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Short communication

Comparative analysis of destruction of the infective forms of *Trichuris trichiura* and *Haemonchus contortus* by nematophagous fungi *Pochonia chlamydosporia*; *Duddingtonia flagrans and Monacrosporium thaumasium* by scanning electron microscopy

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ABSTRACT

The present study aimed to demonstrate by scanning electron microscopy (SEM) the *in vitro* predatory activity of nematophagous fungi *Pochonia chlamydosporia* (VC1 and VC4 isolates) *Duddingtonia flagrans* (AC001 isolate) and *Monacrosporium thaumasium* (NF34a isolate) on eggs of *Trichuris trichiura* and infective larvae (L3) of *Haemonchus contortus*. The work was divided into two experimental tests (A and B). In tests A and B, the predatory activity of nematophagous fungi *P. chlamydosporia, D. flagrans* and *M. thaumasium* on eggs of *T. trichiura and H. contortus* L3 was observed. After 6 h, in test A, isolates *P. chlamydosporia* (VC1 and VC4) had a role in destroying eggs of *T. trichiura*. For fungi *D. flagrans* and *M. thaumasium* the ovicidal activity on *T. trichiura* eggs was not observed. Test B showed that *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were capable of predating *H. contortus* L3, but no predation by the fungus *P. chlamydosporia* was seen. These fungi can offer potential for the biological control of nematodes.

1. Introduction

Alternative measures for the control of endoparasitosis of humans and domestic animals have been widely searched in the world by researchers (Braga et al., 2007; Araújo et al., 2008). *Trichuris trichiura* is a nematode of major importance in public health, infecting approximately 604 million people in the world, being the second greatest prevalent globally. In mild infestations, this nematode causes few symptoms, but in massive infestations it can cause bloody diarrhea and diarrhea with mucus, and it can be associated with rectal prolapse. In the developing countries of Asia, Africa and Latin America the deficient sanitary infrastructure and hot, humid climates provide the necessary conditions for eggs of this parasite to incubate in the soil (Hotez et al., 2008).

Haemonchus contortus is a hematophagous nematode of major prevalence and veterinary medical importance for small ruminants in tropical climate countries, having as infective form a third-stage larvae (L3) (Amarante et al., 2009).

Nematophagous fungi are a viable and promising alternative, which can be an option in controlling gastrointestinal nematodes of humans and domestic animals (Larsen et al., 1998; Braga et al., 2007). Its action is focused on faecal environments where important status changes of gastrointestinal nematode parasites take place. There are different types of nematophagous fungi, which can be characterized as opportunistic or parasitic of eggs, predators and endoparasites (Silva et al., 2009).

Among opportunists or parasites of eggs, the species *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) stands out (Gams and Zare, 2001). According to Lysek and Sterba (1991), the action of this fungus is based on appressorial formation, developed from undifferentiated

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hyphae, which allows the colonization of the egg surface and penetration through both mechanical and enzymatic actions, characterizing a type 3 effect (eggs destruction) (Lysek, 1976, 1978).

Species of predator fungi differ in their ability of capturing larvae of nematodes. They are the most studied organisms in the biological control of nematodes, and present higher potential for marketing (Gronvold et al., 1996). These fungi also show some kind of interaction on the eggs of gastrointestinal helminthes parasites, but without causing its destruction. In this group, the *Duddingtonia* and *Monacrosporium* genera stand out due to their efficacy in the biological control of gastrointestinal nematode parasites (Dimander et al., 2003; Araújo et al., 2008).

The objective of this work was to demonstrate by scanning electron microscopy (SEM) the *in vitro* predatory activity of isolate fungi *P. chlamydosporia, Duddingtonia flagrans* and *Monacrosporium thaumasium* on eggs of *T. trichiura* and infective larvae of *H. contortus*.

