

In vitro efficacy of two commercial products of *Metarhizium anisopliae* s.l. for controlling the cattle tick *Rhipicephalus microplus*

Eficácia *in vitro* de dois produtos comerciais de *Metarhizium anisopliae* s.l. no controle do carrapato bovino *Rhipicephalus microplus*

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Abstract

The effects of two different products - Metarril® SP Organic (dry conidia) and Metarril® SC Organic (emulsifiable concentrated conidia in vegetable oil) - on eggs, larvae and *Rhipicephalus microplus* engorged females were here explored. Three concentrations (10^8 , 10^7 , and 10^6 conidia mL⁻¹) for both products were prepared in water + 0.1% Tween® 80 (v/v); afterward, bioassays were carried out for all *R. microplus* stages by immersion in suspensions (Metarril® SP) or formulations (Metarril® SC). Metarril® SP suspensions showed low efficacy and did not affect biological parameters of treated engorged females; for eggs and larvae, only slight decreases in hatchability and larvae population were observed. Despite a delay in germination, Metarril® SC presented better results; for females, reductions in Egg Mass Weight (EMW) and Egg Production Index (EPI) were reported. On eggs, 10^8 conidia mL⁻¹ increased Incubation Period (IP), shortened Hatching Period (HP) and decreased hatchability by up to 61%; for larvae, 10^7 and 10^8 conidia mL⁻¹ reached 99.6 and 100% larval mortality respectively, 10 days after fungal exposure. Thus, further studies involving the use of oil-based formulations for ticks such as Metarril® SC need to be performed, especially to control the most susceptible stages (eggs and larvae).

Keywords: Biological control, entomopathogenic fungus, oil-based formulation, cattle tick.

Resumo

No presente trabalho, os efeitos de dois diferentes produtos foram avaliados - Metarril® SP Organic (conídios secos) e Metarril® SC Organic (conídios concentrados em óleo vegetal) - para ovos, larvas e fêmeas ingurgitadas de *Rhipicephalus microplus*. Três concentrações (10^8 , 10^7 e 10^6 conídios mL⁻¹) para cada produto foram preparadas em água + Tween® 80 0,1% (v/v); os bioensaios foram realizados para todos os estágios de *R. microplus* por imersão nas suspensões (Metarril® SP) ou formulações (Metarril® SC). Metarril® SP não afetou os parâmetros biológicos das fêmeas, demonstrando assim baixa eficácia; para ovos e larvas, foram observadas discretas diminuições na eclodibilidade e na população de larvas. Apesar de um atraso na germinação, Metarril® SC apresentou melhores resultados; para as fêmeas, foram detectadas reduções no Peso da Massa de Ovos (PMO) e no Índice de Produção de Ovos (IPO). Para os ovos, a concentração de 10^8 conídios mL⁻¹ aumentou o Período de Incubação (PI), reduziu o Período de Ecloração (PE) e também o da eclodibilidade em até 61%; para larvas, 10^7 e 10^8 conídios mL⁻¹ atingiram 99,6 e 100% de mortalidade larval, respectivamente, 10 dias após a exposição fúngica. Com isso, estudos adicionais que envolvem o uso de formulações à base de óleo para carrapatos, como Metarril® SC, precisam ser realizados, especialmente para controlar os estágios mais suscetíveis (ovos e larvas).

Palavras-chave: Controle biológico, fungo entomopatogênico, formulação oleosa, carrapato bovino.

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Introduction

Commonly affecting livestock production, *Rhipicephalus microplus* (Canestrini 1888) (Acari: Ixodidae) infestations lead to huge economic losses in Brazil (Grisi et al., 2014). The main tick control method includes a widespread application of chemicals; however, indiscriminate usage of these molecules not only result in reductions on efficacy and tick resistance (Abbas et al., 2014; Reck et al., 2014; Klafke et al., 2017) but also pose a risk to the environment and human health (Pignati et al., 2017). Regarding some alternatives, new techniques for tick-controlling have been constantly investigated including vaccines (Merino et al., 2013), herbal products (Ghosh et al., 2015), and entomopathogenic fungi (Fernandes et al., 2012).

