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ORIGINAL PAPER

Isolation and characterization of the nematophagous fungus *Arthrobotrys conoides*

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Abstract The spread of organic farming and the development of resistance to anthelmintics by parasites, especially in small ruminants, have necessitated the search for alternative methods of nematode control. Biological control using nematophagous fungi is one option; however, few studies have been conducted with native strains. The present study was divided into two phases. In the first phase, we aimed to isolate, identify, and assess the in vitro predatory activity of nematophagous fungi that had been isolated on *Trichostrongylidae* third-instar larvae. In the second phase, the isolate with superior predatory activity in vitro was molecularly characterized, and its morphological plasticity was observed using scanning electron microscopy (SEM) on *Haemonchus* third-instar larvae. Of the 56 soil samples from different regions of Paraná State, Brazil, 57 fungal strains were recovered, of which four exhibited predatory activity. Two pure isolates were obtained: the CED and LIN strains. After demonstrating 96.35 % predatory activity for the CED strain, this strain was selected and characterized using molecular criteria by sequencing the rDNA internal transcribed spacer and was identified as *Arthrobotrys conoides* (GenBank ID: JN191309). Morphological patterns in this strain during the interaction between the fungus and the nematode were revealed by SEM, in which two extensions of the infection bulb that was used to pierce the nematode's cuticle were clearly visible.

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Introduction

Studies conducted in three major sheep-raising countries (Australia, South Africa, and Uruguay) have classified nematodes as being responsible for most of the damage to this animal species (Waller 2006). In Brazil, this scenario is no different. Among the barriers to controlling these diseases, one of the most significant is the high level of the resistance of gastrointestinal helminths to different compounds, which has been reported since the 1990s (Echevarria et al. 1996; Thomaz-Soccol et al. 1996, 2004; Waller and Faedo 1996). To resolve this problem, new methods for controlling gastrointestinal helminths in ruminants have been studied, with an emphasis on biological control using nematophagous fungi (Waller 2006; Ketzis et al. 2006), which raises expectations for their use in integrated pest management (Waller et al. 2004; Larsen 2006; Maingi et al. 2006).

Research has been conducted on the fungus *Duddingtonia flagrans* (Fernandez et al. 1999; Faedo et al. 2000;

Chandrawathani et al. 2004; Epe et al. 2009; Cruz et al. 2011); however, few studies have isolated and utilized native strains (Manueli et al. 1999; Sanyal 2000), which is crucial because fungi adapt easily to regional climatic conditions.

Therefore, isolating and characterizing fungal strains with predatory activity is essential for further research. One of the steps in selecting strains of interest involves characterizing the interaction between fungi and nematodes (Mendonza-de-Gives et al. 1999), and scanning electron microscopy (SEM) is a tool that shows this interaction between predator and prey in addition to the predatory mechanisms used (Nordbring-Hertz et al. 1989).

In this study, we aimed to select a native nematophagous fungal strain with the potential for use in biological control programs in ruminants, and to this end, this study was divided into two phases. In the first phase, we sought to isolate, identify, and assess the in vitro predatory activity of the isolated strains on *Trichostrongylidae* third-instar larvae. In the second phase, after selecting the best strain based on the results from the in vitro assay, we characterized that strain using molecular markers and characterized its morphological plasticity by SEM during predation on *Haemonchus* sp.

Materials and methods

Phase one

Soil samples

A total of 56 soil samples were collected between August and November 2006 from different regions of Paraná State, including the west-central, north-central, south-central, south-eastern, western and south-western regions, incorporating agricultural, livestock, integrated crop-livestock, and environmentally protected areas. The samples were collected at a depth of 0–5 cm, placed into labeled plastic bags, and stored at 4 °C until the fungi were isolated.

Free-living nematode larvae used in isolation

The free-living nematode larvae of *Panagrellus* sp. that were used to isolate the fungi were maintained in Petri dishes with a medium containing oatmeal that was mixed and moistened with distilled water (Heintz 1978). The nematodes were extracted from the culture medium by immersing small quantities of oats in a Baermann funnel and were collected in hemolysis tubes after 6–8 h of decanting. Next, the larvae were washed seven times with distilled water and recovered after centrifugation at 72.67×*g* for 10 min. The supernatant was discarded after each centrifugation. Subsequently, the larvae were immersed in a solution containing 0.05 % streptomycin and chloramphenicol for 6 h. Following this step, the washing process was repeated, and the total number of larvae was estimated by counting five 20-μL aliquots under a light microscope at 10× magnification (Araújo et al. 1994).

