Volumen 30, Nº 1. Páginas 93-99
IDESIA (Chile) Enero-Abril, 2012

Evaluation of entomopathogenic fungi as biological control agents *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae)

Evaluación de hongos entomopatógenos como agentes de control biológico para Rhipicephalus (Boophilus) microplus *(Canestrini, 1887) (Acari: Ixodidae)*

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ABSTRACT

The aim of this study was to evaluate in the laboratory different isolates and concentrations of conidia of *Metarhizium anisopliae* (Metschnikoff, 1879) Sorokin, 1883 and *Beauveria bassiana* (Balsamo) Vuillemim, 1912 for control of the cattle tick. The conidial suspensions were prepared from fungi grown in rice medium in polypropylene bags. The tests were performed by immersion of engorged females collected from animals not treated with acaricides. The experimental design was completely randomized with 20 treatments and 5 repetitions, each consisting of 5 females. The mortality rate caused by the fungus *M. anisopliae* ranged from 92 to 100%, while for *B. bassiana* it was 44 to 100%; mortality was greater at concentrations of 10^8 and 10^9 conidia mL^{-1} suspension. The isolates *B. bassiana* Fitossan 1 and *M. anisopliae* PL 43 (both at 10^9 conidia mL^{-1}) and Fitossan 4 (10^8 conidia mL^{-1}) within 14 days of treatment killed 100%, 92% and 88% of the engorged females, respectively. In general there was no difference in the weight of eggs from females treated with the same isolate at concentrations of 10^7, 10^8 and 10^9 conidia mL^{-1}, although isolates of *M. anisopliae* had lower weight. The efficiency of control ranged from 0% (control with water) to 31.30% (*M. anisopliae* Fitossan 4, 10^8 conidia mL^{-1}).

Key words: cattle tick, biological control, *Metarhizium anisopliae, Beauveria bassiana*.

RESUMEN

El objetivo de este trabajo fue evaluar diferentes colonias aisladas en concentraciones de Metarhizium anisopliae *(Metschnikoff, 1879) Sorokin, 1883 y de* Beauveria bassiana *(Balsamo) Vuillemim, 1912 para el control de la garrapata común del vacuno. La multiplicación de conidias fue realizada a partir de hongos inoculados en arroz precocido estéril. Los ensayos de inmersión fueron realizados sobre garrapatas adultas ingurgitadas o teleoginas colectadas de bovinos no tratados con acaricidas. El diseño experimental fue completamente al azar con 20 tratamientos y cinco repeticiones, siendo cada una de estas constituidas por cinco hembras. La mortalidad de las teleoginas ocasionada por* M. anisopliae *fue variable entre 92 a 100% y para* B. bassiana *este parámetro varió entre 44 a 100%, observándose que la mortalidad fue superior en las concentraciones de 10^8 y 10^9 conidias mL^{-1}, destacándose las cepas de* B. bassiana *Fitossan 1 e* M. anisopliae *PL 43 (ambos 10^9 conidias mL^{-1}) y Fitossan 4 (10^8 conidias mL^{-1}), que a los 14 días de tratamiento habían eliminado el 100, 92 y 88 % de las teleoginas, respectivamente. En general, no existió diferencia en relación al peso de los huevos provenientes de hembras tratadas con el mismo aislado en las concentraciones de 10^7, 10^8 y 10^9 conidia .mL^{-1}, aunque se obtuvieron pesos inferiores para las cepas de* M. anisopliae. *La eficiencia del control varió de 0% (control) a 31,30% (*M. anisopliae *Fitossan 4, 108 conidias.mL^{-1}).*

Palabras clave: *garrapata bovina, control biológico,* Metarhizium anisopliae, Beauveria bassiana.

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Fecha de Recepción: 28 Julio, 2009.
Fecha de Aceptación: 07 Marzo, 2012.

1. Introduction

In tropical and subtropical regions the tick *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae) is the most important ectoparasite in livestock farms, and is responsible for large economic losses (Athayde *et al*. 2001). As well as producing spoliation, the attack of this species damages the hide of the host and is the principal transmitter of pathogenic agents of cattle (Bittencourt *et al*. 1999).

The control of the parasitic stages of the tick *R. microplus* uses mainly chemical products applied directly on the animal (Wenzel *et al*. 2004). Currently, this type of control is producing a progressive increase in the resistance of this ectoparasite, and consequently an increase in the residues of acaricides in milk and meat (Mendes *et al.* 2007). Studies on *R. microplus* are currently searching for control strategies using a combination of the prudent and rational use of the available acaricides and other forms of control, in order to maintain the parasite populations under the economic damage limit and to produce the minimum environmental impact (FAO 2003). Alternative methods include the use of genetically resistant animals, development of vaccines, management of grasslands with alternation of species and biological control (Leal *et al*. 2003).

