EFFECTS OF TEMPERATURE, MINERAL SALT AND PASSAGE THROUGH THE GASTROINTESTINAL TRACT OF CALVES ON SODIUM ALGINATE FORMULATION OF *ARTHROBOTRYS ROBUSTA*, A NEMATODE-TRAPPING FUNGUS

J.V. ARAÚJO; M.A. STEPHANO & W.M. SAMPAIO

Departamento de Veterinária, Universidade Federal de Viçosa, CEP 36571-000, Viçosa-MG, Brazil, E-mail: jvictor@mail.ufv.br

SUMMARY: Experiments were performed to determine whether the nematode-trapping fungus *Arthrobotrys robusta* is able to survive encapsulation in sodium alginate, and the effect of temperature and mineral salt and if this formulation is able to pass through the gastrointestinal tract of calves without loss of viability, to predate infective *Haemonchus placei* larvae. Pellets of sodium alginate were treated with paraffin, mineral salt or without these elements. The pellets were put in Erlenmeyers flasks of 250 ml at 4°C, room temperature, 25°C, 30°C and 35°C. Every week, for 16 weeks, one pellet was put in the center of 8.5 cm Petri dish containing 20 ml of 2% Potato Dextrose agar and radial growth was followed for 7 days. Four housed male Holstein x Zebu calves, six months old, were randomly separated. Animal 1 - received orally 300 g of fungal pellets with paraffin; Animal 2 - received orally 300 g of fungal pellets without paraffin; Animal 3 - received orally 300 g of fungal pellets without paraffin and fungi and Animal 4 – received orally 300 g of pellets with paraffin and without fungi. The treatment of fungal pellets without paraffin at 4°C was the best ($P < 0.01$) inducing higher growth than those with paraffin in all temperatures, as well as those without mineral salt ($P < 0.01$). The isolation of fungi from the feces varied from 18 to 72 hours after the oral administration, with a peak mainly at the 18th hour.

KEY WORDS: Biological control, nematode-trapping fungi, *Arthrobotrys robusta,* calves, *Haemonchus placei.*

INTRODUCTION

Non-chemotherapeutic approaches to nematode parasite control of livestock are no longer largely of academic interest. Alternatives, or at least supplements, to anthelmintic control of nematode parasites are absolutely necessary. To this end, significant advances have recently been made in the development of ruminant vaccines against parasites (MEEUSEN, 1996), in the breeding of animals for parasite resistance (WOOLASTON & BAKER, 1996) and in the biological control of parasites, particularly by exploiting nematophagous fungi (ARAÚJO, 1996).

Fungi antagonistic to nematodes consist of a wide variety of organisms including nematode-trapping or predatory fungi, endoparasitic fungi, parasites of nematode eggs and cysts, and those producing nematotoxic metabolites. It is notable that fungi belonging to highly divergent orders and families occur in each of the above groups (MANKAU, 1980).

The predatory group, which includes the genera *Arthrobotrys* and *Monacrosporium*, produce an extensive system of hyphae in the environment, carryng organs able to capture living nematodes (BARRON, 1977).

The fungi inhabit a wide variety of substrates including soil, moulds, animal dung decaying plant material, roots plant and others. The most practical use of nematophagous fungi in the control of animal parasitic nematodes is by means of the oral administration of fungal material. After this passes through the gastrointestinal tract of animals and is eliminated together with the feces into the environment, the fecal material is colonized by these fungi and a close contact between the recently hatched larvae and the fungi takes place promoting the production of traps and capture and death of the nematodes. However, most nematode-trapping fungi are very sensitive to destruction by the severe conditions of the gastrointestinal tract. The final challenge is to develop an efficient, low cost means of scaling up production of fungal material and to develop a formulation to satisfy industrial needs for commercial exploitation of this technology.

The present study was designed to determine whether the nematode-trapping fungus *Arthrobotrys robusta* is able to

survive encapsulation in alginate, the effects of temperature, mineral salt and whether this formulation can pass through the gastrointestinal tract of calves without a loss of viability to prey on nematodes.

MATERIALS AND METHODS

One isolate of the nematode-trapping fungus *Arthrobotrys robusta* was obtained from Brazilian soil and kept in small flasks containing 2% Potato-Dextrose-Agar (2% PDA) at 4°C.