Isolation and identification of nematophagous fungi

Fungi were isolated according to the method described by Duddington (1955) and modified by Santos et al. (1991). The 2-g soil samples were distributed in the center of Petri dishes containing 2 % water–agar medium (WA) with 0.05 % chloramphenicol in duplicate. Then, 1 mL of resuspended *Panagrellus* sp. (approximately 1,000 larvae) was added, and the Petri dishes were incubated at 26 °C and monitored daily under a stereoscopic microscope for 10 days. After this initial period, the samples were monitored on a weekly basis for 30 days. Once the nematodes were trapped within the fungal structures, fragments of the culture were transferred to Petri dishes containing Potato Dextrose Agar (PDA) [Himédia®, India] medium until pure fungal cultures were obtained (Saumell et al. 1999).

Morphological characterization of fungal isolates

To morphologically characterize the isolates, the samples were cultured in a PDA medium, and the microculture slides were identified and classified based on a key prepared by Cooke and Godfrey (1964) and Philip (2002).

In vitro assay

The in vitro assay was performed in 8-cm Petri dishes containing 2 % WA medium. The experiment was completely randomized using the two isolated strains, *CED* and *LIN*, and a control with five replicates each.

Fragments of the culture of each isolate were removed from test tubes containing corn meal agar medium [CMA; Difco® (17 gL^{-1})], transferred to 8-cm Petri dishes containing 15 mL of PDA medium and incubated at 26 °C and 95 % humidity for 6 days. After this period, approximately 4-mm fragments of each culture were transferred to dishes containing 2 % WA medium and incubated at 26 °C in darkness for 6 days (Campos et al. 2008). On the sixth day, 350 μL of a solution containing approximately 1,000 infective *Trichostrongylidae* third instar larvae (L3) was added in the following proportions: 64 % *Haemonchus* sp., 22 % *Trichostrongylus* sp., and 14 % *Strongyloides papillosus*.

The control group consisted of Petri dishes containing 2 % WA without fungus to which 350 μL of resuspended 1,000 L3 was added. All of the dishes were incubated again at 26 °C in darkness for 6 days. On the sixth day of the

interaction between the fungi and L3, the dishes were removed from the incubator, and the agar was removed from the Petri dishes using a metal spatula. This agar was subjected to the Baermann method for 6 h as described by Araújo et al. (1994), and the larvae were counted and identified as described by Van Wyk et al. (2004).

For larval counting, the contents of the tubes were standardized to 1 mL, and the estimated number of larvae in five 50-μL aliquots was extrapolated to the tube volumes. The predation rate was estimated using the following formula (Braga et al. 2010)

$$\%\,reduction = [(XC - XT)/XC] \times 100,$$

where

XC average number of larvae recovered in the control group

XT average number of larvae recovered in the treatment group

Phase two

In this phase, the strain that exhibited the greatest predatory activity in the in vitro assay was selected.

Molecular characterization by sequencing the ITS region of rDNA

a) DNA extraction

After 4–5 days of culture on a PDA medium, approximately 1-cm^2 fragments of the fungal culture were transferred to 2-mL Eppendorf tubes containing 300 μL of a cetyltrimethylammonium bromide (CTAB) buffer [2 % CTAB (*w*/*v*); 1.4 M NaCl; 100 mM Tris–HCl, pH8.0; 20 mM EDTA; 0.2 % 2-mercaptoethanol (*v*/*v*)] and 80 mg of silica gel (2:1, *w*/*w*) (Merck, Germany). The cells were manually lysed with a sterile pestle for approximately 5 min. Next, 200 μL of CTAB buffer was added, and the mixture was homogenized and incubated at 65 °C for 10 min. Then, 500 μL of chloroform was added, and the solution was homogenized and centrifuged at 20,500×*g* for 5 min. The supernatant was transferred to new tubes, and two volumes of cold 96 % ethanol were added. The DNA was precipitated at −20 °C for 30 min and centrifuged again at 12,000×*g* for 5 min. Subsequently, the pellet was washed with cold 70 % ethanol. The material was dried at room temperature, resuspended in 97.5 μL of TE containing 2.5 μL of 0.02 U μL^{-1} RNase and incubated at 37 °C for 5 min prior to storage at −20 °C (Gerrits Van Den Ende and De Hoog 1999).