2. Material and methods

2.1. Fungi

Isolates of the nematophagous fungi of the species *P. chlamydosporia* (VC1 and VC4), *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were maintained at 4 °C, protected from light and in assay tubes with corn–meal–agar 2% (2% CMA). These isolates were further peaked for plates in water–agar medium 2% (2% WA) where they grew for 7 days. Culture plates of 4 mm in diameter were extracted from fungal isolates maintained in assay tubes with 2% CMA and were transferred into Petri dishes of 9.0 cm in diameter with 20 ml of potato–dextrose–agar 2% and kept at 25 °C, in the dark for 10 days.

2.2. Obtainment of T. trichiura eggs

T. trichiura unembryonated eggs were obtained from human faeces, descendents from Native Brazilian Amazon societies. They were extracted and concentrated by spontaneous sedimentation for 2–4 h, which were kept in refrigeration from 4 to 8 °C. This material was donated by the Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil. The eggs were later washed with distilled water and centrifuged five times discarding the supernatant.

2.3. Obtainment of H. contortus infective larvae (L3)

H. contortus (L3) were obtained from coprocultures with 100% of *H. contortus* L3 from positive ovine faeces according to Gordon and Whitlock (1939). After stool tests at 26 °C for 10 days, L3 were recovered according to Barçante et al. (2003), washed with distilled water and centrifuged five times discarding the supernatant.

2.4. Experimental assay and tests A and B

This work is constituted by two experimental tests (A and B). In test A, the objective was to observe the ovicidal activity of fungi *P. chlamydosporia* (VC1 and VC4), *D. flagrans* (AC001) and *M. thaumasium* (NF34a) on eggs of *T. trichiura*. In test B, the objective was to observe the predatory activity of nematophagous fungi *D. flagrans* (AC001), *M. thaumasium* (NF34a) and *P. chlamydosporia* (VC1 and VC4) on L3 of *H. contortus*.

After the mycelial grew on the entire surface of the Petri dishes, 100 eggs of *T. trichiura* (test A) and 1000 *H. contortus* of L3 (test B) were poured on the dialysis membranes of the 9.0 cm in diameter Petri dishes with medium 2% WA of treated groups, and in control plates (without fungus). In the first 24 h after the inoculation of nematodes on the Petri dishes, cultures on the plates were observed every 6 h through a light microscope (100×). After observing larvae of *H. contortus* larvae preyed and *T. trichiura* eggs in certain areas of the plate, marks were made on bottom of them with a permanent marker.

2.5. Scanning electron microscopy

Culture plates of fungal isolates *P. chlamydosporia* (VC1 and VC4), *D. flagrans* (AC001) and *M. thaumasium* (NF34a) were transferred into disposable Petri dishes of 6.0 cm × 1.0 cm. Surfaces of the plates were covered with cellulose membrane plates (dialysis membrane), with proteins of a molecular weight over 12,000 Da and with a filtering capacity approx. 640 mL/ft (Sigma–Aldrich®, U.S.A). The dialysis membrane was cut into 6 cm diameter disks that were placed in Erlenmeyer flasks with distilled water. The material was autoclaved at 120 °C for 15 min. After, they were removed from the Erlenmeyer flasks with the aid of a clamp and placed on 2% WA surfaces so that the membrane margins covered all agar surfaces and the edges were attached to the edge of the plates. These edges were also covered with 2% WA. Next, plates were incubated in the dark, at a temperature of 25 °C for 7 days (Nordbring-Hertz, 1983).

After 6 h of observation of the interaction with *T. trichiura* eggs (test A) and with *H. contortus* L3 (test B), pieces of the dialysis membrane with eggs of *T. trichiura* parasitized fungi and L3 samples exposed to capture were cut with the aid of a blade, collected with a fine-tipped clamp and fixed in glutaraldehyde at 2.5% in 0.05 M of phosphate buffer, pH 7.4 and for 24 h. Next, they were washed six times in the same buffer, post-fixed in osmium tetroxide 2% and dehydrated by passing the material in a series of ethyl alcohol (30%, 50%, 60%, 70%, 95% and 100%). The material was dry in critical point dryer BALZERS® using carbon dioxide, recovered with gold plating (Nordbring-Hertz, 1983; Guimarães and Caldeira, 1997) and electron-micrographed in a scanning electronic microscope LEO, model 1430VP at 10–15 kV.

