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Original article

In vitro acaricidal activity of *Metarhizium anisopliae* and *Beauveria bassiana* against *Rhipicephalus microplus*

Actividad acaricida *in vitro* de *Metarhizium anisopliae* y *Beauveria bassiana* sobre *Rhipicephalus microplus*

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ABSTRACT: The tick *Rhipicephalus microplus* represents a sanitary and economic threat to the global cattle industry, exacerbated by the increasing resistance to chemical acaricides. The objective of this study was to determine the effectiveness of the synergistic interaction between the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* for the *in vitro* biological control of this pest. An immersion bioassay was conducted using engorged females of *R. microplus*, distributed into four experimental groups: T1 (*M. anisopliae*), T2 (*B. bassiana*), T3 (1:1 synergistic combination), and T4 (control). Mortality was recorded over a period of 13 days. The results showed that the synergistic treatment (T3) was significantly superior ($p < 0.05$), achieving 100 % mortality within five days. Individually, *M. anisopliae* (T1) reached 100 % mortality after nine days, whereas *B. bassiana* (T2) achieved a final mortality of 73.33 %. No mortality was observed in the control group. Mortality data were analyzed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test ($p < 0.05$). These findings demonstrate a strong synergistic effect between both fungi, accelerating acaricidal activity and confirming the research hypothesis. The combined application of *M. anisopliae* and *B. bassiana* is proposed as an effective and sustainable biological alternative for the integrated management of *R. microplus*, with potential for the development of bioacaricides capable of mitigating chemical resistance.

Key words: Biocontrol, entomopathogenic fungi, synergism.

RESUMEN: La garrapata *Rhipicephalus microplus* representa una amenaza sanitaria y económica para la ganadería bovina mundial, agravada por la creciente resistencia a los acaricidas químicos. El objetivo principal de este estudio fue determinar la efectividad de la sinergia entre los hongos entomopatógenos *Metarhizium anisopliae* y *Beauveria bassiana* para el control biológico in vitro de esta plaga. Se realizó un bioensayo de inmersión con hembras ingurgitadas de *R. microplus*, distribuidas en cuatro grupos experimentales: T1 (*M. anisopliae*), T2 (*B. bassiana*), T3 (sinergia 1:1) y T4 (control). La mortalidad se evaluó durante 13 días. Los resultados demostraron que el tratamiento sinérgico (T3) fue significativamente superior ($p < 0,05$), alcanzando el 100 % de mortalidad en solo cinco días. De manera individual, *M. anisopliae* (T1) logró el 100 % de mortalidad a los nueve días, mientras que *B. bassiana* (T2) alcanzó una mortalidad final del 73,33 %. El grupo control no registró mortalidad. Los datos fueron analizados mediante análisis de varianza (ANOVA) y prueba de comparación de medias de Tukey ($p < 0,05$). Se concluye que la aplicación combinada de *M. anisopliae* y *B. bassiana* presenta un marcado efecto sinérgico que acelera la acción acaricida, constituyéndose en una alternativa biológica eficaz y sostenible para el manejo integrado de *R. microplus*.

Palabras clave: Biocontrol, hongos entomopatógenos, sinergismo.

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INTRODUCTION

Bovine livestock constitutes a strategic pillar for food security and economic development on a global scale, by providing high biological value proteins and sustaining the livelihoods of millions of producers (1,2). However, the productivity and sustainability of this sector are persistently compromised by health factors, among which ectoparasite infestations represent one of the main constraints (3,4). In this context, the tick *Boophilus microplus* (syn. *Rhipicephalus microplus*) has become one of the most impactful pests in livestock systems in tropical and subtropical regions (6), causing economic losses amounting to billions of dollars annually worldwide (6,7).

The damage caused by *R. microplus* is not limited to direct blood loss due to its hematophagous habit, which induces anemia, physiological stress, and reduced productive performance, but is aggravated by its role as a vector of hemoparasites responsible for diseases of high sanitary relevance, such as bovine babesiosis and anaplasmosis (8). These pathologies increase livestock morbidity and mortality, raise production costs, and generate health and trade restrictions that comprehensively affect the bovine production chain.

