THE ANTIFEEDANT ACTION OF *CLIMACODON SEPTENTRIONALIS* AND TWO SPECIES OF *SPHAEROBOLUS* TO HYPHAL GRAZING BY THE FUNGUS-FEEDING NEMATODE *APHELENCHOIDES* SP.

by

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ABSTRACT

Tanney, J.B. 2010. The antifeedant action of *Climacodon septentrionalis* and two species of *Sphaerobolus* to hyphal grazing by the fungus-feeding nematode *Aphelenchoides* sp. 90 pp.

Keywords: Antifeedant, defence mechanism, fungivory, grazing responses, mycology, mycophagy, perennial mycelium.

Fungi are ubiquitous in forest ecosystems and are, collectively, a major force in driving nutrient and organic matter availability and cycling. Some saprotrophic and mycorrhizal fungi are characterized by a perennial vegetative body, delayed sexual reproduction, and a relatively long lifespan. Such fungi are exposed to a barrage of antagonistic forces throughout their existence, one notable stress being invertebrate grazing. It is suggested that fungi with perennial mycelia must have developed means to deter grazing of their hyphal networks by pervasive mycophagist invertebrates.

Controlled inoculation studies with a mycophagous nematode, *Aphelenchoides* sp., and isolates representing 78 fungal species were conducted to investigate the presence of possible antifeedants. These *in vitro* pairings resulted in the discovery of two novel antifeedant mechanisms in three species of fungi. In the presence of the saprotrophic fungi *Sphaerobolus stellatus* and *S. iowensis*, the anterior portion of nematodes became encapsulated in a material of unknown composition. This encapsulation phenomenon effectively prevented further hyphal grazing by obstructing stylet extension, which resulted in the eventual death of the nematode. Nematodes that died as a result of the encapsulation were never colonized or consumed by the fungus. It is hypothesized that the encapsulating material originates from modified hyphal cells, referred to as gloeocystidia, and is liberated when the cells are punctured by the nematode stylet.

The wood-decaying fungus *Climacodon septentrionalis* was found to produce tall, stalked secretory cells in abundance on the aerial mycelia of the colony. Nematodes were enveloped and immobilized by droplets produced at the apices of the secretory cells. Immobilized nematodes were rarely colonized by the fungus and dead individuals persisted for weeks. A media study was employed to investigate the effect of nutrient concentration on the *in vitro* production of secretory cells. The discovery of novel antifeedants which mitigate damage caused to the mycelial network by grazing invertebrates offers a stimulus for further investigation into the interactions between fungi and their co-inhabiting microfauna.

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GENERAL INTRODUCTION

Fungi with perennial mycelia may be characterized by a relatively long existence with delayed sexual reproduction. As a consequence of the ubiquity of fungus-feeding invertebrates in the soil and litter, it is suggested that such long-lived fungi must have been compelled to develop deterrents to combat the constant threat of grazing. The intent of this thesis is to investigate possible antifeedant mechanisms which may be exhibited by various forest soil fungi with perennial mycelia.

Identifying potential antifeedant mechanisms is an important step in elucidating the interactions between fungi and their grazing co-inhabitants. Despite the ancient association between soil invertebrates and fungi since the early colonization of land, there are few examples of, and little clear evidence for, co-evolutionary relationships between fungi and invertebrates feeding on them. The results from this research will provide evidence of co-evolution between these two groups of organisms via the antifeedant responses of fungi to a feeding antagonist. The screening of fungi as possible biological control agents is also of importance in an era which is witnessing the continual introduction of invasive diseases and pests.

The research question was addressed by performing controlled *in vitro* inoculation studies. A mycophagous nematode (*Aphelenchoides* sp.) was isolated from the Thunder Bay District and selected as a model organism representing an antagonistic grazing invertebrate. The nematodes were introduced to isolates representing 78 species of fungi by means of controlled inoculation. Following inoculation, qualitative observations were made every 24 hours to assess for the presence of a possible antifeedant reaction. These reactions included the immobilization of nematodes, a

decrease in nematode population growth, or the direct parasitism of nematodes by the host fungus. Interesting interactions were selected for further investigation.

Of the 78 species of fungi included in this investigation, five exhibited apparent responses to the grazing nematodes. Three of these species were selected for further exploration: *Climacodon septentrionalis* (Fr.) P. Karst, *Sphaerobolus stellatus* Tode, and *S. iowensis* L.B. Walker. The responses of these fungi to the mycophagous nematode represent novel observations, which have not been previously described.

MYCELIAL GRAZING: INTERACTIONS BETWEEN MYCOPHAGIST INVERTEBRATES AND FUNGI

ABSTRACT

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Keywords: Antifeedant, defence mechanism, fungivory, grazing responses, hyphal grazing, mycology, mycophagy.

Fungal mycelium is ubiquitous to the forest ecosystem and provides a potential nutrient source for invertebrate mycophagists such as collembola, mites, and nematodes. The effects of grazing on vegetative fungal mycelium may have cascading consequences for the fungal individual, community, and ecosystem processes driven by fungi. In turn, fungi may alter the effects of grazing by adjusting their growth strategies or by the presence of antifeedant mechanisms. Such deterrents may include physical antifeedant structures and the production of toxic secondary metabolites. A review of the literature concerning grazers and their fungal resources has offered a glimpse into these complex interactions; however, our current understanding is ambiguous at best. Investigating how fungi are able to respond to the relentless threat of grazing and mitigate damage may offer insight into the evolution and success of these robust organisms.

INTRODUCTION

The consumption of any fungal matter, whether it be vegetative mycelium or fruiting body tissue, is frequently referred to as “mycophagy” or “fungivory”. Grazing refers to the consumption of vegetative hyphae through selective cropping or feeding. The grazing of fungal mycelia by microorganisms is an ancient interaction which must have had a significant impact on the evolution of both fungus and grazer (Schmidt *et al.* 2008). Compared to the extensive literature regarding plant herbivory, relatively little is known about the phenomenon of grazing on fungal mycelium. This is despite the incredible diversity and abundance of grazing organisms present in terrestrial ecosystems. The destruction of fungal mycelium by grazing may significantly affect the growth and respiration of the colony and subsequently alter interactions within the fungal community. It is conceivable that such effects may influence fungal-driven processes including decomposition, mycorrhiza formation and function, and phytopathogen interactions.

The focus of this chapter is restricted to the forest soil-, litter-, and woody debris-inhabiting invertebrates which feed on vegetative mycelium. The effects of mycophagy on the fungal community and on the fungus as an individual will be examined with emphasis on responses of fungi with perennial mycelium to such grazing. This literature review attempts to (1) illustrate the ubiquity of fungal grazing in the natural environment and (2) address the defence response of fungi to constant grazing pressures.

FUNGI AS FOOD

As much as 10% of soil and litter may be composed of fungal biomass (Dighton 2003). Flanagan and Van Cleve (1977) estimated the microbial biomass in the forest floor of a black spruce (*Picea mariana* (Mill.) BSP) taiga ecosystem was composed of 85% fungi, while Högberg and Högberg (2002) estimated at least 32% of the soil microbial biomass in a Swedish *Pinus sylvestris* L. forest was contributed by extramatrical ectomycorrhizal mycelium alone. Fungal hyphal length was estimated at 2,500 m/g soil in a lodgepole pine (*Pinus contorta* Douglas ex Loudon) forest (Ingham *et al.* 1989) and up to 26,000 m/g soil in a Swedish *P. sylvestris* forest (Söderström 1979).

Representing over 99% of the fungal colony biomass, vegetative mycelium offers an enticing and valuable source of nutrients for soil fauna (Frankland 1982). Carbohydrates, primarily in the form of polysaccharides, usually comprise half of the dry weight of fungi (Lundgren 2009). The average N and P content of fungal mycelium can range from 3.7 to 5.32% and 0.55 to 0.7%, respectively (Flanagan and Van Cleve 1977; Bååth and Söderström 1979). In a comparison of nutrient content as a percentage of dry weight, Swift (1977) found that *Stereum hirsutum* (Willd.) Pers. mycelium had approximately 8.2 times more nitrogen and 9.4 times more phosphorus than living oak wood. The profusion of mycelia in the soil environment has spurred the development of a plethora of organisms which take advantage of this substantial nutrient source.

MYCOPHAGOUS FAUNA BIODIVERSITY

The abundance and inherent nutritional content of hyphae in soil ecosystems has resulted in the proliferation of organisms which use fungal biomass as food; between 21% and 76% of soil fauna biomass is comprised of mycophagists (McGonigle 1995).

Soil fauna may be grouped into three diameter size classes (Swift *et al.* 1979). Mycophagous macrofauna (>2 mm diameter) include earthworms (Nasim and Zahoor 1994), diplopods (Taylor 1982), and gastropods (Silliman and Newell 2003). This size class may also include some insects and insect larvae (Shaw 1992), which graze mycelium. The mesofauna (0.1 mm to 2 mm diameter) are composed of invertebrates including mites (Mitchell and Parkinson 1976), collembola (Visser and Whittaker 1977; Shaw 1985), and enchytraeids (Hedlund and Augustsson 1995; Jaffee *et al.* 1997). Microfauna (<0.1 mm diameter) include nematodes (Townshend 1964) and protozoa such as amoebae (Chakraborty *et al.* 1985) and ciliates (Foissner 1998). The model organisms used in the majority of fungal grazing studies are primarily collembola, mites, and nematodes. These will be briefly summarized below.

COLLEMBOLA

Collembola (springtails) are extremely abundant in soil, leaf litter, and woody debris. In most terrestrial ecosystems, they occur at densities of 10,000 to 100,000 individuals/m^2, although seasonal population fluctuations between 145,000 and 244,000 individuals/m^2 were recorded in a Norwegian spruce forest (Petersen and Luxton 1982). Despite their abundance, collembola contribute only 1 to 5% to total soil animal biomass and respiration in temperate ecosystems due to their small size, although they may

contribute as much as 33% of the total soil animal biomass and respiration in early stages of succession (Petersen 1994). Regardless of their low biomass, collembola play an important role in the structure of some soils and influence decomposition and soil respiration (Filser 2002).

Collembola are typically omnivorous, although different species are morphologically and ecologically differentiated into feeding guilds (Rusek 1998). Many primarily feed on fungal hyphae or decaying plant material, but others appear to be generalists, also feeding on nematodes, bacteria, algae, detritus, and fine roots and root hairs (De Ruiter *et al.* 1994; Lee and Widden 1996; Hopkin 1997; Endlweber *et al.* 2009). Collembola graze fungi growing on leaf litter, fecal pellets, and soil (Sadaka-Laulan *et al.* 1998; van der Drift and Jansen 1977; Ponge 1991), as well as on mycorrhizal species (Moore *et al.* 1985).

ACARI

Oribatid mites (Acari, Oribatida) may be one of the most abundant forest soil arthropods, reaching densities of up to 400,000 individuals/m^2 in temperate forests and comprise about 10,000 described species worldwide (Schatz 2002). Mites typically dominate forest soils and undisturbed habitats (Filser 2002). All life stages may feed on fungi (Mitchell and Parkinson 1976) and mycophagous mites have been observed to feed on foliar pathogens (Norton *et al.* 2000), soil fungi (Mitchell and Parkinson 1976), leaf litter (Santos *et al.* 1981), and mycorrhizae (Schneider *et al.* 2005).

NEMATODES

Nematodes are the most abundant multicellular organisms in the world and are of great ecological importance (Bongers and Bongers, 1998). It is estimated that there are approximately 29 million nematodes per m^2 of mixed deciduous forest soil, and that their species diversity is exceeded only by the arthropods (Bernard 1992). Nematodes comprise the majority of mycophagous microfauna and feed by piercing hyphae and ingesting fungal cytoplasm (Ruess and Lussenhop 2006).

FEEDING BEHAVIOUR AND STYLE OF MYCOPHAGOUS FAUNA

Mycophagous fauna may be also divided into either of two categories: particulate fungivores or fluid-feeding fungivores (Moore *et al.* 1988). However, there are many examples of diverse feeding methods within both categories. Mandibular mouthparts may be modified for various feeding styles such as cutting, scraping, grinding, sucking, and piercing (Ruess and Lussenhop 2006). Mycelium may be scraped from leaves and ingested, as observed in Lauxaniid flies (Broadhead 1984). Anas and Reeleder (1988) found that *Bradysia coprophila* (Lintner) larvae were able to remove the outer rind of *Sclerotinia sclerotiorum* (Lib.) de Bary sclerotia and consume the contents. Oribatid mites have been observed to sever hyphae and ingest them „like spaghetti" (Ruess and Lussenhop 2006).

Mycophagous amoebae are able to perforate fungal spores and hyphae and ingest the cytoplasm within (Anderson and Patrick 1978). Protura and collembola may penetrate and suck hyphal contents out with modified mandibles (Sturm 1959; Rusek 1998). Mycophagous nematodes feed on fungal hyphae by inserting a retractable stylet

into the fungal cell and actively pumping out cytoplasm (Siddiqui and Taylor 1969). Following feeding, hyphal cells may have virtually all cytoplasm removed, which typically results in cell death and collapse (Doncaster 1966; Siddiqui and Taylor 1969; Avery and Thomas 1997).