As an important biopesticide, *Metarhizium* spp. are applied in the field to control some arthropods (Aw & Hue, 2017); it is known about its low non-target impacts and high safety for mammals, birds, aquatic animals and plants (Zimmermann, 2007). This fungus affects all *R. microplus* stages (Fernandes et al., 2012; Quinelato et al., 2012; Mascarin et al., 2019) and its good performance is totally dependent on the environmental conditions (Jackson et al., 2010; Camargo et al., 2016; Ment et al., 2017; Tomer et al., 2018). In order to minimize these impediments, formulations are investigated by several researchers over the years, and the commercial production has greatly increased (Kaay & Hassan, 2000; Faria & Wraight, 2007; Kaaya et al., 2011; Camargo et al., 2016; Beys-da-Silva et al., 2020). There are 82 microbial pesticide products registered in Brazil, being 60% originally composed of fungi and none officially registered for ticks. Most formulations use conidia as the base, which may have or not some adjuvant added (Mascarin et al., 2019). In this regard, oil-based formulations improve efficacy and promote protection against environmental challenges (Samish et al., 2014).

This paper reports the *in vitro* efficacy in controlling *R. microplus* by testing two products from Koppert® Biological Systems (formerly Itaforte Bioproducts - Piracicaba, São Paulo, Brazil) based on *M. anisopliae* s.l. primarily indicated to agricultural pests. Since Itaforte® was purchased by Koopert® (Alves et al., 2017), many formulations of Metarril® have been revised and currently, Metarril® SP and Metarril® SC are no longer accessible commercially in Brazil (Mascarin et al., 2019). However, all tests of this study were done before Koppert® stopped producing and/or marketing these products. The effects of both formulations at different concentrations on all tick stages were explored, contributing then to better understanding the tick control using commercial products based on fungi.

Material and Methods

The experiments were performed at Federal Rural University of Rio de Janeiro (UFRRJ), Seropédica, RJ, Brazil [Department of Animal Parasitology, Veterinary Institute]. Two different products not yet registered for tick control in Brazil - based on *M. anisopliae* s.l. conidia - were used according to manufacturer's directions: Metarril® SP Organic (a mixture of two *M. anisopliae* s.l. strains - ESALQ 1037 and E9 - suspended in water) and Metarril® SC Organic (ESALQ 1037 strain in emulsifiable concentrated conidia in vegetable oil). For both products, three concentrations of fungi (10^8 , 10^7 and 10^6 conidia mL⁻¹) were prepared in 0.1% Tween® 80 (v/v - diluent); and aqueous suspensions (Metarril® SP) and oil formulations (Metarril® SC) were vigorously homogenized and quantified using a hemocytometer. As a control, in Metarril® SP assays, only the diluent was used; for Metarril® SC, the emulsifiable vegetable oil present in the product was gently supplied by the company and diluted 10 (oil control 3), 100 (oil control 2), and 1000 times (oil control 1) in order to follow the same oil proportions found in the fungal oil suspensions.

Conidial germination was assessed from 10 µL aliquots of each aqueous suspension or oil formulation (10^6 conidia mL⁻¹) dripped on Potato Dextrose Agar (PDA) + 0.05% chloramphenicol (CAP) (Kasvi®). The plates were kept in dark (25 ± 1 °C and relative humidity (RH) $\geq 80\%$). After 24 or 48 hours, 10 µL lactophenol cotton blue was placed directly over the inoculum and covered with a glass coverslip. Then, viability was achieved by counting 3×100 conidia using a light microscope. Conidia were considered germinated if the germ tube was at least twice the width of the conidia (Hywel-Jones & Gillespie, 1990).

In the bioassays, *R. microplus* engorged females were obtained by artificial infestation in calves [Research Permit nº 133/2014 - Animal Ethics Committee/ UFRRJ] and collected from the stall floor, being sanitized in 0.1% sodium hypochlorite solution. For females' assays, ticks were separated into ten groups with ten specimens each according to Yule formula ($nc = 2.5\sqrt{N}$), where nc is the number of classes and N is the number of variables (Sampaio, 2015). The treatment was made by immersion of females in one mL control, aqueous suspension or oil formulation for three min. After, females were kept at 27 ± 1 °C and RH $\geq 80\%$, all eggs from each group weighed daily and the Egg Mass Weight (EMW), Nutrient Index (NI) and Egg Production Index (EPI) assessed (Bennett, 1974). Finally, the efficacy (% Control) were calculated for each treatment taking into account their respective controls (Drummond et al., 1971).

