

Selection of *Metarhizium* spp. Brazilian isolates to control *Rhipicephalus microplus* ticks: *in vitro* virulence tests and conidiogenesis

Seleção de isolados Brasileiros de *Metarhizium* spp. para controle do carrapato *Rhipicephalus microplus*: testes de virulência *in vitro* e conidiogênese

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Abstract

This study aimed to select *Metarhizium* spp. isolates to control *Rhipicephalus microplus* ticks by analyzing their *in vitro* virulence against *R. microplus* eggs, larvae, and engorged females as well as their ability to produce aerial conidia on potato dextrose agar (PDA). After the treatment of *R. microplus* eggs with the highest fungal concentration (10^8 conidia.ml⁻¹), most of the eleven tested isolates reduced the larval hatching compared to the control group, except *M. anisopliae* s.l. ARSEF 2211 and ARSEF 3641. *M. anisopliae* s.l. isolates ARSEF 729, ARSEF 760, ARSEF 929, and ARSEF 3643 exhibited the best results in the egg bioassay. In the bioassay with larvae, the entomopathogenic fungal isolates yielded average larval mortality ranging from 0.1% to 98.9% and from 23.9% to 99.9% five and fifteen days after the treatment, respectively. ARSEF 552, ARSEF 729, ARSEF 929, and ARSEF 3643 yielded the highest larval mortality. Analysis of the bioassay with *R. microplus* engorged females found that the different isolates negatively impacted the egg mass weight, larval hatching percent, egg production index, and nutritional index. The percent of tick control ranged from 5.32% to 70.83%, and the best tick control rates were caused by *M. anisopliae* s.l. ARSEF 3643 (70.83%), ARSEF 3641 (62.87%), and ARSEF 729 (64.27%). The highest conidiogenesis on PDA was observed for *M. anisopliae* s.l. ARSEF 3641 and *M. pingshaense* ARSEF 552. The isolates ARSEF 729 and ARSEF 3643 are considered promising candidates for field applications against *R. microplus* ticks.

Keywords: biological control, entomopathogenic fungi, cattle tick.

Resumo

Este estudo teve como objetivo selecionar isolados de *Metarhizium* spp. para controlar carrapatos *Rhipicephalus microplus* por meio da análise de sua virulência *in vitro* contra ovos, larvas e fêmeas ingurgitadas, bem como sua capacidade de produzir conídios aéreos em ágar dextrose de batata (PDA). Após o tratamento dos ovos de *R. microplus* com maior concentração fúngica (10^8 conídios.ml⁻¹), a maioria dos onze isolados testados reduziu a eclosão larval em relação ao grupo controle, exceto *M. anisopliae* s.l. ARSEF 2211 e ARSEF 3641. *M. anisopliae* s.l. os isolados ARSEF 729, ARSEF 760, ARSEF 929 e ARSEF 3643 exibiram os melhores resultados no bioensaio com ovo. No bioensaio com larvas, os isolados fúngicos entomopatogênicos apresentaram mortalidade larval média variando de 0,1% a 98,9% e de 23,9% a 99,9% cinco e quinze dias após o tratamento, respectivamente. ARSEF 552, ARSEF 729, ARSEF 929 e ARSEF 3643 produziram a maior mortalidade larval. A análise do bioensaio com fêmeas ingurgitadas de *R. microplus* mostrou que os diferentes isolados impactaram negativamente o peso da massa de ovos, a porcentagem de incubação larval, o índice de produção de ovos e o índice nutricional. A porcentagem de controle de carrapatos variou de 5,32% a 70,83%, e as melhores taxas de controle de carrapatos foram causadas por *M. anisopliae* s.l. ARSEF 3643 (70,83%), ARSEF 3641 (62,87%) e ARSEF 729 (64,27%). A maior conidiogênese em PDA foi observada para *M. anisopliae* s.l. ARSEF 3641 e *M. pingshaense* ARSEF 552. Os isolados ARSEF 729 e ARSEF 3643 são considerados candidatos promissores para aplicações de campo contra o carrapato *R. microplus*.

Palavras-chave: controle biológico, fungos entomopatogênicos, carrapato de bovinos.



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Introduction

Rhipicephalus microplus (Canestrini, 1887) (Acari: Ixodidae) Murrel & Barker, 2003, popularly known as the cattle tick, is an important ectoparasite in Brazilian livestock and several other tropical countries. The annual economic losses in Brazil related to the parasitism of this tick are estimated at 3.24 billion dollars and are related to the damages in the leather, transmission of pathogens, reduction in milk and meat production, loss of weight gain, and expenditures on the use of chemical acaricides used to control it (Grisi et al., 2014).