Selective Mycophagy

Grazing organisms with a strong selective preference for certain fungal species over others may have an important effect on the adaptation of target fungi over ecological time. Preferential feeding may affect the fungal community and alter fungal-driven ecological functions by promoting the proliferation of unpalatable species over preferred species. Consequently, this research topic has garnered a substantial amount of interest over the past 30 years. The usual approach is to analyze mycophagist gut contents or investigate food preferences.

Gut Content Analysis

Gut content analysis, through dissection or observations of living specimens, may be used in conjunction with food preference tests. Thimm and Larink (1995) assessed the food preference of 5 collembola species by simultaneously offering them dyed parsley roots infected with one of 5 vesicular arbuscular mychorrhizae (VAM) species. Preference was determined by observing the differentially dyed roots in the collembolan gut with the corresponding VAM species. A benefit of gut content analysis is the ability to evaluate the diet of field specimens, as opposed to food preference tests, which are typically constrained to the laboratory.

Gut content analysis proves difficult when contents are unidentifiable or rapidly digested and also fail to detect the consumption of fungal cytoplasm in the guts of fluid-feeding invertebrates, such as nematodes and some collembola. Bardgett *et al.* (1993) were unable to identify fungal hyphae in the guts of the collembola *Onychiurus procampatus* Gisin collected from the field, or in captive specimens reared on a fungal diet. It is, therefore, difficult to make solid conclusions regarding food preferences based on gut contents alone, as the analysis represents only recent and identifiable feedings.

Food Preference Tests

Food preference tests are conducted in "choice chambers" with 2 or more fungal species or isolates. The numbers of invertebrates occupying the offered fungal colonies are counted over a period of time, offering direct observations of test subject feeding behaviour and preference. Food preference tests may not reflect realistic field conditions for a number of reasons. The movement of individuals may not be considered independent; the presence of feeding microfauna may stimulate others in proximity to feed or increase explorative activity (Doncaster 1966). Collembola are known to aggregate in response to pheromonal cues (Shaw 1988), although Bardgett *et al.* (1993) found their test subjects appeared to disperse randomly and independently of one another. Some studies have used the presence of faecal pellets to determine the preferred food; however pellets may not be deposited on the exact grazing location due to gut-passage time, organism mobility, or coprophagy exhibited by the grazer (Koukol *et al.* 2009).

Food choice studies involving nematodes are rare (Townshend 1964; Pillai and Taylor 1967; Ruess *et al.* 2000; Hasna *et al.* 2007). Nematodes may be attracted to fungal species which cannot support their population growth (Ruess *et al.* 2000) and even to nematophagous fungi (Jansson and Nordbring-Hertz 1979; Wang *et al.* 2009). Evidence suggests that although nematodes demonstrate a marked preference towards certain fungi, they tend to favour a mixed diet (Doncaster 1966; Ruess *et al.* 2000; Scheu and Folger 2004). A mixed diet may be beneficial to a grazer by keeping undesirable toxins inherent to each food type within acceptable limits (Begon *et al.* 1996). Therefore, food preference may be based on the concentration of toxins rather than energy or nutrition content of the potential food choice (Bryant and Kuropat 1980). Scheu and Folger (2004) noted an increase in fitness when collembola were offered a mixed diet, possibly due to the lack of essential nutrients when provided a single food item diet.

The Dematiaceous Preference?

Preferential feeding behaviour has been observed in several food preference experiments concerning collembola (Shaw 1988; Chen *et al.* 1995), nematodes (Ruess *et al.* 2000), oribatid mites (Luxton 1966), and astigmatid mites (Hubert *et al.* 2004). Preferences will most likely differ among species and organisms with varying feeding habits, however a phenomenon frequently observed in food choice tests is the preference for dark-pigmented (dematiaceous) fungi over hyaline forms by collembola (Mills and Sinha 1971; Visser and Whittaker 1977; Klironomos and Kendrick 1995; Sadaka-Laulan *et al.* 1998; Maraun *et al.* 1998) and mites (Luxton 1966; Klironomos and Kendrick 1995; Hubert *et al.* 2004; Koukol *et al.* 2009). Gut content analyses of field specimens

have also yielded a higher proportion of pigmented hyphae in the guts of collembola (Kaneko *et al.* 1995) and oribatid mites (Mitchell and Parkinson 1976). Maraun *et al.* (2003) suggested several possible hypotheses which may explain the preference of dark hyphae as a food source for mycophagous animals including: nutritive value, absence of toxicity, or superior exoenzymes which are exploited by mycophagous fauna.

This is a surprising trend, as melanins are implicated in the protection of fungal hyphae and propagules against a variety of environmental stresses (Bell and Wheeler 1986; Gadd 1993; Jacobson and Tinnell 1993; Butler and Day 1999). Gut content studies involving field animals may not be indicative of feeding preferences towards dark hyphae as melanized hyphal walls may be difficult to digest, therefore appearing more evident and abundant than hyaline hyphae in the animal gut. In many food preference studies hyaline species are represented by *Penicillium* or *Paecilomyces* species (Dash and Cragg 1972; Luxton 1972; Mitchell and Parkinson 1976; Moore *et al.* 1987; Scheu and Simmerling 2004), which have been found to produce secondary metabolites toxic to many soil invertebrates (Cram and Tishler 1948; Cayrol *et al.* 1989; Kwok *et al.* 1992; Chandler *et al.* 2000; Scheu and Simmerling 2004; Rohlfs *et al.* 2007; Liu *et al.* 2009; but see Rusek 1989). Rearing *Aphelenchus avenae* Bastian or *Aphelenchoides composticola* Franklin nematodes on species of *Penicillium* may be unsuitable for population growth and even repel grazing nematodes (Mankau and Mankau 1963; Chen and Ferris 2000). Visser and Whittaker (1977) reported a preference for pigmented fungi by the collembola *Onychiurus subtenuis* Folsom, however the two unknown basidiomycetes which comprised the hyaline species in their study were found to be toxic.

Possible flaws in experimental design suggest the preference of pigmented fungi by mycophagous invertebrates may be a result of representing hyaline fungi with species which are known to produce toxic secondary metabolites. The majority of studies also utilize ubiquitous fungal species, with little attention given to more specialized species, which may be of more interest. Future studies should also incorporate not only food selection as a factor for preference, but also animal fitness (Scheu and Folger 2004).

Food Preference Conclusions

Despite the expected ambiguity in feeding preferences among different mycophagist species at various life stages, evidence suggests collembola, mites, and nematodes are best described as "choosy generalists" (Schneider and Maraun 2005). They are able to feed on a wide variety of resources, but show preference for certain materials over others in food choice experiments. Continued research is expected in this area of mycophagy, as selective grazing pressure may have profound effects on fungal communities and the diversity and spatial distribution of mycophagous organisms.

EFFECTS OF GRAZING ON FUNGI

In a microcosm study with the fungus *Fusarium oxysporum* E.F. Sm. & Swingle, the consumption of fungal cytoplasm by the nematode *Aphelenchus avenae* ranged from 9.2 to 32.8% of the total standing crop per day (Ingham *et al.* 1985). Shafer *et al.* (1981) reported the presence of grazing *Aphelenchoides bicaudatus* (Imamura) Fil. and Sch. Stek nematodes limited the *in vitro* growth of 5 ericoid mycorrhizal fungus isolates from 47 to 70% of the mycelial areas of the ungrazed controls and that grazing caused fragmentation of hyphae. The destruction of aerial mycelia is frequently observed in

cultures inoculated with mycophagous nematodes (Riffle 1967; Sutherland and Fortin 1968; Ruess and Dighton 1996).

PHYSIOLOGICAL EFFECTS

Fungal respiration and growth may increase when a fungus is subjected to low intensity grazing or decrease under high intensity grazing (Hanlon and Anderson 1979; Hanlon 1981; Ingham *et al.* 1985; Moore 1988; Hedlund and Augustsson 1995). This bell-shaped response of fungal activity to increasing grazing intensity suggests that activity is increased up to an optimum value, after which further increases in grazing intensity begin to reduce fungal activity (Hanlon 1981). Grazing at optimal densities may enhance fungal growth and respiration through selective pruning of senescent hyphae, thereby releasing immobilized nutrients (Hanlon 1981; Moore *et al.* 1987). Hanlon (1981) suggested grazing might remove toxin-accumulating hyphae, thus permitting the further utilization of nutrients by new fungal growth. This compensatory growth is discussed in the context of a grazing response strategy later on.

Grazing intensity is not the only factor dictating the fungal response. Hanlon (1981) found that fungal respiration increased by as much as 100% in the presence of grazing collembola on high nutrient agar; however respiration decreased when the grazed fungus was cultured on low nutrient agar. Okada *et al.* (2005) also found that culture media had a significant effect on nematode population growth rates. Other factors such as the fungal species (Ruess and Dighton 1996), spatial distribution of fungal mycelium (Bengtsson and Rundgren 1983), grazing species (Tordoff *et al.* 2008), age (Moore *et al.* 1987), and behaviour (McMillan 1976) may result in differential

effects of grazing on a fungus. Research supports the notion that fungi are not passive organisms simply susceptible to grazing; rather, they are capable of responding to grazing damage to their mycelial network.

Mycelial Growth Responses to Grazing

Compensatory growth, a phenomenon observed in plants subjected to grazing (Paige and Whitham 1987), has also been described in microcosm and *in vitro* studies involving fungi and grazing collembola species (Hanlon and Anderson 1979; Bengtsson and Rundgren 1983; Bengtsson *et al.* 1993; Hedlund and Augustsson 1995; Bretherton *et al.* 2006). This is typically caused by low to moderate densities of grazing animals and is also affected by the nutritional quality of the medium (Hanlon 1981). Bretherton *et al.* (2006) hypothesized that the compensatory growth observed in grazed *Phanerochaete velutina* (DC.) Parmasto may be a result of apical pruning and/or decreased competition for water and nutrients by mycelia. Whether compensatory growth is a specific response to grazing or a strategy evolved to mitigate detrimental effects of all types of damage is debatable (Belsky *et al.* 1993).

Harold *et al.* (2005) found grazing *Folsomia candida* (Willem) significantly reduced the coverage and extension of the cord-forming basidiomycete *Hypholoma fasciculare* (Huds.) P. Kumm. Collembolan grazing is typically concentrated on fine mycelium within the colony and hyphal tips (Wood *et al.* 2006; Tordoff *et al.* 2008; Harold *et al.* 2005). Thick mycelial cords may be less palatable due to a thick outer rind or the possible presence of encrusting crystals and secondary chemicals (Tordoff *et al.*

2006). However, grazing on thick cords has been observed, sometimes completely severing them from their source (Wood *et al.* 2006; Tordoff *et al.* 2006).

Wood *et al.* (2006) conducted a series of relatively large (57 x 57 cm) microcosm experiments exploring the changes in mycelial networks of the cord-forming *Phanerochaete velutina*, quantified by hyphal coverage and estimated mass fractal dimension, following the addition of new resources (2 x 2 x 1 cm *Fagus sylvatica* L. blocks) and/or collembola (*F. candida*). The authors found both the addition of resources and collembola grazing had interactive effects on mycelial morphology. For example, the hyphal coverage and mass fractal dimension of grazed systems without additional resources were not significantly different from ungrazed systems without additional resources. In contrast, grazed fungi with additional resources grew significantly less than ungrazed systems with additional resources.

The mycelial network may spatially respond to grazing pressures. Collembola grazing caused *H. fasciculare* to switch from a growth pattern with a broad, continuous foraging front and uniform growth in all directions to a pattern with fast growing sectors while growth in some sectors completely ceased (Kampichler *et al.* 2004). The authors interpreted this phalanx growth strategy (*sensu* Schmid and Harper 1985) as a fugitive response and a growth strategy employed to quickly escape from localized areas where grazing pressure is present. This differential growth, in conjunction with increased protease activity, was also observed in the grazing of *Mortierella isabellina* Oudem. by the collembola *Protaphorura armata* Tullberg (Hedlund *et al.* 1991).

Hyphal fungi are modular organisms characterized by a complex network, which may effectively diminish the significance of localized damage and senescence (Carlile 1995). Exploring how fungi spatially respond to grazing pressures may offer further insight into the mechanisms which have allowed this group of organisms to thrive in an antagonistic environment. The impact of grazing on the fungal host is complex and becomes even less apparent in the context of the fungal community.

THE FUNGAL COMMUNITY

Selective grazing may alter the species composition and distribution within a site (Wicklow and Yocom 1982). Grazing on *Trametes versicolor* (L.) Lloyd by *Folsomia candida* growing in oak litter reduced fungal biomass while increasing bacterial biomass (Hanlon and Anderson 1979). Newell (1984a,b) found that preferential feeding by the collembola *Onychiurus latus* Gisin suppressed the dominant litter-decomposer *Marasmius androsaceus* (L.) Fr., allowing the proliferation of the less competitive fungus *Mycena galopus* (Pers ex Fr.) Kummer when grazing was present. The grazing on *Marasmius androsaceus* stimulated litter decomposition. Grazing by *Onychiurus subtenuis* reduced the ability of an unknown dematiaceous fungus (Sterile dark form 298) to colonize leaf litter when competing with a basidiomycete fungus (Basidiomycete 290) (Parkinson *et al.* 1977).

The selective grazing behaviour of mycophagists combined with their inherent abundance suggests the ability of grazers to influence the fungal community. This phenomenon must be investigated further as changes to the fungal community may have

cascading effects on the various functions which fungi contribute to in the forest ecosystem.

