Mycoscience (2012) 53:31–35
DOI 10.1007/s10267-011-0128-1

SHORT COMMUNICATION

The production of nematode-immobilizing secretory cells by *Climacodon septentrionalis*

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Received: 3 December 2010 / Accepted: 25 May 2011 / Published online: 12 June 2011

Abstract The ability of *Climacodon septentrionalis* to immobilize and kill a mycophagous nematode (*Aphelenchoides* sp.) in vitro is described for the first time. Two isolates produced droplets (20–45 μm in diameter) that formed at the apices of tall, stalked, and branching secretory cells (700–1,500 μm tall). On 2% modified malt extract agar, nematodes became enveloped in the droplets, which restricted their ability to move and resulted in complete immobilization and death within several hours of contact. The rate of decomposition of the nematodes varied considerably, with most individuals persisting for weeks whereas others were degraded within several days and appeared to be colonized by dense hyphal growth. This study provides the first documentation of a non-agaricoid fungus producing secretory cells that are able to immobilize nematodes.

Keywords *Aphelenchoides* · Fungivory · Mycophagy · Secretory cell · Wood decay

The ability of fungi to immobilize and kill co-inhabiting microorganisms has captured the interests of researchers for more than a century (Barron 1977). Some soil-inhabiting members of the Ascomycota may trap and consume invertebrates such as tardigrades, nematodes, rotifers, and amoebae via the employment of constricting rings or modified adhesive hyphae and conidia (Gray 1987). The list of basidiomycetous fungi that consume invertebrates has continued to grow since it was first discovered that *Nematoctonus* was capable of capturing nematodes with hourglass-shaped adhesive knobs (Drechsler 1949). The stephanocysts of *Hyphoderma* serve a similar function by acting as adhesive trapping devices (Liou and Tzean 1992). Toxic droplets produced by secretory structures ("toxocysts") on hyphae of members of the genus *Pleurotus* immobilize nematodes, enabling rapid penetration and consumption via directional hyphae (Barron and Thorn 1987; Hibbett and Thorn 1994; Mamiya et al. 2005). Sharp crystalline structures, such as the spiny balls produced by *Coprinus comatus* (O.F. Müll.) Pers. and the acanthocytes of *Stropharia rugosoannulata* Farl. ex Murrill, can immobilize nematodes by physically damaging the cuticle and causing the leakage of inner contents (Luo et al. 2006; Luo et al. 2007).

It has been suggested that the ability of wood-decaying basidiomycetous fungi to immobilize and consume nematodes and other invertebrates is analogous to that of carnivorous plants (Thorn and Barron 1984). The relatively nitrogen-poor substrate on which these fungi subsist may be supplemented with exogenous sources of nutrients including yeasts, bacteria, pollen grains, spores, and the various invertebrates that occupy their environment (Fries and Swedjemark 1985; Barron 1988; Hutchison and Barron 1996; Hutchison and Barron 1997).

It is also proposed that the ability to immobilize invertebrates may serve as a defensive function by preventing subsequent grazing by fungivorous invertebrates on the fungus colony. Hutchison et al. (1996) found that the lawn-inhabiting agarics *Conocybe lactea* (J.E. Lange) Métrod and *Panaeolina foenisecii* (Pers.) Maire produced secretory cells that immobilized nematodes. These fungi did not consume the nematodes after immobilizing and killing them, suggesting a defensive action.

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An ongoing study investigating the presence of possible antifeedant mechanisms in fungi with perennial mycelia spurred interest in the interactions between *Climacodon septentrionalis* (Fr.) P. Karst. and a mycophagist nematode. The white rot fungus *C. septentrionalis* causes heart rot in living hardwoods, notably species of *Acer* (Boulet 2003; Sinclair and Lyon 2005). The presence of its very large, shelving hydnoid basidiome indicates extensive decay within the host tree (Coker and Beers 1951; Koski-Kotiranta and Niemelä 1987). Relatively little research has been conducted regarding the biology and ecology of this fungus, presumably because of its low economic impact. However, *C. septentrionalis* may be an aggressive parasite of mature hardwoods in the urban environment (Koski-Kotiranta and Niemelä 1987).

