of the cattle tick; this could help reduce environmental contamination by diminishing the need for chemical acaricides. Two endophytic strains were isolated from maize leaves and characterized by molecular sequencing of 5.8S rDNA ITS1 and ITS2 and morphological analyses of conidia. We found that these two endophytic *Beauveria* isolates, designated B95 and B157, are close to *Beauveria amorpha*.

Key words: Fungal acaricides; *Rhipicephalus* (*Boophilus*) *microplus*; *Beauveria amorpha*; *Beauveria bassiana*; Endophytic fungi

INTRODUCTION

The Asiatic bovine tick, *Rhipicephalus* (*Boophilus*) *microplus* (Acari, Ixodidae), is an ectoparasite that has been introduced into almost all subtropical and tropical countries through the importation of cattle. The ticks cause substantial economic losses due to reduced productivity resulting from anemia, toxicities, physical damage, and the transmission of various diseases to their hosts. Ticks are one of the largest livestock health problems in the world (Bittencourt et al., 1997; Bittencourt, 2000) and their control is mainly limited to the application of synthetic chemical products combined with management measures (Rocha, 1984). Tick control throughout the world is based mainly on the repeated use of chemical acaricides. Unlimited use of these tick-controlling chemicals has resulted in problems related to environmental pollution, milk contamination and resistance development in the target species (Onofre et al., 2001). Entomopathogenic fungi have been widely used for the control of agricultural and forest pests (Kaaya et al., 1996). The laboratory bioassay is an important test to determine the virulence of a fungal pathogen. It considers mortality and other data (egg production, egg hatchability, longevity) that could reduce the growth rate of tick populations in the field. However, comparing assay results is difficult due to variations in methods and use of different fungal isolates. Experiments under laboratory conditions (controlled temperature, humidity, day-light period) show more consistent results than do similar experiments under natural conditions, and laboratory bioassays are expected to indicate the most virulent isolates, and therefore those with greatest potential for biological control (Onofre et al., 2001; Pirali-Kheirabadi et al., 2007; Fernandes and Bittencourt, 2008). There are several groups of organisms that attack not only *R.* (*B.*) *microplus* but other *Rhipicephalus* species such as *R.* (*B.*) *annulatus* and *R.* (*B.*) *decoloratus*, and have potential as biocontrol agents. Among these organisms that are potential biological control agents for ticks, entomopathogenic fungi such as *Beauveria bassiana*, *Metarhizium anisopliae* and *M. flavoviride* appear to be satisfactory acaricides (Bittencourt et al., 1997; Monteiro et al., 1998; Pirali-Kheirabadi et al., 2007; Fernandes and

Bittencourt, 2008; Lubeck et al., 2008; Gindin et al., 2009), being effective against ticks such as *Ixodes scapularis*, *Oligonynchus yothersi*, *Rhipicephalus appendiculatus*, *R. sanguineus*, *Hyalomma excavatum*, and *Amblyomma variegatum* under laboratory and field conditions (Oliveira et al., 2002; Leemon et al., 2008). The entomopathogenic *B. bassiana* is usually found infecting insects. However, members of the genus *Beauveria* have also been isolated from plants, and thus considered to be endophyte strains (Bing and Lewis 1991; Wagner and Lewis 2000), which probably protect the host plant against the attack by insects. Strains of *Beauveria* are able to produce several cuticle-degrading hydrolytic extracellular enzymes such as chitinases and β-1,3-glucanases, all of which are considered to be determinants of fungal pathogenicity (Gupta et al., 1992; St. Leger et al., 1993). Hyphal modifications (appressorium) are also important as physical structures that help *Beauveria* strains to penetrate the cuticle of its hosts (Mendgen et al., 1996; Bastmeyer et al., 2002; Campos et al., 2005). *B. bassiana* and *M. anisopliae s*ecrete toxic metabolites during the infection process that contribute to the establishment and progression of disease (Alves, 1998). Also, *B. bassiana* and *B. amorpha* produce subtilisin-like proteases and chitinases in the presence of *R.* (*B.*) *microplus* tick cuticle (Campos et al., 2005). The use of chemical acaricides for tick control has resulted in problems related to environmental pollution, milk contamination and development of resistance in the target species. Since biological control is an attractive alternative method for controlling ticks, we evaluated the potential of two new endophytic and two entomopathogenic *Beauveria* strains as biological control agents for *R.* (*B.*) *microplus*.

