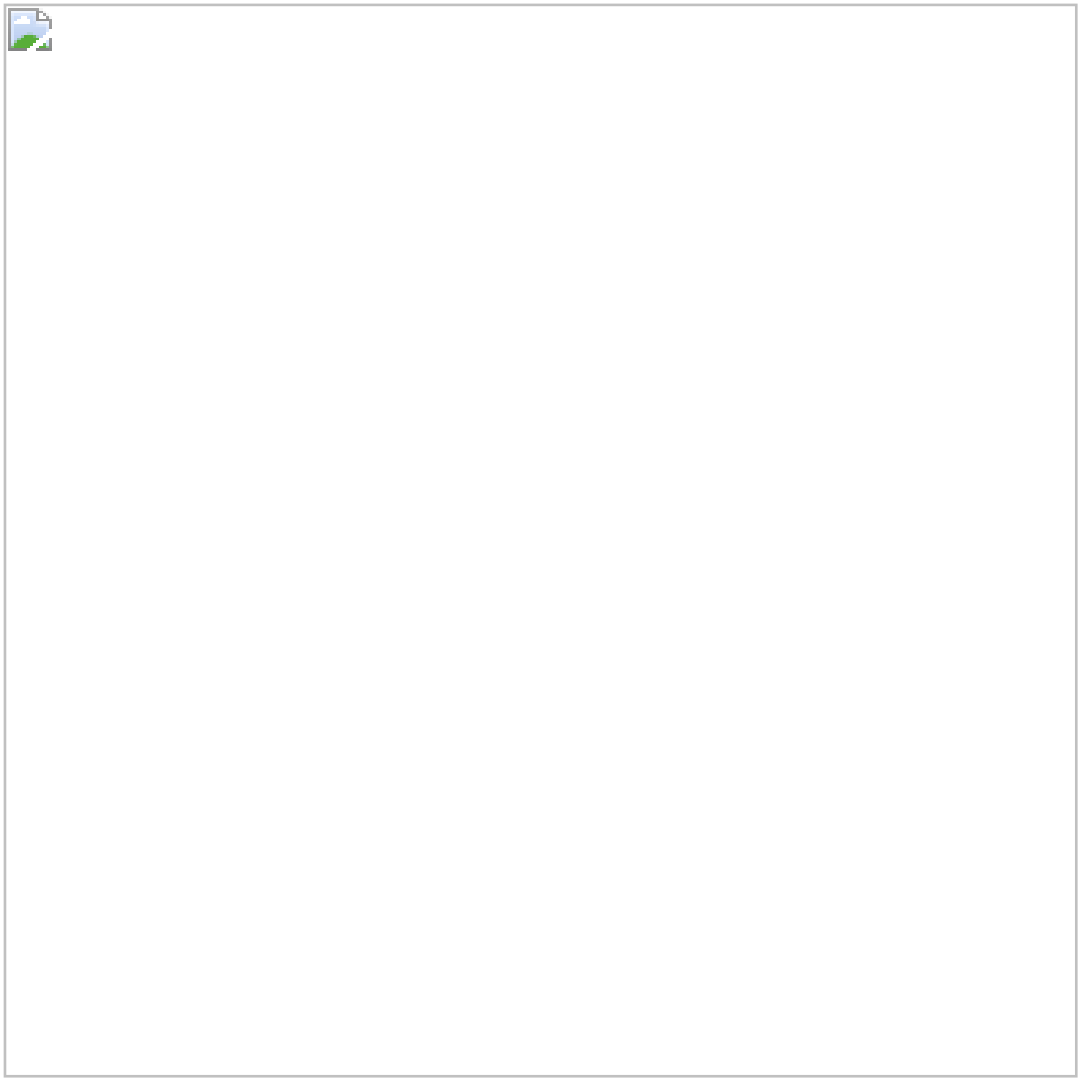

# Effects of Passages Through a Suitable Host of the Fungus, Metarhizium anisopliae, on the Virulence of Acaricide-Susceptible and Resistant Strains of the Tick, Rhipicephalus microplus

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# Effects of passages through a suitable host of the fungus, *Metarhizium anisopliae*, on the virulence of acaricide-susceptible and resistant strains of the tick, *Rhipicephalus microplus*

