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Short communication

Metarhizium anisopliae for controlling *Rhipicephalus microplus* ticks under field conditions

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ABSTRACT

Metarril SP Organic is a product based on the fungus *Metarhizium anisopliae*, which was developed for controlling agricultural pests. The present study evaluated the effect of Metarril SP Organic plus 10% mineral oil, for controlling *Rhipicephalus microplus* under field conditions. Three groups were formed: Control group, which received no treatment; Oil control group, which was bathed only with water, Tween 80 and mineral oil; and Metarril group, bathed in the oil-based formulation Metarril SP Organic. Two treatments per group were performed and to verify the effect of the treatments, all *R. microplus* ticks between 4.5 and 8.0 mm in length on the left side of the cattle were counted on days +7, +14 and +21 after each treatment, and a sample of engorged females was collected for evaluation of biological parameters. The Metarril SP Organic oil formulation showed efficacy ranging from 8.53 to 90.53%. The average efficacy of the oil-based formulation of Metarril SP Organic was 75.09 and 46.59% compared with the groups Control and Oil control, respectively. There were no significant changes in biological parameters of engorged *R. microplus* females collected from animals. Although there was no significant difference in the amount of ticks between the Oil control and Metarril groups, it is believed that the association of mineral oil with Metarril SP Organic product is effective in *R. microplus* tick control in field. Thus, this association has potential to be used in strategic control programs of cattle tick.

1. Introduction

Interest in biological control programs for agricultural pests and parasites in livestock has grown considerably around the world because of new directives for production that promotes conservation and sustainable use of biological resources, in addition to concerns about the food quality and the absence of chemical residues in meat and dairy products. However, the use of chemical insecticides and acaricides is still the predominant method for controlling pests and parasites such as *Rhipicephalus microplus* ticks. This parasite has a major economic impact on livestock because of the direct and indirect losses it causes. It has been estimated that in Brazil alone, these losses reach approximately 3.24 billion dollars a year (Grisi et al., 2014).

Lack of concern about proper use of the chemical bases that are commercially available has reduced the effectiveness of these products through selection and spreading of resistant ticks (Furlong et al., 2007). In this context, biological control using arthropod-pathogenic fungi is a promising method that should be considered in relation to tick control.

The virulence of these fungi against ticks has already been proven in many laboratory studies (Fernandes et al., 2006; Leemon and Jonsson, 2008; Camargo et al., 2012; Quinelato et al., 2012). However, under natural conditions, this virulence decreases because the action of these pathogens is influenced by various

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environmental factors, such as temperature, relative humidity and exposure to solar radiation (Butt et al., 2001; Leemon et al., 2008; Ojeda-Chi et al., 2010; Fernandes et al., 2012). Therefore, it is necessary to evaluate the action of fungi under environmental conditions in order to devise ways of using these pathogens without losing their infectivity and virulence.

The formulation in which the conidia are applied is of fundamental importance to the success of tick biocontrol because this maintains the viability, virulence and effectiveness of the pathogens under field conditions. Some products composed by arthropod-pathogenic fungi were tested against different tick species under laboratory conditions. Oil formulations of a product based on isolates ESALQ 1037 and ESALQ E9 of *Metarhizium anisopliae* (Metarril WP Organic) were tested against *R. microplus* tick and presented significant changes in all evaluated parameters (Marciano et al., 2013). The products Metarril SC (this product is no longer available on market) and Boveril WP were tested against *Amblyomma cajennense* tick (Lopes et al., 2007). Boveril WP was also tested against *R. microplus* (Perinotto et al., 2012a) and *Dermacentor nitens* (Perinotto et al., 2012b). All these products referred to above caused significant changes against ticks tested, especially when elaborated in oil formulations.

The present study was developed through motivation from the results found by Camargo et al. (2014). In assessing the oil-based formulation of Metarril SP Organic, a product based on *M. anisopliae* fungus, against cattle ticks in a pen-based study, these authors concluded that this oil-based fungal formulation might be an important tool for controlling *R. microplus* ticks, and that studies in the field using more than one treatment would be beneficial for increasing the effectiveness of this product for tick control under natural conditions. Therefore, the aim of the current study was to evaluate the formulation of Metarril SP Organic, plus 10% mineral oil, for controlling *R. microplus* ticks in field tests.

