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BIOLOGY, HISTOPATHOLOGY, AND IMPROVEMENTS IN AXENIC CULTURE OF LABYRINTHULA TERRESTRIS, CAUSAL AGENT OF RAPID BLIGHT OF COOL-SEASON TURFGRASSES

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BIOLOGY, HISTOPATHOLOGY, AND IMPROVEMENTS IN AXENIC CULTURE OF *LABYRINTHULA TERRESTRIS*, CAUSAL AGENT OF RAPID BLIGHT OF COOL-SEASON TURFGRASSES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Plant and Environmental Sciences

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by
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May 2012

Accepted by:
Dr. Julia Kerrigan, Committee Chair
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ABSTRACT

Rapid blight is a disease of cool-season turfgrasses caused by *Labyrinthula terrestris*. Disease symptoms include water-soaked lesions, yellowing and browning of foliage, and coalescing patches of dead turf. *L. terrestris* belongs to a group of marine microorganisms in the kingdom Chromista (also known as Stramenopila). Morphological characteristics of *Labyrinthula* spp. include spindle-shaped vegetative cells that move in an ectoplasmic network. The biology and pathology of *L. terrestris* is not clearly understood; therefore, the ultrastructure, life cycle, and histopathology of *L. terrestris* were investigated. In addition, improvements in axenic culture and long-term storage methods were made to better culture *L. terrestris.* Ultrastructure studies showed *L. terrestris* cells contain cytoplasmic contents consistent with relatives; bothrosomes, cell surface organelles that secrete the ectoplasmic network, were also elucidated. Examinations of cultures revealed that cells initially divided repeatedly with little motility, later cells were highly motile in ectoplasmic networks, and finally cells moved in a whirling motion and formed round aggregates. Aggregate formation was also observed on the host. Aggregates gave rise to new colonies and appear to play a role in survival under stress. Asexual reproduction was through mitotic cell division and sexual reproduction or production of zoospores was not observed. Histopathology investigations showed that *L. terrestris* infected the host through stomata, wounds, and trichome bases. *L. terrestris* occupied all foliar cells in the host, multiplied inside the host, and moved across host cell walls. The basic culture medium called SSA (serum saline water agar), typically used for *L. terrestris*, resulted in poor growth and viability. SSA was modified

with additional nutritive sources; this medium was designated as SSA+A (plus additives). Another medium, GESSA (grass extract SSA) was developed by adding grass extract to SSA. Results showed that the growth rate was significantly higher on GESSA followed by SSA+A and SSA. Cultures were successfully stored for up to two years with the modified long-term storage method.

DEDICATION

I dedicate this dissertation to my parents, Mr. Ravi Kumar Yadagiri and Mrs. Sesha Kumari Yadagiri, and my brother, Mr. Prakhyath Yadagiri, for their love, support, and encouragement throughout my education.

I further want to dedicate this work to all my friends and dear ones who made my life very special and meaningful.

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I would like to thank my committee member, Dr. Bruce Martin, for his research collaboration on rapid blight with me and Dr. Julia Kerrigan. I am thankful to him for providing me *Labyrinthula terrestris* isolates and for his valuable suggestions and comments on my research.

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PREFACE

Rapid blight, a disease of cool-season turfgrasses, was first observed in 1995 in southern California, USA (Stowell, 1995). Due to the pathogen's unusual morphological characteristics, it took almost a decade to identify and describe the causal microorganism, *Labyrinthula terrestris* (Bigelow et al., 2005; Olsen et al., 2003; Stowell et al., 2005). Although most *Labyrinthula* species are typically found in coastal marine or estuarine habitats, *L. terrestris* was isolated from a terrestrial habitat (golf courses).

Labyrinthula spp. have characteristic vegetative fusiform or spindle-shaped cells that move within an ectoplasmic double-membraned network in a gliding fashion (Porter, 1967; Porter, 1969; Porter, 1987). Based on morphological features such as tubular mitochondrial cristae (Porter, 1969), production of heterokont biflagellate zoospores (reported from one species, *L. algeriensis*) (Amon and Perkins, 1968), and molecular phylogenetic data (Honda et al., 1999; Tsui et al., 2009), the genus *Labyrinthula* has been placed in the kingdom Chromista, also known as Stramenopila (Dick, 2001; Kirk et al., 2001; Porter, 1987). The current taxonomy and classification of the genus *Labyrinthula* are discussed in Chapter 1.

Numerous studies have been conducted on different *Labyrinthula* species. Of special interest are two *Labyrinthula* spp. that cause plant diseases. Wasting disease of marine eelgrass, caused by *L. zosterae*, devastated populations of marine eelgrass in the early 1930's and 1980's (Cottam, 1933; Muehlstein et al., 1991; Renn, 1942). More recently, rapid blight of cool-season turfgrasses, caused by *L. terrestris*, has been emerging as a problematic disease and thus far has been reported from North America,

South America, and Europe. Rapid blight is a fast spreading disease known to destroy large areas of turf within a few weeks, hence the name 'rapid blight'(Martin et al., 2002b). The major symptoms of the disease include water-soaked lesions and yellowing and browning of the turf foliage, ultimately leading to death of the turf (Martin et al., 2002b; Stowell et al., 2005). The disease is frequently reported from golf courses where the irrigation water is saline in nature (Olsen et al., 2004). Salt-sensitive varieties of turfgrasses, such as *Poa trivialis* (rough bluegrass), *Lolium perenne* (perennial ryegrass), *Poa annua* (annual bluegrass), and *Agrostis tenuis* (colonial bentgrass) are found to be more susceptible to rapid blight (Martin et al., 2002a; Olsen et al., 2003; Stowell et al., 2005). More details on the history and background of rapid blight are discussed in Chapter 1.

In order to better manage or control any disease, a thorough understanding of the pathogen's biology is necessary. *L. terrestris* is a relatively new species that has been shown to differ from other turf diseases as well as other *Labyrinthula* species. Therefore, investigations were conducted on the biology, life cycle, and histopathology of *L. terrestris*. Advanced microscopy techniques such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), and light microscopy (LM) were used to study *L. terrestris*. In Chapter 3, studies on *L. terrestris*' ultrastructure, morphology, and the role of aggregate formation are reported. In Chapter 4 the processes of infection and colonization of host tissues by *L. terrestris* are reported. The axenic culture of *L. terrestris* was challenging when conducting experiments; therefore, improvements in pure culture and long-term storage methods of *L. terrestris* are described in Chapter 2.

Studies on *Labyrinthula* span a long time period but many publications are out of date and no comprehensive review of genus has been published in decades. Therefore, in Chapter 1, a literature review of *Labyrinthula* is presented, including studies on morphology and cytology, physiology and nutrition, and taxonomy and classification, in addition to the history and background of two important diseases caused by *Labyrinthula* spp.

CHAPTER ONE

LITERATURE REVIEW OF *LABYRINTHULA*

An Overview

Cienkowski (1867) was the first scientist to describe and name the genus *Labyrinthula*. He referred to these novel organisms as "marine net-slime molds" as they are mostly found in marine habitats and have slimy ectoplasmic net. Since Cienkowski's description, *Labyrinthula* species are often referred to as "net-slime molds" because of their gelatinous appearance similar to cellular slime molds (phylum Myxomycota); however, they are not cellular slime molds (Alexopoulos et al., 1996). The main characteristics of the genus *Labyrinthula* are the fusiform or spindle-shaped vegetative cells that move within an ectoplasmic net (Cienkowski, 1867; Nakatsuji et al., 1980; Porter, 1987) secreted by cell surface organelles called bothrosomes (Porter, 1969). The cell size or dimensions vary among the species and even within the species of *Labyrinthula* (Pokorny, 1967), usually ranging from 10 – 25 µm in length and 3 – 8 µm in width. Cells are uninucleate and colonies are formed by continuous mitotic cell division. Cells move in a gliding fashion in an ectoplasmic net at a rate of approximately 100 – 175 µm/min (Muehlstein et al., 1991; Porter, 1993; Preston and King, 2005; Young, 1943). Cells move as the ectoplasmic network is being formed by the bothrosomes (Porter, 1969) or move within networks previously created by other cells. This special gliding motility is apparently facilitated by the actin-myosin interactions between the cell surface and ectoplasmic net (Preston and King, 2005). Another important feature of *Labyrinthula* is the production of heterokont biflagellate zoospores

with mastigonemes on an anterior flagellum and a dark eyespot located at the base of the posterior flagellum (Amon and Perkins, 1968; Perkins and Amon, 1969). Zoospore formation has been reported in only one species, *L. algeriensis* (Amon and Perkins, 1968); however, Porter (1993) stated that Perkins and Amon (1968) may have misidentified *L. vitellina* as *L. algeriensis*; therefore it is not certain whether zoosporulation was seen in *L. vitellina* or *L. algeriensis*. This process was reported as a part of sexual cycle (Amon and Perkins, 1968; Perkins and Amon, 1969) but meiosis was not observed.

Morphology and Cytology

Because of its unique characteristics, multiple studies have been conducted on the cytology and ectoplasmic net of *Labyrinthula* using light and electron microscopy (Bartsch, 1971; Bigelow et al., 2005a; Hohl, 1966; Klie and Mach, 1968; Moens and Perkins, 1969; Muehlstein, 1992; Perkins, 1970; Perkins, 1972; Perkins and Amon, 1969; Pokorny, 1967; Porter, 1967; Schwabst.H and Schwab, 1974; Solomon et al., 1978; Stey, 1969). Cienkowski (1867) described the cell features and ectoplasmic slime tracts of *L. vitellina.* Later, Watson (1957) reported a number of interesting microscopic observations on several strains isolated by him which included *L. vitellina* and others which were not identified to the species level. He described the cells shape as "fusiformed" and was the first to state that cells move through filaments of hyaline material, which he referred to as "slimeways". He also made observations describing cell and nuclear division in his isolates. Hohl (1966) conducted a study on the ultrastructure of *Labyrinthula* and was the first to report that the slime pathways are actually tubular and extracellular, and the

spindle cells glide in the slime tube and not on the slime tract, as previously thought. Later, other ultrastructural studies revealed several unique characteristics of *Labyrinthula* such as tubular cristae in the mitochondria, Golgi apparatus with large dictyosomes, a high number of lipid droplets and vacuoles, the trilaminar membrane structure of the "slimeways", and bothrosomes which are a cell surface organelle that secretes the ectoplasmic net (Bartsch, 1971; Porter, 1969). Light and electron microscopic studies made by Klie and Mach (1968) showed that bacteria and yeasts can be digested in the ectoplasmic net by extracellular enzymes. Porter (1972) described the details of cell division and the role of the bothrosome in production of the ectoplasmic net. He reported that protocentrioles appear before each cell division and are visible throughout cell separation until interphase, with cytokinesis occuring after completion of mitosis by accumulation and fusion of vesicles. He showed that bothrosomes arise in between newly formed daughter cells after mitosis and form ectoplasmic sheets around newly divided cells. Ultrastructural studies made by Perkins (Perkins, 1970; Perkins, 1972) supported the studies of Porter (1972). Perkins observed centrioles and centriole-like structures during meiosis and mitosis of *Labyrinthula* cells (Perkins, 1970). Surface organelles, which he referred to as "sagenogenetosomes" (synonym of bothrosomes), were also observed in association with ectoplasmic nets of not only *Labyrinthula* but also in the thraustochytrids, a related group (see taxonomy and classification section below) (Perkins, 1972).

Amon and Perkins (1968; 1969) described zoospore morphology and ultrastructural details of zoospore production in *L. algeriensis.* They reported that the

zoospores were pyriform, 2 – 3 µm wide and 3 – 5 µm long, and laterally biflagellate; the anterior flagellum was uniform in diameter and 13 – 15 µm long with 2 rows of mastigonemes or tinsels (lateral hairs) while the posterior flagellum was tapered distally and 6 – 10 µm long with no mastigonemes. The ultrastructural study of zoosporulation revealed that the initiation of zoosporulation begins with aggregation of spindle cells into sori in which the cells lose their spindle shape and turn oval or round in shape and each round cell (sporocyte) become subdivided into eight cells which eventually differentiate into eight zoospores (Perkins and Amon, 1969). The appearance of synaptinemal complexes in the prophase nucleus during the subdivision indicated that zoosporulation might involve meiosis and each of the eight zoospores is probably haploid giving rise to a haploid spindle cell; however, fusion of haploid cells into diploid cells was not reported (Perkins and Amon, 1969). Analysis of the synaptinemal complexes through ultramicrographs showed that the haploid chromosome number of *Labyrinthula* spp. is nine (Moens and Perkins, 1969).

The unique gliding motility of the labyrinthulids and nature of the ectoplasmic network were studied by several scientists (Dietz and Schnetter, 1999; Nakatsuji and Bell, 1980; Nakatsuji et al., 1978; Nakatsuji et al., 1980; Porter, 1967; Preston and King, 2005; Schwab-Stey and Schwab, 1974; Solomon et al., 1978). Porter (1972), from his extensive microscopic observations on *Labyrinthula*, stated that the ectoplasmic net is important for gliding motility, nutrition, and intercellular communication. Schwab-Stey and Schwab (1974) made one of the first scanning electron microscopy studies on *L. coenocystis*. They studied the germination of the aggregate-cyst, a temporary resting

stage formed by aggregation of cells under unfavorable conditions (Stey, 1969), and the structure of branching pathways in which cells move. They stated that the pathways are ectoplasmic in nature and extend with dicotomous branching further connecting to branches of other major pathways creating a network system. The round evaginations on the cell surface were thought to be lipid droplets because the bothrosomes were not visible as they were covered by the ectoplasmic net (Schwab-Stey and Schwab, 1974). In 1980, Natatsuji and Bell stated that actomyosin plays a role in the gliding motility of *Labyrinthula.* Using fluorescent staining and confocal microscopy, Dietz and Schnetter (1999) suggested that the gliding motility is caused by two actin-myosin complexes, one for the locomotion of the ectoplasmic net and the other for gliding movement of the cells inside the ectoplasmic net. Later, Preston and King (2005) supported the actin-myosin based model of gliding motility using advanced microscopy and biochemical techniques. They reported that at first small and thin filopodial branches of the ectoplasmic net are produced centrifugally from the cell and a few of these radial branches extend to form a major pathway into which the cell glides and moves forward. In addition, it was estimated that the amount of force put out by a spindle cell for efficient gliding is >50 pN (pico Newtons) (Preston and King, 2005)

Physiology and Nutrition

The major studies on the physiology and nutritional requirements of *Labyrinthula* were done by Vishniac (Vishniac, 1955a; Vishniac, 1955b; Vishniac, 1957; Vishniac and Watson, 1953),Watson, and Ordal (Watson, 1957; Watson and Ordal, 1957). Vishniac (Vishniac, 1955b), studied the temperature, pH, and nutritional requirements of three

species, *L. vitellina*, *L. macrocystis,* and *L. minuta*. *L. minuta* has been transferred to the genus *Aplanochytrium* (Dick, 2001; Leander and Porter, 2000); therefore its nutritional requirements are not included. The two other species grew well when incubated for 5 days at 20 – 25° C but at 30° C *L. vitellina* died, *L. macrocystis* showed slow growth, and both died when incubated at 36° C (Vishniac, 1955b). This optimum temperature range was later confirmed by Aschner (1958). However, Watson (1957) reported that his *Labyrinthula* cultures grew well at temperatures ranging from 5 – 25° C.