FUNGAL DEFENCE

Due to the longevity of the mycelial network and delayed sexual reproduction, it is plausible that grazing damage to late-successional fungi with "perennial mycelium" is more detrimental than such damage to ruderal species. Rather than relying on a rapid colonization and reproduction type of life cycle to avoid grazing and other stresses, fungi with perennial mycelia must expend more energy on the maintenance of their persistent vegetative hyphae before they may successfully reproduce. It is conceivable that species which are "bound to be found" (*sensu* Feeny 1976) by grazers must have evolved antifeedant mechanisms to mitigate damage caused to their mycelial network by grazing organisms.

Despite the plausibility of attributing a defensive role to morphological structures and toxic compounds, few papers provide conclusive data. The conclusions of many papers (e.g.: Böllmann *et al.* 2010) are correlative and do not provide conclusive data which show a causal relationship between the proposed defensive traits and their effects on invertebrate or fungal fitness (see Rohlfs *et al.* 2007; Kempken and Rohlfs 2010). The identification of apparent antifeedants provides important evidence for possible defensive mechanisms in fungi; however, experimental verification is the next step which must be fulfilled to confidently address the role of such mechanisms in fungal defence. This step presents many underlying methodological obstacles which may be overcome using transgenic model organisms (Rohlfs *et al.* 2007) to suppress the

production of apparent antifeedant traits, which may enable the validation of their function as defence characters.

Examples of fungal antifeedants will be presented according to the nature of their defence: mechanical or chemical.

MECHANICAL ANTIFEEDANT STRUCTURES

Wicklow (1979) suggested the ascomata of *Chaetomium bostrychodes* Zopf are avoided by larvae of *Lycoriella mali* Fitch. due to the ornamented perithecial hairs which act as a mechanical deterrent against grazing by larger mycophagist arthropods. Mechanical defences may persist over longer periods of time compared to volatile secondary metabolites. Antifeedant mechanisms which physically deter grazing are differentiated by their mode of inhibition and briefly summarized below.

Adhesive Structures

The adhesive structures of nematophagous fungi are one of the most well-known trapping devices and have been reviewed in detail (Barron 1977). These structures are diverse in size and shape, ranging from unmodified adhesive hyphae to adhesive knobs and three-dimensional nets (Gray 1987). Stephanocysts are two-celled appendages, which function as both asexual reproductive propagules and adhesive structures in *Hyphoderma* species (Liou and Tzean 1992). Hourglass-shaped adhesive appendages are found in members of the genus *Nematoctonus* and its teleomorph *Hohenbuehelia* (Barron 1977). The recognition and subsequent attachment of the nematode cuticle to the fungal surface appears to be mediated by a lectin-carbohydrate interaction (Tunlid *et al.* 1992).

Constricting Rings

One of the most dramatic and sophisticated structures employed by fungi to disable nematodes are the constricting rings produced by some species, including *Arthrobotrys dactyloides* Drechsler. Induced by the presence of nematodes, the rings are comprised of three curved cells on a short two-celled stalk. Mechanical stimulation of the rings induces the rapid (0.1 s) inflation of the cells, effectively snaring nematodes which may have entered the rings (Higgins and Pramer 1967). Ring-forming fungi are very efficient nematode predators and were found to have a lower saprotrophic capability than other nematode-trapping fungi, suggesting an ecological adaptation as a predominant predator (Cooke 1963).

Crystalline Structures

Spiny balls, the sharp, crystal structures produced along the hyphae of *Coprinus comatus* (O.F. Müll.) Pers. mechanically damage the cuticle of nematodes (Luo *et al.* 2004; Luo *et al.* 2007). These structures appear to be analogous to thorns in some plants, lacerating the nematode cuticle and, in severe cases, causing the leakage of inner materials through extensive wounds. The authors created „regenerating plates" by removing aerial fungal hyphae with a scalpel, which induced the growth of special aerial hyphae with abundant spiny balls. This suggests that the antifeedant is stimulated by mechanical damage to the colony, possibly indicating a general defence mechanism induced by the destructive presence of nonspecific grazers.

Acanthocytes, the stellate cells produced by *Stropharia rugosoannulata* Farl. ex Murrill, have a similar function to the spiny balls of *C. coprinus*, immobilizing

nematodes via physical damage to the cuticle (Luo *et al.* 2006). The authors observed the colonization and rapid digestion of injured *Panagrellus redivivus* L. nematodes by *S. rugosoannulata*, however the fungus could kill but not consume *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle nematodes.

The formation of crystals, frequently composed of calcium oxalate, along the hyphae has been observed in many species of fungi (Whitney and Arnott 1987; Connolly 1997). Several functions have been attributed to oxalic acid and oxalate, including pathogenicity (Godoy *et al.* 1990; Cessna *et al.* 2000), wood decomposition (Dutton *et al.* 1993), mineral weathering (Wilson *et al.* 1981), and reducing deleterious effects of excess calcium ions (Whitney and Arnott 1987).

Thompson (1984) suggested an antifeedant role in calcium oxalate crystals on fungal hyphae. This is a reasonable assertion, as calcium oxalate crystals have antifeedant properties in plant leaves (Molano-Flores 2001) and the presence of sharp, needle-like raphides encrusting the mature hyphae of some fungi suggests a potential physical barrier to mycophagists. The lack of grazing in pruinose lichen specimens by the coleopteran *Lasioderma serricorne* (F.) was attributed to the presence of a superficial layer of calcium oxalate (Nimis and Skert 2006). In a rare study focusing on fungal defence against grazing, Böllmann *et al.* (2010) attributed an antifeedant function to hyphal crystals against the collembola *F. candida*, although the evidence was largely anecdotal. The presence of crystals on fruiting body cystidia may also serve a protective function (Nakamori and Suzuki 2007). Crystals may physically impede grazing by acting as a protective covering and/or deterring microorganisms via sharp structures.

CHEMICAL ANTIFEEDANTS

Janzen (1977) suggested that the role of toxic secondary metabolites of fungi is analogous to that of the chemical defence systems of higher plants: deter predators and protect substrate territory. Toxins found concentrated in fruiting body tissues possibly play a role in protection from predation (Gloer *et al.* 1988; Wang *et al.* 1995).

The discovery of fungal-derived nematicidal compounds has generated considerable interest from ecological and biological control perspectives. A large number of nematicidal substances have been described (see Li *et al.* 2007b for a review of 179 nematicidal compounds). Despite investigations into the consequences of fungal secondary metabolite formation, a causal link between the biosynthesis of such metabolites and its role in fungal defence is rarely supported by experimental evidence (Rohlfs *et al.* 2007). Possible fungal chemical antifeedants may be presented to the potential grazer in three ways.

Exotoxins

Fungi produce an array of extracellular enzymes and metabolites, some of which have been exploited for our own purposes. Mycotoxins have been the subject of scrutiny due to their deleterious effects on human and livestock health. Toxic secondary metabolites may be secreted extracellularly, or may present in specific tissues such as developing stroma (Stadler *et al.* 2006), or in injured fruiting bodies (Stadler and Sterner 1998). Exotoxins are secreted from hyphae into the surrounding environment, creating a gradient which may prevent grazing before contact occurs. In contrast, some toxins are secreted by specific structures.

Secretory Cells

Rather than excreting toxins directly into the environment surrounding vegetative tissue, concentrated toxins may be produced in specialized structures, equivalent to glandular trichomes in some plants. Many *Pleurotus* species immobilize nematodes via toxin-producing secretory cells called toxocysts (Thorn and Barron 1984; Hibbett and Thorn 1994; Mamiya *et al.* 2005). When a nematode comes into contact with the toxocyst its body adheres to the balloon envelope, typically causing it to burst, which may facilitate the delivery of toxins (Truong *et al.* 2007). Within 30 to 60 s of contact with the toxocyst the nematodes are completely immobilized and soon thereafter directional hyphae invade the body orifices and begin to digest the nematode (Thorn and Barron 1984; Truong *et al.* 2007).

Kwok *et al.* (1992) identified the nematicidal compound trans-2-decenedioic acid, which immobilized 95% of test nematodes (*Panagrellus redivivus*) within 1 hour, and suggested the toxin destroys nerve and muscle function in nematodes by affecting membrane permeability. Immobilized nematodes which are rinsed in deionized water or transferred to a fresh water agar plate rarely recover (Barron and Thorn 1987; Kwok *et al.* 1992). Truong *et al.* (2007) found that nematodes were paralyzed but not consumed when *Pleurotus cystidiosus* O.K. Miller subsp. *abalonus* (Y.H. Han, K.M. Chen & S. Cheng) O. Hilber was grown on a rich medium (potato dextrose agar). These paralyzed nematodes lived for a long period of time, suggesting death is ultimately caused by directional hyphae. Li *et al.* (2007a) identified the toxin linoleic acid in *P. ostreatus*, which appears to reduce the size of the nematode head (Satou *et al.* 2008).

Truong *et al.* (2007) observed the consumption of immobilized nematodes by *Pleurotus cystidiosus* O.K. Mill. when the colony was nutritionally starved on water agar. However, when grown on rich media (PDA) the nematodes were immobilized but not penetrated by hyphae despite the induction of toxocysts on the media. These observations support the idea that the role of the toxocyst is not limited to nutrient acquisition but also protection against mycophagous nematodes and other microfauna. The agarics *Conocybe lactea* (J.E. Lange) Métrod and *Panaeolina foenisecii* (Pers.:Fr.) R. Maire produce droplets of toxins on conspicuous secretory cells (Hutchison *et al.* 1996). These cells were shown to have an antifeedant function against a mycophagous *Aphelenchoides* species. Both fungi did not colonize or consume immobilized nematodes when cultured on water agar, suggesting an antifeedant function in the strictest sense.

Secretory cells occurring at various densities within the fungal colony may allow the fungus to mitigate costs of producing ubiquitous toxins; however the grazer must come into contact with them, whether by attraction or chance, for immobilization to occur. Truong *et al.* (2007) suggested that the storage and production of toxins might be more suitable if restricted within the toxocyst, especially if the toxin is potentially detrimental to the fungus itself (Kwok *et al.* 1992). Secretory cells may therefore be advantageous as a means of defence as they allow the production of more toxic compounds which are delivered to the grazer at higher concentrations.

Endotoxins

Tzean and Liou (1993) described two major modes of nematode immobilization in 18 *Hyphoderma* species. The first mode, involving adhesive stephanocysts, has been previously mentioned. The second mode involves the ingestion of fungal cytoplasmic toxins. Mycophagous nematodes became sluggish and died within 2 h following ingestion, while non-mycophagous, free-living nematodes remained unaffected. Immobilized nematodes were penetrated by hyphae and consumed within 24h. Dong *et al.* (2004) identified 13 freshwater fungi which produced endotoxins that were capable of immobilizing the nematode *B. xylophilus*. Endotoxins require ingestion and therefore expose the fungus to some grazing damage before the grazer is immobilized. However, this strategy may be more efficient in terms of metabolic cost than exotoxins, which must be constantly secreted to maintain an effective concentration in the substrate.

CONCLUSION

An exploration of the current literature reveals a longstanding interest in invertebrate mycophagy. Research interests include assessing the impact of grazing on fungal fitness and on the ability of fungi to perform ecological functions. Despite substantial efforts to understand this phenomenon in both laboratory and natural settings, our insight into the importance and mechanisms of grazing is very limited.

Unravelling the complex interactions between grazing invertebrates and their fungal prey offers many challenges and exciting possibilities. Future approaches should be directed to microcosm studies which investigate the fitness effects of grazing on both the fungus and grazer. Quantifying the impact of grazing on the fungus must

incorporate factors beyond growth rate, such as reproduction capability, competition potential, and nutrient acquisition.

It is evident that neither invertebrates nor fungi are passive participants and that their co-existence over the millennia is evidence of both organisms‟ ability to respond to one another. The tremendous success of filamentous fungi in the face of adversity suggests the employment of various mechanisms to mitigate general and specific damage caused to their hyphal network without risk to the integrity of the entire organism. Robust growth strategies combined with an arsenal of both general and specific chemical and physical defence responses may have enabled fungi as a whole to flourish while subjected to various environmental pressures including grazing.

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THE ABILITY OF TWO *SPHAEROBOLUS* SPECIES TO ENCAPSULATE AND IMMOBILIZE THE MYCOPHAGOUS NEMATODE *APHELENCHOIDES* SP.

ABSTRACT

Tanney, J.B. 2010. The ability of two *Sphaerobolus* species to encapsulate and immobilize the mycophagous nematode *Aphelenchoides* sp. Pp. 40-71.

Keywords: *Aphelenchoides*, defence mechanism, fungivory, gloeocystidium, grazing inhibition, hyphal grazing, mycology, mycophagy, nematode, perennial mycelia.

The presence of a defence mechanism against nematodes produced by cultures of *Sphaerobolus stellatus* Tode and *S. iowensis* L.B. Walker is described for the first time. Within 3 days of introduction, a mycophagous nematode (*Aphelenchoides* sp.) exhibited lethargic behaviour marked by the cessation of feeding and a reduction in locomotion. The anterior portion of the infected nematodes appeared swollen and was observed to be encapsulated with a material of unknown composition. The nematode stylet cannot penetrate the cap matrix, preventing further hyphal grazing and subsequently death of the nematode occurs through starvation. Immobilized nematodes are not consumed by the fungi. It is hypothesized that when the nematode pierces a gloeocystidium with its stylet the oleaginous contents are released and solidify on the nematode"s head. It is proposed that the gloeocystidia function to protect the hyphal system and act as an antifeedant mechanism to prevent hyphal grazing by mycophagous nematodes and possibly other fungus-feeding microfauna. The identification of such a mechanism provides an example of co-evolution between fungi and co-inhabiting microfauna.

