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Studies on the Effect of Temperature, Incubation Time and *in vivo* Gut Passage on Survival and Nematophagous Activity *Arthrobotrys oligospora* Var. *Oligospora* and *A. cladodes* Var. *Macroides*

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Abstract: This work was carried out to study effects of important environmental factors including temperature, incubation time and gut passage on nematophagous activity of *Arthrobotrys oligospora var. oligospora* and *Arthrobotrys cladodes var. macroides* against *Haemonchus contortus*. *H. contortus* third stage larvae (L_3) were added to the fungal cultures in Petri-dishes and incubated for 24-96 hours at 15-25°C. The nematophagous potential of the fungi was determined by counting the number of viable L_3 using Baermann technique. Dung pat bioassay and fecal culture techniques were used for testing the viability and nematophagous activity of the fungi after gut passage through calve in a mixture with barley grains. Results indicated that both tested fungi were able to survive at all temperatures and incubation times and no differences were reported for their nematophagous activity in this regard. Also, predacious activity of fungi after gut passage in reduction of L_3 of *H. contortus* were 79.7 and 83.3% in faecal cultures method, and 85.4 and 78.8% in dung pat bioassay method, respectively. In conclusion, these native fungi are promising for use in biological control of gastrointestinal nematodes of ruminants under field conditions.

Key words: Biological control · *Arthrobotrys cladodes* var. *macroides* · *A. oligospora* var. *oligospora* · *Haemonchus contortus* · Nematophagous activity · Gut passage

INTRODUCTION

After first description of *Arthrobotrys oligospora* as a member of Hyphomycetales in 1850 by Fresenius, the ability of this fungus for producing circular traps and its nematode-trapping capability were further characterized [1]. Investigations on the nematophagous effects of *Arthrobotrys* species revealed the importance of these fungi as potential biocontrol agents of various parasitic nematodes of ruminants [1-3]. Further studies showed that some parameters including temperature, pH, incubation time and other environmental factors could affect the predacious activity of nematode-trapping fungi by various extends [4,5]. Previous studies led to the isolation of two subspecies in the genus *Arthrobotrys*; *A. oligospora var. oligospora* and a rare nematode-trapping fungus, *A. cladodes var. macroides* from compost samples in Iran with potent nematophagous activity against *Haemonchus contortus* infective larvae [6,8]. It has been shown that gastrointestinal nematode involvement is a major problem in about 100 millions of ruminants in Iran [9,10]. The nematode-related infections

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of ruminants are generally treated by routine anthelmintic compounds which they may suffer from large limitations including adverse effects on animal health, persistence residues in animal products; meat and milk and in the natural sources such as drinking water [9]. So, any attempt to replace anthelmintic therapy by other means is extremely important not only for public health but also for preserve natural ecosystem. Despite the reports on potent nematophagous activity of many *Arthrobotrys* species isolated so far; a major problem in the use of nematophagous fungi as biocontrol agents is the deposition of the fungal material in dung pats, whereas the entrapment of the parasite larvae should take place [11]. So, the most obvious possibility would be to add nematophagous fungi to the animal feed, provided the fungus is able to pass the gastro-intestinal tract without loss of viability and activity [12].

In the present study, the effects of temperature and incubation time as two important environmental factors on nematophagous activity of native fungi *A. cladodes var. macroides* and *A. oligospora var. oligospora*, as well as, their ability for gut passage through alimentary tract of cattle were evaluated.

MATERIALS AND METHODS

Fungal Material: Two native *Arthrobotrys* strains including *A. oligospora var. oligospora* IRAN 679 C and *A. cladodes var. macroides* IRAN 677C (CBS 113565) isolated from compost samples in Mazandaran province (north of Iran) were used through out the study.

Donor Animals: Six male calves (three to six months old, 112-163 kg weight) treated with albendazole (5 mg/kg B.W. twice during one-week period) were used for the gut passage experiment. Three calves were used for each of the fungal isolates. Calves were kept indoor during the experiment and fed with fresh hay. Fifteen days after anthelminthic treatment, no infection to parasites and nematophagous fungi was confirmed by faecal examination and culture.