3. Results

In the experimental test A, by observing SEM, it was found that *P. chlamydosporia* isolates (VC1 and VC4) had a role in destroying *T. trichiura* eggs after a 6 h observation period (Fig. 1a and b). The formation of appressoria of *P. chlamydosporia* on *T. trichiura* eggs caused the formation of a halo on the eggs surface, suggesting enzymatic and mechanic actions, later causing its penetration and destruction (Fig. 2a and b).

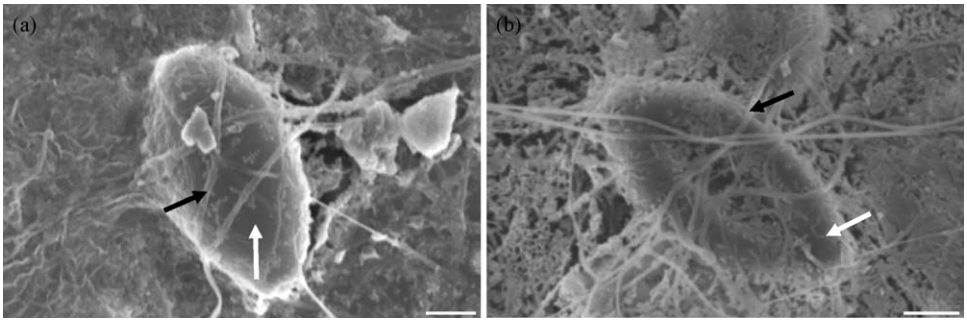

Fig. 1. (a and b) Destruction of *Trichuris trichiura* eggs (white arrow) after the 6-h observation period by isolates of *Pochonia chlamydosporia* (VC1 and VC4) (black arrow). SEM. Bar: (a) 10 μm; (b) 10 μm.

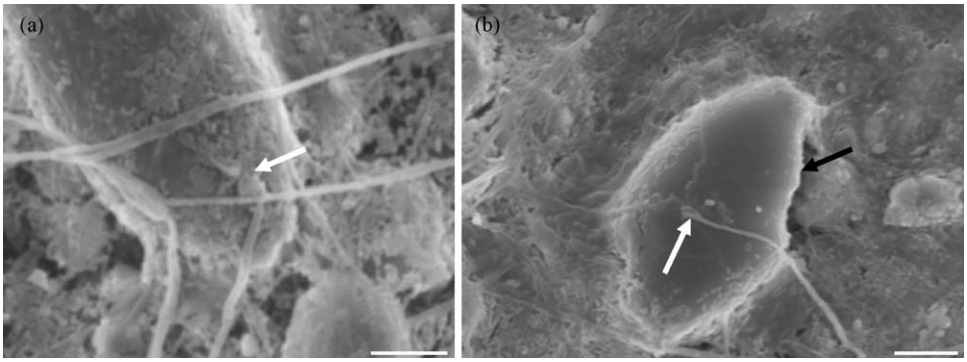

Fig. 2. (a and b) Formation of appressoria of *Pochonia chlamydosporia* (white arrow) on *Trichuris trichiura* eggs (a and b), causing its destruction (black arrow) (b). SEM. Bar: (a) 10 μm; (b) 10 μm.

For predator fungi *D. flagrans (*AC001*)* and *M. thaumasium* (NF34a), it was noticed by light microscope the interaction of predator fungi in the first 6 h of the test with *T. trichiura* eggs where hyphae of these isolates were attached to the surface of eggs, without causing destruction was noticed by light microscope. Likewise, scanning electron microscopy revealed hyphae of *D. flagrans* (AC001*)* and *M. thaumasium* (NF34a) attached to the surface of the eggs during the observation period, but without destroying the eggs (Fig. 3a and b). No predation of *T. trichiura* eggs in the control plates was observed (Fig. 4).