2. Material and methods

2.1. Location and period of the experiment

The field test was carried out between the months of April and June 2014, in the field area of the "W.O. Neitz" Experimental Station for Parasitological Research of the Federal Rural University of Rio de Janeiro (UFRRJ), Seropédica campus, Rio de Janeiro, Brazil, which is located at 22° 44′ South, 43° 42′ West. This region has a tropical climate, an altitude of 33 m, average annual temperature of 23.5 °C and average annual rainfall of 1483 mm. The evaluation of the biological parameters of *R. microplus* females collected from animals during the test was carried out from May to July 2014 in the Microbial Control Laboratory of UFRRJ.

2.2. Cattle used in the experiment

A total of 30 Angus cattle, Red Angus variety, aged approximately between 1 and 4 years (mean age 1.87 years) and weighing between 155 and 461 kg (average weight of 313.1 kg), were used. The groups were kept in three separate paddocks, with an area of about 2 ha per paddock with grass and water freely available. This study was approved by the Ethics Committee for Animal Use (CEUA) of UFRRJ, under the protocol number 017/2014.

2.3. Formulations evaluated in the experiment

A formulation only containing water, 10% mineral oil (Vetec Fine Chemicals Ltda., Rio de Janeiro, RJ, Brazil) and 1% Tween 80 was prepared for the treatment administered to the oil control group. A fungal formulation was prepared using Metarril SP Organic, water, 10% mineral oil and 1% Tween 80. The product Metarril SP Organic was developed by Koppert Biological Systems to control agricultural pests and consisted of the isolates ESALQ 1037 and ESALQ E9 of *M. anisopliae*. The fungal formulation used in the field test contained 1×10^8 conidia/mL.

2.4. Experimental design

All the methodology used in the field test was performed in accordance with to the methodology described by Holdsworth et al. (2006) and the methodology recommended by the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) for production, control and use of antiparasitic drugs for veterinary purposes (Ordinance No. 48 of May 12, 1997; MAPA) (Brasil, 2012).

The animals used in the field test were naturally infested with *R. microplus* ticks, i.e., all animals had sufficient natural parasite load for the field test development, not being necessary the artificial infestation of animals with tick larvae (Holdsworth et al., 2006). Before the experiment was started, ticks between 4.5 and 8.0 mm long were counted weekly so as to assure the presence of at least 20 ticks on one side of each animal. The 30 cattle were distributed among the groups according to the infestation degree, determined according to the number of ticks between 4.5 and 8.0 mm present on one side of the body of each animal over the three days prior to treatment (−3, −2 and −1). The three animals with the highest average tick counts were randomly allocated, one to each group. The next three animals were then allocated, and so on until the three groups of ten animals each had been completed (Holdsworth et al., 2006). Thus, the groups began the test with very similar average number of ticks.

Two treatments with the formulations were performed on the cattle: the first treatment on day zero and the second on day +3. There were three test groups: a group treated with the oil-based formulation of Metarril SP Organic (Metarril); a group treated only with a formulation of water, mineral oil and Tween 80 (Oil Control) and a control group that received no treatment (Control). Using a backpack pump sprayer system, the formulations were sprayed over the animals' bodies, from the bottom up and in the opposite direction to how the fur lies, giving greater attention to the areas most affected by ticks, such as the inner thighs, dewlap, ears and perineum. Each animal was sprayed with 4 L of the corresponding formulation, which was enough to bathe the entire body surface of an animal with the physical characteristics listed above.