The effect of pH on *Labyrinthula* spp. growth was first studied by Young (1943) using hanging-drop preparations in sea water. He concluded that *Labyrinthula* can easily grow at a pH range of 4 – 9. Vishniac (1955b) reported that *L. vitellina* and *L. macrocystis* showed best growth at pH 7.5. Watson (1957) stated that his cultures did not show a good growth at pH < 7.8. Pokorny (1967) suggested pH 8 for optimal stock culture maintenance.

The growth at various salinities has been studied for *L. macrocystis*. Young (1943) reported that *L. macrocystis* can tolerate salinities from twice the seawater salinity to zero salinity or freshwater salinity. However, he reported that the cells suspended in distilled water did not form slime tracks or ectoplasmic pathways. The highest activity was observed from 24‰ – 42‰ (‰ = ppm) salinity range; Young (1943) expressed the salinity in % chlorinity but the conversion to ppm is taken from Pokorny (1967). Aschner (1958) was able to grow his *L. macrocystis* isolate on tap-water medium after transfer from a saline medium.

The most in depth study on inorganic and organic nutritional requirements of *Labyrinthula* spp. was made by Vishniac (1955b). She suggested a semi-synthetic medium on which to grow *Labyrinthula* spp. based on her isolates of *L. vitellina* and *L. macrocystis*, which required marine conditions for growth. Vishniac's (1955b) studies showed that the vitamin thiamine was essential for growth, B vitamins did not affect growth, and that several of the amino acids present in gelatin hydrosylate, an additive in the growth medium, provided the necessary carbon and nitrogen requirements. Other amino acids were effective for growth; *L. vitellina* and *L. macrocystis*; grew well in the presence of L-glutamic acid and L-proline, *L. macrocystis* grew well in the presence of L-aspartic acid but *L. vitellina* did not. No species responded to the presence of DL-alanine, L-arginine, and L-leucine. DL-phenylalanine, L-histidine, DL-isoleucine, L-tyrosine, DL-methionine, and DL-valine were found to decrease or totally prevented the growth (Vishniac, 1955b). Despite these findings, Vishniac (1955b) stated that the nutritional requirements of *Labyrinthula* are only partially understood and certain salts, micronutrients, and other organic nutrient requirements naturally in seawater or host plants are unknown.

In a study on the steroid requirements of *L. vitellina* by Vishniac and Watson (1953), active steroids such as cholesterol, Δ^4–cholestenone, fucosterol, and β-sitosterol were reported as essential growth factors. They stated that in nature *Labyrinthula* may obtain these steroid metabolites from host plants, diatoms, or bacteria. In order to understand more about the nutritional requirements of *Labyrinthula*, Sykes and Porter (1973) conducted studies using a growth medium that was completely defined (all the

nutrients are known and added in precise quantities), designed by modifying Goldstein's medium A (Goldstein and Moriber, 1966). They determined optimal concentrations of all the major mineral nutrients and found that sodium, although required in high concentrations for growth, does not play a role as an osmoticum and phosphate uptake might be involved in osmotic pressure regulation. It was reported that *Labyrinthula* can utilize a wide range of sources for carbon requirement but among all the nitrogen sources tested, glutamic acid supported better growth (Sykes and Porter (1973). Thiamine was found to be essential for growth but B12, biotin, and cholesterol are not (Sykes and Porter (1973).

Taxonomy and Classification of Genus *Labyrinthula*

In 1867, Cienkowski reported and described the first two *Labyrinthula* species, *L. vitellina* and *L. macrocystis,* isolated from algae growing on rotted wooden pilings at Odessa Harbor, Ukraine. By 1967, about 10 species were included in the genus *Labyrinthula* but due to some contradicting morphologies some of the members were moved to another genus or considered a synonym (Dick, 2001). Currently, there are approximately 14 species described under the genus *Labyrinthula* according to the Index Fungorum (Dick, 2001) and 16 according to MycoBank (Table. 1.1.). Most of the *Labyrinthula* species were found near estuarine or near-shore marine habitats (Alexopoulos et al., 1996; Porter, 1987) with a few exceptions. *L. cienkowskii* was isolated from a fresh water alga, *Vaucheria sessilis* (Zopf, 1892). Aschner (1958) isolated *L. macrocystis,* originally isolated from marine algae, from saline soils around roots of a diseased *Carica papaya* in Israel. An undescribed *Labyrinthula* sp. was isolated from

inland saline soils near Great Salt Lake, Utah, USA (Amon, 1978). Koske (1981) isolated an unknown *Labyrinthula* sp. from spore cases of a vesicular-arbuscular mycorrhizal fungus, *Gigaspora gigantean*. *L. terrestris* was isolated from turfgrasses on golf courses (Bigelow et al., 2005a). A species called *L. apis* was found to be entomopathogenic, isolated from the abdomen of *Apis millifera* (honey bee) (Stejskal, 1976). *Labyrinthula* spp. are primarily saprotrophic and are usually associated with marine microalgae and marine vascular plants such as *Zostera, Thalssia,* and *Spartina,* in addition to dead leaf material (Porter, 1987). Two species are pathogenic to plants; *L. zosterae* causes wasting disease of marine eelgrass, *Zostera marina* (Muehlstein et al., 1991) and *L. terrestris* causes rapid blight of cool-season turfgrasses. Because of these two diseases, *Labyrinthula* has received more attention. Below both diseases are discussed in detail.

The taxonomy and classification of *Labyrinthula* and related organisms have gone through changes and reconsideration. These organisms were initially thought to be Protozoa (Calkins, 1934; Corliss, 1984; Honigberg et al., 1964; Levine et al., 1980), then as Mycetozoa in Fungi (Bessey, 1950; Olive, 1975; Pokorny, 1967), and finally, based on characteristics such as tubular mitochondrial cristae (Pokorny, 1967; Porter, 1969), biflagellate heterokont zoospores (Amon and Perkins, 1968), and 18s rDNA studies (Cavalier-Smith et al., 1994; Honda et al., 1999), they were classified as stramenopiles under the phylum Heterokonta, subphylum Labyrinthista, family Labyrinthulaceae, order Labyrinthulales, class Labyrinthulomycetes under the kingdom Chromista (Dick, 2001; Kirk et al., 2001). According to Porter (1987) and a molecular phylogenetic study made by Honda et al. (1999), Labyrinthulomycetes consist of two groups or families,

Labyrinthulaceae (the labyrinthulids) and Thraustochytriacea (the thraustochytrids). Labyrinthulaceae consists of only one genus, *Labyrinthula* (Cienkowski, 1867) and Thraustochytriacea consists of seven genera, *Thraustochytrium* (Sparrow, 1936), *Japonochytrium* (Kobayasi and Ookubo, 1953), *Shizochytrium* (Goldstein and Belsky, 1964), *Althornia* (Jones and Alderman, 1971), *Ulkenia* (Gaertner, 1977), *Aplanochytrium* (Bahnweg and Jackle, 1986; Sparrow, 1972) and *Labyrinthuloides* (Perkins, 1973).

There are several unique characteristics between these two families that unite them into the Labyrinthulomycetes. Most members are saprotrophic and are commonly found in marine estuarine habitats and have an obligate sodium requirement. They also produce heterokont biflagellate zoospores with a longer anterior flagellum, contain mitochondria with tubular cristae, and have cell walls with dictyosome-derived scales composed of L-galactose. A characteristic feature of the Labyrinthulomycetes is the production of a branching and anastomosing membrane-bound, wall-less ectoplasmic net or extracellular network, used in substrate attachment, cell motility, and nutrient absorption, that is produced by a specialized cell surface organelle referred to as a sagenogen (Olive, 1975) or sagenogenetosome (Perkins, 1972) or bothrosome (Porter, 1969) (Alexopoulos et al., 1996; Dick, 2001; Porter, 1987). However, there are a few members (e.g. *Corallochytrium*) that do not produce zoospores or contain cell surface organelles such as sangenogenetosomes and therefore their classification as Labyrinthulomycetes is doubtful (Dick, 2001).

Several major morphological differences separate the Labyrinthulaceae and Thraustochytriacea. The cells are fusiform or spindle-shaped in the labyrinthulids

whereas the thraustochytrids have ovoid, spherical, or ellipsoidal cell shapes. The ectoplasmic net envelopes the cells in the labyrinthulids which makes the cells move in it in a gliding fashion (Nakatsuji et al., 1980), whereas in the thraustochytrids the cells are not fully enveloped or covered by the ectoplasmic net. Although a few genera have gliding motility, it is not aided by ectoplasmic net. The thraustochytrids contain multiple layers of scales in their cell walls whereas the labyrinthulids only have a single layer of scales (Dick, 2001).

In 2001, based on specific morphological differences and molecular phylogenetic studies using small subunit ribosomal DNA, Celeste and Porter stated that the Labyrinthulomycota (Labyrinthulomycetes) is comprised of not two but three phylogenetic groups or lineages – the labyrinthulids, the thraustochytrids, and the labyrinthuloids. The classification of the Labyrinthulomycetes has always been challenging and confusing because of their unique biology. In the future, more changes and revisions in the classification of *Labyrinthula* are expected as scientists gather more morphological and molecular data (Bigelow et al., 2005b; Olsen, 2007).

Labyrinthula zosterae and Wasting Disease of Marine Eelgrass, *Zostera marina*

Populations of *Zostera marina*, a marine eelgrass species that lives along the Atlantic coasts of North America and Europe, were devastated in huge proportions in the early 1930's due to an epidemic of wasting disease (Cottam, 1933; Muehlstein, 1989; Renn, 1934). Wasting disease reoccurred in the 1980's causing a significant decline of *Zostera marina* populations in North America and Europe (Muehlstein et al., 1988; Short et al., 1987). The major cause or causal agent of the outbreak in the early 1930's was not

understood. There were several causes hypothesized, ranging from biotic agents such as fungi and bacteria to abiotic causes such as drought, sunshine level, temperature changes etc.; these hypotheses were discussed in detail by Muehlstein (1989).

In 1938, Tutin described the symptoms of wasting disease and reported that two parasites, *Labyrinthula* and *Lulworthia*, were consistently associated with the diseased tissue. In addition, it was hypothesized that low sunlight levels could have made the eelgrass more susceptible to wasting disease (Tutin, 1938). However, other studies showed that there was no significant relationship between sunshine levels and wasting disease occurrence (Stevens (1939). Renn (1934; 1935; 1936a; Renn, 1936b; Renn, 1942) and Young (1943; 1937), through their extensive research, showed that *Labyrinthula* was consistently associated with wasting disease and they described *Labyrinthula* cells forming net-like aggregates in eelgrass leaf cells. Renn (1936a) showed that when small diseased eelgrass pieces are attached to healthy plants, more than 50% of the healthy plants showed wasting disease symptoms after two days. He concluded that this rapid spread of the disease could only be caused by *Labyrinthula*. Renn (1936b; Renn, 1937) observed that *Labyrinthula* had decreased activity in winter which corresponded to lower disease symptoms and the symptoms increased in early summer when the activity or vigor of *Labyrinthula* increased. Young (1938; 1943; 1937) also concluded that *Labyrinthula* was the causative agent of wasting disease and correlated the disease occurrence with the higher seawater salinities which resulted due to high evaporation during dry summers of 1935, 1936 and 1937. Young (1938; 1937) gave a detailed description of the *Labyrinthula* that he isolated and identified it as *L. macrocystis*. He

reported two other *Labyrinthula* seagrass hosts, *Ruppia maritima* and *Zanichellia palustris* along with two other algal hosts, *Cladophora hirta* and *Chaetomorpha linnum* (Young, 1943).

Labyrinthula was not only found in diseased eelgrass tissue but was also isolated from healthy eelgrass leaves (Pokorny, 1967; Watson, 1957) and observations of Renn (1936a) and Young (1938) showed that *Labyrinthula* cells were found in the healthy eelgrass plants ahead of the wasting disease symptoms. Due to these reasons and incomplete evidence that *Labyrinthula* was the causal agent, Rasmussen (1977) strongly argued that *Labyrinthula* was not the pathogen of wasting disease. He concluded that *Labyrinthula* could be easily found in healthy eelgrass leaves and it was a 'secondary invader'.

In the 1980's wasting disease of eelgrass reoccurred, generating interest in studying *Labyrinthula* and this disease (Muehlstein, 1989; Muehlstein et al., 1988; Short et al., 1987). Short et al.(1987) and Muehlstein et al. (1988) proved that *Labyrinthula* sp. was indeed causing the symptoms of wasting disease in eelgrass. In 1991, Muehlstein et al. identified and characterized a new species of *Labyrinthula, L. zosterae.* They carried out Koch's postulates to show that *L. zosterae* was responsible for wasting disease symptoms in eelgrass. Muehlstein (1992) made studies on the host-pathogen interactions of wasting disease using light and transmission electron microscopy. Her studies showed that *L. zosterae* cells were frequently located in the lesion margins and cells rapidly moved through the leaf tissue, directly penetrating host cell walls. She concluded that the ectoplasmic network played a key role in enzymatic degradation of host cell walls and

also destroyed the host cell cytoplasm. She also stated that wasting disease spreads by direct contact of diseased leaves with uninfected plants.

Labyrinthula terrestris and Rapid Blight of Cool-Season Turfgrasses

Another important plant pathogenic species of *Labyrinthula* is *L. terrestris*, the causal agent of rapid blight on cool-season turfgrasses (Bigelow et al., 2005b; Stowell et al., 2005). The disease was first observed in 1995 in southern California on *Poa annua* (Stowell, 1995). The disease symptoms were unusual with turf showing yellow to brown colored lesions with a water-soaked appearance. The disease was found to spread very fast destroying large areas of turfgrass, and subsequently was "rapid blight" (Martin et al., 2002).

The signs of the pathogen, fusiform cells (6 – 16 μm) in turfgrass leaves, were unlike known fungal or bacterial pathogens (Stowell, 1995). In 2002 the pathogen was identified as a *Labyrinthula* sp., similar to *L. zosterae* the causal organism of wasting disease of eelgrass (Stowell et al., 2005). After completion of Koch's postulates which supported that a *Labyrinthula* species was responsible (Olsen et al.2003), the pathogen was identified as a new species, *Labyrinthula terrestris*, by means of morphological and molecular identification tools (Bigelow et al., 2005b; Craven et al., 2005).

Rapid blight has a broad distribution. It has been reported from more than 100 golf courses in eleven states throughout the United States of America (Stowell et al., 2005). Recently, the disease has been found for the first time in Colorado (Hyder et al., 2010). It has also been found in Europe, including the United Kingdom (Entwistle et al., 2006), Spain, and Argentina (Olsen, 2007).

