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Article

In vitro evaluation of the acaricidal potential of *Beauveria bassiana* DS3.17 on the common tick (*Rhipicephalus microplus*) in Oaxaca, Mexico

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Abstract:

The tick *Rhipicephalus microplus* causes damage to cattle farming, has a great economic impact, and significantly influences the productivity and commercial competitiveness of the sector, mainly in tropical and subtropical areas of the country, generating annual losses of millions. Currently, the control of the ectoparasite using biological agents (mainly entomopathogenic fungi) has a high potential to reduce tick populations. The objective of the present study was to evaluate *in vitro* the acaricidal potential of *Beauveria bassiana* DS3.17 on engorged *R. microplus* females, by immersion of the mite in different concentrations of conidia of the fungus to determine the effects on reproductive parameters (total egg weight, hatching percentage, oviposition and nutrition indices). The evaluated concentrations of *B. bassiana* DS3.17 showed an acaricidal effect of up to 93.2 % in adults and a change in the behavior of the biological parameters of adult females, inhibiting oviposition between 75.84 and 79.60 %. As for the estimated reproduction index, a control of 45.23 to 83.79 % was obtained, the nutrition index was 10.21 and 10.67 %. The study showed a change in the behavior of reproductive parameters when *B. bassiana* DS3.17 was used, and each of the results showed significant differences; the concentrations of conidia affected the reproduction of the parasite with respect to the control, therefore, *B. bassiana* DS3.17 presented an acaricidal potential for the control of the tick.

Keywords: Biological control, *Beauveria bassiana*, *Rhipicephalus microplus*.

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Introduction

The damage caused by *Rhipicephalus microplus* to cattle has a great economic impact and significantly influences the productivity and commercial competitiveness of the sector in the tropical and subtropical areas of the country, generating annual losses of 573 million dollars(1,2,3).

The application of synthetic ixodicides plays an important role in the control of this pest, being the most used method to reduce its populations(4,5). Nevertheless, the indiscriminate use of these products generates a negative impact on the environment, contributing to the emergence of secondary pests, reduction of natural enemies and development of resistance by mites(2,6,7).

On the other hand, biological control that uses entomopathogenic fungi is one of the multiple strategies focused on solving the problem of synthetic ixodicides, this methodology is increasing due to the awareness that society has acquired about environmental safety and human health, together with the increase in the cost of applying chemical control(8). In addition, biological control agents stand out for their wide distribution, low risk to animal and human health, compatibility with the environment and high virulence(1,4).

Strains of the genera *Beauveria bassiana* and *Metarhizium anisopliae* have been evaluated against ticks; due to their virulence, infection mechanism, mortality on adults, larvae, for altering egg hatching and generating changes in the lipid metabolism of their host(9-12). Despite the favorable results obtained *in vitro* and in the field, these microorganisms show erratic behavior because their activity is reduced when applied in the field, so their adaptation to environmental conditions is a determining factor on their pathogenicity and virulence. In addition, it has been described that some entomopathogenic fungi are more susceptible to abiotic factors, particularly temperature and ultraviolet radiation[13]. In this sense, endemic fungi are better adapted to these conditions[1,14] and play an important role in the natural control of pest populations. The objective of the present study was to determine the acaricidal effect of *B. bassiana* DS3.17 on the viability and reproductive parameters of engorged *R. microplus* females.

Material and methods

Microorganism

B. bassiana DS3.17 belonging to the collection of strains of the academic body Sustainable Biotechnology of the University of Papaloapan Tuxtepec Oaxaca, Mexico, was used. The strain was isolated from Rancho Grande (latitude, 17.844256, longitude, -96.333192), San Juan Bautista Valle Nacional, Oaxaca, Mexico. All coordinates were obtained using an eTrex Legend Handheld Navigator (Garmin Ltd, USA). The strain was incubated at 25 °C for 14 d in Sabouraud Dextrose agar medium at 4 % (w/v) supplemented with 0.05 % (w/v) of yeast extract (Bioxon, Mexico). The production of the fungal inoculum was carried out by biphasic fermentation using the methodology described by López-Sosa *et al*[15]. At the end of fermentation, the conidia were recovered by sieving (300 µm, TEST SIEVE Germany), suspended in sterile distilled water (1:10 g ml^{-1}) to a final concentration of $4.2x10^9$ conidia ml^{-1}; from this, dilutions were made to obtain suspensions with concentrations of $1x10^6$, $1x10^7$, $1x10^8$ and $1x10^9$ conidia ml^{-1}, which were used for bioassays. The dilution values were terminated based on the literature; seeking to cover the range of concentrations used in it.

