German Journal of Veterinary Research

eISSN:2703-1322

Research Article

Evaluation of nematophagous fungal mycelial growth and interactions with bovine gastrointestinal parasitic nematodes

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Article History:
Received: 05-Apr-2022
Accepted: 21-Apr-2022
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Abstract

Previous research has shown an increased action on helminth biological control by fungal combinations. This study characterized the temperature and pH conditions necessary for better mycelial growth of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4). In addition, electron and optical microscopy showed the fungal structures that benefit from their use in the biological control of nematodes and interactions with infective larvae of helminths. Nematode larvae held by *P. chlamydosporia* mycelium confirm its ability to prey upon larvae stages, despite being classified in the "ovicidal" group. *P. chlamydosporia* showed the highest growth rate in water agar medium at 20°C, whereas *M. sinense* showed numerically better growth at 30°C. Fungi did not grow at 35 or 40°C. Surprisingly, the mycelial growth of both isolates was inhibited by temperatures above 35°C for 6 days and resumed when temperatures were reduced to 25°C. The pH observation was important to show that the pH variations in the gastrointestinal tract of bovines will not be harmful to fungi since offering oral formulations to the animals is the most practical way of dispersing fungi in the fecal pats. *In-vitro* studies facilitate the exploration of biological control agents. The use of nematophagous fungi is a viable solution in the control of gastrointestinal nematodes and needs to be further improved.

Keywords: Biological control, Environmental conditions, Helminths, *Monacrosporium sinense*, *Pochonia chlamydosporia*, Predation

Citation: Oliveira, I. C., Vieira, I. S., Freitas, S. G., Campos, A. K., Paz-Silva, A., C. F. C., de Gives, P. M. and de Araújo, J. V. 2022. Evaluation of nematophagous fungal mycelial growth and interactions with bovine gastrointestinal parasitic nematodes. Ger. J. Vet. Res. 2 (2): 39-45. https://doi.org/10.51585/gjvr.2022.2.0035

Introduction

For long-term effective parasite control in animal production systems, a combination of management strategies is required. Nematophagous fungi can be an important adjunct to the traditional gastrointestinal nematode control (Araújo et al., 2021; Canhão-Dias et al., 2020; Holsback et al., 2021; Szewc et al., 2021). Nematophagous fungi are natural nematode predators in soil (Braga and de Araújo, 2014; Zhang et al., 2014), where they develop parasitic or predatory relationships with nematodes and are classified as nematode-trapping, opportunistic or ovicidal, endoparasitic, toxin producers and producers of specific attack devices (Araújo et al., 2021).

The fungus *Pochonia chlamydosporia* parasitizes eggs, female nematodes (Dallemole-Giaretta et al., 2013; Vieira et al., 2019), belongs to the nematophagous fungal group known as "ovicidal fungi" (Araújo et al., 2021), and the species *Monacrosporium sinense* predates nematode larvae through adhesive networks (Campos et al., 2007). Each fungus attacks nematodes at different stages, and a compatibility study between *M. sinense* and *P. chlamydosporia* has demonstrated greater success in nematode control by their combined use (Oliveira et al., 2021a). However, fungi need to find suitable environmental conditions, such as an optimum temperature, for the efficient predatory activity of nematode infecting forms (Vieira et al., 2020). Thus, basic and applied studies about the biological variables influencing the development of

these microorganisms are required (Vieira et al., 2020; Ocampo-Gutiérrez et al., 2021).

Studies on nematophagous fungi controlling gastrointestinal nematode infective stages, particularly in pasturing ruminants (Mendoza-de Gives et al., 2018; Oliveira et al., 2018; Vieira et al., 2019; Voinot et al., 2020), have provided substantial evidence that these microorganisms can efficiently be used in nematode control (ABCBIO, 2016; Araújo et al., 2021; Braga et al., 2020; Oliveira et al., 2021b). In addition to their predatory activity, their rapid growth is another important factor for their survival and spread in the environment (Anan'ko and Tepliakova, 2011; Vieira et al., 2020), resulting in significant commercial interest (Braga et al., 2020; Oliveira et al., 2021b); however, related data are scarce. In this context, this study investigated the optimal pH and temperature conditions of *M. sinense* and *P. chlamydosporia* growth under laboratory conditions, with the aim to enhance the use of fungi in biological nematode control strategies.

