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An intensive search for promising fungal biological control agents of ticks, particularly *Rhipicephalus microplus*

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ABSTRACT

Entomopathogenic fungi have been investigated worldwide as promising biological control agents of the cattle tick *Rhipicephalus microplus*. The current study evaluates the virulence of several fungal isolates to *R. microplus* larva in the laboratory as part of an effort to identify isolates with promise for effective biocontrol of *R. microplus* in the field. Sixty fungal isolates, encompassing 5 *Beauveria* spp. and 1 *Engyodontium albus* (=*Beauveria alba*), were included in this study. In addition to bioassays, the isolates were characterized morphologically and investigated as to their potential for conidial mass production. These findings were correlated with previous reports on the same fungal isolates of their natural UV-B tolerance (Fernandes et al., 2007), thermotolerance and cold activity (Fernandes et al., 2008), and genotypes (Fernandes et al., 2009). *R. microplus* larvae obtained from artificially infested calves were less susceptible to *Beauveria bassiana* infection than ticks acquired from naturally infested cattle from a different location. Isolates CG 464, CG 500 and CG 206 were among the most virulent *Beauveria* isolates tested in this study. All fungal isolates presented morphological features consistent with their species descriptions. Of the 53 *B. bassiana* isolates, five (CG 481, CG 484, CG 206, CG 235 and CG 487) had characteristics that qualified them as promising candidates for biological control agents of *R. microplus*, viz., mean LC_{50} between 10^7 and 10^8 conidia ml^{-1}; produced 5000 conidia or more on 60 mm^2 surface area of PDAY medium; and, in comparison to untreated (control) conidia, had the best conidial tolerances to UV-B (7.04 $kJ\,m^{-2}$) and heat (45 °C, 2 h) of 50% or higher, and conidial cold (5 °C, 15 d) activity (mycelial growth) higher than 60%. The current study of 60 *Beauveria* spp. isolates, therefore, singles out a few (five) with high potential for controlling ticks under field conditions.

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1. Introduction

The tick *Rhipicephalus* (*Boophilus*) *microplus* Canestrini, 1887 (Acari: Ixodida) (Murrell and Barker, 2003), formerly *Boophilus microplus*, is one of the most important bovine ectoparasites in Brazil and several other countries worldwide. Economic losses to the cattle industry in Brazil alone are estimated at two billion dollars per year (Grisi et al., 2002). These economic losses include costs associated with the use of chemical acaricides for tick control. The continual use of these chemicals, however, has many negative

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side effects; including the development of chemical resistance in tick populations, as well as food and environmental contamination if the products are improperly used. Public concerns about the environmental impacts and safety to vertebrates of widespread chemical acaricide use are driving research towards alternative, sustainable methods for tick control, including biological control (Chandler et al., 2000).

Entomopathogenic fungi (EPF) are natural enemies of arthropods, and they have been investigated worldwide as promising biological control agents of ticks (Chandler et al., 2000; Fernandes and Bittencourt, 2008; Samish and Rehacek, 1999). *Beauveria* (Balsamo) Vuillemin (Ascomycota: Hypocreales) is one of the EPF groups most commonly studied; primarily due to their cosmopolitan geographical distribution, wide host range, and capacity to cause enzootic and epizootic outbreaks in several arthropod pests (Alves, 1998; Roberts and Campbell, 1977).

Due to environmental factors, especially the exposure of conidia to strong solar irradiation, reduced viability and/or conidia germination delay is fully expected to reduce the bioinsecticidal efficacy of fungal inocula in field situations (Braga et al., 2002; Fargues et al., 1997). In addition, conidia in the environment also are exposed to indirect solar effects, such as heat and desiccation (Luz and Fargues, 1997; Magalhães and Boucias, 2004; Rangel et al., 2005). Through selection of the isolates most tolerant to UV-B radiation as well as incorporating UV protectants in formulations, it may be possible to significantly prolong the persistence and increase efficacy of these fungi in highly insolated habitats (Fargues et al., 1996). Also, effective use of insect pathogens within integrated tick management programs necessitates the selection of fungal pathogens tolerant to the temperature range found in the host arthropod's ecosystem, including on the skin of these warm-blooded animals.

The current study investigates the virulence of 60 *Beauveria*-like fungal isolates to *R. microplus* larva (in a search for isolates with high potential for biological control of this tick species). Bioassay ticks originated from two different locations, and from either artificially or naturally infested cattle. In addition, the fungal isolates were characterized morphologically; and the isolates with the highest potential for conidial mass production identified. The findings were compared with previous studies of the same isolates which evaluated UV-B tolerance (Fernandes et al., 2007), elevated heat tolerance and cold activity (Fernandes et al., 2008), and their genotypes (Fernandes et al., 2009). Comparisons of these findings allowed the selection of a short list of isolates most promising for further investigation as tick biocontrol agents.

2. Materials and methods

2.1. Fungal isolates

Fifty-three *Beauveria bassiana* isolates were included in this study: 49 originating from different regions of Brazil and 4 from USA. In addition, 6 isolates of other *Beauveria* spp. (3 *Beauveria amorpha*, 1 *Beauveria brongniartii*, 1 *Beauveria velata* and 1 *Beauveria vermiconia*), and 1 isolate of *Engyodontium albus* (= *B. alba*) were included. The fungal isolates were originally from Acari, six orders of insects, or soil. This study emphasized Brazilian *B. bassiana* isolates; with isolates of other *Beauveria* species or from other geographic origins being used primarily as general references. The fungal isolates investigated in the current study were known to have considerable genotypic variation (Fernandes et al., 2009). The designation of the isolates, the culture collections from which they were obtained, their geographical origins, and hosts or substrates from which they were isolated are available at Fernandes et al. (2007, 2008, 2009).

2.2. Bioassays

2.2.1. R. microplus larvae from eggs from engorged females collected on artificially infested calves (Group A)

Engorged females of *R. microplus* were manually collected directly from naturally tick-infested cattle at the Universidade Federal Rural do Rio de Janeiro (UFRRJ) dairy farm, Seropédica, Rio de Janeiro State, Brazil. Ticks were surface sterilized by immersion in a 1% sodium hypochlorite solution for 3 min, rinsed with sterile distilled water and dried with sterile tissue paper. The engorged females were held in polystyrene Petri plates (95 mm × 15 mm, BD Falcon®, São Paulo, SP, Brazil) incubated in the dark at $27 \pm 1\,^{\circ}$C and ≥80% relative humidity (RH) for oviposition. Eggs were separated in aliquots of 100 mg (approximately 2000 eggs) and incubated at the same temperature and RH conditions to allow them to hatch. Four calves were held in individual pens at the UFRRJ W.O. Neitz Station for Parasitology Reseach, and each calf was artificially infested once with 6000 larvae. Twenty-one days after infestation, engorged females were manually collected directly from the calves and from the floor of each pen. Ticks were taken to the laboratory for cuticle antisepsis and oviposition as described above. Ten days after the beginning of oviposition, the eggs were divided into 50 mg aliquots (approximately 1000 eggs) and placed in glass test tubes (150 mm × 15 mm, Pyrex®, São Paulo, SP, Brazil). The test tubes were sealed with hydrophilic cotton plugs and incubated at $27 \pm 1\,^{\circ}$C and RH ≥ 80%. Since *R. microplus* larvae have strong negative geotropism and positive phototropism, the tubes were held vertically with the cotton plugs down (in the shadows), and the glass end in the light. The bioassays were carried out 10 days after total hatch. The tubes that did not have complete hatch were discarded to ensure that each test tube had approximately 1000 live *R. microplus* larvae.

2.2.2. R. microplus larvae from eggs from engorged females collected on naturally infested cattle (Group B)

Five *B. bassiana* isolates from among the 60 isolates previously tested against Group A ticks, due to their low virulence, were chosen to be tested on *R. microplus* larvae obtained from engorged females collected directly from naturally tick-infested cattle (Group B) on a private farm located on highway Presidente Dutra, Km 201, in Seropédica, RJ. This farm is approximately 10 km from the UFRRJ dairy farm where Group-A ticks were collected. Engorged females were surface sterilized and held in the

lab following exactly the same methods and conditions (temperature and RH) used with ticks from Group A. Also, as before, the eggs were divided into 50 mg aliquots in glass test tubes, and the bioassays were carried out 10 days after total hatch of larvae to ensure that each test tube had approximately 1000 vigorous *R. microplus* larvae. These bioassays were conducted immediately after the second repetition of the bioassay with Group A ticks, but with new batches of conidia.

