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The extracellular bioactive substances of *Arthrobotrys oligospora* during the nematode-trapping process

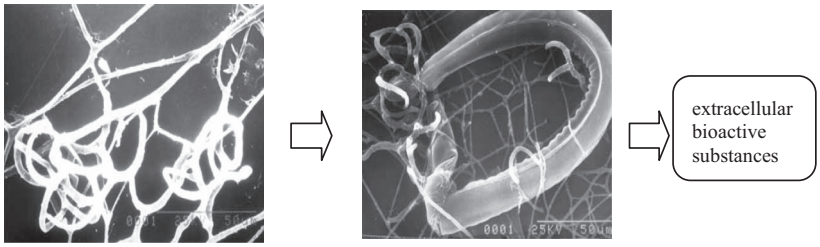
Rui Wang [a,b], Jun Wang [a,*], Xiaoye Yang [b]

[a] College of Life Science, Inner Mongolia University, Hohhot 010021, China
[b] College of Veterinary Medicine, Inner Mongolia Agricultural University, Hohhot 010018, China

HIGHLIGHTS

- *Arthrobotrys oligospora* prey nematode larvae on livestock.
- The extracellular polymeric substances of *A. oligospora* play the key role in the process of capturing nematodes.
- Proteases are required for *A. oligospora* to catch and feed on nematodes.

GRAPHICAL ABSTRACT



ring and network of nematode-trapping fungi

A.oligospora prey third stage larvae of nematode.

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ABSTRACT

To investigate the role of the extracellular polymers present on the traps of the predatory fungus *Arthrobotrys oligospora* in the nematode-trapping process, five solutions with different chemical properties, two enzymes, and seven protease inhibitors were used to treat the extracellular polymers. The effects of these treatments on mycelial growth and on the ability of the fungus to trap and kill nematodes were subsequently observed. The results demonstrate that 5 mol L^{-1} LiCl and pronase E can significantly reduce the trapping of the fungus to nematodes, while 0.1 mol L^{-1} NaOH, SDS–mercaptoethanol–urea PBS solution and 1.5% SDS can completely prohibit the trapping and mycelial growth. Enzyme inhibitors (except phenanthroline) had no significant impacts on the adhesion to the nematodes, while serine protease inhibitors can inhibit the killing of the fungi to the nematodes. It had no effect on the adhesion when treated nematodes with the inhibitors. Taken together, the extracellular polymers on *A. oligospora* traps that are critical for adhering to and capturing nematodes are proteins or protein-containing substances. Moreover, it was indirectly confirmed that although proteases are not essential for the adhesion and capture stages of nematode trapping, they play an important role in the subsequent killing of nematodes.

1. Introduction

Nematode-trapping fungi are a heterogeneous group of organisms broadly distributed in terrestrial and aquatic ecosystems, and they are capable of developing specific trapping devices (traps) such as adhesive networks, adhesive knobs, and constricting rings (Yang et al., 2011). These fungi utilize traps to capture and kill nematodes, then extract nutrients from them (Paraud and Chartier, 2003; Terrill et al., 2004). The nematode-capturing process is known to involve recognization, adhesion, infection, and digestion stages, and among these, the nematode adhesion and capture stage are critical steps for the infection process. It has been

* Corresponding author.
E-mail addresses: nmgdxwj@163.com (J. Wang), xiaoyeyang122@sohu.com (X. Yang).

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proposed that the adhesion of the fungi to nematodes is mediated by extracellular polymers present on the traps (Gold and Mendgen, 1984; Man, 2004; Mims and Richardson, 1989). Tunlid et al. observed that the surface of the nematode-contacting ring on predatory fungi is significantly rougher than the surface of regions that do not make contact with nematodes; additionally, a layer of mucinous material is present on the surface of the rings (or nets) of the fungal traps (Tunlid et al., 1991; Yang et al., 2004). However, few studies have addressed the functions of this mucinous layer during the adhesion phase of the nematode-trapping process, and even fewer studies have investigated these functions in detail. The present experiment was therefore designed to investigate the nature of the extracellular polymers on the traps of the predatory fungus *Arthrobotrys oligospora* and the functions of these polymers during the adhesion stage of the nematode-trapping process, with the goal of elucidating the fundamental mechanisms of nematode capture by predatory fungi.

2. Materials and methods

2.1. Reagents

Solutions of 5 mol L^{-1} LiCl, 12 mmol L^{-1} dithiothreitol (DTT), sodium dodecyl sulfate (SDS)–mercaptoethanol–urea in phosphate-buffered saline (PBS), 1.5% (w/v) SDS, and 0.1 mol L^{-1} NaOH were prepared and filter-sterilised for subsequent use.

2.2. Culture of A. oligospora and third-stage nematode larvae

A culture medium containing 0.4 g L^{-1} corn meal agar (CMA) was prepared (Wang, 2008; Yang et al., 2004). Samples of the predatory fungus *A. oligospora* (CGMCC No.5508) and third-stage *Strongylus equinus* larvae (The laboratory) were cultured and harvested (Deng et al., 2009; Wang, 2008; Yang et al., 2005).

