EVALUATION OF THE PREDATORY CAPACITY OF THE FUNGI *Arthrobotrys robusta* AND *Monacrosporium thaumasium* SUBMITTED TO DIFFERENT PRESERVATION METHODS AGAINST GASTROINTESTINAL PARASITIC NEMATODES OF BOVINES

Avaliação da capacidade predatória dos fungos *Arthrobotrys robusta* e *Monacrosporium thaumasium* sobre nematóides gastrintestinais parasitos de bovinos, submetidos a diferentes métodos de preservação

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SUMMARY: Biological control of helminth parasites of livestock is an alternative to the anti-helminthic use and has the principal objective of reducing the numbers of infective larvae on pasture. The nematophagous fungi produce structures that trap and kill the pre-parasitic stages of nematodes. The continuous maintenance of predatory activity in fungal isolates is one of the basic prerequisites for the success of this control method. In this study *in vitro* predation of nematodes by isolates I31 of *Arthrobotrys robusta* and NF34a of *Monacrosporium thaumasium* were evaluated, after these had been submitted to storage at 4°C, cryopreservation with or without the addition of DMSO or glycerol or maintenance in silica gel. Storage of NF34a at 4°C or freezing with the addition of DMSO were the treatments for which greatest reduction of infective larvae was obtained. No difference was seen between the *in vitro* predatory capacity of the I31 isolate stored at 4°C and those when frozen with DMSO or glycerol. Maintenance of the isolates in silica gel and freezing without cryoprotection reduced the predatory capacity to a similar extent, although the numbers of infective larvae recovered from cultures after both treatments were significantly lower than those seen in the control group.

KEY WORDS: biological control, nematophagous fungi, gastrointestinal helminth parasites of bovines, preservation of microorganisms.

INTRODUCTION

Parasitism by gastrointestinal nematodes is considered to be one of the major obstacles to the development of cattle raising (MACRAE, 1993). The high cost of chemical treatments and the appearance of cases of anti-helminthic resistance provide incentives to look for alternative ways to the control these infections (WALLER & FAEDO, 1996). The predatory fungi are saprophytic organisms, widely encountered in nature, that possess a capacity to produce traps along their hyphae, allowing them to capture nematodes (DIJKSTERHUIS et al., 1994). Development of these structures is triggered by limiting nutritional conditions or even by the presence of excreta produced by the nematodes in substrate (BALAN & GERBER, 1972). The fungi adhere firmly to the cuticle by means of a substance rich in acid phosphatase and then initiate penetration and digestion of the nematode internal structures (VEEHNIUS et al., 1995). The fungal species *Arthrobotrys robusta* and *Monacrosporium thaumasium* predate larvae of gastrointestinal helminth parasites *in vitro* (ARAÚJO et al., 1992, CHARLES et al., 1995, MENDONZA de GIVES et al., 1992, RODRIGUES et al., 2001). Although choice of the ideal species for the control of nematode parasites of livestock cannot be based solely on the results of *in vitro* assays, these may reveal which isolates have the potential to reduce the parasite population *in vivo* after passage through the gastrointestinal tract (LARSEN, 1999). To guarantee the stability of the isolates, methodologies

for extended preservation of selected characteristics of these organisms should be studied (SMITH & ONIONS, 1994). The objective of this study was to evaluate the effects of five preservation methods on the capacity of the isolates I31 of *A. robusta* and NF34a of *M. thaumasium* to predate larvae of bovine parasites.

MATERIAL AND METHODS

Culture of infective larvae of gastrointestinal nematodes

Infective larvae of the nematode genera *Cooperia* and *Haemonchus* were obtained from coprocultures made with faeces of naturally infected bovines (GUIMARÃES, 1971). Larvae were recovered from coprocultures and quantified (ROBERTS & O'SULLIVAN, 1950).

Maintenance of Fungal Cultures in Maize Agar Extract at 4°C

Samples of the isolates I31 of *A. robusta* and NF34a of *M. thaumasium* were maintained in five culture tubes containing 2% maize agar extract (MAE) and stored in the dark at 4°C. The cultures for renewed after three intervals of six months each.

Maintenance of Fungal Cultures in Silica Gel

Samples of the isolates I31 of *A. robusta* and NF34a of *M. thaumasium* originating from culture tubes filled with 2% MAE at 25°C were inoculated into YPSSA sporulation medium (4g yeast extract; 1g K_2HPO_4; 0.5g $MgSO_4.7H_2O$; 20g soluble amide, 20g agar; 1l distilled water). After seven days, a 5% solution of sterilised skimmed milk was added to the surface of each plate on 1 cm^2 pieces of filter paper. The pieces of paper thus moistened with a suspension of fungal material were immediately transferred to five flasks containing grains of sterile silica gel. The samples were stored at 4°C in the absence of light and humidity. After 18 months the cultures were recuperated by transferring pieces of paper from the flasks with silica to the surface of Petri dishes containing 2% MAE and maintaining the dishes at 25°C.

