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Overwinter survival of *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, screening hosta for resistance to *S. rolfsii* var. *delphinii*, and phylogenetic relationships among Sclerotium species

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Overwinter survival of *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, screening hosta for resistance to *S. rolfsii* var. *delphinii*, and phylogenetic relationships among *Sclerotium* species

by

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in partial fulfillment of the requirements for the degree of

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This dissertation is dedicated to my family.
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ABSTRACT

In this study, three aspects of plant-pathogenic *Sclerotium* fungi were investigated: overwinter survival of *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*; development of a rapid method for detecting resistance of hosta cultivars to petiole rot disease; and clarification of phylogenetic relationship among pathogenic *Sclerotium* species. To test the hypothesis that differential tolerance to climate extremes affects the geographic distribution of *S. rolfsii* and *S. rolfsii* var. *delphinii*, overwinter survival of these fungi was investigated in the northern and southeastern U.S. At each of four locations, nylon screen bags containing sclerotia were placed on the soil surface and buried at 20-cm depth. Sclerotia were recovered six times from November 2005 to July 2006 in North Dakota and Iowa, and from December 2005 to August 2006 in North Carolina and Georgia. Survival was estimated by quantifying percentage of sclerotium survival on carrot agar. Sclerotia of *S. rolfsii* var. *delphinii* survived until at least late July in all four states. In contrast, no *S. rolfsii* sclerotia survived until June in North Dakota or Iowa, whereas 18.5% survived until August in North Carolina and 10.3% survived in Georgia. The results suggest that inability to tolerate low temperature extremes limits the northern range of *S. rolfsii*. In the second study, a rapid assay was developed to assess hosta cultivars for resistance to petiole rot caused by *S. rolfsii* var. *delphinii*. The leaf-petiole junction of excised leaves of greenhouse-grown hosta (*Hosta kikutii* and *H. spp.* cultivars Lemon Lime, Munchkin, Tardiflora, Pearl Lake, Zounds, Honeybells, Gold Drop, and Haleyon) were treated with 20 µl of oxalic acid (50 mM) on a cotton swab, then incubated at 100% relative humidity and 27 °C. After 4 days, incidence of leaves with lesions was evaluated. Cultivar resistance rankings were generally consistent with those of field and greenhouse cultivar screening tests in which whole plants were inoculated with the pathogen.
In the third study, genetic evidence and morphological features were used to determine the taxonomic placement of eight plant-pathogenic \textit{Sclerotium} species and transfer one \textit{Ceratorhiza} species to the genus level. Sequences of rDNA large subunit (LSU) and internal transcribed spacer (ITS) regions were generated for isolates of each species. Parsimony analysis grouped two species, \textit{S. denigrans} Pape and \textit{S. perniciosum} Slogt. et K.S. Thomas, within the Ascomycota. Mycelium morphology generally matched with results of the parsimony analysis. \textit{S. hydrophilum} Sacc and \textit{S. rhizodes} Auersw. were transferred to \textit{Ceratorhiza hydrophilum} (Sacc.) Xu, Harrington, Gleason, et Batzer, \textit{comb. nov.} and \textit{Ceratorhiza rhizodes} (Auersw.) Xu, Harrington, Gleason, et Batzer, \textit{comb. nov.}, respectively.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation consists of an abstract and five chapters. The first chapter is an introduction to hosta petiole rot, caused by *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, and sets the stage for the research presented in the dissertation. This chapter starts with a literature review of hosta, hosta petiole rot, ecology, and management of the disease, and phylogenetic relationships among plant-pathogenic *Sclerotium* species and ends with the research rationale and objectives. The second chapter is a manuscript, published in 2008 in the journal *Plant Disease*, which investigated overwinter survival of *S. rolfsii* and *S. rolfsii* var. *delphinii* in the midwestern and southeastern U.S. The third chapter is a manuscript submitted to the journal *Plant Health Progress*, summarizing development of a technique to assess hosta cultivar resistance to petiole rot. The fourth chapter is a manuscript in preparation for *Mycologia*, summarizing research on phylogenetic placement of plant-pathogenic *Sclerotium* species. The fifth chapter summarizes results and provides overall conclusions to the dissertation.

Literature Review

Hosta

Hostas (*Hosta* spp) are shade-tolerant ornamentals that have become the world's most popular herbaceous perennials during the past decade (The American Hosta Society 2001). Native to China, Japan, and Korea, hostas were first imported from Japan and grown in Europe in the 1700s. They were grown in North America by the mid-1800s (Linneman
Currently, there are about 50 species and nearly 4,000 cultivars (cultivated varieties) available in the trade (The American Hosta Society 2001). Hostas are grown primarily for their foliage attributes.

A Hosta species population is a group of hosta composed of interbreeding plants that have certain features in common but are diverse in appearance (Schmid 2006).

_H. plantaginea_, the first species to reach Europe in the early 1780s, was one of the first Hosta species to receive formal taxonomic placement. As most hosta species have the same chromosome number, interspecific hybridization is generally possible, with the exception of _H. ventricosa_, a natural tetraploid that sets seed through apomixis (Schmid 2006).