After the cattle had been treated with the formulation, all female ticks between 4.5 and 8.0 mm in length, present on the left side of each animal were counted on days +7, +14 and +21 after each treatment, i.e. a total of six counts (+7, +10, +14, +17, +21 and +24). A sample of ten engorged females per group per day was randomly collected to evaluate the biological parameters. They were taken to the laboratory, individually weighed, fixed in Petri dishes and incubated at 27 ± 1 °C and relative humidity ≥80%. The eggs produced by the females were weighed and incubated under the same temperature and humidity conditions for further evaluation of the larvae that hatching.

To evaluate the efficacy of the oil-based formulation of Metarril SP Organic on *R. microplus* ticks in the field tests, the following biological parameters were investigated: initial weight of the female, weight of the egg mass, larvae hatching percentage, and nutritional and egg production indexes (Bennett, 1974).

The efficacy of the Metarril SP Organic formulation was calculated using the following formula (Henderson and Tilton, 1955; Holdsworth et al., 2006; Brasil, 2012):

$$\text{Efficacy} = \left(1 - \frac{T_a \times C_b}{T_b \times C_a}\right) \times 100 \quad (1)$$

where T_a = average number of ticks that fell from treated animals after the day of treatment; T_b = average number of ticks that

Table 1
Average number of *Rhipicephalus microplus* ticks, according to the day of evaluation, obtained after treatments of the animals with the formulation of Metarril SP Organic plus 10% mineral oil, and in the two control groups, in the field test.

Days	Average number of ticks		
	Control	Oil Control	Metarril
Day 0	65.3 a[a] ± 35.0	65.4 a ± 31.0	65.4 a ± 28.5
Day +7	41.6 a ± 24.22	45.3 a ± 36.69	19.5 a ± 17.10
Day +10	38.5 a ± 22.74	55.6 a ± 51.52	34.7 a ± 17.17
Day +14	30.9 a ± 23.71	28.9 a ± 23.32	16.7 a ± 8.83
Day +17	65.5 a ± 42.22	25.3 b ± 26.49	20.0 b ± 13.70
Day +21	129.6 a ± 97.89	23.0 b ± 23.48	12.3 b ± 7.65
Day +24	165.3 a ± 132.04	42.0 b ± 43.46	17.4 b ± 7.35

[a] Means followed by the same letter on the same line do not differ significantly from each other ($p \geq 0.05$), according to the Student-Newman-Keuls test.

Table 2
Daily and average efficacies (%) of the Metarril SP Organic formulation plus 10% mineral oil for controlling *Rhipicephalus microplus* ticks, in relation to the control groups, in the field test.

Days	Daily efficacy	
	Control[a]	Oil Control[b]
Day +7	52.43%	56.24%
Day +10	8.53%	36.59%
Day +14	45.15%	41.29%
Day +17	69.01%	19.68%
Day +21	90.53%	45.53%
Day +24	89.29%	57.81%
Average efficacy	75.09%	46.59%

[a] Efficacy of the oil-based formulation of Metarril SP Organic calculated relative to the Control group.
[b] Efficacy of the oil-based formulation of Metarril SP Organic calculated relative to the Oil Control group.

fell from treated animals over the three days prior to treatment; C_a = average number of ticks that fell from control animals after the day of treatment; C_b = average number of ticks that fell from control animals over the three days prior to treatment.

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed, followed by the Student-Newman-Keuls (SNK) test for parametric data analysis. To evaluate the nonparametric data, the Kruskal-Wallis test followed by SNK was used. In all analyses, the significance level was 5% ($p \leq 0.05$) (Sampaio, 2002).

3. Results

There was a reduction in the average number of ticks in the Metarril group, compared with the Control group, starting from the second count after the second treatment (day +17). However, there was also a reduction in the number of ticks in the Oil Control group, compared with the Control group, starting from day +17 (Table 1). The average numbers of ticks in the Oil Control and Metarril groups remained lower than the number in the Control group until the last day, with reductions in the number of ticks in Oil Control and Metarril groups of up to 5.63 and 10.54 times, respectively, in relation to the Control group, in the last week (Table 1).

During the first week after treatment, the three groups continued to present similar average numbers of ticks. From the second week on, significant differences in the average number of ticks began to be seen between the groups, and these differences increased considerably in the third week after the treatments.