Traditionally, the control of *R. microplus* has depended almost exclusively on the use of chemical acaricides, such as organophosphates, synthetic pyrethroids, and amidines. Although these compounds have shown high initial efficacy, their intensive and prolonged use has favored the emergence of tick populations resistant to multiple active ingredients (9). Concurrently, the indiscriminate use of these products has raised growing concern about environmental contamination and the presence of chemical residues in meat and milk, with direct implications for public health and export markets. This scenario has highlighted the limited sustainability of conventional chemical control and has driven the search for safer, more efficient, and environmentally responsible alternatives (10).

In this context, biological control using entomopathogenic fungi has positioned itself as a promising strategy for the integrated management of *R. microplus* (10,11). Species such as *Metarhizium anisopliae* and *Beauveria bassiana* have been widely studied for their ability to infect and cause mortality in various arthropods, including ticks of veterinary importance (12). The infectious process of these fungi begins with the adhesion of conidia to the host's cuticle, followed by germination and penetration through the combined action of mechanical pressure and the secretion of hydrolytic enzymes, such as proteases, chitinases, and lipases. Once in the hemocoel, the mycelium disseminates, evades the host's immunological defenses, and produces toxic secondary metabolites that ultimately lead to the death of the parasite (13,14).

Several studies have documented the individual efficacy of *M. anisopliae* and *B. bassiana* against *R. microplus* under laboratory conditions and, to a lesser extent, in field trials. However, most of this research has focused on evaluating each fungus independently, with limited and fragmentary information still existing on the possible synergistic effects derived from their combined application. In particular, few studies have controlledly analyzed whether the combination of both entomopathogens can enhance acaricidal activity, accelerate mortality, and improve the efficiency of biological control compared to individual treatments.

In this context, the present research addresses this knowledge gap by evaluating the effectiveness of the synergy between *Metarhizium anisopliae* and *Beauveria bassiana* in the *in vitro* biological control of *Rhipicephalus microplus*. It is hypothesized that the combined application of both fungi increases acaricidal virulence and significantly reduces the time required to reach lethal mortality levels, compared to their individual use. The results of this study aim to provide scientific evidence supporting the development of more effective and sustainable bioacaricide formulations. All this contributing to the integrated management of this pest and the reduction of dependence on chemical acaricides in bovine livestock.

MATERIALS AND METHODS

Study area

The field phase of the research was carried out at the "Don Julio" cattle farm, located in the rural parish of Pancho Negro, La Troncal canton, Cañar province, Ecuador. The sampling site is georeferenced at coordinates 2°29'34.1"S and 79°27'08.5"W, at an altitude ranging between 40 and 200 meters above sea level. The area has a tropical climate, with an average annual temperature of 30 °C, average annual rainfall of 1239 mm, and relative humidity of 77 %.

The collection of *Rhipicephalus microplus* specimens was carried out through direct manual sampling on naturally infested crossbred cattle. 150 adult female ticks in the engorgement stage were selected, characterized by their maximum degree of engorgement and low mobility. The selection was based on criteria of size, physical integrity, and absence of visible damage. The collected specimens were carefully placed in sterile plastic vials and were transported under refrigeration conditions to the laboratory of the Faculty of Health and Life Sciences at the Universidad Internacional del Ecuador, Guayaquil campus, for subsequent processing within a period not exceeding 24 hours.

Isolation and reactivation of fungal strains

Commercial strains of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* were used. For their reactivation and purification, Potato Dextrose Agar (PDA) culture medium, supplemented with chloramphenicol (0.1 g L) to inhibit bacterial contamination, was employed (14). Aliquots of the strains were plated on Petri dishes containing PDA medium and incubated in an oven at a controlled temperature of 25 ± 2 °C with a 12:12 hour photoperiod (light:dark) for 15 days. This period allowed complete mycelial development and sporulation of the fungi. Fungal growth was monitored and measured daily during the first three days using a vernier caliper to record colony diameter.

Morphological characterization and preparation of suspensions

Confirmation of the morphological identity of the strains was carried out using a microculture technique. Small portions of the sporulated mycelium were taken with transparent adhesive tape, which were then mounted on a slide with a drop of lactophenol blue as a contrast stain. The preparations were observed under an optical microscope with a 40x objective, analyzing the distinctive characteristics of the hyphae, conidiophores, and conidia of each species.