Hybridizing is an important way to develop new hosta cultivars. Hybridization within and among species and cultivars has produced numerous cultivars. Hybrids can occur either naturally in the garden through pollination by insects, or from crosses by breeders (The American Hosta Society 2001). Hybridization usually leads to changes in leaf size or color, clump size or shape, or bloom time or color. Another way to develop new cultivars is through “sports” that are discovered in gardens, nurseries and tissue-culture labs (The American Hosta Society 2001). Natural sports arise from mutations that impart new patterns of variegation in leaf color but are unlikely to affect disease susceptibility or resistance to a particular pathogen (Royal Horticultural Society 2008; Kevin Hurd, personal communication, Walters Gardens, Inc., Zeeland, MI). Sports are a source of leaf pigment alterations; for example, a hosta with entirely green leaves may have a sport with white leaf margins or center variegation (The American Hosta Society 2001). The cultivar name is written as _Hosta_ ‘Name’ or _H._ ‘Name’. Rules for naming cultivars are determined by the International Code of Nomenclature for Cultivated Plants (ICNCP), and the cultivar names
are registered with the International Registrar for the Genus *Hosta* under the auspices of The American Hosta Society (AHS) (Schmid 2006).

Hosta plants vary in size from a few cm to 3 m in diameter. Some hostas cultivars develop a vase-like shape, narrowing at the base. Leaves of hosta also differ greatly in size and shape. Most hostas have linear, oval, spade-shaped, heart-shaped, or round leaves, whereas others may have bowl-shaped leaves with surfaces that are smooth or puckered (The American Hosta Society 2001).

Hostas are also variable in color and color pattern. Leaf colors may be green, yellow, gold, or blue. Some hostas have leaves that are variegated. The types of variegation may be “marginal-variegated” (along the leaf margins), or “medio-variegated” (misted with fine dots or speckles) (The American Hosta Society 2001).

Substance is the thickness or rigidity of the leaf. In general, hostas with thicker leaves are more resistant to damage from slugs and snails (The American Hosta Society 2001).

**Hosta petiole rot**

Petiole rot, caused by *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, has caused substantial losses in hosta plantings in the U.S. Previously known only from the southern U.S. (Mullen 2001; Punja 1985; Tian 2006), hosta petiole rot was reported in the midwestern U.S. with increasing frequency during the 1990s (Edmunds 2003). Petiole rot is difficult and expensive to control in nurseries and landscapes, since the causal fungi can survive in the soil for many years and spread from hosta to dozens of popular annuals and perennials (Edmunds 2000).
Most research on hosta petiole rot has focused on *S. rolfsii*, and behavior of *S. rolfsii* var. *delphinii* has largely been inferred from studies of *S. rolfsii*.

**Symptoms and signs**

Symptoms appear on hosta after extended periods of warm and rainy weather (Mullen 2001). *S. rolfsii* and *S. rolfsii* var. *delphinii* infect hosta at the base of petioles. The lower, outermost leaves of a crown begin to turn yellow, then brown, and wilt from the margin back toward the base. The inner, upper leaves may soon soon collapse as well. Wilted leaves can be detached easily from infected crown because the tissue at the petiole base develops a mushy texture. White, fan-shaped mycelium typically develops on infected tissue and surrounding soil along with sclerotia. Sclerotia are spherical and 1.0 to 3.5 mm in diameter; they are white when immature, turning tan and then reddish brown when mature. Sclerotia are the primary dispersal form of the pathogens, and allow them to survive prolonged periods of unfavorable conditions.

**Disease cycle**

Sclerotia of *S. rolfsii* and *S. rolfsii* var. *delphinii* are easily spread in soil attached to shoes, hand tools, vehicle tires, or machinery, or in splashing water. Long-distance movement occurs by means of sclerotia in plant material or soil during shipment (Mullen 2001). During favorable weather conditions, sclerotia resume activity by either eruptive or hyphal germination (Punja and Grogan 1981). Eruptive germination means that aggregates of white mycelium burst out of the sclerotial rind. An external food source is not required for this type of germination. Sclerotia can germinate eruptively only after being induced by dry conditions or volatile compounds (Punja and Grogan 1981). Sclerotia can germinate hyphally...
more than once. Growth of individual hyphae from sclerotia is in response to availability of exogenous nutrients (Punja and Damiani 1996).

*Infection mechanism of the pathogens*

During infection, *S. rolfsii* and *S. rolfsii* var. *delphinii* secrete oxalic acid and tissue-degrading enzymes such as cellulase (Aycock 1966; Punja and Damiani 1996). Oxalic acid is corrosive to tissues of hundreds of genera of plants (Ghaffar 1976). Oxalic acid can combine with calcium in plant tissues, removing it from association with the pectic compounds in plant cell walls, lowering cell wall pH, and thereby favoring activity of the cell wall-degrading enzymes endopolygalacturonase and cellulase (Deacon 2006). Massive mycelial growth on plant tissues produces large quantities of oxalic acid that facilitate penetration of hyphae into tissue (Aycock 1966). Oxalic acid and tissue-degrading enzymes work together to break down cell walls, resulting in tissue maceration. The pathogens then absorb nutrients from the macerated tissue (Aycock 1966). Maceration interrupts transport of water and nutrients in plant tissues, thereby causing wilting, yellowing, and necrosis (Bateman and Beer 1965).