Entomological pathogens are considered to be an important factor in the reduction of pest species; these agents may occur naturally or may be introduced into the agroecosystems (Bahiense *et al*. 2007). The fungi *Metarhizium anisopliae* (Metschnikoff, 1879) Sorokin, 1883 (Ascomycota: Nectriaceae) and *Beauveria bassiana* (Balsamo) Vuillemim, 1912 (Ascomycota: Clavicipitaceae) are promising agents for the biological control of ticks, thus in recent years a number of studies have investigated the relationships between entomopathogenic fungi and different species of tick (Bittencourt *et al*. 1994, Bittencourt *et al*. 1996, Bittencourt *et al*. 1997, Correia *et al*. 1998, Bittencourt *et al*. 1999, Frazzon *et al*. 2000, Bahiense y Bittencourt 2004, Basso *et al*. 2005, Lopes *et al*. 2007, Bahiense 2007).

The objective of this study was to evaluate, using immersion tests, different isolates and concentrations of *M. anisopliae* and *B. bassiana* in terms of the mortality of engorged females, number of eggs laid, egg weight (mg), percentage eclosion and efficiency of the product (%).

2. Methods and Materials

The experiment was performed between November, 2006 and January, 2007 in the Laboratorio de Entomología of the Unidad Académica Centro de Ciencias Agrarias of the Universidad Federal de Alagoas, Brazil (9°27'S, 35°27'W, altitude 127 m). The mean temperature and mean relativity during the experiment were 26.6 ± 1° C and 66.5 ± 5%, respectively.

In the Beira Rio Farm, located in the city of Batalha (09°40'40"S, 37°07'29"W, altitude 120 m) we collected engorged females of from cattle of the Zebu breed, naturally infected and with no application of pesticides in more than 30 days. Ticks were collected from the pelvic, inguinal and auricular regions of the cattle, placed in Petri dishes and transported to the laboratory in thermal containers. To reduce the mobility and avoid premature oviposition of the engorged ticks, they were maintained in the laboratory at 3 ± 2º C until the beginning of the experiments.

The bioassays were based on the techniques described by Drummond (1973) and Campos Júnior & Oliveira (2005); engorged ticks collected no more than 24 hours earlier and in optimum conditions for oviposition were weighed and separated randomly into groups for the treatments. The fungal isolates Fitossan 1, Fitossan 2 and Fitossan 3 of *B. bassiana* were used at concentrations of 10^7, 10^8 and 10^9 conidia mL^{-1}); the isolates PL 43, UOD and Fitossan 4 of *M. anisopliae* were used at the same concentrations. We used two controls; distilled water + Tween-80 (Polyoxethylene Sorbitan) 0.01% and distilled water alone, for a total of 20 assays.

All the fungal isolates used in this study are part of the fungal culture library of the Fitossan Laboratory – Asistencia Fitosanitaria y Control Biológico, Ltda. The isolates Fitossan 1, Fitossan 2 and Fitossan 3 of *B. bassiana* were collected, respectively, from *Orthezia praelonga* Douglas, 1891 (Hemiptera: Orthezidae), *Castnia licus (*Drury, 1773) (Lepidoptera: Castniidae) and *Cosmopolites sordidus* (Germar, 1824) (Coleoptera: Curculionidae), while the isolates of *M. Anisopliae* were collected from *Mahanarva posticata* (Stal, 1855) (Hemiptera: Cercopidae).

For multiplication and thinning, the strains were cultivated for 7 days in Petri dishes with potato dextrose, the antibiotic (PDA+A) and Nujol® mineral oil (previously autoclaved at 120° C for 20

Table 1. Evaluation of the efficiency of control of different treatments on *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887).

Treatments	
Beauveria bassiana (conidia mL^{-1})	*Metarhizium anisopliae* (conidia mL^{-1})
Control	Control
Controle+ Tween 80	Controle + Tween 80
Fitossan 1 $(10)^9$	PL43 $(10)^9$
Fitossan 1 $(10)^8$	PL43 $(10)^8$
Fitossan 1 $(10)^7$	PL43 $(10)^7$
Fitossan 2 $(10)^9$	UOD $(10)^9$
Fitossan 2 $(10)^8$	UOD $(10)^8$
Fitossan 2 $(10)^7$	UOD $(10)^7$
Fitossan 3 $(10)^9$	Fitossan 4 $(10)^9$
Fitossan 3 $(10)^8$	Fitossan 4 $(10)^8$
Fitossan 3 $(10)^7$	Fitossan 4 $(10)^7$

minutes). Strains were transferred to similar Petri dishes and cultivated for 7 days in a BOD-type incubator at 26 ± 1° C with a 12h:12h light-dark cycle (Alves, 1998; Alves *et al.*, 1998). Conidial suspensions were prepared by adding 100 mL of distilled water and 0.01% Tween-80 (Polyoxethylene Sorbitan). Conidial densities were estimated in a Neubauer chamber under an optical microscope and then adjusted to concentrations of 10^7, 10^8 and10^9 conidia mL^{-1}.

