EVALUATION OF *IN VITRO* EFFECT OF THE FUNGI *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* ON EGGS AND LARVAE OF *AMBLYOMMA CAJENNENSE*.

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SUMMARY: This work was conducted to verify the *in vitro* pathogenicity of two *Beauveria bassiana* and three *Metarhizium anisopliae* isolates on eggs and larvae of *Amblyomma cajennense*. The alterations determined by the fungi on both developmental stages were observed and the lethal concentrations for 50 and 90% of population (LC_{50} and LC_{90}) were calculated. Four suspensions with different conidia concentrations were prepared (10^5, 10^6, 10^7 and 10^8 conidia/ml) with the help of a Neubauer chamber, as stated in previous experiments. Significative differences between treatments ($p<0.05$) were found for hatching percentage, where the hatching rate found for the groups treated with the different conidia suspensions was lower that found for control group. The hatching rates presented by the treated groups were inversely proportional to the concentration of conidia used. For the larval assay, alterations were found regarding larval mortality rates, being the values found for treated groups higher than those for controls. Lethal concentrations 50% (LC_{50}) for hatching inhibition ranged from 3.23×10^7 until 3.82×10^8 conidia/ml among the different isolates, and for larval mortality from 1.8×10^5 until 3.9×10^7 conidia/ml.

KEY WORDS: *Amblyomma cajennense*, *Beauveria bassiana*, *Metarhizium anisopliae*, entomopathogenic fungi, biological control.

INTRODUCTION

Amblyomma cajennense (Fabricius, 1787), also know as "star tick", is an heteroxenous tick found mainly on horses, although can also feed on other mammals as cattle, deer, dogs and even the man. Besides the direct damage it causes through their blood-sucking habits, as the consequent skin lesions, this species is considered as major vector for several infectious diseases, as bovine *erlichiosis* (MASSARD, 1984) and exantematous typhus (MONTEIRO *et alii*, 1931).

Tick mortality due fungi among laboratory colonies was observed by BOYCEV & RIZVANOV (1960). Later on LIPA (1971) listed 15 fungi found on tick eggs and larvae. GORSKOVA (1966) used *Beauveria bassiana* to artificially infest *Ixodes ricinus*, and demonstrated a diminution in the hatching rates and a mortality rate from 86 until 100 %.

Artificial infection of eggs, larvae and engorged females of *Boophilus microplus* by the fungi *Metarhizium anisopliae* was described, when a high larval mortality rates were found. The development of this fungi in the haemolinph of this tick was verified and led to alterations of the following parameters of the non parasitical phase: pre-egg laying period, egg laying period, egg production index, incubation period, hatching period and hatching rates (BITTENCOURT, 1992).

This work was aimed at: the verification of the *in vitro* pathogenicity of the fungi *B. bassiana* and *M. anisopliae*, which were inoculated as conidia suspensions in eggs and non fed larvae of *A. cajennense*; the observation of the biological alterations determined by the fungi and finally, the calculation of the lethal concentrations (LC_{50} and LC_{90}) for both eggs and larvae of this tick.

MATERIALS AND METHODS

Collection of eggs and larvae

Engorged *A. cajennense* females were collected from naturally infested animals. They had no recent contact with tickcides. Thereafter they were washed with water, dried, identified, weighted and placed into Petri dishes using an adhesive tape. Once egg laying started, eggs were weighted and 100 mg samples were put into test vials properly identified and then incubated under controlled environmental conditions (27°C and >80% relative humidity).

After the oviposition, a 25 mg sample was taken from the egg mass and placed into test vials to originate the larvae to be used in the tests. Each vial produced an average offspring of 390 larvae (PRATA & DAEMON, 1997).

Collection and use of *Metarhizium anisopliae* e *Beauveria bassiana* samples

Some of the isolates of *M. anisopiliae* and *B. bassiana* used in this experiment were donated by the Entomology Department, Escola Superior de Agricultura Luiz de Queiroz, São Paulo University. Three isolate of *M. anisopliae* were evaluated: 959 (isolated from ticks), 319 (isolated from ants) and E9 (standard isolated). Two isolates of *B. bassiana* were analyzed: 986 (isolated from ticks) and 747 (isolated from ants).