Parasite Material: A parasite-free male sheep (twelve months old, weighing 17 kg), was used for producing third stage larvae (L_3) of *Haemonchus contortus*. One hundred and fifty adult helminthes (mixed male and female) were inserted into the abomasums directly using surgical method. The sheep was fed manually during the experiment. Faecal samples were collected directly from the rectum and were examined for the number of eggs per gram of feces (EPG). Fresh faeces contained eggs were prepared for both the dung pat bioassay and the faecal culture techniques. Also after incubation of faecal samples for 3 weeks at room temperature, third stage larvae (L_3) were harvested by a modified Baermann technique [9].

Plate Fungal Culture: An agar block method was used for culturing of fungi on 2% water-agar (WA). A 2×2 mm agar block of each fungus from initial cultures on potato-dextrose agar plates were transferred into the WA plates. The cultures were incubated for 10 days at 25°C. After this period, 600 *H. contortus* L_3 were added to each test plate. For control plates, fungi were cultured in absence of L_3. Different sets of plates were incubated for 24, 48, 72 and 96 hours at 15, 20 and 25°C. A set of 3 plates was considered for each incubation time in each temperature. Finally, each plate was placed on a Baermann funnel for 12 hours in order to collect the live larvae. The percentage of larvae reduction was calculated from 0.5 ml aliquots using a stereomicroscope after comparing the tests with the controls.

Cultivation of Fungi: Portions of barley grains (200 g) were mixed with 10% H_2O_2 solution for 30 min and washed three times in sterile water. The mixture was transferred into the flasks and then autoclaved after adding 200 ml water. The flasks were inoculated with three to five blocks (5×5 mm) from a 10 days old pure cultures of fungi, grown on 1:10 CMA. Flasks were shaken thoroughly two to three times per week and incubated in room temperature (20-22°C). So, fungus-grain mixture was ready for passage experiment.

Feeding to Fungus-grain Mixture: Twenty one days after anthelmintic treatment, calves were fed four days with fungus-barely grains mixture (two times in day), half of them before the morning fodder and the remaining half before feeding in the afternoon. Then, faecal samples were collected on 4th day (morning and afternoon) and 5th day (only morning).

Re-Isolation of Fungi: After passage of fungi, collected faecal samples from calves were washed by means of a kitchen sieve and isolated identifiable barely grains. Three TCC-WA plates (Tetracycline chloride-water agar) were used for each isolate and in each plate were inoculate with five grains isolated from faeces. After one week and incubation in 25°C, re-isolation of fungi was inspected on the basis of specific trapping structures and conidia.

Nematophagous Activity

Dung Pat Bioassay Method: Faecal samples from calves infected with a monoculture of *H. contortus* were diluted with faeces from experimental calves which were containing fungal material. This mixture was shaped into dung pats (approx. 3 cm high and 8 cm diameter) and the number of eggs per gram (EPG) in this mixture was 1900. Five dung pats was made for each fungal isolate and were incubated at 22°C and 60-80% relative humidity for 4 weeks. Then, third stage larvae were extracted using a Baermann technique. Five dung pats with same number of eggs, but without fungal material used as control [12,13].

Fecal Cultures Method: For each fungal isolate, five faecal cultures (10 g faeces) were made. Also, five cultures containing eggs but without fungal material served as control. The cultures were incubated similar to *2.8.1* and third stage larvae were calculated after 4 weeks.

Statistical Analysis: One-way ANOVA in Tukey range was used to compare the means of the groups.
P values of <0.05 were considered as significant.

RESULTS

The effects of incubation time and temperature on predatory activity of both *A. cladodes* var. *macroides* and *A. oligospora* var. *oligospora* are shown in Table 1.

Our findings revealed that both subspecies were capable for reduction of third stage larvae of *H. contortus* significantly at different incubation times (P<0.0001). The L_3 reduction values for *A. oligospora* var. *oligospora* in comparison with that of control were 81.26 to 99.86%. No significant differences were shown regarding the reduction of L_3 of *H. contortus* at different temperature. The maximum effects were obtained 14th day post culture (96 h. after adding larvae) as 95.95 to 97.55%. Therefore, more time was needed for demonstration of predatory activity of *A. cladodes* var. *macroides*. Meanwhile no significant difference was found between test and control groups regard the effects of incubation time and temperature. On the other hand nematophagous activity of *A. oligospora* var. *oligospora* at 25°C and at 11th day post culture (24 h. after adding larvae) was significantly higher than that of control (P<0.019). The predatory activity of *A. oligospora* was found to be higher than *A. cladodes* only on 11th day post culture (24 h. after adding larvae) (P<0.0001). For other incubation times no significant differences were shown. Study on the passage of fungi through gastro-intestinal tract of cattle showed that both of fungi were able to survive gut passage. Nematophagous activity of *A. oligospora* var. *oligospora* and *A. cladodes* var. *macroides* in reduction of third stage larvae of *H. contortus* were 79.7 and 83.3% in faecal cultures method, and 85.4 and 78.8% in dung pat bioassay method, respectively (Table 2).