Two Holstein calves, 100 kg of live weight each and free of helminths, were given with 1,000 infective *Haemonchus placei* larvae per kg of body weight. After 30 days, and for a period of one month thereafter, daily fecal samples from the animals were obtained and mixed with along with sterile charcoal and kept at 26°C for two weeks. Larvae were recovered in a Baermann apparatus, with water at 42°C, washed three times, with 0.9% NaCl physiological solution and stored at 4°C for one to two months. To obtain *H. placei* larvae free of bacteria and fungi, they were washed ten times in distilled water by centrifugation at 1,000 rpm for five minutes. The larvae were stored at 4°C for one week in a solution containing 0.05% of streptomycin sulfate, 0.01% of chloramphenicol and 0.05% of amphotericin B and washed twice with distilled water. Before their use, they were held at 25°C in day light for six hours, and their viability checked under a stereoscopic microscope.

Mycelium was grown in the liquid medium of KADO & HESKET (1970), after 7 days of incubation at 25°C in the dark. Sodium alginate pellets were made as described by WALKER & CONNICK (1983) and modified by LACKEY *et alli.*, (1993). A part of pellets was treated with paraffin in their surface and 100 g of mineral salt was added for 7.5 g of pellets. They were weighed and divided in 7.5 g lots and put in Erlenmeyer flasks of 250 ml at 4°C, room temperature, 25°C, 30°C and 35°C. Every week, for 16 weeks, one pellet was put in the center of 8.5 cm Petri dish containing 20 ml of 2% PDA and radial growth was followed for 7 days.

Four housed male Holstein x Zebu calves, six months old, were randomly separated:

- Animal 1 - received orally 300 g of fungal pellets with parafin.
- Animal 2 - received orally 300 g of fungal pellets without parafin.
- Animal 3 - received orally 300 g of pellets without parafin and fungi.
- Animal 4 - received orally 300 g of pellets with parafin and without fungi.

Five days before and five days following administration of the pellets, each animal was fed daily a special autoclaved ration of 1 kg of crushed corn and 3 kg of *Pennisetum purpureum* grass to control other nematodes and fungi.

Fecal samples were collected from each animal at 15, 18, 21, 24, 48, 72, 96 and 110 hours after the administration of the pellets. Two grams of feces were removed from these samples and added to 9 cm Petri dishes containing 2% wateragar at 25°C, in the dark. These dishes were baited with 1,000 infective *H. placei* larvae, and the amount of the conidiophore growth was observed daily. Counting was performed at 24 h intervals from the time when the material was placed on the Petri dishes, during 12 days. These assays were repeated three times and analized by the minimal significant difference test at the level of ($P < 0.01$).

RESULTS AND DISCUSSION

Figures 1, 2, 3, 4 and 5 show the radial growth of mycelia from pellets with paraffin, and without paraffin, at 4°C, room temperature, 25°C, 30°C and 35°C, respectively. The treatment of pellets without paraffin at 4°C was the best ($P < 0.01$), inducing higher growth than those with paraffin at all temperatures, as well as pellets without mineral salt ($P < 0.01$). Mycelia growth occurred in all temperatures up to 16 weeks. Figure 6 shows the number of conidiophores in the fecal samples after administration of pellets without paraffin containing *Arthrobotrys robusta*. Twelve days after the administration of infective *Haemonchus placei* larvae on the dishes, no larvae free from the fungal trapping were observed. No fungal growth was found in the other fecal samples belonging to the calf after the administration of the pellet with paraffin. There was no fungal growth in the