Suspensions preparation

Conidial suspensions of each isolate were prepared from fungi raised in rice medium. Polypropylene bags filled with 50 ml of distilled water and three drops of spreading adhesive Tween 80®.

Egg assay

Each egg test consisted of one group kept under controlled environmental conditions (27°C and >80% r.h.) with four different treatments (10^5, 10^6, 10^7 e 10^8 conidia/ml). The suspensions concentrations were determined in previous experiments, and prepared with the help of a Neubauer chamber (BITTENCOURT *et alii*, 1994). A control group was used for each one of the isolates tested. Three replicates were used for each treatment, with 100 mg of eggs per replicate.

The test vials were corked with hydrophilic cotton and identified showing the concentration used, and the isolate used. After shaking the suspension to be tested, 1 ml was added to each group of eggs. An immersion time of 5 minutes was allowed, when the tubes were turned upside down, in order to let cotton plugs absorb then the excess of suspension. The tubes were then returned to the previously described controlled environmental conditions.

For the evaluation of the effect of the different samples of the fungi *B. bassiana* and *M. anisopliae* on eggs of *A. cajennense* were evaluated: a) egg incubation period, from the first day of egg-laying until the beginning of hatching (PEREIRA, 1980); b) hatching period, from the first until the last day of larval hatching (BELLATO, 1995); and c) hatching percentage, that was recorded 30 days after the beginning of the hatching period by visual evaluation (DAVEY *et alii*, 1984).

Larval assay

Larvae used in these tests were derived from the test tubes with 25 mg of eggs of *A. cajennense* each. The methodology used was the same than that used for eggs, except by the effect evaluation, which in this case, was done through the mortality evaluation, carried out ten days after the test, using a stereo microscope. The test tubes were opened in a Petri dish (100 mm x 20 mm), that was place into another Petri dish (150 mm x 20 mm) with alcohol 70°, in order to prevent escape of alive larvae.

A light spot act as a heat source that stimulates larvae. Alive larvae tried to escape from the smaller Petri dish and fell into the alcohol. After some time the number of larvae in the alcohol was counted, the mortality rate calculated with a correction using the values of the control group (ABBOTT, 1925).

ANOVA analyzes were performed to investigate if variation inside as well as among the treatments with different isolates and concentrations. After ANOVA a TUKEY test was performed for comparison of the averages and for calculation of the variation coefficient (to verify the precision of data). To calculate the lethal concentrations LC_{50} and LC_{90} probit analysis was used, in agreement with LITCHFIELD & WILCOXON (1949).

RESULTS AND DISCUSSION

Egg assay

Regarding to the parameters mean incubation period and mean hatching period no significative differences were found ($p<0.05$) between treatments (10^8, 10^7, 10^6, 10^5 and control) for any of the isolates tested, despite some differences in the results had been observed ($p>0.10$). For hatching percentage however, significative differences were found ($p<0.05$) between treatments for some isolates (Table 1).

The results for hatching percentage for the isolates *M. anisopliae* 959, *M. anisopliae* 319 and *B. bassiana* 986 presented differences in the comparison of the control and the concentration with 10^5 conidia/ml with the suspension with 10^8 conidia/ml. For the isolates *M. anisopliae* E9 and *B. bassiana* 747, no significative differences ($p>0.10$) were observed between treatments regarding hatching percentage.

The mean hatching percentage was lower for the treated groups when compared with controls. BOYCEV & RIZVANOV (1960) found a hatching percentage of 6.5% for the groups of *I. ricinus* eggs treated with *Beauveria bassiana*. GORSKOVA (1966) also reported a very low hatching percentage when *I. ricinus* was treated with *B. bassiana*. Such findings confirm the action of this microorganism in the hatching percentage, although the authors had not told the concentrations used.

DAVEY *et alii* (1980), BITTENCOURT *et alii* (1990) and BITTENCOURT *et alii* (1996) used the similar environmental conditions and observed results similar to those of the controls

groups in this experiment. Treated groups presented a hatching percentage inversely proportional to the conidia concentration, *i.e.* the greater the concentration, lower is the hatching percentage for treated larvae.