MATERIAL AND METHODS

Fungal strains

Two entomopathogenic *Beauveria bassiana* (Bals.-Criv) Vuillemin (Ascomycetes, Hypocreales) strains, CG166, isolated from *Schrius* sp (Lepidoptera: Saturnidae), and CG478, isolated from *Anthonomus grandis* (Coleoptera: Curculionidae) were supplied by CENARGEN Laboratories, EMBRAPA, Brasília, Brazil. Two endophytic *Beauveria* strain isolates from leaves of maize, *Zea mays*, designated B95 and B157, were also used.

Isolation of endophytic fungi

Hybrid maize (*Zea mays* L. OC 705) was grown in the field and in a greenhouse in the Canguiri Experimental Station at the Federal University of Paraná, Brazil, using non-fertilized soil free from insecticides and other chemicals. Only plants exhibiting healthy vegetative growth were used. Leaves and stems were collected from field-grown and greenhouse-grown plants 3 weeks after emergence and at the end of the reproductive phase, about 7 weeks after seed germination. The plant samples were washed twice in sterile water, surface-disinfected by immersion in 70% ethanol for 1 min followed by 3% sodium hypochlorite for 4 min and 70% ethanol for 30 s, and rinsed three times with sterile water. After surface disinfection, leaves and stems were peeled and cut into 10 fragments (5-7 mm), which were placed on potato dextrose agar (PDA) culture medium supplied with 100 µg/mL tetracycline. The plates were incubated at 28°C and checked every day for 30 days. The number of fragments showing fungal growth was determined and recorded. The hyphal tip of each morphologically different

mycelium that emerged from a branch fragment was transferred to PDA slants for later identification (Pimentel, 2001). Following incubation, fungal isolates recovered from each plant fragment were selected at random, purified and grouped on the basis of phenotypic characteristics such as colony morphology, color and growth rate. Isolates representing each fungal group of interest were selected for further identification by morphological characteristics and rDNA sequencing.

DNA extraction and rDNA sequencing of endophytic *Beauveria* strains

The DNA of *Beauveria* isolates was extracted according to Raeder and Broda (1985) and further characterized by sequencing 5.8S rDNA, internal transcribed spacers 1 and 2 (ITS-1 and ITS2), using the primers ITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), which are universal primers for the fungal group. Polymerase chain reaction (PCR) was carried out in a 50-mL final volume containing 2 mL template DNA (5-10 ng), 0.4 mM of each primer, 200 mM of each dNTP, 3.7 mM $MgCl_2$ and 2 U Taq DNA polymerase (Invitrogen) in 10 mM Tris-HCl, pH 8.3, and 10 mM KCl. The reaction conditions were as follows: 94°C for 1 min for initial denaturation and 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, followed by a final extension at 72°C for 7 min. A negative control was included in all PCR experiments. The purified PCR products (GFX PCR DNA Kit, GE Healthcare Life Science) were sequenced using ITS-1 and ITS-4 primers and the sequences compared to the GenBank database using the BLASTn program (Altschul et al., 1990).

Nucleotide sequence analysis and accession numbers

The rDNA sequence alignment was performed using ClustalX (Thompson et al., 1999). The phylogenetic analyses were performed with the Geneious Program Tree Builder Release 4.6.4, neighbor-joining method (Saitou and Nei, 1987), and Jukes-Cantor correct distance model (Jukes and Cantor, 1969). The nucleotide sequences obtained in this study were submitted to GenBank, accession numbers AY388629 and AY388630.

