

Screening of Soil and Sheep Faecal Samples for Predacious Fungi: Isolation and Characterization of the Nematode-Trapping Fungus *Arthrobotrys oligospora*

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ABSTRACT

Over one-year period, 150 pasture soil samples and 138 sheep faecal samples, collected from different parts of Iran were screened for the presence of nematophagous fungi. The samples were cultured at 25°C on chloramphenicol-2% water agar (CHF-WA) plates in the presence of *Haemonchus contortus* third stage larvae (L3) and checked over a two-month period for characteristic conidia, conidiophores and hyphal traps of nematophagous fungi. Suspected nematophagous fungi were isolated by periodic transfer of the fungi on CHF-WA plates using the agar block method. Overall, 11 soil samples were found to harbour the nematode-trapping fungus *Arthrobotrys* from which 3 pure isolates were made and consequently identified as *Arthrobotrys oligospora* IRAN 877 C, IRAN 878 C and IRAN 879 C. Nematophagous fungi were not found in any tested sheep faecal samples. The predatory capacity of the isolates was tested against *H. contortus* infective larvae and then compared to reference strains *A. oligospora* CBS 111.37, *A. oligospora* CBS 251.82 and *Duddingtonia flagrans* CBS 583.91. The local strains of *A. oligospora* reduced the development of *H. contortus* L3 by 75-85%, whereas, the predatory capacity of reference *A. oligospora* and *D. flagrans* strains was measured in the range of 51-85% compared to the fungus free controls. Study of the effect of temperature on predatory activity of *A. oligospora* strains IRAN 877 C and CBS 111.37 revealed a reduction of more than 95% in infective larvae of *H. contortus* at temperature levels between 15 to 25°C. This reduction was significantly decreased to 30% and 50% at 10°C and 30°C, respectively. The nematode-trapping fungus *A. oligospora* is reported from soil of Iran for the first time and its potential role in biocontrol of gastrointestinal nematodes of ruminants is discussed. *Iran. Biomed. J.* 8 (3): 135-142, 2004

Keywords: Screening, Soil, Predacious fungi, *Arthrobotrys oligospora*, Nematophagous activity

INTRODUCTION

A large number of organisms including fungi, bacteria, viruses, insects, mites and some invertebrates have been found to invade or prey the nematodes [1, 2]. Among these organisms, the fungi have increased value as potential candidates for biocontrol of livestock nematodes [3, 4]. Fungi are considered as the major microbial biomass in many soils [5]. These organisms have a significant association with nematodes in rhizosphere and thus, they can constantly destroy

nematodes in nearly all soils at different geographical areas [2]. Although association of more than 70 genera and 160 species of fungi with nematodes have been established, only a few of them, known as nematophagous fungi, were considered as suitable biocontrol agents [3, 6]. These fungi have several characteristics including highly specific parasitism to nematodes, not to plants and higher animals, and growth at suitable pH and temperature ranges on natural or synthetic media [7, 8]. Nematophagous fungi have been classified into three major classes: endoparasitic,

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Table 1. Geographical sites of collected soil samples investigated for the presence of nematophagous fungi in Iran*.

Region	Locality	Number of positive samples	Nematophagous fungal isolates
Mazandaran	Savadkooh	2	<i>A. oligospora</i> IRAN 877 C <i>Arthrobotrys</i> sp. (lost)
	Ghaemshahr	1	<i>Arthrobotrys</i> sp. (lost)
	North-east Noor	2	<i>A. oligospora</i> IRAN 878 C <i>Arthrobotrys</i> sp. (lost)
	Amirkela	1	<i>Arthrobotrys</i> sp. (lost)
	South-west Noor	2	<i>Arthrobotrys</i> sp. (lost)
	South-east Noor	1	<i>A. oligospora</i> IRAN 879 C
	Sisangan forest	1	<i>Arthrobotrys</i> sp. (lost)
Garmsar	Hosseinabad	1	<i>Arthrobotrys</i> sp. (lost)

*Five samples collected from each location in both regions. The number of 13 and 9 locations from Mazandaran and Garmsar were negative for nematophagous fungi, accordingly (details not included in Table).