The results showed in test B that *H. contortus* L3 visualized by SEM, were preyed after 6 h by the predator fungi *D. flagrans (*AC001*)* and *M. thaumasium* (NF34a) that produced traps and caused its destruction (Figs. 5a–c and 6). It was noticed in all Petri dishes that specific structures (conidia and traps) of predator fungi attached to nematodes and later caused its destruction (Fig. 7a and b). In addition, chlamydospores of fungus *D. flagrans* were observed on Petri dishes of the treated groups (Fig. 8a and b). Concerning the fungus *P. chlamydosporia* (VC1 and VC4), no predatory activity on *H. contortus* L3 throughout the experimental test was noticed.

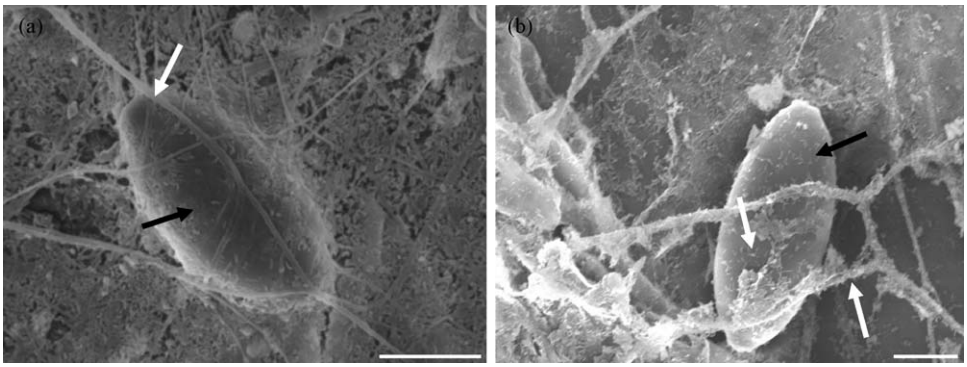

Fig. 3. (a and b) Hyphae of fungi (a) *Duddingtonia flagrans* (AC001) and (b) *Monacrosporium thaumasium* (NF34a) (white arrow) attached to the surface of eggs (black arrow) during the entire observation period, without destruction of eggs. SEM. Bar: (a) 20 μm; (b) 20 μm.

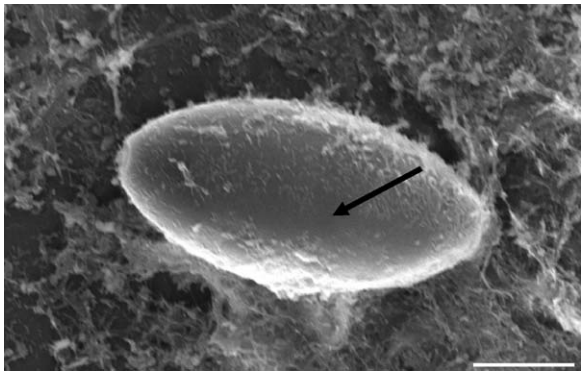

Fig. 4. *Trichuris trichiura* eggs (black arrow) without fungus (control). Bar: 20 μm.

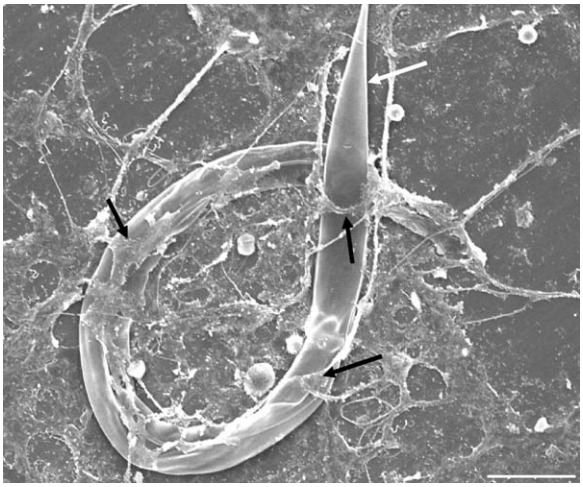

Fig. 6. SEM of infective larvae (L3) of preyed *Haemonchus contortus* (white arrow) by predator nematophagous fungi stimulating the production of traps (black arrow). Bar: 40 μm.