Material and methods

Fungal species

The fungi *Monacrosporium sinense* (SF53 isolate) and *Pochonia chlamydosporia* (VC4 isolate) used in this study are part of the collection of the Parasitology Laboratory of the Veterinary Department, Federal University of Viçosa, where they are kept at 4°C in dark in test tubes containing 2% corn meal agar medium (2% CMA). These isolates have been obtained from Brazilian agricultural soil in the municipality of Viçosa (Zona da Mata, Minas Gerais), and were collected using the soil-sprinkling method of Duddington (1955).

Assessing the mycelium growth of the nematophagous fungi

Disks (5 mm in diameter) of the fungi *M. sinense* and *P. chlamydosporia*, obtained from pure cultivation in a 2% CMA culture medium, were transferred separately to the center of 9-cm diameter Petri dishes containing 20 mL of 2% water agar medium (2% WA) (Kasvi® Laboratory Products, Brazil) at pH 7. The plates were kept in a biochemical oxygen demand incubator ((BOD, Prolab Materials, Brazil) at temperatures of 15, 20, 25, 30, 35, and 40°C in the dark. The same procedure was performed using 2% potato dextrose agar medium (2% PDA) (Kasvi® Laboratory Products, Brazil) and 2% corn meal agar medium (2% CMA) (Sigma-Aldrich, Brazil) at pH 7. These treatments consisted of a 2×3 factorial arrangement, with two isolates of nematophagous fungi and three culture media. The experimental design was completely randomized with six replications, with each replication consisting of one Petri dish.

Observations were started 24 hrs after starting the experiment. The mycelial growth of the isolates was determined by measuring the diameter of the colonies at 24 hrs intervals, using a millimeter ruler, and taking two measurements in perpendicular directions on each plate, as shown in Figure 1. For 6 days, measurements of mycelial growth were carried out until the growth of the colony of one of the isolates occupied the entire surface of the medium (Lilly and Barnett, 1951).

The mycelial growth rate index was calculated according to the formula described by Oliveira (1991): MGRI = Σ (D - Da)/N, where "MGRI" (cm/day) is the mycelial growth rate index; "D" is the current average colony diameter; "Da" is the mean diameter of the colony from the previous day; "N" is the number of days after inoculation. After the measurements, when a certain temperature totally inhibited fungal growth on the 6th day in certain plates, these plates were transferred to a BOD at 25°C and continuously observed daily for another nine days, resulting in 15 observations and no measurements were made over these nine days.

In parallel, 5-mm diameter disks of VC4 and SF53 strains were inoculated in the center of Petri dishes containing a 2% PDA medium to analyze the effect of pH on growth. The pH values of the 2% PDA medium were adjusted from 4 to 10 using an acidometer and 3.0 M NaOH solution. The Petri dishes were incubated for 6 days, at 25°C, in the dark in a BOD chamber. The mycelial growth rate index was evaluated as described above in the temperature test, and six repetitions were performed for each fungal strain at each pH value.

The mean values of the MGRI for each temperature and pH condition were submitted to the Kruskal-Wallis nonparametric statistical test at a significance level of 5%. The analyses were performed using the IBM SPSS Statistics 2.0 software.

Electron and optical microscopy of the nematophagous fungi and interactions with bovine gastrointestinal nematodes

Images of the fungal mycelia, their structures, and interactions with bovine gastrointestinal nematode larvae were obtained under optical and scanning electron microscopes (SEM). The preparations for SEM followed the technique proposed by Nordbring-Hertz (1983), with modifications. Cultures of SF53 and VC4 isolates were separately plated on dialysis membranes in Petri dishes containing 2% WA medium and incubated in the dark at 25°C for 21 days. Subsequently, approximately 500 infective larvae (L3) of bovine gastrointestinal parasites previously obtained by coprocultures were dripped onto the cultures of each fungus grown on a dialysis membrane surface.

After 72 h of fungi-nematode interactions, cultures were fixed on plates with 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, for 72 h. Subsequently, the plates were washed six times in the same buffer. With the aid of a scalpel, dialysis membrane flaps were cut and collected with fine-tipped forceps and then dehydrated through an alcohol serial passage (30, 50, 60, 70, 95, and 100%). Then, the samples were dried in a BALZERS® critical point dryer using carbon dioxide, covered with gold in a metalliser, and electronmicrographed in a LEO scanning electron microscope at 10–15 kV at the Center for Electron Microscopy and Microanalysis at the Federal University of Viçosa.