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## Abstract

The aim of this work was to assess the virulence of strain M379 of the fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) after different passages through a suitable host and at different concentrations for the control of both acaricide-susceptible and resistant strains of the tick, *Rhipicephalus* (formerly *Boophilus*) *microplus* Canestrini (Ixodida: Ixodidae) *in vitro*. The highest value of $LC_{50}$ for the susceptible strain corresponded to zero passage with $7.68 \times 10^7$ conidia/ml followed by the fourth passage with $2.68 \times 10^7$, which reduced 2.87-fold the lethal concentration. When comparing LC50 values of the fourth vs. the seventh passage ($2.59 \times 10^5$ conidia/ml**)**, the lethal concentration was reduced 103.47-fold by the seventh passage. In addition, in the resistant strain the $LC_{50}$ highest value corresponded to zero passage with $4.95 \times 10^7$ conidia/ml followed by the fourth passage with $7.86 \times 10^6$, which reduced 6.30-fold the lethal concentration. When comparing LC50 values of the fourth vs. the seventh passage ($1.04 \times 10^5$ conidia/ml) in the resistant strain, the lethal concentration was reduced 75.58-fold by the seventh passage. These results suggest that the number of passages on *M. anisopliae* through a suitable host increased its virulence on both *R. microplus* strains. When comparing $LC_{50}$ of the zero passage through a suitable host of both acaricide-susceptible and resistant strains, the highest $LC_{50}$ values corresponded to the susceptible strain with $7.68 \times 10^7$ conidia/ml followed by the resistant one with $4.95 \times 10^7$, showing that on the resistant strain the lethal concentration is reduced by 1.55-fold. When comparing the fourth passage, the highest values of $LC_{50}$ corresponded to the susceptible strain with 2.68 x $10^7$ conidia/ml followed by the resistant one with $7.86 \times 10^6$ conidia/ml, showing for the resistant strain a 3.41-fold reduced lethal concentration. Moreover, when comparing the seventh passages, the highest values of $LC_{50}$ corresponded to the susceptible strain with $2.59 \times 10^5$ followed by the resistant with $1.04 \times 10^5$ conidia/ml, revealing for the resistant strain a 2.49-fold reduced lethal concentration. These results suggest that the resistant strain needs a lower concentration of conidia than the susceptible

strain. In this case, the acaricide-resistant strain is more susceptible to *M. anisopliae* of zero- and seven-passage strains.

**Keywords:** biocontrol, ectoparasite, pathogen, oviposition inhibition
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## Introduction

One of the most important cattle pests in tropical regions is the tick, *Rhipicephalus (Boophilus) microplus* (Canestrini) (Acari: Ixodidae), which is a strict ectoparasite of bovines. To control this parasite, around 7 billion dollars are spent each year worldwide (Bull et al. 1996). *R. microplus* causes several cattle diseases such as anemia, flesh wounds, and abortive events (Yeruham et al. 1998). The effects of these diseases cost up to 100 million dollars a year in North America alone, but in Central and South America the costs rise to 1 billion dollars per year (Acosta-Rodriguez et al. 2005).

*Rhipicephalus microplus* is a vector for infectious cattle diseases. Parasitic protozoa, like *Babesia bovis* and *B. bigemina*, are transmitted to both domestic and wild animals (Alonso et al. 1992). In Mexico, the control of *R. microplus* is based on the use of chemical acaricides applied to animals. The most commonly used chemicals are organophosphorics (OP), synthetic pyretroids (SP), amidines, and macrocyclic lactones (Taylor 2001). This kind of control was effective in the past, but it has become too expensive and the rise of resistant strains together with the intrinsic pollution of this practice has rendered this strategy unviable (Norval et al. 1992; Kaaya and Hassan 2000).

In Mexico, some populations of *R. microplus* have developed resistance to different acaricides, such as pyretroids (Miller et al. 2007), carbamates, formamidines, and organophosphates (Li et al. 2004). Entomopathogenic fungal species are being evaluated for use as an alternative control for *R. microplus*; among these, some strains of *Metarhizium anisopliae* are promising (Fernandez-Ruvalcaba et al. 2005; Leemon and Jonsson 2008).