Ticks

Males and females of *R*. (*B*.) *microplus* were collected, according to the method described in Bittencourt et al. (1997), from naturally infested adult Holstein-Friesian cows pastured at 3 dairy farms (coded A, B and C) located close to the town of Serafina Correia (S28°42'42''/W51°56'06'') in Rio Grande do Sul State, southern Brazil. The ticks were transported to the laboratory, washed in a 0.25% (v/v) sodium hypochlorite solution, rinsed with 0.85% NaCl (w/v) twice and dried. Females were selected and weighed to obtain the initial female weight (IFW), and homogenous weight groups were placed on Petri dishes.

Fungal growth and preparation of conidial suspensions

Stock cultures of the fungal strains were maintained on Sabouraud dextrose agar (SDA). To prepare conidial suspensions, the individual stock cultures were transferred to Petri dishes containing SDA and incubated for 15 days at 28°C, after which conidia from the

grown colonies were transferred to test tubes containing 10 mL aqueous 0.1% (v/v) Tween 80. The number of conidia was estimated using a Neubauer hemocytometer. For the bioassays, the conidial suspensions were decimally diluted to produce pure-strain suspensions containing 10^5, 10^7, and 10^9 conidia/mL. For the field tests, 25 mL of a single-strain suspension containing about 10^6 conidia/mL was prepared in aqueous 0.1% (v/v) Tween 80, added to 250 g sterilized rice in conical flasks and incubated for 15 days at 28°C, 60-80% relative humidity and a 16:8-h photoperiod. After incubation, cultures were pooled in a 25-L receptacle with 20 L aqueous 0.1% (v/v) Tween 80 and 0.05% (w/v) Mineral Adhesive Attach (Novartis). The receptacles were shaken to form a conidial suspension, which was decanted from rice and transferred to clean receptacles. This procedure was repeated several times to produce the amount of conidial suspension necessary for the tests under field conditions. For both laboratory bioassays and field tests, the procedures above were repeated for each strain.

Laboratory bioassays

Beauveria strain suspensions (10^5, 10^7 and 10^9 conidia/mL) were bioassayed (in triplicate) for *R.* (*B.*) *microplus* pathogenicity by immersing 10 engorged females (360 in total) in each of the suspensions for 1 min and then transferring the specimens to sterile Petri dishes containing moist filter paper, where they were kept for up to 20 days at 28°C and 90 ± 5% relative humidity. Three control groups of 10 engorged *R.* (*B.*) *microplus* females (30 in total) were treated in the same way, except that they were immersed in sterile aqueous 0.1% (v/v) Tween 80 solution instead of conidial suspension. Both *Beauveria*-treated and control ticks were evaluated for infection and mortality every 24 h. The Stendel (1980) method was used to obtain the IFW in grams of each tick before immersion in the conidial suspensions. Egg weight (EW) was obtained by separating the eggs from the females and weighing the eggs, which were then placed in test tubes and the hatching percentage (H%) was visually determined. Reproductive efficiency (RE) was calculated as RE = EW / IFW x H%, and the reproductive efficiency of the *Beauveria*-treated group was evaluated. Mortality percentage (M%), represented by the number of dead female ticks, was calculated as $M\% = RE_c - RE_t / RE_c \times 100$ (t = treated group; c = control group) (Stendel, 1980). Data were subjected to analysis of variance (ANOVA) and means compared using the Duncan test ($P < 0.05$). Median lethal dose (LD_{50}) values were calculated using the probit analysis (Finney, 1971).

Tests under field conditions

The test under field conditions was carried out in southern Brazil between September and February of 2006 and 2009, the spring-summer seasons in Brazil, where the incidence of parasites on cattle is higher. Holstein-Friesian cows infested with *R.* (*B.*) *microplus*, from the same three dairy farms (A, B and C) used before for tick-collection for the laboratory bioassays, were used for field tests. The group of cows used for each fungal strain and an additional group were used as the control, which was sprayed with the same volume of aqueous 0.1% (v/v) Tween-80. The treated groups were sprayed with 3 L *Beauveria* suspension containing 10^6 conidia/mL and then stabled for 72 h after which all ticks were collected both from cows and the adjacent stable floor. Dead ticks were counted and washed for 1 min in a 0.25% (v/v) sodium hypochlorite solution and placed on SDA plates, which were incubated at 28°C for up to 15 days to determine the presence of *Beauveria*.