Some females were used to obtain eggs and larvae to the other trials. Fifty mg were placed into test tubes and sealed with hydrophilic cotton to assess the efficacy for eggs. One mL of each product or control was added to each tube and the egg masses were submerged for three min. After, all tubes were turned upside down and the excess absorbed (Quinelato et al., 2012). Eggs were kept at 27 ± 1 °C and RH \geq 80% and Incubation Period (IP), Hatching Period (HP), and Hatchability (%) were evaluated up to thirty days after fungal exposure.

The effects on larvae were assessed from 50 mg of eggs (approximately 1000 larvae) weighed into test tubes. Only tubes with hatchability greater than 95% were used in the trials. Larvae were also treated with one mL of each product or control and submerged for three min. The excess was after drained and larval mortality evaluated every five days until 15 days (Quinelato et al., 2012).

The fungal re-isolation from eggs (those from which larvae did not hatch), dead larvae and colonized engorged females were performed on PDA + 0.05% CAP and kept at 25 ± 1 °C and RH \geq 80% for 14 days. Fungal colony growth was examined and morphologically classified as *Metarhizium* species (Bischoff et al., 2009).

Data were submitted to statistical analysis using InStat® 3.0 (GraphPad Software, San Diego, California). After normality tests (Shapiro-Wilk), variance analysis (one-way ANOVA) followed by Tukey's *post hoc* test (parametric data) or Kruskal-Wallis followed by Dunn's Multiple Comparison Test (non-parametric data) were applied for determining the differences ($p < 0.05$).

Results

A delay in germination was observed to Metarril® SC; although conidia from Metarril® SP germinated up to 100% 24 h post-inoculation, conidia from Metarril® SC only fully germinated 48 h after incubation.

Metarril® SP did not affect the biological parameters of treated engorged females, showing low efficacy. In contrast, Metarril® SC, in some concentrations, was able to reduce EMW and/or EPI (Table 1). Regarding NI, Metarril® SP at 10^7 and 10^8 conidia mL⁻¹ was able to decrease this parameter and for Metarril® SC, NI were not altered at any concentration tested. Percent Control is demonstrated in Figure 1, where Metarril SC at 10^8 conidia mL⁻¹ had the highest percent control.

On eggs, Metarril® SP only decreased hatchability by 12 to 24% after treatment. For Metarril® SC, 10^8 conidia mL⁻¹ demonstrated the best results, increasing IP, shortening HP around 2 days and decreasing hatchability by up to 61% (Table 2).

The larval stage was the most affected by fungal exposure. Metarril® SP showed reductions on larvae population by two (10th day) and five (15th day) times at its highest concentration. Metarril® SC achieved the most significant results, reaching at 10^7 and 10^8 conidia mL⁻¹ complete or near-complete larval mortality within 10 days of treatment (Table 3).

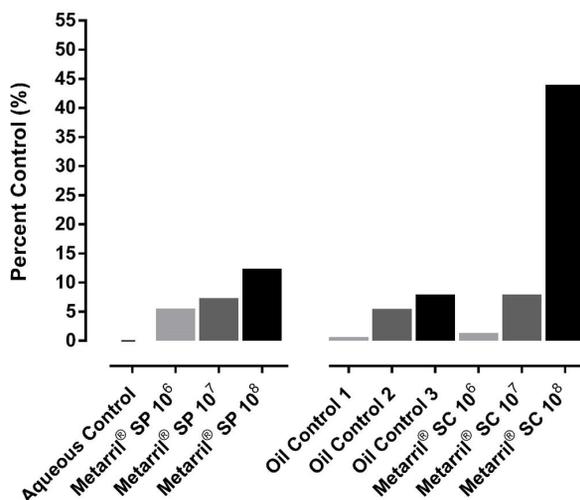


Figure 1. Percent Control (% Control) of *Rhipicephalus microplus* engorged females exposed to Metarril® SP suspensions and Metarril® SC formulations (10^6 , 10^7 and 10^8 conidia mL⁻¹). The emulsifiable vegetable oil was diluted 10 times (Oil Control 3), 100 times (Oil Control 2), and 1000 times (Oil Control 1).