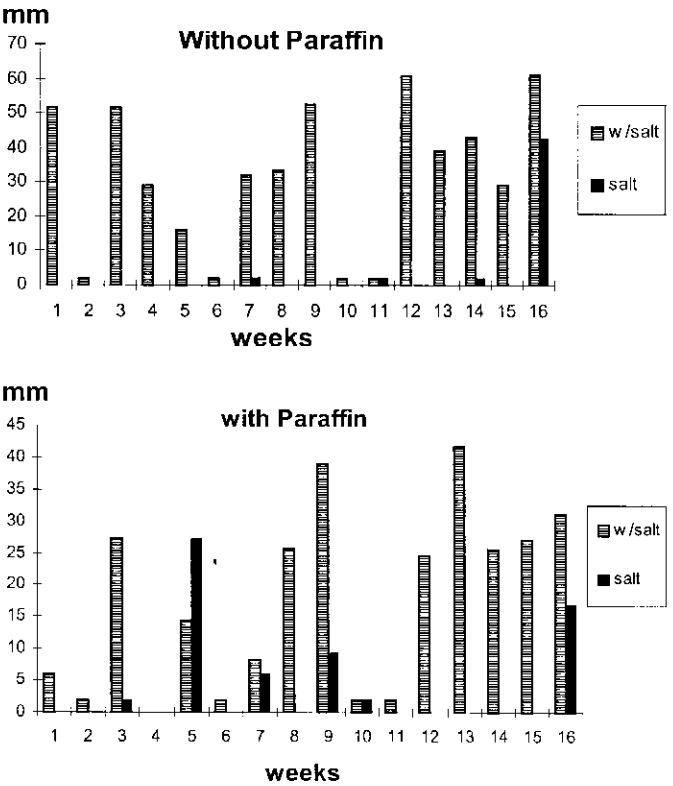

Fig. 1. Radial growth of the mycelia from pellets with *Arthrobotrys robusta* without paraffin, with mineral salt (salt) or without mineral salt (w/salt) (above) and with paraffin, with mineral salt (salt) and without mineral salt (w/salt) (below) at 4°C, during 16 weeks.

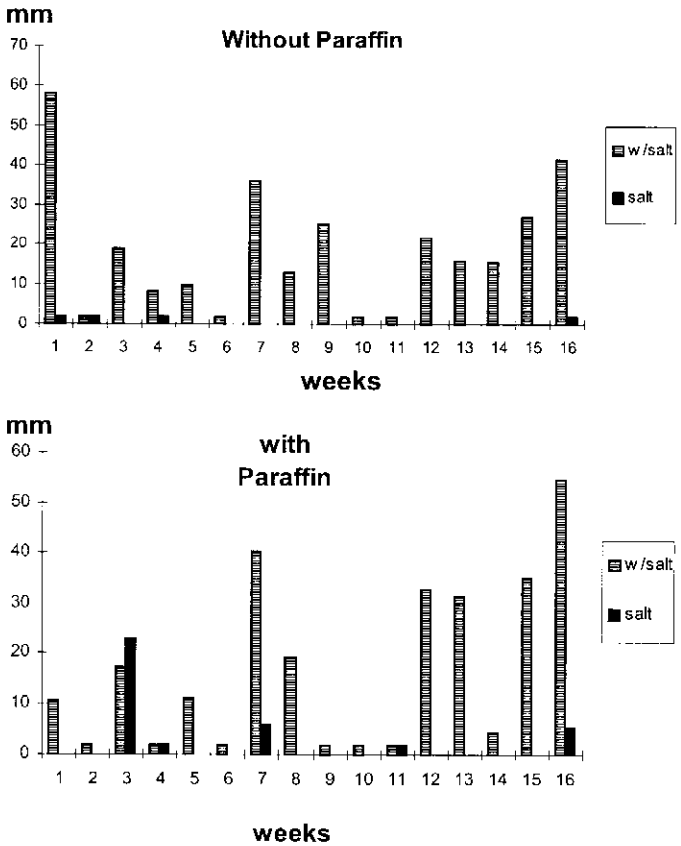

Fig. 2. Radial growth of the mycelia from pellets with *Arthrobotrys robusta* without paraffin, with mineral salt (salt) or without mineral salt (w/salt) (above) and with paraffin, with mineral salt (salt) and without mineral salt (w/salt) (below) at room temperature, during 16 weeks.