INTRODUCTION

Fungi fulfill critical roles which drive terrestrial ecosystem processes. Decomposition of organic matter provides saprotrophic fungi with an energy source while providing fertility for primary productivity. Members of the Basidiomycota and Ascomycota that colonize litter and wood are the primary organisms responsible for the biological decay of organic carbon compounds (most abundant being cellulose, hemicellulose, and lignin) found in these substrates under well oxygenated conditions (Rayner and Boddy 1988).

The extensive hyphal systems produced by these fungi may persist for considerable periods of time. Due to the nutritive value of hyphae, lignicolous fungi may be subjected to intensive grazing by invertebrates during this extended mycelial phase. Mycophagous invertebrates include nematodes (Townshend 1964; Riffle 1971), enchytraeids (Hedlund and Augustsson 1995; Jaffee *et al.* 1997), mites (Mitchell and Parkinson 1976), collembola (Visser and Whittaker 1977; Shaw 1985), insects (Weber 1966; Newton 1984), insect larvae (Fletcher *et al.* 1989; Shaw 1992), mollusks (Silliman and Newell 2003), and amoebae (Chakraborty *et al.* 1985).

Grazing is an important factor in regulating higher plant community structure and biomass. Despite the wealth of knowledge accrued over several decades of plant ecology research, relatively little attention has been paid to fungal grazing until recently. Fungal grazers may alter fungal species richness by selectively eliminating or promoting certain species over others (Newell 1984ab; McGonigle 2007). Invertebrate grazing has been shown to impact fungal growth and respiration (Hedlund and Augustsson 1995;

Hanlon and Anderson, 1979; Bengtsson *et al.* 1993; Bretherton *et al.* 2006; Tordoff *et al.* 2007). Reproduction may also be adversely affected due to colony grazing (Melidossian *et al.* 2005; Rohlfs 2005) or sporophagy (Gochenaur 1987; Nakamori and Suzuki 2007).

Nematodes are among the most abundant multicellular organisms in the world and are of great ecological importance (Bongers and Bongers 1998). It is estimated that there are approximately 29 million nematodes per m^2 of mixed deciduous forest soil, and that their species diversity worldwide is exceeded only by the arthropods (Bernard 1992; Liang *et al.* 2000). Mycophagous nematodes feed on fungal hyphae by inserting a retractable stylet into the fungal cell and actively pumping out cytoplasm (Siddiqui and Taylor 1969). Extensive feeding by mycophagous nematodes can detrimentally affect mycelial networks and, consequently, ecological functions such as decomposition and mycorrhizal relationships (Ingham 1988; Gera Hol and Cook 2005; Boddy and Jones 2008).

The selection pressures exerted by mycophagous nematodes and other invertebrates must have caused fungi to evolve antifeedant mechanisms and other appropriate responses to grazing. These responses may include compensatory growth, avoidance, decrease in palatability, or the production of toxic secondary metabolites or appendages which physically deter grazing. Both chemical and physical nematode immobilizing and/or killing structures have been described, including almost 200 nematicidal substances of fungal origin (Li *et al.* 2007). With few exceptions, the research premise has been that the role of these devices in fungi is to trap nematodes as a supplementary nutritional source. The utilization of nematodes as an exogenous source

of nitrogen has been proposed, which may be of importance in relatively nitrogen poor woody substrates (Barron and Dierkes 1977; Thorn and Barron 1984).

Hutchison *et al.* (1996) identified toxin-producing secretory cells on hyphae of *Conocybe lactea* (J.E. Lange) Métrod and *Panaeolus foenisecii* (Pers.) Maire but found that the fungi did not colonize immobilized nematodes, suggesting an antifeedant function. Rohlfs *et al.* (2007) found that the collembola *Folsomia candida* (Willem) preferentially grazed upon a mutant *Aspergillus nidulans* (Eidam) G. Winter strain, which was unable to produce many secondary metabolites including mycotoxins, over the wild-type strain which was capable of producing an array of secondary metabolites. Repellent metabolites and the presence of crystals on the surface of hyphae were suggested to be part of the defensive strategies of fungi subjected to grazing by *Folsomia candida* (Böllmann *et al.* 2010).

While conducting antifeedant studies with mycophagous nematodes and various decay and mycorrhizal fungi, a novel defence mechanism was observed in *Sphaerobolus stellatus* Tode and *S. iowensis* Walker. The genus *Sphaerobolus* caught the attention of early mycologists who recognized its novel and dramatic spore dispersal method (Buller 1933; Ingold 1972), and has recently attracted notoriety as a nuisance among home and vehicle owners (Davis *et al.* 2005; Grossman 2005).

Following inoculation onto cultures of *Sphaerobolus stellatus* and *S. iowensis*, the feeding nematodes soon developed swollen heads which became encapsulated with an unknown material that effectively prevented stylet extension and subsequent feeding

behaviour. This study investigates the efficacy and mechanistic evidence of this unique antifeedant strategy.

MATERIALS AND METHODS

FUNGI

Cultures were obtained from peridioles of fresh basidiomata of *Sphaerobolus stellatus* and *S. iowensis*. The isolate of *S. iowensis* (DAOM 232081) and one isolate of *S. stellatus* (DAOM 2343082) originated in Guelph, Ontario and additional cultures of *S. stellatus* (DAOM XXXXXX [#589] and DAOM XXXXXX) were isolated from specimens collected in Thunder Bay, Ontario. Peridioles were removed, surface sterilized in 30% hydrogen peroxide for one minute, then washed several times in sterile water before being transferred to individual Petri dishes containing modified 2% malt extract agar (MEA) (20 g malt extract, 1 g yeast extract, 15 g agar, 1 L distilled water). Cultures were maintained on MEA at 20°C and transferred using 7 mm plugs cut with a sterile cork borer. A culture of *Coprinopsis macrocephala* (Berk.) Redhead, Vilgalys & Moncalvo (DAOM 232080) was obtained from tissue pieces of basidiomata originating from Guelph, Ontario and maintained on MEA. Voucher specimens for material from Guelph and Thunder Bay were deposited in the University of Guelph Mycological Herbarium and the Lakehead University Mycological Herbarium, respectively. All isolates were deposited in the Canadian Collection of Fungal Cultures, Ottawa.

NEMATODES

The mycophagous *Aphelenchoides* sp. (Figure 2.1) was isolated from forest soil on the campus of Lakehead University (Thunder Bay, Ontario). Basidiomata of

Piptoporus betulinus (Bull.) P. Karst. were cut into pieces and used to bait for nematodes. Nematodes were extracted from the fruiting body pieces using a modified Baermann funnel technique (Barron 1977). Contaminating organisms were overcome using a combination of an antibiotic solution (1000 mL water, 300 mg penicillin G, 30 mg streptomycin) and surface sterilization using 1% NaClO. Nematodes were selected and reared monoaxenically on cultures of *Coprinopsis macrocephala* and transferred once a month. A culture of nematodes was submitted to Agriculture and Agri-Food Canada (Ottawa, Ontario) to confirm the identification of the taxon at the genus level based on morphological characteristics.

FUNGUS-NEMATODE PAIRING

All isolates of *S. stellatus* and *S. iowensis* were transferred to 1.5% water agar (WA) (15 g agar, 1 L tap water) to facilitate initial observations. After reaching approximately two-thirds diameter of the Petri dish, cultures were inoculated with a suspension of nematodes in sterile tap water and observed every 24 hours using a dissecting microscope.

Nematodes used for detailed inoculation studies were extracted from *C. macrocephala* cultures using a modified Baermann funnel method (Ruess 1995) and nematode concentrations were calculated and adjusted using a haemocytometer. Fifty µl of the nematode suspension in sterile tap water, containing approximately 250 nematodes, were inoculated onto 4-week-old cultures of DAOM 232082 growing on MEA and incubated at 20° C. Three plates were harvested every 10, 20, and 30 days using the modified Baermann funnel method to quantify the number of infected

nematodes over time. Both healthy and encapsulated individuals were counted using a Nikon Eclipse E400 phase contrast light compound microscope. The proportion of encapsulated-to-healthy nematodes was calculated by dividing the number of encapsulated nematodes by the total number of observed nematodes for each sampling.

The effect of encapsulation on nematode locomotion was quantified by counting the number of lateral head movements per minute of infected and healthy adult nematodes. Approximately 250 nematodes were inoculated on five MEA plates containing 4 week-old cultures of DAOM 232082. Two weeks after inoculation the number of movements per minute of 10 encapsulated and 10 healthy individuals was recorded for each plate.

SCANNING ELECTRON MICROSCOPY

Infected nematodes were extracted using the modified Baermann funnel method (Ruess *et al.* 1995). The nematode suspension was transferred to 2 mL Eppendorf tubes and centrifuged for 12 minutes at 2000 rpm. After the supernatant was removed, a solution of 4% glutaraldehyde and 0.2 M phosphate buffer (pH 7.0) was added to the nematode pellet to obtain a final concentration of 2% glutaraldehyde. The tubes were shaken for 5 minutes and nematodes were fixed in the glutaraldehyde solution for 3 hours at 4°C. The suspension was centrifuged for 12 minutes at 2000 rpm and the supernatant was removed and replaced with buffer (pH 7.0). This washing process was repeated three times to remove residual glutaraldehyde. Following washing, the suspension was pelletized again and dehydrated in a graded series of ethanol (10, 20, 40, 60, 80, 100, 100, 100% for 15 minutes each). Drops of the nematode suspension were

placed directly on aluminum stubs and air-dried in a dessicator. Specimens were sputter-coated with gold and examined using a JEOL JSM-5900LV scanning electron microscope located at the Lakehead University Instrumentation Laboratory (LUIL).

LIGHT MICROSCOPY

Observations of fungal colonies and interactions with nematodes were made using a Wild Heerbrugg M5 stereoscope, a Nikon Eclipse E400 phase contrast compound microscope, and an Olympus IX51 inverted differential interference contrast microscope. Various mounting media, including Phloxine B (1% aqueous solution), lactophenol cotton blue, and Melzer‟s reagent, were used to stain samples (Malloch 1981). Measurements were made on material mounted in Phloxine B. Digital micrographs were captured with an Olympus EVOLT E-330.

STATISTICS

The experimental design investigating the effect of time on the proportion of encapsulated nematodes is illustrated in the following linear model .

$$Y_{ijk} = \mu + D_i + S_{(i)j} + \varepsilon_{(ij)k} \qquad \text{Equation (1)}$$

$$i = 1, 2, 3 \quad j = 1, 2, 3, 4, 5 \quad k = 3$$

Where:

- $Y_{ijk(l)}$ = the proportion of encapsulated nematodes from the l^{th} replicate in the k^{th} sample of the j^{th} Petri dish on the i^{th} day.
- μ = the overall mean.
- D_i = the fixed effect of the i^{th} of three levels of factor D.
- $S_{(ij)k}$ = the nested effected of the k^{th} of five levels of factor S in factors D and P.

$\varepsilon_{(ijk)l}$ = the random effect of the l^{th} of 1 experimental unit in the ijk^{th} treatment combination. The $\varepsilon_{(ijk)l}$ are assumed to be i.i.d. $N(0, \sigma^2)$.

Residuals (ε_{ijk}) were explored visually with mean frequency histograms and quantile-quantile (Q-Q) plots and further tested using the Shapiro-Wilk test for normality. The dependent variables (Y_{ijk}) which were not normally distributed were transformed respectively. The Mann-Whitney U-Test ($\alpha = 0.05$) was used to determine the significance of treatments when data were non-normal and could not be sufficiently transformed into a normal distribution. All tests were performed using SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA).