*Comparison of the pathogens*

*S. rolfsii* infects >600 species of vegetable, grain, and ornamental crops in >100 families (Holcomb 2004; Farr 2006). On crops other than hosta, diseases incited by *S. rolfsii* are often referred to as southern blight. Over 270 host genera have been reported in the U.S. alone (Fichtner 2005; Farr 2006). *S. rolfsii* var. *dolphinii* attacks at least 27 herbaceous ornamental species worldwide (Farr 2006). While these fungi are similar with regard to life cycle and disease management, *S. rolfsii* is distinct from *S. rolfsii* var. *dolphinii* in morphology of sclerotia *in vitro*, geographic distribution, and probably host range (Harlton et
Anecdotal information suggests that *S. rolfsii* is more prevalent in relatively warm regions such as the southern U.S., Brazil, and South Africa, whereas *S. rolfsii* var. *delphinii* has been reported primarily from cooler areas such as the northern and midwestern U.S. (Aycock 1966; Farr 2006). The cause of this apparent difference in geographic distribution is not known (Aycock, 1966; Harlton et al. 1995). It is possible that differential tolerance to climatic extremes could affect the survival of *S. rolfsii* and *S. rolfsii* var. *delphinii* and thereby influence their geographic distribution, but no evidence supporting or refuting this hypothesis has been published.

**Survival of sclerotia**

Sclerotia are the primary structures that enable *S. rolfsii* and *S. rolfsii* var. *delphinii* to survive periods of unfavorable conditions. Sclerotia are formed from masses of hyphal strands. Mature sclerotia include an outer layer (rind), an underlying cortical layer, and a central medullary region comprised of loosely interwoven hyphae (Chet 1967; Deacon 2006). Sclerotia of *S. rolfsii* survived from 2 months to 7 years in field soil depending on experimental conditions (Aycock 1966).

**Temperature and moisture**

Temperature and moisture may interact in influencing survival of *S. rolfsii* under field conditions (Beute and Rodriguez-Kabana 1981). To isolate the impact of specific environmental variables, survival of sclerotia has also been studied under controlled conditions. In one such study, high moisture plus high temperature adversely affected survival of sclerotia of *S. rolfsii* (Beute and Rodriguez-Kabana 1981). Matti (1988) reported little difference in the proportion of viable sclerotia of *S. rolfsii* recovered under a range of
controlled temperature regimes (0 to 40 °C) or under moderate to low soil water holding capacity. In saturated soil, however, sclerotia lysis and mortality increased significantly, and survival was < 2 weeks. Similarly, in moist soil (0.033 MPa) survival of sclerotia was significantly lower when temperature was >20 °C than ≤20 °C, whereas no significant difference between these temperatures was observed in drier soil (-0.5 MPa).

Cycles of drying and wetting, as well as cycles of freezing and thawing, may decrease survival of sclerotia of *S. rolfsii* (Punja and Jenkins 1984). Stimulation of eruptive germination during drying following wetting was associated with rind cracking and leakage of nutrients, permitting activity of lytic microorganisms (Smith 1972). Therefore, Smith (1972) suggested attempting biological control of *S. rolfsii* through alternate wetting and drying of soil. Matti (1988) found that only 11% of sclerotia survived on the soil surface, whereas 94% survived at 10-cm soil depth, after alternating 7-day cycles of wetting and drying over 8 weeks. The relative absence of soil drying at 10-cm depth might account for greater survival than at the soil surface (Matti 1988). Unlike sclerotia of *S. cepivorum*, *Botrytis cinerea* and *B. tulipae*, sclerotia of *S. rolfsii* tend to leak large quantities of nutrients into soil after a succession of wetting and drying periods, resulting in increased microbial colonization (Coley-Smith 1974).

**Soil characteristics**

Survival of sclerotia can be affected by depth of burial in the soil. Smith et al. (1989) found that survival of *S. rolfsii* decreased when depth of burial was greater than 2.5 cm, and that survival decreased in proportion to depth of burial (Smith et al. 1989). Punja and Jenkins (1984) attributed this trend in part to increasing gravitational pressure at greater depths, which may enhance substrate leakage from sclerotia.
Soil texture and pH may also affect survival of sclerotia. For *S. sclerotiorum*, Mitchell et al. (1990) and Alexander and Stewart (1994) showed more rapid sclerotial degradation and reduced survival in soil with higher clay content and relatively low pH (~6), and lower survival in clay loam than in sandy loam. Alexander and Stewart (1994) attributed lower survival in clay loam to greater water holding capacity, which affected drying and wetting of soil, resulting in greater microbial activity. Factors such as drying, wetting, and heating that increase activity of soil microorganisms near sclerotia and predispose sclerotia to antagonism may accelerate their mortality rate (Alexander and Stewart 1994). Ubiquitous soil microorganisms such as *Trichoderma* spp., *Fusarium* spp., *Penicillium* spp. and *Aspergillus* spp. can penetrate the rind and destroy the inner sclerotial tissues (Coley-Smith 1974; Elad et al. 1984). For *Trichoderma*, this process is facilitated by production of the enzymes β–1,3 glucanase and chitinase (Elad et al. 1982).