Rapid blight is known to affect salt-sensitive cool-season turfgrasses and is frequently associated with poor quality irrigation water or saline irrigation water and salty soils (Peterson et al., 2005a) but has not been reported from residential or commercial lawns (Stowell et al., 2005). Rapid blight is more severe on cool-season turfs such as rough bluegrass (*Poa trivialis*), perennial ryegrass (*Lolium perenne*), annual bluegrass (*Poa annua*), and colonial bentgrass (*Agrostis tenuis*) (Olsen, 2007). All of these grasses are used extensively on golf courses in the winter to early spring seasons and are of great commercial value in the golf course industry. Research has shown that other plants such as rice, wheat, and barley also showed rapid blight symptoms when the pathogen was inoculated artificially on these plants (Bigelow & Olsen, 2004). This indicates that *L. terrestris* is a potential threat to other monocot species.

A direct relationship between salinity, salinity tolerance, and rapid blight has been documented (Camberato et al., 2005a; Camberato et al., 2005b; Peterson et al., 2005a). Several cool-season turfgrasses were screened for tolerance against rapid blight (Peterson et al., 2005b). Fescues, creeping bentgrass, alkaligrass, and Kentucky bluegrass were most tolerant to *L. terrestris* and bluegrasses cultivars such as Canada (*Poa compressa* L.), (*Poa pratensis* L.), annual bluegrasses and some perennial ryegrass varieties such as ‘Hawkeye’ and ‘Peregrine’ were moderately tolerant (Peterson et al., 2005b). Supporting this study, other previous studies have shown that these rapid blight tolerant grasses are also very salt tolerant et (Grueb and Drolsom, 1985; Harivandi, 1982; Hughes, 1975; Humphreys, 1981; Lunt, 1961; Marcum, 2001).

Control and management of the rapid blight pathogen is challenging. *L. terrestris* is not easy to control using regular fungicides; however, studies have shown that trifloxystrobin (Compass), pyraclostrobin (Insignia), and mancozeb (Fore) help control or prevent the spread of rapid blight (Martin et al., 2004; Olsen and Gilbert, 2004). In order to better control the pathogen, more details about its biology, lifecycle, and pathology need to be elucidated.

Table. 1.1. List of current species in the genus *Labyrinthula*

Species	Location	Habitat	Host	Reference
L. algeriensis	Algeria	marine/estuarine	*Laminaria iberica* (algae)	A. Hollande and M. Enjumet, 1955
L. apis	Venezuela	entomopathogenic	*Apis mellifera* (Honey bee)	M. Stejskal, 1976
*L. chattonii**	France	marine	*Cladophora refracta* (algae)	P. A. Dangeard, 1932
L. cienkowskii	Germany	marine	*Vaucheria terrestris* (algae)	W. Zopf, 1892
L. coenocystis	Germany	marine	*Cladophora* sp. (algae)	H. Schmoller, 1960
*L. jeremarina**	Texas, USA	marine	*Littorina ziczac* (marine gastropod)	L. Rolf (1967)
L. macrocystis	Ukraine	marine	marine algae	L. Cienkowski, 1867
L. magnifica	Bulgaria	marine	diatoms and marine algae	(A. Valkanov) L. S. Olive, 1975
L. pohlia	Germany	marine	*Chorda filum* (algae)	(H. Schmoller) M. W. Dick, 2001
L. roscoffensis	France	marine	*Taonia atomaria* (algae)	M. Chadefaud, 1797 (1956)
L. terrestris	Arizona, USA	terrestrial	*Poa trivialis* (rough bluegrass) & *Lolium perenne* (perennial ryegrass)	D. M. Bigelow et al., 2005
L. valkanovii	Bulgaria	marine	marine algae	J. S. Karling, 1944
L. vitellina	Ukraine	marine	marine algae	L. Cienkowski, 1867
L. zosterae	Washington, USA	marine	*Zostera marina* (marine eel grass)	D. Porter et al., 1991

**L. chattonii* and *L. jeremarina* are still considered as doubtful species because apart from their original description, they were not again studied or described by other scientists (Dick, 2001)

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CHAPTER TWO

IMPROVED METHODS FOR AXENIC CULTURE AND LONG-TERM STORAGE OF *LABYRINTHULA TERRESTRIS*, CAUSAL AGENT OF RAPID BLIGHT OF TURFGRASSES

Introduction

Labyrinthula terrestris is an unusual plant pathogenic microorganism that causes a disease called rapid blight on cool-season turfgrasses (Bigelow et al., 2005; Martin et al., 2002; Olsen et al., 2003). Members of genus *Labyrinthula* are mostly found in coastal marine or estuarine habitats and they have characteristic vegetative spindle-shaped cells that move within an ectoplasmic network in a gliding fashion (Porter, 1972). Due to these unique morphological features, production of heterokont biflagellate zoospores (Amon and Perkins, 1968), and molecular phylogenetic data (Hondal et al., 1999; Tsui et al., 2009), the members of genus *Labyrinthula* (labyrinthulids) have been placed in the phylum Labyrinthulomycota, along with their sister group thraustochyrids, under the kingdom Chromista (Kirk et al., 2001; Porter, 1987).

The necessity to study *Labyrinthula* species has become more important because of two plant pathogenic species, *L. terrestris,* which is pathogenic to terrestrial plants (Bigelow and Olsen, 2004; Bigelow et al., 2005), and *L. zosterae,* which causes wasting disease on marine eelgrass, *Zostera marina* (Muehlstein et al., 1991). Less is known of *L. terrestris* because it was discovered more recently than *L. zosterae*; in 2003 a *Labyrinthula* species was identified as the causal agent of rapid blight of turfgrass (Martin et al., 2002; Olsen et al., 2003) and in 2005 the species *L. terrestris* was formally described (Bigelow et al., 2005). The disease has since been reported from golf courses

across the United States (Stowell et al., 2005), in addition to reports from the United Kingdom (Entwistle et al., 2006), southern Europe, (Olsen, 2007) and Argentina (S. B. Martin, unpublished). It is believed that *L. terrestris* has emerged as a turfgrass pathogen because of increased use of relatively high salinity water or reclaimed water for irrigation on golf courses.

Maintaining *L. terrestris* in axenic culture is important for studying its biology and pathology, but it is difficult because *Labyrinthula* cells cannot survive more than 10 – 14 days on an agar plate. Although there are some previously suggested culture media and techniques to isolate and grow *Labyrinthula*, it is still challenging. In 1957, Watson and Ordal suggested a technique to isolate and maintain axenic cultures of *Labyrinthula* (Watson and Ordal, 1957). They stated that their medium resulted in an optimal growth of *Labyrinthula* when compared to the media suggested previously by Vishniac and Watson (1953) and Vishniac (1955a). However, Sykes and Porter (1973) cited many cases of poor *Labyrinthula* growth on Watson and Ordal's serum seawater medium. In order to study the nutritional requirements of *Labyrinthula*, Sykes and Porter (1973) created a completely defined medium (all the nutrients are known and added in precise quantities), which was designed by modifying Goldstein's medium A (Goldstein and Moriber, 1966). Although this medium is good for optimal growth of *Labyrinthula*, it can be cumbersome to prepare because of its high number of ingredients. To maintain a collection of *Labyrinthula* isolates or to make frequent transfers to keep cells actively growing, researchers commonly use an easy-to-prepare medium suggested by Porter (Porter, 1987), known as SSA (serum sea water agar).

SSA contains horse serum, agar, and natural seawater fortified with antibiotics (Porter, 1972; 1987), similar to the medium reported by Watson and Ordal (1957).

In order to better maintain *Labyrinthula* cells on agar medium, supplementary food sources have been added. For example, for optimal growth on SSA, yeast cells such as *Rhodotorula rubra* are streaked on the agar to serve as a food source (Muehlstein et al., 1991; Porter, 1987). Amon (1978) reported a method to successfully isolate and culture sporulating *Labyrinthula* using modified Vishniac's medium (Fuller et al., 1964) and this method also suggested use of live yeast cells (*Rhodotorula rubra*) as a food source. Yokochi et al. (Yokochi et al., 2001) have suggested a better isolation method for labyrinthulids using a bacterium, *Psychrobacter phenylpyruvicus* as a food source. Therefore, it is evident that optimal growth of *Labyrinthula* is difficult unless it is supported by some kind of food source.

For *L. terrestris* isolation and culture, Olsen et al. (2003) have used 10 ml horse serum (Porter, 1987) per liter of saline irrigation water (salinity or Electrical conductance (EC) = 4.0 decisiemens/meter (dS/m)) obtained from a golf course where rapid blight was reported. This is due to the fact that *L. terrestris*, as the causal organism of rapid blight, has always been associated with saline irrigation water with salinity higher than 1.5 dS/m (Olsen et al., 2003). Presently, the most common medium used to isolate and culture *L. terrestris* is a modified SSA which contains agar, antibiotics, and artificial seawater (saline water), with EC = 4.0 dS/m (Bigelow et al., 2005; Douhan et al., 2009; Olsen, 2007). Note that the salinity level of this artificial seawater (4.0 ds/m) is much lower when compared to natural seawater salinity (50 – 55 dS/m). Therefore, here we used the term 'saline water' instead of artificial seawater. For better culture of *L.*

terrestris, 1 - 2 cm long autoclaved pieces of perennial rye grass (*Lolium perenne*, Brightstar SLT) are often placed on the agar as a supplement (Douhan et al., 2009). However, with this technique we were unsuccessful in getting an optimal growth of *L. terrestris* after a few transfers to fresh media and several isolates lost their viability.

When handling *L. terrestris* isolates, a long-term storage technique is helpful to avoid the time-consuming transfer of cells to fresh medium every 10 – 14 days, especially when numerous cultures are being maintained. No reports of any long-term storage technique for *L. terrestris* or other *Labyrinthula* species have been published. One technique involves growing *L. terrestris* cells on SSA, cutting out small agar plugs with cells, and suspending them in serum saline water broth (SSB) and autoclaved pieces of perennial ryegrass in sterile glass vials. After incubation at room temperature, these glass vials are stored long-term in a refrigerator at 4°C (S. B. Martin and P. Peterson, unpublished). However, with this technique, we can maintain viable cultures of *L. terrestris* for up to only 6 months.

As stated above, it has always been difficult to maintain *Labyrinthula* cells without any additional food sources such as yeasts, bacteria, and grass pieces, but for research it is important to have axenic cultures without other organisms that may compromise or alter results. Therefore, in this paper we describe two alternative, more effective culture media for *L. terrestris* that do not require another organism for nutrition and report a study comparing the growth of *L. terrestris* on these media in addition to a previously published medium (SSA). In addition, we report an improved long-term storage.

Materials and Methods

Labyrinthula terrestris isolates and maintenance – Eighteen isolates of *L. terrestris* were obtained from different locations in the U.S.A where rapid blight was reported. Isolates were from the collection of S. B. Martin; the location and the host from which each was obtained are listed in Table 2.1.

Media preparation:

Serum saline water agar (SSA) or broth (SSB) – This medium was prepared by adding 12 g Bacto™ agar (no agar added in case of SSB), 10 ml pre-filter sterilized horse serum (Hema-Resources & Supply Inc, Aurora, OR), 0.25 g streptomycin sulfate (MP Biomedicals, LLC., Solon. OH), 0.25 g penicillin G and 0.25 g of ampicillin (both Research Products International Corp. Mount Prospect, IL) in a liter of saline water (1.765 g NaCl, 0.21 g $MgSO_4$, 0.164 g $MgCl_2$, 0.071 g $CaCl_2$, 0.015 g $NaHCO_3$, 0.014 g KCl per liter of distilled water) and salinity (electrical conductance) was adjusted to 3.5 – 4.0 dS/m (Bigelow et al., 2005; Porter, 1987). For convenience, the three antibiotics were mixed into one stock solution of higher concentration and were used in appropriate volumes when preparing medium. In order to avoid precipitation of horse serum and heat inactivation of antibiotics, the temperature of the medium should not be more than 45°C when adding them.

SSA with additives (SSA+A) – In addition to the regular ingredients in SSA, 4 g D-glucose anhydrous, 1 g gelatin hydrolysate (both MP Biomedicals, LLC., Solon, OH), 0.25 g Bacto™ peptone, 0.25 g Difco™ yeast extract, and 0.02 g vegetable extract No. 2 (Fluka Biochemika, Buchs, Switzerland) were added per liter of medium. The additives are similar to that of modified Vishniac's medium (Fuller et al., 1964) except that the

liver extract is replaced with vegetable extract, peptone was added, and the amounts of other ingredients were modified slightly.

Grass Extract SSA (GESSA) – The grass extract was prepared by thoroughly blending 30 – 40 g of fresh two-week-old perennial ryegrass (*Lolium perenne*, Bright Star SLT) in 200 ml of saline water (described above) using a commercial blender for 1 min to form a crude homogenate. This homogenate was strained through 4 – 6 layers of grade #10 Purewipe® cheesecloths (American Fiber & Finishing, Inc., Albemarle, NC). The filtered homogenate was transferred into 28 ml Oak Ridge centrifuge tubes (Nalgene®, Rochester, NY) and centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was collected and centrifuged the same way; this step was repeated once. The final supernatant was autoclaved for 10 min at 121°C. After autoclaving, the extract was shaken thoroughly to suspend the precipitate and 20 ml of this grass extract was added to 1 liter of SSA, along with horse serum and antibiotics, as described above, when the temperature of the medium was not more than 45°C.

Comparative assessment of SSA, SSA+A, and GESSA:

A comparative study was conducted to determine the efficiency of SSA, SSA+A, and GESSA. *L. terrestris* isolates were grown on SSA for 4 – 5 days and the cells were gently scraped from the agar using a sterile Scienceware® cell scraper (Bel-Art Products, Pequannock, NJ) and suspended in 2 – 3 ml SSB. In order to reduce clumping of the cells, a non ionic surfactant,Tween® 80 (Fisher Scientific, Fair Lawn, NJ) (2.5 µL /ml), was added to cell suspensions. Cell densities were adjusted to 25 x 10^4 cells/ml using a hemacytometer (VWR International, LLC.). The suspensions were vortexed well and a 50 µl drop was carefully pipetted onto the center of the agar plates (100 mm X 15 mm)

and the lid was placed on top. The plates were left undisturbed so that the drop remained intact until it had dried, creating a uniform circular area of cells. Plates were incubated at 25°C. Plates in which the drop was not intact at the center were discarded. Each isolate was grown on all three media in three replicates. After 5 days, the colony edges were traced using a fine permanent marker and the plates were digitally scanned using an Epson® digital scanner (Longbeach, CA). Using Adobe® Photoshop® CS5 Extended software (San Jose, CA), the colony area was measured with the options of custom calibration, quick selection and analysis (record measurement) tools. All of the colony areas were measured in cm^2 units. The data were statistically tested with factorial two-way analysis of variance (ANOVA) using IBM® SPSS®, statistics software (Armonk, NY). The graphs were prepared using Microsoft® Excel 2007 (Redmond, WA).