It has been reported that cultures of entomopathogenic fungus in artificial media cause phenotypic alterations associated with the degeneration of entomopathogenic cultures (Nagaich 1973; Ibrahim et al. 2002; Ryan et al. 2002). These alterations include changes in color, growth, and morphology. Another important issue is that culture degeneration leads to a strong decay in sporulation, metabolites, and virulence (Butt et al. 2006). The degeneration of entomopathogenic fungi has been identified by the biological insecticide manufacturers as a cause for decay in the quality of stocks, thereby rendering the product commercially unviable (Butt et al. 2006).

Strains are different in their virulence decay rates; some strains lose their virulence in a single subculture (Butt et al. 2006). Nagaich (1973) reports the loss of virulence of *Verticillium lecanii* (Zimm.) after two or three subcultures. Some strains need to be successively subcultured 10 to 12 times before a significant decline in virulence is observed (Morrow et al. 1989; Hajek et al. 1990). Brownbridge et al. (2001) reported that one strain of *B. bassiana* did not lose its virulence after 15 successive subcultures in artificial media, and the ectoparasitic fungi *Paecilomyces fumosoroseus* did not lose its virulence after 30 successive subcultures (Vandenberg and Cantone 2004).

Very few studies have been carried out to determine the capabilities of the host in restoring or increasing fungal virulence of attenuated fungal cultures in artificial media (Wasti and Hartman 1975; Butt and Goettel 2000). Guedes–Frazzon et al. (2000) found

that after single-step re-isolation of *M. anisopliae* strains (M5 and E6S1) previously employed on engorged *R. microplus* females strongly elevates their virulence. However, this work was not conclusive in determining the effects of different passages through a suitable host in increasing fungal virulence because the overall effect was only measured in the zero and first passages through a suitable host.

*Rhipicephalus microplus* strains have been reported to have oscillating susceptibility to a specific kind of acaricide, highlighting the importance of determining the optimal concentration of *M. anisopliae* needed to control different strains of *R. microplus*. Establishing the best concentration for the control of *R. microplus* is of critical interest in dealing with this ectoparasite because over-concentration would increase the cost of the formulation.

Another important point is that the number of passages through a suitable host increases fungal virulence, and this strategy reduces the total time needed to control *R. microplus*. This specific strategy would result in an improved method for dealing with this ectoparasite in Mexican cattle.

The objective of this work is to compare the virulence of strain M379 of *M. anisopliae* with different passages through a suitable host on both susceptible and resistant strains of *R. microplus*.

## Materials and Methods

### Study Location

The present work was carried out at the Centro de Investigación en Biotecnología of the Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, Mexico. *M. anisopliae* M379 strain was isolated from cadavers of *Aeneolamia postica* (Hemiptera: Cercopidae). Engorged female individuals of acaricide-susceptible *R. microplus* strain Media Joya and an OP- and SP-resistant amidines San Alfonso strain were employed in this work (Soberanes-Céspedes et al. 2000). Both strains were cultured on bovines and stored at the Instituto de Investigaciones Forestales, Agrícolas and Pecuarias in Progreso, Morelos, Mexico.

### Culture Medium

*M. anisopliae* strain M379 was cultured in a Sabouraud-dextrose-agar (SDA) that included 5 g/l of mild peptone, 5 g/l casein peptone, 40 g/l dextrose, and 1.5 g/l agar. The culture was adjusted to pH 5.6 ± 0.2 and incubated in a dark room at 27 ± 1° C for 15 days to induce sporulation and then preserved at 4 ± 2° C .

### Conidia Collection

Conidia were recovered from Petri dishes using distilled water with 1% adhesive Tween 20 in a laminar flux chamber (CFLV-80; Aparatos de Laboratorio BG). Conidia were counted in a Newbauer chamber.

### Immersion Bioassay

An immersion assay was employed for engorged ticks (Drummond and Whestone 1970). Increasing concentrations of conidia of the M379 strain were used ($10^4$-$10^8$ conidia/ml), and distilled water was used as a negative control. Immediately after treatment, ticks were allowed to dry on paper towels and placed in clean snap-cap vials with 15 ticks per vial (the caps had a hole covered with screening). Vials were incubated for 15 days in a humid chamber at 90% RH and 25 ± 2° C. The high relative humidity is required for optimal survival of ticks (Drummond and Whestone 1970).

**Multiple Passages through a Suitable Host**
After inoculation of *M. anisopliae* into susceptible ticks, conidia were isolated from the cadavers and cultured in an SDA medium using successive tick-SDA-tick-SDA cycles. The successive isolation of conidia from the cadavers of ticks determined the number of consecutive subcultures of *M. anisopliae*.