For the preparation of working suspensions, conidia were harvested from the surface of the PDA cultures by gentle scraping with a sterile Digralsky loop. The collected fungal material was suspended in a sterile distilled water solution with 0.05 % (v/v) Tween 80 as a surfactant agent to facilitate homogeneous dispersion of the conidia. The concentration of the suspensions was determined by counting in a Neubauer chamber and adjusted to a final standardized concentration of $1x10^8$ conidia/mL for all treatments (13,15).

In vitro synergism bioassay

The bioassay was designed following previously reported immersion methodologies for evaluating the acaricidal activity of entomopathogenic fungi (16). The 150 collected ticks were randomly distributed into four experimental treatments, each with three independent biological replicates, composed of 10 ticks per replicate, constituting 30 ticks per treatment.

The evaluated treatments were as follows:

- T1: Immersion in a suspension of *Metarhizium anisopliae* (1×10^8 conidia/mL).
- T2: Immersion in a suspension of *Beauveria bassiana* (1×10^8 conidia/mL).
- T3: Immersion in a combined suspension of *M. anisopliae* and *B. bassiana* in a 1:1 ratio, with a final concentration of 1×10^8 conidia/mL.
- T4 (control): Immersion in sterile distilled water with 0.05 % Tween 80.

Ticks were immersed in their respective suspensions for 30 seconds, subsequently they were dried at room temperature on sterile absorbent paper, and placed individually in 90 mm diameter Petri dishes with moistened filter paper to maintain relative humidity above 80 %. The dishes were sealed with parafilm and incubated at 27 ± 1 °C, under a 12:12 h photoperiod.

Evaluation of mortality and statistical analysis

Tick mortality was evaluated daily from day 3 to day 13 post-treatment. A tick was considered dead when it showed no motor response to stimulus with a fine brush and characteristic mycelial growth of the entomopathogenic fungus was evident on the cuticle (14).

Cumulative mortality percentages were transformed using the arcsine square root function to meet normality assumptions. Subsequently, the data were analyzed using analysis of variance (ANOVA), followed by Tukey's mean comparison test, considering a significance level of $p < 0.05$. Statistical analysis was performed using InfoStat software version 2020.

RESULTS AND DISCUSSION

Growth and characterization of entomopathogenic fungi

The reactivation of *Metarhizium anisopliae* and *Beauveria bassiana* strains on Potato Dextrose Agar (PDA) medium confirmed their viability, purity, and adequate growth behavior for use in bioassays. In the case of *Metarhizium anisopliae*, initial whitish mycelial growth was observed, which progressively evolved towards a dark olive-green color as the sporulation process advanced. The colony presented a velvety and compact texture, reaching an average diameter of 3.5 cm after three days of incubation, evidencing a high capacity for colonization of the culture medium (Figure 1).

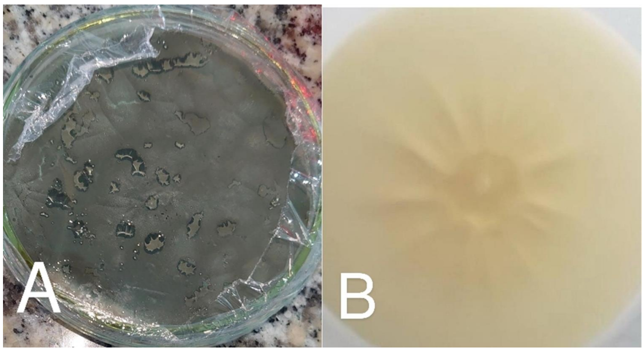


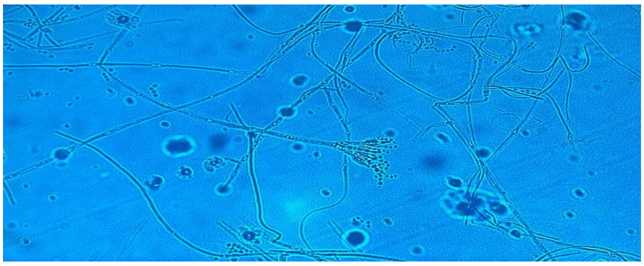
Figure 1. Macroscopic culture of *Metarhizium anisopliae* on PDA, top view (A) and bottom view (B)

Microscopic observation confirmed the morphological identity of ***M. anisopliae,*** revealing septate hyaline hyphae, branched conidiophores, and cylindrical conidia arranged in dense chains, characteristics consistent with the taxonomic descriptions reported for the species (Figure 2).