Long-term culture technique:

Perennial rye grass (*Lolium perenne*, Bright Star SLT) was established in 15.24 cm diameter pots (6-inch pots) in the greenhouse for two weeks and then treated with 100 ml 3.5 dS/m artificial sea water (described above) per pot daily for 7 – 10 days. The treatment with artificial seawater makes the rye grass more susceptible to infection by *L. terrestris* (Olsen et al., 2004). To sterilize the grass, blades were cut into 5 – 8 cm pieces, suspended in artificial sea water, and then autoclaved for 10 min in 200 ml glass bottles. For storage, 28 ml screw-cap glass vials (25 mm x 95 mm) were used. These were dry autoclaved before pipetting 15 ml sterile SSB with additives (SSA+A without agar) into them. The autoclaved grass was then transferred to the vials containing broth. From plates containing *L. terrestris*, small agar plugs (3 – 5 mm) with cells were cut using a sterile scalpel and transferred to the vials with grass and SSB+A medium. The vials were

kept shaking in an incubator at 25°C for 2 – 3 days to allow for *L. terrestris* cell proliferation and then maintained at 4°C for long-term storage. To start a culture from stored cells, 2 – 5 grass pieces were taken out from the screw-cap vials using sterile technique, blotted on sterile paper towels to remove excess moisture, and placed on any of the above mentioned agar media.

Results

Effect of media – The factorial analysis of variance of the data showed that there was a significant main effect of the type of medium on *L. terrestris* colony growth, $F_{(2,34)} = 80.489$, $P < 0.001$. The Bonferroni *post hoc* test showed that the growth means on three media (SSA, SSA+A, and GESSA) were significantly different from each other (all $P < 0.001$). The mean growth area was greatest on GESSA followed by SSA+A, and then SSA (Fig. 2.1).

Effect of isolate – There was a significant main effect among isolates, depending on their origin, on the overall growth, $F_{(17, 34)} = 10.887$, $P < 0.001$ (Fig. 2). The Bonferroni *post hoc* test showed that with a few exceptions, there were significant differences in the growth among most of the isolates (*post hoc* tests results are not shown here because of too much data). This means that the optimal growth of *L. terrestris* varies among isolates and depends on their individual growth characteristics.

Effect of interaction between isolates and media – There was a significant effect of the interaction between the different isolates and the type of medium on the overall growth, $F_{(34, 108)} = 31.681$, $P < 0.001$. However, this significant effect was only in magnitude of the media comparisons but not a change in order. The mean growth was always greatest on

GESSA followed by SSA+A, and was always least on SSA among all the isolates (Fig 2.2).

Long-term storage – With the improved long-term storage technique, we were able to successfully store *L. terrestris* cultures for at least two years. Older *L. terrestris* colonies tend to form yellow to pale yellow colored aggregates on the agar and on the grass blades in the long-term storage vials. Although aggregation was seen in almost all the isolates, some isolates showed relatively greater aggregate formation (in terms of aggregate number and size). When grown on agar plates, isolates with greater aggregate formation in long-term storage vials gave rise to new cells even after two years. However, after one year of storage, all the isolates showed an apparent decrease in the rate of growth when plated on agar medium.

Discussion

The objective of this study was to develop improved methods to culture *L. terrestris*. It is evident from our results that better growth and culture maintenance is possible by enriching the basic SSA medium with additional nutrients. The two media developed in this study, SSA+A and GESSA, showed better growth of *L. terrestris* compared to the basic SSA medium, and the modified long-term storage method has allowed cultures to remain viable in excess of two years.

Among the three media, the GESSA medium showed the fastest growth rate of *L. terrestris* colonies after five days (Fig. 2.1) due to greater extracellular network formation and cell motility. As the causal agent of rapid blight, *L. terrestris* is known to spread quickly from diseased to healthy plants; therefore, the greater growth and motility rate recorded on GESSA might be due to the presence of grass extract. Previously, scientists

studying the nutritional requirements of *Labyrinthula* spp. speculated that *Labyrinthula* can survive and grow better in nature by obtaining its required nutrients from plants, diatoms, bacteria, or natural seawater (Vishniac, 1955b; Vishniac and Watson, 1953). Although *L. terrestris* appears to be getting the essential nutrients from the grass extract and horse serum in GESSA, all of the nutrients in the grass extract might not be available in their natural form. This is because, for sterility, the grass extract was autoclaved and this could be detrimental to some temperature-sensitive nutrients such as proteins and vitamins. Methods of non-heat sterilization, such as filtration, could be tried to potentially achieve even better growth results.

Although the GESSA medium showed highest growth rate, cells survived the longest on SSA+A, typically living 20 – 25 days compared to about 15 – 18 days on GESSA. SSA+A is more nutrient rich than GESSA, which may have attributed to the longer cell life. Although cell motility on SSA+A was relatively slower compared to GESSA, cell density, cell division, and aggregate formation appeared higher on SSA+A compared to the other two media. This makes it a better medium if the goal of culturing is to collect the maximum number of cells, such as for DNA extraction. SSA+A also lasts the longest in storage. Plates can be stored for up to about 10 days at 4°C, while GESSA medium, like SSA, should always be used immediately after preparation. It is likely that the nutrients are more stable for a longer period of time in SSA+A. Another limitation of the GESSA medium is that it involves the establishment of grass and preparation of the grass extract. The major limitation of SSA+A is that it can be expensive compared to GESSA or SSA because of the price of horse serum, in addition to other ingredients such as vegetable extract, gelatin hydrolysate, peptone, and yeast extract.

SSB+A was used to improve the long-term storage method by lengthening the storage life of *L. terrestris* cells. This protocol includes ingredients from both new media described above, the additional nutrients that are added in SSA+A and grass as used in GESSA. Thus far cultures have been stored for at least two years and cells have been viable when plated on a suitable medium. The formation of aggregates is believed to be associated with longer cell survival. Cell aggregates might be a means of dormancy, an anti-stress strategy in abnormal growth conditions. A gradual decrease in aggregate formation was observed after about one year; therefore, it is recommended that isolates are transferred to fresh vials containing long-term storage media on an annual basis.

Although our results indicate that GESSA and SSA+A are excellent media to culture *L. terrestris*, these media are not completely defined thus are not appropriate for studies on nutritional requirement experiments or specific nutrient-based studies.

This paper contains techniques and methods suggested specifically to culture *L. terrestris;* however, we expect that these methods or their modifications can be used to culture other species of *Labyrinthula* as well.

Table 2.1 Isolates of *Labyrinthula terrestris* used in our experiments

	Location	**Host Turfgrass**	**Date Received**
AZ-1	Univ. of Arizona	*Poa trivialis*	unknown
AZ-2	Phoenix, AZ	*Lolium perenne*	12/1/2003
AZ-3	Lake Havasu City, AZ	*Lolium perenne*	2/18/2004
AZ-4	Phoenix, AZ	*Poa trivialis*	4/15/2004
AZ-5	Phoenix, AZ	*Lolium perenne*	12/3/2004
CA-3	San Jose, CA	*Poa annua*	10/10/2003
CA-5	Irvine, CA	*Poa annua*	11/14/2003
CA-9	Burbank, CA	*Poa annua*/*Agrostis stolonifer*a (80/20)	2/10/2004
CA-11	Oxnard, CA	*Poa annua*	10/26/2004
CA-12	Laguna Hills, CA	*Poa annua*	10/31/2005
CA-13	Tustin, CA	*Poa annua*	11/3/2005
CA-14	Atascadero, CA	*Poa annua*	11/3/2005
CA-19	Hollister, CA	*Poa annua*	12/15/2005
CA-20	Whittier, CA	Unknown	12/19/2005
NC-12-07	Shallot, NC	*Agrostis stolonifer*a	10/10/2007
TX-1	Pottsboro, TX	*Poa annua*/*Agrostis stolonifer*a (80/20)	1/13/2004
UT-1-3	Wahington, UT	*Agrostis stolonifer*a	10/1/2003
UT-1-4	Washington, UT	*Poa annua*	10/1/2003

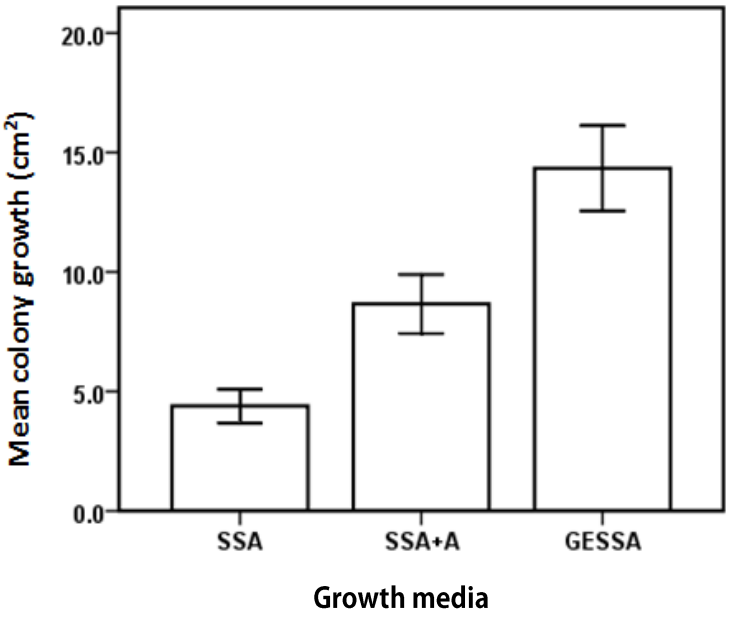

Fig. 2.1. Mean colony growth (cm^2) (bars = +/- 2 standard errors) of *Labyrinthula terrestris* after 5 days on three different media: serum saline water agar (SSA), serum saline water with additives (SSA+A) and grass extract serum saline water agar (GESSA). Each media was tested with 18 isolates and 3 replications (n=54). Differences between growth media (GESSA>SSA+A>SSA) were significant based on the analysis of variance (ANOVA) $F_{(2, 34)} = 80.489$; $P < 0.001$.

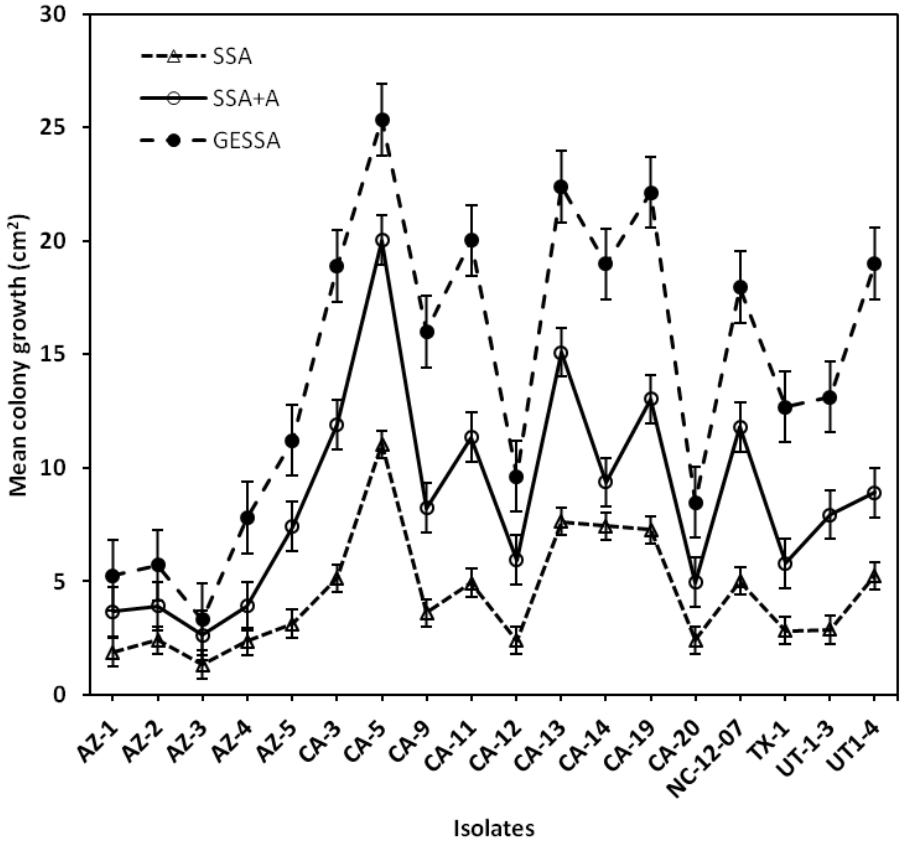

Fig. 2.2. Means of colony growth (cm^2) (bars = +/- 2 standard errors) after 5 days on three different media, serum saline water agar (SSA), serum saline water agar with additives (SSA+A) and grass extract serum saline water agar (GESSA) separated for the eighteen isolates of *L. terrestris* on three different media. Each point is based on three replicates. Factorial analysis of variance (two-way ANOVA) indicated a significant effect of isolate type on the growth; $F_{(17, 34)} = 10.887$, $P < 0.001$ and a significant interaction between isolate and medium; $F_{(34, 108)} = 31.681$, $P < 0.001$. However, the comparison of the three media (GESSA>SSA+A>SSA) on growth is consistent among all isolates, so the presence of interaction does not affect the results in Fig. 1.

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CHAPTER THREE

ULTRASTRUCTURE AND LIFE CYCLE OF *LABYRINTHULA TERRESTRIS,* CASUAL AGENT OF RAPID BLIGHT OF COOL-SEASON TURFGRASSES

Introduction

Labyrinthula terrestris is an unusual plant pathogen that causes rapid blight of cool-season turfgrasses (Bigelow et al., 2005; Martin et al., 2002b; Olsen et al., 2003; Stowell et al., 2005). Typical symptoms of rapid blight include initial water-soaked lesions and browning or yellowing of foliage that leads to irregular patches of dead turf on golf courses (Martin et al., 2002b; Stowell et al., 2005). The disease is known to spread quickly from diseased to healthy turf, hence the name "rapid blight" (Martin et al., 2002b). It is also geographically widespread; thus far it has been reported from eleven states across the U.S.A (Hyder et al., 2010; Stowell and Gelernter, 2003), in addition to other countries such as the United Kingdom (Entwistle et al., 2006), parts of southern Europe (Olsen, 2007), and Argentina (S. B. Martin unpublished). The disease is associated with saline irrigation water and soil on golf courses (Kohout et al., 2004; Olsen et al., 2006; Olsen et al., 2004). It is more severe on salt-sensitive varieties of turfgrasses which are mostly cool-season turfgrasses such as *Agrostis tenuis* (colonial bentgrass), *Lolium perenne* (perennial ryegrass), *Poa annua* (annual bluegrass), and *Poa trivialis* (rough bluegrass) (Martin et al., 2002a; Martin, 2002; Olsen et al., 2003; Stowell et al., 2005).