**Experimental Design and Analysis**
Fifteen individuals from *R. microplus* for each treatment were used as an experimental group. To standardize data, each individual was weighed using an analytical balance (OHAUS AS 120; OHAUS Corporation, www.ohousscale.com) and randomly distributed. Three replications were used for each treatment.

A totally randomized experimental design was employed for the concentration ($10^4$-$10^8$ conidia/ml and distilled water) and passage (0, $4^{th}$, and $7^{th}$) factors through a suitable host Each combination was tested on Media Joya and San Antonio tick strains (Soberanes-Céspedes et al. 2002). The assay was replicated three times and the mortality percentages were calculated at 3, 5, 7, and 9 days after treatment. The concentration, lethal to 50% of tick populations ($LC_{50}$ value), was computed on the basis of the mortality percentage recorded at each concentration through Probit analysis within 95% confidence limits. Tukey's multiple range tests was used to separate passage means and Student's *t* test was used for the pairwise comparisons of means given in the text. SAS® software (Version 9.0) was employed for statistical analyses.

**Pathogenicity**
To determine the values of this dependent variable, a stereoscopic microscope (DM143; Motic Digital Microscope, www.motic.com) was used to measure the susceptibility of ticks to *M. anisopliae* strains in each treatment. A binomial measurement (death vs. survival) was employed over 24 h of observation.

**Virulence**
The virulence of the *M. anisopliae* strain on *R. microplus* was measured using the following parameters:

**Mortality Percentage of *Rh. (Bo.) microplus*.** Dead and live ticks were counted at 3, 5, 7, and 9 days after inoculation. Ticks were analyzed using a stereoscopic microscope and were considered dead when they remained motionless, were moribund, and had fungal mycelia emerging through the cuticle.

**Inhibition Percentage of Oviposition.** To measure this variable, after 15 days the total mass of oviposited tick eggs was weighed using an analytical balance (OHAUS AS 120; OHAUS Corporation, Florham Park, NJ). The formula employed (Drummond and Whestone 1970) was:

$$\% \text{ I. O.} = ( PQLt/PQLT - PHLt/PHLT ) * 100$$

where PQLt = weight of female ticks in the experimental stock, PQLT = weight of female ticks in the control stock, PHLt = weight of oviposited eggs in the experimental stock, and PHLT = weight of oviposited eggs in the control stock.

## Results

**Susceptible Ticks**
**Virulence.** The effects of the different *M. anisopliae* passages through a suitable host and of the conidia concentration on the virulence of susceptible Media Joya female ticks are shown in Table 1. A higher virulence

value was obtained using a concentration of 1 × $10^8$ conidia/ml, with 71.11% mortality in the seventh passage, followed by a 66.67% in the fourth passage at the same dose. Zero passage was less effective at the same dose with a mortality value of 53.33%.

When comparing the $LC_{50}$ values of the different passages through a suitable host (Table 2), the highest value of $LC_{50}$ for the susceptible strain corresponded to the zero passage with 7.68 × $10^7$ conidia/ml followed by the fourth passage with 2.68 × $10^7$, which reduced 2.87-fold the lethal concentration with statistical differences between the two ($df = 2, 6$ $p < 0.05$). When comparing $LC_{50}$ values of the fourth vs. the seventh passage (2.59 × $10^5$ conidia/ml), the lethal concentration was reduced 103.47-fold by the seventh passage showing a significant statistical difference ($df = 2, 6$ $p < 0.05$) with respect to the other passages.

**Oviposition Inhibition.** The effects of the different *M. anisopliae* passages through a suitable host and of the conidia concentration on the inhibition of oviposition of susceptible Media Joya female ticks are shown in Table 3. The results show an inhibition of oviposition proportionally related to the conidia concentration, which correlated positively with the different passages through a suitable host.

In the seventh passage through a suitable host, the percentage of inhibition of oviposition increased for the lower conidia concentrations; at a 1 × $10^4$ conidia/ml concentration a 17.65% mortality was obtained versus 1.28% and 5.68% mortality rates at the fourth and zero passages, respectively. An identical behavior was observed using a concentration of 1 × $10^5$ conidia/ml. However, when zero and fourth passage factors were used with increased concentrations of conidia per milliter, a different behavior for oviposition inhibition was observed: a 77.09% oviposition inhibition for a concentration of 1 × $10^7$ conidia/ml with the zero passage factor versus a 62.69% inhibition for the fourth passage through a suitable host, and a 64.47% rate for the seventh passage. However, when using the highest concentration (1 × $10^8$ conidia/ml) the proportional inhibition of oviposition revealed an identical response for each passage through a suitable host.