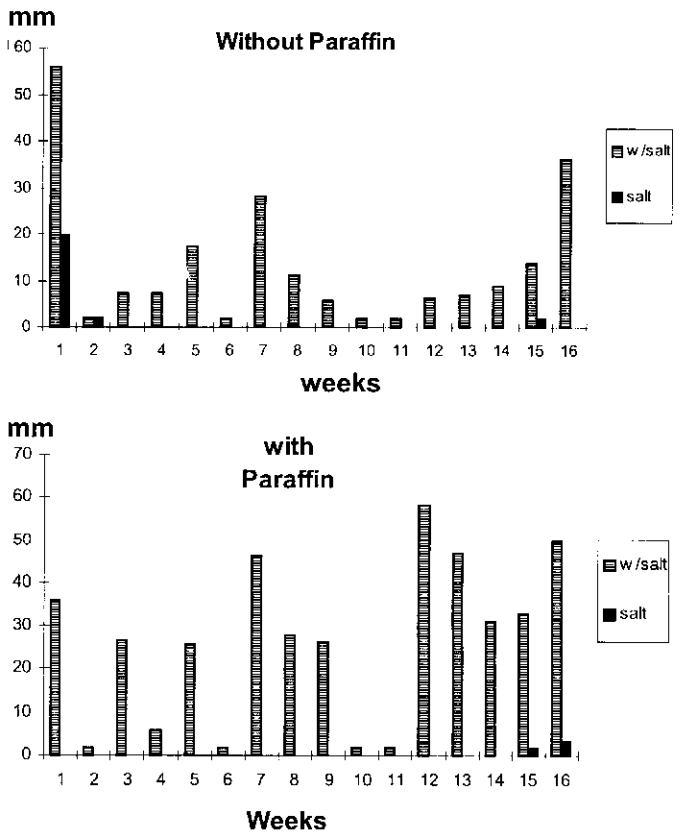

Fig. 3. Radial growth of the mycelia from pellets with *Arthrobotrys robusta* without paraffin, with mineral salt (salt) or without mineral salt (w/salt) (above) and with paraffin, with mineral salt (salt) and without mineral salt (w/salt) (below) at 25°C, during 16 weeks.

dishes containing feces of the calves used as Control (Groups 3 and 4).

These results demonstrate the importance of screening nematode-trapping fungi according to their ability to passage through the gastrointestinal tract before administering them in biocontrol. HASHMI & CONNAN (1989) were first to mention this passage after performing oral administration of *A. oligospora* conidia to calves. Further, LARSEN *et alli.*, (1992) achieved this goal using isolates of *A. oligospora, A. superba* and *Duddingtonia flagrans* as well as GRONVOLD *et alli.*, (1993), WOLSTRUP *et alli.*, (1994), NANSEN *et alli.*, (1995) using *D. flagrans and* ARAÚJO *et alli.*, (1996) using *A. robusta.*

WALLER *et alli.*, (1994) demonstrated *in vivo* that the average time spent in the passage of conidia through the digestive system of ovines, after oral administration, is about 24 hours. In the present study, this time varied from 18 to 72 hours after oral administration of fungi in calves, with peak isolation mainly at the 18th hour (Figure 6). It is very important to screen nematophagous fungi at a local level because they can be effective, or even be reatreated to certain ecological niches. Future experiments will demonstrate whether the fungus isolates used here can be used for biological control of gastrointestinal nematodes of grazing calves under natural conditions. According to LARSEN *et alli.*, (1991) the major problem in the use of nematophagous fungi as biocontrol agents is the deposition of the fungal material in dung pats where the entrapment of the parasite larvae should take place. The most obvious possibility would be to add nematophagous fungi to the alimentary tract without loss of viability. The present study aimed at selecting a formulation of *Arthrobotrys robusta* as detailed above.

The final challenge is to develop an efficient, low cost method of scaling up production of fungal material to satisfy industrial needs for commercial exploitation of this technology. This formulation of the fungi proved to be a powerfull tool to be used for biological control of gastrointestinal nematodes of grazing calves under natural conditions.

SUMÁRIO

Experimentos foram realizados para determinar se o fungo predador de nematódeos *Arthrobotrys robusta* sobrevive ao encapsulamento em alginato de sódio, efeito de temperatura, sal mineral e se esta formulação é capaz de passar através do trato gastrintestinal de bezerros sem haver perda de viabilidade para