4. Discussion

During the experimental test A, it was noticed that *P. chlamydosporia* isolates (VC1 and VC4) were found to have a role in destroying *T. trichiura* eggs after the 6 h observation period. This result is consistent with the work of Braga et al. (2008) who recorded by SEM the destruction of *Schistosoma mansoni* eggs throughout the experimental test. However, in the present work, the ovicidal activity of *P. chlamydosporia* was observed throughout the experimental test by SEM. This information is important, because its ovicidal action is characterized after 6 h of interaction with the eggs. In addition, it was observed the formation of appressoria of *P. chlamydosporia* causing the penetration and destruction on *T. trichiura* eggs was observed in the present work. This is the first work analyzing the interaction of nematophagous fungi on *T. trichiuria* eggs.

In the experimental test B the production of traps, non-adhesive constricting rings, non-constrictive rings, adhesive 3D networks and buttons on *H. contortus* L3 was

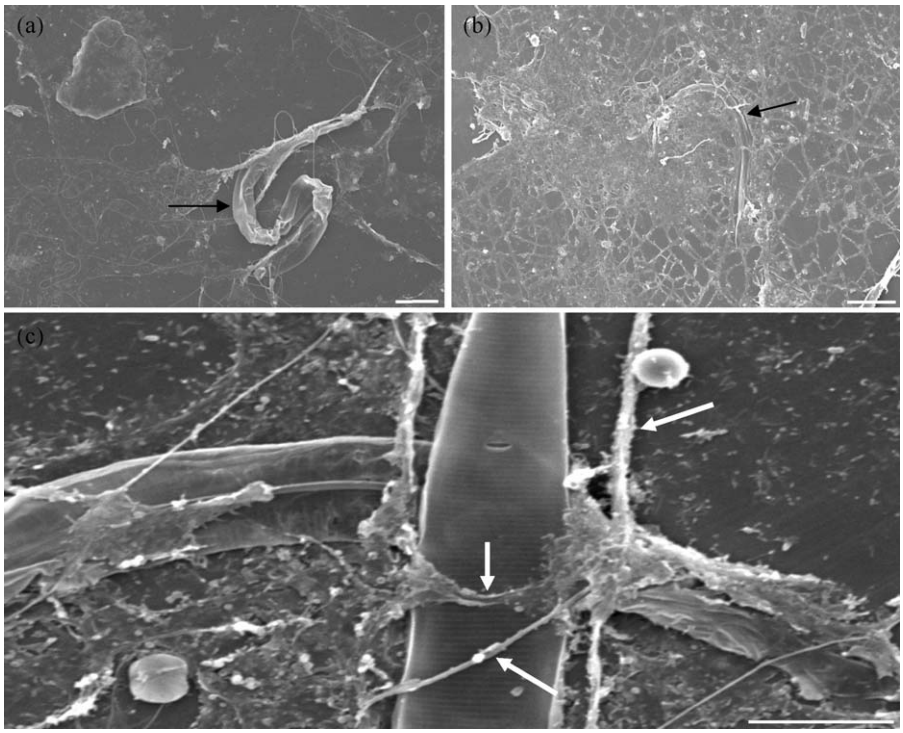

Fig. 5. (a and c) Infective larvae (L3) of preyed *Haemonchus contortus* (black arrow) by predator fungi *Duddingtonia flagrans* (AC001) (a and c) and *Monacrosporium thaumasium* (NF34a) (b) with production of traps (white arrow), causing its destruction, after 6 h. Scanning electron microscopy (SEM). Bar: (a) 40 μm; (b) 100 μm; (c) 20 μm.