L. terrestris is the only terrestrial species (Bigelow et al., 2005) in the genus *Labyrinthula*, most species are typically found in coastal marine and estuarine habitats (Porter, 1987). The genus was first described by Cienkowski (1867) who referred to them

as “marine net-slime molds” because of their marine habitat and gelatinous appearance similar to the true slime molds; however, they do not belong to the cellular or true slime molds (Alexopoulos et al., 1996). *Labyrinthula* species have characteristic fusiform, uninucleate vegetative cells which move within an enveloping double-membraned ectoplasmic network that aids in cell motility, surface adhesion and nutrition (Cienkowski, 1867; Nakatsuji et al., 1980; Porter, 1987). The ectoplasmic net is secreted by specialized cell surface organelles called bothrosomes (Porter, 1969). The cell size varies among different species ranging from 10–25 µm in length and 3–8 µm in width (Bigelow et al., 2005; Pokorny, 1967). Cells glide or move in the ectoplasmic network at a rate of 100–175 µm/min (Muehlstein et al., 1991; Porter, 1993; Preston and King, 2005; Young, 1943). This gliding motility is facilitated by an interaction between myosin present on the cell surface and F-actin present on the inner membrane of the ectoplasmic net (Preston and King, 2005). Production of heterokont, biflagellate zoospores with mastigonemes on the anterior flagellum and a dark eyespot on posterior flagellum is an important feature, although it was only reported in one species, *L. algeriensis* (Amon and Perkins, 1968; Perkins and Amon, 1969). Zoospore production was reported as part of the sexual state in *L. algeriensis* due to the appearance of synaptinemal complexes in the prophase nucleus during its subdivision, indicating the occurrence of meiotic cell division resulting in eight haploid zoospores (Perkins and Amon, 1969). However, meiosis, germination of haploid zoospores into haploid spindle cells, and fusion of haploids cells into diploids was not observed directly (Perkins and Amon, 1969).

Taxonomy and classification of *Labyrinthula* spp. has gone through number of changes because of their unique biology (Dick, 2001; Pokorny, 1967; Porter, 1987;

Porter, 1993). Based on morphological features such as tubular mitochondrial cristae (Porter, 1969), production of heterokont, biflagellate zoospores (Amon and Perkins, 1968), and molecular (18s rDNA) phylogenetic data (Honda et al., 1999), the genus *Labyrinthula* has been placed in the kingdom Stramenopila or Chromista, *Labyrinthula* is the only genus in the Labyrinthulaceae. Recent phylogenetic studies showed that *Labyrinthula* spp. are closely related to oomycetes and the bicoceans; it is believed that in the course of evolution they lost their photosynthetic ability and became heterotrophic (Tsui et al., 2009).

Of the approximately dozen of currently accepted *Labyrinthula* species, two are plant pathogens. *L. zosterae,* the causative agent of wasting disease of eelgrass (*Zostera marina*) (Muehlstein et al., 1991), devastated the populations of eelgrass along the Altantic coasts of North America and Europe in the early 1930's and 1980's (Cottam, 1933; Muehlstein, 1989; Renn, 1934). Because of this, the majority of the previous studies on *Labyrinthula* biology focused on *L. zosterae*; little is known about the biology of *L. terrestris* because of its more recent discovery. The objective of this study is to examine the basic biology and ultrastructure of this organism using light, transmission electron, and scanning electron microscopy.

Materials and Methods

Specimen and culture conditions. —An isolate of *L. terrestris* obtained from a golf course in Shallotte, North Carolina (MycoBank nnnnn; NC-12-07) was used for this study. Although various isolates from different locations across the United States were obtained and observed (Yadagiri et al. in review), this isolate was used because of its fast

growth and virulence. All isolates were cultured and maintained on modified 1% horse serum saline water agar (SSA+A) or broth (SSB+A) as described in Yadagiri et al. (in review).

Inoculation.—A suspension of *L. terrestris* cells, adjusted with a hemocytometer to ~500 000 cells/ml in SSB+A, was used as inoculum. Perennial ryegrass, *Lolium perenne* (Bright Star SLT cultivar), a common host, was used for studies on plant material. Plants were grown from seed and watered with saline water (salinity 3.5-4.0 dS/m) for 2-3 wk to reproduce conditions on golf courses from which *L. terrestris* had been isolated. The inoculum was sprayed onto seedlings until runoff with a hand atomizer. Inoculated plants were covered with plastic bags for 2 days to maintain the humidity and promote infection. Plants were kept at 25 C under 12 hr light and 12 hr dark regime in a plant growth chamber.

Transmission Electron Microscopy (TEM).—L. terrestris cells were chemically fixed and prepared using methods described by Porter (1972) and Muehlstein et al. (1991) except that 10 mM HEPES buffer (Sigma-Aldrich Co. LLC, St. Louis, MO) in saline water (Yadagiri et al., in review) was used instead of 0.05 M cacodylate buffer in distilled water. Briefly, *L. terrestris* cells were grown for 2 days in 60 x 15 mm Permanox® Petri dishes (Electron Microscopy Sciences, Hatfield, PA) containing SSB+A medium. The SSB+A was replaced with a prefixative mixture of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), and 1% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) in 10 mM HEPES buffer, pH 6.8–7.5, for not more than 2 min. The prefixative was replaced with 2.5% glutaraldehyde in 10 mM HEPES buffer four times each for 5 min. The cells were then gently washed with 10 mM HEPES

buffer four times at 20 min intervals. The cells were treated with 1% OsO_4 in 10 mM HEPES buffer for 20 min and washed with 10 mM HEPES buffer four times at 20 min intervals. Cells were dehydrated using a graded ethanol series ranging from 10% to 100% ethanol at 15 min intervals, 100% ethanol was replaced twice before the infiltration process. Infiltration was done by replacing 100% ethanol with Embed 812 (Epon-812) resin mixture (Electron Microscopy Sciences, Hatfield, PA) and left overnight on a shaker. For embedding, the resin mixture was replaced with a fresh mixture for 8 h and then cured at 60 C for 24 h. Areas in the dishes that contained numerous intact cells were chosen, cut out, and glued onto tips of resin blocks (casted from plastic capsules) for added support while sectioning (Mims et al. 1988). Sections 60-80 nm thick were cut with a DiATOME® ultra 45° diamond knife (Electron Microscopy Sciences, Hatfield, PA) on a Zeiss HM 355 S ultra microtome. Sections were collected on copper grids and stained with 5% methanolic uranyl actetate (10 min) and stable lead staining solution (3 min) (Hanaichi et al., 1986). The samples were examined with a Hitachi H7000T TEM operated at 60kV accelerating voltage.

Scanning electron microscopy (SEM).—L. terrestris cells were grown either on UV-sterilized 0.1 % poly L-lysine (Electron Microscopy Sciences, Hatfield, PA) coated coverslips or autoclaved cut pieces of dialysis membrane (Sigma-Aldrich Co. LLC, St. Louis, MO) which served as growth substrates. In 50 x 9 mm Petri dishes (Electron Microscopy Sciences, Hatfield, PA), coverslips or dialysis membranes were immersed in SSB+A broth (Yadagiri et al., in review) in which a drop of *L. terrestris* cell suspension (~500 000 cells/ml in SSB+A) was inoculated. Prepared plates were incubated at room temperature for 1-3 days until there were considerable amounts of cells on coverslips or

dialysis membranes. Infected host leaf samples with *L. terrestris* aggregates were directly processed for SEM. All samples were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), pH 7.5, in 10 mM HEPES buffer for 2 h at room temperature. The fixative was replaced with 10 mM HEPES buffer three times at 20 min intervals and the samples were post fixed in 1% OsO_4 in 10mM HEPES buffer for 1 h. Samples were then washed two times, 20 min each in 10mM HEPES. The samples were dehydrated with graded ethanol series ranging from 25% to 100% ethanol. Instead of specimens being critical point dried, they were dipped in 100% HMDS (hexamethyldisilazane) (Electron Microscopy Sciences, Hatfield, PA) solution for 5-10 sec and air dried overnight in a desiccator. The samples were coated for 60 sec with gold-palladium (coating thickness 2 nm) using a sputter coater. Samples were examined using SEM-Hitachi S3500N and FESEM-Hitachi S4800 at the Electron Microscopy Facility of the Clemson University Advanced Materials Center.

Light microscopy.—L. terrestris cells were grown in Analyslide® Petri dishes (Pall Life Sciences, Port Washington, NY) or 50 x 9 mm Petri dishes (Electron Microscopy Sciences, Hatfield, PA) containing SSA+A medium. Cells on agar were observed directly and analyzed with an Olympus BX60 (Olympus, Japan) compound microscope and micrographs were taken with a Prog Res® C5 (Jenoptik, Germany) digital camera.

Results

Ultrastructure.—Transmission electron micrographs of longitudinal sections of cells (Fig. 3.1a-c) revealed that each *L. terrestris* cell contained a single round nucleus (1.0–1.5 µm diameter) with a nucleous, mitochondria with tubular cristae, Golgi

apparatus with long and dark dictyosomes, and round lipids containing vesicles, liposomes, or fat bodies. However, other organelles such as the endoplasmic reticulum and ribosomes were not clearly visible. The cross-sections of *L. terrestris* cells (Fig. 3.1d-e) showed the double membrane of the ectoplasmic net and its association with the bothrosome, a specialized cell surface organelle known to secrete the ectoplasmic net from studies of other species (Fig. 3.1e).

The ellipsoid-fusiform shape of *L. terrestris* cells and orientation in rows within the ectoplasmic net was clearly visible with SEM (Fig. 3.2a-c). Longitudinal cell division was also visible with SEM (Fig. 3.2d). In some instances cell surfaces appeared uneven with round, ball-like projections (Fig. 3.2b-f) which might be fat bodies or lipid droplets (Schwab-Stey and Schwab, 1974) inside the cell. The structure of the bothrosome on the cell surface and the secretion of the ectoplasmic network through bothrosomes were apparent in figure 3.2e-f.

Aggregate formation.—Older *L. terrestris* cultures often formed spherical aggregates typically ranging from 200–800 μm in diameter (sometimes larger) (Figs. 3.3a, 3.4b). On agar media aggregates were usually formed within 5 to 10-days by cells that seeded other cells to collect in a spiral configuration (Fig. 3.4a). These cell aggregates were also seen on fully infected leaves of host plants 15–20 days after infection (Fig. 3.3). SEM studies showed that the aggregates were formed on the host leaf surface by cells that emerged from stomatal openings of the infected plant (Fig. 3.3e-f). The ectoplasmic network appeared to play a major role in binding the cells together into a defined structure (Fig. 3.3a) and at least partially covered the aggregates (Fig. 3.3b-c). Aggregates one-month old or older gave rise to new, actively dividing cells when placed

on fresh culture agar medium. These old aggregates eventually gave rise to new colonies on the agar medium (Figs. 3.3g-h, 3.4c-d).

L. terrestris growth on agar medium — Four different states of *L. terrestris* growth were observed on agar culture medium: (i) actively dividing state; (ii) highly motile state; (iii) aggregate formation; and (iv) old and declining state. These states were sometimes found simultaneously in the same culture plate. In the initial stages of colony formation, cells were typically found actively dividing and increasing cell numbers (Fig. 3.5a-d). These cells tended to be stationary or move little and the shape of the cells was more ovoid than the regular ellipsoid-fusiform shape of motile cells. The ectoplasmic network surrounding these actively dividing zones of cells was usually observed as a thin film, spreading evenly on the agar medium (Fig. 3.5b). After the cell number increased significantly over 3–4 days, the majority of cells tended to move rapidly in a streaming fashion, in rows, gliding through the tube-like enveloping ectoplasmic networks (Fig. 3.5e). Although the rate at which they moved was not recorded, movement was easily observed under a compound microscope. Another common state was the aggregation of cells after 5–10 days, discussed above under aggregate formation. Just a few days before death, old cells which did not form aggregates showed a high percentage of fat bodies and vacuoles in the cytoplasm (Fig. 3.5f).

Discussion

Observations were made on *L. terrestris* cells in culture to study its biology and ultrastructure and eliminate host-related factors. While the overall results of ultrastructure are comparable to those in the species description of *L. terrestris* (Bigelow et al. 2005) and other studies on marine *Labyrinthula* species (Porter 1969), some organelles were

more prominent. The bothrosome and its association with the ectoplasmic network were clearly visible with both TEM (Fig. 3.1e) and SEM (Fig. 3.2e-f). These images provide further support that bothrosomes are the sources of the ectoplasmic network. Vesicles were not observed in the ectoplasmic network as described by Porter (1969) in his specimens. No special structures or organelles were observed in the ectoplasmic network; however, it showed dense granular content of unknown composition (Fig. 3.1e). The tube-like structure of the ectoplasmic network was evident with both TEM (Fig. 3.1d) and SEM (Fig. 3.2a-c). The structure of the ectoplasmic network with both its inner and outer membranes was clearly visible and well preserved with our fixation method (Fig. 3.1e).

Portions of mitochondria were evident in a high proportion of the cytoplasm, indicating cells were actively growing or moving. This can be related to why some strobilurin fungicides were found effective in controlling this pathogen (Martin et al., 2004; Olsen and Gilbert, 2004), as strobilurin fungicides are known to inhibit mitochondrial respiration. The tubular nature of the mitochondrial cristae was more prominent with our fixation method (Fig. 3.1b) compared to previous reports. The Golgi complexes were distinctly dark with large dictyosomes (Fig. 3.1c) and were comparable to those images documented by Porter (1969). Older cells contained a high percentage of lipid vesicles or fat bodies, which may act as food reserves during unfavorable conditions. However, other cellular structures such as endoplasmic reticula and ribosomes were not clearly visible with our chemical fixation. The round electron dense structures that appeared to have been pulled away from the organelle edge (Fig. 3.1a, c) might be artifacts due to chemical fixation.

This is the first detailed report of *L. terrestris* structure using SEM. Apart from two SEM micrographs of *L. terrestris* inside the host, (Stowell and Gelernter, 2003), to our knowledge there were no other previous SEM studies made on *L. terrestris.* With SEM studies the surface structure and the function of the ectoplasmic network in cell motility and aggregate formation were clearly demonstrated (Figs. 3.2-3.3). The ectoplasmic network was membranous in structure when serving as track way for fusiform cells to glide (Fig. 3.2a-c) and was in a network form of branching patterns apparently holding cells together in an aggregate (Fig. 3.3d), and also partially covering the aggregate as means of protective layer (Fig. 3.3b-c). These structural variations of any *Labyrinthula* species' ectoplasmic network have not been reported before.

Although individual cells were enclosed by the ectoplasmic network, the cells were clearly visible inside which suggests that ectoplasmic network membranes are fairly thin and adhere close to the surface of the cells. Cell surface structure contained round, ball-like evaginations which are presumed to be the round lipid bodies present inside the cell near the plasma membrane. These structures were observed earlier with SEM studies on *L. coenocystis*, the author presumes they are lipid bodies inside the cell (Schwab-Stey and Schwab, 1974). The cell surface was relatively smooth in younger cells (Fig. 3.1a) when compared to older cells (Fig. 3.1b-f). This can be related to the accumulation of lipid bodies in old declining cells observed using light microscopy (Fig. 3.5f).

It was challenging to keep the cells and ectoplasmic network intact for SEM with general fixation methods. Our modified SEM protocol and the use of HMDS resulted in good preservation of cell shape and ectoplasmic network structure. In addition, the surface structure of the bothrosome and the secretion of ectoplasmic network through the

bothrosome were clearly demonstrated with our SEM studies (Fig. 2f). However, the bothrosomes were not always visible because the ectoplasmic net usually covered most of the cell surface, the number of bothrosomes per cell was not clear. It appears that bothrosomes are not permanent structures and their formation and function may be dynamic, depending on the physiological state of the cells, by invagination of the plasma membrane.