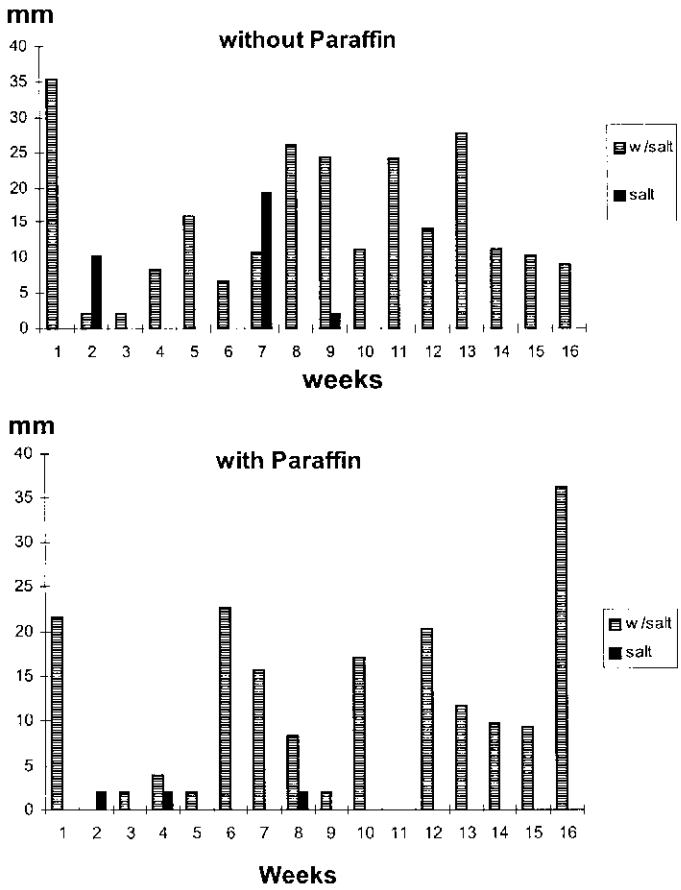

Fig. 4. Radial growth of the mycelia from pellets with *Arthrobotrys robusta* without paraffin, with mineral salt (salt) or without mineral salt (w/salt) (above) and with paraffin, with mineral salt (salt) and without mineral salt (w/salt) (below) at 30°C, during 16 weeks.

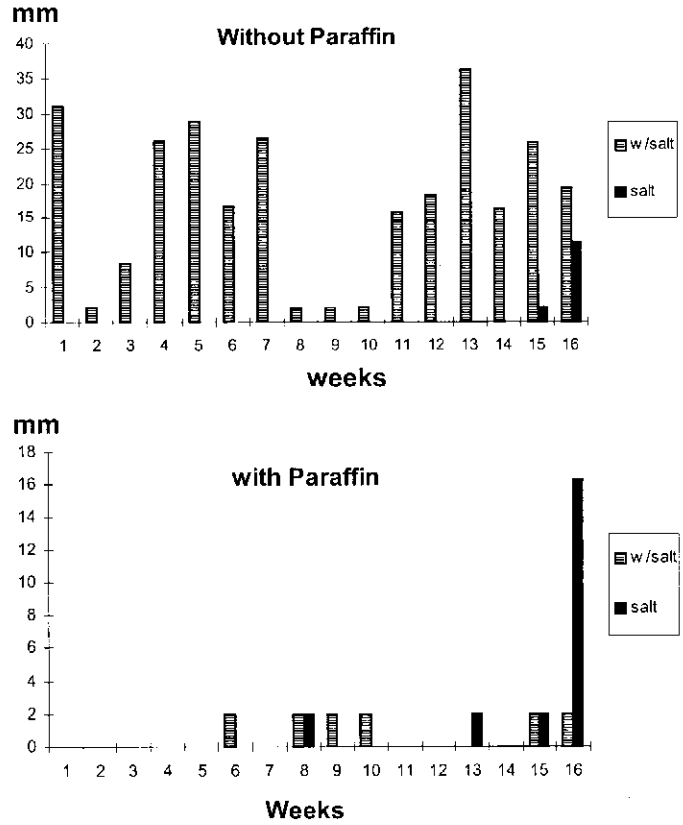

Fig. 5. Radial growth of the mycelia from pellets with *Arthrobotrys robusta* without paraffin, with mineral salt (salt) or without mineral salt (w/salt) (above) and with paraffin, with mineral salt (salt) and without mineral salt (w/salt) (below) at 35°C, during 16 weeks.

predar larvas infectantes de *Haemonchus placei*. "Pellets" de alginato de sódio foram tratados com parafina, sal mineral e sem esses elementos. Eles foram colocados em frascos Erlenmeyers de 250 ml a temperatura de 4°C, ambiente, 25°C, 30°C e 35°C. Semanalmente, por 16 semanas, um "pellet" foi colocado no centro de placas de Petri de 8,5 cm contendo 20 ml de batata dextrose agar a 2% e o crescimento radial foi medido durante 7 dias. Quatro bezerros machos, holandês x zebu, de seis meses de idade, foram aleatoriamente separados em quatro grupos de um animal cada: Grupo 1 - o animal recebeu 300 g de "pellets" do fungo com parafina por via oral; Grupo 2 - O bezerro recebeu 300 g de "pellets" do fungo sem parafina por via oral; Grupo 3 - O animal recebeu 300 g de "pellets" sem parafina e sem fungo por via oral e Grupo 4 - O bezerro recebeu 300 g de "pellets" com parafina e sem fungo por via oral. O tratamento de "pellets" fúngicos sem parafina a temperatura de 4°C foi o melhor ($P < 0,01$). Os "pellets" sem parafina induziram maior crescimento do que "pellets" com parafina em todas as temperaturas assim como os "pellets" sem sal mineral ($P < 0,01$). O isolamento de fungos das fezes variou de 18 a 72 horas depois da administração oral, principalmente na 18ª hora.