Historically the most common and effective practice to control *R. microplus* ticks in cattle farming has been the use of chemical acaricides, but the evolution of *R. microplus* populations resistant to these acaricides has caused great concern among livestock farmers and government agencies (Andreotti et al., 2011). Several studies have reported resistance of *R. microplus* to the chemical acaricides in almost all classes of products available for tick control in Brazil, including multiple resistance in some field populations (Klafke et al., 2017; Reck et al., 2014). The factors that lead to this development include the indiscriminate use of acaricides, applications with incorrect doses, and delay in initiating treatment (Klafke, 2008).

The growing global concern about environmental contamination and the market for chemical-free foods has contributed to the development of alternative control methods for the *R. microplus* tick (Samish et al., 2004). One of the alternatives may be the use of entomopathogenic fungi against different life stages of this tick (Wassermann et al., 2016). Fungi are more studied and used microorganisms for biological control than viruses or bacteria (Thomas & Read, 2007).

The genus *Metarhizium* is composed of entomopathogenic fungi that are generally greenish, often isolated from soils in tropical and temperate regions, and can colonize arthropods (Bischoff et al., 2009). *In vitro* (Bahense et al., 2006; Bittencourt et al., 1994a, 1994b; Perinotto et al., 2014; Perinotto et al., 2017; Quinelato et al., 2012) and *in vivo* studies (Camargo et al., 2014, 2016; Marciano et al., 2020; Mesquita et al., 2020; Samish et al., 2014; Webster et al., 2015) have already demonstrated its efficiency to control *R. microplus* with positive perspectives for its use in the field.

Considering the problems associated with the massive use of chemical acaricides and the current knowledge about the entomopathogenic fungi, screening non-exotic fungal isolates with outstandingly biocontrol traits is crucially important to obtain increased effectiveness in tick biocontrol to support the increase of fungal-based biological products developed exclusively to be used against ticks.

Most mycoinsecticides produced currently in Brazil are based on aerial conidia produced by solid substrate fermentation technologies (Mascarin et al., 2019). The capacity of isolates to produce conidia is an important trait for field use as biological control of ticks is challenged by the need for high concentrations of fungal propagules (Fernandes & Bittencourt, 2008). Accordingly, the present study aimed to select *Metarhizium* spp. isolates to control *R. microplus* ticks through the analysis of their *in vitro* virulence and capacity for conidial production.

Material and methods

Fungal isolates and suspensions

Metarhizium spp. native Brazilian isolates (Table 1) were obtained from the National Center for Genetic Resources-CENARGEN, EMBRAPA, Brazil and are also deposited at the Agriculture Research Service Collection of Entomopathogenic Fungi (ARSEF) at the Laboratory of Plants, Soil, and Nutrition (Ithaca, NY, USA). As the present study accessed Brazilian genetic heritage, the research was registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (Sis-Gen) under the code AACFDD5.

The fungal isolates were produced on potato dextrose agar (PDA) at $25 \pm 1^\circ\text{C}$ and $\geq 80\%$ relative humidity (RH) for 14 days. Conidia were harvested from culture plates by scraping the medium surface with a scalpel blade and suspended in 30 mL of polyoxyethylene sorbitan monooleate (Tween 80®, Sigma Chemical Co., St. Louis, MO, USA) solution [0.01% (v/v)]. The conidial suspension was homogenized for 1 min using a vortex mixer, quantified in a hemocytometer and adjusted to 1.0×10^8 conidia mL^{-1} . Serial dilutions were made from the concentration of 10^8 conidia mL^{-1} to obtain the other concentrations (10^7 , 10^6 , and 10^5 conidia mL^{-1}).

Table 1. Fungal isolates used in the experiments.

Isolate Code	Species	Substrate/Host	Origin
ARSEF	CG/CP		
ARSEF 552	-	<i>Metarhizium pingshaense</i>	Lepidoptera
ARSEF 724	CP 25	<i>Metarhizium robertsii</i>	<i>Cerotoma arcuata</i> (Coleoptera:Chrysomelidae)
ARSEF 729	CP 24	<i>Metarhizium anisopliae</i> sensu lato (s.l.)	<i>Deois flavopicta</i> (Homoptera: Cercopidae)
ARSEF 760	CP 31	<i>M. anisopliae</i> s.l.	<i>Cerotoma arcuata</i> (Coleoptera: Chrysomelidae)
ARSEF 929	CP 67	<i>M. anisopliae</i> s.l.	<i>Chalcodermus aeneus</i> (Coleoptera: Curculionidae)
ARSEF 1885	CP 174	<i>M. anisopliae</i> s.l.	<i>Diabrotica</i> sp. (Coleoptera: Chrysomelidae)
ARSEF 2211	CP 207	<i>M. anisopliae</i> s.l.	Soil
ARSEF 2521	CP 225	<i>M. anisopliae</i> s.l.	<i>Deois</i> sp. (Homoptera: Cercopidae)
ARSEF 3479	CG 339	<i>M. anisopliae</i> s.l.	(Coleoptera: Scarabaeidae)
ARSEF 3641	CG 347	<i>M. anisopliae</i> s.l.	Soil
ARSEF 3643	CG 349	<i>M. anisopliae</i> s.l.	Soil

ARSEF: Agricultural Research Service Entomopathogenic Fungus Collection, USDA, NY, USA; CG: National Center for Genetic Resources-CENARGEN, EMBRAPA, Brazil. CP: CNPAF National Center for Agricultural Research on Rice and Beans, EMBRAPA, Goiânia, Goiás, Brazil.