When nematodes were introduced to cultures of *C. septentrionalis*, they quickly became immobilized in the aerial mycelia. This article describes the previously unknown ability of *C. septentrionalis* to immobilize nematodes in vitro and the possible mechanisms involved.

Experiments were conducted using two isolates of *C. septentrionalis* deposited in the Canadian Collection of Fungal Cultures, Ottawa, Ontario, Canada. Isolate DAOM 241183 was derived from tissue of a fresh basidiome growing on a declining *Acer saccharinum* L. tree in Thunder Bay, Ontario, Canada. The second isolate (DAOM 241184) originated from basidiome tissue collected from the base of a mature *Acer nigrum* Michx. tree in London, Ontario, Canada. Cultures were grown on modified 2% malt extract agar (MEA) (20 g malt extract, 15 g agar, 1 g yeast extract, 1,000 ml sterile distilled water) in 9-cm-diameter Petri dishes and transferred monthly.

The mycophagous nematodes used in this study were a species of *Aphelenchoides* isolated from forest soil in Thunder Bay, Ontario, Canada. Basidiomata of *Piptoporus betulinus* (Bull.) P. Karst. were cut into pieces and used to bait the soil for nematodes. Nematodes were extracted from the fruiting body pieces using a modified Baermann funnel technique (Barron 1977) and surface sterilized using a combination of an antibiotic solution (300 mg penicillin G, 30 mg streptomycin sulfate, 1,000 ml water) and 1% NaClO. Nematodes were selected and reared monoaxenically on cultures of *Coprinopsis macrocephala* (Berk.) Redhead, Vilgalys & Moncalvo (DAOM 232080) and transferred monthly.

Nematodes were harvested from *C. macrocephala* colonies 1 month after inoculation using a modified Baermann funnel technique described by Ruess (1995). The nematode and tap water suspension was calculated and adjusted using a hemocytometer. Fifty microliters of the suspension containing approximately 250 nematodes was inoculated onto 4-week-old cultures of the two *C. septentrionalis* isolates growing on both MEA and water agar (WA) (15 g agar, 1,000 ml distilled water). Five plates of each isolate were inoculated with nematodes; this experiment was replicated an additional five times to facilitate observations. Nematodes were inoculated on the agar beyond the colony margin. Observations were made every 24 h following nematode inoculation. Dead nematodes were collected by increasing the Baermann funnel extraction time to 48 h, which resulted in the death of nematodes from asphyxia.

Specimens were mounted in several stains including phloxine B, lactophenol cotton blue, and Melzer's reagent. Observations were made using a Wild Heerbrugg-M5 stereoscope (Wild Heerbrugg, Gais, Switzerland) and a Nikon Eclipse E400 phase-contrast light compound microscope (Nikon, Tokyo, Japan). Measurements were made on material mounted in phloxine B. Digital micrographs were captured with an Olympus EVOLT E-330 (Olympus, Tokyo, Japan).

Secretory cells were produced on aerial mycelia in great abundance by the two *C. septentrionalis* isolates on MEA. The cells were first produced in the center of young colonies and later covered the entire surface of mature colonies. The secretory cell apices were swollen and obovoid to spathulate, approximately 8–18 µm (11 µm on average) wide, and produced on tall, septate, single-celled stalks that branched 1–3 times (Fig. 1). Individual stalks were 700–1,500 µm tall; although the branches were often many times taller, they were difficult to measure because of their dense growth. Large, transparent droplets, approximately