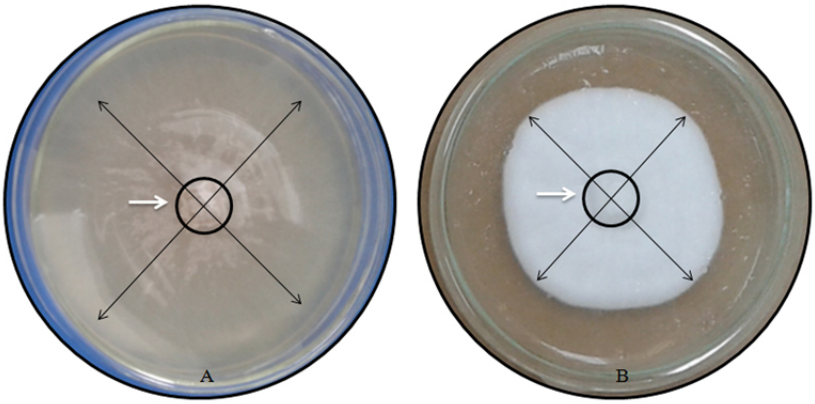

Figure 1: Petri dishes used in the growth bioassay of the fungi *Monacrosporium sinense* (A) and *Pochonia chlamydosporia* (B). White arrow indicates the edge of initial inoculum. Black arrows represent measurements of fungi mycelium radial growth.

Results

As shown in Table 1, *M. sinense* had the numerically highest growth rate at 30°C, regardless of the culture medium used, and the growth rate at 15°C was significantly lowest ($p<0.05$) in the 2% WA medium. The growth rate index of *P. chlamydosporia* was higher in 2% WA at 20°C and in 2% PDA and 2% CMA at 30°C. There were significant differences in MGRI values of *P. chlamydosporia* between 15 and 25°C in 2% WA and between 15 and 30°C in 2% PDA and 2% CMA ($p<0.05$). The two fungi grew at all tested pH levels, as presented in Table 2.

Figure 2 and Figure 3 illustrate Monacrosporium sinense morphology with subglobose conidia with one to three septa 23–30 x 17–25 µm, spherical chlamydospores with thick-walled and intercalated along the hyphae and adhesive networks capable of trapping nematode larvae. Figure 4 and Figure 5 show conidia and chlamydospore of Pochonia chlamydosporia and its predation ability on nematode larvae.

Discussion

Both investigated fungi showed high conidium and chlamydospore production, which are important structures to facilitate their survival and establishment in the environment. The predator fungus *M. sinense* modified its hyphae in adhesive networks capable of trapping the nematode, as reported by Campos et al. (2007). Nematode larvae held by *P. chlamydosporia* mycelium were seen by scanning electron microscopy, confirming its ability to colonize the nematode larvae stage and to penetrate the larvae by mechanical and enzymatic actions, despite belonging to the "ovicidal" group.

Recently, the possibility of obtaining high, efficient parasite control by the union of these distinct fungal isolates and compatible predatory abilities has been demonstrated (Oliveira et al., 2021a), making it important to consider *P. chlamydosporia* larvae predation. In this sense, Vieira et al. (2019) showed a reduction of 92.67% in infective bovine larvae when associating *P. chlamydosporia* with *Arthrobotrys cladodes*. These studies open possibilities for new commercial formulations. In the last 5 years, commercial formulations containing *Duddingtonia flagrans* have started to become commercially available in Brazil (Bioverm®), Australia, and New Zealand (BioWorma®), and these products are already used in animal feed (Araújo et al., 2021). Thus, this is an opportunity to explore the wide and promising field of research in parasitic biological control to find and analyze other factors that can ensure even more successful performance of these agents (Vieira et al., 2020; Araújo et al., 2021; Oliveira et al., 2021a).