Conidial viability was determined by plating an aliquot (~20 µL) of the conidia suspension at 10^5 conidia.mL⁻¹ on PDA medium plus 0.05% chloramphenicol, followed by incubation at 25 ± 1 °C and $\geq 80\%$ RH. Conidial germination was observed by microscope ($\times 200$) after 24 h (Alves et al., 1998).

Eggs, larvae, and female ticks were treated with fungal suspensions at 10^8 or 10^7 conidia.mL⁻¹. A Tween 80® solution (0.01% v/v), without fungus, was used to treat the control group. All bioassays (eggs, larvae, and female ticks) were conducted twice (in different days) with new batches of conidia each time.

Rhipicephalus microplus ticks

Engorged *R. microplus* females were collected from the floor of cattle pens holding infested calves with approval of the ethics committee for the use of animals in research - CEUA/IV/UFRRJ - protocol number O37/2014. The calves had no recent (more than 3 months) contact with any chemical acaricides. Female ticks were taken to the laboratory and washed in a 0.05% sodium hypochlorite solution for cuticle asepsis. Then they were rinsed in sterile distilled water and dried with sterile paper towels. Part of these female ticks were used for the bioassay, and the other portion was incubated at 27 ± 1 °C and relative humidity $\geq 80\%$ for oviposition and larval hatching.

Bioassays

Fungal virulence against *Rhipicephalus microplus* eggs

Aliquots containing 50 mg of *R. microplus* eggs were weighed and placed into test tubes, which were then sealed with hydrophilic cotton. Each bioassay consisted of three groups (two treatments and one control). Each group had ten test tubes, each containing approximately 1000 *R. microplus* eggs. Experiments were conducted by injecting 1 mL of conidial suspension into each test tube. The eggs were kept immersed in the injected fluid for 3 min, and the test tube

was then inverted until all of the conidial suspension was absorbed by the cotton plug. The tubes were maintained at 27 ± 1 °C and $\geq 80\%$ RH in the dark. The percent of larval hatchability for each tube was visually estimated by microscopic observation ($\times 20$), with the estimates expressed as percentages varying from 0 to 100% in 1% intervals.

Fungal virulence against *Rhipicephalus microplus* larvae

The methodology used in the larval bioassay was similar to the methodology described for the bioassay with eggs. Aliquots with 50 mg of eggs were collected from day 1 to day 10 of oviposition, placed in test tubes sealed with cotton plugs and observed daily for 20–25 days to estimate percentage of hatched eggs. Tubes with less than 95% hatched were discarded. The larval treatment with fungal suspensions was performed on the 15th day after larval hatching (approximately 40 days after oviposition). Each group had ten test tubes, each containing approximately 1000 *R. microplus* larvae. Larval mortality was recorded at days 5 and 15 after the treatment. The percent of larval mortality for each tube was visually estimated by microscopic observation ($\times 20$), with the estimates expressed as percentages varying from 0 to 100% in 1% intervals. Larvae that were unable to move were recorded as dead.

Fungal virulence against *Rhipicephalus microplus* engorged females

Females ticks were weighed individually and homogeneously distributed according to their weight into the groups of ten females. Each female was immersed individually for three minutes in test tubes with one ml of the fungal suspension. After that, each female was fixed by the dorsal part of the idiosome on Petri dishes using double-sided adhesive tape and then the plates were conditioned in a climatic chamber at 27 ± 1 °C and $\text{RH} \geq 80\%$.

Biological parameters used to evaluate the effects of the different isolates on the engorged females were the initial female weight (IFW), egg mass weight (EMW), and larvae hatching percentage (LHP). The average of each parameter was used to calculate the egg production index (EPI) and the nutritional index (NI) using the equations from Bennett (1974). The percentage of *R. microplus* controlled by the fungal isolates was obtained by the calculation of the estimated reproduction according to Drummond et al. (1971).

Fungal infection was confirmed by incubating dead ticks at 25 ± 1 °C. Dead ticks were surface sterilized and placed into Petri dishes with moistened filter paper until fungal externalization to verify post-mortem sporulation.