2.2.3. Preparation of conidial suspensions

The fungal isolates were cultured on 23 ml potato dextrose agar medium (Difco Laboratories, Sparks, MD, USA) supplemented with $1\,g\,l^{-1}$ yeast extract (Technical, Difco) (PDAY) in polystyrene Petri plates (95 mm × 15 mm) in the dark at 25 ± 1 °C for 15 days. Conidia were harvested with a microbiological loop and suspended in polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Co., St. Louis, MO, USA) solution (0.01%, v/v). Conidial suspensions were quantified using the hemacytometer. A suspension at 1×10^8 conidia ml^{-1} was prepared, and suspensions at 10^7, 10^6 and 10^5 conidia ml^{-1} were obtained through serial dilutions. Sterile aqueous Tween 80 (0.01%) solution was used to treat the control groups. The conidial viability was evaluated according to Alves (1998).

2.2.4. Treatments

The bioassay consisted of four treatment groups ($1 \times 10^8, 10^7, 10^6$ and 10^5 conidia ml^{-1}); each dosage group had 10 test tubes containing *R. microplus* larvae. The test tubes were turned to cotton-plug-up orientation, and 1 ml of conidial suspension was injected into the tube using a hypodermic syringe with the needle inserted between the cotton plug and glass-tube wall. The larvae were held immersed in the inoculum for 5 min, and thereafter the tubes were inverted until all conidial suspension had been absorbed by the cotton plug. Tubes were maintained in this position for the remainder of the experiment. Due to their phototropism, living larvae remained at the clear tip of tubes where they were easily inspected at 10× magnification for signs of viability (movement). Each of the 10 test-tube groups had one additional control group of 10 test tubes containing 1000 larvae each; each tube received only 1 ml of 0.01% Tween 80 solution with no conidia, and followed the same procedure described above. The test tubes were incubated at 27 ± 1 °C and RH ≥ 80% in the dark. Larval mortality was recorded at 5-day intervals for 30 days. Percentage of larval mortality for each tube was visually estimated through microscopic observation; with the estimates expressed as varying from 0% to 100%, with 5% intervals. The bioassay methodology was based on Bittencourt et al. (1996). Each of the two bioassays was repeated once on a different day, with new conidial preparations used for each assay.

2.2.5. Statistical analysis of bioassays

The virulence of fungal isolates to *R. microplus* larvae was assessed using the non-parametric analysis of Kruskal–Wallis because the data obtained (percentage of larval mortality) were qualitatively estimated and represented a discrete variable. The means of the ranks were compared by the SNK test (Student–Newman–Keuls) to determine significant variations among treatments. *P*-values less than 0.05 were considered as significant (Sampaio, 2002). Data analyses were generated using Kruskal–Wallis software, Version 1.0 for Linux. Virulence among fungal isolates was compared by calculation of the median lethal concentration (LC_{50}) using probit analysis (Finney, 1971) generated by Probit or Logit Analysis, POLO-PC (LeOra Software, 1987, Berkeley, CA, USA).

2.3. Morphological characterization of fungal isolates

All fungal isolates were inoculated on 23 ml oatmeal agar (OA [60 g oatmeal flour (Quaker, Pepsico do Brazil), 12.5 g agar (Technical, Difco), 1000 ml distilled water]) in Petri plates (95 mm × 15 mm) using a 0.25mm-width-steel needle. Every isolate was inoculated in the center of three Petri plates, and incubated in the dark at temperatures and time periods that varied according to the literature on each species: *B. amorpha*: 25 ± 1 °C for 14 days, *B. velata*: 25 ± 1 °C for 7 days (Samson and Evans, 1982), and *B. bassiana*, *B. brongniartii*, *B. vermiconia* and *E. albus*: 20 ± 1 °C for 8 days (De Hoog, 1972, 1978; De Hoog and Rao, 1975). Colonies of each isolate were evaluated as to growth rate, general aspect, color of conidial masses and colony reverses. The micromorphology of each isolate also was evaluated according to the microculture technique described by Rivalier and Seydel (1932). Briefly, OA medium was solidified in Petri plates, blocks of medium were cut and placed on sterile microscope slides, inoculated with conidia, a sterile coverslip placed on the agar block. The slides were incubated at high humidity in dishes lined with wet filter paper using the same temperature conditions described above. For microscopic observation, the coverslip was placed on a drop of lactophenol cotton blue on a new slide. Also, a drop of lactophenol cotton blue was placed on the slide where the fungus had been growing, followed by a clean coverslip. Micromorphology observations were made at 1000× magnification.

2.4. Production of conidia

Dry conidia of all fungal isolates were inoculated by spreading with a microbiological loop on 23 ml PDAY in Petri plates (95 × 15). The isolates were cultured at 25 ± 1 °C in the dark for 20 days. Each culture was randomly punched (cut) 3 times with a 5-mm-diameter cork borer, and the plugs of medium and fungus were transferred to a 2-ml-microcentrifuge tube containing 1 ml of Tween 80 solution (0.1%, v/v). The tubes were vortexed for 30 s, and the conidial concentrations were quantified using a hemacytometer to estimate the number of conidia produced on $60\,mm^2$ of PDAY surface. The test was repeated at least twice on different days with new batches of conidia each day.

2.5. Pearson correlation analysis

Traits examined for each fungal isolate included: size, texture and color of fungal colony; smallest and largest conidial diameter; smallest and largest conidial length;

smallest and largest lateral-cell diameter; smallest and largest lateral-cell length; smallest and largest diameter of conidiogenous cell base; smallest and largest length of conidiogenous cells; dominance of clusters of conidiogenous cells; dominance of single conidiogenous cells; lethal concentration (LC_{50}) of conidia to control *R. microplus* larva; conidial production on PDAY medium; conidial tolerance to UV-B irradiation ($7.04\,kJ\,m^{-2}$) (Fernandes et al., 2007); conidial tolerance to heat (45 °C, 2 h) (Fernandes et al., 2008); and conidial cold (5 °C, 15 d) activity (Fernandes et al., 2008). Basic Pearson correlations coefficients were generated for each trait using the CORR procedure in SAS/STAT software, Version 9.1.3 of the SAS system for Windows. *P* values less than 0.01 were considered as significant.

3. Results

3.1. Virulence of fungal isolates to R. microplus larva

Conidial viability of all the isolates was high, viz., 98–100%. No tick mortality was observed in control groups, whereas mortality in the treated groups ranged between 0% and 100%, depending on the isolate and conidial concentration. There was high variability among the fungal isolates (Table 1). In general, mortality was proportional to the conidial concentration, i.e., the higher the conidial concentration, the higher the larval mortality (Table 1). The *B. bassiana* isolates CG 464, CG 500 and CG 206 were among of the most virulent to *R. microplus* larva; they had low median LC_{50s} and narrow confidence intervals. Some *B. bassiana* isolates, however, viz. UFPE 496 and UFPE 479, did not cause larval mortality at any of the conidial concentrations. Isolates other than *B. bassiana* had low virulence, with the exception of *B. amorpha* isolate ARSEF 4755 (Table 1).

The bioassay with Group-B larvae showed higher mortality within a shorter period of time (Fig. 1). The mean lethal doses (LC_{50}) of the five *B. bassiana* isolates tested (Bb 23, Bb 44, CG 408, ESALQ 747 and ESALQ 986) with Group B larvae were considerably lower than with ticks from Group A, indicating greater susceptibility of Group B larvae. For example, isolates Bb 44 and ESALQ 986 tested with Group B ticks had lower LC_{50} at day 10 after treatment, than with Group A ticks at day 30 after treatment (Table 2). As the bioassay with ticks from Group A, mortality with group-B ticks tended to be proportional to the conidial concentration; and no mortality was observed in the control groups.

3.2. Morphological characterization of fungal isolates

All fungal isolates presented the key morphological features consistent with their published species descriptions: *B. bassiana* and *B. brongniartii* according to De Hoog (1972), *B. amorpha* and *B. velata* according to Samson and Evans (1982), *B. vermiconia* according to De Hoog and Rao (1975), and *E. albus* according to De Hoog (1972, 1978) (Table 3).

The colony sizes of *B. bassiana* isolates at 8 days varied from 9.48 mm (CG 66) to 22.93 mm (Bb19), with an average of 19.63 ± 3.16 mm; the colonies were lanose, floccose or velvety in texture. In general, the colonies were white, but some were slightly yellow. The colonies' reverses were mostly yellow or pink. Micromorphologically, *B. bassiana* conidial surfaces were smooth, and their shapes were globose or subglobose. The smallest *B. bassiana* conidium measured was $1.0\,\mu m \times 1.0\,\mu m$ (CG 228), the largest conidium measured was $3.0\,\mu m \times 5.0\,\mu m$ (CG 367). Lateral cells of *B. bassiana* were numerous in some isolates and rare in others. The smallest lateral cell measured $2.0\,\mu m \times 3.0\,\mu m$ (viz., GHA, CG 234, CG 235, CG 251 and UFPE 479), while the largest lateral cell measured $3.0\,\mu m \times 6.0\,\mu m$ (CG 154). Conidiogenous cells of *B. bassiana* were globose or flask-shaped and geniculated. The base of conidiogenous cells of *B. bassiana* varied from $1.5\,\mu m$ to $3.0\,\mu m$, and the length varied from $4.0\,\mu m$ to $27.0\,\mu m$ among the isolates; also, the length of conidiogenous cells varied markedly in many isolates (viz., CG 149, CG 206, CCT 4641). Many *B. bassiana* isolates had clusters of conidiogenous cells; some isolates, however, had mostly individual conidiogenous cells (see Table 3).