RESULTS

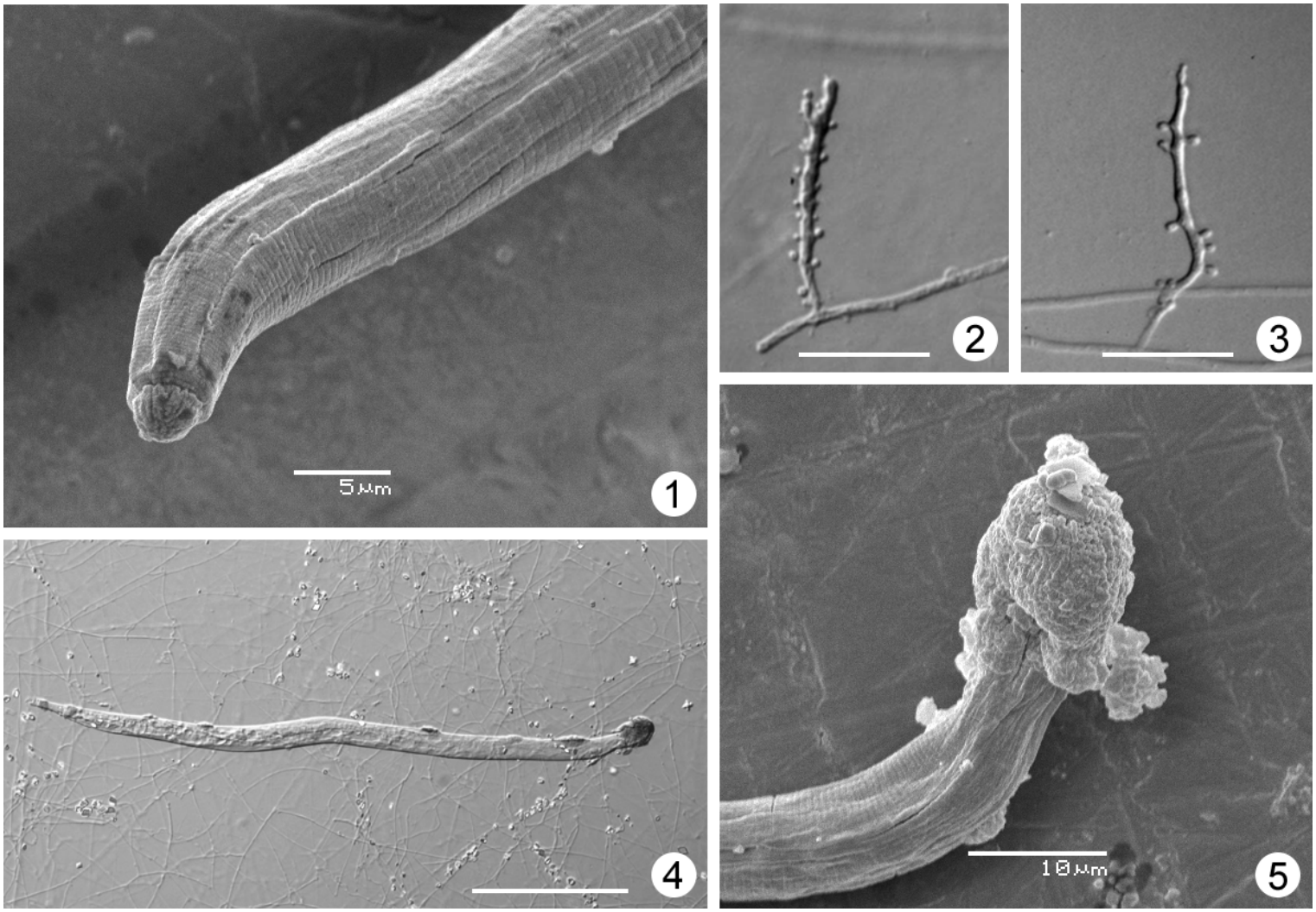
NEMATODE-*SPHAEROBOLUS* OBSERVATIONS

Observations on the feeding habits of *Aphelenchoides* sp. showed that nematodes tended to move along an individual hypha while simultaneously using their heads to probe the hypha, presumably testing the turgidity which is indicative of the presence of cytoplasm. *Aphelenchoides* sp. possess a specialized mouthpart called a stylet, which they use to penetrate the hyphal wall and actively pump out the cytoplasm. After feeding, the stylet is retracted and the hyphal cell may appear empty or shrunken. Colonies of *C. macrocephala* supported the rapid population growth of *Aphelenchoides* sp., with subsequent feeding causing the destruction of virtually all aerial mycelium, resulting in a characteristic moist and appressed colony appearance. These observations on the impact of grazing nematodes were parallel to those made by other authors (Riffle 1967; Sutherland and Fortin 1968; Ruess and Dighton 1996).

Following inoculation onto cultures of *S. stellatus* and *S. iowensis*, nematodes migrated to the margins of colonies or into the agar below the colonies. Within 24 hours after introduction, a few of the nematodes occupying the colony surface exhibited a decrease in locomotion, a complete cessation of feeding behaviour, and the encapsulation of their anterior portion with a material of unknown composition (Figure 2.5). The encapsulating material was always found concentrated on the anterior end of the nematode with a lesser amount occasionally found adhering along the lateral portion (Figure 2.4). The caps were accentuated when mounted in lactophenol cotton blue and phloxine B, indicating the absorption of both dyes by the encapsulating matter.

Surface hyphae of *S. stellatus* and *S. iowensis* growing on MEA produced conspicuous upright cells, similar in morphology to gloeocystidia (Figures 2.2 and 2.3). The gloeocystidia were erect and narrow, approximately 40.0-110.0 µm tall and 2.0-4.0 µm wide. The apex of the gloeocystidia possessed numerous bulbous, knob-like projections (3-25 per gloeocystidium), with each knob being 1.5-2.0 × 2.0-8.0 µm. The gloeocystidia were variable in shape and found at concentrations of up to 8000/cm^2 on water agar. Contents of the gloeocystidia possessed a refractive index different from the subtending hyphae, appearing oily. Crystals were found encrusting both hyphae (Figure 2.4) and the knob-like projections of the gloeocystidia when cultures were grown on water agar for several weeks. Gloeocystidia were sometimes found detached and adhering to the globular material encapsulating the infected nematodes.

FIGS 2.1-2.5. 2.1. SEM micrograph of control *Aphelenchoides* sp. reared on *C. macrocephala* (bar = 5 µm). 2.2-2.3. Gloeocystidia on aerial hyphae of *S. stellatus* (bar = 20 µm). 2.4. Encapsulated nematode with adhering material along the body (bar = 120 µm). 2.5. SEM micrograph of encapsulated nematode head (bar = 10 µm).


5µm
1
2
3
4
10µm
5

Rate of Encapsulation

Variable proportions of encapsulated nematodes were observed among dishes and the three time treatments (Table 2.1). The total observed nematodes on day 10, 20, and 30 were 962, 6359, and 10240 individuals, respectively, indicating a rapid population growth.

Table 2.1. Nematode encapsulation rates observed with time treatments.

Day	Dish	Proportion of Encapsulated Nematodes (%)
10	1	21.80
10	2	11.12
10	3	14.92
Average		**15.95**
20	1	31.82
20	2	16.09
20	3	12.43
Average		**20.12**
30	1	21.74
30	2	20.58
30	3	17.97
Average		**20.09**

The data representing the proportion of encapsulated nematodes over time was explored prior to further analysis. A visual test using a data frequency histogram and quantile-quantile plot (Q-Q plot) shows that the residuals do not appear to conform to a normal distribution (Figure 2.6 and 2.7).

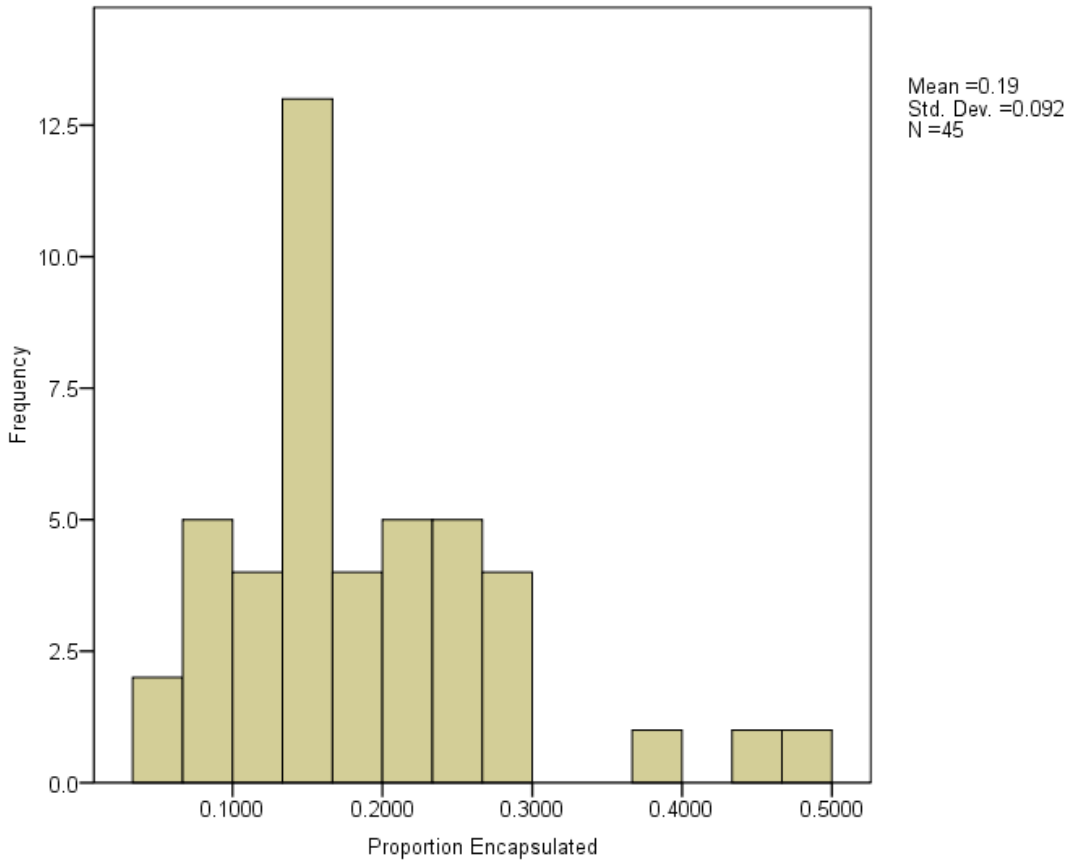


Figure 2.6. Mean frequency histogram for proportion of encapsulated nematodes.

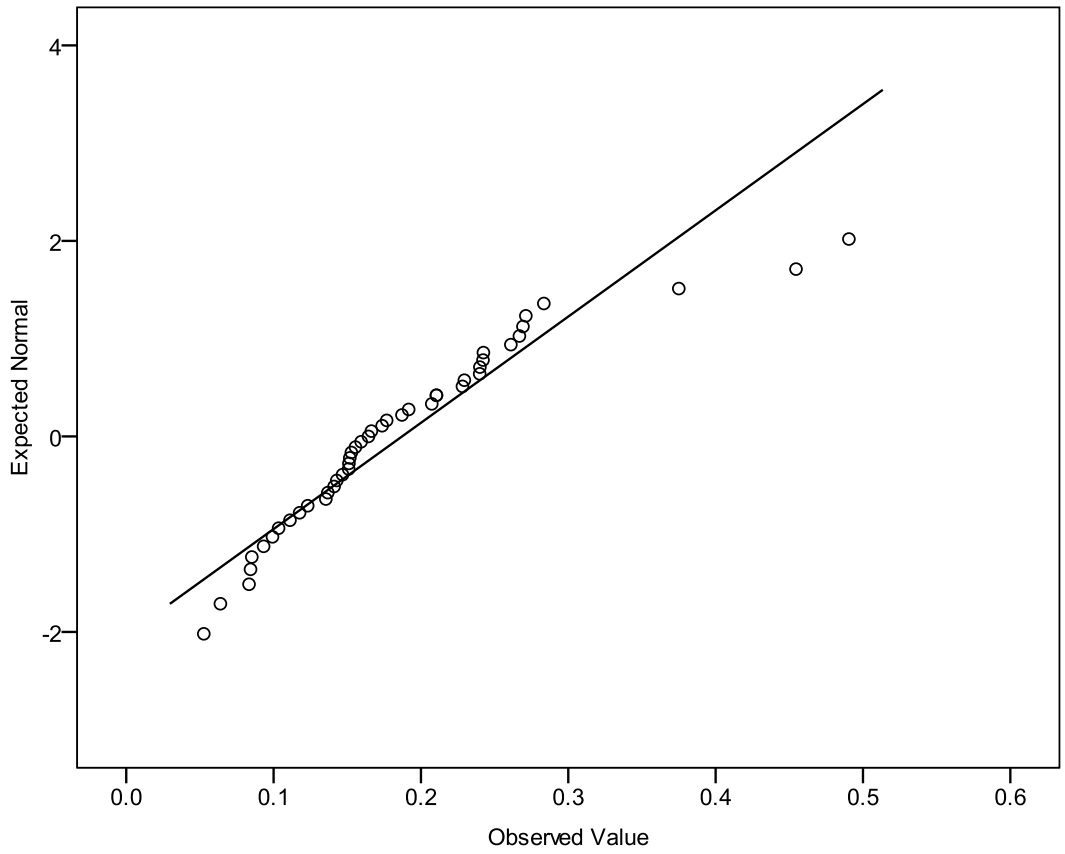


Figure 2.7. Normal Q-Q plot for proportion of encapsulated nematodes data.

The Shapiro-Wilk test for normality was used to quantitatively assess the distribution of residuals. The skewness value indicated a highly positive skew, indicative of a non-symmetric distribution. The Shapiro-Wilk test with a p-value < 0.05 rejected the null hypothesis that the residuals are normally distributed. The proportional data are therefore not normally distributed and do not meet the assumptions of an ANOVA test.

Table 2.2. Normality test statistics for the proportion of encapsulated nematodes data.

Skewness	Kurtosis	Shapiro-Wilk		
Statistic	Statistic	Statistic	df	Sig.
1.400	2.703	.893	45	.001

The data (proportion of encapsulated nematodes %) were transformed using a base-10 logarithmic transformation. The mean frequency histogram and Q-Q plot demonstrated an acceptable degree of normality for the transformed data (Figure 2.8 and 2.9).