The eggs incubation period and larvae hatching period did not presented significative variations ($p>0.10$) in the comparison among the groups treated with different suspensions of *M. anisopliae* and *B. bassiana*, and also when compared with controls. These findings disagree with BOYCEV & RIZVANOV (1960), who found an increase in the incubation period after the treatment of *I. ricinus* with *B. bassiana*. GORSKOVA (1966) also verified that the treatment with some fungi species (*B. bassiana* e *Penicillium insectivorum*) also increased the incubation period in eggs of *I. ricinus* in the comparison with controls. BITTENCOURT (1992) immersed *B. microplus* eggs in different suspensions of *M. anisopliae* isolates and observed an increase in the incubation period for the treated groups. A positive relationship between concentration used and incubation period was also found.

The values obtained for LC_{50} and LC_{90} for the different isolates, that prove to be able to inhibit larval hatching in treated eggs were high (Table 3), if compared to the values cited by BITTENCOURT *et alii*, (1994b and 1996) and MONTEIRO (1997), who worked with the same *B. bassiana* and *M. anisopliae* isolates on the ticks *B. microplus* and *R. sanguineus*, respectively. Anatomophisiological differences between these tick species, different methodology for data collection could explain such differences.

Larval assay

Regarding larval surviving, it was observed that the mortality rates were higher for those groups treated with the different fungi suspensions when compared with controls (Table 2). Significative ($p<0.05$) differences were found for all isolates testes, except by *B. bassiana* 986 and 747 which did not presented differences in the comparison of the controls with the 10^5 conidia/ml suspension.

Treated groups presented larval surviving rates inversely proportional to the conidia concentration used, what confirms the pathogenicity of these fungi for non fed larvae of *A. cajennense*.

Several authors also verified the pathogenicity of these fungi on non-fed tick larvae. BITTENCOURT *et alii* (1994) and

Table 1 - Hatching rates of *Amblyomma cajennense* larvae, derived from eggs treated with different concentrations of conidia from the entomopathogenic fungi *Metarhizium anisopliae* (Ma) and *Beauveria bassiana* (Bb); (Temperature 27°C ± 1, Relative humidity ≥ 80% e light period of 14/10 hours).

Concentration (conidia/ml)	Hatching Rate (%)				
	Ma 959[1]	Ma 319[1]	Ma E9[1]	Bb 986[1]	Bb 747[1]
Control	100.00 a	100.00 a	100.00 a	100.00 a	100.00 a
10^5	100.00 a	100.00 a	100.00 a	100.00 a	100.00 a
10^6	67.66 ab	65.33 ab	66.67 a	68.33 ab	85.00 a
10^7	65.00 ab	35.33 ab	66.67 a	33.33 ab	75.00 a
10^8	0.00 b	1.66 b	15.00 a	0.33 b	24.33 a

[1] Means in the same row, followed by the same letter are not significatively different ($p<0.05$).

Table 2 - Larval surviving rates of non fed larvae of *Amblyomma cajennense* treated with different concentrations of conidia from the entomopathogenic fungi *Metarhizium anisopliae* (Ma) and *Beauveria bassiana* (Bb); (Temperature 27°C ± 1, Relative humidity ≥ 80% e light period of 14/10 hours).

Concentration (conidia/ml)	Hatching Rate (%)				
	Ma 959[1]	Ma 319[1]	Ma E9[1]	Bb 986[1]	Bb 747[1]
Control	100.00 a	93.33 a	76.67 a	85.00 a	90.00 a
10^5	48.33 b	56.67 b	30.00 b	83.33 a	88.33 a
10^6	20.00 c	26.67 c	16.67 b	60.00 b	26.67 b
10^7	13.33 c	16.67 c	6.67 bc	30.00 c	6.67 c
10^8	3.33 c	13.33 c	3.33 bc	5.00 d	0.00 c

[1] Means in the same row, followed by the same letter are not significatively different ($p<0.05$).