b) Sequencing of the ITS segments of rDNA

The rDNA ITS was amplified using the primers V9G and LS266 (Gerrits Van Den Ende and De Hoog 1999) and sequenced with the primers ITS5 and ITS4 (White et al. 1990). The amplicons were purified using the GFX PCR DNA purification kit (GE Healthcare, U.K.). Sequencing was performed on an ABI 3130 automated sequencer. The sequences were edited and aligned using the Staden sequence analysis package v1.6.0 (Staden 1996). Sequence analysis was performed using the sequence alignment software BLASTn with the NCBI database (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/]. Phylogenetic analyses were performed by Mega 4.0.2 (Tamura et al. 2007) using the neighbor-joining method (Saitou and Nei 1987) and the Jukes-Cantor correct distance model (Jukes and Cantor 1969). The nucleotide sequence obtained in this study was deposited in GenBank under accession number JN191309.

Scanning electron microscopy

To observe the interactions between larvae and fungi, the materials were prepared for scanning electron microscopy (SEM) using the techniques described by Nordbring-Hertz (1983) and Campos et al. (2008). Dialysis membranes (Sigma®) were cut to a 6-cm diameter, placed inside Erlenmeyer flasks with distilled water, and autoclaved at 121 °C for 15 min. After autoclaving, six 6-cm diameter Petri dishes containing 2 % WA medium were covered with the membranes. Then, fragments of cultures of the CED strain were transferred to the dishes, and the edges were sealed with 2 % WA medium, thereby preventing the larvae from traveling down to the dialysis membrane (Campos et al. 2008). Subsequently, the plates were incubated at 26 °C in darkness for 4 days (Nordbring-Hertz 1983).

After a 4-day incubation, the Petri dishes were removed from the incubator, and 50 μL of a suspension of approximately 500 *Haemonchus* sp. third-instar larvae was added. Six control Petri dishes containing 2 % WA without fungi were used to assess the viability of the larvae and contamination with other fungi. During the first 8 h, the dishes were observed hourly under an optical microscope at 10× and 40× magnifications; the next observations were performed at 12, 18, 24, and 36 h post-inoculation. The backs of the Petri dishes were marked with a permanent marker, and the dialysis membranes within the marked areas were excised using a sterile scalpel.

The samples were fixed with 2.5 % glutaraldehyde in 0.05 M phosphate buffer at pH7.4 for 24 h. Then, they were washed six times in the same buffer and dried in a series of increasing ethanol concentrations of 30 %, 50 %, 60 %, 70 %, 95 %, and 100 % for 10 min each, with the exception of the 100 % concentration, in which the samples were immersed three times for 5 min (Nordbring-Hertz 1983).

The samples were dried to critical point under CO_2, coated in gold, and observed on a scanning electron microscope (JEOL-JSM 6360).

Isolation of infective Haemonchus sp. larvae for use in SEM

Sheep feces that were naturally infected with gastrointestinal nematodes predominantly composed of *Haemonchus* sp. (Cruz et al. 2011) were collected directly from the rectum to perform a coproparasitological assay. After obtaining *Trichostrongylidae* eggs, coprocultures were prepared with 20 g of feces mixed with sterile vermiculite at a 1:1 ratio (Roberts and O'Sullivan 1950) and were incubated at 28 °C. After 10 days, the infective larvae (L3) were recovered using the Baermann method, washed seven times with distilled water to prevent bacterial contamination, and centrifuged at 72.67×*g* for 10 min; we discarded the supernatant after each centrifugation. Subsequently, the nematodes were incubated overnight in a solution containing 0.05 % streptomycin sulfate and 0.05 % chloramphenicol. The washing process was repeated as previously described (Araújo et al. 1994). For larval counting, the contents of the tubes were standardized to 1 mL, and the estimated number of larvae in five 50-μL aliquots was extrapolated to the tube volumes.

These procedures are in agreement with the ethics committee and animal Universidade Estadual do Centro Oeste do Paraná—Brazil.

Results

Phase one—isolation, identification, and in vitro testing

Of the 56 soil samples collected from different regions of Paraná State, Brazil, 57 fungi were isolated. Of these, four isolates exhibited predatory activity against *Panagrellus* sp. in 2 % WA culture medium, although only two pure isolates were obtained; these were the CED and LIN strains.

Morphological identification using the microculturing technique revealed that the CED strain possessed conidia that ranged from 18.6 to 25.3 μm in length and were approximately 9.7 μm in width, and they were divided by a septum. The basal cell was at least two times smaller than the distal cell. The traps that formed were three-dimensional, and each ring was approximately 22.6 μm in diameter, which was suggestive of either *Arthrobotrys conoides* or *Arthrobotrys oligospora.*

The other strain, called LIN, identified as *Arthrobotrys musiformis* by the morphological characteristics of the conidia, which exhibited an elongated ellipsoidal to musiform shape, was bicellular with an inframedial septum and formed traps consisting of two-dimensional adhesive networks.