The viability of the strains was evaluated using two Petri dishes with dextrose agar and antibiotic PDA+A for each treatment. With a Drigalsky spatula we added 0.1 mL of the suspensions of 10^7, 10^8 and 10^9 conidia mL^{-1}. After 24 hours, the percentage of germinated conidia was estimated using an optical microscope, observing 100 conidia per plate.

Engorged female ticks were washed with distilled water, dried on absorbent paper and immersed in 100 ml of fungal suspension for 10 minutes. Beginning 72 hours after treatment, we evaluated daily the percentage mortality, number of eggs, egg weight (mg), percentage eclosion and product efficiency (%). The percentage mortality (M) was analyzed according to Schneider & Orelli (Nakano *et al.* 1981) and the reproductive efficiency (RE) and product efficiency (PE) with the formula of Drummond (1973).

$$M = \frac{\%\text{ Mortality in a treatment} - \%\text{ Mortality in the control}}{100 - \%\text{ Mortality in the control}} \times 100$$

$$RE = \frac{\text{egg weight} \times \%\text{ eclosion} \times 20{,}000^{1}}{\text{Weight of females}}$$

$$PE = \frac{RE\text{ (control)} - RE\text{ (treatment)}}{RE\text{ (control)}} \times 100$$

The experimental design was completely randomized with 20 treatments and 5 repetitions. The data were analyzed by analysis of variance and Tukey *a posteriori* tests using the program Assistat 7.4 (Ferreira 2000).

3. Results and Discussion

The viability of the conidia of the isolates was greater than 95% for all experiments.

Mortality varied from 16% (water + Tween 80) to 100% (*B. bassiana* Fitossan 1 with 10^9 and 10^7 conidia mL^{-1}; *M. anisopliae* PL 43 with 10^9 and 10^8 conidia mL^{-1}; *M. anisopliae* UOD with 10^9 and 10^8 conidia mL^{-1} and *M. anisopliae* Fitossan 4 with 10^9, 10^8 and 10^7 conidia mL^{-1}) during the 21 days of observation (Table 2). Fitossan 1 at 10^9 conidia mL^{-1} produced 48% mortality of *B. bassiana* within 4 days and 100% mortality within 14 days, notably more than the other treatments. Treatment with PL 43 at 10^8 and 10^9 conidia mL^{-1} produced 60% and 44% mortality, respectively, after 11 days, while after 14 days PL 43 at 10^9 conidia mL^{-1} produced 92% mortality. These values were greater than the mean mortality of 16% and 24% obtained after 21 days in the controls with water + Tween 80 and water, respectively (Table 2), which demonstrates the pathogenic effect of these strains on engorged females of *R.* (*Boophilus*) *microplus*.

Overall the mortality of engorged females produced by *B. bassiana* was significantly less than that produced by *M. anisopliae*, comparing the controls with and without Tween 80 and *B. bassiana* Fitossan 2 (10^8 and 10^7 conidia mL^{-1}) and *B. bassiana* Fitossan 3 (10^7 conidia mL^{-1}) (Table 3). This difference may be related to the species and strains of the studied pathogens; these results are similar to those obtained by LOPES *et al.* (2007). The high activity of *M. anisopliae* was also found by Kaaya *et al.* (1996) for the species *Amblyomma variegatum* (Fabricius, 1794) (Acari: Ixodidae); these authors reported an adult mortality

[1] According to Campos Júnior (2005), there are about 20,000 cattle tick eggs in one gram.

Table 2. Mean[1] ± (PE) mortality (%) of engorged females, number of eggs oviposited, egg weight (g), eclosion (%) and efficiency of the product (%) in *Rhipicephalus* (*Boophilus*) *microplus* after treatment with isolates of *Beauveria bassiana* and *Metarhizium anisopliae*. Experimental conditions were temperature 26.6 ± 1 °C and RH 66.5 ± 5%.