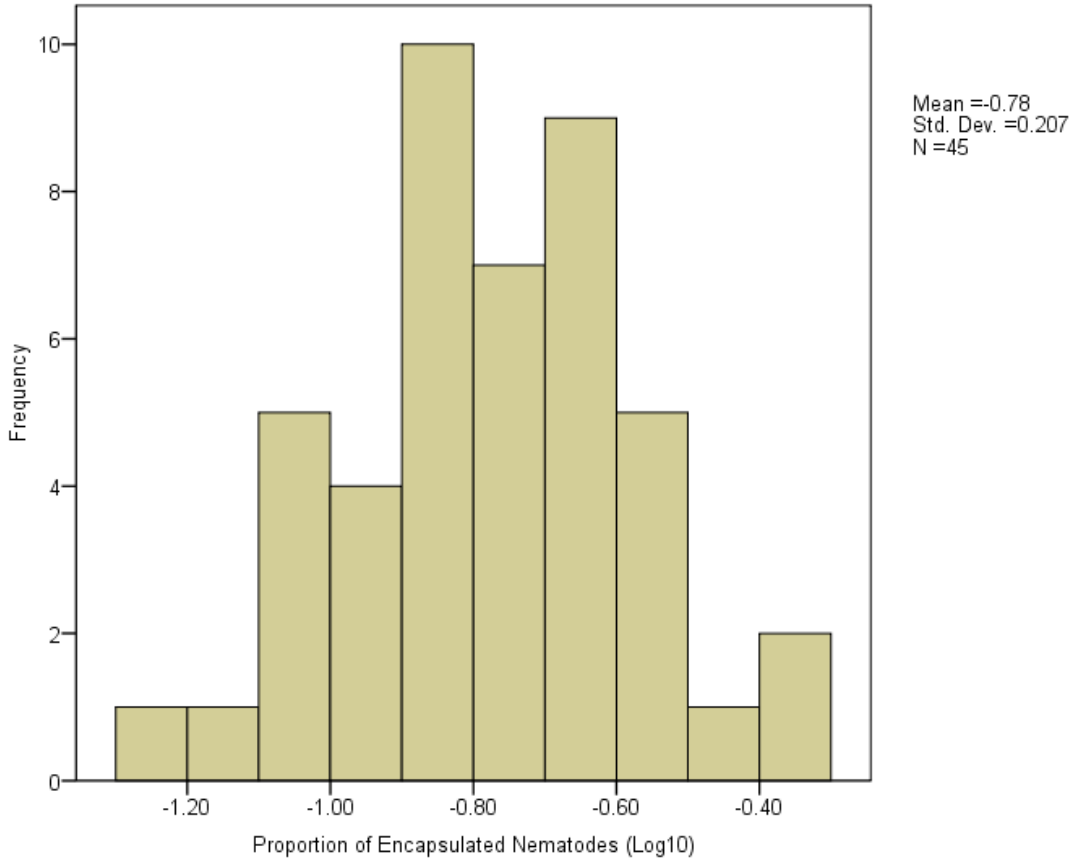


Figure 2.8. Mean frequency histogram for transformed proportion of encapsulated nematodes.

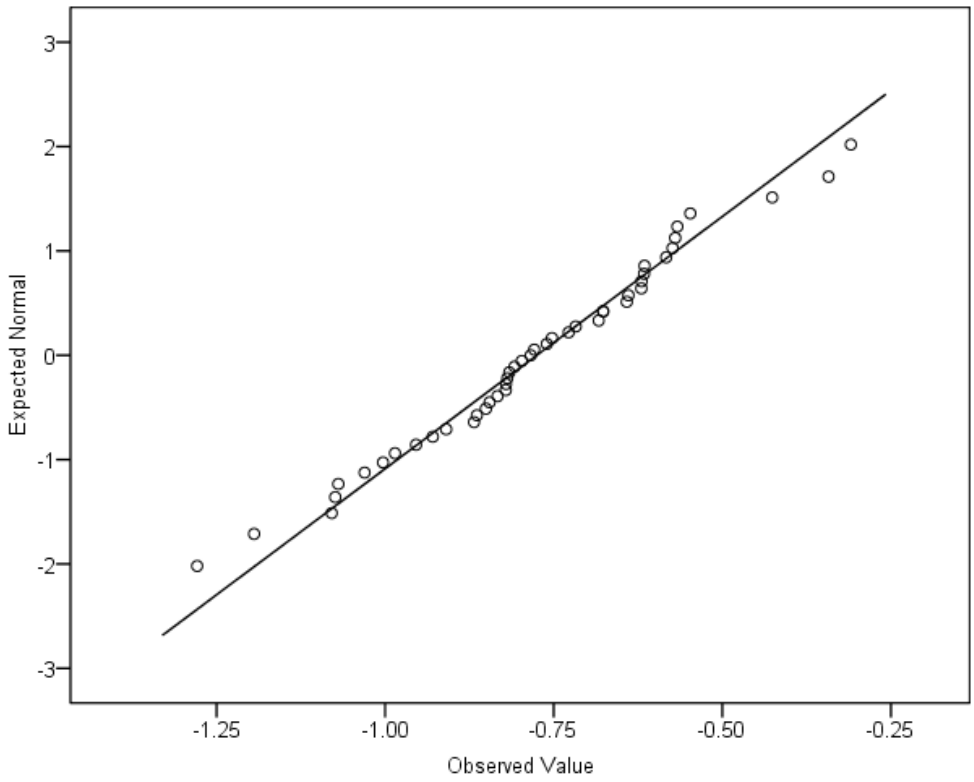


Figure 2.9. Normal Q-Q plot for transformed proportion of encapsulated nematodes data.

The skewness and kurtosis values indicated an improvement in the normality of the data (Table 2.3). The Shapiro-Wilk test with a p-value > 0.05 fails to reject the null hypothesis that the residuals are normally distributed.

Table 2.3. Normality test statistics for the transformed proportion of encapsulated nematodes data.

Skewness	Kurtosis	Shapiro-Wilk		
Statistic	Statistic	Statistic	df	Sig.
-.111	.197	.986	45	.858

The number of days after nematode inoculation did not have a significant effect on the proportion of encapsulated nematodes (Table 2.4).

Table 2.4. ANOVA results for proportion of encapsulated nematodes data.

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	Hypothesis	27.038	1	27.038	786.572	.000
	Error	.412	126	.034		
Day	Hypothesis	.180	2	.090	2.623	.113
	Error	.412	2	.034		
Sample(Day)	Hypothesis	.412	12	.034	.799	.648
	Error	1.291	30	.043		

An increase in the proportion of encapsulated nematodes over time is observed in Figure 2.10, however this trend is not as readily apparent when the average proportion of infected nematodes per dish over time are viewed (Figure 2.11). An increase in the number of replicates used in the experiment may help reduce the error associated with the dish effect.

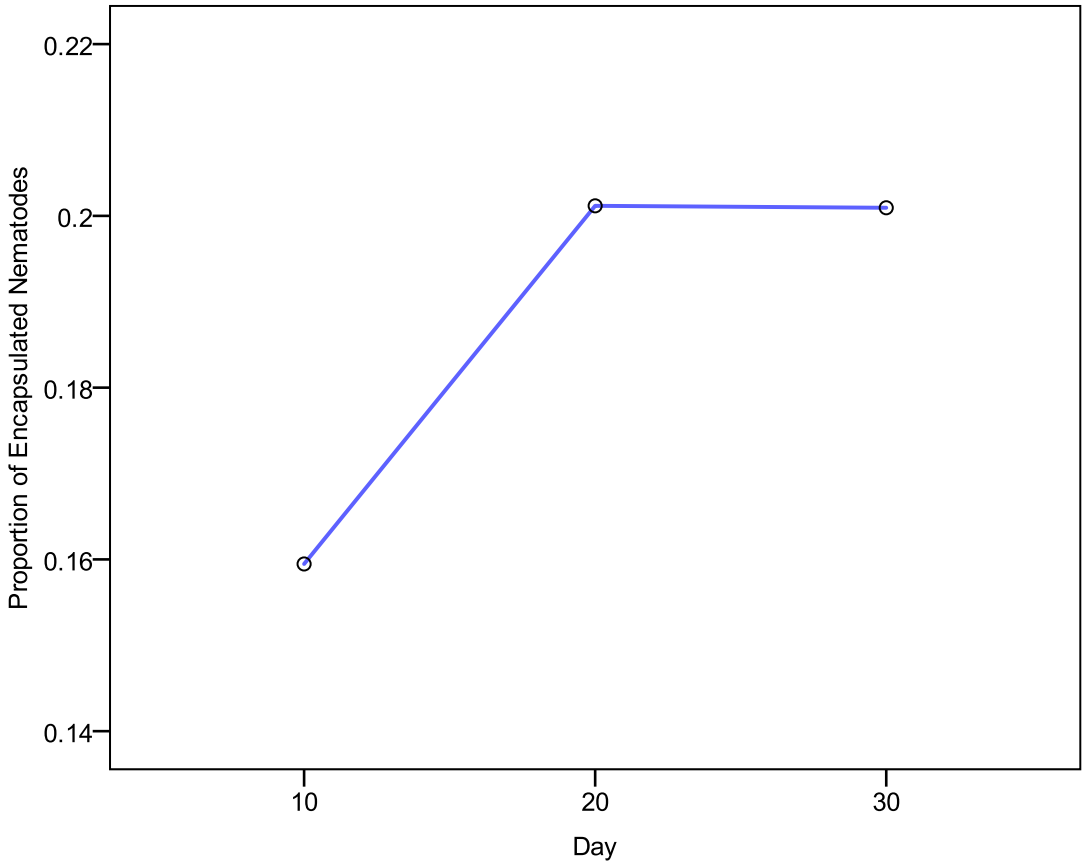


Figure 2.10. Proportion of encapsulated nematodes over 3 sample periods.

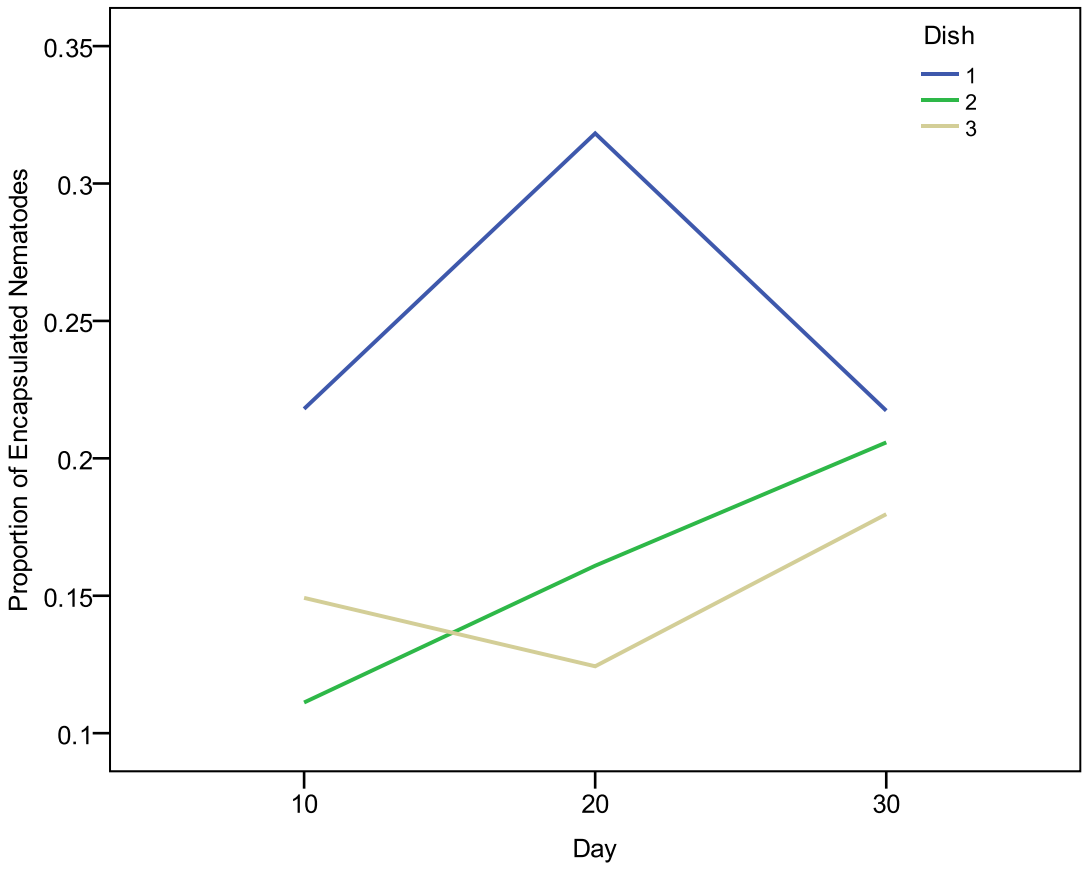


Figure 2.11. Proportion of encapsulated nematodes over time for each Petri dish count.

The matrix of the cap obstructed stylet extension, preventing infected nematodes from feeding on fungal hyphae. Affected nematodes demonstrated reduced activity and eventually appeared dead when physical stimulation did not incite a physical response. There was no reaction by the hyphae of either *S. stellatus* or *S. iowensis* to the presence of dead nematodes. Nematodes eventually degraded over time; however, the encapsulating material persisted. The infection rate was not high enough to prevent the reproduction and subsequent development of low and apparently sustainable nematode populations over time. The encapsulation phenomenon was never observed with an unidentified bacterial-feeding nematode introduced onto *Sphaerobolus* cultures.