When assessing the in vitro predatory activity against *Trichostrongylidae* larvae in the Petri dishes, the CED strain exhibited 96.35 % efficacy, whereas the LIN strain exhibited 85.75 % efficacy (Table 1). Therefore, the CED strain was selected for the second phase of this study.

Phase two—molecular characterization and SEM

Because the classical methods of species identification did not lead to conclusive results, the ITS regions of rDNA were sequenced. The 601-bp sequence that was obtained was aligned and submitted to GenBank for comparison with other sequences. The nucleotide sequence obtained in this study was deposited in GenBank under accession number JN191309.

The molecular identification suggests that the CED isolate belongs to the species *A. conoides* (AY773455.1, with 97 % similarity). We used 15 strains of *Arthrobotrys* sp. deposited in GenBank with similarities ranging from 90 % to 100 % to the CED isolate for the phylogenetic analyses in this study (Table 2).

The tree based on the rDNA ITS sequencing was built by neighbor joining and by applying the Jukes-Cantor correct distance model implemented in Mega 4.0.2 with 1,000 rapid bootstrap inferences. According to the clustering, two major groups were obtained with high bootstrap values. The phylogenetic analysis showed that the CED isolate was closer to *A. conoides* than to *A. oligospora* (Fig. 1).

Using SEM, the fungal morphological plasticity was monitored at different predation stages, from trap formation to adhesion, capture and digestion and culminating in the death of the nematode, thereby confirming this isolate's predatory activity.

Trap formation was observed at 7 h after the addition of larvae. At 12 h, three-dimensional adhesive structures were observed (Fig. 2a) that trapped the third-instar larvae of *Haemonchus* sp. (Fig. 2b). Furthermore, two extensions in the infection bulb (Fig. 2c, d) were used to pierce the nematode's cuticle. In addition to the mechanical pressure exerted by the fungus (Fig. 2e) during penetration, it was also possible to observe the nematode's cuticle after penetration, suggesting the activity of hydrolytic enzymes that solubilize the cuticle (Figs. 2f and 3h).

Table 1 Average number of *Trichostrongylidae* (L3) infective larvae recovered by the Baermann method after incubation for 6 days in the in vitro assay conducted in Petri dishes with the fungi *A. conoides* and *A. musiformis* and the percent reduction relative to the control

Group	Nematode larvae Average per dish	Reduction (%)
A. conoides	26.4a	96.35
A. musiformis	103.2a	85.75
Control	724b	–

Different letters (a; b) indicate significant differences between the groups ($p<0.01$)

Table 2 GenBank sequences of *Arthrobotrys* spp. used for phylogenetic analysis

Species	Strain	Source	Region	GenBank Accession
Arthrobotrys conoides	CED	Soil	Brazil, Paraná state	JN191309
Arthrobotrys conoides	Strain 670	Soil	China	AY773455
Arthrobotrys javanica	CBS 534.63	Soil	Indonesia, Java island	U51947
Arthrobotrys oligospora	Isolated 109	Decaying wood	–	EU977526
Duddingtonia flagrans	NZ48C	–	–	AY095103
Duddingtonia flagrans	NZ144jR	–	–	AY095102
Arthrobotrys flagrans	CBS 583.91	Greenhouse soil	Germany, Berlin-Dahlem	AF106520
Arthrobotrys javanica	Isolated 105	Soil	–	EU977514
Arthrobotrys vermicola	Isolated 103	Decaying leaf	–	EU977508
Arthrobotrys oligospora	Isolated 128	Decaying wood	–	EU977563
Arthrobotrys thaumasia	Isolated 112	Decaying wood	–	EU977535
Arthrobotrys oligospora	Isolated 108	Dung	–	EU977523
Arthrobotrys flagrans	ATCC MYA-4124	Vineyard soil	USA, California state	EF445995
Arthrobotrys thaumasia	CBS 322.94	Cold greenhouse	Germany, Berlin-Dahlem	AF106526
Arthrobotrys amerospora	AA1	–	–	EF192178
Arthrobotrys amerospora	BBA 68701	–	Germany	AF106533

Figure 3g, h shows a large quantity of bacilli at the bulb's penetration site.