**Impact of size of sclerotia on survival**

Alexander and Stewart (1994) found that the relatively large sclerotia of *S. rolfsii* and *Sclerotinia sclerotiorum* survived longer than the smaller sclerotia of *Sclerotinia minor* and *Sclerotium cepivorum*. They related greater durability to the fact that larger sclerotia may contain more nutrients in the cortex and thus be more resistant to prolonged periods of unfavorable conditions (Alexander and Stewart 1994; Chet 1975). Smolinska and Horbowicz (1999) documented much higher resistance to plant-derived toxic volatiles from plants by

*S. sclerotiorum* than *S. cepivorum* sclerotia, concluding that larger size of *S. sclerotiorum* sclerotia may impede penetration of volatiles into the cortex. Similarly, *Botrytis elliptica*, which has larger sclerotia than *B. cinerea* or *B. tulipae*, appeared more resistant to fungicides than sclerotia of the latter two species (Hsiang and Chastagner 1992).
Larger sclerotia size confers smaller surface to volume ratio, which means that sclerotia are less exposed to the environment, and may provide a protective effect by shielding the cortex from temperature extremes and microbial attack (Schmidt-Nielson 1984).

**Impact of source of sclerotia on survival**

In previous studies, sclerotia of *S. rolfśii* survived 2 months to 3 years in field soil (Aycock 1966), whereas those of *S. rolfśii var delphinii* survived 6 months to 2 years (Coley-Smith et al. 1974; Javed et al. 1977; Javed et al. 1973; Williams and Western 1965). It is possible, however, that conditions under which sclerotia are produced can impact their durability in survival trials. Some of the cited studies used sclerotia that were either produced on agar media or air dried before burial. Sclerotia produced from natural infections differ physiologically and structurally from those produced on agar media (Beute Rodriguez-Kabana 1981; Linderman and Gilbert 1973; Punja 1985; Punja et al. 1984; Smith et al. 1989). Sclerotia produced on nutrient-rich media also have a thicker cortex than those produced under natural conditions, which may enable sclerotia to survive for a longer period of time. Sclerotia formed under natural conditions are likely to be more adversely affected by the soil environment and to have a lower rate of survival than those formed on agar plates (Alexander and Stewart 1994; Coley-Smith et al. 1990). Linderman and Gilbert (1973) suggested that naturally produced sclerotia are more uniform in size and should be used in survival experiments.

**Summary**

Previously known only from the southern U.S., hosta petiole rot has appeared in northern states during the past 15 years. Available information suggests that
S. rolfsii var. delphinii is the predominant pathogen in the North (Harlton et al. 1995), whereas S. rolfsii is believed to be most prevalent in the southern U.S. (Aycock 1966; Harlton et al. 1995; Punja, 1996). The critical factors causing the difference in the geographic range of the two fungi are unknown. It is possible, however, that differential overwinter survival ability may have contributed to these biogeographic differences. Research on overwinter survival of S. rolfsii and S. rolfsii var. delphinii has focused primarily on S. rolfsii. Beute (1981) reported that 28 to 73% of sclerotia of S. rolfsii in soil survived up to 10 months from fall through summer in the southern states Alabama and North Carolina, respectively. In another study, sclerotia at the soil surface and buried in the soil survived overwinter at least 9 months in North Carolina and Georgia (Smith 1989). Using sclerotia produced under controlled conditions that mimicked natural infections, Edmunds and Gleason (2003) found that sclerotia of S. rolfsii var. delphinii survived 10 months in the northern U.S. (Iowa), until at least July of a subsequent growing season - long enough to have caused hosta petiole rot in the latter season.

It is unclear, however, whether S. rolfsii var. delphinii can overwinter in the southern U.S. or S. rolfsii can overwinter in the northern U.S.

Disease management

Current disease management practices

Management recommendations for diseases incited by S. rolfsii and S. rolfsii var. delphinii are similar (Edmunds, et al. 2003), and integrate a combination of strategies. One of the best ways to avoid the diseases is to exclude the pathogens, infected plants, or infested soil from a planting area. If disease has been found in a planting area,
management becomes a challenge, since the pathogens can survive as sclerotia for years (Mueller et al. 2005). Management techniques have focused on fungicide application and cultural techniques (Edmunds, et al. 2003).

**Chemical control**

Numerous fungicides inhibit the germination of sclerotia or the mycelial growth of *S. rolfsii* (Punja et al. 1982). The soil fungicide pentachloronitrobenzene (PCNB) has been used on peanuts and some other crops since the 1940s. A relatively new fungicide, flutolanil, is also registered for use against hosta petiole rot (Edmunds 2000). However, applying fungicides to soil may not be practical or cost effective in many landscape situations, and some fungicides, such as PCNBs, are suspected human carcinogens. Also, fungicide effectiveness is not always consistent from year to year (Mullen 2001). Furthermore, fungal isolates that were tolerant to PCNB were reported in some Texas peanut fields (Shem et al. 1998).

**Cultural practices**

Cultural management techniques against *S. rolfsii* and *S. rolfsii var. delphinii* include excluding the pathogen from an area, soil removal and replacement, and roguing of infected plants (Mullen 2001). In some situations, deep plowing may be done to prevent sclerotia from contacting plant tissues (Mullen 2001). In warm and humid regions, such as the southern U.S., soil solarization has been effective in control of *S. rolfsii* (Hagan 2004). Incorporating organic amendments such as compost, oat or corn straw, and cotton-gin trash reduced the incidence of southern blight and also enhanced populations of beneficial soil microbes (Bulluck and Ristaino 2002). Neem oil and pine bark extracts or pine bark powder also has reduced growth of *S. rolfsii* (Kokalis-Burelle and Rodriguez-Kabana 1994).
Although *S. rolfsii* and *S. rolfsii var. delphinii* have wide host ranges, rotating to non-host crops such as corn, cotton, or switchgrass was reported to reduce disease incidence (Rodriguez-Kabana et al. 1994). Other cultural practices that suppressed *S. rolfsii* growth include adjusting the soil pH to about 6.5 by adding lime (Bulluck and Ristaino, 2002) and aerification of the soil (Mullen 2001). Some cultural practices, such as soil replacement and solarization, are labor-intensive and expensive, and can be impractical in many commercial settings (Edmunds et al. 2003).