PALAVRAS-CHAVE: Controle Biológico, fungo predador de nematóides, *Arthrobotrys robusta*, nematódeo, bezerros, *Haemonchus placei*..

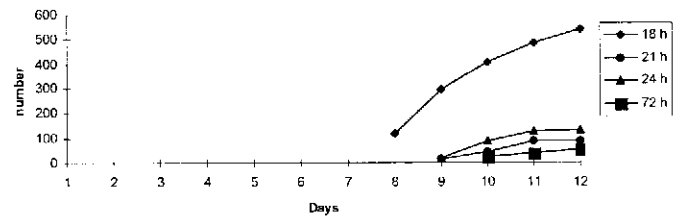

Fig. 6. The number of conidiophores of *Arthrotrys robusta* which apperead on 2% wateragar after a administration of fungal pellets of sodium alginate without paraffin.

REFERENCES

ARAÚJO, J.V. (1996). Interação entre larvas infectantes de *Cooperia punctata* e fungos predadores do gênero *Arthrobotrys*, caracterização de isolados de *Arthrobotrys* e seu uso no controle biológico de nematódeos parasitos gastrintestinais de bovinos. 110p. [Tese de Doutorado em Parasitologia/Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais].

ARAÚJO, J. V., NETO, A. P. & AZEVEDO, M. H. F. (1996). Screening parasitic nematode-trapping fungi *Arthrobotrys* for passage through the gastrointestinal tract of calves. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 48:* 543-552.

BARRON, G. L. (1977). *The Nematode-Destroying Fungi.* Canadian Biological Publications, Canada: Guelph, 140.

GRONVOLD, J., WOLSTRUP, J. & LARSEN, M. (1993). Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. *Journal of Helminthology. 67*: 31-36.

HASHMI, H. A. & CONNAN, R. M. (1989). Biological control of ruminant Trichostrongylids by *Arthrobotrys oligospora,* predacious fungus. *Parasitololology Today.* 5: 28-30.

KADO, C. I. & HESKET, M. G. (1970). Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas* and *Xanthomonas. Phytopathology. 60*:969-976.

LACKEY, B.A., MULDOON, A. E. & JAFFE, B.A. (1993). Alginate pellet formulation of *Hirsutella rossiliensis* for biological control of plant-parasitic nematodes. *Biological Control. 3*: 155-160.

LARSEN, M., WOLSTRUP, J. & HENRIKSEN, S. A. (1992). *In vivo* passage through calves of nematophagous fungi selected for biocontrol of parasitic nematodes. *Journal of Helminthology. 66*: 137-141.

LARSEN, M., WOLSTRUP, J. & HENRIKSEN, S. A. (1991). *In vitro* stress selection of nematophagous fungi for biocontrol of parasitic nematodes in ruminants. *Journal of Helminthology. 65*: 193-200.

MANKAU, R. (1980). Biological control of nematode pests by natural enemies. *Annual Review of Phytopathology. 18*: 415-440.

MEEUSEN, E. (1996). Rational design of nematode vaccines-natural antigens. *International Journal for Parasitology. 26*: 813-818.

NANSEN, P., LARSEN, M. & GRONVOLD, J. (1995). Prevention of clinical trichostrongylidosis in calves by strategic feeding with the predacious fungus *Duddingtonia flagrans. Parasitology Research. 81*: 371-374.

WALKER, H.L. & CONNICK, Jr. (1983). Sodium alginate for production and formulation of mycoherbicides. *Weed Science. 31*:333-338.

WALLER, P. J., LARSEN, M. & FAEDO, M. (1994). The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: *in vitro* and *in vivo* studies. *Veterinary Parasitology. 51*: 289-299.

WOLSTRUP, J., GRONVOLD, J. & HENRIKSEN, S. A. (1994). An attempt to implement the nematode-trapping fungus *Duddingtonia flagrans* in biological control of trichostrongyle infections of first year grazing calves. *Journal of Helminthology. 68*: 175-180.

WOOLASTON, R.R. & BAKER, R.L. (1996). Prospects of breeding for parasite resistance. *International Journal for Parasitology. 26*: 845-855.

(Received 7 April 1999, Accepted 8 September 1999)