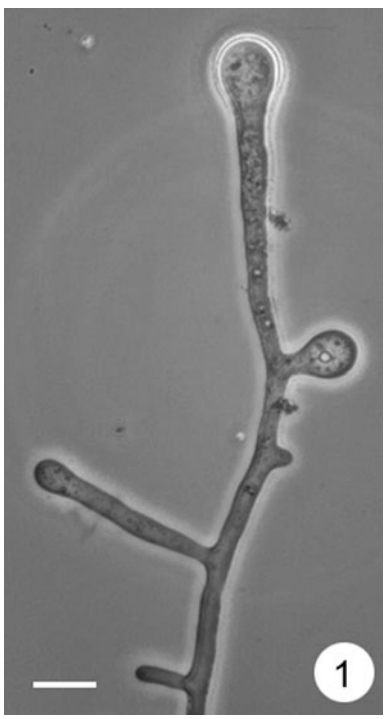


Fig. 1 Secretory cell produced on aerial mycelia of *Climacodon septentrionalis* on malt extract agar (MEA). *Bar* 10 µm

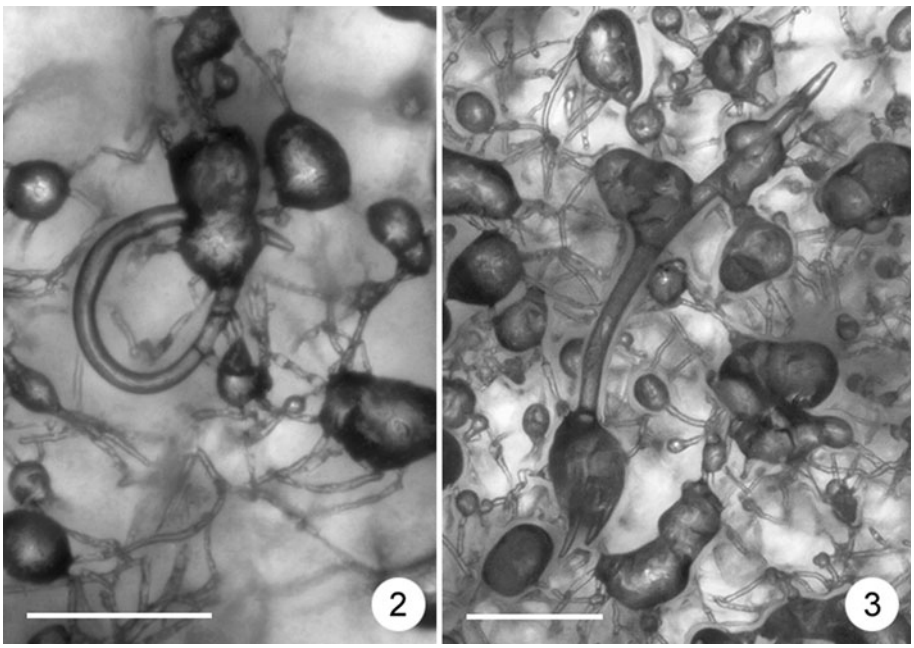
20–45 μm (33 μm on average) in diameter, were produced at the apex of the secretory cells and appeared to be insoluble in water. These droplets were found in densities up to 235/mm^2 on MEA. In the presence of nematodes or when cultures were mechanically damaged, the droplets became reddish in color as the colony matured and appeared to solidify when the stalks collapsed and came into contact with the agar surface. A change in the color of droplets was not observed in cultures not inoculated with nematodes or not mechanically damaged.

Following inoculation, nematodes descended into the agar substrate or moved along aerial hyphae. Nematodes occupying the younger colony margins, where secretory cells were absent, were able to move freely and feed on hyphae. When nematodes came into contact with secretory cell droplets, their movement was restricted. The droplets enveloped portions of the nematode body, resulting in struggling motions and a decrease in locomotion by the nematode. Head-waving was frequently observed in the confined nematodes in an attempt to locate adjacent hyphae. The movement of nematodes frequently caused droplets to coalesce, further ensnaring them (Fig. 2, 3). Individuals enveloped by a small number of droplets were sometimes able to liberate themselves.