Maintenance of Fungal Cultures in Liquid Nitrogen (–196°C)

Samples of the isolates I31 of *A. robusta* and NF34a of *M. thaumasium* originating from culture tubes filled with 2% MAE at 25°C, were transferred to YPSSA medium. After seven days, discs from the periphery of the colony were distributed among 30 cryotubes each containing one of three different treatments: 10 filled with 1.5 ml of 10% glycerol solution, 10 containing 1.5 ml of 10% dimethyl sulphoxide (DMSO) solution and 10 containing 1.5 ml of distilled water. The process was carried out in two stages: slow freezing (-1°C/h), until the samples reached a temperature de -20°C and then direct immersion in liquid nitrogen and storage at -196°C. After 18 months the samples were thawed in a water bath at 37°C and sown onto Petri dishes containing 2% MAE maintained at 25°C.

Predatory activity of isolates I31 and NF34a against infective larvae of nematode parasites of bovines

Assays of predation were carried out in Petri dishes 5 cm in diameter filled with 2% AA. Eleven groups were set up, each with five replicates. A disc of culture from isolate I31 or NF34a that had either been stored at 4°C, preserved in silica gel or cryopreserved with DMSO, glycerol or without cryoprotectant was placed on the agar surface of each dish. A control group was also set up, in which no fungal material was inoculated onto the plates. Approach five hundred infective larvae of the nematode genera *Cooperia* and *Haemonchus* were added to each dish. After seven days, infective larvae that had not been predated were recovered using a Baermann funnel and quantified by examination of 20ml aliquots of the initial samples and extrapolating the counts to the total volume. The predation rate was estimated by comparing the number of larvae recovered from the treated and control groups using the following formula:

$$\% \text{ Reduction} = \frac{X_t - X_c}{X_c}$$

Where: X_c= mean no. of larvae recovered from control; X_t = mean no. of larvae recovered from the treatment group

Statistical analysis

Mean numbers of infective larvae recovered at the end of the experiment by Baermann's method and percentage reductions of larval populations by each fungal species after exposure to the different treatments were submitted to ANOVA. They were then compared by Tukey's test, using the SAEG statistical package developed by the Universidade Federal de Viçosa.

RESULTS

Predation rates of the isolates I31 of *A. robusta* and NF34a of *M. thaumasium* on infective larvae of gastrointestinal nematode parasites are shown in Fig. 1. Samples of NF34a stored at 4°C and those frozen after addition of DMSO were more efficient in predating larvae than those subjected to the other treatments ($p < 0.05$), giving a percentage reduction of 92.5%. Preservation of I31 whether stored at 4°C, frozen with DMSO or preserved with glycerol did not differ significantly among each other at $p < 0.05$, presenting percentage reductions compared to the control group of 88.99%; 86.92% and 85.06% respectively.

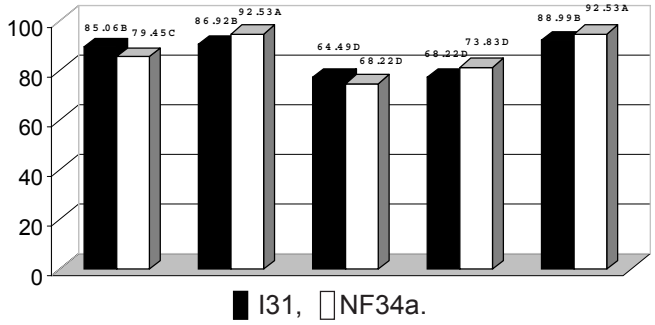

Figure 1. Percent reduction of mean of infective larvae of gastrointestinal nematode parasites recovered after seven days interaction with fungi *A. robusta* (I31) and *M. thaumasium* (NF34a) submitted to different preservation methods. Percents means followed by the same letter do not differ statistically by Tukey test ($p<0.05$).

Preservation of the I31 and NF34a samples in silica gel and those frozen without cryoprotection did not differ significantly at $p < 0.05$. These were the treatments from which the greatest numbers of infective larvae were recovered (Table 1). However the quantity of live nematodes recovered from these groups was much less than that obtained from the control ($p < 0.05$).

Table 1. Mean of infective larvae of *Cooperia spp.* and *Haemonchus spp.* recovered using a Baermann funnel after seven days of interaction with fungi *A. robusta* (I31) and *M. thaumasium* (NF34a) submitted to storage at different methods of preservation.