3.3. Production of conidia

The *B. bassiana* isolates had high variability in conidial production. About one third of the *B. bassiana* isolates produced less than 2.0×10^7 conidia on $60\,mm^2$ of PDAY medium, another third produced between 2.0×10^7 and 5.0×10^7 conidia, and the last third produced more than 5.0×10^7 conidia on the same area of PDAY (Table 4). Isolates other than *B. bassiana* were all low conidial producers, with exception of the isolate ARSEF 4755 (*B. amorpha*) that produced more than 4.0×10^7 conidia on $60\,mm^2$ of PDAY (see Table 4).

3.4. Connecting the results

Based on this screening of 60 isolates, five *B. bassiana* isolates (CG 481, CG 484, CG 206, CG 235 and CG 487) show promise as candidates for biological control agents of *R. microplus* larva. These isolates had mean LC_{50} values between 10^7 and 10^8 conidia ml^{-1} with narrow confidence intervals, produced an average of 5000 conidia (or more) on $60\,mm^2$ surface area of PDAY medium, had conidial tolerances to UV-B and heat equal to or higher than 50%, and conidial cold activity higher than 60%. The three of the five promising isolates were similar by isozyme and DNA analyses: CG 484, CG 206 and CG 235 grouped together (Group 4) based on Multilocus Enzyme Electrophoresis (MLEE) with approximately 96% similarity (Fernandes et al., 2009), and isolates CG 206 and CG 235 were grouped together (Group 17) based on Amplified Fragment Length Polymorphism (AFLP) with 100% similarity (Fernandes et al., 2009) (see Table 4).

Strong Pearson correlation (r) among *B. bassiana* isolates was observed between conidial production and conidial tolerance to heat ($r = 0.41$, $P = 0.002$). In addition, there was correlation between high conidial production and presence or absence of conidiogenous-cell clusters ($r = 0.35$, $P = 0.009$).

Table 1
Mean mortality of *Rhipicephalus microplus* Group A larvae and mean lethal concentration (LC_{50}) of *Beauveria* spp. and *Engyodontium albus* (= *Beauveria alba*) isolates at day 30 after treatment.[a]

Species and isolates	Mean mortality of *Rhipicephalus microplus* larvae (%)[b]					LC_{50} and confidence intervals (conidia ml^{-1})	
	Control	10^5	10^6	10^7	10^8		
Beauveria bassiana							
Bb 02	0.0 a	3.0 a	14.5 b	41.3 c	26.6 c	6.31×10^9	(1.05×10^8–3.80×10^{11})
Bb 09	0.0 a	0.3 a	1.3 a	22.8 b	24.8 b	6.55×10^9	(3.35×10^8–1.28×10^{11})
Bb 13	0.0 a	0.0 a	3.4 b	12.6 c	9.2 c	1.60×10^{12}	(6.54×10^6–3.88×10^{17})
Bb 15	0.0 a	1.0 a	1.8 ab	7.5 b	17.5 c	6.88×10^{10}	(1.49×10^8 –3.18×10^{13})
Bb 19	0.0 a	4.5 b	6.0 b	25.0 c	25.0 c	2.82×10^{10}	(1.16×10^8 –6.88×10^{12})
Bb 21	0.0 a	3.5 b	9.8 c	10.5 cd	45.0 d	3.07×10^9	(2.46×10^8–3.83×10^{10})
Bb 23	0.0 a	0.8 a	2.0 ab	11.3 b	8.8 b	3.11×10^{12}	(2.48×10^6–3.90×10^{18})
Bb 27	0.0 a	0.0 a	0.5 a	5.3 b	6.8 b	1.20×10^{12}	(3.21×10^7–4.50×10^{17})
Bb 31	0.0 a	4.8 b	6.5 b	16.0 c	34.3 c	1.10×10^{10}	(2.01×10^8–5.00×10^{11})
Bb 35	0.0 a	0.0 a	6.0 b	6.0 b	9.0 b	2.94×10^{13}	(2.84×10^4–5.45×10^{22})
Bb 38	0.0 a	1.3 a	5.8 b	24.0 c	25.0 c	1.21×10^{10}	(2.22×10^8–6.60×10^{11})
Bb 44	0.0 a	13.0 b	16.5 bc	23.9 cd	36.8 d	2.63×10^{10}	(2.40×10^7–2.88×10^{13})
Bb 46	0.0 a	8.8 b	12.0 bc	18.0 c	58.4 d	1.00×10^9	(1.38×10^8–7.23×10^9)
LCM 01	0.0 a	6.8 b	13.0 b	42.1 c	48.8 c	6.82×10^8	(1.02×10^8–4.55×10^9)
ESALQ 986	0.0 a	12.3 b	12.8 b	17.3 b	58.5 c	1.21×10^9	(1.17×10^8–1.26×10^{10})
CG 66	0.0 a	0.0 a	3.3 b	6.8 b	21.3 c	2.20×10^{10}	(2.80×10^8–1.73×10^{12})
CG 222	0.0 a	3.8 b	4.5 b	12.8 c	40.3 d	4.00×10^9	(2.82×10^8–5.66×10^{10})
CG 227	0.0 a	5.8 b	8.9 bc	13.8 c	46.1 d	3.02×10^9	(2.09×10^8–4.35×10^{10})
CG 228	0.0 a	1.5 ab	3.5 b	13.0 c	38.8 d	3.00×10^9	(3.39×10^8–2.59×10^{10})
CG 319	0.0 a	8.5 b	23.3 b	60.0 c	70.8 c	9.21×10^7	(3.14×10^7–2.70×10^8)
CG 464	0.0 a	13.5 b	32.5 c	56.5 cd	95.0 d	3.23×10^7	(1.37×10^7–7.63×10^7)
CG 481	0.0 a	17.5 b	29.0 bc	44.0 c	56.0 c	3.29×10^8	(3.22×10^7–3.36×10^9)
CG 484	0.0 a	11.8 b	21.6 bc	34.7 cd	50.0 d	9.95×10^8	(6.75×10^7–1.47×10^{10})
CG 495	0.0 a	1.8 ab	4.8 b	18.3 c	50.8 c	1.10×10^9	(2.49×10^8–4.89×10^9)
CG 500	0.0 a	31.8 b	35.3 bc	61.0 cd	69.3 d	3.53×10^7	(5.99×10^6–2.08×10^8)
ARSEF 252	0.0 a	3.5 b	4.0 b	17.0 c	22.0 c	6.87×10^{10}	(1.04×10^8–4.53×10^{13})
GHA	0.0 a	2.0 a	4.5 a	25.0 b	23.5 b	1.63×10^{10}	(1.98×10^8–1.33×10^{12})
CG 02	0.0 a	8.9 b	15.5 bc	22.0 cd	30.3 d	7.41×10^{10}	(1.95×10^7–2.81×10^{14})
CG 138	0.0 a	3.0 ab	13.0 bc	31.0 cd	74.0 d	2.38×10^8	(8.94×10^7–6.33×10^8)
CG 367	0.0 a	0.0 a	4.0 b	20.8 c	30.0 c	4.45×10^9	(3.26×10^8–6.08×10^{10})
CG 471	0.0 a	2.5 ab	6.0 b	18.8 c	75.8 d	3.35×10^8	(1.39×10^8–8.07×10^8)
CG 478	0.0 a	11.8 b	17.8 b	47.5 c	78.8 d	1.04×10^8	(3.64×10^7–2.95×10^8)
CG 483	0.0 a	4.8 b	10.3 b	27.1 c	41.6 c	2.32×10^9	(1.72×10^8–3.14×10^{10})
EP 01	0.0 a	15.5 b	19.3 b	40.8 c	65.6 c	2.32×10^8	(4.70×10^8–1.15×10^9)
CG 17	0.0 a	5.5 ab	17.4 b	35.8 c	56.0 c	4.90×10^8	(9.65×10^7–2.48×10^9)
UFPE 496	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	–	–
CG 01	0.0 a	6.1 b	6.8 b	7.0 b	10.0 b	1.91×10^{23}	Excessive variation
CG 149	0.0 a	4.0 b	4.3 b	6.8 b	21.9 c	2.74×10^{10}	(4.18×10^7–1.80×10^{15})
CG 154	0.0 a	2.0 ab	2.0 ab	7.0 bc	26.0 c	2.61×10^{11}	(3.84×10^7–1.78×10^{15})
CG 234	0.0 a	0.0 a	0.0 a	0.0 a	0.5 a	7.92×10^{21}	(5.88×10^{-39}–1.70×10^{38})
CG 206	0.0 a	4.0 ab	14.0 bc	60.0 cd	93.0 d	6.10×10^7	(3.03×10^7–1.23×10^8)
CG 235	0.0 a	4.5 ab	16.0 bc	30.0 c	50.6 c	8.92×10^8	(1.29×10^8–6.18×10^9)
CG 479	0.0 a	1.0 a	2.5 ab	6.5 b	12.3 b	8.63×10^{11}	(1.33×10^7–5.61×10^{16})
ESALQ 747	0.0 a	1.0 ab	1.7 abc	3.5 bc	4.75 c	2.79×10^{11}	(2.93×10^5–2.65×10^{37})
CCT 4641	0.0 a	2.0 ab	2.0 ab	5.0 bc	13.9 d	1.12×10^{12}	(9.83×10^6–1.28×10^{17})
CG 251	0.0 a	2.5 b	7.8 b	19.0 b	20.0 b	1.91×10^{23}	Excessive variation
CG 480	0.0 a	1.3 a	0.8 a	5.0 b	13.5 c	2.81×10^{11}	(4.08×10^7–1.94×10^{15})
CG 487	0.0 a	3.8 b	6.3 b	36.8 c	51.3 c	6.66×10^8	(1.48×10^8–2.99×10^9)
UFPE 479	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	–	–
CG 307	0.0 a	2.5 a	8.5 b	19.4 c	55.0 c	8.85×10^8	(1.99×10^8–3.94×10^9)
CG 309	0.0 a	1.0 a	12.0 b	18.0 bc	58.2 c	7.12×10^8	(1.85×10^8–2.74×10^9)
CG 310	0.0 a	1.5 a	1.8 a	10.7 b	29.0 b	8.25×10^9	(3.53×10^8–1.93×10^{11})
CCT 3161	0.0 a	2.0 ab	2.3 ab	5.3 bc	8.8 c	1.89×10^{14}	(7.56×10^2–4.71×10^{25})
Beauveria amorpha							
ARSEF 656	0.0 a	0.0 a	0.0 a	0.8 a	1.3 a	7.34×10^{15}	(1.53×10^{12}–1.70×10^{38})
ARSEF 1682	0.0 a	0.0 a	1.0 a	2.3 ab	9.3 b	2.57×10^{11}	(1.85×10^7–3.59×10^{15})
ARSEF 4755	0.0 a	18.0 b	28.9 b	66.0 c	64.7 c	6.94×10^7	(1.71×10^7–2.81×10^8)
Beauveria brongniartii							
ATCC 58798	0.0 a	0.0 a	0.0 a	1.5 a	3.5 a	5.78×10^{12}	(2.53×10^3–1.32×10^{22})
Beauveria velata							
ARSEF 2998	0.0 a	0.0 a	0.0 a	0.0 a	9.2 b	2.04×10^{10}	(6.16×10^7–6.78×10^{12})
Beauveria vermiconia							
ARSEF 2922	0.0 a	2.8 b	6.0 c	8.6 cd	10.0 d	2.48×10^{15}	(1.14×10^{30}–5.34×10^{30})
Engyodontium albus (=*Beauveria alba*)							
UFPE 3138	0.0 a	0.0 a	1.3 ab	2.5 bc	3.5 c	1.04×10^{15}	(0.72×10^6–1.52×10^{31})