**Biological control**

Biological control of *S. rolfsii* has been attempted using bacteria (*Bacillus subtilis*), actinomycetes, a mycorrhizal fungus, and certain *Trichoderma* species (Punja 1985). Many studies have shown control of *S. rolfsii* by biological agents in laboratory and greenhouse tests, but disease control was less effective in the field (Cattalan et al. 1999; Tsahouridou and Thanassoulopoulos 2002). Biological control agents have not been evaluated for hosta petiole rot control (Mullen 2001).

**Resistance breeding**

The use of resistant varieties or cultivars is a potentially preferable method of disease management (Mullen 2001). Identifying cultivars with high levels of resistance to petiole rot would benefit breeders, growers, marketers, and hosta enthusiasts by providing a durable, cost-effective option to combat this difficult-to-manage disease. Preliminary results from recent laboratory studies with transgenic carrots indicated reduced susceptibility to *S. rolfsii* (Punja 2005). Similarly, transgenic hosta resistance could supplement conventional breeding methods to fight the disease in the future.
Cultivar differences in resistance to *S. rolfsii* have been noted for several host species, including peanut, peppers, soybean, cowpea, apple, and potato (Aycock 1966; Branch and Brenneman 1999; Fery et al. 2002). Information on levels of genetic resistance to *S. rolfsii* and *S. rolfsii* var. *delphinii* among ornamental plants, including hosta, was almost nonexistent before Edmunds et al. (2003) completed a greenhouse evaluation of 18 hosta cultivars. In this study, disease development varied significantly among cultivars. Overall, ‘Lemon Lime’, ‘Munchkin’, ‘Nakaiana’, ‘Platinum Tiara’, and ‘Tardiflora’ showed severe symptoms, whereas ‘Halcyon’ and ‘Zounds’ remained much healthier. The mechanism of resistance is unknown, although breaking down or inhibiting penetration of oxalic acid and tissue-degrading enzymes could reduce the pathogenicity of *S. rolfsii* (Punja 1985).

Developing a rapid screening technique could make it cost-effective to screen hundreds of hosta cultivars for petiole rot resistance. This step would pave the way for breeders to incorporate petiole rot resistance into popular commercial cultivars.

Although little resistance screening work has been done with *S. rolfsii* and *S. rolfsii* var. *delphinii*, several techniques have been used to identify resistance to *Sclerotinia sclerotiorum*, the fungus that causes white mold in soybean, sunflower, and common bean. *S. sclerotiorum*, like *S. rolfsii* and *S. rolfsii* var. *delphinii*, releases oxalic acid during infection. Screening techniques for *S. sclerotiorum* include detached leaf inoculation, an oxalate resistance test, and stem inoculation of intact seedlings in controlled environments (Hunter et al. 1981, Kolkman and Kelly 2000, Kull et al. 2003; Nelson et al. 1991, Sedun and Brown 1989; Wegulo et al. 1998, Zhao et al. 2004). Compared to resistance evaluations on intact plants in the field or greenhouse, a detached tissue assay has several potential advantages. For example, a detached tissue assay could yield results in a relatively short time.
– a few days compared to months for intact plants. Furthermore, assays performed on detached tissues require much less space than those using intact plants.

Most detached-tissue inoculation techniques utilize wounded host tissue. Researchers used a pipette tip method developed for screening canola seedlings (del Rio 2001). In evaluating oilseed rape resistance to *S. sclerotiorum*, Zhao et al. (2004) forced the cut stub of a petiole through an agar plug containing mycelium at the end of a 1-ml pipette tip. Wegulo et al. (1998) developed an oxalic acid test to screen soybean resistance to stem rot caused by *S. sclerotiorum* in which the excised ends of soybean petioles were placed in test tubes containing 40 mM oxalic acid in a growth chamber. Kolkman and Kelly (2000) confirmed that an oxalic acid assay was useful for determining resistance of common bean resistance to white mold caused by *S. sclerotiorum*. A detached shoot technique was developed to evaluate the reaction of soybean to *S. rolfsii*: after individual soybean shoots supported by a cotton plug were immersed in Hoagland’s solution, each shoot was inoculated by placing a *S. rolfsii*-infested mycelium plug adjacent to leaf axils (Akem 1991). Results correlated well with field screening data. Results of these quick and simple techniques do not always match field results, however. Nelson et al. (1991) found no correlation between field evaluations of resistance in 15 soybean cultivars to *S. sclerotiorum* and laboratory evaluations (inoculating cut stems with tissue paper infested with *S. sclerotiorum*) of the same cultivars. No rapid, simple techniques have been developed for screening hosta for resistance to *S. rolfsii* or *S. rolfsii* var. *delphinii*. In greenhouse trials, Edmunds et al. (2003) inoculated whole plants with carrot disks infested with mycelium of *S. rolfsii* var. *delphinii* at the base of each plant; this assay required 18 weeks to complete.
In a preliminary trial, Koehler et al. (2003) adapted an oxalic acid test using excised leaves, originally developed for screening soybean resistance to *S. sclerotiorum* (Wegulo et al. 1998), screening for hosta petiole rot resistance. However, the results did not correlate with cultivar differences in resistance that were determined by whole-plant inoculations (Edmund et al. 2003).