Table 1. Egg Mass Weight (EMW), Egg Production Index (EPI) and Nutrient Index (NI) of engorged *Rhipicephalus microplus* females exposed to Metarril® SP suspensions and Metarril® SC formulations (10⁶, 10⁷ and 10⁸ conidia mL⁻¹). The emulsifiable vegetable oil was diluted 10 times (Oil Control 3), 100 times (Oil Control 2), and 1000 times (Oil Control 1).

Groups	EMW (g) ¹	EPI (%) ²	NI (%) ¹
Aqueous Control	0.1664 ± 0.025 a	62.94 ± 4.94 a	80.91 ± 4.73 a
Metarril® SP 10 ⁶	0.1591 ± 0.019 a	60.68 ± 5.54 ab	79.12 ± 3.76 a
Metarril® SP 10 ⁷	0.1617 ± 0.022 a	58.76 ± 5.80 ab	75.36 ± 5.31 b
Metarril® SP 10 ⁸	0.1665 ± 0.022 a	62.47 ± 4.20 a	74.56 ± 8.20 ab
Oil Control 1	0.1689 ± 0.015 a	63.09 ± 3.39 ab	81.61 ± 3.59 a
Oil Control 2	0.1594 ± 0.023 a	59.76 ± 6.09 ab	76.00 ± 4.41 ab
Oil Control 3	0.1658 ± 0.022 a	61.88 ± 3.30 ab	73.89 ± 3.52 ab
Metarril® SC 10 ⁶	0.1695 ± 0.022 a	63.04 ± 4.25 a	78.37 ± 6.57 ab
Metarril® SC 10 ⁷	0.1465 ± 0.026 ab	55.18 ± 8.98 b	75.66 ± 8.48 ab
Metarril® SC 10 ⁸	0.1262 ± 0.026 b	45.74 ± 9.17 c	68.40 ± 3.12 ab

Mean ± standard deviation with the same letter, in the same column, did not differ by variance analysis (one-way ANOVA) followed by Tukey's post hoc test¹ or by Kruskal-Wallis followed by Dunn's Multiple Comparison Test² (p ≥ 0.05). Bioassays were repeated twice (10 females each).

Table 2. Incubation Period (IP), Hatching Period (HP), and Hatchability (%) of *Rhipicephalus microplus* eggs exposed to Metarril® SP suspensions and Metarril® SC formulations (10⁶, 10⁷ and 10⁸ conidia mL⁻¹). The emulsifiable vegetable oil was diluted 10 times (Oil Control 3), 100 times (Oil Control 2), and 1000 times (Oil Control 1).

Groups	IP (days) ¹	HP (days) ²	Hatchability (%) ²
Aqueous Control	24.80 ± 1.14 a	14.70 ± 0.95 a	98.00 ± 1.70 a
Metarril® SP 10 ⁶	24.70 ± 0.95 a	15.40 ± 0.84 a	86.00 ± 3.94 b
Metarril® SP 10 ⁷	25.30 ± 0.48 ab	14.80 ± 0.63 a	84.00 ± 5.16 b
Metarril® SP 10 ⁸	24.90 ± 0.99 a	15.20 ± 0.92 a	74.00 ± 3.94 c
Oil Control 1	24.60 ± 0.84 a	15.30 ± 0.95 a	89.40 ± 3.44 a
Oil Control 2	25.50 ± 0.71 ab	15.50 ± 0.71 a	93.00 ± 3.17 a
Oil Control 3	24.80 ± 0.63 a	14.80 ± 0.92 a	93.00 ± 4.04 a
Metarril® SC 10 ⁶	25.60 ± 0.70 ab	14.50 ± 0.71 a	85.00 ± 3.33 b
Metarril® SC 10 ⁷	24.70 ± 0.95 a	15.20 ± 0.92 a	82.00 ± 4.12 b
Metarril® SC 10 ⁸	26.60 ± 1.58 b	12.60 ± 2.76 b	36.00 ± 8.43 d

Mean ± standard deviation with the same letter, in the same column, did not differ by variance analysis (one-way ANOVA) followed by Tukey's post hoc test¹ or by Kruskal-Wallis followed by Dunn's Multiple Comparison Test² (p ≥ 0.05). Bioassays were repeated twice (10 test tubes each).