Effect of Encapsulation on Nematode Movement

To determine the effect of encapsulation on the number of lateral turns, a Mann-Whitney U-Test was performed due to the non-normal dataset and inability to transform the data into a normal distribution. The data were ranked accordingly prior to the test. The encapsulation phenomenon had a significant effect on the number of lateral head turns made by the affected nematodes (Table 2.5).

Table 2.5. Mann-Whitney U-Test results for effects of encapsulation on number of lateral head turns.

	Health Status	N	Mean Rank	Sum of Ranks
Movement	Healthy	50	75.23	3761.50
	Encapsulated	50	25.77	1288.50
	Total	100		

Test Statistics	Movement
Mann-Whitney U	13.500
Wilcoxon W	1288.500
Z	-8.532
Asymp. Sig (2-tailed)	.000

Nematodes with encapsulated heads displayed a 75% decrease in lateral head turns per minute (5.1 ± 3.4) compared to healthy specimens (20.1 ± 6.0) (Figure 2.12). Head turns often resulted in a full sigmoid wave in healthy individuals but rarely so in infected nematodes, whose movements were restricted to feeble gestures of the head, infrequently resulting in actual locomotion. Encapsulated nematodes were always found occupying the agar surface and appeared unable to penetrate and move into the substrate, which healthy nematodes frequently inhabited.

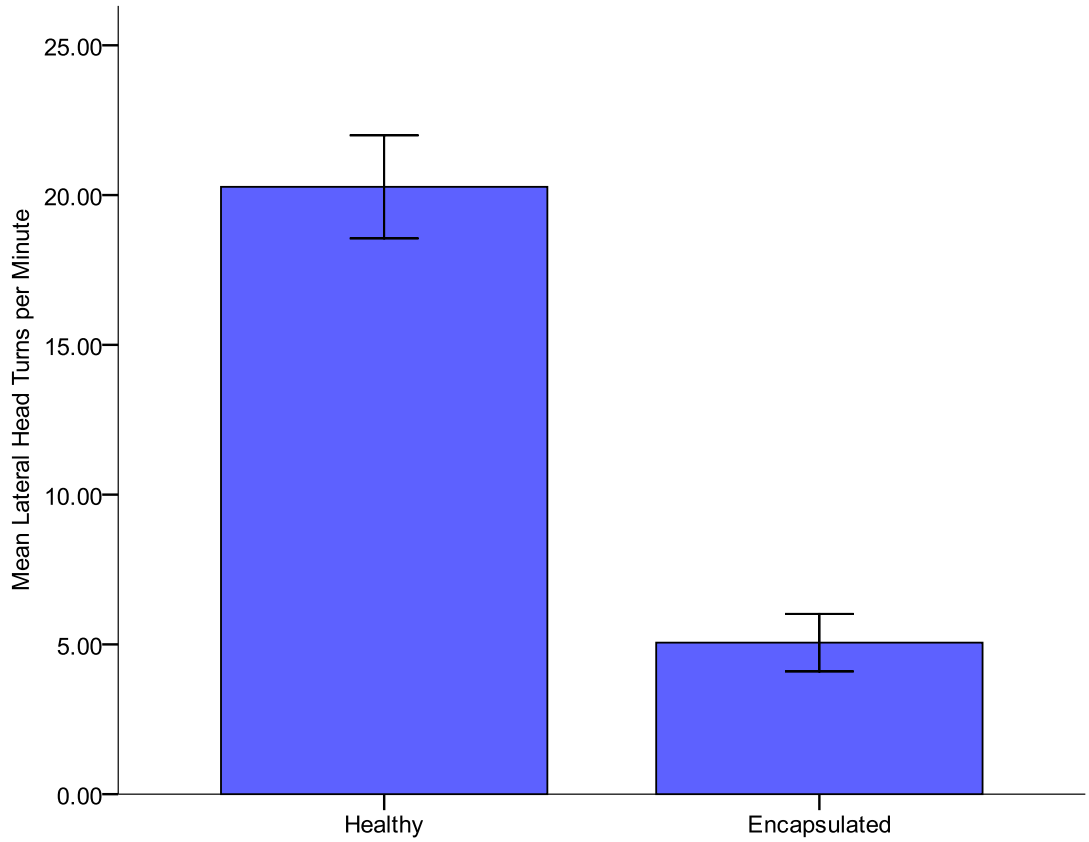


Figure 2.12. Effect of encapsulation on nematode movement (error bars = 2 standard errors).

DISCUSSION

It is hypothesized that the encapsulating material originates from the gloeocystidia produced on the surface mycelium of *Sphaerobolus* colonies. When a fungus-feeding nematode such as *Aphelenchoides* sp. inserts its stylet into the gloeocystidium, the oleaginous-like contents are liberated and come into contact with the nematode head. The oleaginous contents solidify and form a persistent cap on the head of the nematode. Although the composition of the cap is unknown at present, the matrix is of a nature which incapacitates the stylet so that it is no longer functional. As the nematode can no longer feed and movement is restricted, it is eliminated as a threat

to the hyphal network. The free-living bacterial-feeding nematodes possess sucking mouthparts for consuming bacteria and detritus as nutrient sources and are unable to pierce fungal hyphae. These nematodes were never encapsulated in the presence of gloeocystidia, suggesting the feeding behaviour of the *Aphelenchoides* sp. may play a key role in the antifeedant process. The gloeocystidia are therefore presented as a novel antifeedant mechanism which enables *S. iowensis* and *S. stellatus* to protect their hyphal network from grazing mycophagous nematodes.

Although gloeocystidia occur at relatively high densities on MEA and WA, the infection rates were not observed to be proportionally high. Gloeocystidia were restricted to the agar surface, permitting the relative safety of grazing nematodes when feeding on hyphae submerged in the agar. The observed immobilization rates over time do not represent an absolute proportion of infected individuals, as the fecund nematodes are able to rapidly reproduce on *Sphaerobolus* cultures, which also indicates that *Sphaerobolus* hyphae is of sufficient nutritional content to sustain *Aphelenchoides* sp. The majority of immobilized nematodes appeared to be adults. It is plausible that juvenile nematodes are able to shed the encapsulating material through moulting, which might account for the low number of infected juveniles. Moulting has been found to facilitate the removal of parasites in other organisms (Whitney 1982), an inadvertent or deliberate benefit of moulting which may also be applicable to nematodes. The high variability found in the plate counts over the three time periods suggests a larger sample size is required to more accurately determine the effect of time on the proportion of infected nematodes.

Agerer *et al.* (1994) reported that the cystidia on the ectomycorrhizae formed by

species of *Russula* exuded an oily substance when the apical knob was broken off. These ectomycorrhizal cystidia were suggested by Agerer *et al.* (1994) to function as a possible defence mechanism against grazing soil invertebrates. The sticky cystidial secretions on *Russula bella* Hongo fruiting bodies were implicated in the deaths of two grazing collembolan species, although the exact mechanism remains unclear (Nakamori and Suzuki 2007). Because gloeocystidia have evolved independently among many different taxonomic groups within the Basidiomycota, particularly among members of the Corticiaceae *s.l.* (Talbot 1954; Jülich and Stalpers 1980, Maekawa 1994), it is suggested that they possibly play some sort of similar defensive role as that observed with *S. stellatus* and *S. iowensis*. Future research directions may involve investigating the interactions between grazing invertebrates and the lactiferous hyphae of some fungi, including species of *Lactarius*.

Although the ability of *Sphaerobolus* to defend itself against grazing by obstructing the mouthparts of the grazer may be the only known example among fungi, this strategy has been observed in other organisms. Darby *et al.* (2002) found that the bacterium *Yersinia pestis* (Lehmann & Neumann) produced an extracellular biofilm which obstructed the mouthparts and inhibited feeding in the bacteria-feeding nematode *Caenorhabditis elegans* Maupas. Almost 8% of all plant species bear laticiferous structures, which have been implicated in both chemical and physical defence roles, including the gumming up of feeding insect mouthparts (Lewinsohn 1991). Such defensive phenomena are referred to as “antifeedants”, which are counter predation measures that are either chemical or physical in nature, and are utilized by one organism to prevent or deter being eaten by another. The occurrence of antifeedants among

animals (e.g.: Eisner *et al.* 1996) and vascular plants (e.g.: Cooper and Owen-Smith 1986, Dalin and Björkman 2003, Rhoades 1979) is well recognized, but such observations among Ascomycetous and Basidiomycetous fungi have been few. Apart from the present report on the function of gloeocystidia, other such antifeedant mechanisms also include the presence of setae on perithecia of *Chaetomium* spp. (Wicklow 1979), the production of toxin droplets from secretory cells of *Conocybe lactea* and *Panaeolina foenisecii* (Hutchison *et al.* 1996), the production of cytoplasmic toxins in fungal hyphae (Shaw 1985; Tzean and Liou 1993), the release of toxic volatiles and antibiotics from fungal hyphae (e.g.: Hayashi *et al.* 1981, Riffle 1971, Stadler *et al.* 1993; Stadler *et al.* 1994; Rohlfs *et al.* 2007), and the presence of calcium oxalate crystals on hyphae (Böllmann *et al.* 2010). All of these types of known or possible antifeedants among fungi must have evolved in response to grazing by fungus-feeding invertebrates including mycophagous nematodes.

There are many more facets of this interaction which demand further investigation. The model organism used in this study, a species of *Aphelenchoides*, is most likely one of several "target organisms" of this defence mechanism, as there are many other mycophagists which may graze on *Sphaerobolus* colonies *in situ*. The effects of gloeocystidia on other mycophagist microorganisms with various feeding mouthparts, such as collembola and mites, might offer more insight into this defensive interaction. The chemical composition of the encapsulating material on infected specimens must also be identified to further the understanding of this unique defence mechanism. The evidence of antifeedants in fungi offers an exciting opportunity to

investigate potentially novel mechanisms which are of systematic, ecological, and possibly applied significance.

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THE PRODUCTION OF NEMATODE-IMMOBILIZING SECRETORY CELLS BY *CLIMACODON SEPTENTRIONALIS*

ABSTRACT

Tanney, J.B. 2010. The production of nematode-immobilizing secretory cells by *Climacodon septentrionalis*. Pp. 72-87.

Keywords: *Aphelenchoides*, defence mechanism, fungivory, grazing inhibition, hyphal grazing, mycophagy, nematode, perennial mycelia, secretory cell, wood-decay.

The ability of *Climacodon septentrionalis* (Fr.) P. Karst to immobilize and kill a mycophagous nematode (*Aphelenchoides* sp.) *in vitro* is described for the first time. Two isolates produced droplets which formed at the apices of tall, stalked, and branching secretory cells. 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. Immobilized or killed nematodes were rarely colonized by dense hyphal growth, with some individuals persisting for weeks while others were degraded within several days. The exact mechanism which causes the immobilization of nematodes is currently unknown. This study, involving 14 growth media, demonstrated the effect of the growth media composition and concentrations on secretory cell production. This study provides the first documentation of a non-agaricoid fungus producing secretory cells which are able to immobilize nematodes.

INTRODUCTION

The ability of fungi to immobilize and kill co-inhabiting microorganisms has captured the interests of researchers for over a century (Zopf 1888). 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 which 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.:Fr.) Pers. and the acanthocytes of *Stropharia rugosoannulata* Farlow ex Murrill, can immobilize nematodes by physically damaging the cuticle and causing the leakage of inner contents (Luo *et al.* 2007; Luo *et al.* 2006).

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 which these fungi subsist on may be supplemented with exogenous sources of nutrients including yeasts, bacteria, pollen grains, spores, and the various invertebrates which

occupy their environment (Hutchison and Barron 1996; Barron 1988; Hutchison and Barron 1997; Fries and Swedjemark 1985).

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

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* (Farr *et al.* 1989; Ginns and Lefebvre 1993). The presence of its very large, shelving hydnoid basidiome is indicative of 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 hemerophilous fungus, presumably due to its low economic impact. However, *C. septentrionalis* may be an aggressive parasite of mature hardwoods in the urban environment (Koski-Kotiranta and Niemelä 1988).

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

MATERIALS AND METHODS

SOURCE OF FUNGAL CULTURES

Experiments were conducted using two isolates of *Climacodon septentrionalis* (DAOM XXXXXX, XXXXXX). Isolate DAOM XXXXXX was derived from tissue of a fresh basidiome growing on a declining *Acer saccharinum* L. tree in Thunder Bay, Ontario. The second isolate originated from basidiome tissue collected from the base of a mature *Acer nigrum* Michx. tree in London, Ontario. Cultures were grown on modified 2% malt extract agar (MEA) (20 g malt extract, 15 g agar, 1 g yeast extract, 1000 mL sterile distilled water) and transferred monthly. All isolates were deposited in the Canadian Collection of Fungal Cultures, Ottawa.

NEMATODE ISOLATION AND COLLECTION

The mycophagist nematodes used in this study were a species of *Aphelenchoides* isolated from forest soil in Thunder Bay, Ontario. 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 (1000 ml water, 300 mg penicillin G, 30 mg streptomycin) 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 using a modified Baermann funnel technique described by Ruess (1995). The nematode and tap water

suspension was calculated and adjusted using a haemocytometer. Fifty µl of the suspension containing approximately 250 nematodes were inoculated onto cultures of the two *C. septentrionalis* isolates growing on both MEA and water agar (WA) (15g agar, 1000 mL distilled water).