predacious and opportunistic groups. There are more than 50 species of predacious fungi that able to capture and kill nematodes in soil [2, 4]. These fungi have mainly been classified in some different genera of Hyphomycetes, which are susceptible to antagonism from other soil fungi [9]. The genera *Arthrobotrys* and *Duddingtonia* are considered to be the most important nematophagous fungi which intensively studied regarding their ecology and pathophysiology, especially in nematode management programmes [2-4]. Because of drug resistance and other problems, such as drug residues in animal tissues and also pastures which resulted from intensive use of anthelmintic treatment in livestock farms, the demands for application of nematode biocontrol agents i.e. nematophagous fungi in field managing programmes have been increased rapidly in recent years [10-17]. Since nematophagous fungi are common soil habitants, they can be ingested by animals grazing in pastures and then recycle in the environment after excretion into the animal feces. There are some reports on the isolation and characterization of nematophagous fungi from various sources including soil, dung, compost and fresh feces of some animal species at different geographical areas [10, 18-23]. This communication presents the results of a survey conducted in two temperate-moist (Mazandaran) and warm-dry (Garmsar) regions of Iran with the aim of isolation of nematophagous fungi from soil and fresh sheep faecal samples and characterization of their predatory activity *in vitro*. This is the first report on the isolation and characterization of a nematode-trapping fungus named *Arthrobotrys oligospora* from Iran.

MATERIALS AND METHODS

Sampling sites. One-hundred and fifty pasture soil samples (100 g each) were collected from different parts of Mazandaran and Garmsar, which belonged to the moist and dry regions of Iran, respectively during April 2002 (Table 1). Five samples were taken from each location in both regions and then rapidly transferred into the nylon bags and sent to the laboratory. Also, one-hundred and thirty eight faecal samples (50 g each) were collected directly from rectum of different sheep herds in both regions.

Culture media. Primary isolation of nematophagous fungi was achieved using chloramphenicol-2% water agar (CHF-WA) medium. This medium consisted of chloramphenicol (0.05%, dissolved in ethanol), Difco agar (2%) and demineralized water (1 L). All ingredients were mixed in an Erlenmeyer flask and divided into 10-cm diameter Petri dishes after autoclaving for 15 min at 121°C. CHF-WA was also used for subsequent isolation and subculturing of the fungi. The fungal isolates were maintained on potato-dextrose agar (PDA, Difco) plates.

Nematodes. An experimentally infected lamb donor was used as a source of *H. contortus* third stage larvae (L3). This donor was first treated with albendazole (0.5 mg/kg twice a week) and immediately after treatment, a total number of 150 adult male and female *H. contortus* were surgically putted into the abomasum. The faecal samples contained only *H. contortus* eggs. After 1 week

incubation of the faecal cultures at 25-27°C, L3 was harvested by Baermann technique [24]. *H. contortus* L3 was thoroughly washed in sterile water before adding to the fungal cultures.

Isolation and identification procedures. Isolation of the nematophagous fungi from soil was performed according to Larsen *et al.* [10]. Each soil sample (10 g) was suspended in 50 ml distilled water in a 250-ml Erlenmeyer flask. The flasks were vigorously shaken for 30 min and maintained for an extra 10 min in static condition at room temperature. Consequently, 4 supernatant samples (1 ml each) were separately spread on CHF-WA plates. For faecal samples, 2 g of each sample was thoroughly homogenized and then directly cultured on 2% water agar plates. After 3 days incubation of the soil and faecal cultures at 25°C, approximately 500-1000 *H. contortus* larvae were added to each plate. The plates were maintained at 25°C for 2 months in order to demonstrate growth of nematophagous fungi. During this period, the plates were monitored 3-4 times a week for the presence of fungi using a stereomicroscope. Characteristic conidia or traps of nematophagous fungi were repeatedly subcultured on fresh CHF-WA plates by transferring 2 × 2 mm culture pieces using agar block technique [25]. Pure cultures were obtained for each isolate using a method named hyphal tipping [25]. The identification of nematophagous fungi was based on the morphology of trapping structures and conidia [26, 27]. The fungi *A. oligospora* CBS 111.37, *A. oligospora* CBS 251.82 and *D. flagrans* CBS 583.91 were obtained from Centraalbureau voor Schimmelfcultures, Baarn (The Netherlands) and were used as reference strains for confirmation of identity.

Mycological studies. Three local isolates of *A. oligospora* which were successfully isolated in this study, were encoded as *A. oligospora* IRAN 877 C, IRAN 878 C and IRAN 879 C by Culture Collection of Mycology Department, Pasteur Institute of Iran. These strains and the reference strains *A. oligospora* CBS 111.37, CBS 251.82 and *D. flagrans* CBS 583.91 were cultured on petridishes containing potato-dextrose agar (PDA). Some important mycological features including the rate of growth and conidiogenesis, chlamidospore formation and conidia dimensions, were noted after 3 weeks incubation at 25°C.