Table 3. Larval Mortality (%) on 5th, 10th and 15th days after *Rhipicephalus microplus* larvae exposure to Metarril® SP suspensions and Metarril® SC formulations (10⁶, 10⁷ and 10⁸ conidia mL⁻¹). The emulsifiable vegetable oil was diluted 10 times (Oil Control 3), 100 times (Oil Control 2), and 1000 times (Oil Control 1).

Groups	5 th day	10 th day	15 th day
Aqueous Control	0.30 ± 0.24 ab	4.00 ± 3.41 a	6.90 ± 4.95 a
Metarril® SP 10 ⁶	0.00 ± 0.00 a	3.40 ± 2.80 a	8.50 ± 6.41 a
Metarril® SP 10 ⁷	0.00 ± 0.00 a	4.10 ± 2.39 a	12.60 ± 5.24 a
Metarril® SP 10 ⁸	1.00 ± 1.18 ab	8.90 ± 5.36 b	39.70 ± 22.15 b
Oil Control 1	0.70 ± 0.75 ab	1.20 ± 0.60 a	1.90 ± 1.58 a
Oil Control 2	3.20 ± 3.85 ab	3.50 ± 1.32 a	13.13 ± 16.11 a
Oil Control 3	3.20 ± 6.31 ab	3.11 ± 2.88 a	15.13 ± 11.49 a
Metarril® SC 10 ⁶	1.60 ± 2.81 b	8.60 ± 1.96 b	32.50 ± 13.46 b
Metarril® SC 10 ⁷	69.50 ± 26.97 c	99.50 ± 0.92 c	99.60 ± 0.92 c
Metarril® SC 10 ⁸	86.67 ± 9.72 c	100.00 ± 0.00 c	100.00 ± 0.00 c

Mean ± standard deviation with the same letter, in the same column, did not differ by Kruskal-Wallis followed by Dunn's Multiple Comparison Test (p ≥ 0.05). Bioassays were repeated twice (10 test tubes each).

Discussion

Even few agricultural policies encouraging the tick biocontrol in Brazil, it has noticeably increased the use of entomopathogenic fungi as biopesticides (Faria & Wraight, 2007; Mascarín et al., 2019; Beys-da-Silva et al., 2020). Facing this challenge, progress has been achieved using different Metarril® formulations in *in vitro* trials for different tick species such as *R. sanguineus* (Alves et al., 2017), *Dermacentor nitens* (Perinotto et al., 2013) and *Amblyomma sculptum* (= *A. cajennense* s.l.) (Lopes et al., 2007). Additionally, our findings endorse that in the laboratory, the oil formulation (Metarril® SC) outperforms the aqueous suspension (Metarril® SP) for *R. microplus*, particularly at 10^8 conidia mL⁻¹. Even though the relatively low *Metarhizium* spp. speed to kill ticks (Mascarín et al., 2019), the use of oil-based formulations has promoted host-pathogen interaction (Prior et al., 1988; Polar et al., 2005). Furthermore, oil-based formulations can protect the conidia of *M. anisopliae* from the adverse effects of high temperatures (Oliveira et al., 2018). Despite these advances and the recent review made on all products developed by Koppert® (Alves et al., 2017; Mascarín et al., 2019), improvements in tick fungal formulations are expected particularly adding oils in their compositions, while, on the one hand, large amounts of conidia may make bioproduction more expensive, but on the other, oily fungal products have shown to enhance efficacy. As an example, the addition of mineral oil to Metarril® SP allowed a percent control higher than 45% on *R. microplus* engorged females after two treatments on naturally infested animals (Camargo et al., 2016). In a different approach, the spread of fungal pellets on soil - against engorged females that drop from the host - has been innovatively developed, but these results need yet to be improved before large-scale applications (Mascarín et al., 2019).