Table 1: Effects of temperature and incubation time on the nematophagous activity of *A. oligospora var. oligospora* and *A. cladodes var. macroides*

		Arthrobotrys cladode Var. macroides(CBS 113565)		*Arthrobotrys oligospora Var. oligospora* (IRAN 679C)		Control
		Alive larvae	Reduction (%)	Alive larvae	Reduction (%)	Alive larvae
11th day (The first day after adding of larvae)	15°C	413.7±43.23	24.78	103.06±20.26	81.26	550.1±32.05
	20°C	268.43±4.47	37.61	18.06±3.44	95.80	430.26±29.32
	25°C	208.36±37.04	23.66	16.2±3.50	94.06	272.96±24.40
12th day (The second day after adding of larvae)	15°C	241.06±39.57	59.21	23.2±3.89	96.07	591±25.10
	20°C	92.13±4.81	84.56	9.5±1.23	98.40	597±23.94
	25°C	41.43±7.60	92.89	7.66±2.01	98.68	583.26±4.35
13th day (The third day after adding of larvae)	15°C	95.86±16.38	76.75	9±1.42	97.81	412.36±41.94
	20°C	34±0.65	92.23	10.2±0.41	97.66	437.73±9.59
	25°C	36.66±2.10	92.32	4.4±0.95	99.07	477.56±47.58
14th day (The fourth day after adding of larvae)	15°C	14.2±3.38	97.55	7.4±0.22	98.72	580.03±4.37
	20°C	16.1±3.69	96.71	0.66±0.08	99.86	489.8±33.78
	25°C	11.56±1.33	95.93	0.33±0.04	99.88	284.66±41.18

Table 2: Re-isolation of native isolates of *Arthrobotrys* from faeces and study on its nematophagous activity on the third stage larvae of *Haemonchus contortus* after passage through the gastro-intestinal tract of cattle.

	Re-isolation of fungus	Isolated third stage larvae of *Haemonchus contortus* by Dung Pat bioassay method	Reduction (%)	Sig.	Isolated third stage larvae of *Haemonchus contortus* by Faecal cultures method	Reduction (%)	Sig.
Arthrobotrys oligospora *Var. oligospora* IRAN 679C	+	238±22	85.41	*	311±17	79.75	*
Arthrobotrys cladode *Var. macroides* CBS 113565	+	346±15	78.79	*	257±27	83.26	*
Control	-	1632±19	-	-	1536±31	-	-

* P<0.001

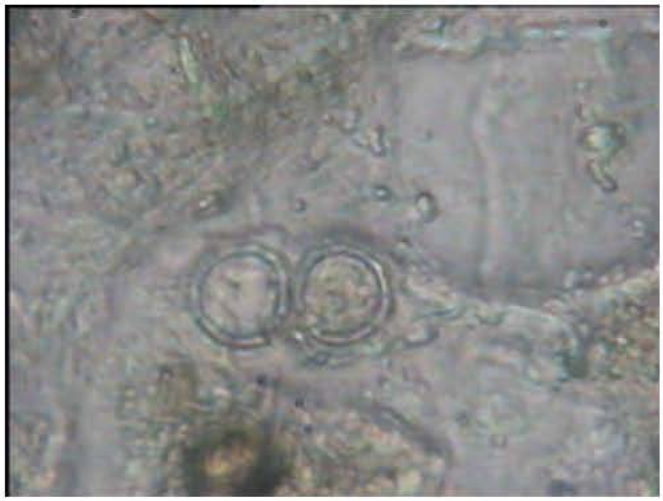

Fig. 1: Producing trapping structures by fungal hypha after passage through the gastro-intestinal tract of cattle (× 10 objective lens)