**Resistant Ticks**

**Virulence.** The effects of the different *M. anisopliae* passages through a suitable host, and of the conidia concentration on the virulence of acaricide-resistant *R. microplus* strain, are shown in Table 1. A higher virulence value was obtained using a concentration of 1 × $10^8$ conidia/ml with a 66.67% mortality in the seventh passage, followed by a 55.56% in the fourth passage at the same dose. However, in the zero passage of acaricide-resistant *R. microplus* strain, a concentration of 1 × $10^8$ conidia/ml yielded a 37.38% mortality, i.e. it was less effective

**Table 1.** Mortality percentage of different passages through a suitable host of M. anisopliae on female susceptible and acaricide-resistant ticks at the 7th day of incubation.

| | | Susceptible Ticks | | | Resistant Ticks | |
|---|---|---|---|---|---|---|
| Concentrations | Zero Passage | Fourth Passage | Seventh Passage | Zero Passage | Fourth Passage | Seventh Passage |
| (conidia/ml) | Mortality (%) | Mortality (%) | Mortality (%) | Mortality (%) | Mortality (%) | Mortality (%) |
| $1x10^8$ | 53.33 ± 6.67* | 66.67 ± 7.46 | 71.11 ± 7.26 | 37.78 ± 7.84 | 55.56 ± 6.42 | 66.67 ± 3.67 |
| $1x10^7$ | 37.78 ± 6.67 | 35.56 ± 7.46 | 66.67 ± 7.26 | 67.78 ± 7.84 | 55.56 ± 6.42 | 56.67 ± 3.67 |
| $1x10^6$ | 15.56 ± 6.67 | 26.67 ± 7.46 | 55.56 ± 7.26 | 25.00 ± 6.79 | 50.00 ± 7.86 | 55.00 ± 3.67 |
| $1x10^5$ | 13.33 ± 6.67 | 6.67 ± 7.46 | 44.44 ± 7.26 | 23.33 ± 6.79 | 26.67 ± 6.42 | 42.22 ± 4.24 |
| $1x10^4$ | 6.67 ± 6.67 | 11.11 ± 7.46 | 37.78 ± 7.26 | 20.00 ± 7.84 | 26.67 ± 7.86 | 50.00 ± 3.67 |
| $H_2O$ | 11.11 ± 6.67 | 6.67 ± 9.13 | 10.00 ± 8.89 | 12.77 ± 7.84 | 13.33 ± 6.42 | 13.00 ± 4.24 |

*Standard Error

**Table 2.** Mortality by concentration of *M. anisopliae* conidia concentration in susceptible and acaricide-resistant female *B. microplus* at the 7th day of incubation, and the negative control (H2O).

| Ticks | Passages | $LC_{50}$ (Conidia/ml) | $X^2$ | CI (95%)*** | Slope |
|---|---|---|---|---|---|
| | Zero | $7.68 \times 10^7$ A* | 0.3969 | $2.11 \times 10^7$-$7.20 \times 10^8$ | 0.411 ± 0.074** |
| Susceptible | Fourth | $2.68 \times 10^7$ B | 0.0135 | $5.69 \times 10^6$-$5.94 \times 10^8$ | 0.457 ± 0.104 |
| | Seventh | $2.59 \times 10^5$ C | 0.5700 | $1.93 \times 10^4$-$1.39 \times 10^6$ | 0.231 ± 0.061 |
| | Zero | $4.95 \times 10^7$ A | 0.0010 | $3.14 \times 10^6$-$1.79 \times 10^{18}$ | 0.241 ± 0.097 |
| Resistans | Fourth | $7.86 \times 10^6$ B | 0.4584 | $1.29 \times 10^6$-$2.02 \times 10^8$ | 0.231 ± 0.067 |
| | Seventh | $1.04 \times 10^5$ C | 0.9793 | $1.33 \times 10^4$-$2.24 \times 10^{10}$ | 0.118 ± 0.052 |

*Means in each column within each treatment followed by the same letter did not differ significantly ($p<0.05$, Turkey's Test).
**Standard Error
***Confidents Intervals

than $1 \times 10^7$ conidia/ml with a 37.38% mortality.