RESULTS AND DISCUSSION

Our aim was the characterization and identification of two new endophytic *Beauveria* isolates designated B95 and B157. These isolates and two entomopathogenic fungi of *B. bassiana* were evaluated for biological control of *R.* (*B.*) *microplus* by laboratory bioassay and under field conditions. The results show that the *Beauveria* strains tested in the laboratory bioassays reduced EW and significantly reduced RE, where in both cases, the EW and RE values decreased progressively with increase in the concentration of conidia used (Table l). The mortality showed that endophytic *Beauveria* strains (B95 and B157) and the entomopathogenic strain CG478 were equally efficient at killing *R.* (*B.*) *microplus* females (Figure 1). The LD_{50} values differed for each *Beauveria* strain tested (Table 2).

Table 1. Laboratory bioassays of parameters evaluated: initial female weight, egg weight, reproductive efficiency, and mean percent mortality, for two different concentrations of conidial suspension of *Beauveria* strains.

Fungal strain	Conidia/mL	Initial female weight (g)	Egg weight (g)	Reproductive efficiency	Mortality percentage
B95	10^9	2.11 ± 0.08	0.56 ± 0.01	10.31 ± 0.25^{a}	86.54 ± 0.34^{a}
	10^7	2.07 ± 0.06	0.65 ± 0.01	14.80 ± 0.36^{bc}	80.28 ± 0.48^{bc}
	10^5	2.04 ± 0.03	0.83 ± 0.01	27.66 ± 0.40^{d}	66.42 ± 2.59^{d}
B157	10^9	2.08 ± 0.04	0.57 ± 0.03	13.19 ± 0.98^{ab}	82.77 ± 1.29^{ab}
	10^7	2.05 ± 0.05	0.69 ± 0.02	18.36 ± 1.44^{c}	75.55 ± 1.91^{c}
	10^5	2.04 ± 0.05	0.85 ± 0.03	30.17 ± 0.99^{de}	61.83 ± 1.25^{e}
CG166	10^9	2.05 ± 0.06	0.63 ± 0.05	13.56 ± 1.30^{b}	82.29 ± 1.69^{b}
	10^7	2.19 ± 0.03	0.85 ± 0.03	20.14 ± 1.07^{c}	73.17 ± 1.43^{c}
	10^5	2.13 ± 0.06	0.88 ± 0.02	32.65 ± 0.46^{e}	58.70 ± 0.58^{e}
CG478	10^9	2.13 ± 0.11	0.67 ± 0.05	12.69 ± 0.97^{ab}	83.42 ± 1.23^{ab}
	10^7	2.15 ± 0.12	0.82 ± 0.06	17.79 ± 0.92^{bc}	76.31 ± 1.22^{bc}
	10^5	2.07 ± 0.07	0.95 ± 0.03	30.49 ± 0.51^{de}	61.44 ± 0.64^{e}
Control	0	2.07 ± 0.04	1.70 ± 0.02	76.94 ± 2.00^{f}	0

Different superscript letters in the same column indicate a significant difference between concentrations by the Duncan test ($P < 0.05$).