Treatment	Mean[1] ± PE				
Beauveria bassiana (conidia mL^{-1})	Mortality	Oviposition	Eclosion	Egg weight (gr)	Efficiency of the product
Water	24.00 ± 10.95e	27.20 ± 0.58a	100.00 ± 0.00a	0.40 ± 0.58bcd	0.00 ± 0.00b
Water + Tween 80	16.00 ±11.66e	26.80± 0.73a	100.00 ± 0.00a	0.41 ±0.71bcd	9.42 ± 0.71ab
Fitossan 1 $(10)^9$	100.00 ± 0.00a	10.80 ± 0.49c	90.00 ± 6.12a	0.42 ± 0.71bcd	24.62 ± 0.71ab
Fitossan 1 $(10)^8$	96.00 ± 4,.0ab	14.20 ± 1.11bc	90.00 ± 6.12a	0.55 ± 0.51ab	14.74 ± 0.71ab
Fitossan 1 $(10)^7$	100.00 ± 0.00a	15.20 ± 1.28bc	100.00 ± 0.00a	0.63 ± 0.51a	2.04 ± 0.49b
Fitossan 2 $(10)^9$	84.00 ± 7.48abc	1740± 1.29b	90.00 ± 10.00a	0.64 ± 0.71a	12.77 ± 0.49ab
Fitossan 2 $(10)^8$	56.00 ± 9.80cd	18.60 ± 0.98b	100.00± 0.00a	0.64 ± 0.71a	1.01 ± 0.40b
Fitossan 2 $(10)^7$	64.00 ± 11.66bcd	19.80 ± 0.97b	100.00 ± 0.00a	0.66± 0.31a	5.40 ± 0.32ab
Fitossan 3 $(10)^9$	92.00 ± 14.70ab	19.40 ± 1.78b	85.00 ± 10.00a	0.63 ± 0.71a	20.42 ± 0.71ab
Fitossan 3 $(10)^8$	96.00 ± 4.00ab	18.00 ± 2.02b	100.00 ± 0.00a	0.65 ± 0.71a	7.81 ± 0.20ab
Fitossan 3 $(10)^7$	44.00 ± 4.90de	17.00 ± 0.95b	95.00 ± 5.00a	0.69 ± 0.51a	7.27 ± 0.51ab
Metarhizium anisopliae (conídia mL^{-1})					
PL43 $(10)^9$	100.00 ± 0.00a	15.00 ± 0.84bc	95.00 ± 5.00a	0.31 ± 0.71cd	31.60 ±0.71a
PL43 $(10)^8$	100.00 ± 0.00a	16.80 ± 1.01b	100.00 ± 0.00a	0.28 ± 0.71cd	23.35 ± 0.71a
PL43 $(10)^7$	96.00 ± 0.20ab	16.80 ± 0.66b	100.00 ± 0.00a	0.28 ± 0.71cd	24.02 ± 0.71ab
UOD $(10)^9$	100.00 ± 0.00a	19.00 ± 0.45b	100.00 ± 0.00a	0.41 ± 0.58bcd	14.88 ± 0.71ab
UOD $(10)^8$	100.00 ± 0.00a	17.60 ± 0.60b	100.00 ± 0.00a	0.38 ± 0.51bcd	7.84 ± 0.71ab
UOD $(10)^7$	92.00 ± 0.24ab	17.20 ± 1.32b	100.00 ± 0.00a	0.40 ± 0.37bcd	10.20 ±0.71ab
Fitossan 4 $(10)^9$	100.00± 0.00a	17.60 ± 0.51b	100.00 ± 0.00a	0.41 ± 0.71bcd	13.61± 0.71ab
Fitossan 4 $(10)^8$	100.00± 0.00a	14.40 ± 1.63bc	100.00 ± 0.00a	0.24 ± 0.71d	31.30 ± 0.71a
Fitossan 4 $(10)^7$	100.00 ± 0.00a	17.40 ± 1.02b	100.00 ± 0.00a	0.31 ± 0.71cd	13.04 ±0.71ab

[1] Means followed by the same letter in a column were not significantly different ($p > 0.05$) by Tukey's test.

of 37%, while *B. bassiana* did not cause mortality in the same period. Similar results were obtained by for engorged females of *Amblyomma cajennense* (Fabricius, 1787) (Acari: Ixodidae); the percentage control of the studied strains of *M. anisopliae* was superior to those of *B. bassiana*.

The mean number of eggs oviposited was greater for the two controls than for all the treatments. Fitossan 1 at 10^9 conidia mL^{-1} *B. bassiana* significantly reduced the oviposition, to 10.8. Egg weight varied from 0.24g (*M. anisopliae* Fitossan 4, 10^8 conidia mL^{-1}) to 0.69g (*B. bassiana* Fitossan 3, 10^7 conidia mL^{-1}), indicating that the isolates had different action on the females (Table 2). All the isolates of *M. anisopliae* in the three concentrations studied had lower egg weight, especially Fitossan 4 (10^8 conidia mL^{-1}), which weighed 0.24g (Table 2).