Adult ticks (*R. microplus*) were collected at the La Guadalupe ranch located in the community of Peñarrubia (17º 50’ 39”N, 96º 19’ 59”W) Tuxtepec, Oaxaca, Mexico. The specimens were deposited in polypropylene vials with a capacity of 50 ml, those that were considered non-viable (loss of limbs, lack of mobility or death of the organism) were discarded for the study. The selected ticks were disinfected with sodium hypochlorite at 1 % (v/v) for 1 min, performing three consecutive washes with sterile distilled water, then placed on sterile absorbent paper to remove excess water. Finally, they were incubated in a wet chamber at 26 ± 1 °C and relative humidity of 70 to 80 %, monitoring them for three days to rectify their viability. At the end of the period, the engorged females were weighed and classified into groups of 10 individuals to perform the bioassays.

Bioassays

Acaricidal effect of *B. bassiana* DS3.17 on adult *R. microplus* females

The acaricidal effect of *B. bassiana* DS3.17 was evaluated with the methodology of adult immersion using four concentrations of conidia ($1x10^6$, $1x10^7$, $1x10^8$, and $1x10^9$ conidia ml^{-1}) and a control (sterile distilled water); in each one, 10 fully engorged females weighing approximately between 200 and 300 mg were immersed for 3 min. Subsequently, the ticks were placed on sterile absorbent paper until removing excess water. Each group was placed in wet chambers, at 26 ± 1 °C and 70 to 80 % relative humidity, making observations every 48 h for 23 d to evaluate daily mortality. Bioassays were concluded when the control group exceeded 10 % mortality(16).

Acaricidal effect of *B. bassiana* DS3.17 on the reproductive potential of *R. microplus*

The acaricidal effect of the fungus on the reproductive parameters of the tick was evaluated in five groups (replications), plus 1 control of 10 ticks for each concentration of the fungus; through the methodology described in the previous section. After 10 d of immersing the mites in the dilutions of conidia, the eggs from each of the groups were removed and weighed; of the quantity collected, aliquots of 50 mg were taken from each group and incubated in wet chambers until their hatching under the conditions already described. The parameters evaluated were initial and final tick weight, egg weight, hatched eggs; with these, the percentage of hatching (%H), nutrition index (NI), reproductive efficiency (RE) and oviposition (OP) were determined, using the equations proposed by Bennett(17). From the estimated parameters, the indices of oviposition inhibition (OPI) and reproductive efficacy inhibition (REI) were calculated.

Equations

Percentage of mortality: $= \frac{\text{N°of dead ticks}}{\text{N° of evaluated ticks}} X100$

Estimated oviposition: $= \frac{\text{(egg weight in grams)x(20000}*\text{)}}{\text{weight of female ticks in grams}}$

*Constant of the number of eggs in one gram of egg mass.

Nutrition Index: $= \frac{We}{WET-RWT} x100$
We= weight of eggs; WET= weight of engorged ticks; RWT= residual weight of ticks.

Oviposition inhibition: $\%OI = \frac{(OPT-OPC)}{(OPT)} X100$
OPT= oviposition of the treatment; OPC= oviposition of the control group.

Reproductive efficiency: $= \frac{egg\ weight}{female\ weight} x\ 20000\ x\ C.F. of\ the\ \%H$
C.F.= centesimal fraction

Reproductive efficiency inhibition: $\%REI = \frac{RET-REC}{RET}\ x\ 100$
RET= Reproductive effectiveness of the treatment; REC= reproductive effectiveness of the control.

Statistical analysis

For the analysis of adult mortality, the data were transformed with the equation: Y=asin (√(p). Where: "Y" is the transformed mortality, "asin" is the arcsine and "p" is the mortality in proportion[18].