[a] Data reported here were obtained with Group A tick larvae (= larvae obtained from eggs from engorged female ticks developed on artificially infested calves in barn stables). Each dosage group had 10 test tubes with each tube containing approximately 1000 *R. microplus* larvae. The bioassay was conducted twice, on two different days, using new conidial preparations each day.
[b] Means with the same letter in the same row do not differ significantly at $P > 0.05$ (Kruskal–Wallis test followed by SNK test).

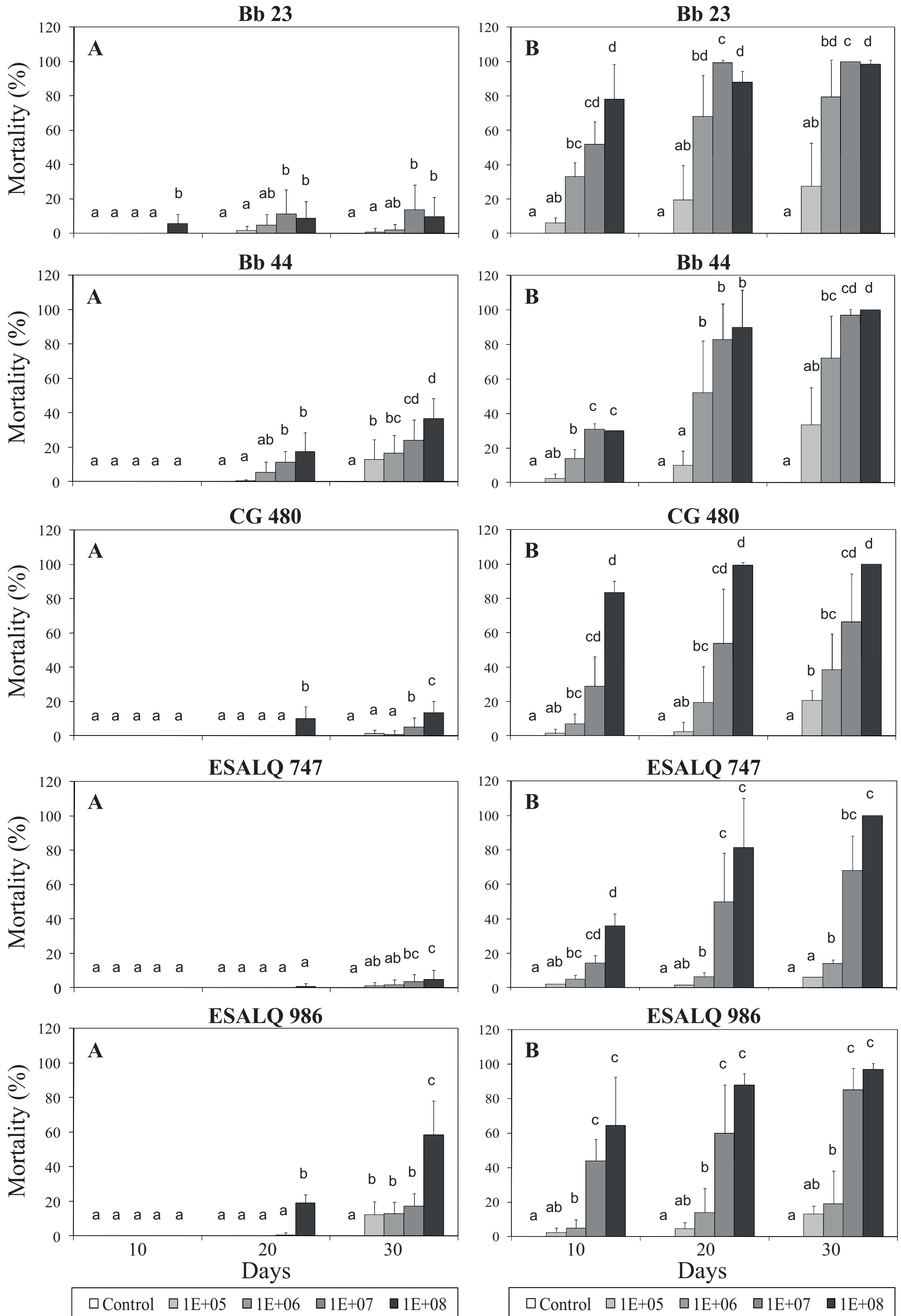
Bb 23
A
B
Bb 44
CG 480
ESALQ 747
ESALQ 986
Mortality (%)
0
20
40
60
80
100
120
10
20
30
Days
Control
1E+05
1E+06
1E+07
1E+08

Table 2
Mean lethal concentrations (LC_{50} and LC_{90}) of *Beauveria bassiana* isolates in bioassays with *Rhipicephalus microplus* Group-B larvae at days 10, 20 and 30 after treatment.[a]