When comparing the $LC_{50}$ values of the different passages through a suitable host (Table 2), the highest value of $LC_{50}$ corresponded to the zero passage with $4.95 \times 10^7$ conidia/ml followed by the fourth passage with $7.86 \times 10^6$, which reduced 6.30-fold the lethal concentration, with statistically significant differences between the two ($df = 2, 6$ $p < 0.05$). When comparing $LC_{50}$ values of the fourth vs. the seventh passage ($1.04 \times 10^5$ conidia/ml) in the resistant strain, the lethal concentration was reduced 75.58-fold by the seventh passage; showing statistically significant differences ($df = 2, 6$ $p < 0.05$) with respect to the other passages.

When comparing $LC_{50}$ values of the zero passage through a suitable host for Media Joya vs. San Alfonso strains, the lowest $LC_{50}$ values corresponded to San Alfonso with $4.95 \times 10^7$ conidia/ml and $7.68 \times 10^7$ for Media Joya showing significant differences between both strains ($df = 16$ $p<0.05$), reducing 1.55-fold the lethal concentration for the San Alfonso resistant strain. When comparing the fourth passage, the lowest $LC_{50}$ values corresponded to San Alfonso with $7.86 \times 10^6$ conidia/ml and $2.68 \times 10^7$ to the Media Joya strain, reducing the San Alfonso strain the lethal concentration 3.41-fold and showing both statistically significant differences. Moreover, when comparing the seventh passage, the lowest values of $LC_{50}$ corresponded to the San Alfonso strain with $1.04 \times 10^5$ followed by Media Joya with $2.59 \times 10^5$, reducing the San Alfonso strain the lethal concentration 2.49-fold and showing significant differences for both passages.

**Percentage of inhibition of oviposition.** The effects of the different passages through a suitable host and the concentration of *M. anisopliae* conidia on the oviposition inhibition of the resistant *R. microplus* are shown in Table 3. In the seventh passage through a suitable host, the percentage of inhibition of oviposition increased with the lower conidia concentrations; at a $1 \times 10^4$ conidia/ml concentration 25.27% mortality was obtained versus 10.10% and 1.08% mortality rates at the fourth and zero passages, respectively. An identical behavior was observed using a concentration of $1 \times 10^5$ conidia/ml. However, when zero, fourth, and seventh passage factors were used with increased concentrations of conidia per milliliter the proportional inhibition of oviposition revealed an identical response for each passage through a suitable host. The results for the zero and fourth passages through a suitable host show an increment in the oviposition inhibition, which is highly dependent on the conidia concentrations employed; however, this dependence was less evident for the seventh passage through a suitable host. Similar trends were observed when comparing the highest value of conidia

concentration for each kind of passage through a suitable host.

**Table 3.** Oviposition (g) and inhibition of oviposition (O.I.) of different passages through a suitable host of *M. anisopliae* on female susceptible (ST) and acaricide-resistant (RT) ticks.