Development of a rapid and simple screening technique would accelerate screening hundreds of hosta cultivars for petiole rot resistance, and thereby help nurseries, wholesalers, and retailers to minimize petiole rot losses, increase sales, and preserve hosta’s reputation as a trouble-free perennial.

**Phylogenetic relationships among plant-pathogenic Sclerotium species**

The name *Sclerotium* was first introduced by Tode (1790) to describe eight species. In 1821, Fries accepted the genus *Sclerotium* but listed it as *Sclerotium* Tod. The genus *Sclerotium Tod.* was sanctioned by Fries in 1822 and the first-listed species *Sclerotium complanatum* was suggested as the type species for *Sclerotium* “Tode” by Clements and Shear (1973). It is now agreed that this type species belongs to *Typhula phacorrhiza* Reichard ex Fr., a basidiomycota genus that produces basidiocarps from sclerotia (Remsberg 1940). Fungi in this genus formed sclerotia and sterile mycelia (Saccardo 1899; Punja 2001). There are more than 40 plant-pathogenic *Sclerotium* species (Farr 2006). Many *Sclerotium* species do not or rarely reproduce sexually and are known only from their asexual stage (Kohn 2004; Punja 1988; Punja and Rahe 2001). Based on the taxonomically correct position of the type species of the genus, some *Sclerotium* species in the genus were assigned to the Basidiomycota (Kirk, CABI, UK, personal communication).
Some species in the genus *Sclerotium* were confirmed to have Basidiomycete affinities (Tu and Kimbrough 1978; Punter 1984). For example, *Sclerotium rolfsii*, the best known species in this genus, has the teleomorph *Athelia rolfsii* (Curzi) Tu and Kimbrough (1978). Genus *Athelia* forms resupinate hymenia and has hyphal strands emerging from sclerotia (Tu and Kimbrough 1978).

Some *Sclerotium* species were known to be related to *Rhizoctonia* species. *Rhizoctonia* D.C., a basidiomycetous genus forming sclerotia and sterile mycelia, was first described by de Candolle (1815) and accepted by Fries in 1821 (Donk 1962). The most well-known teleomorph genera connected with *Rhizoctonia* anamorphs include *Ceratobasidium* D.P. Rogers and *Thanatephorus* Donk. Binucleate *Rhizoctonia* species have a *Ceratobasidium* teleomorph, whereas multinucleate *R. solani* have a *Thanatephorus* teleomorph. The two teleomorph species also differ in the shape of their basidia (Staplers, 1996).

Although no sexual stage is known for *S. cepivorum*, based on similarity of the sclerotia to those of in the ascomycetous *Stromatinia gladioli* (Drayt.) Whetzel, *S. cepivorum* was transferred into genus *Stromatinia* Boud. by Whetzel (Whetzel 1945). *Stromatinia* species form two kinds of sclerotia, “apothecia arising from a thin, black, subcuticular, effuse sclerotium covering or manteling, or small black sphaerules borne free on the mycelium and not giving rise to apothecia” (Whetzel 1945). However, *Stromatinia cepivorum* form only the latter sclerotia.

Descriptions of seven species for which cultures were obtainable from The Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, are as follows:
Sclerotium rolfsii is the best known species in this genus. The first published report of losses due to this pathogen in the U.S. was made by Rolfs in 1892 on tomato in Florida (Aycock 1966). The sexual state of *S. rolfsii* is *Athelia rolfsii* (Curzi) Tu and Kimbrough (Tu and Kimbrough 1978; Punja 1988). The biology, ecology and management of *S. rolfsii* have been studied extensively during the ensuing 116 years (Aycock 1964; Punja 1985; Punja and Sun 2001; Mullen 2001). However, we still lack a through understanding of basic biology, mechanisms of host-pathogen interaction, and genetic relationship with other *Sclerotium* species.

The fungus believed to be most closely related to *S. rolfsii* is *S. rolfsii* var. *delphinii* (Punja and Damiani 1996). *S. rolfsii* var. *delphinii*, isolated from diseased delphinium in New York, Pennsylvania, Indiana and New Jersey, was initially classified as *S. delphinii* (Welch 1924). The binomial was changed to *S. rolfsii* var. *delphinii* by Boerema and Hamers (1988).

The taxonomic relationship between *S. rolfsii* and *S. rolfsii* var. *delphinii* remains uncertain, however. *S. rolfsii* differed from *S. rolfsii* var. *delphinii* in optimal *in vitro* temperature for sclerotia production (35 and 20 °C, respectively) (Punja and Damiani 1996). These researchers also found that *S. rolfsii* mycelium grew most rapidly on V8 agar, whereas *S. rolfsii* var. *delphinii* grew most rapidly on PDA. The diameter of *S. rolfsii* sclerotia produced in culture (0.5 to 1.0 mm) was smaller than that of *S. rolfsii* var. *delphinii* (1.2 to 3.5 mm) (Punja and Damiani 1996). The two fungi probably also differ in host range (approximately 600 host species for *S. rolfsii* vs. 27 host species for *S. rolfsii* var. *delphinii* (Farr 2006), although comparative host range trials have not been reported. On PDA, a
basidial stage (*Athelia rolfsii*) could be induced in *S. rolfsii* but not in *S. rolfsii var. delphinii* (Punja and Damiani 1996).