Fungi	Preservation methods				
	Freezed + Glycerol	Freezed + DMSO	Freezed without cryoprotector	Silica gel	4°C
I31	53.28^{B}	46.62^{B}	126.64^{D}	113.32^{D}	39.26^{B}
NF34a	73.28^{C}	26.64^{A}	113.32^{D}	93.32^{D}	26.64^{A}
Control	356.66^{E}				

Means followed by the same letter do not differ statistically by Tukey test ($p<0.05$).

DISCUSSION

Predation *in vitro* by *M. thaumasium* isolates of nematodes has already been demonstrated against larvae of the genera *Cooperia* and *Haemonchus* (GOMES et al., 1999). The results of tests using *Arthrobotrys* and *Cooperia* species showed *A. robusta* to be effective in diminishing larval populations of the parasite (GOMES, et al., 2001). In the present study, both the I31 and NF34a isolates maintained their predatory capacity on infective larvae of nematode gastrointestinal parasites even after storage under different preservation methods.

There was no difference between storage at 4°C and freezing with addition of either of the cryoprotectants for the I31 isolate samples. Maintenance of NF34a at 4°C and freezing with DMSO were the treatments that least affected the predatory capacity of this isolate, however freezing with glycerol did produce a reduction. Although DMSO and glycerol belong to the same class of cryoprotectants, they possess different modes of action, explaining the differences in predation rates between NF34a samples frozen with either of these agents (BROWN & GILBERT, 1995). Cryopreservation with the addition of glycerol has already been related to slow growth and a fall in the fungal sporulation rate, although these phenomena were observed after the addition of higher concentrations than those tested in the experiments (SANDSKÄR & MAGALHÃES, 1994).

Slow freezing and rapid thawing were unable to prevent cellular injury to the samples frozen in water alone, demonstrating that the addition of cryoprotectants is indispensable for adequate preservation. During freezing of the samples, the absence of DMSO, glycerol or any other cryoprotectant may result in breakdown of the osmolarity of the fungal cell membrane. Cryoprotectants act by reducing the formation of ice crystals inside the cells and impeding excessive cellular dehydration (SMITH et al., 1986). A combination of these two phenomena may have reduced recoverability of samples from both isolates I31 and NF34 frozen without cryoprotection.

Storage of filamentous fungi in silica gel is the most practical of the methods tested, due to its low cost and proven effectiveness in preserving isolates for extended periods (GENTLES & SCOTT, 1979, SHARMA & SMITH, 1999). However in the present study it produced a significantly greater loss in predatory capacity than the other treatments. Only resistant structures are preserved by this method of preservation, mycelium and thin-walled spores being unable to resist the stress of water removal from their cellular constituents (SMITH & ONIONS, 1994). Despite causing alterations in the initial characteristics of fungi, preservation in silica gel continues to be recommended, especially when there is no available source of liquid nitrogen for cryopreservation (DHINGRA & SINCLAIR, 1995).

Storage of the samples in silica gel and freezing without cryoprotection reduced the predatory capacity of the isolates, although these were still able to reduce markedly the population of infective larvae in relation to the control group. Storage of samples at 4°C was the preservation method that yielded the best percentage reductions of the parasite population. This method would also be feasible for culture maintenance and causes least fungal stress. Recuperation of the isolates is easy and yields rapid results with respect to the initial characteristics of the samples. However, the appearance of contamination due to continuos subculturing is common

and may induce loss of pathogenicity after several generations (SMITH & ONIONS, 1994).

RESUMO

O controle biológico de parasitos gastrintestinais de bovinos é uma alternativa ao uso de anti-helmínticos e tem como principal meta reduzir o número de larvas infectantes disponíveis no ambiente. Os fungos nematófagos produzem estruturas capazes de capturar e matar os estágios pré parasitários dos nematóides. A manutenção contínua da atividade predatória dos isolados fúngicos selecionados é fundamental para o sucesso desta forma de controle. Neste experimento avaliou-se a capacidade predatória *in vitro* dos isolados I31 de *Arthrobotrys robusta* e NF34a de *Monacrosporium thaumasium*, sobre larvas infectantes de *Cooperia spp. e Haemonchus spp.*, após armazenamento em 4°C, criopreservação com e sem adição de DMSO, glicerol e mantidos em sílica-gel. O armazenamento de NF34a a 4°C ou congelamento com adição DMSO foram os tratamentos em que se obteve a maior redução das larvas infectantes. Não foi observada diferença na capacidade predatória do isolado I31 armazenado a 4°C e quando congelado com adição de DMSO ou glicerol. A manutenção dos isolados em sílica-gel e o congelamento sem crioproteção foi responsável pela diminuição da capacidade predatória dos isolados, porém o número de larvas recuperadas nestes tratamentos foi inferior ao observado no controle.

PALAVRAS-CHAVE: Controle biológico, fungos nematófagos, helmintos gastrintestinais parasitos de bovinos, preservação de microorganismos.

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