| Concentrations | Susceptible Ticks | | | Resistant Ticks | | | Susceptible Ticks | | | Resistant Ticks | | | Susceptible Ticks | | | Resistant Ticks | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Zero Passage | | | Zero Passage | | | Fourth Passage | | | Fourth Passage | | | Seventh Passage | | | Seventh Passage | | |
| (conidia/ml) | Ticks (g)* | Oviposition (g)** | O. I % | Ticks (g) | Oviposition | O. I % | Ticks (g) | Oviposition | O. I % | Ticks (g) | Oviposition | O. I % | Ticks (g) | Oviposition | O. I % | Ticks (g) | Oviposition | O. I % |
| $1 \times 10^4$ | 5.552 ± 0.395* | 1.091 ± 0.157 | 10.36 ± 2.52*** | 5.868 ± 0.098 | 1.841 ± 0.341 | 1.08 ± 1.52 | 4.956 ± 0.133 | 0.920 ± 0.202 | 1.28 ± 1.09 | 5.761 ± 0.257 | 1.362 ± 0.298 | 10.10 ± 6.53 | 5.027 ± 0.110 | 0.860 ± 0.185 | 17.65 ± 2.27 | 5.690 ± 0.075 | 1.270 ± 0.277 | 25.27 ± 7.58 |
| $1 \times 10^5$ | 5.380 ± 0.395 | 0.934 ± 0.071 | 21.30 ± 4.58 | 6.060 ± 0.257 | 1.693 ± 0.213 | 12.09 ± 0.96 | 4.646 ± 0.636 | 0.639 ± 0.205 | 23.44 ± 1.58 | 4.853 ± 0.170 | 1.142 ± 0.172 | 17.61 ± 3.29 | 5.171 ± 0.046 | 0.591 ± 0.112 | 46.42 ± 9.23 | 6.478 ± 0.249 | 1.366 ± 0.125 | 33.45 ± 3.94 |
| $1 \times 10^6$ | 4.907 ± 0.238 | 0.474 ± 0.045 | 53.39 ± 1.17 | 6.156 ± 0.202 | 1.356 ± 0.007 | 31.45 ± 2.19 | 5.257 ± 0.067 | 0.489 ± 0.055 | 49.80 ± 2.79 | 5.121 ± 0.129 | 0.729 ± 0.055 | 43.52 ± 2.63 | 5.327 ± 0.257 | 0.530 ± 0.100 | 55.18 ± 2.55 | 6.312 ± 0.131 | 1.070 ± 0.333 | 46.06 ± 2.97 |
| $1 \times 10^7$ | 5.191 ± 0.142 | 0.272 ± 0.164 | 77.09 ± 2.72 | 6.099 ± 0.169 | 0.786 ± 0.204 | 60.53 ± 1.66 | 5.015 ± 0.131 | 0.318 ± 0.139 | 62.69 ± 1.85 | 6.162 ± 0.078 | 0.729 ± 0.055 | 60.02 ± 1.54 | 5.246 ± 0.157 | 0.428 ± 0.102 | 64.47 ± 3.21 | 6.138 ± 0.224 | 0.801 ± 0.077 | 57.14 ± 3.77 |
| $1 \times 10^8$ | 5.238 ± 0.168 | 0.206 ± 0.102 | 83.94 ± 1.78 | 5.985 ± 0.186 | 0.523 ± 0.194 | 72.48 ± 2.01 | 5.522 ± 0.125 | 0.158 ± 0.027 | 87.72 ± 1.36 | 6.282 ± 0.146 | 0.679 ± 0.213 | 64.61 ± 3.56 | 4.880 ± 0.157 | 0.120 ± 0.017 | 86.49 ± 1.36 | 6.409 ± 0.109 | 0.571 ± 0.104 | 73.74 ± 2.12 |
| $H_2O$ | 5.113 ± 0.341 | 1.113 ± 0.193* | | 5.982 ± 0.207 | 1.898 ± 0.030 | | 5.339 ± 0.304 | 1.005 ± 0.263 | | 6.27 ± 0.266 | 1.913 ± 0.029 | | 4.981 ± 0.191 | 1.032± 0.069 | | 6.235 ± 0.182 | 1.939 ± 0.217 | |

*Average Weight of 45 ticks by treatments
**Average eggs Weight of 45 ticks by treatments
***Standard Error

## Discussion

Several reports have suggested that *M. anisopliae* is susceptible to attenuation when continuously sub-cultured in artificial media (Guedes-Frazzon et al. 2000; Shah et al. 2005; Butt et al. 2006). Some authors consider that passaging enhances virulence, but it is not clear if they are simply restoring the pathogen's original insecticidal activity. Virulence is restored, or enhanced, usually after a single passage through a suitable host, but some authors report that virulence is increased after two or more successive passages (Brownbridge et al. 2001; Vandenberg and Cantone 2004). Taking into consideration both hypotheses, *M. anisopliae* was continuously sub-cultured on *R. microplus* to elucidate the effect on its virulence.

One important finding is that as the number of passages increased on a suitable host of *M. anisopliae*, the mortality percentage incremented proportionally for both susceptible and resistant *R. microplus* strains. Morrow et al. (1989) found differences in the adhesion of virulent and attenuated conidia of *Nomuraea rileyi* on the surface of the host cuticle. The attenuation of fungal strains could be affecting the germination capability and conidia adhesion to the cuticle. A faster rate of germination is correlated with high virulence in *M. anisopliae* and *P. fumosoroseus* (Inglis et al. 2001). The last finding indicates that continuous passage through a suitable host may be correlated with better conidia adhesion on the host's cuticle and a faster germination rate, leading to a faster invasion of the host's hemocele. One possible explanation for these phenomena is a higher level of the membranal protease Pr1, which is responsible for the degradation of the insect cuticle, found in spores obtained from insect cadavers in *in vitro* experiments (Shah et al. 2005).