Throughout the process, the three-dimensional networks increased in addition to the imprisonment of the nematode at various points on its body, which became completely immobilized and overtaken by hyphal growth (Fig. 3i, j). After 18 h, many larvae were dead, whereas others were "exhausted", i.e., they moved slowly in the presence of

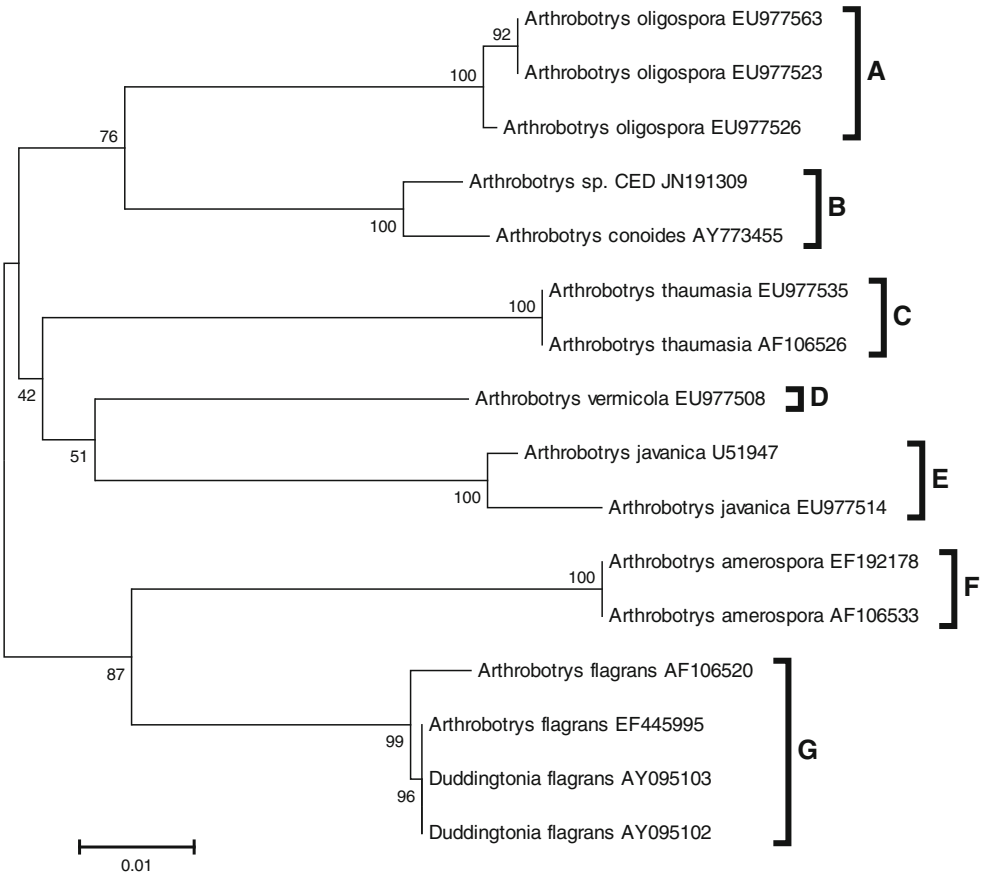

Fig. 1 Phylogenetic tree based on confidently aligned ITS sequences constructed using the neighbor-joining method. The numbers on the tree branches indicate the bootstrap values from 10,000 replicates using Mega 4.0.2. Sequence available under GenBank accession number JN191309