B. bassiana isolates	Mean lethal concentrations (LC_{50} and LC_{90}) and confidence intervals	
	LC_{50} (conidia ml^{-1})	LC_{90} (conidia ml^{-1})
Day 10		
Bb 23	2.98×10^6 (1.46×10^6–6.09×10^6)	2.89×10^7 (1.01×10^7–9.78×10^7)
Bb 44	3.02×10^6 (1.38×10^6–6.60×10^6)	3.83×10^7 (1.25×10^7–1.40×10^8)
CG 480	2.01×10^7 (8.97×10^6–4.51×10^7)	4.99×10^8 (1.27×10^8–2.44×10^9)
ESALQ 747	5.24×10^7 (2.99×10^7–9.20×10^7)	2.92×10^8 (1.23×10^8–7.99×10^8)
ESALQ 986	2.72×10^7 (1.40×10^7–5.27×10^7)	2.84×10^8 (1.00×10^8–9.53×10^8)
Day 20		
Bb 23	6.43×10^6 (1.97×10^6–2.01×10^7)	6.30×10^8 (9.03×10^7–6.03×10^9)
Bb 44	1.78×10^7 (7.99×10^6–3.97×10^7)	4.19×10^8 (1.10×10^8–1.97×10^9)
CG 480	6.36×10^7 (3.43×10^7–1.18×10^8)	5.01×10^8 (1.89×10^8–1.56×10^9)
ESALQ 747	1.65×10^8 (8.30×10^7–3.30×10^8)	1.77×10^9 (5.19×10^8–7.36×10^9)
ESALQ 986	9.07×10^7 (4.57×10^7–1.80×10^8)	1.04×10^9 (3.26×10^8–4.00×10^9)
Day 30		
Bb 23	1.11×10^7 (3.17×10^7–1.56×10^8)	1.56×10^9 (3.61×10^8–8.59×10^9)
Bb 44	9.68×10^9 (1.89×10^8–4.95×10^{11})	6.86×10^{12} (1.25×10^9–1.52×10^{17})
CG 480	2.48×10^8 (1.27×10^8––4.82×10^8)	2.16×10^9 (6.47×10^8–8.75×10^9)
ESALQ 747	4.28×10^9 (4.06×10^8–4.52×10^{10})	2.07×10^{11} (1.65×10^9–5.67×10^{13})
ESALQ 986	3.40×10^8 (1.38×10^8–8.41×10^8)	6.69×10^9 (1.05×10^9–5.75×10^{10})

[a] Data reported here were obtained with Group B tick larvae (= larvae obtained from eggs from engorged female ticks collected directly from naturally infested cattle).

4. Discussion

Considerable variation in virulence to *R. microplus* larva was detected among the *Beauveria* spp. isolates. In addition to differing genetic backgrounds, other factors may determine the virulence of entomopathogenic fungal isolates towards a specific host. For example, reduction of virulence has been reported among isolates of *B. bassiana* and *Metarhizium anisopliae* sensu lato (s.l.) after repeated cultivation on artificial media (Alves, 1998). Some *B. bassiana* isolates, however, seem to be less susceptible to virulence reduction on successive cultivation on artificial culture media and retain their virulence after repeated transfer (Brownbridge et al., 2001). On the other hand, the passage *in vivo* (through host arthropods) of isolates of *B. bassiana* or *M. anisopliae* may increase their virulence to arthropods (Fargues and Robert, 1983; Frazzon et al., 2000; Schaerffenberg, 1964; Wasti and Hartmann, 1975). Previous studies suggested that isolates originating from naturally infected arthropods are more virulent to arthropods that belong to the same order (Goettel et al., 1990; Vicentini et al., 2001). In the present study, however, several *B. bassiana* isolates obtained from naturally infected ticks (viz., Bb 13, Bb 23, Bb 27, Bb 35) were not significantly more virulent to *R. microplus* larvae than isolates obtained from other arthropod orders, such as CG 464 or CG 206 isolated from Coleoptera and Hymenoptera, respectively. Also, variation in virulence among entomopathogenic fungal isolates has been reported in several studies with no apparent correlation to other traits (Barci et al., 2009; Luz et al., 1998; Paião et al., 2001; Posadas and Lecuona, 2009).

Tick larvae obtained from Group A were less susceptible to *B. bassiana* infection than larvae from Group B. Greater than 30% mortality of Group A larvae did not occur until 30 days after treatment, and then only with two isolates at the highest dose; whereas 30% or higher mortality of Group B larvae with the same dose was observed at 10 days (see Fig. 1).

Previous studies have obtained high mortality of *R. microplus* larva at day 10 after treatment with *B. bassiana* or *M. anisopliae* s.l. conidia; most of these studies used larvae obtained from engorged females from artificially infested calves (Barci et al., 2009; Bittencourt et al., 1994, 1996; Fernandes et al., 2003; Leemon and Jonsson, 2008; Paião et al., 2001; Posadas and Lecuona, 2009). These studies concluded that the variations in tick mortality were directly related to the isolates' differences in virulence. In the current study, *R. microplus* larva obtained by different methods and from different locations differed markedly in susceptibilities to infection by *Beauveria* spp., as evidenced by bioassays of five isolates using both Groups A and B larvae. Similarly, a study reported by Devi et al. (2008) demonstrated variation in virulence of a *B. bassiana* isolate to different populations within a single insect species. The

Fig. 1. Percent mortality of *Rhipicephalus microplus* larvae. Larvae were treated with *Beauveria bassiana* (isolates: Bb 23, Bb 44, CG 480, ESALQ 747 or ESALQ 986) at 0 (Control), 1×10^5, 10^6, 10^7 or 10^8 conidia ml^{-1}. Mortality was assessed at day 10, 20 and 30 after treatment. (A) Engorged females were collected from naturally tick-infested cattle at dairy farm at Universidade Federal Rural do Rio de Janeiro. Engorged females were held in glass tubes for oviposition and the eggs pooled. Larvae hatched from these eggs in the lab were used to infest calves artificially. The larvae used for bioassay were obtained from eggs from engorged females collected directly from artificially infested calves as well as from the floor of pens (= Group A larvae). (B) Engorged females were collected from cattle naturally infested at a private farm located beside highway Presidente Dutra, Km 201, in Seropédica, RJ. Engorged females from these cattle were held for oviposition and larvae that hatched from these eggs in the lab were used in the bioassay (= Group B larvae). Note that Group A larvae were from eggs produced on artificially infested (by single application of larvae) cattle; whereas Group B larvae were from eggs produced by engorged females that had naturally infested cattle at a location ∼10 km from the type A larval source. Bars are standard error of two bioassays.

Table 3
Morphology of *Beauveria* spp. and *Engyodontium albus* (=*Beauveria alba*) isolates.