MEDIA STUDY

Isolate DAOM XXXXXX was cultured on various MEA and Czapek‟s solution agar (CSA) (Malloch 1981) concentrations, where either the sucrose and/or $NaNO_3$ levels were altered (as in CSA) or the malt extract levels were altered (as in MEA), to assess the effect of nutrients on secretory cell production. Individual water agar plugs containing 4-week-old mycelia were placed in the centre of Petri dishes containing the various media concentrations (Table 3.1). For each media treatment, two 1 mm^2 areas of the colony were randomly selected and the number of droplet-forming secretory cells was counted using a dissecting microscope. The experiment was conducted in triplicate for each medium and the results expressed as means. The effect of media on the number of observed secretory cells was explored using the Kruskal-Wallis *H*-Test and Mann-Whitney U-Test with SPSS Statistics 17.0 software (SPSS Inc. 2008).

MICROSCOPY

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 and a Nikon Eclipse E400 phase contrast light compound microscope. Measurements were made on material mounted in Phloxine B. Digital micrographs were captured with an Olympus EVOLT E-330.

RESULTS

SECRETORY CELL OBSERVATIONS

Secretory cells were produced on aerial mycelia in great abundance by the two *Climacodon septentrionalis* isolates on MEA. The cells were first produced in the centre 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) wide, and produced on tall, septate, single-celled stalks which branched 1-3 times (Figure 3.2). Individual stalks were 700-1500 µm tall; however the branches were often many times taller but difficult to measure due to their dense growth. Large, transparent droplets, approximately 20-45 µm (= 33 µm) in diameter, were produced at the apex of the secretory cells and appeared to be insoluble in water. In the presence of nematodes or when cultures were mechanically damaged, the droplets became reddish in colour as the colony matured and appeared to solidify when the stalks collapsed and came into contact with the agar surface. A change in the colour of droplets was not observed in cultures not inoculated with nematodes or not mechanically damaged.

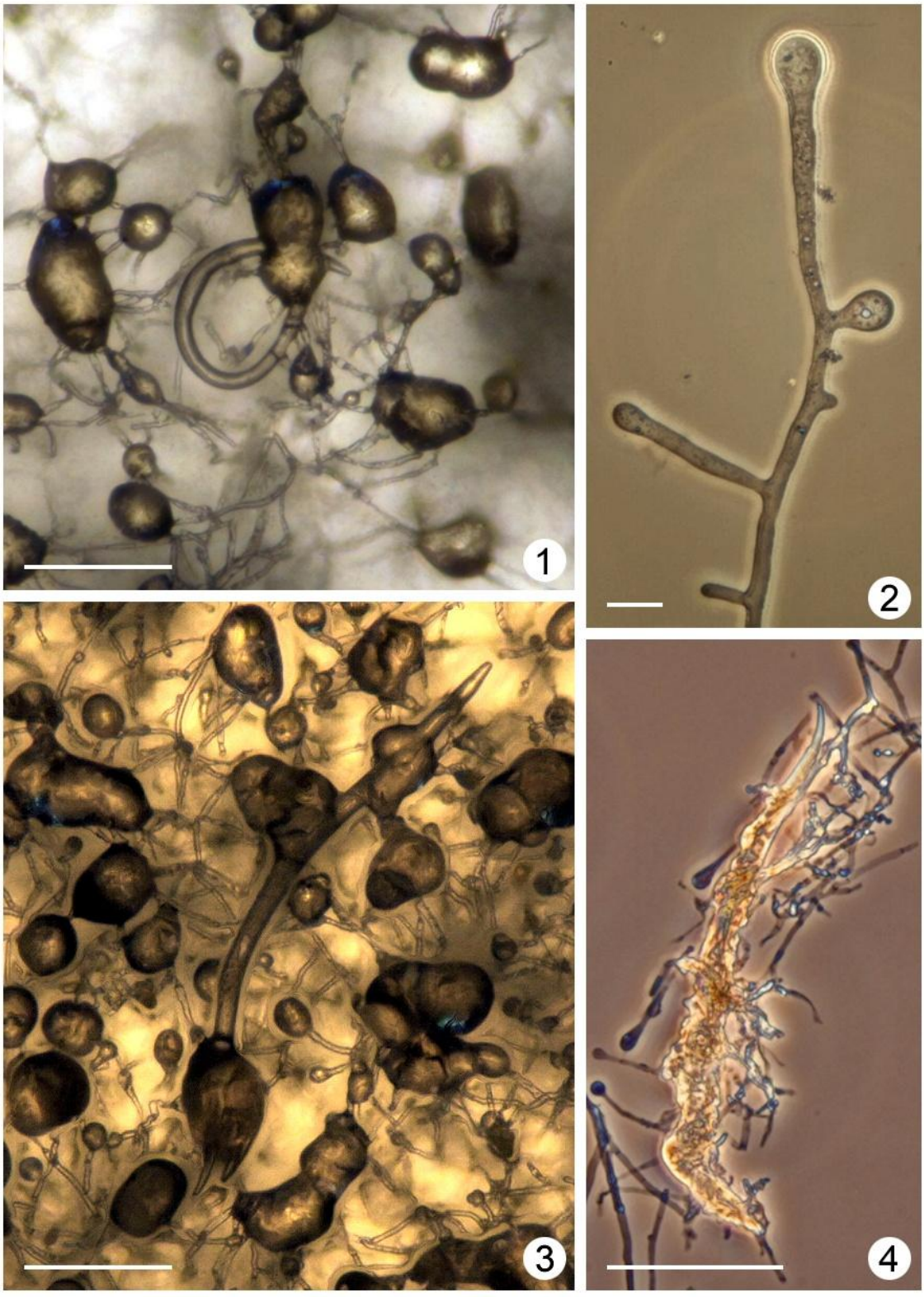
NEMATODE-FUNGUS INTERACTIONS

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. 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 (Figures 3.1 and 3.3). 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 which were put into 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 hours after immobilization. Nematodes degraded at a variable rate, with some persisting for weeks while those enveloped in hyphae becoming unrecognizable within 72 hours. The production of secretory cells was not observed when isolates were when grown on Czapek Solution Agar (Malloch 1981) or WA media. When dead nematodes were place on *C. septentrionalis* colonies reared on WA, an increase in nematode colonization or the occurrence of directional hyphae growth was not observed.

FIGS 3.1-4. 3.1,3. Nematodes immobilized within droplets exuded from secretory cells (bar = 100 μm). 3.2. Secretory cell produced on aerial mycelia of *C. septentrionalis* (bar = 10 μm). 3.4. Hyphal growth enveloping dead nematode (bar = 100 μm).


1
2
3
4

MEDIA STUDY

The residuals obtained from the media study were not normally distributed and attempts to transform the data were unsuccessful. The Kruskal-Wallis *H*-Test, a non-parametric method, was therefore selected to compare the treatments (Table 3.2). The data were ranked accordingly prior to the test.

The results of the Kruskal-Wallis *H*-Test rejected the null hypothesis that the probability distributions of the number of secretory cells are the same for all media treatments (Table 3.1).

Table 3.1. Kruskal-Wallis *H*-test results for the effect of medium on secretory cell production.

	Media
Chi-Square	79.785
df	13
Asymp. Sig.	.000

To determine which media were significantly different than one another, Mann-Whitney U Tests were performed for every combination of media pairs. The groups of media which were not significantly different were placed into 3 subsets for comparison (Table 3.2). The nine media concentrations which produced zero secretory cells were grouped together. All concentrations of CSA media resulted in appressed growth with sparse aerial mycelium. The production of secretory cells was not observed in isolate DAOM XXXXXX when grown on CSA, WA, or 0% MEA media. The second group consisted of four growth media which produced a moderate number of secretory cells within the aerial mycelia. The greatest production of secretory cells was observed in 2% MEA, which was significantly different than all other media concentrations.

Table 3.2. Media subgroups categorized via Mann-Whitney U tests.

Media	Subset (mean # cells / mm^2)		
	1	2	3
0% MEA (yeast)	0.0		
CSA	0.0		
CSA 0 g $NaNO_3$	0.0		
CSA 0 g sucrose	0.0		
CSA 0 g sucrose 0 g $NaNO_3$	0.0		
CSA 0.5 g $NaNO_3$	0.0		
CSA 0.5 g sucrose	0.0		
CSA 1.5 g sucrose 0.5 g $NaNO_3$	0.0		
WA	0.0		
2% MEA (no yeast)		113.5	
1% MEA		140.0	
1.5% MEA		146.3	
0.5% MEA		170.3	
2% MEA			235.3

DISCUSSION

The presence of nematode-immobilizing secretory cells in *C. septentrionalis* offers the first example of such a structure in a non-agaricoid fungus. The cells present in *C. septentrionalis* are produced on relatively tall, branching support stalks when grown on MEA, compared to similar but shorter and non-branched structures produced by *Pleurotus* spp., *Conocybe lactea*, and *Panaeolina foenisecii*. The occurrence of secretory cells at various densities within the fungal colony may offer several advantages over the production and excretion of extracellular toxins. Producing small quantities of concentrated toxins may be less metabolically costly than the continuous production of larger quantities of excreted toxins. The storage and production of toxins

might also be more suitable if restricted within a structure such as a secretory cell droplet, especially if the toxin is potentially detrimental to the fungus itself (Truong *et al.* 2007; Kwok *et al.* 1992).

The precise mechanism which causes the immobilization and subsequent death of the nematodes is currently unknown. The viscosity of the liquid droplet appears to play a role in restricting the movement of nematodes; however nematodes which 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.

Immobilized nematodes degraded at inconsistent rates. This was suspected to be a result of inoculation experiments being conducted 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 raised on a nutrient rich medium (PDA), the 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 protection against mycophagous nematodes and other microfauna. However, the present author found that when dead nematodes were placed on *C. septentrionalis* colonies which 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. Ex Fr.) Kummer, 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 model 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 chapter offer a stimulus for continued surveying of fungi and examining their interactions with their co-inhabiting fauna. Future experiments should include screening various invertebrate species with *C. septentrionalis* cultures, such as mites, Collembola, and other organisms which may share the same habitat. Fungi that produce similar structures in culture should be examined to determine if they have similar functions.

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APPENDIX I

LIST OF FUNGI USED IN NEMATODE PAIRINGS

	Species	Culture
1	*Agaricus bitorquis*	
2	*Agrocybe dura*	198
3	*Alpova mollis*	416
4	*Amanita muscaria var. alba*	500
5	*Amanita muscaria var. formosa*	224
6	*Amanita pantherina*	228
7	*Armillaria ostoyae*	254
8	*Asterophora lycoperdoides*	
9	*Auriscalpium vulgare*	307
10	*Baeospora myosura*	080
11	*Baeospora sp.*	
12	*Bisporella citrina*	239
13	*Bjerkandera adusta*	297
14	*Boletus edulis*	415
15	*Bovista sp.*	426
16	*Calvatia utriformis*	448
17	*Chalciporous piperatus*	456
18	*Chlorociboria aeruginascens*	141
19	*Clavariadelphus ligula*	203
20	*Climacodon septentrionalis*	
21	*Climacodon septentrionalis*	086
22	*Coprinus ephemerus*	422
23	*Coprinus heptemerus*	488
24	*Coprinus stercoreus*	141
25	*Crinipellis stipitaria*	203
26	*Crucibulum laeve*	419
27	*Entoloma abortivum*	
28	*Entoloma sp.*	89
29	*Flammulina rossica*	441
30	*Flammulina velutipes*	123
31	*Fomes fomentarius*	312
32	*Fomitopsis pinicola*	230
33	*Fomitopsis roseum*	403
34	*Galerina autumnalis*	078
35	*Ganoderma applanatum*	
36	*Grifola frondosa*	197
37	*Gyromitra gigas*	489
38	*Hebeloma crustuliniforme*	079
39	*Hebeloma mesophaeum*	397
40	*Hericium coralloides*	

	Species	Culture
41	*Heterobasidion annosum*	
42	*Inonotus tomentosus*	
43	*Laccaria bicolor*	227
44	*Lactarius deliciosus*	
45	*Laetiporus sulphureus*	189
46	*Lentinellus montanus*	430
47	*Lepista nuda*	420
48	*Leucoagaricus naucina*	
49	*Lycoperdon pyriforme*	292
50	*Lyophyllum decastes*	
51	*Morchella elata*	
52	*Mutinus caninus*	207
53	*Panus serotinus*	512
54	*Paxillus involutus*	231
55	*Peniophora rufa*	232
56	*Phaeolepiota aurea*	589
57	*Phaeolus schweinitzii*	222
58	*Phellinus tremulae*	427
59	*Pholiota aurivella*	294
60	*Piptoporus betulinus*	90
61	*Plicaturopsis crispa*	292
62	*Polyporus varius*	
63	*Psilocybe sp. 1*	
64	*Psilocybe inquilina*	223
65	*Psilocybe sp. 2*	224
66	*Rhizopogon ellenae*	413
67	*Rhizopogon rubescens*	297
68	*Sphaerobolus iowensis*	415
69	*Sphaerobolus stellatus*	123
70	*Sphaerobolus stellatus*	237
71	*Suillus granulatus*	206
72	*Suillus umbonatus*	192
73	*Trametes suaveolens*	095
74	*Tricholoma vaccinum*	423
75	*Tuber sp.*	308
76	*Urnula craterium*	199
77	*Xeromphalina campanela*	210
78	*Xylaria polymorpha*	417