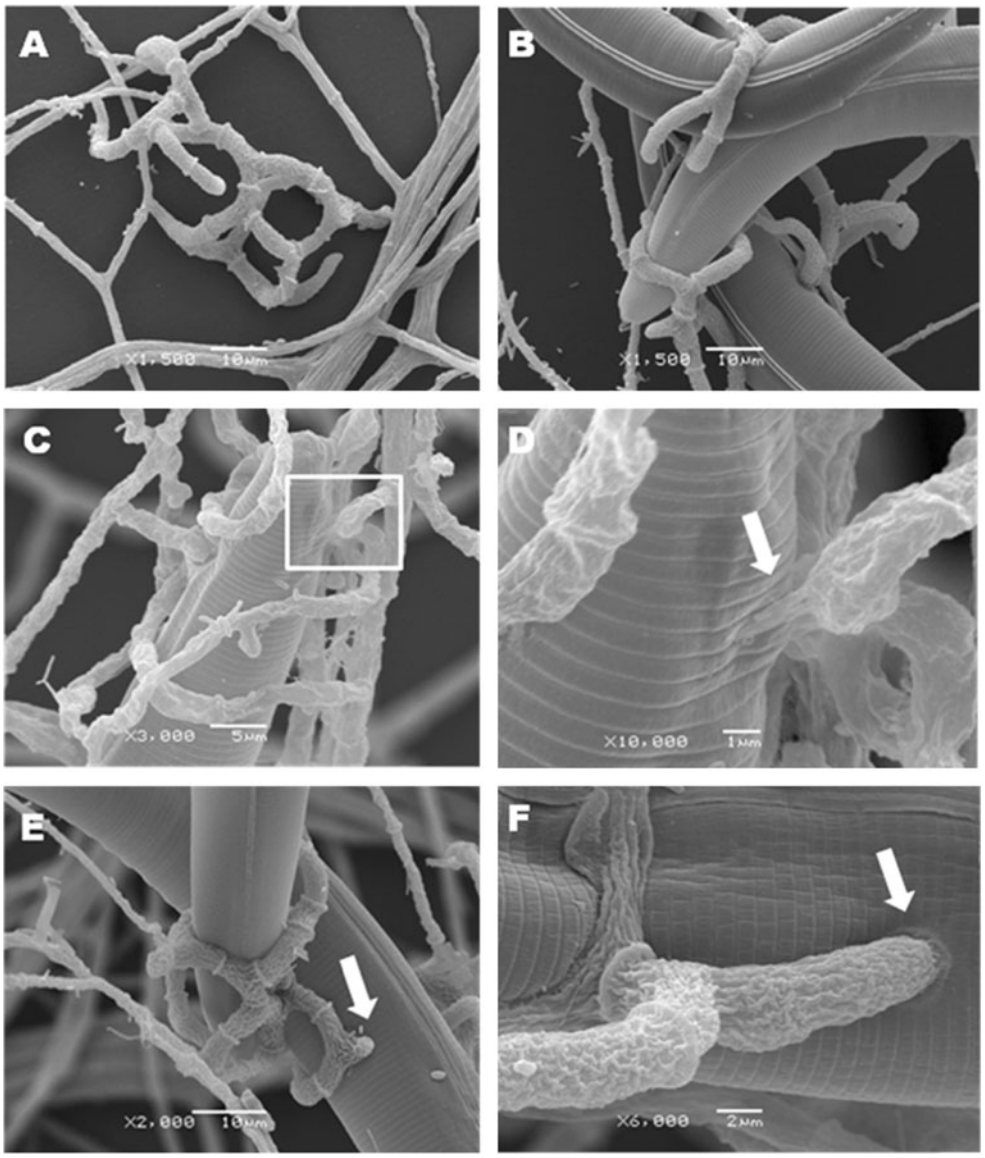

Fig. 2 SEM of the trap formation process and interaction between the nematophagous fungus, *A. conoides*, and L3 of *Haemonchus* sp. at the dialysis membrane surface. **a** Formation of three-dimensional networks 12 h after inoculation with nematode larvae. **b** Infective larvae of *Haemonchus* sp. captured after 12 h of interaction between the nematode and the fungus. **c** L3 captured and immobilized in three-dimensional networks. **d** Magnification of the area highlighted in panel **c** showing the extension of the infection bulb. **e** Hyphal pressure point on the nematode's cuticle; *arrow* indicates the pressure point. **f** Hyphal penetration site (*white arrow*) on the nematode's cuticle

light. Other newly captured worms struggled vigorously, thereby demonstrating the force involved in the processes of larval adhesion and trapping.

At 24 h post-inoculation, most of the trapped L3 exhibited no further movement in the presence of light, and at 36 h, some larvae appeared "shriveled", i.e., empty, indicating absorption by the fungi (Fig. 3l, m).

Discussion

It is known that season, temperature, and the amount of organic matter in the soil are factors that may correlate with successful strain isolation. However, the greatest difficulty we encountered was in isolating pure strains; often, the trap structures were observed, but the faster growth of other fungi, especially *Fusarium* and *Aspergillus*, prevented successful isolation for transplanting. It is possible that the increased pH of the culture medium prevented the development of other microorganisms (Gardner et al. 2000), or it may be that other isolation methods lead to better results (Kelly et al. 2009) .

The two strains isolated in this study, CED and LIN, are from the south-central region of Paraná state, Brazil, a region that is characterized by a mesothermal, humid subtropical climate with no dry season, cool summers, moderate winters, and an average temperature of 14 °C in the coldest month and 20.3 °C in the hottest month from 1976 to 2009 (Iapar 2009). Annual rainfall varies from 1,400 to 2,000 mm, with the lowest rainfall levels in April, May, and August. The altitude of the region is approximately 1,100 m (Maak 1968).