Species and isolates	Macromorphology (colony)				Micromorphology (microscopy)		
	Size (mm)	Texture	Color	Conidia (μm)[a]	Lateral cell (μm)[a]	Conidiogenous cell (μm)[a]	Cluster[b]
Beauveria bassiana							
Bb 02	22.86	Lanose, smooth	White	1.5–2.0 × 1.0–3.0	3.0–5.0 × 3.0	2.0–3.0 × 4.0–18.0	+
Bb 09	19.45	Floccose, zonate	White	1.5–3.0 × 1.5–4.0	3.0 × 3.0	2.0–3.0 × 4.0–11.0	+
Bb 13	20.10	Floccose, sulcate	Yellowish	1.5–3.5 × 1.5–2.5	3.0 × 4.0	2.0–2.5 × 4.0–13.0	+
Bb 15	20.39	Velvety, smooth	White	2.0–2.5 × 2.0–3.0	3.0 × 4.0–5.0	2.0–2.5 × 2.0–8.0	+
Bb 19	22.93	Velvety, smooth	White	1.5–3.0 × 1.5–4.0	3.0 × 3.0	2.0–2.5 × 4.0–15.0	+
Bb 21	20.02	Velvety, smooth	White	1.5–3.0 × 1.5–4.0	3.0 × 3.0	2.0–2.5 × 4.0–8.0	–
Bb 23	17.83	Floccose, smooth	White	1.5–2.0 × 1.5–3.0	3.0–4.0 × 4.0	2.0–2.5 × 4.0–13.0	+
Bb 27	22.07	Velvety, smooth	White	1.5–2.5 × 1.5–4.0	2.5 × 4.0	2.0–2.5 × 4.0–13.0	+
Bb 31	20.20	Floccose, smooth	White	1.5–2.0 × 1.5–3.0	3.0 × 3.0	2.5–3.0 × 5.0–10.0	–
Bb 35	16.57	Velvety, smooth	White	1.5–2.5 × 1.5–3.5	3.0 × 3.0	2.0–2.5 × 4.0–13.0	+
Bb 38	19.60	Velvety, smooth	White	1.5–2.5 × 1.5–3.0	3.0 × 3.0	2.0–2.5 × 4.0–10.0	+
Bb 44	19.98	Floccose, zonate	White	1.5–3.0 × 1.5–4.0	3.0 × 3.0	2.0–2.5 × 5.0–7.0	–
Bb 46	19.49	Velvety, smooth	White	1.5–2.5 × 1.5–3.0	3.0–4.0 × 4.0	2.0–3.0 × 4.0–9.0	+
LCM 01	19.71	Lanose, smooth	White	1.5–2.0 × 1.5–2.5	3.0 × 3.0	1.5–2.0 × 4.0–15.0	–
ESALQ 986	22.57	Lanose, zonate	Yellowish	1.5–2.0 × 1.5–3.0	3.0 × 6.0	2.0–2.5 × 4.0–5.0	+
CG 66	9.48	Lanose, smooth	White	1.5–3.0 × 1.5–4.0	3.0–3.5 × 3.0–4.0	2.0–2.5 × 4.0–15.0	+
CG 222	20.96	Floccose, smooth	White	1.5–2.0 × 1.5–2.0	3.0 × 6.0	1.5–2.0 × 4.0–7.0	+
CG 227	16.77	Velvety, smooth	White	1.5–2.5 × 1.5–3.0	3.0 × 3.0–5.0	2.0–3.0 × 4.0–12.0	+
CG 228	20.57	Floccose, smooth	White	1.0–2.0 × 1.0–2.0	3.0–3.5 × 4.0–5.0	1.5–2.0 × 5.0–11.0	+
CG 319	22.19	Lanose, smooth	White	1.5–2.0 × 1.5–2.0	3.0 × 3.0–4.0	2.0–2.5 × 4.0–8.0	+
CG 464	22.80	Lanose, smooth	White	1.5–2.0 × 1.5–2.5	3.0 × 3.0	2.0–2.5 × 4.0–9.0	+
CG 481	20.73	Lanose, smooth	White	1.5–2.0 × 1.5–3.0	3.0 × 6.0	1.5–2.5 × 5.0–8.0	+
CG 484	21.18	Floccose, zonate	White	1.5–2.5 × 2.0–3.0	3.0 × 5.0	2.0–2.5 × 4.0–8.0	+
CG 495	22.82	Lanose, smooth	White	1.5–2.0 × 1.5–3.0	3.0 × 5.0	2.0–2.5 × 4.0–15.0	+
CG 500	22.85	Floccose, sulcate	Yellowish	1.5–2.5 × 1.5–3.0	3.0 × 4.0	1.5–2.5 × 4.0–11.0	+
ARSEF 252	18.21	Lanose, smooth	White	1.5–2.0 × 1.5–2.0	2.0–3.0 × 3.5–4.0	3.0 × 6.0–10.0	+
GHA	17.68	Velvety, smooth	White	2.0–2.5 × 2.0–3.0	2.0 × 3.0	2.0–3.0 × 5.0–13.0	+
CG 02	22.28	Floccose, smooth	White	2.0–2.5 × 2.0–2.5	3.0 × 5.0	2.0–2.5 × 5.0–11.0	+
CG 138	21.35	Lanose, smooth	White	1.5–2.0 × 1.5–3.0	3.0 × 3.0	2.0–2.5 × 9.0–17.0	+
CG 367	12.53	Floccose, smooth	White	1.5–3.0 × 2.0–5.0	3.0 × 3.0	2.0–3.0 × 4.0–11.0	+
CG 471	17.80	Floccose, smooth	White	1.5–2.0 × 1.5–3.0	3.0 × 5.0	2.0–2.5 × 5.0–13.0	+
CG 478	22.37	Floccose, sulcate	White	1.5–2.0 × 1.5–2.0	3.0 × 3.0	2.0–2.5 × 5.0–10.0	+
CG 483	14.73	Floccose, smooth	White	1.5–2.0 × 1.5–4.0	3.0 × 3.0	2.0–2.5 × 4.0–15.0	+
EP 01	17.75	Floccose, smooth	White	1.5–2.5 × 1.5–3.0	3.0 × 3.5	2.0–3.0 × 6.0–15.0	–
CG 17	20.05	Velvety, zonate	White	1.5–3.0 × 1.5–4.0	3.0 × 5.0	2.0–3.0 × 4.0–14.0	+
UFPE 496	12.04	Velvety, smooth	White	1.5–2.5 × 1.5–3.0	3.0 × 3.0	1.5–2.0 × 9.0–15.0	–
CG 01	21.38	Lanose, smooth	White	1.5–2.5 × 1.5–3.0	3.0 × 3.0–4.0	2.0–2.5 × 4.0–9.0	+
CG 149	21.33	Floccose, sulcate	White	1.5–2.5 × 1.5–4.0	3.0 × 3.0–4.0	2.0–2.5 × 4.0–12.0	+
CG 154	19.99	Floccose, smooth	Yellowish	1.5–2.5 × 1.5–3.0	3.0 × 4.0–6.0	1.5–2.5 × 4.0–8.0	+
CG 234	12.86	Velvety, smooth	White	1.5–2.0 × 1.5–2.5	2.0 × 3.0	1.5–2.0 × 4.0–10.0	+
CG 206	22.88	Floccose, smooth	Yellowish	1.5–2.0 × 1.5–2.5	3.0 × 5.0	2.0–3.0 × 4.0–16.0	+
CG 235	21.47	Floccose, sulcate	Yellowish	1.5–2.0 × 1.5–3.0	2.0 × 3.0–4.0	2.0–2.5 × 4.0–14.0	+
CG 479	22.92	Floccose, smooth	Yellowish	1.5–2.0 × 1.5–2.0	2.5 × 3.0	2.0–2.5 × 4.0–10.0	+
ESALQ 747	17.33	Lanose, zonate	White	1.5–2.0 × 1.5–2.0	3.0 × 5.0	2.0–2.5 × 5.0–7.0	+
CCT 4641	19.22	Velvety, smooth	White	1.5–2.0 × 1.5–4.0	3.0 × 3.0	2.0–2.5 × 4.0–15.0	–
CG 251	22.65	Floccose, smooth	Yellowish	1.5–2.0 × 1.5–3.0	2.0–3.0 × 3.0–3.5	2.0–2.5 × 4.0–10.0	+
CG 480	21.90	Lanose, smooth	White	1.5–2.0 × 1.5–2.0	3.0 × 3.0	2.0–2.5 × 4.0–10.0	+
CG 487	22.48	Velvety, smooth	White	1.5–2.5 × 1.5–4.0	3.0 × 3.0	1.5–2.5 × 4.0–10.0	+
UFPE 479	20.65	Velvety, smooth	White	1.5–2.0 × 1.5–3.0	2.0 × 3.0	1.0–2.0 × 6.0–27.0	–
CG 307	22.86	Floccose, sulcate	Yellowish	1.5–3.0 × 1.5–3.0	3.0 × 6.0	2.0–2.5 × 4.0–8.0	+
CG 309	16.81	Lanose, smooth	White	1.5–2.0 × 1.5–2.0	3.0 × 3.0–5.0	2.0–2.5 × 4.0–8.0	+
CG 310	14.21	Floccose, zonate	Yellowish	1.5–2.0 × 1.5–3.0	3.0 × 5.0	2.0–2.5 × 4.0–8.0	+
CCT 3161	18.62	Lanose, smooth	White	1.5–2.0 × 1.5–3.0	3.0 × 4.0	1.5–3.0 × 5.0–10.0	–
Beauveria amorpha							
ARSEF 656	26.92	Velvety, smooth	White	1.5–2.0 × 2.0–5.0	–	2.0–2.5 × 4.0–19.0	+
ARSEF 1682	19.18	Lanose, smooth	White	1.5–2.0 × 3.0–5.0	–	1.5–2.0 × 8.0–17.0	–
ARSEF 4755	20.27	Cottony, zonate	Yellowish	1.5–2.0 × 3.0–6.0	–	1.5–2.0 × 4.0–12.0	+
Beauveria brongniartii							
ATCC 58798	18.01	Floccose, smooth	Yellowish	1.5–2.5 × 2.5–5.0	2.0–3.0 × 4.0–7.0	2.0–3.5 × 5.0–20.0	+
Beauveria velata							
ARSEF 2998	15.22	Velvety, smooth	White	3.0–4.0 × 3.0–4.0	–	2.0–2.5 × 4.0–8.0	–
Beauveria vermiconia							
ARSEF 2922	16.52	Lanose, smooth	White	1.0–1.5 × 2.0–4.0	2.0–3.0 × 5.0–6.0	1.0–2.5 × 5.0–9.0	+
Engyodontium albus (=*Beauveria alba*)							
UFPE 3138	12.95	Floccose, zonate	White	1.5–2.0 × 1.5–2.0	2.0 × 6.0–12.0	1.5 × 10.0–35.0	–

[a] Smallest to largest width × smallest to largest length.
[b] (+) dominance of clusters of conidiogenous cells and (–) dominance of single conidiogenous cells.