In-vitro tests have shown that temperature was a limiting factor in the growth of nematophagous fungi *M. sinense* and *P. chlamydosporia*. No growth of fungal isolates was observed at 35 or 40°C over the 6 days. However, interestingly, the mycelial growth of both strains inhibited at 35°C started after these plates were incubated again at 25°C. This result shows that the initial temperature for the mycelial growth of fungi is of essential importance in their establishment in the environment. In the present study, the highest growth rate of *M. sinense* was similar to observations reported by Xue et al. (2018), who analyzed the temperature influence on the growth of *Arthrobotrys sinense* from China and observed mycelial growth between 11 and 35°C, with maximum levels at 30°C. These findings allow predicting in which microclimate it will be possible to keep the fungus abundant in the environment and to facilitate nematode infective form predation.

The MGRI results for *P. chlamydosporia* were similar compared to those reported by Vieira et al. (2020), who showed that *P. chlamydosporia* colony growth was higher at intermediate temperatures (20, 25, and 30°C) than at temperature extremes of 15 and 35°C in PDA medium over 10 days. In addition, understanding the temperature influence is crucial for storing process improvement of these microorganisms. In the current assay, the temperature influenced the extensive hyphae system formation more than the culture media composition. This observation supports the statement about

Table 1: Mean values and standard deviations (between parentheses) of mycelial growth rate index (MGRI) of the fungi *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4) grown in plates containing 2% water agar medium (2% WA), 2% potato dextrose agar medium (2% PDA) and 2% cornmeal agar medium (2% CMA) at temperatures of 15, 20, 25, 30, 35 and 40°C, for six days.

Temperature (°C)	***Monacrosporium sinense*(SF53)**			***Pochonia chlamydosporia*(VC4)**		
	2% WA	2% PDA	2% CMA	2% WA	2% PDA	2% CMA
15	0.28(0.06)^{a}	0.17(0.08)^{a}	0.35(0.07)^{a}	0.12(0.11)^{a}	0.14(0.15)^{a}	0.12(0.09)^{a}
20	0.61(0.26)^{b}	0.49(0.55)^{ac}	0.61(0.32)^{a}	0.25(0.16)^{ab}	0.19(0.16)^{ab}	0.27(0.20)^{ab}
25	0.73(0.34)^{b}	0,57(0.21)^{bc}	0.77(0.50)^{a}	0.23(0.07)^{b}	0.24(0.08)^{ab}	0.27(0.10)^{b}
30	0.84(0.44)^{b}	0.74(0.21)^{b}	0.92(0.65)^{a}	0.20(0.09)^{ab}	0.33(0.12)^{b}	0.30(0.10)^{b}
35	0	0	0	0	0	0
40	0	0	0	0	0	0

Same lowercase letters in the same column indicate no significant difference (p>0.05) between the data.

Table 2: Mean values and standard deviations (between parentheses) of the mycelial growth rate index (MGRI) of *Monacrosporium sinense* (SF53) and *Pochonia chlamydosporia* (VC4), grown in 2% potato dextrose agar medium (2% PDA), at pH values from 4 to 10.

pH	***Monacrosporium sinense*(SF53)**	***Pochonia chlamydosporia*(VC4)**
4	0.33(0.25)^{a}	0.24(0.22)^{a}
5	0.54(0.46)^{ac}	0.28(0.28)^{ab}
6	0.58(0.29)^{ac}	0.35(0.26)^{ab}
7	0.68(0.34)^{bc}	0.36(0.30)^{ab}
8	0.68(0.32)^{bc}	0.23(0.06)^{ab}
9	0.68(0.30)^{bc}	0.22(0.06)^{ab}
10	0.61(0.34)^{ac}	0.31 (0.10)^{b}

Same lowercase letters in the same column indicate no significant difference (p>0.05) between the data.

the capacity of these microorganisms to adapt to different environmental conditions, allowing them to be found dispersed in a variety of ecosystems (Braga and de Araújo, 2014; Zhang et al., 2014), which increases their potential use. Despite the interference observed in the present study in some temperature conditions, these fungal isolates are promising agents in nematode control.