Production of conidia on PDA

Forty μl of fungal suspensions at 10^6 conidia. mL^{-1} were applied on Petri plates with 23 mL of PDA and distributed throughout the plate using a Drigalski handle. Six plates were prepared for each isolate. The plates were incubated at 25 ± 1 °C and $\text{RH} \geq 80\%$ for 14 days. Three random cut-offs of 1.256 cm^2 were made on the fungal plates and deposited in a test tube with one mL of Tween 80[®] aqueous sterile solution at 0.1% (v/v). Tubes were vigorously vortexed for 60 seconds. Conidia quantification was performed using a Neubauer chamber under an optical microscope. The procedure was repeated with all six plates of each fungal isolate. Analysis of conidiogenesis was repeated three times.

Statistical analysis

The tick bioassays (with eggs, larvae, and engorged females) were installed using a factorial arrangement $(11 \times 2) + 1$ from the combination of 11 entomopathogenic fungal isolates applied in two fungal concentrations (10^7 and 10^8 conidia. mL^{-1}) and an additional treatment without the use of fungus (control treatment). The conidiogenesis experiment (production of conidia on PDA) was carried out using a completely randomized design, with 11 “treatments” (eleven entomopathogenic fungal isolates), six replicates and two samples per experimental unit.

Data were submitted to the Kolmogorov-Smirnov test ($P > 0.05$) to verify the residual normality and to the Bartlett test ($P > 0.05$) to determine the homogeneity of variance. Having verified these assumptions, the analysis of variance (ANOVA) was conducted. The averages obtained for each fungal isolate and their respective concentrations were compared

and grouped by the Scott-Knott test at 5% probability. The comparison between the factorial treatments and the control treatment used the Dunnett test. All analyzes were performed using the software R, version 3.5.2.

Results

Viability of fungal suspensions

Conidia of *Metarhizium* spp. isolates used to treat eggs, larvae, and adult females had approximately 100% germination after incubation for 24 h at 25±1 °C and ≥80% RH.

Fungal virulence against *Rhipicephalus microplus* eggs

Analysis of larval hatching of *R. microplus* eggs exposed or not (control) to the different *Metarhizium* spp. isolates demonstrated that the different entomopathogenic fungal isolates (F), concentrations (C), and the interaction between these two factors (F × C) significantly interfered in the larval hatching (Tables 2 and 3). At the lowest concentration, only *Metarhizium* spp. ARSEF 552 and ARSEF 760 significantly reduce the larval hatching in comparison with the control group (untreated eggs). At the highest concentration (10⁸ conidia.ml⁻¹), most fungal isolates reduced the larval hatching compared to the control group, except ARSEF 2211 and ARSEF 3641. ARSEF 729, ARSEF 760, and ARSEF 929, and ARSEF 3643 exhibited the best results among the tested isolates (Table 3).

Bioassay with *Rhipicephalus microplus* larvae

A summary of the variance analysis of larval mortality five and fifteen days after the treatment with 11 fungal isolates and 2 different concentrations is reported in Table 2. Entomopathogenic fungal isolates yielded average larval mortality ranging from 0.1% to 79.3% and 7.42% to 98.9% five days after the treatment with 10⁷ and 10⁸ conidia.ml⁻¹, respectively. Five days after the treatment, *M. anisopliae* s.l. ARSEF 729 applied at 10⁷ conidia.ml⁻¹ yielded the best result of the studied fungal isolates; on the same day, but at the highest concentration, ARSEF 729, ARSEF 760, and ARSEF 3479 caused the highest mortality rates (Table 4).

Table 2. Analysis of variance of *Rhipicephalus microplus* larval hatching, larval mortality at 5 and 15 days after the fungus treatment, and production of conidia by 11 *Metarhizium* spp. isolates.

Sources of variation	Larval hatching		Larval mortality		Production of conidia		
	Degrees of freedom	Mean squares	Degrees of freedom	Mean squares	Degrees of freedom	Mean squares	
	Days after the treatment				Number of conidia		
			5	15			
Fungal isolates (F)	10	653.4**	10	8341.7**	3959.1**	10	350748.8**
Concentration (C)	1	9164.4**	1	52245.0**	19738.9**		
F × C	10	540.4**	10	3713.0**	896.6**		
Additional treatment (control) × factorial (fungal treatment in each concentration)	1	1165.7**	1	7161.6**	45540.0**		
Residual	138	22.9	138	31.02	38.7	55	2474.8
Total	160		160			65	
Coefficient of variation		7.1%		4.52%	5.2%		12.3%

** = significant effect at 1% probability.

Table 3. Average percent of hatching of *Rhipicephalus microplus* larvae and standard error after the treatment of the eggs with *Metarhizium* spp. isolates.