Study of predatory activity. The predatory effects of the local and reference strains of *A. oligospora* and *D. flagrans* were studied on *H. contortus*

infective larvae [3]. The strains were cultivated on PDA plates at 25°C for 15 days. The surface of the cultures was washed thoroughly with distilled water containing 0.02% Tween 80, and the number of conidia was calculated using a Neubauer (Haemocytometer) slide under the microscope. Conidia of each strain (20×10^3) were separately added to 1 gram faecal samples of a lamb experimentally infected with a monoculture of *H. contortus* [egg per gram (EPG) = 200]. Control feces (without fungal material) were also cultured at the same condition. All faecal cultures were incubated at 25°C for 8 days and the number of alive L3 was determined using Baermann technique [24]. In order to examine the effect of temperature on predatory activity, the selected strains *A. oligospora* IRAN 877 C and CBS 111.37, which had shown the highest nematophagous activity in our previous experiments, were cultivated for 10 days on corn meal agar (CMA) plates and then, incubated with 600 infective larvae of *H. contortus* for 96 h at 10, 15, 20, 25 and 30°C.

RESULTS

Nematophagous fungi from soil and feces.

Among a total of 150 pasture soil samples examined in this study, 11 samples were noted positive for nematophagous fungi initially based on the observation of characteristic conidia and traps around the immobilized larvae (Fig. 1). From these, 3 pure cultures were made and identified as *A. oligospora* IRAN 877 C, IRAN 878 C and IRAN 879 C (Table 1). Unfortunately, we could not purify other 8 isolates of the genus *Arthrobotrys* because

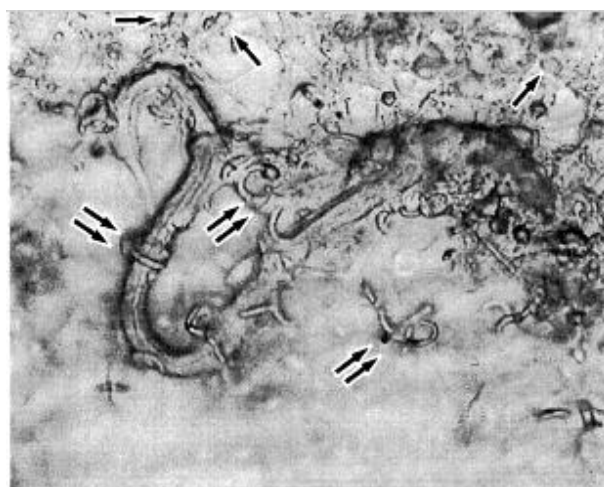


Fig. 1. Characteristic egg-like two celled conidia (single arrows) and mycelial traps (double arrows) around the *H. contortus* larvae on CHF-WA plates inoculated with a contaminated soil sample.

of the heavily contamination with saprophytic fungi which were present in the samples, specially the fungus *Fusarium oxysporum* (Fig. 2). Also, we could not isolate any nematophagous fungi in sheep faecal samples. Concurrent study of the total fungal flora of soil samples of Mazandaran and Garmsar using selective isolation media showed that the genus *Arthrobotrys* comprised 2% and 0.4% of

the total fungal isolates, respectively. The percent of the fungus *Arthrobotrys* contamination determined as 1.5% of the total fungi in both regions studied (data not shown in details). *D. flagrans* and other genera of nematophagous fungi were not found in this study. Some mycological features of local *A. oligospora* strains compared with the reference strains are shown in Table 2.