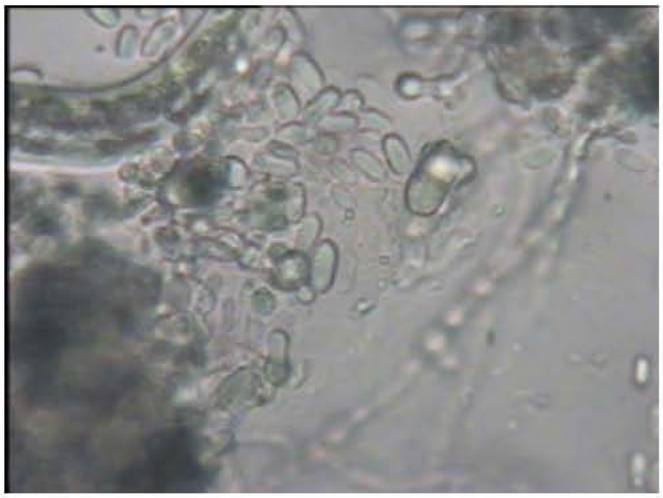

Fig. 2: Conidium of *Arthrobotrys* in faecal samples after passage through the gastro-intestinal tract of cattle (× 10 objective lens

In both methods for predacious activity of fungi were observed all of fungal structures including; conidia, trapping structures and trapped larvae (Fig. 1 and 2).

DISCUSSION

In past decades, biological control of gastrointestinal nematodes of livestock by predatory fungi has been received major consideration regarding the remarkable difficulties in chemotherapy of gastrointestinal nematodes such as resistance, high cost, their residues in tissues and milk [14]. About 160 fungal species belonging to 70 genera were found to be associated with nematodes of which more than 50 species exert killing properties [15]. Among these, the genus *Arthrobotrys* is considered as the most effective species, which is capable to control gastrointestinal nematodes of ruminants by producing specific circular mycelial traps [1]. Recently, two nematophagous fungi were isolated from compost samples of Iran, namely *A. oligospora* var. *oligospora* and *A. cladodes* var. *macroides* and the predatory activity of them was shown for the first time [6-8]. The results of present study are in line with Sanial [5] and Virat and Peloille [16] who showed the optimum temperature for the growth of *Arthrobotrys oligospora* was 25°C and 15°C-22°C after 24 hours contact period, respectively. So with regard to the effects of various environmental parameters such as temperature, incubation time and pH and even genera, species and strain on the predatory activity of nematophagous fungi, we achieved the object of this study [1,4,17]. Regard to the high sheep and goat population of Iran (about 75 millions), large amount of anthelminthics used and the harmful affects of chemotherapy, both native isolates of *Arthrobotrys* isolated in this study can be considered as potential alternatives for chemotherapy. According to Skerman *et al.* [10] Iran can be divided climatologically into four different zones of which zone 4 (central and salt desert) is not convenient for animal breeding, but the temperature in this experiment (15-25°C) coincide with the natural temperature condition of spring to mild summer of zone 1: Caspian Sea zone includes the Coastal plains and northern aspect of Alborz range and zone 2: the Mountain Plateau zone includes the southern aspect of the Alborz range, The Zagros range and late winter and early spring of zone 3: the Persian Gulf Lowlands comprising the low altitude country bordering the Persian Gulf and extending northwards along the basin of the Tigris River bordering Iraq, when the peak of gastrointestinal nematode infections of ruminants occur [10]. But, the major problem in the using of nematophagous fungi is the deposition of them in dung pats and the best way for that is adding of fungal material to the animal feed and it is necessary for survive passage of fungi through alimentary tract. In this study, both of native isolated fungi were successfully passed through gastro-intestinal tract of cattle without loss of viability and predacious activity. This is in accordance with other reports on survival *in vitro*, through cattle, sheep and in contrast with the Sanial [5] report that couldn't isolate *Arthrobotrys* conidia from faeces [5,12,18-20]. Of course, Graminha *et al.* [11] determined presence and predatory activity of some species of *Arthrobotrys* e.g. *A. musiformis* and *A. conoides* during passage through the gastrointestinal tract of sheep after oral administration of conidia. So, this study showed that passage of *Arthrobotrys* through alimentary tract is species dependent. In the present study, for the first time, survival of the predatory fungus: *A. cladodes* var. *macroides* for gut passage was showed and with regard to successful gut passage of both fungi through the gastro-intestinal tract, their presence of in dung pats and their good nematophagous activity at 15-25°C for a long time period (24-96 hours), which coincides with the temperature suitable for development of gastrointestinal nematodes of sheep and goats in Iran.

In conclusion, these fungi are potential candidates for use as biocontrol agents in management of parasitic nematodes infections of ruminants in field conditions. However, it is essential to continue with further studies, for establish the influence of other factors.

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