Degrees of inter- and intraspecific variation in nuclear rDNA [internal transcribed spacer (ITS) region] have been analyzed to determine the phylogenetic relationship between these fungi. The size of the ITS region for each fungus is the same: about 720 bp. The RFLP patterns of the ITS region digested with AluI, HpaII, RsaI, and MboI and sequence information of the ITS region supported the view that *S. rolfsii var. delphinii* should be designated as a subspecific variety of *S. rolfsii* (Harlton 1995). Genetic diversity among isolates of *S. rolfsii var. delphinii* was less than that observed in *S. rolfsii* by using mycelial compatibility groups and RAPD analysis (Punja and Sun 2001). Okabe and Matsumoto (2003) cloned isolates of both fungi and sequenced three different ITS types: r1, r2, and r3. Japanese strains and one U.S. strain of *S. rolfsii* contained types r-1 and r-2, whereas another U.S. strain and one from Chile had only one ITS type, r-2. In contrast, *S. delphinii* (the name used by these authors rather than *S. rolfsii var. delphinii*) strains had types r-1 and r-3. Okabe and Matsumoto (2003) argued that hybridization between *S. rolfsii* and *S. delphinii* was possible, although it was not observed. Further studies using molecular markers are needed to clarify the evolutionary history and taxonomic status of both fungi.

*Sclerotium cepivorum*

Known as the pathogen of white rot of onions (*Allium cepa* L.), *Sclerotium cepivorum* also causes disease on chives, garlic, leek, shallot, and other species of *Allium*. White rot of onion is the most economically devastating disease of onions worldwide (Happer et al. 2002).
S. cepivorum was first described by Berkeley (Saccardo 1899; Whetzel 1945). Although no teleomorph is known, based on similarity of the sclerotia for those seen in the ascomycetous Stromatinia gladioli (Drayt.) Whetzel, S. cepivorum was transferred into genus Stromatinia Boud. by Whetzel (1945). Sequence analysis suggested that S. cepivorum seems closely related to family Sclerotiniaceae (Carbone and Kohn 1993; Holst-Jensen et al. 1998).

Sclerotium coffeicola

The fungus was first isolated from an infected coffee plant by Stahel in Surinam (Petrak 1921). A comparative study of S. coffeicola, S. rolfsii, and S. delphinii focusing on colony characteristics, sclerotial formation, growth response to different temperature and media, and ability to produce oxalic acid and pectinase enzymes was done by Punja and Damiani (1996). The result showed that the three species are closely related, but can be differentiated by the parameters measured. S. coffeicola belongs to phylum Basidiomycota because of the presence of clamp connections (Punja and Damiani 1996).

Sclerotium denigrans

S. denigrans causes dying-off of lily of the valley (Convallaria majalis) and was first isolated by Pape in Germany (Boerema and Hamers 1988). Information on S. denigrans is almost non-existent. Kirk (2004) assumed that it belongs to Basidiomycota.

Sclerotium hydrophilum

First described by Saccardo (1899), S. hydrophilum has been reported on wild rice (Zizania aquatica), rice (Oryza sativa), fragrant waterlilies (Nymphaea odorata), and Eurasian water milfoil (Myriophyllum spicatum). It has been suggested that S. hydrophilum might be a potential biological control agent of undesirable aquatic plants, including M.
spicatum (Hausner 1999). Evidence suggesting that it belongs to Basidiomycota includes the presence of a well-defined dolipore septum (Punter et al. 1984).

_Cerarhiza oryzae-sativae_ (Basionym: _S. oryzae-sativae_)

_S. oryzae-sativae_, first described by Sadawa (1922) in Japan, causes sheath rot of rice (_Oryza sativa_). _S. oryzae-sativae_ was first transferred to _R. oryzae-sativae_ (Sawada) Mordue and then _R. oryzae-sativae_ was renamed as _Cerarhiza oryzae-sativar_ (Sawade) Moore, comb.nov. (Moore 1987; Stalpers and Andersen 1996). _Cerarhiza oryzae-sativae_ often occurs in association with _S. hydrophilum_ on _Zizania_ as well as _Oryza_ (Punter et al. 1984).

_Sclerotium perniciosum_

The causal organism of smoulder disease of tulip, _S. perniciosum_ was first described in 1925 and has also been recorded as a pathogen of _Fritillaria_ and _Allium_ (Boerema and Hamers 1988). The lack of any form of fructification makes it difficult to classify. Disease symptoms in the field strongly resemble those of _Sclerotium tuliparum_, which belongs to Basidiomycota (Boerema and Hamers 1988). As long as no spores are produced, the fungus was considered to be a 'Mycelium sterile' in the genus _Sclerotium_ and given the preliminary name _Sclerotium perniciosum_ nov. spec. (Petrak 1930).

_Sclerotium rhizodes_

The fungus was first isolated from reed canary grass (_Phalaris arundinacea_) by Auerswald in German (Saccardo 1899). Kirk (2004) suggested that it belongs to Basidiomycota.