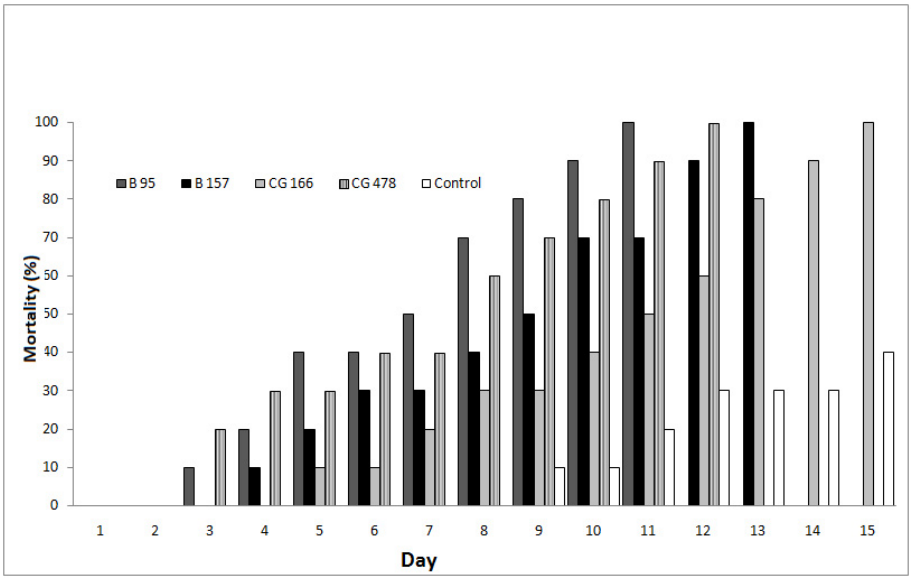

Figure 1. *Rhipicephalus* (*Boophilus*) *microplus* female mortality caused by different fungal strains of *Beauveria*.

Table 2. Lethal dose (LD_{50}) for engorged *Rhipicephalus* (*Boophilus*) *microplus* females.

Beauveria strain	LD_{50} (conidia/mL)	95% confidence limits	Probit x-axis*
B95	5.4×10^5	1.9×10^5 - 3.5×10^6	-2.23 + 0.405 [log10{dose$_i$}]
B157	7.6×10^8	3.5×10^8 - 1.3×10^9	-2.21 + 0.34 [log10{dose^i}]
CG166	4.6×10^9	2.3×10^9 - 8.6×10^9	-2.47 + 0.24 [log10{dose$_i$}]
CG478	2.3×10^7	8.6×10^6 - 5.7×10^7	-2.45 + 0.31 [log10{dose$_i$}]

*All values were calculated using the probit analysis (Finney, 1971).

The field tests showed that the mortality of *R.* (*B.*) *microplus* varied from 18.6 to 32.4%. The endophytic *Beauveria* strain B95 was the most efficient, followed by the entomopathogenic strain CG478 (Table 2). The endophytic *Beauveria* strain B95 induced the highest mortality percentage, and the entomopathogenic *B. bassiana* strain CG166 produced the lowest *R.* (*B.*) *microplus* mortality percentage (Figure 1). It has been reported that *B. bassiana* is an effective biological control agent for several *Rhipicephalus* species, including *R.* (*B.*) *microplus* and *R.* (*B.*) *annulatus*, with *R.* (*B.*) *annulatus* showing an *in vitro* mortality around 45% 7 days after treatment with *B. bassiana* (Bittencourt et al., 1997; Paião et al., 2001). Species of the fungus *Metarhizium* have also been used to control *R.* (*B.*) *microplus* based on similar procedures and variables (Onofre et al., 2001) as those used in bioassays previously reported. Our results using *Beauveria* strains are comparable to those obtained with *M. anisopliae* (Onofre et al., 2001) indicating that, in laboratory bioassays, *Beauveria* and *Metarhizium* are equally effective in controlling *R.* (*B.*) *microplus*, with the LD_{50} values for the *Beauveria* strains (Table 1) being similar to those recorded for *Metarhizium* strains. Endophytic fungi are commonly found inhabiting grasses (Graminae) (Azevedo et al., 2000) and *Beauveria* is also found in the soil (Hughes et al., 2004). The strains used in the present study were isolated from maize, and it is likely that they may also be encountered in association with pasture grasses, where they may act as a natural tick control agent. In fact, a combination of plant extracts and pasture grasses able to control ticks (Kaaya, 2000) may be a way to develop some form of integrated control (Kaaya, 1994). To our knowledge, endophytic fungi have not been previously evaluated for their pathogenicity against ticks. The results of the field tests showed that the mean mortality caused by strains B95, CG478 and B157 was superior to that produced by strain GC166 (Table 2). These are the first tests under field conditions in Brazil using endophytic fungi for the control of *R.* (*B.*) *microplus*. Field tests using *B. bassiana* and *M. anisopliae* for the biological control of the African tick *R.* (*B.*) *appendiculatus* resulted in 41% tick mortality 6 days after treatment, and some reports have indicated that mortality can be as high as 76-85% (Mwangi et al., 1995; Kaaya et al., 1996; Kaaya, 2000).