The soil sample from which the CED strain was isolated was collected in late spring (November) from an area of crop-livestock integration where sheep are consigned in the

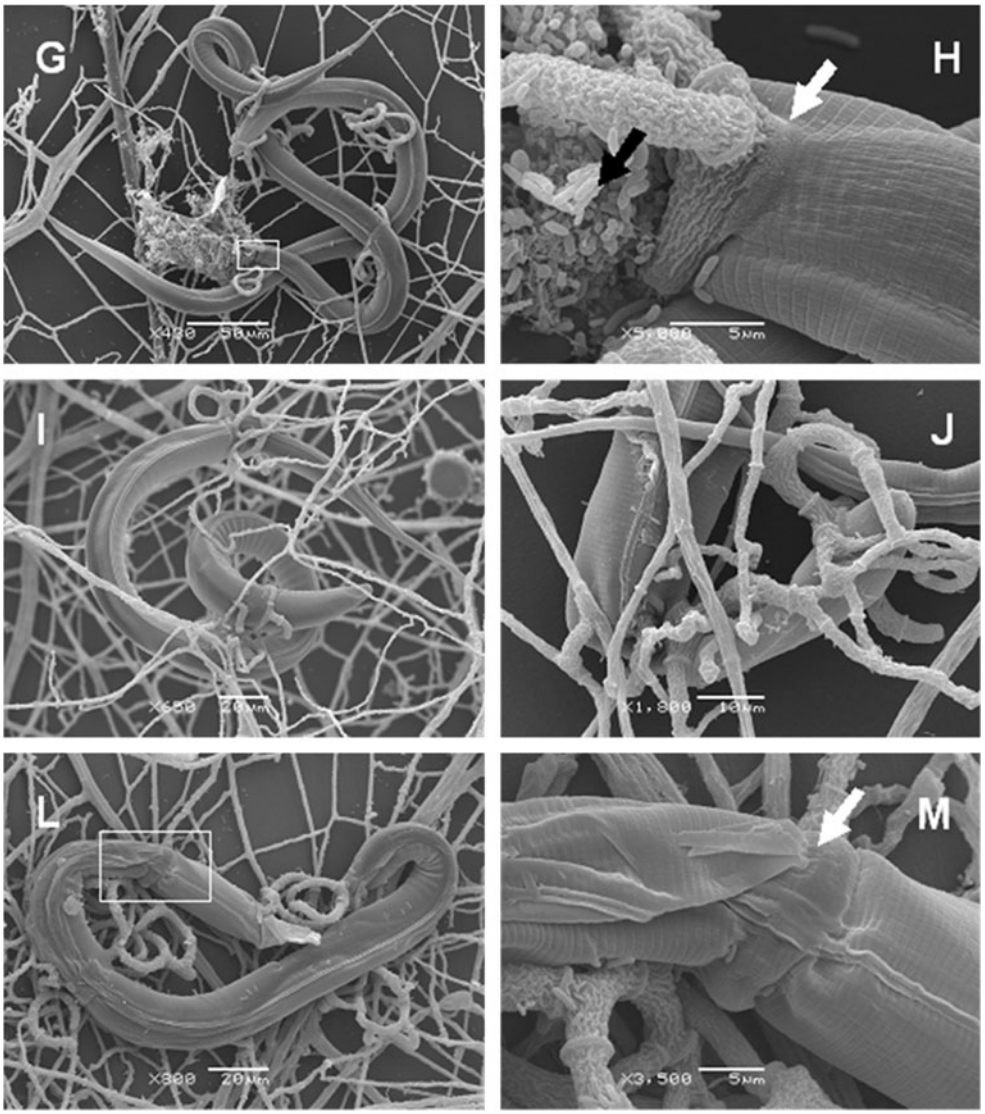

Fig. 3 SEM of the trap formation process and interaction between the nematophagous fungus, *A. conoides*, and L3 of *Haemonchus* sp. at the dialysis membrane surface. **g** Trapped larvae. **h** Magnification of the area highlighted in panel **g** showing the presence of a large number of NHB (*black arrow*, panel **h**). Note the site of penetration by the fungus and the hydrolysis of the nematode's cuticle (*white arrow*, panel **h**). **i** and **j** Larvae trapped in three-dimensional networks at various points on the body. **l** After 36 h, the turgor of the L3 is decreased. **m** Magnification of the area highlighted in panel **l** showing the presence of hyphae on the larva's body (*white arrow*), suggesting the digestion of the larva

winter. The soil sample from which the LIN strain was isolated was collected in October from a commercial sheep property, which is considered ideal for isolating agents used in the biological control of nematode parasites because there is a higher probability of these agents surviving the passage through the gastrointestinal tract of ruminants (Kelly et al. 2009).