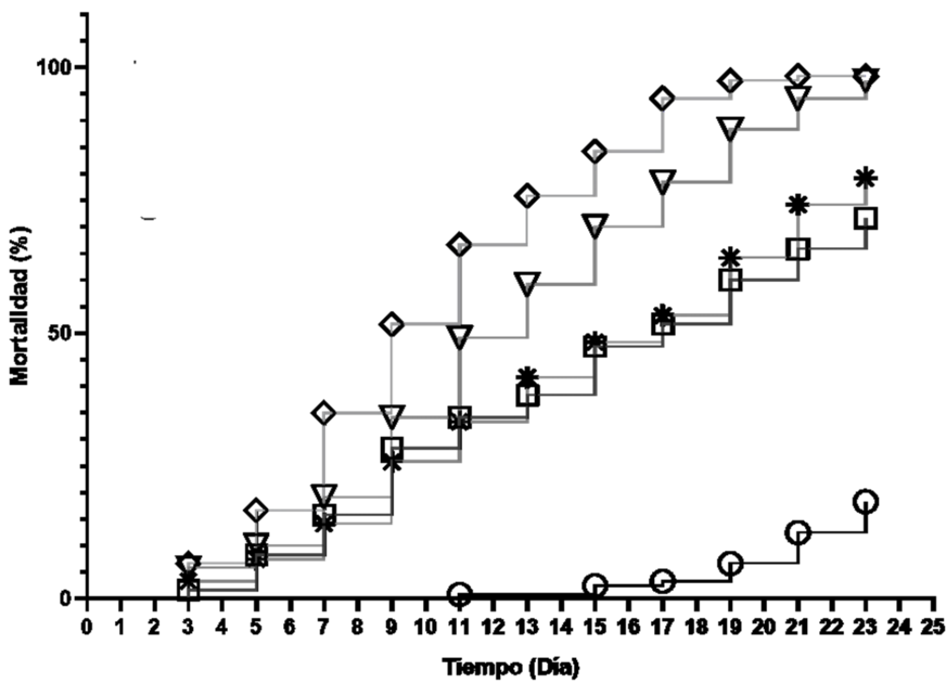
Statistical analysis was performed using the statistical packages "Rcmdr", "drc", "MASS", "ggplot2" and "ecotox" of the free software "R" (R Development Core Team. USA). An analysis of variance and a comparison between means with the Tukey test (α≤0.05) were performed on the mortality, final weight and egg weight data obtained. Nonparametric data such as hatching percentage, nutrition index, oviposition inhibition and reproductive effectiveness were analyzed with the Kruskal-Wallis test and the Student-Newman-Keuls comparison test (α≤0.05).

Results

Acaricidal effect of *B. bassiana* DS3.17 on adult *R. microplus* females

The daily mortality produced by *B. bassiana* DS3.17 on engorged *R. microplus* females (Figure 1) started on the third day after immersion for all concentrations evaluated, presenting mortality percentages of 1.66 % ($1x10^6$ conidia ml^{-1}), 3.33 % ($1x10^7$ conidia ml^{-1}), 5.83 % ($1x10^8$ conidia ml^{-1}), 6.66 % ($1x10^9$ conidia ml^{-1}), in the control group the beginning of mortality was observed on d 11. The speed of mortality was directly proportional to the concentration of conidia. The maximum mortality was reached on d 23 (97.5 and 98.3 %) with concentrations of $1x10^8$ and $1x10^9$ conidia respectively, at the same time mortalities with concentrations of $1x10^6$ and $1x10^7$ conidia were 71.6 and 79.16 %, for the control; at this time, a mortality percentage of 18.33 % was reached. The mortality values obtained with the concentrations of $1x10^8$ and $1x10^9$ conidia did not show significant differences; however, they showed them with the control and the rest of the concentrations.

Figure 1: Curve of daily (23 days) mortality of engorged adult *R. microplus* females after immersion in suspensions of conidia of *B. bassiana* DS3.17 at different concentrations ⊖ control, ⊟ $1x10^6$, ✱ $1x10^7$, ▽ $1x10^8$, ◇ $1x10^9$



Acaricidal effect of *B. bassiana* DS3.17 on the reproductive potential of *R. microplus*

The evaluated concentrations of *B. bassiana* DS3.17 had an effect on the reproductive parameters of adult *R. microplus* females (Table 1). The weights of the eggs immersed in the concentrations of $1x10^8$ and $1x10^9$ conidia ml^{-1} (0.200 and 0.131 g respectively) were significantly lower ($\alpha \leq 0.05$) than obtained with the control (0.650 g) and with the concentration of $1x10^7$ conidia ml^{-1} (0.588 g). The same trend was observed for the nutrition index. In the case of the percentage of hatching, an inversely proportional relationship was observed with respect to the concentration of conidia of the fungus, also, no significant differences were found for the concentrations of $1x10^8$ and $1x10^9$ conidia.