The oil-based formulation of Metarril SP Organic presented daily efficacy ranging from 8.53% to 90.53% and from 19.68% to 57.81%, compared with the Control and Oil Control groups, respectively. The average efficacy of the Metarril SP Organic formulation plus 10% mineral oil was 75.09% and 46.59%, in relation to the Control and Oil Control groups, respectively (Table 2).

The efficacy of the oil-based formulation of Metarril SP Organic began to increase in the second week after treatment, reaching peaks of 90.53% over the Control group and 57.81% over the Oil Control group, respectively, in the third week.

There were no significant changes in biological parameters of engorged *R. microplus* females collected from the animals' bodies in the field test on any of the days on which ticks were collected.

4. Discussion

Numerous studies have demonstrated the in vitro pathogenicity of arthropod-pathogenic fungi in relation to different species of ticks (Reis et al., 2001; Fernandes and Bittencourt, 2008). However, few studies have tested the action of these pathogens under environmental conditions. One important factor that aids in consolidating microbial control programs is to evaluate the influence not only of environmental factors, but also of the fungus/tick/cattle relationship under realistic conditions, within which all factors exist and contribute. With this in mind, the aim of the present study was to evaluate the action of Metarril SP Organic associated with mineral oil, against *R. microplus* tick under ambient conditions.

A downward trend in the average number of ticks in the group treated with the Metarril oil formulation when compared to Control and Oil control groups was observed. However, there was no significant difference between the Oil control and Metarril groups (Table 1). The high standard deviation values observed in this study, probably caused by differences between the animals, may have influenced the statistical analysis of the average number of ticks, making it difficult to detect significant difference between the Oil control and Metarril groups. Factors, such as age and animal weight may have interfered in obtaining the data and hindered the results interpretation. For example, older animals may have had more contact with ticks and consequently developed greater resistance when compared with younger animals. It is believed that the standardization of animals and its appropriate distribution between the groups has utmost importance for the assays development. Therefore, in view of the difficulty of having a large number of animals available with similar characteristics (weight, age, breed, blood level, infestation degree, etc.) and the importance of the development of the field test for the best understanding of the effect of the fungal formulation on the tick in environmental conditions, it was decided in this study the animals standardization according to the characteristics that are believed to have more influence on the results. These characteristics were: (1) Breed and degree of blood: animals were selected from the same breed (Angus cattle, Red Angus variety) and very similar blood level due to the large variability in susceptibility to tick *R. microplus* presented by different cattle breeds. (2) Infestation degree: animals were distributed among the groups according to the infestation degree, making the three groups present an initial average number of ticks very similar (Table 1). Therefore, even the cattle showed different resistance degree to the ticks due to differences in age, for example, it was expected that this difference was diluted with stratification by the infestation degree. (3) High replication: it was decided to use of a large number of animals per group (10 animals per treatment), thus aiming to reduce the effect of other factors that might have interfered, including individual susceptibility of animals to tick. Thus, the importance of standardization to the maximum characteristics of animals to be used in field trials is noted.

Development of a treatment protocol for cattle using the oil-based formulation of Metarril SP Organic may be an important step for adding to the potential effectiveness of the product for controlling ticks under natural conditions. Through establishing an ideal number of treatment baths for cattle using fungal formulations, and an ideal interval between treatments, it ought to be possible to increase the efficacy of the product and maintain the tick population at an acceptable level. In assessing the product Metarril SP Organic plus mineral oil against cattle ticks in a pen study, Camargo et al. (2014) observed average efficacy of 47.74% from a single treatment applied to the animals. In the present study, the same fungal formulation was used, in field tests comprising two treatments applied to cattle, and average efficacy of 75.09% was achieved. In other words, just a single additional round of spraying with fungal formulation increased the efficacy of the product by 57%. Alonso-Díaz et al. (2007) observed efficacy of between 0 and 91.2% from a suspension of *M. anisopliae* that was used to bathe cattle presenting natural infestation with *R. microplus*, every 15 days for two months, under field conditions. Kaaya et al. (2011) assessed the effect of an oil-based formulation of *M. anisopliae* that was sprinkled onto cattle infested with *R. evertsi* and *R. decoloratus* ticks, once every three weeks for one year, under field conditions, and observed a reduction in the tick population of up to 83%. These results emphasize that there is a need to develop a cattle treatment protocol focusing on enhancing the action of fungal formulations for controlling ticks under field conditions.