The formation of aggregates appears to be a major part of the life cycle of *L. terrestris*. Aggregation was reported in other species of *Labyrinthula* (Nakatsuji et al., 1981) and in the case *L. algeriensis*, in which zoosporulation was observed in cell aggregates (Amon, 1978). Although zoosporulation was not observed in *L. terrestris*, our studies suggest that the aggregates might play a major role in *L. terrestris* survival during stressful or unfavorable conditions. It seems possible that when cells are aggregated they conserve energy by restricting their motility and production of ectoplasmic network, and old cells that die in the aggregate might be used as food source for younger cells to survive. However, our observations indicate that moisture is important for aggregates to survive, as dryer aggregates did not give rise to new cells when placed on fresh agar while moist aggregates did. Additional studies on these aggregates should reveal their role and importance in the *Labyrinthula* life cycle.

The contents of the ectoplasmic network and chemistries that are involved in cell-to-cell communication are still unknown, as are motility and nutrient absorption. Our observations on *L. terrestris* on the agar medium suggest that the rapid cell division and the faster gliding motility using ectoplasmic network can be related to quick disease spreading nature of rapid blight. *L. terrestris* is a challenging pathogen to control;

therefore, further research to understand its ecology, evolution, physiology, cell and molecular biology is definitely important.

This is the first comprehensive study on the biology of *L. terrestris*. TEM and SEM studies provided a better understanding of the ultrastructure and surface structure of *L. terrestris*. Studies showed that *L. terrestris* reproduces by mitotic cell division and no meiosis or sexual reproduction has been observed. The methods and protocols described here may be helpful in understanding the biology of such inadequately described species as well as those that are newly reported.

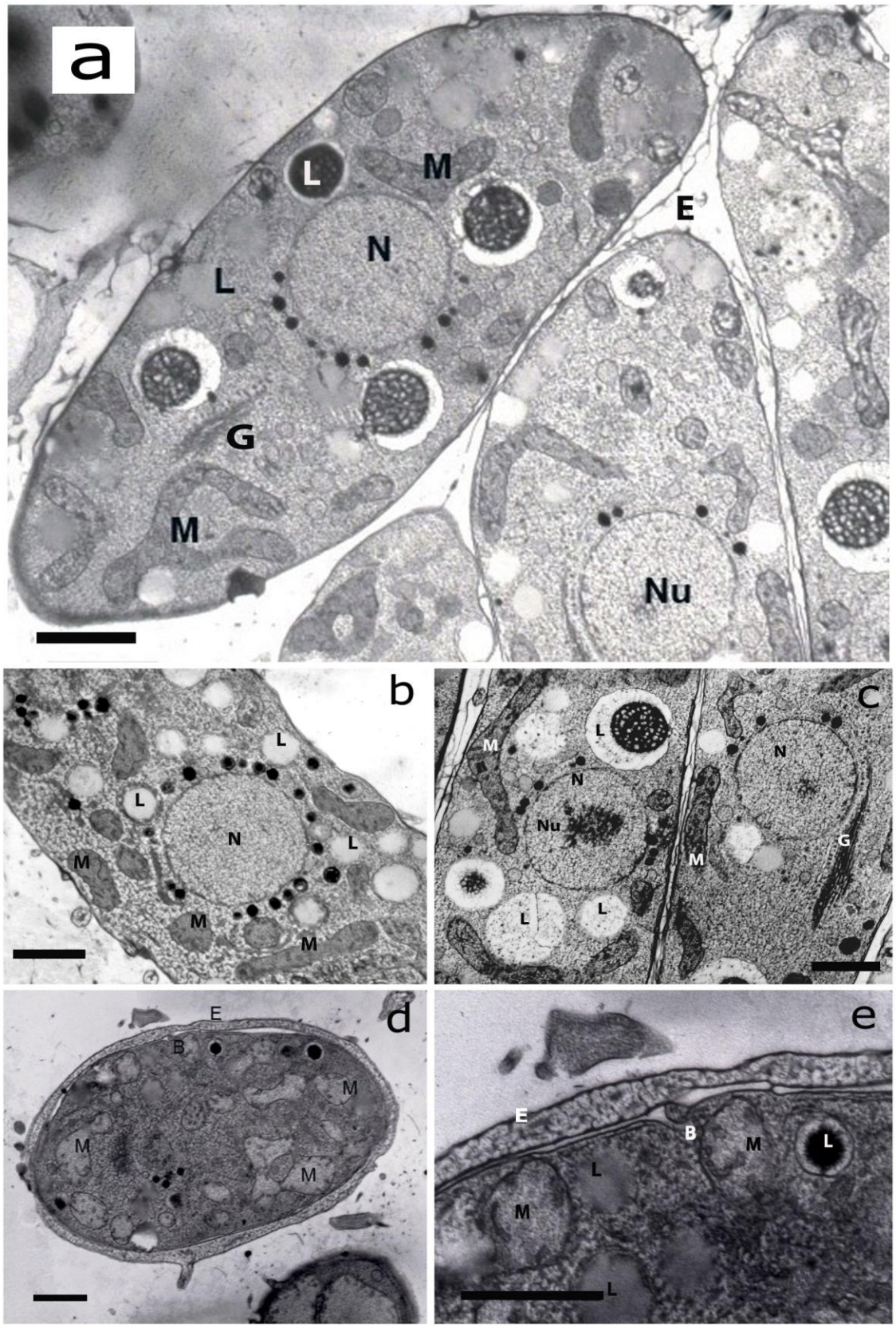
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Fig. 3.1. Transmission electron micrographs showing the ultrastructure of *L. terrestris* cells. a-c. Longitudinal sections of cells, each with a distinct nucleus (N), nucleous (Nu), mitochondria with tubular cristae (M), liposomes (L), long and dark Golgi complex (G), and double-membraned ectoplasmic network (E). d. Cross section of a cell within a double-membraned ectoplasmic network (E). e. Higher magnification showing the bothrosome (B) and ectoplasmic network (E). Bars = 1 µm.

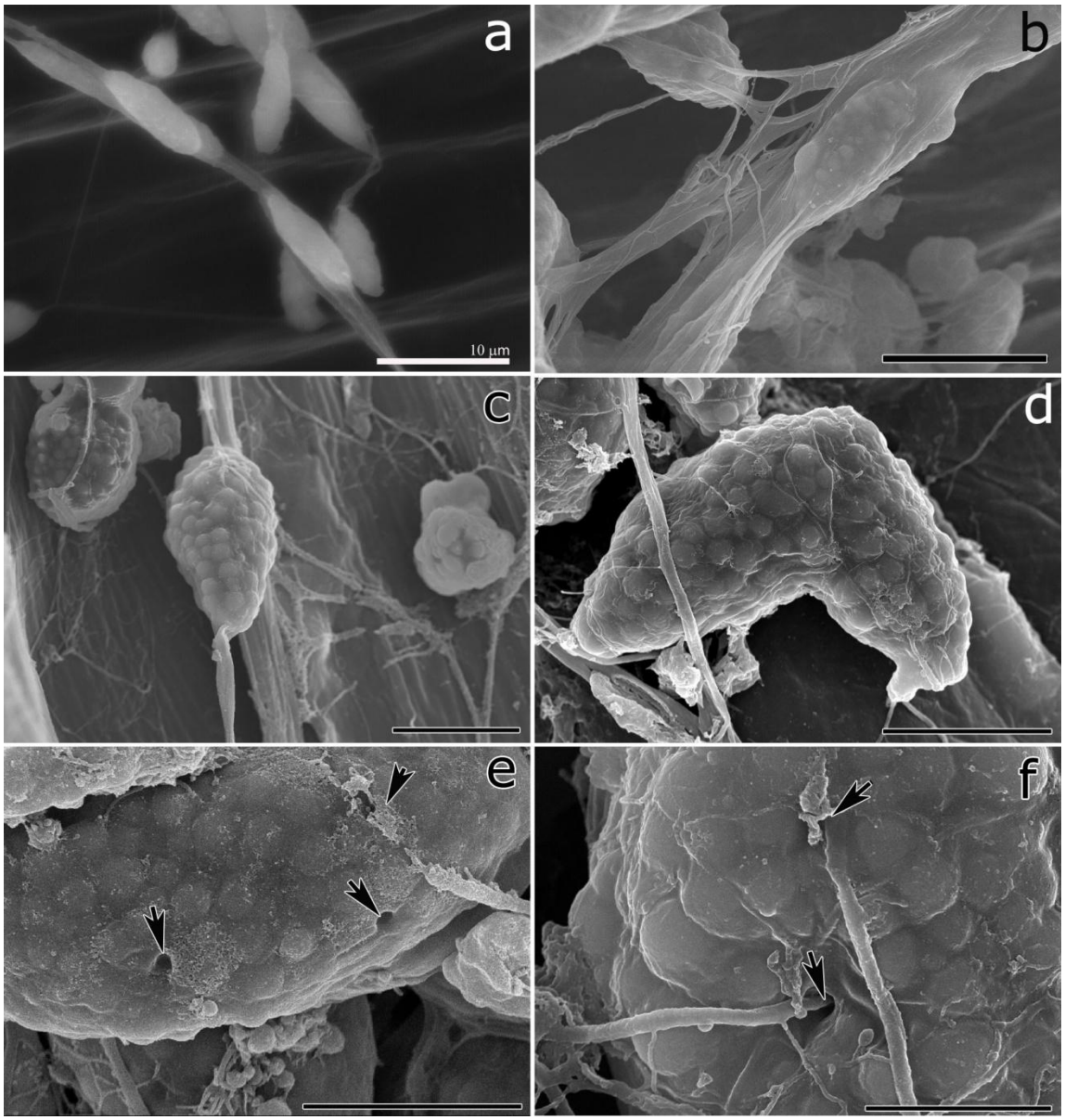

Fig. 3.2. Scanning electron micrographs showing the surface structure of *L. terrestris* cells. a-c. Fusiform cells within the ectoplasmic network. a. One-day-old cells showing smoother cell surface. b-f. Two to three-day-old cell surfaces showing ball-like projections (presumably lipid bodies inside cells). d. The formation of two cells by mitotic division. e-f. Cell surfaces with bothrosomes (arrows) and their associated ectoplasmic networks. Bars: a-b =10 µm; c-d = 5 µm; e = 4 µm; f = 2 µm

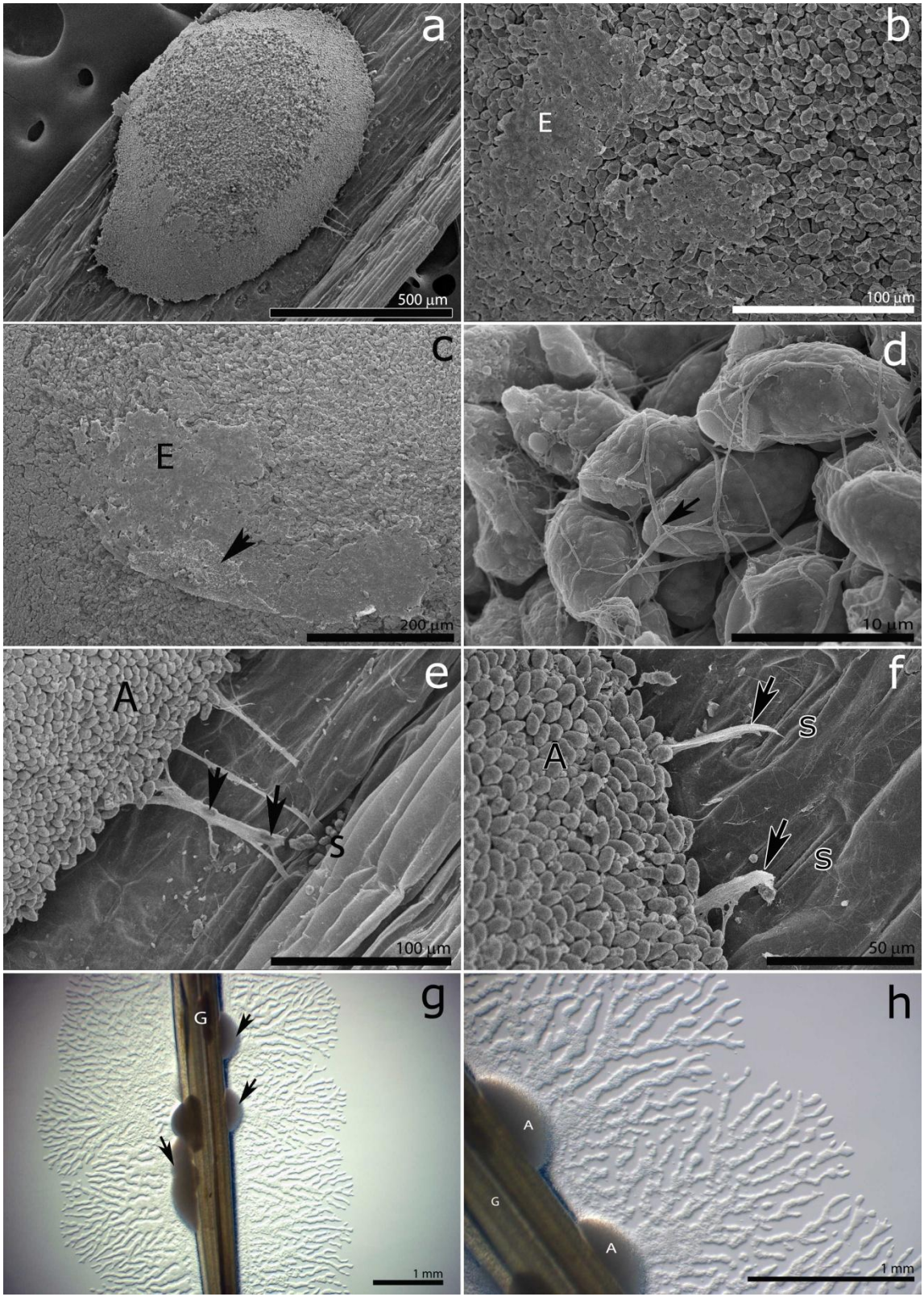
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Fig. 3.3. Scanning electron (a–f) and light (g-h) micrographs showing the structure of the *L. terrestris* aggregates on infected grass. a. An aggregate of *L. terrestris* cells on an infected grass blade, 15 days post inoculation. b. The ectoplasmic network (E) partially covering the aggregate. c. A layer of the ectoplasmic network (E) (arrow) peeling off from the aggregate. d. Higher magnification image showing *L. terrestris* cells packed in an aggregate with filaments of the ectoplasmic net (arrow). e-f. *L. terrestris* cells coming out from the infected grass leaf through stomata (S) to form an aggregate (A) using ectoplasmic network (arrows). g & h. Infected grass leaf (G) with aggregates (A) when placed on fresh agar medium, giving rise to new cells forming digitate colonies by active cell division. Bars: g & h = 1 mm; a = 500 µm; c = 200 µm; b & e = 100 µm; f = 50 µm; d =10 µm.