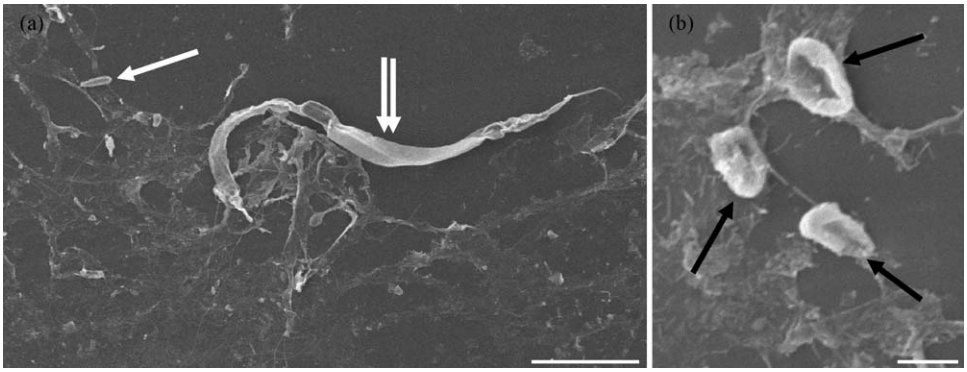

Fig. 7. (a and b) Observation of specific structures of the predator fungus *Duddingtonia flagrans* attached to infective larvae (L3) of *Haemonchus contortus* (white double arrow) and later causing its destruction. (a) Conidia (white arrow); (b) traps (black arrow). SEM. Bar: (a) 100 μm; (b) 10 μm.

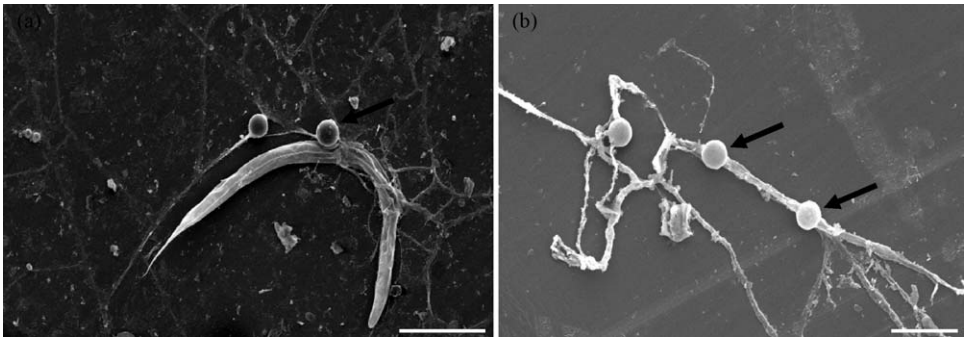

Fig. 8. (a and b) Production of chlamydospores (black arrow) of fungus *Duddingtonia flagrans* in Petri dishes. SEM. Bar: (a) 100 μm; (b) 50 μm.

observed. Specific structures (conidia and chlamydospores) of fungus *D. flagrans* and *M. thaumasium* were observed attached to *H. contortus* L3. According to Araujo et al. (2004), the production of chlamydospores is the main requirement for a fungus to be used as a possible biological controller. For predator fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a), hyphae of these isolates attached to the surface of eggs using light microscope and SEM were noticed. These results are consistent with Braga et al. (2007) who mention that predator fungi show effect without causing the destruction of parasitized eggs. Concerning the fungus *P. chlamydosporia* (VC1 and VC4), it did not destroy *H. contortus* L3 throughout the experimental test. This information is consistent with works performed with this fungus on eggs of gastrointestinal helminthes (Braga et al., 2010) where its ovicidal action, and not larvicidal action, was proven.

Predator fungi formed traps produced at intervals throughout the hyphae. In pure cultures, many of these fungi did not form traps. The formation of these structures is a response to the presence of nematodes or their substances (Larsen, 1999). They are also induced by adverse culture conditions, such as scarcity of water and/or nutrients. The hyphae differentiation in this work occurred after the 6 h interval where numerous trapping structures were produced. According to Araujo et al. (2004), after capturing larvae through capture structures of predator fungi a process of penetration of hyphae in the cuticle occurs, followed by the digestion of the internal content of the nematode. The most observed differentiated structures of these fungi throughout the mycelium are non-adhesive constricting rings, non-constrictive rings, adhesive 3D networks and buttons.

5. Conclusion

Using scanning electron microscopy, the results presented in this work showed that fungus *P. chlamydosporia* destroyed the *T. trichiura* eggs, showing for the first time its ovicidal activity. *D. flagrans* and *M. thaumasium* destroyed *H. contortus* L3. Therefore, these fungi could be used as possible biological controls of these nematodes.

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