The movements of nematodes became feeble within an hour after immobilization occurred. When movement ceased, the nematodes appeared dead and did not react to contact stimuli. Nematodes that were put in contact with droplets and removed to a clean water agar Petri dish rarely recovered and appeared immobilized or dead. Hyphae were never found penetrating the nematode cuticle or orifices; however, dense hyphal growth around the immobilized nematodes was infrequently observed 24–72 h after immobilization. Nematodes degraded at a variable rate, with some persisting for weeks whereas those enveloped in hyphae became unrecognizable within 72 h. The production of secretory cells was not observed when isolates were grown on Czapek solution agar (Malloch 1981) or WA media. When dead nematodes were placed on *C. septentrionalis* colonies reared on WA, there was no observed increase in nematode colonization or the directional growth of hyphae toward the nematodes compared to that seen in cultures grown on MEA.

The presence of nematode-immobilizing secretory cells in *C. septentrionalis* offers the first example of such a structure in a non-agaricoid fungus. In *C. septentrionalis*, these secretory cells are produced on relatively tall, branching support stalks when grown on MEA. These structures are significantly taller than the unbranched secretory structures described by Hutchison et al. (1996) in cultures of *P. foenisecii* (7.0–13.0 μm) and *C. lactea* (6.0–10.0 μm) and those described by Barron and Thorn (1987) in *Pleurotus ostreatus* (1.5–3.0 μm). The droplets formed by these species are typically less than 10 μm across, compared to the droplets 20–45 μm wide produced by *C. septentrionalis*.

The precise mechanism causing the immobilization and subsequent death of the nematodes is unknown. The



Figs. 2, 3 Nematodes immobilized within droplets exuded from secretory cells on MEA. *Bars* 100 μm

surface tension of the liquid droplet appears to play a role in restricting the movement of nematodes; however, nematodes that were removed from the droplets and placed on blank WA plates seldom recovered. Investigating the chemical composition of the droplets and conducting bioassays on several species of free-living nematodes should be the next step in the attempt to elucidate the immobilization effect of the secretory cells. The change in droplet color, from transparent to a red hue, appeared to be correlated with colony disturbance. This phenomenon may indicate degradation of the droplet or a change in metabolite production of the fungus and should be investigated further.

Immobilized nematodes degraded at inconsistent rates. This observation was suspected to be a result of conduction of the inoculation experiments on a nutrient-rich medium, which may negate the requirement to consume exogenous nutrient sources by the fungus. Truong et al. (2007) observed the consumption of immobilized nematodes by *Pleurotus cystidiosus* O.K. Mill. when the colony was nutritionally starved on WA. However, when colonies were raised on a nutrient-rich medium (potato dextrose agar, PDA), these authors noted that the nematodes were immobilized but not penetrated by hyphae despite the induction of toxocysts on the media. These observations may support the concept that the role of immobilizing structures is not limited to nutrient acquisition but also serves as protection against mycophagous nematodes and other microfauna. However, we found that when dead nematodes were placed on *C. septentrionalis* colonies that were nutritionally starved on WA, no increase in nematode colonization or degradation was observed. Although it is tempting to therefore describe the function of the secretory cells as purely defensive, it is possible that other nematode or invertebrate species may be readily consumed.

Okada et al. (2005) found that *Pleurotus ostreatus* (Jacq.) P. Kumm., a recognized nematophagous fungus, was a viable food source for six *Filenchus* species. Nematodes are one of the most diverse groups of organisms on our planet. It is not surprising that results from investigations utilizing specific nematode experimental organisms may not be applicable to all nematode species. The relatively tall stalks and varying rate of nematode immobilization and decomposition suggest other invertebrate groups may be targeted by the secretory cells of *C. septentrionalis.*

The results presented in this communication offer a stimulus for continued surveying of fungi and examining their interactions with their co-inhabiting fauna. Future experiments should include screening *C. septentrionalis* cultures with various invertebrates such as mites, Collembola, and other organisms that may share the same habitat. Fungi that produce similar structures in culture should be examined to determine if they have similar functions.

Acknowledgments We thank Dr. Qing Yu, Curator of the Canadian National Collection of Nematodes in Ottawa, Ontario, Canada, for confirming the nematode identification and Dr. R.G. Thorn for generously supplying the *Climacodon septentrionalis* isolate (DAOM 241184) used in this study. Financial support was provided by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to Dr. L.J. Hutchison.

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