Table 3 - Lethal concentrations (LC_{50} and LC_{90}), observed in eggs and larvae of *Amblyomma cajennense* treated with different isolates of *Beauveria bassiana* and *Metarhizium anisopliae* (Temperature 27°C ± 1, Relative humidity ≥ 80% e light period of 14/10 hours).

	Hatching		Mortality	
Isolates	CL 50[1]	CL 90[1]	CI 50[1]	CL 90[1]
Ma 959	4.1E7	2.7E9	6.5E5	1.1E8
Ma 319	3.2E7	3.7E8	1.8E5	1.2E9
Ma E9	1.1E8	3.4E9	2.3E5	8.6E7
Bb 986	3.2E7	3.2E8	3.9E7	5.7E9
Bb 747	3.8E8	1.5E10	7.9E5	5.7E7

[1] Concentrations shown in conidia/ml.

BITTENCOURT *et. alii.* (1996), evaluated the effect of *M. anisopliae* and *B. bassiana* on *B. microplus* larvae, and found a high pathogenicity for all isolates tested. MONTEIRO (1997) verified high mortality rates of non fed *Rhipicephalus sanguineus* larvae exposed to these same fungi isolates. BOYCEV & RIZVANOV (1960) found a mortality rate of 93.5% for larvae of *I. ricinus* after their immersion in a suspension of *B. bassiana*.

The LC_{50} and LC_{90} values for each one of the isolates that were able to promote larval mortality can be found in Table 3. On probit analysis done with *B. bassiana* mortality data, it was verified that the LC_{50} ranged 7.9×10^5 until 3.9×10^7 conidia/ml. For *M. anisopliae* isolates it ranged from 1.8×10^5 until 6.5×10^5 conidia/ml. BITTENCOURT *et alii* (1996) treated *B. microplus* larvae with *B. bassiana* suspensions and found mortality rates from 6.83×10^6 until 1.01×10^7 conidia/ml. MONTEIRO (1997) evaluated LC_{50} data for *R. sanguineus* larvae treated with *B. bassiana* and found values from 2.2×10^5 until 2.15×10^6 conidia/ml; for *M. anisopliae* this index ranged from 7.8×10^4 until 1.4×10^5 conidia/ml. These differences between the values found by several authors, could be explained by anatomophysiological differences between the tick species used in these experiments, once the fungi isolates are the same.

Based upon the results presented herein one can conclude that the entomopathogenic fungi tested can cause detrimental effects on this tick species, presenting therefore, a potential to be used in tick microbial or integrate control programs.

SUMÁRIO

Este trabalho teve como objetivo verificar a patogenicidade *in vitro* de dois isolados de *Beauveria bassiana* e de três isolados de *Metarhizium anisopliae* para ovos e larvas de *Amblyomma cajennense*, observando as alterações causadas nesses estágios de desenvolvimento do carrapato e calcular a concentração letal (CL) 50 e 90. Foram preparadas quatro suspensões com concentrações diferentes de conídios/ml (10^5, 10^6, 10^7e 10^8), estabelecidas em pré experimento e quantificadas em câmara de Neubauer. Observamos diferenças significativas ($p<0,05$) entre os tratamentos no parâmetro percentual de eclosão, onde o percentual médio de eclosão de larvas oriundas dos ovos tratados com as diferentes suspensões foi inferior ao observado nos grupos controle, ou seja, os grupos tratados apresentaram uma porcentagem de eclosão inversamente proporcional à concentração de conídios da suspensão. No bioensaio com larvas observamos alteração biológica no índice de mortalidade de larvas, onde os grupos tratados apresentaram índices superiores ao observado no grupo controle. As concentrações letais (CL) 50 % encontradas para promover a inibição da eclosão dos ovos variaram de $3,23 \times 10^7$ até $3,82 \times 10^8$conídios / ml entre os diferentes isolados e para mortalidade de larvas variaram de $1,80 \times 10^5$ até $3,9 \times 10^7$conídios / ml.

PALAVRAS-CHAVE: *Amblyomma cajennense*, *Beauveria bassiana*, *Metarhizium anisopliae*, fungos entomopatogênicos, controle microbiano.

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