Fungal isolates	Larval hatching (%) after the treatments	
	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹
ARSEF 552	88.1 ± 2.3 Aa*	83.4 ± 2.9 Ab*
ARSEF 724	93.1 ± 0.7 Ba	81.8 ± 2.0 Ab*
ARSEF 729	88.6 ± 0.9 Ba	52.9 ± 4.1 Aa*
ARSEF 760	87.7 ± 1.7 Ba*	61.4 ± 0.9 Aa*
ARSEF 929	88.9 ± 1.2 Ba	63.5 ± 3.2 Aa*
ARSEF 1885	92.3 ± 0.5 Ba	78.6 ± 3.0 Ab*
ARSEF 2211	90.1 ± 0.7 Aa	91.3 ± 0.5 Ab
ARSEF 2521	91.1 ± 0.5 Ba	74.3 ± 1.8 Ab*
ARSEF 3479	93.5 ± 0.5 Ba	85.1 ± 1.6 Ab*
ARSEF 3641	89.9 ± 0.5 Aa	91.1 ± 0.4 Ab
ARSEF 3643	95.1 ± 0.8 Ba	65.7 ± 2.5 Aa*
Control	96.3 ± 0.5%	
MSD Dunnett	7.83%	

Averages followed by the same lowercase letters in the columns and uppercase letters in the lines do not differ statistically by Skott-Knott test (P ≥ 0.05); * = significant difference between the fungus treatment and the control treatment. MSD Dunnett: minimal significant difference for the Dunnett test (P ≤ 0.05).

Table 4. Average percent of mortality of *Rhipicephalus microplus* larvae and standard error 5 and 15 days after the treatment with *Metarhizium* spp. isolates.

Fungal isolates	Fungal concentration and days after the treatment			
	5 days		15 days	
	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹
ARSEF 552	8.1 ± 0.4 Bb	64.5 ± 2.9 Ab*	97.4 ± 1.3 Aa*	99.9 ± 0.07 Aa*
ARSEF 724	2.4 ± 0.3 Bd	67.6 ± 2.9 Ab*	62.1 ± 4.5 Bb*	99.8 ± 0.2 Aa*
ARSEF 729	79.3 ± 2.1 Aa*	84.3 ± 5.1 Aa*	84.6 ± 1.3 Aa*	91.1 ± 0.5 Aa*
ARSEF 760	11.1 ± 0.8 Bb*	98.9 ± 0.5 Aa*	55.7 ± 1.1 Bb*	99.8 ± 0.09 Aa*
ARSEF 929	15.9 ± 1.3 Bb*	63.2 ± 2.0 Ab*	97.6 ± 0.5 Aa*	99.7 ± 0.2 Aa*
ARSEF 1885	3.5 ± 0.4 Ab	7.4 ± 1.1 Ac	68.9 ± 4.3 Bb*	99.4 ± 0.3 Aa*
ARSEF 2211	0.1 ± 0.02 Ab	7.8 ± 0.8 Ac	23.9 ± 1.3 Bc*	51.6 ± 1.3 Ab*
ARSEF 2521	3.9 ± 0.5 Ab	15.0 ± 1.1Ac*	72.1 ± 6.9 Bb*	97.0 ± 0.5 Aa*
ARSEF 3479	8.6 ± 1.2 Bb*	92.8 ± 1.0 Aa*	60.4 ± 3.0 Bb*	99.4 ± 0.34 Aa*
ARSEF 3641	4.8 ± 1.1 Ab	13.5 ± 1.5 Ac*	65.4 ± 4.1 Bb*	96.1 ± 1.2 Aa*
ARSEF 3643	19.3 ± 2.1 Bb*	47.0 ± 5.0 Ab*	96.0 ± 1.4 Aa*	99.6 ± 0.4 Aa*
Control	0.0 ± 0.0%		0.2 ± 0.06%	
MSD Dunnett	8.4%		9.4%	

Averages on the same day followed by the same lowercase letters in the columns and uppercase letters in the lines do not differ statistically by Skott-Knott test (P ≥ 0.05); * = significant difference between the fungus treatment and the control treatment; MSD Dunnett: minimal significant difference for the Dunnett test (P ≤ 0.05).

Fifteen days after the treatment, entomopathogenic fungal isolates yielded average larval mortality ranging from 23.9% to 97.6% and 51.6% to 99.9% followed by the treatment with 10^7 and 10^8 conidia.ml⁻¹, respectively. *Metarhizium* spp. ARSEF 552, ARSEF 729, ARSEF 929, and ARSEF 3643 applied at 10^7 conidia.ml⁻¹ yielded the highest larval mortality, 15 days after the treatment. On the same day, but at the highest concentration, almost all fungal isolates caused larval mortality greater than 90%; ARSEF 2211 was the exception, causing significantly less mortality than the other isolates (Table 4).