Table 4
Overview of traits of *Beauveria* spp. and *Engyodontium albus* (=*Beauveria alba*) isolates that may indicate their potential for use as biological control agents of *Rhipicephalus microplus*.[a]

Species and isolates	Virulence (LC_{50})	Conidia yield × 10^{7}	UV-B tolerance Fernandes et al. (2007)	Heat tolerance Fernandes et al. (2008)	Cold activity	MLEE Fernandes et al. (2009)	AFLP
Beauveria bassiana							
Bb 02	6.31×10^{9}	0.133 ± 0.006	0.0 ± 0.0	3.4 ± 2.1	82.2 ± 7.8	1	1
Bb 09	6.55×10^{9}	0.334 ± 0.007	7.1 ± 3.1	1.8 ± 1.1	72.0 ± 5.3	4	1
Bb 13	1.60×10^{12}	2.838 ± 0.878	9.6 ± 3.0	72.9 ± 6.0	77.6 ± 10.0	1	1
Bb 15	6.88×10^{10}	0.071 ± 0.007	44.8 ± 8.1	38.1 ± 3.3	86.5 ± 8.5	1	1
Bb 19	2.82×10^{10}	8.613 ± 0.350	37.4 ± 4.5	28.3 ± 14.4	90.4 ± 8.6	1	1
Bb 21	3.07×10^{9}	0.679 ± 0.189	22.9 ± 4.0	11.7 ± 4.5	96.4 ± 2.5	1	1
Bb 23	3.11×10^{12}	1.415 ± 0.048	13.6 ± 7.6	51.9 ± 18.4	93.4 ± 3.3	1	1
Bb 27	1.20×10^{12}	5.191 ± 1.291	49.5 ± 6.8	28.3 ± 10.2	92.5 ± 2.2	1	1
Bb 31	1.10×10^{10}	2.988 ± 0.918	18.9 ± 3.1	65.0 ± 3.9	78.1 ± 4.8	1	1
Bb 35	2.94×10^{13}	2.354 ± 0.242	47.6 ± 11.2	19.3 ± 9.3	63.4 ± 15.7	11	1
Bb 38	1.21×10^{10}	4.707 ± 1.512	16.1 ± 4.3	21.0 ± 13.0	93.2 ± 3.9	1	1
Bb 44	2.63×10^{10}	1.113 ± 0.538	15.0 ± 8.4	20.6 ± 8.1	59.2 ± 10.1	5	1
Bb 46	1.00×10^{9}	0.405 ± 0.230	6.9 ± 2.4	9.2 ± 7.3	90.7 ± 2.3	1	1
LCM 01	6.82×10^{8}	0.911 ± 0.039	6.3 ± 2.4	16.4 ± 7.0	85.7 ± 8.2	1	1
ESALQ 986	1.21×10^{9}	0.350 ± 0.058	46.9 ± 6.8	10.2 ± 2.9	87.5 ± 9.1	1	1
CG 66	2.20×10^{10}	3.900 ± 0.050	36.8 ± 5.6	11.7 ± 3.5	20.5 ± 7.0	–	20
CG 222	4.00×10^{9}	0.113 ± 0.014	64.7 ± 8.3	2.1 ± 0.6	76.6 ± 22.7	6	11
CG 227	3.02×10^{9}	13225 ± 1463	46.4 ± 12.6	83.2 ± 10.7	42.3 ± 4.8	–	4
CG 228	3.00×10^{9}	6.522 ± 0.066	75.0 ± 2.7	69.0 ± 8.6	55.6 ± 7.4	15	21
CG 319	9.21×10^{7}	7.309 ± 1.691	24.5 ± 4.8	22.4 ± 4.3	70.8 ± 6.6	4	6
CG 464	3.23×10^{7}	5.638 ± 0.919	36.8 ± 11.5	62.6 ± 17.2	90.9 ± 5.0	4	6
CG 481[b]	3.29×10^{8}	5.416 ± 0.203	49.0 ± 6.1	43.3 ± 17.3	91.4 ± 2.5	3	14
CG 484[b]	9.95×10^{8}	10.34 ± 2.143	55.7 ± 5.7	54.4 ± 14.2	92.1 ± 4.1	4	6
CG 495	1.10×10^{9}	3.959 ± 0.522	55.4 ± 9.2	36.8 ± 17.4	93.2 ± 1.8	1	6
CG 500	3.53×10^{7}	3.771 ± 1.391	34.0 ± 7.6	27.0 ± 10.8	99.1 ± 0.6	4	7
ARSEF 252	6.87×10^{10}	6.502 ± 1.140	28.9 ± 7.2	88.2 ± 3.9	99.0 ± 0.9	22	23
GHA	1.63×10^{10}	11.06 ± 1.650	54.8 ± 3.5	68.5 ± 17.5	99.3 ± 0.3	12	26
CG 02	7.41×10^{10}	4.922 ± 1.178	14.0 ± 2.3	58.6 ± 11.7	44.4 ± 10.3	1	8
CG 138	2.38×10^{8}	3.800 ± 0.568	57.1 ± 4.6	86.3 ± 5.8	95.0 ± 4.1	23	24
CG 367	4.45×10^{9}	0.877 ± 0.020	25.5 ± 8.5	3.5 ± 3.5	80.1 ± 8.4	10	16
CG 471	3.35×10^{8}	1.616 ± 0.674	60.0 ± 5.4	27.3 ± 5.0	86.5 ± 2.3	8	3
CG 478	1.04×10^{8}	3.644 ± 0.088	16.2 ± 5.3	61.2 ± 5.9	97.8 ± 0.9	1	5
CG 483	2.32×10^{9}	6.581 ± 0.294	18.2 ± 5.7	57.6 ± 12.16	65.4 ± 17.3	16	18
EP 01	2.32×10^{8}	0.085 ± 0.026	3.7 ± 2.5	12.8 ± 7.5	91.5 ± 3.2	1	1
CG 17	4.90×10^{8}	1.975 ± 0.143	39.9 ± 4.7	35.5 ± 15.9	55.6 ± 13.9	1	1
UFPE 496	–	1.546 ± 0.173	42.7 ± 4.4	18.8 ± 10.8	85.8 ± 7.4	24	26
CG 01	1.91×10^{23}	1.861 ± 0.526	70.1 ± 6.6	78.1 ± 5.0	88.6 ± 1.9	4	6
CG 149	2.74×10^{10}	12.26 ± 1.125	64.6 ± 2.4	46.3 ± 10.5	90.5 ± 3.4	4	17
CG 154	2.61×10^{11}	11.65 ± 0.650	32.3 ± 6.4	10.8 ± 4.4	65.5 ± 22.0	6	18
CG 234	7.92×10^{21}	0.014 ± 0.003	34.5 ± 8.1	36.9 ± 18.6	99.1 ± 0.5	10	9
CG 206[b]	6.10×10^{7}	13.37 ± 0.213	55.3 ± 13.6	47.6 ± 14.6	75.2 ± 11.5	4	17
CG 235[b]	8.92×10^{8}	5.359 ± 0.903	59.1 ± 6.8	56.1 ± 5.6	92.8 ± 2.3	4	17
CG 479	8.63×10^{11}	3.584 ± 0.716	34.4 ± 11.6	43.0 ± 13.7	74.3 ± 9.0	17	15
ESALQ 747	2.79×10^{11}	2.613 ± 0.555	25.7 ± 7.1	42.3 ± 6.5	91.1 ± 3.4	1	1
CCT 4641	1.12×10^{12}	0.016 ± 0.001	27.6 ± 5.2	36.1 ± 13.9	92.7 ± 0.6	1	10
CG 251	1.91×10^{23}	6.752 ± 0.065	58.5 ± 4.5	60.0 ± 20.1	87.3 ± 5.3	18	22
CG 480	2.81×10^{11}	8.367 ± 0.542	49.5 ± 2.8	37.1 ± 5.6	90.0 ± 3.3	13	19
CG 487[b]	6.66×10^{8}	4.721 ± 0.171	56.5 ± 4.9	72.6 ± 6.5	96.1 ± 1.3	2	2
UFPE 479	–	2.476 ± 0.606	44.8 ± 11.5	3.4 ± 1.6	42.9 ± 19.1	7	13
CG 307	8.85×10^{8}	4.155 ± 0.055	42.4 ± 12.8	69.8 ± 11.6	80.6 ± 4.6	2	11
CG 309	7.12×10^{8}	3.504 ± 0.354	17.5 ± 3.9	65.1 ± 8.4	77.0 ± 10.7	9	1
CG 310	8.25×10^{9}	2.077 ± 0.694	50.3 ± 11.0	26.9 ± 13.4	94.1 ± 3.3	9	12
CCT 3161	1.89×10^{14}	2.309 ± 0.516	27.6 ± 6.5	61.2 ± 5.9	97.3 ± 1.4	20	25
Beauveria amorpha							
ARSEF 656	7.34×10^{15}	2.118 ± 0.237	29.1 ± 5.2	70.9 ± 6.2	79.7 ± 7.5	19	27
ARSEF 1682	2.57×10^{11}	2.378 ± 0.935	14.6 ± 3.2	1.8 ± 1.2	99.8 ± 0.1	21	28
ARSEF 4755	6.94×10^{7}	4.638 ± 0.188	7.0 ± 3.1	13.6 ± 4.2	99.3 ± 0.2	25	29
Beauveria brongniartii							
ATCC 58798	5.78×10^{12}	0.462 ± 0.133	3.9 ± 1.3	0.3 ± 0.3	98.4 ± 1.2	14	30
Beauveria velata							
ARSEF 2998	2.04×10^{10}	0.013 ± 0.004	27.7 ± 14.0	0.0 ± 0.0	69.4 ± 7.0	26	33
Beauveria vermiconia							
ARSEF 2922	2.48×10^{15}	1.557 ± 0.064	20.2 ± 9.8	0.1 ± 0.1	99.1 ± 0.5	26	31
Engyodontium albus (=*Beauveria alba*)							
UFPE 3138	1.04×10^{15}	1.194 ± 0.264	1.6 ± 1.1	0.2 ± 0.2	0.0 ± 0.0	27	32