Figure 2. Lactophenol blue staining of *Metarhizium anisopliae*, 40×

For its part, *Beauveria bassiana* showed cottony and powdery mycelial growth, maintaining a homogeneous white coloration throughout the incubation period. Mycelial development was slightly slower compared to *M. anisopliae* , reaching an average colony diameter of 2.8 cm by the third day. These macroscopic characteristics confirm stable growth suitable for experimental application.

Microscopic identification of *B. bassiana* revealed septate hyaline hyphae and conidiophores widened at the base, with a zigzag rachis where globose conidia were arranged, confirming the identity of the strain before its use in the bioassays (Figure 3).



Beauveria bassiana fungus was observed under an OMAX brand optical microscope, using a 40x magnification objective and lactophenol blue stain for better visualization

Figure 3. Lactophenol blue staining of *Beauveria bassiana*

Acaricidal effectiveness of treatments against *Rhipicephalus microplus*

The *in vitro* bioassay demonstrated marked acaricidal activity of the evaluated entomopathogenic fungi, with statistically significant differences observed between treatments ($p < 0.05$). The control group (T4), treated only with sterile distilled water and 0.05 % Tween 80, showed no mortality during the 13 days of evaluation, with ticks maintaining normal mobility and absence of signs of mycosis. This result confirms that the experimental conditions were not lethal and that the mortality recorded in the experimental treatments was exclusively attributable to the action of the fungi.

The treatment with *Beauveria bassiana* (T2) showed a progressive acaricidal effect, with mortality onset starting from the fourth day post-treatment. Cumulative mortality reached 33.33 % by day 7 and a final value of 73.33 % ± 5.77 % by day 13. Dead individuals exhibited characteristic whitish mycelial colonization, confirming the pathogenic action of the fungus on the ticks (Figure 4).

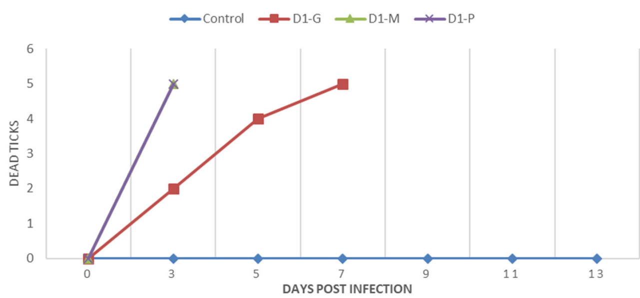


Figure 4. Cumulative mortality curve of *R. microplus* treated with *Beauveria bassiana*

The treatment with *Metarhizium anisopliae* (T1) demonstrated significantly higher virulence compared to *B. bassiana*. The first dead ticks were observed on the third day. The mortality rate was considerably faster, reaching 80 % by day 7 and achieving 100 % mortality by day 9. This result is consistent with the findings (14), who also reported high pathogenicity of M. anisopliae strains against this same tick species. Colonization of the cadavers was evident, with dark green mycelium completely covering the bodies.

Remarkably, the combined treatment (T3), which used a suspension of both fungi, exhibited a potent synergistic effect, surpassing the efficacy of the individual treatments. Mortality in this group was the fastest and most decisive, beginning on the second day and reaching 100 % of the tick population in just 5 days. Statistical analysis (Tukey, $p<0.05$) confirmed that the efficacy of the synergy was significantly superior to that of *M. anisopliae* (T1) and B. bassiana (T2) from the third day of evaluation. The rapid lethality suggests that the combination of the pathogenic mechanisms of both fungi, possibly through a more diverse enzymatic and toxic repertoire, overcame the tick's defenses more efficiently. Post-mortem colonization in this group showed mixed mycelial growth, with white and green areas on the tick teguments. The order of effectiveness of the treatments was clearly T3 > T1 > T2 > T4.