2.3. Culture of the trap-containing mycelia of A. oligospora

A treated dialysis membrane was spread on the surface of the CMA media (Wang, 2008; Yang et al., 2004), and 0.25 mL volumes of both 0.05 g L^{-1} valine and phenylalanine were sterilely applied to the membrane. An agar block approximately 0.5 cm × 0.5 cm in size containing *A. oligospora* was inoculated at the center of the dialysis membrane with the mycelia facing down. The fungus was cultured in a 20 °C incubator, and the growth of mycelia was observed every day for 3 d. When a large number of fungal rings and nets appeared (5–7 d), the dialysis membrane was cut into 2 cm^2 pieces, with 40–60 fungal rings per square centimeter.

2.4. Solutions for processing the extracellular polymers and their impact on A. oligospora growth and the adhesion function of nematode-capturing mycelial traps

Upward-facing dialysis membranes containing mycelia and fungal rings were treated with 5 mol L^{-1} LiCl, 12 mmol L^{-1} DTT, or 0.1 mol L^{-1} NaOH at 4 °C for 10 min; SDS–mercaptoethanol–urea PBS solution at room temperature for 4 h; or 1.5% SDS (w/v) overnight at 4 °C. For the control groups, distilled water or the corresponding buffers alone were used. Six replicates were established for each experimental group, and three replicates were established for each control group. After the treatments, the solutions were aspirated and discarded, and the mycelia were washed three times with Tris–HCl (pH 7.4) for 3 min per wash. The mycelia-containing dialysis membranes (with the mycelia facing down) were then inoculated into CMA media and cultured in a 25 °C incubator. To detect the effects of the treatments on mycelial growth, the mycelial growth rates were measured at a set time each day beginning on the first day after treatment. For each solution that had been used to treat the extracellular polymers, three culture plates were analyzed, and the distances from the edge of the dialysis membrane to the ends of the mycelia were measured on each plate. The mean distance was calculated using three data points for each plate, and analysis of variance was performed.

To determine the effects of the treatments on the adhesion function of the nematode-capturing mycelial traps, three plates were used for each solution that had been used to treat the extracellular polymers. Before culturing, approximately 50 third-stage nematode larvae were added to each piece of dialysis membrane. At 6 and 24 h after the addition of nematodes, larvae that adhered to the traps on the dialysis membrane and larvae that were able to move freely were picked out and counted. The above experiment was repeated once, and the capture rates were calculated and analyzed by SAS.

2.5. The impact of cellulase and pronase E on the growth and nematode-capturing ability of A. oligospora mycelia

The dialysis membranes containing mycelial traps were divided into two major groups according to the different enzymes that had been used for treatment, and two control groups were established for each major experimental group. One of the major groups was the cellulase (2.7 mg mL^{-1}) experimental group. The previously prepared dialysis membranes containing mycelial traps were incubated with the enzyme solution in a 30 °C water bath for 4 h. Inactivated cellulase (boiled at 100 °C for 60 min) and distilled water served as the controls for this group. The other major group was the pronase E (2.9 mg mL^{-1}) experimental group. The prepared dialysis membranes containing the mycelial traps were incubated together with the enzyme solution in a 30 °C water bath for 1 h. Inactivated pronase E (boiled at 100 °C for 60 min) and a 10 mmol L^{-1} Tris–HCl solution at pH 7.5 served as the controls for this group. After the reactions were complete, the dialysis membranes containing mycelia were washed with the corresponding solutions, inoculated into CMA media, and cultured in a 25 °C incubator. Mycelial growth rates were measured at a set time each day beginning the day after treatment, and analysis of variance was performed by SAS.

To assess the impact of the tested treatments on the adhesion function of nematode-capturing mycelial traps, the dialysis membranes were inoculated into CMA media following the enzyme treatments described above. Approximately 50 third-stage nematode larvae were added to each sample, and the membranes were cultured in a 25 °C incubator. At 6 and 24 h after the addition of larvae, the samples were observed and analyzed by SAS.

2.6. The impact of protease inhibitors on A. oligospora growth and the ability of the mycelial traps to adhere to, capture, and kill nematodes

The following seven protease inhibitor solutions were prepared in 0.5% methanol: 1 mmol L^{-1} phenylmethylsulphonyl fluoride (PMSF), 1 mmol L^{-1} phenanthroline, 0.01 mmol L^{-1} antipain, 0.01 mg L^{-1} aprotinin, 0.01 mmol L^{-1} E-64, 0.01 mmol L^{-1} pepstatin, and 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA). These protease inhibitor solutions were used to treat the previously described dialysis membranes containing mycelial traps at room temperature for 20 min. Assays were set up in triplicate for each group, with a 0.5% methanol solution used as the control. After the reaction was complete, the solution was aspirated and discarded, and the mycelia were washed three times with 0.5% methanol solution for 3 min per wash. The samples were inoculated into CMA media and cultured in a 25 °C incubator. Mycelial