The combination of *M. sinense* and *P. chlamydosporia* demonstrated a 98.90% reduction in the number of bovine nematodes infective larvae under *in-vitro* conditions at 25°C (Oliveira et al., 2021a). Surely, rapid colonization in the environment where the fungi will be inserted is an important step to provide them a competitive advantage, and Vieira et al. (2020) emphasized that fungal strains which demonstrate greater resistance to the adversities found in the environment would be most suitable for the use in biological nematode control strategies. Similar to the temperature, the pH can affect fungal metabolism, resulting in changes in their growth. The observations made here are important to show that animal gastrointestinal tract pH variations will not be harmful to fungi. Resistance to passage through the gastrointestinal tract is an important characteristic in fungi to be used in biological control. Additionally, using oral formulations in animals is the most practical way for these fungi to be dispersed and to colonize the fecal pats to act on nematode infective stages.

Interestingly, ruminant saliva has an alkaline pH of around 8.1, whereas the ruminal pH of grazing cattle remains close to neutrality (5.5 and 7) (Oliveira et al., 2019), corroborating with pH variations found in our study. However, characterizing the environmental conditions favoring the growth capacity at different pH values appears necessary for implementing suitable conditions in the laboratory and commercial production of nematophagous fungi. The species *P. chlamydosporia* showed a higher growth rate at pH 7, and there were significant differences in MGRI values only between pH 4 and 10 (p<0.05). The species *M. sinense* showed the highest growth rates in a pH range from 7 to 9 and lowest values at pH 4.

In addition, pH evaluation in the present study seemed realistic regarding the natural environment where fungi will be inoculated to establish growth. Xavier et al. (2016) reported that beef cattle kept in a pasture of *Brachiaria brizantha* and receiving only protein salt had a faecal pH of 7.4. This leads us to infer that fungal isolates, after passing through the animal gastrointestinal tract, will find favorable pH conditions for growth on faecal pats and, consequently, will be able to prey upon nematodes. Although this study focused on macroscopic fungal growth assessment, the limited

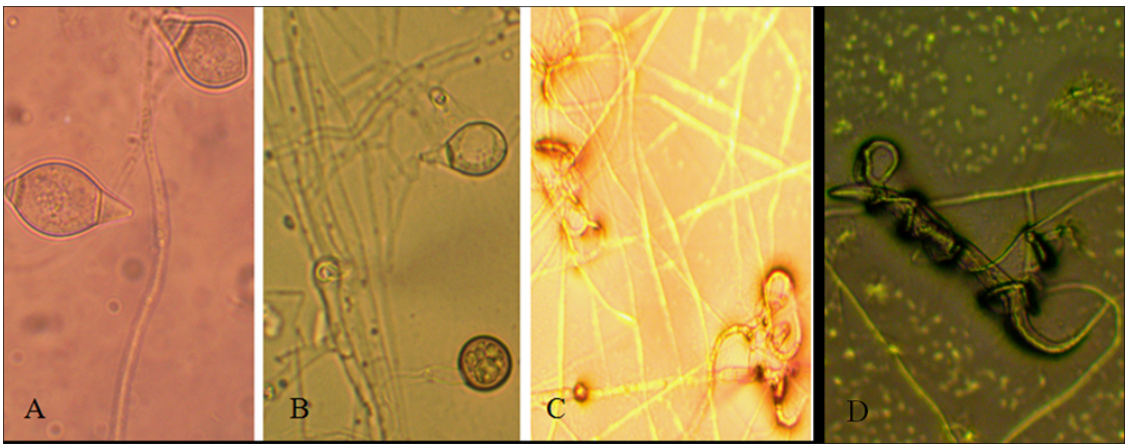

Figure 2: Images of the fungus *Monacrosporium sinense* under light microscope. (A) conidia with 2 and 3 septa respectively and conidiophore at 40× magnification, (B) conidia (top) and chlamydospore (bottom) at 40× magnification, (C) adhesive network trap at 10× magnification, (D) trapped bovine gastrointestinal nematode larvae at 10× magnification.