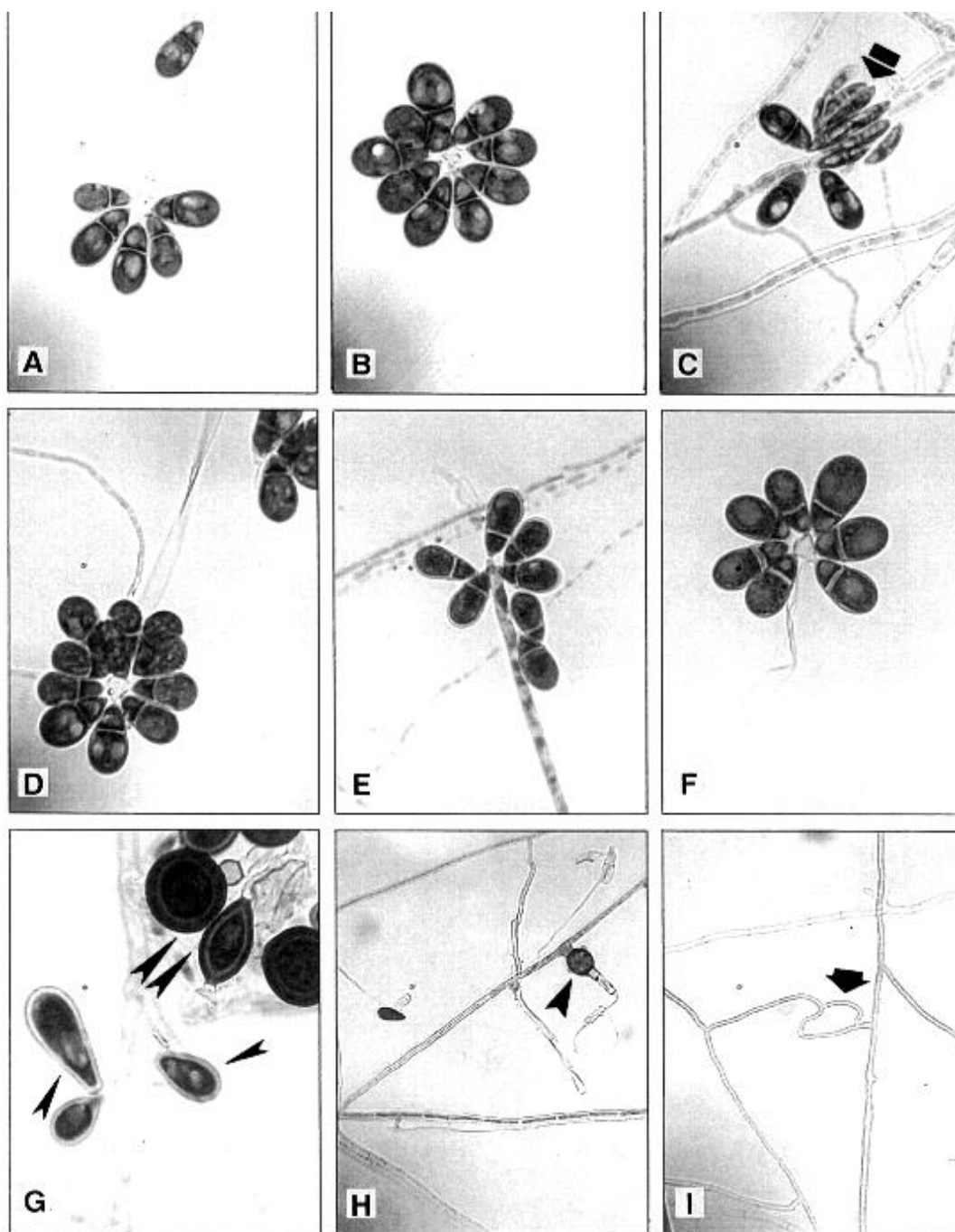


Fig. 2. Microscopic morphology of nematophagous fungi. Characteristic conidial structures including egg-like two celled conidia arranged together on conidiophore denticles are shown in *A. oligospora* strains IRAN 877 C (A), IRAN 878 C (D), IRAN 879 C (B), CBS 111.37 (E) and CBS 251.82 (F). Intercalary chlamidospore (arrow in H) and trap formation (arrow in I) are indicated for IRAN 877 C strain. Contamination with *Fusarium* multi-cell curved macroconidia is shown (arrow in C). Tear-drop shaped conidia (single arrows in G) and characteristic round to ovoid chlamidospores of *D. flagrans* CBS 583.91 (double arrow in G) are also emphasized.

Table 2. Some mycological features of local and reference strains of nematophagous fungi after 3 weeks cultivation on PDA at 25°C*.