Bioassay with *Rhipicephalus microplus* engorged females

Analysis of the biological assays with *R. microplus* engorged females exposed to different *Metarhizium* spp. isolates and concentrations are displayed in Tables 5 and 6. The analysis of variance showed that the different fungal isolates and the two concentrations used negatively impacted the EMW, LHP, EPI, and NI (Table 5) but not the larval hatching. There was no statistical difference in the initial weight of *R. microplus* engorged females used in the groups, which demonstrates that the changes observed in their biological parameters were a result of the treatments with entomopathogenic fungi.

Metarhizium spp. ARSEF 552, ARSEF 724, ARSEF 929, ARSEF 3641, and ARSEF 3643 significantly reduced the EMW from *R. microplus* females treated with 10^7 conidia.ml⁻¹ in comparison to the other isolates and the control (untreated) group. At the highest fungal concentration, the isolates ARSEF 552, ARSEF 729, ARSEF 3641, and ARSEF 3643 yielded the best results for EMW reduction (Table 6). The fungal treatments similarly affected EPI and NI. For both fungal concentrations, ARSEF 552, ARSEF 3641, and ARSEF 3643 significantly reduced the EPI and NI in comparison to the control (untreated) group. *M. anisopliae* s.l. ARSEF 729 used at 10^7 conidia.ml⁻¹ or 10^8 conidia.ml⁻¹ yielded a lower EPI than the control group; however, this isolate only reduced the NI at the highest fungal concentration (Table 6). The isolates ARSEF 724 and ARSEF 929 significantly reduced these indexes only when used at 10^7 conidia.ml⁻¹. On the other hand, ARSEF 1885 and ARSEF 2521 reduced the EPI only when used at the highest concentration (Table 6).

The percent of tick control ranged from 5.32% to 70.83%, and the fungal isolates that yielded the best tick control rates were ARSEF 3643, ARSEF 3641, and ARSEF 729 with tick control of 70.83%, 62.87%, and 64.27%, respectively (Table 6).

Table 5. Analysis of variance for the biological parameters of female *Rhipicephalus microplus* tick after the treatment with 11 fungal isolates and two different concentrations.

Sources of variation	Degrees of freedom	Mean squares			
		EMW	LHP	EPI	NI
Fungal isolates (F)	10	0.008**	1223.6**	1438.13**	1573.14**
Concentration (C)	1	0.005**	4719.8**	936.36**	1137.39**
F × C	10	0.002**	787.8 ^{ns}	490.36**	697.87**
Additional treatment (control) × factorial (fungal treatment in each concentration)	1	0.013**	952.2 ^{ns}	22.84**	2080.95**
Residual	207	0.0008	440.1	117.46	158.74
Total	229				
Coefficient of variation (%)		13.07	16.20	16.41	14.26

EMW: egg mass weight; LHP: larval hatching percent; EPI: egg production index; NI: nutritional index.

** = significant effect at 1% probability. Ns = not significant.

Table 6. Average and standard error of biological parameters of *Rhipicephalus microplus* female ticks and percent of tick control after the treatment with *Metarhizium* spp. isolates.