The morphological identification of the LIN strain was conclusive for *A. musiformis*, but the morphological identification of the CED strain was only suggestive for *A. conoides* and *A. oligospora*. According to Cooke and Godfrey (1964), conidia of *A. conoides* are conical, 19–42 μm in length and 8–15 μm in width and constricted at the septum with a flattened base and larger distal cell. In contrast, the conidia of *A. oligospora* are ovoid and more globose, have a length of 22–32 μm and a width of 12–20 μm and have a spiked base. According to Philip (2002), *A. oligospora* conidia are 16–30 μm in length and 8–16 μm in width, with a distal cell that is twice as large, they are flattened at the septum and the traps are 20–59 μm in diameter. Oliveira et al. (2002) conducted morphological and isoenzyme characterization of several nematophagous fungal isolates isolated from various regions of Brazil and found that Brazilian strains of *A. conoides* had conidia of 31–44 μm in length and 11–14 μm in width, and *A. oligospora* had conidia of 17–28 μm length and 11–14 μm width, which does not agree with what was proposed by Cooke and Godfrey (1964) and Philip (2002). Therefore, from the references consulted and microscopic observation of the morphological characteristics, the CED strain was suggestive of *A. conoides* and *A. oligospora.* Perhaps, the difficulty of identifying this species is related the medium culture used (Singh et al. 2005).

Because this strain exhibited better in vitro predatory activity against third-instar larvae of *Trichostrongylidae*, it was selected for the second phase of the study. Molecular

identification and phylogenetic analysis confirmed that the CED strain was *A. conoides*. This is the fourth strain that had an ITS region sequence that was deposited in GenBank under JN191309.

The *Arthrobotrys* genus is distributed worldwide and in Brazil, where it is researched as fungi with predatory activity against nematodes (Dalla Pria et al. 1991; Naves and Campos 1991; Nordbring-Hertz et al. 2002). The fungus can utilize two different carbon sources, one in organic matter as a saprophyte and the other from predatory activity against nematodes, which makes it adaptable to various habitats (Wachira et al. 2009), and thereby produces a smaller impact on the environment when used in biological control programs.

The plasticity of the *A. conoides* strain JN191309 was verified in predation against the third instar larvae of *Haemonchus* sp. by SEM. Despite several studies of the predatory mechanisms used by nematophagous fungi, in this study, it was possible to clearly document the extensions in the infection bulb that were used to pierce the nematode's cuticle. Nordbring-Hertz et al. (2006) studied the predatory mechanism using SEM; however, in the present study, the morphological plasticity of the fungus while penetrating the nematode was clearly visualized by SEM.

According to Araújo et al. (2001), the recognition of the larval cuticle by the fungus appears to be mediated by a lectin–carbohydrate interaction. Nordbring-Hertz et al. (2006) suggested that this process may be affected by proteases with high homology to subtilisin PII, which have demonstrated nematotoxic activity, in addition to penetration and digestion. According to Wang et al. (2006), the fungal penetration of the nematode's cuticle is a combination of physical force and hydrolytic enzymes.

The presence of nematophagous fungus helper bacteria (NHB) was previously observed by other authors who reported their association with *D. flagrans* and *Haemonchus contortus* larvae (Campos et al. 2008) and with *A. conoides* and *A. musiformis* (Graminha et al. 2001). Dupponois et al. (1998) hypothesized that these bacteria are involved in the processes of fungal predation, sporulation, and pathogenicity because they produce substances that act as molecular bridges between the fungus and the nematode.

According to Mankau (1980), the *Arthrobotrys* genus is as complex as the *Penicillium* genus; among 50 strains of *A. conoides* that were isolated, different patterns of sporulation, chlamydospore production, growth, and predation were reported. Therefore, to facilitate its industrial-scale production, a detailed study of the physiology, ecology, and biochemistry of this strain is necessary to assess its predatory activity in the field and to investigate which physical and chemical factors in the soil favor or inhibit its development.

The results obtained in this study show that *A. conoides* strain JN191309, which was isolated in Brazil, is a promising alternative for use in the control of infective larvae of trichostrongylides.

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