In the field test, there were no significant changes in biological parameters of engorged *R. microplus* females on any of these days which ticks were collected. Kaaya et al. (2011) observed 93–100% mortality of *R. evertsi* and *R. decoloratus*, respectively, among cattle bathed using a formulation of *M. anisopliae* plus 20% sunflower oil, in a field test. However, these researchers collected the ticks from the bodies of the cattle immediately after treatment with the fungal formulation, which differed from the methodology used in the present study, in which ticks were collected on days +7, +14 and +21 after each treatment. Although the fungus was able to kill a large proportion of the larvae and nymphs present on the cattle at the times of the treatments, given that there was a considerable decrease in the number of ticks in the group treated with the fungal formulation in the present study, it is believed that over time, some of the fungus may have been removed from the animals' bodies through their licking action and through contact with the ground when lying down. Another factor that may have influenced this result is the possibility of natural re-infestation of cattle, since they remained unrestrained in the pasture. Larvae that may have become attached to the animals after the treatments possibly had less contact with the fungal formulation than did the larvae that were on the cattle at the time of the treatment. Thus, both the ticks that survived the treatment and those that climbed onto the animals after the treatments were collected as adults for evaluation of biological parameters and their development was unaffected.

A decrease in the average number of ticks on the cattle in the Oil Control group, compared with the animals in the Control group, was observed in this study. Deleterious effects on ticks caused by different oils had previously been reported (Camargo et al., 2012, 2014; Samish et al., 2014). It is believed that the oils interfere with the physiology of arthropods, through penetrating into tissues and causing cell death, locomotor disability and dehydration (Najar-Rodríguez et al., 2008), and also through interfering in the breathing of the arthropods. It would be necessary to conduct a thorough analysis on the oil constituents and their effect on ticks in order to assert that there could be a possible toxic effect. However, it is possible that the interference of the oil in tick physiology may have caused the changes that were observed in the group treated only with mineral oil in the present study. However, the action of the mineral oil used singly is not satisfactory so that it is used alone in *R. microplus* tick control.

Previous studies have shown the action of commercial products based on fungi from different tick species under laboratory conditions (Lopes et al., 2007; Perinotto et al., 2012a,b; Marciano et al., 2013). The product Metarril WP Organic, a wettable powder also based on isolates ESALQ 1037 and ESALQ E9 of *M. anisopliae* was tested against *R. microplus* ticks under laboratory conditions, and it significantly reduced all parameters of eggs, larvae and females when compared to aqueous control and oil control groups. Control groups containing 1, 3 and 5% oil showed 1.85, 6.59 and 16.34% control percentage, respectively; instead of the product formulations containing 1, 3 and 5% oil showed 22.66, 85.26, 97.35% control percentage, respectively (Marciano et al., 2013). Similar results were observed in other studies that evaluated oily fungal formulations against ticks (Camargo et al., 2012; Perinotto et al., 2012b). These studies demonstrated that the products marketed for the control of agricultural pests can take action also against other parasites such as ticks. Moreover, these results support the idea that the oil may have an effect against ticks, however, it does not possess sufficient efficacy to be used alone in the control of ticks, for this reason it should be used as an adjuvant in fungal formulations.

5. Conclusions

It is believed that the standard deviations occurred in the field assay have hampered the observation of difference between Metarril and Oil control groups. However, there was significant reduction in an average number of tick observed in relation to the Control group, in addition to high efficacies caused by oil formulation of product. Thus, it is believed that the association of the product Metarril SP Organic with mineral oil is more effective in the control of *R. microplus* than these used singly, and this association shows potential for the biological control of cattle tick under field conditions.

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