Fungal isolates	EMW (g)		EPI		NI		Percent of tick control	
	Fungal concentration							
	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹	10 ⁷ conidia.mL ⁻¹	10 ⁸ conidia.mL ⁻¹
ARSEF 552	0.090 ± 0.006 Aa*	0.072 ± 0.007 Aa*	39.22 ± 2.66 Aa*	31.58 ± 3.46 Aa*	50.36 ± 2.78 Aa*	41.85 ± 3.53 Aa*	34.02%	51.64%
ARSEF 724	0.097 ± 0.012 Aa*	0.108 ± 0.006 Ab	41.50 ± 5.27 Aa*	46.80 ± 2.08 Ab	51.24 ± 6.00 Aa*	57.29 ± 1.99 Ab	35.26%	34.36%
ARSEF 729	0.104 ± 0.007 Bb	0.062 ± 0.012 Aa*	45.17 ± 2.79 Bb*	26.27 ± 4.96 Aa*	60.98 ± 2.85 Bb	36.27 ± 6.47 Aa*	23.09%	64.27%
ARSEF 760	0.115 ± 0.011 Ab	0.099 ± 0.008 Ab	49.24 ± 4.25 Ab	43.81 ± 4.06 Ab	62.25 ± 4.14 Ab	59.45 ± 5.05 Ab	15.20%	32.19%
ARSEF 929	0.082 ± 0.014 Aa*	0.123 ± 0.007 Bc	34.90 ± 6.09 Aa*	53.29 ± 2.46 Bc	43.48 ± 7.45 Aa*	64.92 ± 2.49 Bb	43.90%	21.07%
ARSEF 1885	0.111 ± 0.005 Ab	0.098 ± 0.006 Ab*	48.31 ± 0.97 Ab	42.51 ± 2.23 Ab*	58.39 ± 1.03 Ab	53.71 ± 2.12 Ab	18.47%	26.84%
ARSEF 2211	0.129 ± 0.004 Ab	0.129 ± 0.006 Ac	55.59 ± 1.39 Ab	55.47 ± 0.99 Ac	67.17 ± 1.32 Ab	64.26 ± 1.31 Ab	5.53%	5.32%
ARSEF 2521	0.109 ± 0.007 Ab	0.094 ± 0.006 Ab*	47.34 ± 2.75 Ab	41.28 ± 2.58 Ab*	58.73 ± 2.84 Ab	53.42 ± 3.07 Ab	20.09%	34.23%
ARSEF 3479	0.120 ± 0.006 Ab	0.113 ± 0.009 Ac	52.33 ± 1.84 Ab	49.92 ± 4.39 Ac	63.85 ± 2.70 Ab	60.65 ± 5.27 Ab	16.73%	15.19%
ARSEF 3641	0.081 ± 0.011 Aa*	0.062 ± 0.009 Aa*	34.46 ± 4.28 Aa*	26.46 ± 3.60 Aa*	44.32 ± 4.40 Aa*	36.34 ± 4.86 Aa*	45.04%	62.87%
ARSEF 3643	0.082 ± 0.009 Ba*	0.049 ± 0.010 Aa*	35.56 ± 3.95 Ba*	20.85 ± 3.91 Aa*	46.59 ± 4.12 Ba*	29.16 ± 5.20 Aa*	44.08%	70.83%
Control	0.134 ± 0.005		58.65 ± 0.86		67.69 ± 1.48			
MSD Dunnett	0.036		13.09		15.21			

Averages on the same biological parameter followed by the same lowercase letters in the columns and uppercase letters in the lines do not differ statistically by Skott-Knott test (P ≥ 0.05); * = significant difference between the fungus treatment and the control treatment; MSD Dunnett: minimal significant difference for the Dunnett test (P ≤ 0.05).

Production of conidia on PDA

Analysis of the production of conidia on PDA by the *Metarhizium* spp. isolates used in the present study is reported in tables 2 and 7. Analysis of variance demonstrated a significant difference in the production of conidia between the different fungal isolates analyzed (Table 2). Among the studied isolates, the ones with the highest conidial production on PDA were ARSEF 3641 and ARSEF 552 (Table 7), followed by ARSEF 1885, ARSEF 2521, ARSEF 3643, and ARSEF 2211, the last three with similar conidiogenesis. The lowest conidial production on PDA was observed for ARSEF 3479 and ARSEF 929 (Table 7).

Discussion

Alternative control of ticks has become an attractive approach due to increased concerns about populations that are resistant to chemical acaricides as well as environmental, meat, and milk contamination due to the inappropriate use of these chemicals (Klafke et al., 2017; Samish et al., 2004). The search for alternative methods to control *R. microplus* is currently a major challenge for researchers due to its importance in the world livestock industry. In this context, biological control of arthropods using entomopathogenic fungi has received great prominence.

Several studies have demonstrated the effectiveness of the fungi *Metarhizium* for tick control (Angelo et al., 2010; Beys-da-Silva et al., 2020; Camargo et al., 2012; Fernandes & Bittencourt, 2008; Mesquita et al., 2020; Perinotto et al., 2017), including under field conditions (Camargo et al., 2016; Kaaya et al., 2011; Marciano et al., 2020; Samish et al., 2014). This fungus acts mainly through the germination of conidia on the arthropod's cuticle followed by the formation of germ tube and appressorium on the cuticle. After a series of enzymatic reactions and the mechanical apparatus of fungal structures, the fungus penetrates the host's hemocele. Once inside the tick, hemocele colonization reduces the function of ticks' body, which can lead to its death (Bittencourt et al., 1999). Establishing how well a fungal isolate can infect and cause disease in ticks is an important step to identify isolates that may be effective in the field. Accordingly, the experimental method used here provided a general estimate as to how well Brazilian native *Metarhizium* spp. isolates could infect tick eggs, larvae, and females.

The viability of the conidia that were used to treat the ticks is extremely important, since it allows the successful onset of fungus penetration of the tick cuticle (Bittencourt et al., 1999; Schrank & Vainstein, 2010). The suspensions used in the present study had 100% conidial germination, supporting the infective capacity of the conidia that were used in the treatments.

Table 7. Average and standard error of production of aerial conidia from *Metarhizium* spp. isolates on potato dextrose agar medium.