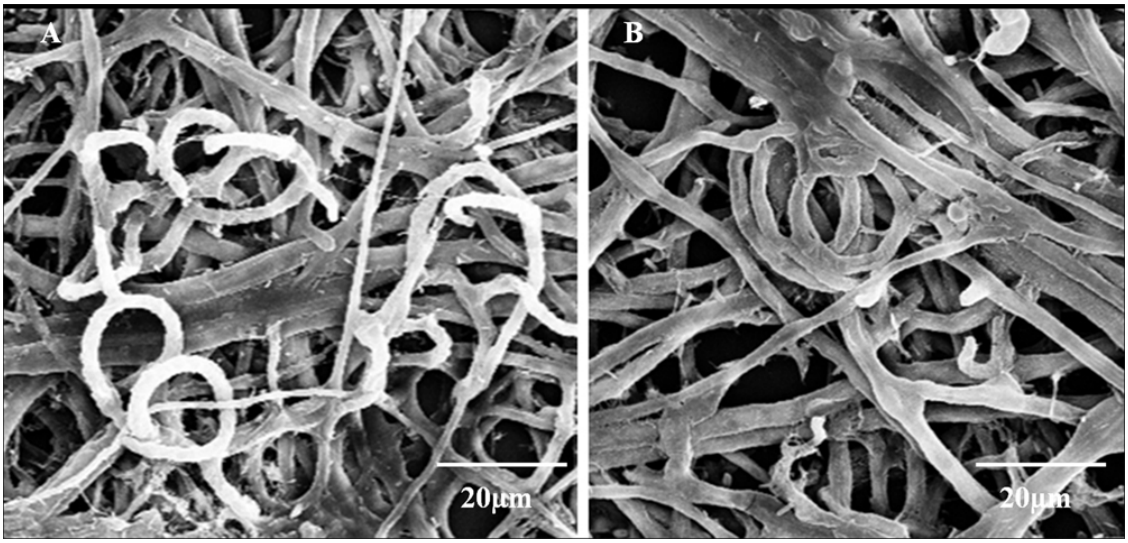

Figure 3: Images of the fungus *Monacrosporium sinense* mycelium and adhesive network under electron microscope.

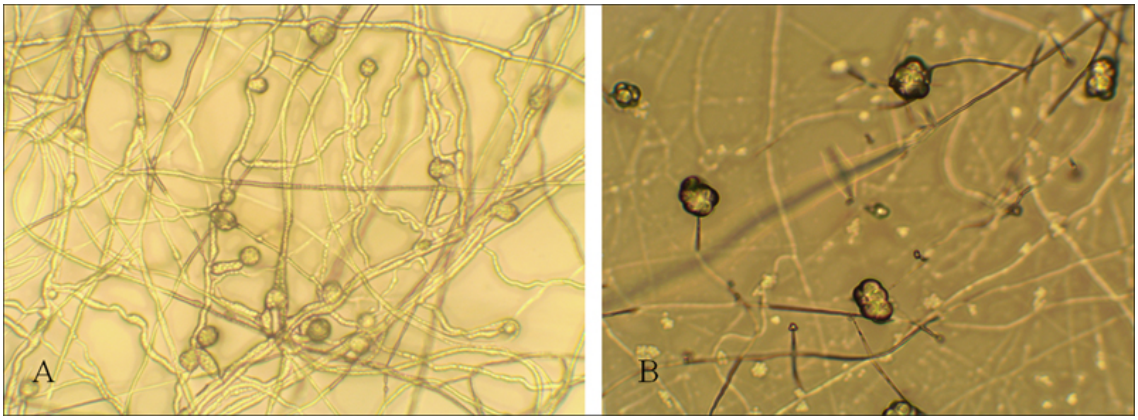

Figure 4: Images of the fungus *Pochonia chlamydosporia* under light microscope. (A) conidia and mycelium at 10× magnification, (B) chlamydospores at 40× magnification.

use of nematophagous fungi for controlling animal gastrointestinal nematodes seems partly due to the lack of the elucidation of chemical, physical and biological factors that affect the development of these microorganisms in the environment. Vieira et al. (2020) emphasized that bioassays under laboratory conditions are an