[a] Conidia yield on 58.9 mm^2 surface area of PDAY medium. UV-B tolerance, heat tolerance and cold activity are expressed in conidial relative germination (%); UV-B irradiation = 7.04 kJ m^{-2}; heat exposure = 45 °C, 2 h; cold activity = 5 °C, 15 days. Genetic grouping based on ≥0.92 similarity coefficient on Multilocus Enzyme Electrophoresis (MLEE), and ≥0.90 on Amplified Fragment Length Polymorphism (AFLP).
[b] Isolates that are promising candidates for biological control of *R. microplus*.

cause(s) of variations in susceptibilities of the different host-tick populations is not known; but the observation of reduced mortality was unexpected since the bioassay utilizing Group A larvae was conducted, to the best of our knowledge, precisely as was done in our laboratory over a period of >10 years (Bittencourt et al., 1996; Fernandes et al., 2003, 2006). Nevertheless, a different larval tick population exhibited different levels of susceptibility to five *B. bassiana* isolates (see Fig. 1).

Natural fungal epizootics apparently have not been reported in tick populations. Ticks may be physiologically or structurally more resistant or tolerant to entomopathogenic fungi than other arthropods (Polar et al., 2005), as evidenced by the high conidial concentrations needed in bioassays to reach high mortality levels or short survival periods (Maniania et al., 2007; Polar et al., 2005). Also, different tick species may display different susceptibility to entomopathogenic fungi due to fungistatic compounds present in the epicuticle of certain tick species (Kirkland et al., 2004). The tick *Dermacentor variabilis*, when associated with the fungus *Scopulariopsis brevicaulis*, survives dosages of topically applied *M. anisopliae* conidia that are normally lethal to other tick species (Yoder et al., 2008). According to these authors, the association *D. variabilis*/*S. brevicaulis* is a mutually advantageous symbiotic relationship, and this type of ecological interaction is consistent with a strategy observed in other fungal groups where the capturing of a substrate by one kind of fungus blocks the occurrence of a secondary fungus. Studies are needed to determine if similar associations between fungi or other organisms with *R. microplus* may be responsible for increased or decreased susceptibility to infection by entomopathogenic fungi. *R. microplus* ticks in Brazil are frequently associated with protozoa, such as *Babesia bovis* and *B. bigemina*. These protozoa cause bovine babesiosis, a tick-borne parasitic infection which is endemic throughout Brazil. In the current study, no investigation of *Babesia* infection on *R. microplus* or cattle was conducted.

The potential of EPF to control ticks has been demonstrated in many laboratory bioassays; conversely, few tests have been conducted under field conditions (Fernandes and Bittencourt, 2008). Solar radiation is one of the most important stress factors encountered in field use of entomopathogenic fungi, and this negatively affects their effectiveness as agents for programs of biological control of arthropods (Rangel et al., 2004). Although massive spore production is considered feasible, the frequent need for reapplications may render the process economically nonviable. On the other hand, selection of isolates with high heat and UV-tolerance and development of fungal formulations that increase conidial persistence in the field may provide effective new fungus-based biocontrol agents (Fargues et al., 1996; Rangel et al., 2005). The current study identifies some *B. bassiana* isolates with high potential use for *R. microplus* control programs. Conversely, *E. albus* and *Beauveria* spp. other than *B. bassiana*, appear to not be promising agents for tick control.

The current study detected considerable variation in morphology and conidial yield among *B. bassiana* isolates. The isolates with their conidial production apparatus tightly clustered tended to yield more conidia than isolates with less tight branching and with their conidiogenous cells occurring in small groups or solitarily. Isolates with tightly clustered conidial apparatus or occurring in small groups or solitarily were reported by De Hoog (1972) in fresh and older isolates, respectively. Also, Liu et al. (2003) reported morphological variation among *B. bassiana* isolates; and the isolates that produced larger conidia had higher spore production. This correlation, however, was not observed in the present study. The mass production of *Beauveria* spp. isolates on PDAY gives a general idea about the conidial mass-production potential of each isolate; but PDAY is not an economically feasible substrate for commercial proposes. Since conidial production may vary according the substrate provided, PDAY-based studies may not be appropriate for final selection of isolates for commercial mass conidial production.

In general, genetic groups of *B. bassiana* (Fernandes et al., 2009) seem to not be associated with virulence to *R. microplus* because more- or less-virulent isolates grouped together with high similarity coefficients, ≥0.92 and ≥0.90 based on MLEE and AFLP, respectively. In agreement, Riba et al. (1986) and Bidochka et al. (2002) also did not find a correlation between genetic variation and virulence of fungal isolates. Bidochka et al. (2002), however, showed that *B. bassiana* genotypes are associated with habitat of origin; moreover, specific habitats of origin were associated with the ability of isolates to grow at higher temperatures and tolerate UV exposure. Conidial tolerance of *B. bassiana* isolates to UV-B irradiation and the ability of conidia to germinate at cold temperature appear to be associated with latitude of origin; viz. the closer the isolate's origin is to the equator, the higher its tolerance to UV-B and the lower its activity at cold temperature (Fernandes et al., 2007, 2008). Additionally, populations of *B. bassiana* from North Brazil differed genotypically from populations from South or Southeast Brazil (Fernandes et al., 2009).

In order to develop an effective biological control program for tick control using a fungus, many avenues of research must be traversed. As has been mentioned, selecting isolates with high UV-B and heat tolerance may be important to increased persistence of microbial control agents in environments with high solar exposure (Fargues et al., 1996; Fernandes et al., 2008; Rangel et al., 2005). In addition, successful biological control programs also must address the development of efficient conidial formulation and application strategies. Effective formulations may include the addition of adjuvants to facilitate conidial adhesion to the tick surface and/or provide UV protection. Because very high conidial doses of most *B. bassiana* isolates are needed to kill high percentages of *R. microplus* larvae when the conidia are applied to tick larvae in bioassays, it is apparent that spraying conidial suspensions onto ticks while on cattle skin will be much more effective in tick control than dispersing the inoculum by spraying it instead onto the pasture vegetation. Although the cattle skin microenvironmental factors may potentially affect the pathogenicity of topically applied EPF, improved formulations of suitable strains may be developed to overcome the host skin challenge (Polar et al., 2008). A fungus-treated trap baited with semiochemicals can be an alternative to minimize the area treated with mycoacaricides onto the

pasture vegetation to control tick populations in the field (Nchu et al., 2010). This method demonstrates effectiveness for controlling ticks of the genus *Amblyomma*, which have heteroxenous life cycle (Maniania et al., 2007; Nchu et al., 2010); they spend a large part of their life off of the host during ecdysis and while they search for the next host. On the other hand, this method may not be appropriate for controlling ticks with monoxenous life cycle, e.g., *R. microplus*. A further consideration may be whether the combination of EPF and low doses of chemical acaricides would be beneficial (Bahiense et al., 2006; Batista Filho et al., 2001). While all of these areas of research are important to developing a successful biological control program, the primary and fundamental emphasis should be finding an isolate that is highly virulent towards the target arthropod.

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