Fungal isolates	N × 10 ⁵ conidia.mL ⁻¹
ARSEF 552	706.8 ± 33.7 a
ARSEF 724	293.7 ± 7.9 d
ARSEF 729	267.6 ± 13.0 d
ARSEF 760	282.3 ± 11.9 d
ARSEF 929	21.4 ± 1.9 e
ARSEF 1885	608.9 ± 35.9 b
ARSEF 2211	493.0 ± 22.9 c
ARSEF 2521	518.5 ± 15.9c
ARSEF 3479	37.8 ± 2.6 e
ARSEF 3641	712.1 ± 24.3 a
ARSEF 3643	498.3 ± 19.6 c

Averages followed by the same lowercase letters do not differ statistically by Skott-Knott test ($P \geq 0.05$).

Aos autores:

Metarhizium spp. used here were able to infect *R. microplus* eggs, reducing the larval hatchability, particularly at the highest concentration. Nevertheless, compared to previously published studies (Fernandes & Bittencourt, 2008) these isolates were not as virulent as expected for *R. microplus* eggs. The tick larval stage is considered the life stage most susceptible to entomopathogenic fungi. In the present study, most isolates (except *M. anisopliae* s.l. ARSEF 2211) greatly affected *R. microplus* larvae survival fifteen days after the treatment with 10^8 conidia.ml⁻¹, although five days after the treatment with this same dose some isolates already achieved remarkably good results (i.e., ARSEF 760, ARSEF 3479, and ARSEF 729).

The virulence survey was very useful to identify isolates with high virulence for *R. microplus* ticks. Four isolates showed exceptional results as biocontrol agents of female ticks (i.e., ARSEF 552, ARSEF 729, ARSEF 3641, and ARSEF 3643). These isolates negatively impacted all biological parameters that were analyzed and exhibited the best tick control percentage. Interestingly, some isolates exhibited excellent results for specific life stages (for example, ARSEF 3641 for *R. microplus* females, but not eggs or larvae); nevertheless, one of these isolates (i.e., *M. anisopliae* s.l. ARSEF 729) was virulent for all stages (eggs, larva, and females) when applied at the highest concentration. Accordingly, this isolate can be considered a promising candidate to be used against *R. microplus* parasitic phases (i.e., applied on the host) and against the non-parasitic phases (i.e., applied to control the life stages that are found on the ground). Note that the fungal conidia concentration usually tested against ticks, which is 10^8 conidia ml⁻¹, is higher than the ones tested for insects, which is often 10^4 or 10^5 conidia.ml⁻¹ (Roberts & St Leger, 2004). In the present study, considering both virulence for *R. microplus* ticks and the capacity of conidial production on PDA, ARSEF 3643 stands out as a promising isolate for tick control.

Most mycoinsecticides produced currently in Brazil are based on aerial conidia (Mascarin et al., 2019). Several variables, including the type of substrates, the application of modified atmospheres, temperature, and light can impact aerial conidia production (number and quality of conidia) of *Metarhizium* (Barra-Bucarei et al., 2016; Garcia-Ortiz et al., 2015; Lopes et al., 2018). The capacity for conidia production is important especially for tick control that requires high concentration of conidia; however, highly virulent isolates do not always have high rates of conidiogenesis as observed for ARSEF 729. Thus, searching for technologies that improve aerial conidia production is necessary to afford the commercial availability of such mycoacaricides.

New strategies for tick control are necessary and the use of entomopathogenic fungi against different stages of tick life is one of them (Wassermann et al., 2016). The genetic variability among the entomopathogenic fungal isolates explains the different virulent potentials for insect and arachnid pest control, highlighting the importance of studies that select isolates with efficient characteristics for biological control programs of agricultural and veterinary pests (Barci et al., 2009). The present study explored the conidiogenesis capacity of different Brazilian *Metarhizium* spp. isolates and their virulence to *R. microplus* eggs, larvae, and engorged females. This is extremely important, since studies involving the selection of convenient isolates for field application is imperative for the successful biological control of ticks.

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Ethics statement

Engorged females of *Rhipicephalus microplus* used in the present study were collected from the floor of cattle pens holding artificially infested calves. This received approval of the ethics committee for the use of animals in research - CEUA/IV/UFRRJ- protocol number 037/2014.

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Conflict of interests

The authors declare that there is no conflict of interest.

Authors' contributions

GAJ and VREPB - Conceived and designed the experiments. GAJ and MGC - Performed the experiments. GAJ, PSG, MGC and WMSP - Analyzed the data. VREPB - Contributed with reagents/materials/analysis tools. GAJ, MGC, PSG and WMSP - Contributed to the writing of the manuscript.

Availability of complementary results

There are no complementary results.

This study was carried out at Estação Parasitológica W. O. Neitz, no Departamento de Parasitologia Animal do Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica, RJ, Brasil.

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