Table 1: Effect of the concentration of conidia of *B. bassiana* DS3.17 on initial weight, final weight, egg weight, hatching and nutrition index of adult *R. microplus* females

Concentration (conidia ml^{-1})	Initial weight (g)	Final weight (g)	Egg weight (g)	Nutrition index (%)	Hatching (%)
Control	0.2127±0.053	0.333±0.045^d	0.651±0.123^a	37.08±4.93^a	87.86±4.46^a
$1x10^7$	0.2504±0.047	0.547±0.045^c	0.588±0.121^a	29.93±4.97^b	62.70±8.24^b
$1x10^8$	0.2790±0.054	0.814±0.040^b	0.201±0.08^b	10.18±3.69^c	31.53±4.74^c
$1x10^9$	0.2321±0.063	0.964±0.017^a	0.131±0.069^b	9.63±2.27^c	28.29±3.97^c

^{abc} Equal letters in the same column do not show significant differences (P>0.05).

Table 2 shows the estimated oviposition, percentage of oviposition inhibition, reproductive efficacy (RE) and reproductive efficacy inhibition (REI) for each of the concentrations evaluated; where it is observed that the values of the oviposition presented a decrease inversely proportional to the concentrations evaluated, with the control (6,174 eggs) being the group with the highest estimated number of eggs, unlike the concentrations of $1x10^8$ (1,434) and $1x10^9$ conidia ml^{-1} (1,083). In the variable of oviposition, inhibition percentages greater than 40 % were observed with the concentrations evaluated; with the concentration of $1x10^9$ conidia being the one that presented the highest percentage of inhibition (94.36 %). When comparing the means obtained, significant differences were found between the highest ($1x10^8$ and $1x10^9$ conidia) and the lowest ($1x10^7$ conidia) concentrations, in the same way the values of the evaluated concentrations were significantly different from the values obtained in the control group.

Table 2: Effect of the concentration of conidia of *B. bassiana* DS3.17 on the parameters of oviposition and reproductive potential

Concentration (conidia ml^{-1})	Estimated oviposition	Oviposition inhibition	Reproductive efficacy	Reproductive efficacy inhibition
Control	6174±889^{c}	0^{c}	5427±628^{c}	0^{c}
1x10^{7}	4689±530^{b}	45.99±7.33^{b}	2944±602^{b}	45.99±7.33^{b}
1x10^{8}	1434±432^{a}	91.60±3.19^{a}	460.9±204.4^{a}	91.60±3.19^{a}
1x10^{9}	1083±667^{a}	94.36±2.15^{a}	310.8±134.5^{a}	94.36±2.15^{a}

[abc] Equal letters in the same column do not show differences (P>0.05).

The values calculated for the reproductive efficiency index were 32,640 viable larvae (1x10^{9} conidia ml^{-1}), which meant a control percentage of 83.79 %. For the concentration of 1x10^{8} conidia ml^{-1}, 44,074 larvae were quantified, which represented an 81.59 % of control. Finally, for the lowest concentration of 1x10^{7} conidia, the values were 299,546 viable larvae and 45.23 % (Table 2). When comparing means of the values obtained for the inhibition of reproductive efficacy, significant differences were found with respect to the control and between the concentrations evaluated.

Discussion

The mortality values obtained with the concentrations of 1x10^{9} and 1x10^{8} conidia ml^{-1} were similar to those reported by García-Corredor *et al*(19), who, with a concentration of 1x10^{8} conidia, obtained a mortality percentage of 90 % at 21 d with *B. bassiana*, observing the beginning of mortality after d 4. Similarly, Alcalá-Gómez *et al*(20) obtained from 7 to 12 % mortality with *B. bassiana* isolates 5 d after treatment, in addition, the maximum mortality varied between 84 and 100 % at d 20, a period that coincides with that shown in this work, with the strain of *B. bassiana* 115 being the one that produced 100 % mortality in ixodicide-resistant tick colonies.