The results of this study confirm the high susceptibility of *Rhipicephalus microplus* to infection by the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* under *in vitro* conditions, evidencing significant differences in the speed and magnitude of mortality induced by each treatment. The high virulence observed for *M. anisopliae*, which reached 100 % mortality in nine days, agrees with previous reports highlighting the efficiency of this species against *R. microplus* and other ticks of veterinary importance (12,14). For its part, *B. bassiana* showed a more gradual action, reaching a final mortality of 73.33 %, a behavior consistent with studies that describe moderate but sustained pathogenicity of this fungal species (12,16).

The most relevant finding of the present research is the marked acceleration of mortality observed in the synergistic treatment, in which the combination of *M. anisopliae* and *B. bassiana* achieved 100 % mortality in a significantly shorter period (five days) compared to individual treatments. This result suggests that the combined action of both entomopathogens not only increases acaricidal efficacy but also substantially reduces the time required for control of the parasitic population, an aspect of high relevance for its practical application.

The observed synergistic effect can be explained by the complementarity of the infection and pathogenicity mechanisms of both fungi. It has been described that *M. anisopliae* produces a broad repertoire of hydrolytic enzymes, such as proteases, chitinases, and lipases, as well as secondary metabolites from the destruxin group, which facilitate rapid cuticle penetration and alter the physiological homeostasis of the host (14). In contrast, *B. bassiana* is characterized by the production of metabolites such as beauvericin and a colonization pattern that, although slower, progressively compromises the arthropod's tissue integrity (16). The simultaneous application of both fungi could generate more efficient degradation of the cuticle. In addition, it presented a toxic overload that more quickly overcomes the immune defenses of *R. microplus*.

Previous studies have independently evaluated the use of *M. anisopliae* and *B. bassiana* for the control of *R. microplus* both under laboratory conditions and in field trials, reporting variable levels of efficacy (15). However, most of these works do not directly address the evaluation of synergies between entomopathogens, limiting themselves to individual comparisons of strains or concentrations. In this sense, the results of the present study provide experimental evidence supporting the hypothesis that the combination of both fungi enhances acaricidal activity, representing a relevant advance in the development of more efficient biocontrol strategies.

The total absence of mortality in the control group confirms that the experimental conditions did not negatively influence tick survival and that the mortality recorded in the experimental treatments was due exclusively to the action of the entomopathogenic fungi. This aspect reinforces the internal validity of the bioassay and the reliability of the results obtained.

From an applied perspective, the rapid action observed in the synergistic treatment acquires special importance in the face of current problems of resistance to chemical acaricides, widely documented in populations of *R. microplus* (19). The formulation of bioacaricides based on the combination of *M. anisopliae* and *B. bassiana* could contribute to reducing the selection pressure exerted by conventional chemical products, decreasing environmental contamination, and improving the sustainability of livestock systems. However, it is important to consider that the results obtained correspond to *in vitro* conditions, so their extrapolation to field scenarios should be done with caution.

In this sense, future studies should focus on evaluating this synergy under *in vivo* conditions, considering environmental variables, interaction with the host, and persistence of the fungal inoculum, in order to validate its efficacy and viability as an integrated management tool for *R. microplus*.

CONCLUSIONS

The present study fulfilled the general objective by demonstrating that the combined application of *Metarhizium anisopliae* and *Beauveria bassiana* presents a significant synergistic effect for the *in vitro* biological control of *Rhipicephalus microplus*. The results showed that both fungi possess acaricidal activity when applied individually; however, their combined use notably increases control efficacy and substantially reduces the time required to reach lethal mortality levels.

The observed synergy manifested as an acceleration of the acaricidal response, achieving 100 % mortality in a considerably shorter period compared to individual treatments. This behavior confirms the hypothesis that the combination of entomopathogens enhances biological activity, possibly through the complementary action of their infection mechanisms and production of toxic metabolites.

Although the results obtained are highly promising, it is important to note that the study was conducted under *in vitro* conditions, which constitutes a limitation for the direct extrapolation of the findings to real production scenarios. Environmental factors, interaction with the host, and conditions specific to agricultural management could influence the efficacy observed under field conditions.

Overall, the findings of this research support the potential of the synergy between *M. anisopliae* and *B. bassiana* as an effective and sustainable biological alternative to the use of conventional chemical acaricides. This approach represents a solid foundation for the future development of integrated bioacaricides, aimed at mitigating chemical resistance and contributing to more sustainable agricultural systems.

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