There were no significant differences in eclosion rate among the different treatments; the observed values ranged from 85% to 100%, the lowest value was obtained for engorged females with the isolate *B. bassiana* using Fitossan 3, 10^9 conidia mL^{-1}. According to Magalhães & Lima (1991), entomopathogenic fungi do not have the capacity to synthesize enzymes or produce proteases which could make eggs infertile or even retard their metabolism. Evaluating suspensions of *B. bassiana* on eggs and larvae of *R.* (*Boophilus*) *microplus*, Bittencourt *et al*. (1996) obtained a significant reduction and an increase in larval mortality.

The efficiency of the products evaluated varied from 0% to 31.60%; the treatments *M. anisopliae* Fitossan PL 43 (10^9 conidia mL^{-1}) and *M. anisopliae* Fitossan 4 (10^8 conidia mL^{-1}) produced the best results in the control of engorged females, since they had the highest efficiency (Table 2).

Comparing all the variables, *M. anisopliae* Fitossan 4 (10^8 conidia mL^{-1}), for the mortality produced in less time and for the efficiency in the control of *R.* (*Boophilus*) *microplus*, may be considered as the strain which produced the best result at lower concentrations.

Table 3. Cumulative mortality (%) of engorged females over time for the different treatments. Entomology Laboratory CECA, UFAL, Rio Largo, AL. Experimental conditions were temperature 26.6 ± 1 °C and RH 66.5 ± 5%.

Treatment	Cumulative mortality (%)					
	Days after treatment					
	4	7	11	14	18	21
Water	0	7	0	0	12	24
Water + Tween 80	0	7	0	4	4	16
Beauveria bassiana (conidia mL^{-1})						
Fitossan 1 $(10)^9$	48	68	84	100	100	100
Fitossan 1 $(10)^8$	0	8	60	84	96	96
Fitossan 1 $(10)^7$	0	0	52	60	88	100
Fitossan 2 $(10)^9$	4	4	12	44	84	84
Fitossan 2 $(10)^8$	0	8	12	28	48	56
Fitossan 2 $(10)^7$	0	0	8	32	44	64
Fitossan 3 $(10)^9$	4	8	16	20	88	92
Fitossan 3 $(10)^8$	4	12	16	20	76	96
Fitossan 3 $(10)^7$	4	4	8	16	28	44
Metarhizium anisopliae (conidia mL^{-1})						
PL43 $(10)^9$	4	4	44	92	92	100
PL43 $(10)^8$	0	0	60	64	96	100
PL43 $(10)^7$	0	0	8	56	92	96
UOD $(10)^9$	0	0	16	16	92	100
UOD $(10)^8$	0	12	36	56	68	100
UOD $(10)^7$	4	4	16	36	64	92
Fitossan 4 $(10)^9$	0	0	0	44	100	100
Fitossan 4 $(10)^8$	4	4	16	88	92	100
Fitossan 4 $(10)^7$	0	8	36	44	80	100

Bahiense *et al*. (2007) suggested that lower percentages of control are expected with biological control methods than with methods of chemical control. It is important to note that this difference is compensated, since the objective of biological control is to maintain pests at economically acceptable levels, while protecting the environment and the native enemies of a given pest.

Recently it has become more urgent to develop new methods of microbial control of pests of veterinary importance, since the results so far indicate the high pathogenic potential of entomopathogens to control organisms considered as key pests. Many of these biological agents may increase the efficiency of control in combination with chemical methods due to additive or synergistic effects, thus obtaining a significant reduction in the use of chemical acaricides (Leal *et al*. 2003). Wenzel *et al*. (2004) concluded that the products Butox® (deltametrina), Ectomin® (cipermetrina) and Triatóx® (amitraz), in the doses recommended by the manufacturers, are compatible with strain IBCB 66 of *B. bassiana* for the control of *B. microplus*.

The population growth of entomopathogenic fungi in an environment depends, among other factors, on the climatic conditions and the type of grass utilized; these factors may have significant importance in the success of biological control (Gauss & Furlong 2002).

Acknowledgements

We thank FINEP/SECT/FAPEAL/CNPq for the financial support of this research and for providing student scholarships. We thank the Phytossan Laboratory, (Assistência Fitossanitária e Controle Biológico Ltda) located in Ingenio Sinimbu, in Jequié da Praia, AL, for providing the fungal strains and cooperating in the research.

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