Fungal isolates capable of inducing such high mortalities at all stages in the life cycle of ticks are likely to significantly reduce tick populations and, consequently, the incidence of tick-borne and tick-associated diseases. Several sequences of *Beauveria* species from the GenBank database were compared with sequences of two endophytic *Beauveria* isolates, B95 and B157. Phylogenetic analysis showed that the isolates are closer to *Beauveria amorpha* (Höhn.) than *B. bassiana* (Figure 2). Optical and scanning microscopy assays using isolates B95 and B157 to measure conidial size and shape were performed to verify the *Beauveria* endophytic isolates (Sia, 2006): in particular, B157 conidia are much larger than *B. bassiana* conidia, showing a flattened shape.

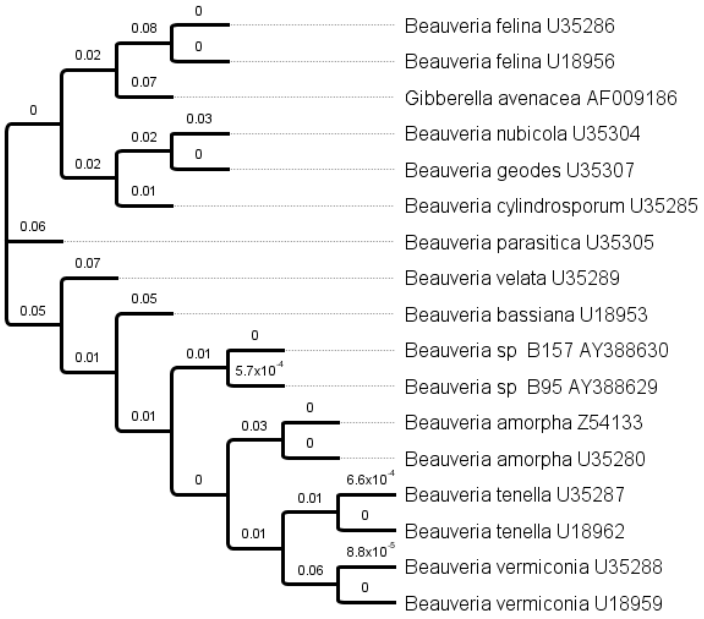

Figure 2. Phylogenetic analysis of two endophytic isolates belonging to the *Beauveria* genus. Neighbor-joining method. Numbers on the tree branches indicate the Jukes-Cantor distance. Geneious Program Tree Builder, Release 4.6.4. Entophytic strains available in GenBank accession Nos. AY388629 and AY388630.

These results are in agreement with results described by Campos et al. (2005), where scanning electron microscopy was used in order to find morphologic differences between the *Beauveria* species studied and their form of penetration of the cattle tick cuticle. The conidial morphology of *B*. *bassiana* showed a spherical shape, whereas *B*. *amorpha* conidia on ticks were often flattened on the side as in the original descriptions by Glare and Inwood (1998), indicating that isolates B95 and B157 are closer to *B*. *amorpha*. The use of *Beauveria* in a biological control program in Brazil and other countries could reduce the use of chemical acaricides and could result not only in lower costs to the farmer but also in a lower environmental impact and less damage to nature.

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