On the other hand, Tofiño-Rivera *et al*(4) reported 100 % mortality on d 7 with a concentration of 1x10^{8} conidia, while with 1x10^{6} conidia, they obtained 90 % mortality at d 10. Broglio-Forti *et al*(21) observed 100 % mortality of ticks with concentrations of 1x10^{8} and 1x10^{9} conidia over a period of 14 d. Similarly, Del Pozo *et al*(22) reported mortality values of 87.2 to 96.4 % after 12 d. In this sense, similar mortality percentages but with longer times were reached in the present study. This could be due to the distinctive complexity of the physiological and enzymatic processes of entomopathogenic fungi, which are influenced by factors such as variation in enzymatic excretion during the infection process, as well as genetic variability between strains of the same genus, which favors the differential expression of virulence or specific genes(23,24,25).

Regarding the effect on reproductive parameters, the values obtained were similar to those presented by Pulido-Medellín *et al*(26), who determined the effect of *M. anisopliae* on *R. microplus*, reporting that the reproductive index decreased by up to 91 %. On the other hand, Alcalá-Gómez *et al*(20) emphasized that *Metarhizium* strains, such as Ma136, inhibited 73 % (endemic colony) and 64 % (tick "Media joya") of the oviposition, interrupting hatching in 73 and 86 %, respectively, with respect to the control. Based on the above, it can be affirmed that the behavior of the evaluated strain was similar to that of other entomopathogenic fungi; this is due to the secretion of proteins that present orthologous relationships between entomopathogenic fungi, it has been considered that entomopathogenic activity is related to proteins that have evolved from a common ancestor(27,28).

In studies of *B. bassiana*, Fernández-Ruvalcaba *et al*(9) mention percentages of 36 to 83 % of oviposition inhibition with an estimated reduction in reproduction of 46 to 95 % with different strains, with *B. bassiana* Bb-5J5 being the one that presented a higher percentage of control for the parameters described, with concentrations of $5x10^7$ conidia ml^{-1}. Ren *et al*(29) found that the reproductive efficacy index was affected by strains of *Beauveria* (BbAT01, BbAT03 and BbAT13) and *Metarhizium* (MaAT04), observing that, at a higher concentration of conidia, the reduction in reproductive efficacy of *R. microplus* decreased, a trend also observed in this work. The reduction in oviposition could be due to infection of the tick ovary as indicated by Paulo *et al*(30); nevertheless, the metabolic imbalance generated during the infection process by the entomopathogen plays an important role, due to the production of derivatives of reactive oxygen species that are capable of causing damage to various cellular components(24,31).

Likewise, when comparing the results of this study with the effect of *B. bassiana* Bb115 on the reproductive parameters of two colonies of ticks with different susceptibility to ixodicides(20), inhibition percentages similar to those obtained for *B. bassiana* DS3.17 were reported, the oviposition index was 0.23 for the native colony and 1.94 for the "media joya" colony (resistant to ixodicides), values that represented a reduction of oviposition of 98 and 79 % respectively. On the other hand, the reproduction index for the other colonies was 0.11 and 0.49 for each, values that meant percentages of control of reproductive potential of 98 and 89 %.

Bernardo *et al*[5] obtained nutrition indices with *Beauveria bassiana* from 58.89 to 59.32 % for the strains IP361 and CG307, these values represented between 35,086 and 182,061 viable larvae, meaning control percentages of 63.29 to 92.92 %.

On the other hand, the virulence of fungi with the potential to regulate tick populations depends on their susceptibility to factors such as temperature, time of exposure to UV radiation[1,5,8], the origin of the strain[32,33] and, in some cases, high concentrations of conidia increased mortality, increasing the control of the pest insect[34]. The latter was observed with the strain DS3.17 in this work, where the highest concentration ($1x10^9$ conidia ml^{-1}) was more effective in the control of *R. microplus*.

Conclusions and implications

B. bassiana DS3.17 had a significant control effect on the reproductive parameters of *R. microplus*; observing that the indices of oviposition and hatching were inversely proportional to the concentration of conidia. This increases the interest for the use of this strain in the biological control of *R. microplus* in cattle in the region, thus decreasing the use of ixodicides. The results obtained open the possibility of modeling the effect of *B. bassiana* D3.17 on the reproductive parameters of the tick, optimizing the most relevant for its control.

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All authors declare that they have no conflicts of interest in carrying out the present work.

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