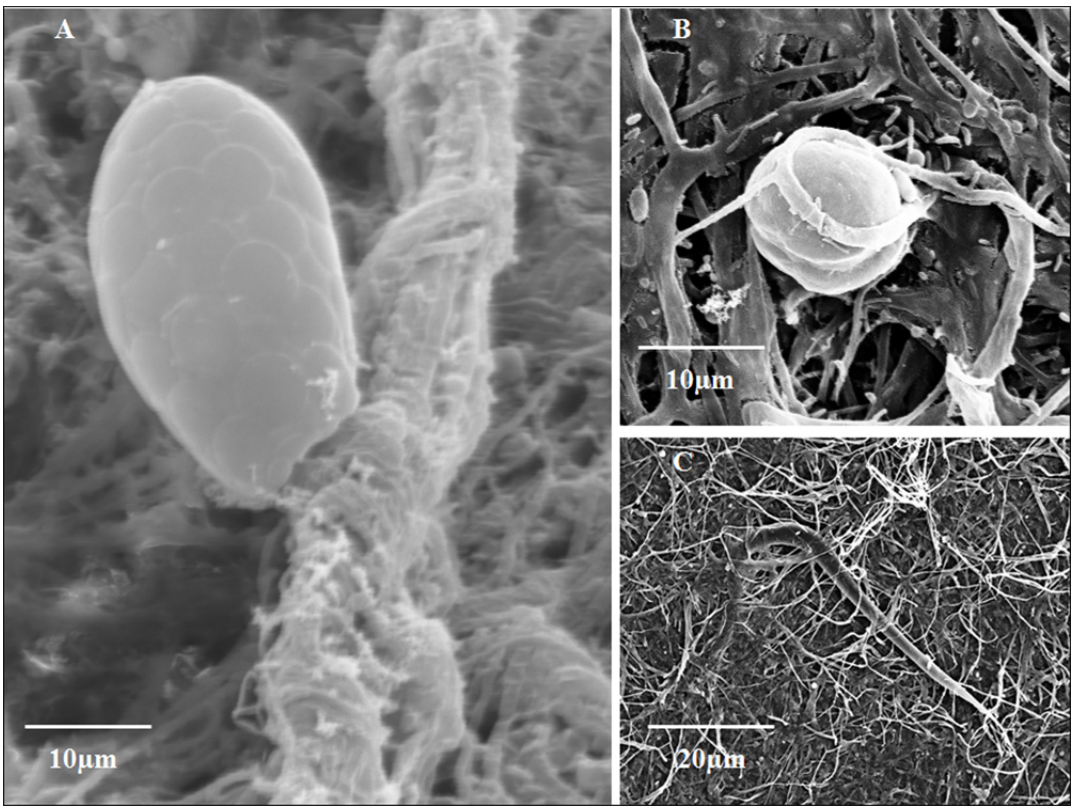

Figure 5: Images of the fungus *Pochonia chlamydosporia* under electron microscope. (A) conidia, (B) chlamydospore, (C) bovine gastrointestinal larvae trapped in the mycelium.

important step in the selection of potential candidates to be used in the control of gastrointestinal parasitic nematodes.

Though *M. sinense* and *P. chlamydosporia* showed synergistic action, suggesting that the joint application of fungi increases the effectiveness of the biological control of bovine infectious larvae (Oliveira et al., 2021a), it is relevant to obtain a better understanding of factors that can influence their growth to enhance the availability of promising fungi. Grazing systems are generally complex, with several biotic and abiotic factors, and fungi can therefore show variations in growth and predation percentages. In this study, the temperature was a decisive variable in the mycelial distribution in the culture medium, and consequently, the study of each strain must be specific. However, field evaluations with experimental formulations by a sodium alginate matrix with different fungal isolates (Mendoza-de Gives et al., 2018; Oliveira et al., 2018; Vieira et al., 2020; Voinot et al., 2020) and commercial formulations containing structures of the fungus *Duddingtonia flagrans* (Braga et al., 2020; Oliveira et al., 2021b) demonstrated excellent results in reducing the infective forms of gastrointestinal nematodes, but scientific data are still scarce. Furthermore, Ocampo-Gutiérrez et al. (2021) emphasized the need for future work with nematophagous fungal mycelia to elucidate the presence of intracellular products that may be crucial in their nematocidal activity.

In conclusion, defining the physiological requirements of the fungi *M. sinense* and *P. chlamydosporia* is essential to ensure their success as biocontrolers, and *in-vitro* studies can facilitate the exploration of these agents. Further studies should focus on understanding other fundamental aspects of the nematophagous fungal ecology of biotechnological interest for controlling gastrointestinal nematodes in animals.

Article Information

Funding. This research received no funds.

Acknowledgments. The authors thank 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior' (CAPES), 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq) and 'Fundação de Amparo à Pesquisa do Estado de Minas Gerais' (FAPEMIG) for support in this study, in the form of a doctoral scholarship.

Conflict of Interest. The authors have no conflict of interest to declare.

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