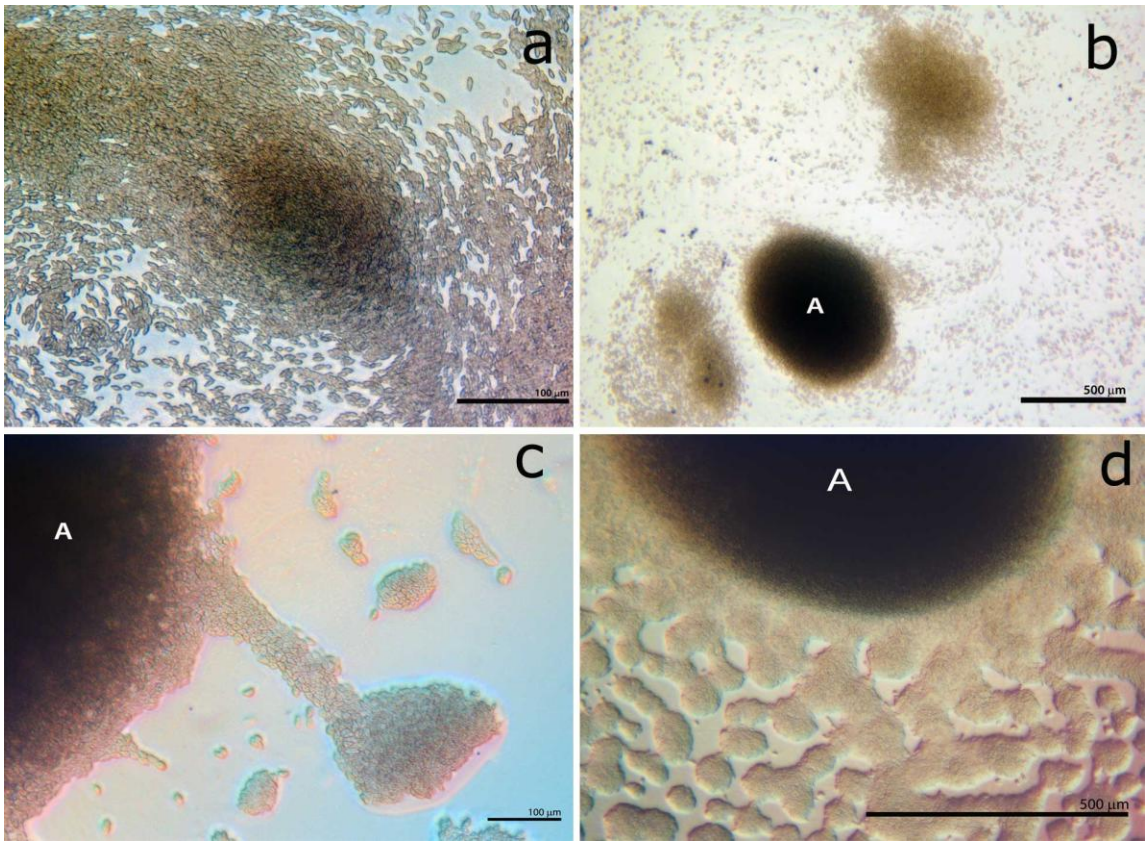
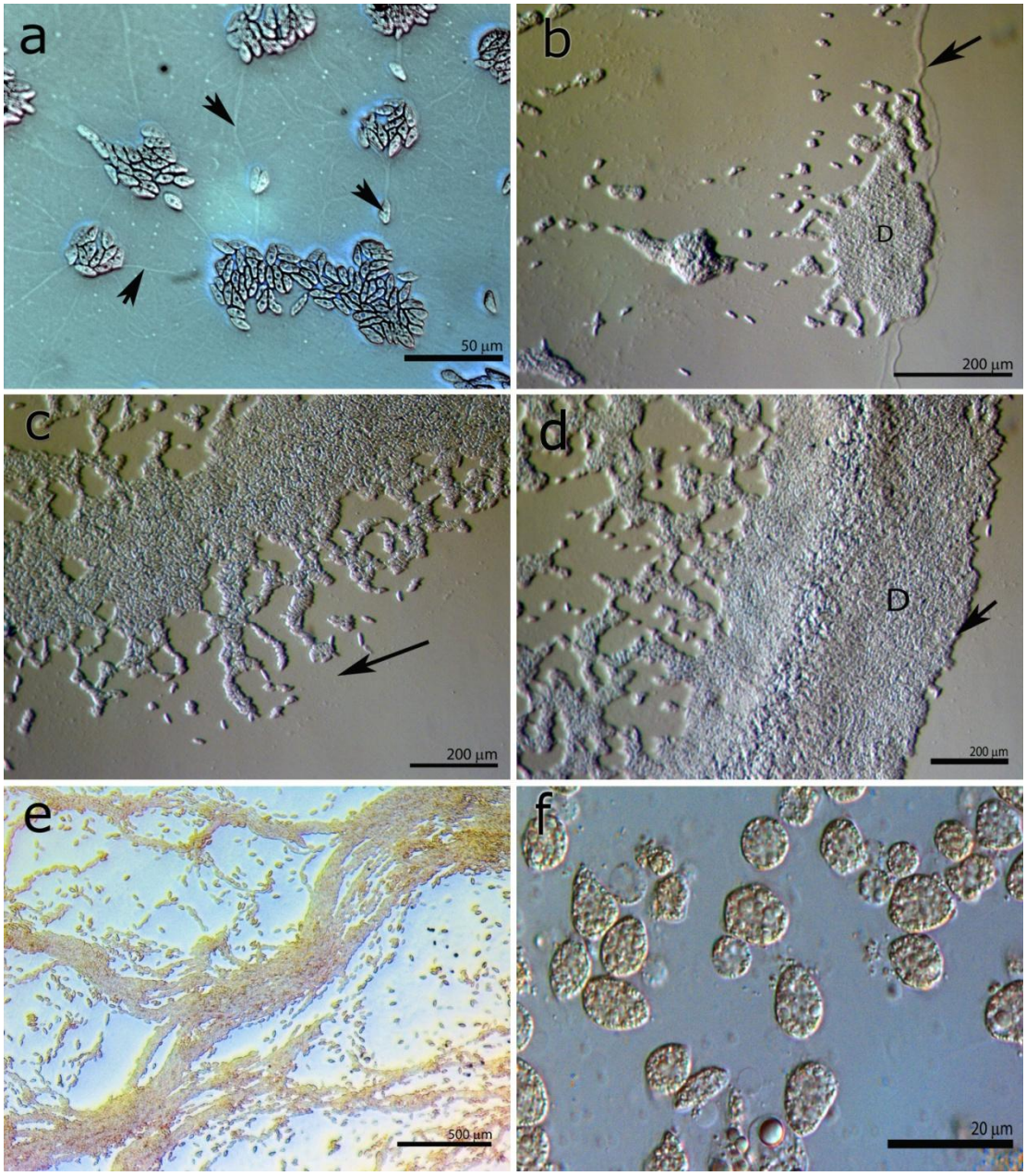


Fig. 3.4. Light micrographs showing different stages of aggregate formation by *L. terrestris* cells on agar media. a. Initial stage of aggregate formation, noted by the whirling configuration of cells. b. Fully formed aggregate (A). c. One-month-old aggregate (A) giving rise to new actively diving cells. d. Actively dividing and motile cells developing from the same aggregate (A). Bars: a & c = 100 µm; b & d = 500 µm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D
200 µm
c
200 µm
d
D
200 µm
e
500 µm
f
20 µm

Fig. 3.5. Light micrographs showing different morphological states of *L. terrestris* growth on an agar medium. a. Initial state of colony formation; small groups of cells simultaneously dividing and moving (arrow) with anatomosing ectoplasmic networks (arrows). b. The actively dividing state (D) with the ectoplasmic network (arrow) visible as a thin film over the dividing cells. c-d. Actively dividing, less motile or stationary state (D) with colony growth and expansion (arrow indicates outer colony border). e. Actively motile state with cells moving in the tube-like ectoplasmic network. f. Old, declining cells with a high percentage of fat bodies or vacuoles in their cytoplasm. Bars: a = 50 µm; b-d = 200 µm; e = 500 µm; f = 20 µm.

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CHAPTER FOUR

HISTOPATHOLOGY OF INFECTION AND COLONIZATION OF COOL-SEASON TURFGRASS BY *LABYRINTHULA TERRESTRIS*, CAUSAL ORGANISM OF RAPID BLIGHT

Introduction

Rapid blight is an unusual disease of cool-season turfgrasses such as *Agrostis tenuis* (colonial bentgrass), *Lolium perenne* (perennial ryegrass), *Poa annua* (annual bluegrass), and *Poa trivialis* (rough bluegrass) (Martin et al., 2002a; Olsen et al., 2003; Stowell et al., 2005). Rapid blight was first described in 1995 in California (Stowell, 1995) and the causative organism was identified as a *Labyrinthula* species in 2003 (Olsen et al., 2003). Based on specific features such as cell size and shape, colony morphology and color, terrestrial habit, and molecular evidence, the pathogen was described as *Labyrinthula terrestris*, a new species (Bigelow et al., 2005; Craven et al., 2005). The major symptoms of rapid blight include water-soaked lesions and yellowing and browning of turf foliage that ultimately leads to death of the turf. The disease is known to spread very fast affecting large areas of turf within few weeks, hence the name 'rapid blight' (Martin et al., 2002a; Martin et al., 2002b; Stowell et al., 2005).

The genus *Labyrinthula* has been placed in the kingdom Stramenopila, also known as Chromista (Dick, 2001) based on characteristics such as the production of biflagellate heterokont zoospores (Amon and Perkins, 1968), tubular mitochondria (Porter, 1969), and molecular studies (Honda et al., 1999; Leander and Porter, 2000). Recent phylogenetic studies showed that it is closely related to oomycetes, it is believed that they lost photosynthetic ability and became heterotophic during the course of evolution (Tsui et al., 2009). *Labyrinthula* species are characterized by their unique

fusiform to ellipsoid shaped vegetative cells that move within an enveloping ectoplasmic network (Cienkowski, 1867; Nakatsuji et al., 1980; Porter, 1987) secreted by specialized cell surface organelles called bothrosomes (Porter, 1969). Only two of the approximately 14 currently accepted species (Dick, 2001, Index Fungorum) are plant pathogens. Most of the *Labyrinthula* species are saprotrophic, found in association with marine algae and vascular plants (Porter, 1987) in near-shore marine and estuarine ecosystems (Alexopoulos et al., 1996; Porter, 1987). One notable exception is *L. terrestris*, which has been shown to be pathogenic on turfgrasses and causes the disease rapid blight (Bigelow et al., 2005).

Rapid blight has been reported from more than 100 golf courses in eleven states throughout the United States of America (Stowell and Gelernter, 2003; Hyder et al., 2010). The disease is widespread; it has also been found in Europe, including the United Kingdom (Entwistle et al., 2006), southern Europe, (Olsen 2007) and Argentina (S. B. Martin unpublished). *L. terrestris* has been primarily observed on golf courses and landscape turfs where irrigation water is highly saline in nature (Olsen et al., 2004); however, it has not been reported in residential or commercial lawns (Stowell et al., 2005).*L. terrestris* is a challenging pathogen of turf because of its unusual taxonomic affiliation and only partially understood biology. For control measures, there are moderately tolerant cool-season turf cultivars available (Peterson et al., 2005) and fungicides such as trifloxystrobin (Compass), pyraclostrobin (Insignia), and mancozeb (Fore) have been somewhat effective in controlling or preventing the spread of the pathogen (Martin et al., 2004, Olsen and Gilbert, 2004). To better manage and control any disease, a thorough understanding of the causal agent of the disease is needed.

Relatively little is known about *L. terrestris* and rapid blight; therefore, the histopathology of rapid blight, including the pathogen's behavior on the host leaf surface, infection, and colonization processes are presented.

Materials and Methods

L. terrestris culture: Various isolates of *L. terrestris* were obtained from different locations in the U.S.A where rapid blight was reported. The isolates were cultured on modified 1% horse serum saline water agar (SSA+A) or broth (SSB+A) as described in Yadagiri et al. (in review). Of the isolates available, an isolate obtained from a golf course in Shallotte, North Carolina (NC-12-07) was used for this study due to its faster growth rate and virulence on inoculated turf plants.

Host plant and inoculation: Two to three-week old perennial ryegrass (*Lolium perenne,* Bright Star SLT cultivar) was used as the host plant. Autoclaved sand was used as growth medium for the plants. It was grown from seed and treated with saline water (salinity 3.5 – 4.0 dS/m) for irrigation. A suspension of *L. terrestris* cells in SSB+A was used as inoculum. Cell density was quantified with a hemocytometer (VWR International, LLC.) and adjusted to a final concentration of ~500,000 cells/ml with SSB+A. Using a hand atomizer the suspension was sprayed gently on live plants until run off. Sterile SSB+A was sprayed on control plants. Samples were collected at 24 h intervals for up to ten days post inoculation to study the histopathology.

Light microscopy (LM): To examine *L. terrestris* on and in whole grass blades under a light microscope, the chlorophyll was removed by putting the samples in a 1:1 solution of glacial acetic acid (Fisher Scientific, Fair Lawn, NJ) and 95% ethanol and gently shaken overnight. This solution also served as a chemical fixative for the samples.

The leaves were then gently washed in sterile distilled water for 5 min and transferred to a staining solution of 0.01% trypan blue (Fisher Scientific, Fair Lawn, NJ) in 67% lacto-phenol (Fisher Scientific, Fair Lawn, NJ) and shaken for 2 – 4 h. Excess stain was removed by washing samples in 70% glycerol (Fisher Scientific, Fair Lawn, NJ). The samples were then mounted on glass slides in mounting medium (Poly-Mount®, PolySciences, Inc., Warrington, PA) or 100% glycerol (Fisher Scientific, Fair Lawn, NJ) and covered with a coverslip. To examine cross-sections of the host-pathogen interactions, samples were chemically fixed, dehydrated (explained below under scanning electron microscopy) and embedded in Spurr's resin (Electron Microscopy Sciences, Hatfield, PA). Sections of 1 µm thickness were cut using 45° glass knives, a Leica RM2165 microtome, and stained using aqueous solution of 0.01% Toluidine blue (Electron Microscopy Sciences, Hatfield, PA) with 0.01% sodium borate (Electron Microscopy Sciences, Hatfield, PA). Olympus BX60 (Olympus, Japan) compound microscope was used to observe and analyze the samples and Prog Res® C5 (Jenoptik, Germany) digital camera was used to capture the images.