Fungus	Growth rate** (colony diameter, cm)	Conidiogenesis **(conidia/plate)	Chlamidos- pore formation	Conidium dimentions (µm)	Predatory activity (% of L3 reduction)
<i>A. oligospora</i> Iran 877 C	3.91 ± 0.42	88.33 × 10 ⁴	+	10-14 × 15-25	84.57
<i>A. oligospora</i> Iran 878 C	4.18 ± 0.35	65.14 × 10 ⁴	+		78.19
<i>A. oligospora</i> Iran 879 C	2.86 ± 0.30	73.12 × 10 ⁴	-		75.49
<i>A. oligospora</i> CBS 111.37	2.90 ± 0.18	115.87 × 10 ⁴	+	10-15 × 15-27	81.17
<i>A. oligospora</i> CBS 251.82	3.75 ± 0.31	84.75 × 10 ⁴	+		51.57
<i>D. flagrans</i> CBS 583.91	4.50 ± 0.29	33.25 × 10 ⁴	+	10-15 × 18-45	85.66

*, the results are the means of 3 experiments each in triplicate; **, the values are related to 2 weeks cultures.

In general, all of the strains produced whitish and loose colonies on CHF-WA plates. Hyaline septate hyphae with not defined branching patterns, hyphal anastomosis and trap formation were noted for all strains (Fig. 2). Conidiophores were usually straight with a few branches and multiple sites of denticle formation for conidia production. The large amount of one or usually two celled egg-like conidia were produced on conidiophore denticles with up to 10 conidia per one geniculation (Fig. 2). Round to ovoid intercalary chlamidospores were noted in very limited numbers in old cultures (Fig. 2).

Predatory activity. The ability of both local and reference strains of *A. oligospora* and *D. flagrans* CBS 583.91 to *in vitro* killing of third-stage larvae of *H. contortus* at a concentration of 20×10^3 conidia/g feces is shown in Table 2. All of the strains reduced the number of infective larvae at a considerable rate in comparison with the controls ($P < 0.001$). It is interested to note that local strains of *A. oligospora* had relatively higher predatory activity (75-85%) as compared with the reference strains (51-81%). The effect of temperature on predatory activity of *A. oligospora* strains IRAN 877 C and CBS 111.37 is shown in Figure 3. More than 95% reduction in the number of *H. contortus* L3 was reported for both strains at temperatures of 15, 20 and 25°C, after comparing with controls ($P < 0.00001$). This reduction for IRAN 877 C and CBS 111.37 strains was measured in the range of 95.93-97.55% and 98.72-99.88%, respectively. The predatory activity of these isolates was sharply diminished to 28-31% and 51-55% at 10 and 30°C, respectively.

DISCUSSION

In the recent years, the demand for biocontrol agents in nematode management programmes has been dramatically increased because of occurring

anthelmintic drug resistance and other problems associated with wide use of these treatments including chemical residues in livestock products and the environment [2-4]. Since nematophagous fungi can predate the infective larvae of nematodes and even survive gut passage of ruminants, they have been suggested as biocontrol agents for ruminant helminthosis [3]. In the present study, we screened 150 pasture soil samples and 138 sheep faecal samples from Iran for the presence of nematode-trapping fungi. Then, we examined the predatory activity of the isolated fungi against nematode *H. contortus*, *in vitro*. It is of interest that 11 isolates of the genus *Arthrobotrys* were noted in soil samples, but no nematophagous fungi were obtained from faecal samples. These fungi comprised 1.5% of the total fungal isolates in both Mazandaran and Garmsar regions. The majority of isolates (10 of 11) were reported from Mazandaran which belong to the temperate and moist regions of Iran. In contrast, only 1 *Arthrobotrys* isolate was found in soil samples of Garmsar which has a warm and dry climate. Among *Arthrobotrys* isolates, which were noted in early screening on CHF-WA

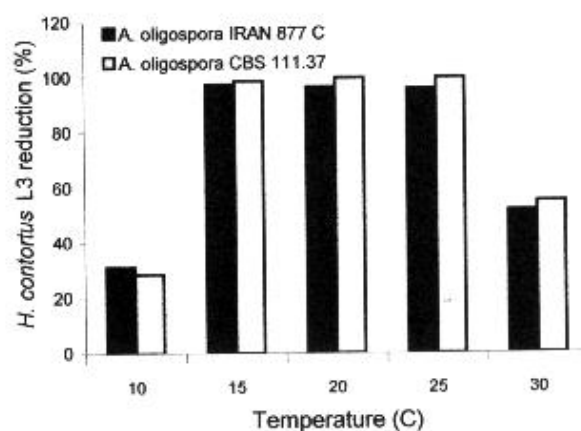


Fig. 3. The effect of temperature on predatory activity of selected local and reference strains of *A. oligospora* against *H. contortus* infective larvae at 96 h cultures.