Viability is one of the fungal efficacy predictors; it is advisable that conidial germination should be evaluated (even for commercial products) before the trials to guarantee reliable results. Although both products were under the same storage instructions (kept in the fridge at 4°C), an expected delay in germination was observed to Metarril® SC. Similar effects were found when *M. anisopliae* s.l and *B. bassiana* s.l suspensions plus 10, 15, or 20% mineral oil were used to control *R. microplus* (Camargo et al., 2012). Interestingly, this delay did not influence the effects on ticks; we assume for this fact that oil may form a barrier between fungi and media, resulting in a long time for conidia to fully germinate. In tandem, oils added in tick fungal formulations by producing micelles can provide moisture to conidia due to its low volatility (Kaaya et al., 2011; Beys-da-Silva, et al., 2020) as well as help protecting against high temperatures (Barreto et al., 2016) and UV radiation (Hedimbi et al., 2008). By inserting mineral oil into Metarril® SP suspensions to control *R. sanguineus*, attractive results ensuring high germination percentages even after heat-stress were attained (Alves et al., 2017). Here, we certified the germination delay on culture medium; however, it could be assumed the same occurred on the cuticle surface. Clearly, the culture medium can provide several nutrients and favorable conditions to fungal development and growth, instead of cuticle, that biologically acts as a physical barrier (Ment et al., 2012; Barreto et al., 2016). Similar results were found by Barreto et al. (2016), where they analyzed conidial germination not only on the culture media but also on the cuticle surface. Additionally, oils can help conidia adhesion since its absence may limit aggregation (i.e., attachment among conidia rather than tick cuticle) and increase the number of free conidia that would germinate on the arthropod cuticle (Ment et al., 2010). Moreover, tick cuticle can contain natural inhibitors and fungistatic molecules (Kirkland et al., 2004) that could also hinder fungal germination (Sosa-Gomez et al., 1997; Kirkland et al., 2004) and make the penetration time longer. For these and other reasons, oil formulations, here exemplified by Metarril® SC, can be a choice to tick control and have the potential to be applied in further field tests.

A single engorged female can lay thousands of eggs after total engorgement; hence, one of the targets of fungal formulations is to diminish novel tick populations that will eventually infest the animals. Our assays demonstrate that the relevant results were observed on eggs and larvae for both products. Notably, Metarril® SC strongly reduced hatchability and increased larval mortality at the highest concentration tested. In agreement, many researchers have observed that the use of oil as an adjuvant enhances the fungal effect on eggs (Polar et al., 2005; Angelo et al., 2010; Camargo et al., 2012, 2014; Perinotto et al., 2017), perhaps by not only covering all the surface and reducing gas exchange but also by protecting the conidia and improving its action. For larvae - the most vulnerable stage to fungal infection (Kaay & Hassan, 2000; Wassermann et al., 2016) - Metarril® SC at 10^7 and 10^8 conidia mL⁻¹ presented similar results. As here, tests with Metarril® SC to control unfed *A. sculptum* nymphs documented that 10^7 conidia mL⁻¹ was able to kill up to 60% of the nymphal population 10 days after treatment (Lopes et al., 2007). Its susceptibility might occur due to the cutaneous respiration and less or non-sclerotized exoskeleton present in larvae (Sonenshine & Roe, 2014), however, further studies should be done to clarify these points and better understand its action prior to developing new tick fungal formulations. These outcomes lead the tick biocontrol to future focus on non-parasitic stages, either by spreading fungi directly onto the soil and acting as a reservoir or by using formulations that will scatter fungi from cattle feces on pasture.

Conclusions

The current study demonstrated that the use of the oil-based product (Metarril® SC) is recommended instead of the dry conidia to be suspended in water (Metarril® SP) to all *R. microplus* stages. In addition, eggs and larvae were the most susceptible stages to the treatments, although high concentrations of conidia are still needed to ensure consistent results for ticks.

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