Scanning electron microscopy (SEM): Samples were chemically fixed for 2 h at room temperature using 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 10 mM HEPES buffer (Sigma-Aldrich Co. LLC, St. Louis, MO) at a pH ~7.5. In order to wash of unused glutaraldehyde, the samples were treated with 10 mM HEPES buffer for 60 min period during which the buffer was replaced at least three times. The samples were then post-fixed for 1 h at room temperature using 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 10mM HEPES buffer followed by rinsing in 10mM HEPES (twice, 20 min each). Dehydration of the samples was done

using graded ethanol series ranging from 25% to 100% ethanol. Instead of using a critical point dryer for total dehydration, 100% HMDS (hexamethyldisilazane) (Electron Microscopy Sciences, Hatfield, PA) solution was used to dip the samples for 5 – 10 sec followed by overnight drying in a desiccator. Using a sputter coater, the samples were coated with gold-palladium (coating thickness 2 nm) for about 60 sec. SEM-Hitachi S3500N and FESEM-Hitachi S4800 were used to examine the samples.

Results

L. terrestris on host surfaces: SEM allowed for detailed examination of *L. terrestris* on the host surfaces. Before infecting the host, *L. terrestris* cells first attached themselves to the host leaf surface using the ectoplasmic net (Fig. 4.1a-c). The cells divided and propagated in large numbers mainly in the adaxial grooves or ridges of the grass leaf epidermal cells (Fig. 4.1d). Cells also colonized the stomatal pits, fully covering the stomata of the leaf prior to infection (Fig. 4.1e-f). These events were observed within 1 – 3 days after inoculation.

Leaf infection: Observations with SEM and LM showed that after establishment of *L. terrestris* cells on the leaf surface, *L. terrestris* infected its host in multiple ways within next 2 – 3 days. Stomatal openings were one of the major entry points for *L. terrestris* to infect its host; this was observed repeatedly, as shown in Fig. 4.2a-c. Infection also appeared to occur through the bases of trichomes (Fig. 4.2d-f), as cells were observed inside the host immediately under the trichome bases. However, direct penetration of the host surface was not observed. Another mode of infection was through artificial wounds (Fig. 4.2g) and cut ends (Fig. 4.2h-i) of the grass blade.

Host leaf tissue colonization: Light micrographs of cross-sections of 8 – 10-day-old infected leaves show that *L. terrestris* cells colonized all types of host cells including the epidermis, mesophyll, bundle sheath, vascular bundle, and sclerenchyma (Fig. 4.3a). Observations indicate that *L. terrestris* cells can divide inside the host (Fig. 4.3b) and there was repeated observations of *L. terrestris* cells moving between host epidermal cells, suggesting possible host cell wall penetration or digestion (Fig. 4.3c-d).

Discussion

We report for the first time the means of host infection by a species of *Labyrinthula*. Although a host-pathogen interaction study was done on another plant pathogenic species, *L. zosterae*, the mode of infection was not understood (Muehlstein 1992). Our results indicate that *L. terrestris* uses multiple modes to infect host leaves (Fig. 4.2). Infection through stomatal openings, wounds, and cut ends might be the easiest way for infection because indirect penetration through natural openings requires less energy than host surface penetration. Given that the presence of cut ends can increase pathogen infection and the spread of the pathogen by mowers has been observed (based on disease symptoms), a reduction in mowing frequency and increase in mowing height might help lower rapid blight severity on a turf field.

Direct penetration of intact host leaf surfaces has not been observed. Evidence of infection through trichome bases (Fig. 4.2d-f) suggests that there is a possibility of direct penetration, as there are no natural openings found near trichome bases. Trichomes are modifications of leaf's epidermal cells, thus the structure of the cell wall and the surface waxy cuticle might be different (weaker) compared to other areas of the leaf. Considering

this fact and that trichomes serve as a means for the pathogen's attachment on the host, it appears that direct penetration at the bases of trichomes is possible.

Examinations of host colonization suggest that *L. terrestris* does not show host cell specificity during pathogen proliferation, as it colonized all of the different host cell types (Fig. 4.3a). It was notable that *L. terrestris* cells were able to multiply inside the host (Fig. 4.3b) and move across the host cell walls (Fig. 4.3c-d). This movement between cells suggests that *L. terrestris* has the ability to digest the host cell wall or weaken the area around the plasmodesmata. Our attempts to determine how *L. terrestris* cells move inside its host with TEM were not successful due to difficulties preparing infected leaves with chemical fixations. However, evidence of host cell wall digestion inside the host was observed previously in *L. zosterae* (Muehlstein 1992). Although it seems probable that *L. terrestris* can digest at least parts of host cell walls, further examination is still needed.

Investigations were carried out on above-ground tissues, i.e. grass leaf blades, because disease symptoms and pathogen dispersal have been observed on these tissues. However, evidence of below-ground dispersal has been observed (M. Olsen pers. comm.) and is a topic for future investigation. The ecological aspects and disease epidemiology of *L. terrestris* need further study to answer questions such as how it persists in the field when susceptible cool-season turfgrasses are not present (i.e. summer), how it interacts with other organisms in an ecosystem, and whether it is capable of zooporulation. We have also examined the biology and life cycle of *L. terrestris* (Yadagiri et al., in review) which showed that older *L. terrestris* cells frequently formed aggregates. It was noteworthy that aggregates one to two months old were able to give rise to new cells

when placed on fresh agar medium; this suggests that aggregates play an important role in survival under stress. Our histopathology studies presented above and observations of *L. terrestris* in culture (Yadagiri et al., in review) suggest that the rapid cell division both inside and outside the host, fast gliding motility via the ectoplasmic network, and multiple modes of infection all contribute to the rapid disease development and quick spread of *L. terrestris*.

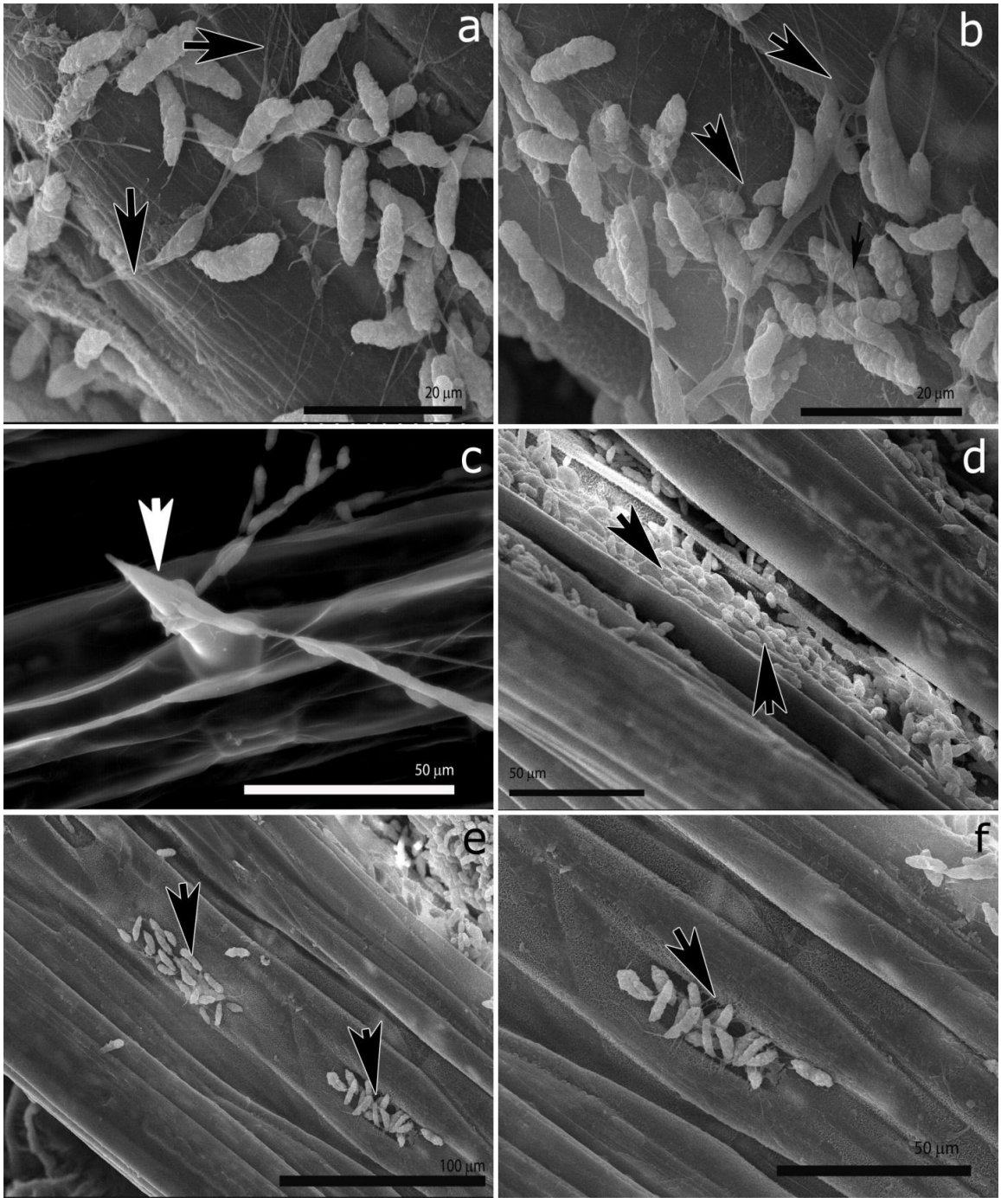
a
20 µm
b
20 µm
c
50 µm
d
50 µm
e
100 µm
f
50 µm

Fig. 4.1. Scanning electron micrographs showing the attachment and surface colonization of *L. terrestris* (MycoBank nnnn) on leaves of host, perennial ryegrass (*Lolium perenne*, cultivar Bright Star SLT). a-b. Cells attached to grass blade surfaces via ectoplasmic networks (arrows) 1 day after inoculation. c. Cells presumably moving within ectoplasmic networks, anchored to a trichome (arrow); 1 day after inoculation. d. Adaxial surface ridges of a ryegrass blade colonized by *L. terrestris* cells (arrows) 2 – 3 days after inoculation. e-f. Stomatal pits colonized by *L. terrestris* cells (arrows) 3 days after inoculation. Scale bars: (a) & (b) = 20 µm; (c) & (d) = 50µm; (e) = 100 µm & (f) = 50 µm

a
b
c
10 µm
d
e
f
50 µm
g
h
50 µm
i
50 µm

Fig. 4.2. Scanning electron (a-b & d-e) and light (c, f-i) micrographs showing process of infection by *L. terrestris* (MycoBank nnnn) on the host (*Lolium perenne* cultivar Bright Star SLT) leaf. a-b. Surface view of *L. terrestris* cells (arrow) infecting the host leaf through stomatal openings. c. Cross section of a *L. terrestris* cell (brown in color) infecting through a stomatal opening (arrow). d-e. *L. terrestris* cells infecting through the base of a trichome, near the trichome bases are areas that may have been degraded (arrows) by *L. terrestris*. f. Unstained *L. terrestris* cells inside the host (black arrow), which presumably gained entrance via the trichome base (white arrow). Trypan blue stained cells are outside of the host. g. Host leaf tissue with an artificial wound and *L. terrestris* cells stained with trypan blue; stained cells (arrows) are just outside the leaf around the wound. h. Cut end of a grass blade with trypan blue stained *L. terrestris* cells visible on the cut area (arrow). i. Colonization of the host's epidermal cells by *L. terrestris* just after infection through a cut end of a leaf (arrows). Scale bars: (a) = 30 µm; (b) = 5 µm; (c) = 10 µm; (d) = 20 µm; (e) = 10 µm; (f) = 50 µm; (g) = 100 µm (h) & (i) = 50 µm

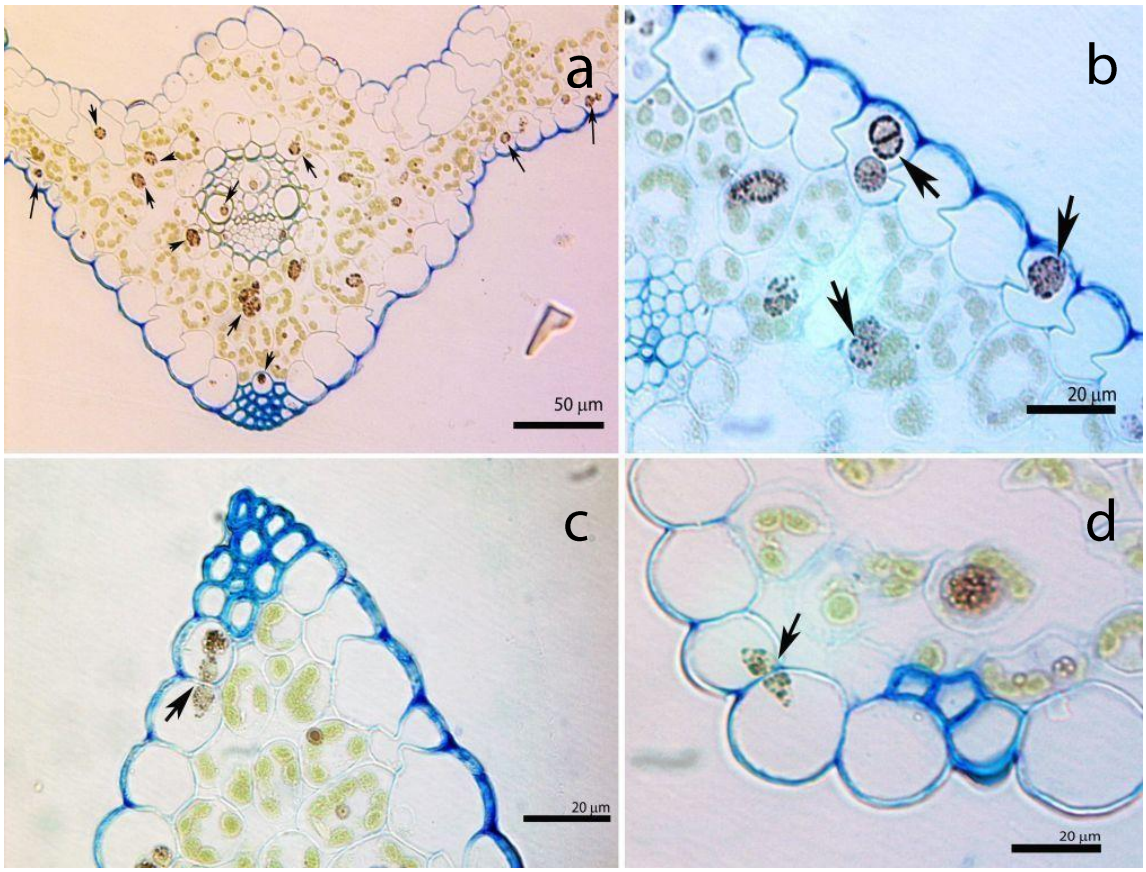

Fig. 4.3. Light micrographs of cross sections through host (*Lolium perenne* cultivar Bright Star SLT) leaf tissues containing *L. terrestris* (MycoBank nnnn) cells. a. *L. terrestris* cells occupying different host cells including the epidermis, mesophyll, bundle sheath, vascular bundle, and sclerenchyma (arrows). b. *L. terrestris* cells diving inside the host (arrows). c-d. *L. terrestris* cells in two cells, presumably moving across the host cells and penetrating the host cell walls (arrows). Scale bars: (a) = 50 µm; (b), (c) & (d) = 20 µm

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