Guedes-Frazzon et al. (2000) reported that the different passages through a suitable host of *M. anisopliae* on *R. microplus* are correlated with increased virulence and reduced attenuation. A proportional influence on the virulence of different passages through a suitable host was shown in M5 and E6S1 cultivated over engorged female ticks of *R. microplus*. M5 strain increased its mortality from 1.8% to 84% in a single subculture. The E6S1 strain showed a similar trend, increasing its mortality by 53%, but these results were not conclusive because the experiment was performed with only a single subculture.

In the present study, when comparing the different concentrations (conidia/ml) of *M. anisopliae* on the *R. microplus*-susceptible strain (Media Joya) through a suitable host, the fourth passage reduced the lethal concentration 2.87-fold in respect to the zero passage, and the seventh passage reduced it by 103.47-fold in respect to the fourth passage. A low $LC_{50}$value indicates a high virulence of the fungus, requiring lower amounts of infective conidia to eliminate half of the plague's population (Bittencourt et al. 1997). In addition, the zero passage strain showed the highest $LC_{50}$value, the fourth passage reduced the lethal concentration 6.30-fold in respect to the zero passage, and the seventh passage reduced it 75.58-fold in respect to the fourth passage. These results suggest that the number of passages through a suitable host increase the mortality of both *R. microplus* strains.

Previously, some researchers reported that the entomopathogenic fungus acts on invertebrates through a mechanism involving invasion of the hemocele (Kaaya and Tanada

1993), and suggested that the hemolymph is employed as substrate, causing nutritional deficiency and integrity damage. In the present study, it was found that the surviving female tick's oviposition was diminished in proportion to the concentration. This phenomenon was attributed to the effect of the fungal colonization of the tick causing a severe reduction in the nutritional reserves available to the tick. These data are in accordance with the results of Fernandez-Ruvalcaba et al. (2005), who showed that conidia concentrations reduced the oviposition of female ticks by a factor of five. Guedes-Frazzon et al. (2000) found a similar trend in the E6S1 *M. anisopliae* strain, in which oviposition was reduced by 17% and by 38% in the CG423 *M. anisopliae* strain.

In this study the oviposition of female *R. microplus* was significantly reduced and maximal inhibition values of 87.72% were obtained when combining conidia concentrations and different passages through a suitable host. However, these results contrast with the results of Barcelos-Correia et al. (1998), who did not find any inhibition of oviposition in female ticks in an *in vivo* experiment on cattle.

In this study, when comparing $LC_{50}$ values of the zero passage through a suitable host for Media Joya vs. San Alfonso strains, the San Alfonso resistant strain was reduced to 1.55-fold the lethal concentration. In addition, when comparing the fourth passage, the San Alfonso strain reduced the lethal concentration 3.41-fold. Moreover, when comparing the seventh passage, San Alfonso strain reduced the lethal concentration 2.49-fold. These results indicate that resistant ticks require a lower concentration to be controlled than the susceptible strain. In this case, the San Alfonso acaricide-resistant strains showed more susceptibility to *M. anisopliae*.

The molecular strategies employed by resistant ticks to hydrolyze xenobiotics are exerted through sterases or by abducting cytochrome P 450 (Dary et al. 1990; Devonshire and Field 1991). In the present study, the acaricide-resistant tick strain showed an increased susceptibility to *M. anisopliae* probably because the contact strategies of acaricide-resistance strains decrease the expression level of enzymes (such as protease inhibitors and chitinases), or because they present a lower mechanical resistance to cuticle penetration by the fungus.

On the other hand, acaricide-susceptible ticks had a higher *M. anisopliae* resistance possibly because the principal natural control for tick are biological entities, such as fungi and bacteria present in the environment and their own adaptation mechanisms, are better prepared to avoid biological attacks. This mechanism could include the expression of protease inhibitors, chitinase inhibitors, or increased mechanical protection.

From the results of this study, we conclude that the use of the seventh passage strain through a suitable host of *M. anisopliae* may be a promising method for the control of acaricide-resistant *R. microplus*.

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