plates, we could purify only 3 isolates that were subsequently characterized as *A. oligospora* IRAN 877 C, IRAN 878 C and IRAN 879 C. Unfortunately, we lost 8 *Arthrobotrys* isolates because of high contamination with spreading fungi i.e. Mucorales and also *Fusarium oxysporum*. There are some reports on the isolation of *Arthrobotrys* and other predatory fungi from soil, dung, and compost samples with different geographical distribution [10, 18-23]. Manuelli et al. [23] reported that they could purify only 12 isolates of the genus *Arthrobotrys* among 23 positive samples which were found to be positive for the fungus in the initial screening in Fiji. They believed that this problem was partly due to technical difficulties and the heavy overgrowth with vigorously growing saprophytic fungi in cultures, as also seen on our samples. In our study, an interesting correlation was noted between two genera *Arthrobotrys* and *Fusarium*, which were concurrently present in nearly all positive samples. The presence of the overgrowing fungus *Fusarium* in our samples was considered to be one of the most important problems for purification of *Arthrobotrys* and possibly other nematophagous fungi. We were unable to identify any nematophagous fungi in feces prepared directly from the sheep rectum. In spite of the some other reports on the isolation of different nematophagous fungi (mainly *Arthrobotrys*) from faecal samples [18, 20, 23], some investigators believe that *Arthrobotrys* conidia are susceptible to rumen fluid of animals *in vitro* and *in vivo* [10, 19, 28, 29]. They have shown the strain dependent disruption and also decrease of viability in these conidia after passing through the alimentary canal in a variety animal species. On the other hand, as a few nematophagous fungi have been isolated from large number of animal feces in most studies [18-21, 23], our inability to isolate these fungi from only 136 faecal samples is not a surprising subject. This means that we need to examine more faecal samples for nematophagous fungi in further researches. The study of nematophagous activity of local and reference strains of nematophagous fungi against *H. contortus* infective larvae revealed predacious capacity of 75.49-84.77% and 51.57-85.66 % compared with the controls, respectively. The nematophagous potential of *A. oligospora* isolates reported in this study is well correlated with the results of other researchers on the reduction of infective larvae of parasitic nematodes by various nematode-trapping fungi at different conditions [10, 19, 30-32]. For example, Larsen et al. [10] reported a reduction of 76-99% in third stage larvae of

Ostertagia ostertagi by *Arthrobotrys* and *Duddingtonia*, *in vitro*. Also, Menduza De Gives and Vazquez-prats [30] showed that *A. oligospora*, *Monacrosporium acermatoum* and *A. robusta* reduced *H. contortus* infective larvae with 93.30%, 95.70% and 10.10%, respectively. Study of the effect of temperature on predatory activity of *A. oligospora* strains Iran 877 C and CBS 111.37 showed that these fungi can significantly reduce *H. contortus* L3 at all temperature regimens from 10 to 30°C, *in vitro*. This reduction for both strains was measured to be more than 95% at temperatures between 15 to 25°C as compared with controls ($P<0.001$). The predatory activity of these isolates decreased sharply to 28-31% and 51-55% at 10 and 30°C, respectively. Sanyal [19] reported *in vitro* predatory activity of more than 70% for *A. oligospora* isolates against *H. contortus* L3 after 72-96 h incubation at 25°C. Fernandez et al. [32] showed that the predatory activity of *A. oligospora* and *D. flagrans* against *Cooperina oncophora* larvae was a temperature dependent process with maximum range of 63-98% at 15-20°C. This activity was diminished to 18-25% at 10°C. Our data is well correlated with this information, which indicates that a temperature range between 15 to 25°C is necessary for maximum predatory activity of *A. oligospora* and *D. flagrans* against *H. contortus* infective larvae. This optimal temperature is well correlated with environmental conditions of the most parts of Iran in grazing seasons.

This is the first report on the isolation and characterization of a nematode-trapping fungus (*A. oligospora*) from Iran. To our knowledge, there is not any published data on description of these amazing group of fungi from Iran now. Although predatory activity of local *A. oligospora* isolates against infective larvae of a parasitic nematode of ruminants *H. contortus* has been established in this study, further trials should also be considered for the evaluation of passing ability of these isolates through the alimentary canal of ruminants. The isolates that preserve their viability after passing *in vivo* can be used as potential candidates for biological control of parasitic nematodes of ruminants in field management programmes.

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