Although sporadic reports on morphological features of sclerotia and the characteristics of colonies have been published, these _Sclerotium_ species have not been studied extensively, and no comparative studies include all of them. Furthermore, little is
known about the variation among this group of fungi in terms of morphology, physiology, and phylogenetic relationship. A study is needed in order to clarify the taxonomic placement of plant-pathogenic *Sclerotium* species. Clarifying taxonomic status among *Sclerotium* species could pave the way for more effective management of the diseases they incite. Disease management strategies are often similar within genera, but differ considerably among classes (Kohn, 2004). If some *Sclerotium* species incorrectly classified as Basidiomycota are in fact Ascomycota, for example, management of disease they cause could have been compromised by applying fungicides that are active primarily against Basidiomycota (Kohn, 2004). Moreover, if some current *Sclerotium* species are actually in the phylum Ascomycota, they need to be renamed.

Several regions of ribosomal DNA, including the ITS region, large subunit, and small subunit regions, are often used in taxonomy and molecular phylogeny studies because they are comparatively easy to amplify even from small quantities of DNA, and they show distinct evolution rates among species, family, or even higher levels in fungal systematic hierarchy (Kendrick, 2001). As a result, regions of ribosomal DNA can provide information about almost any systematic level.

**Research Rationale**

Hostas are the most popular herbaceous perennials in the U.S. Hosta petiole rot, once thought to be restricted to the southern U.S. but increasingly common in the Midwest during the past decade, has caused increasing concern among hosta breeders, producers, and gardeners. Hosta petiole rot is difficult to manage mainly because the causal agents, *S. rolfsii* and *S. rolfsii* var. *delphinii*, have resistant structures for survival and relatively wide host
ranges. Research on hosta petiole rot has been minimal. No studies have focused on genetic variation between *S. rolfsii* and *S. rolfsii* var. *delphinii*, nor among other species within the genus *Sclerotium*, and the taxonomic placement of several *Sclerotium* species is unclear. For these reasons, this dissertation compared overwinter survival of *S. rolfsii* and *S. rolfsii* var. *delphinii*, developed new methods to screen for resistance to hosta petiole rot, and investigated phylogenetic relationships among plant-pathogenic *Sclerotium* species.

Anecdotal information suggests that *S. rolfsii* is more prevalent in relatively warm geographic regions, whereas *S. rolfsii* var. *delphinii* has been reported primarily from cooler zones. The cause of this apparent difference in geographic distribution is not known. It is possible that regional differences in winter temperatures could affect the survival of *S. rolfsii* and *S. rolfsii* var. *delphinii* and influence their geographic distribution, but no evidence supporting or refuting this hypothesis has been published.

A whole-plant greenhouse screening technique that identified hosta cultivars with high levels of resistance to *S. rolfsii* var. *delphinii* (Edmunds and Gleason, 2003) was too time-consuming (3 months) and labor-intensive to screen large numbers of cultivars. Development of a rapid resistance screening method could dramatically speed up identification of resistant germplasm and ultimately help hosta breeders, growers, and marketers to manage petiole rot more effectively.

For most *Sclerotium* species, taxonomic status remains uncertain. Little is known about the variation within this group of fungi in terms of morphology and phylogenetic relationships. A study is needed in order to clarify the taxonomic placement, particularly on the genus level, of plant-pathogenic *Sclerotium* species. Clarifying taxonomic status among
*Sclerotium* species could pave the way for more effective management of the diseases they incite.

**Research Objectives**

The objectives of the present research were to: 1) Compare the survival of sclerotia of *S. rolfsii* and *S. rolfsii* var. *delphinii* in the northern and southeastern U.S.; 2) develop a rapid method to assess resistance among hosta cultivars to petiole rot caused by *S. rolfsii* var. *delphinii*; and 3) reassess taxonomic status of eight plant-pathogenic fungi assigned to the genus *Sclerotium*, based on DNA sequence analysis and morphological features.

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CHAPTER 2. OVERWINTERING OF SCLEROTIUM ROLFSII AND S. ROLFSII VAR. DELPHINII IN DIFFERENT LATITUDES OF THE UNITED STATES

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**ABSTRACT**

Previously known only from the southern U.S., hosta petiole rot recently appeared in the northern U.S. *Sclerotium rolfsii* var. *delphinii* is believed to be the predominant petiole rot pathogen in the northern U.S., whereas *S. rolfsii* is most prevalent in the southern U.S. In order to test the hypothesis that different tolerance to climate extremes affects the geographic distribution of these fungi, the survival of *S. rolfsii* and *S. rolfsii* var. *delphinii* in the northern and southeastern U.S. was investigated. At each of four locations, nylon screen bags containing sclerotia were placed on the surface of bare soil and at 20-cm depth. Sclerotia were recovered six times from November 2005 to July 2006 in North Dakota and Iowa, and from December 2005 to August 2006 in North Carolina and Georgia. Survival was estimated by quantifying percentage of sclerotium survival on carrot agar. Sclerotia of *S. rolfsii* var. *delphinii* survived until at least late July in all four states. In contrast, no *S. rolfsii* sclerotia survived until June in North Dakota or Iowa, whereas 18.5% survived until August in North Carolina and 10.3% survived in Georgia. The results suggest that inability to tolerate low temperature extremes limits the northern range of *S. rolfsii*

*Additional keywords: Ornamental crops, Hosta spp.*

**INTRODUCTION**

Petiole rot, caused by *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, has caused significant economic losses to hosta (*Hosta* spp.) plantings in the U.S. Previously known only from the southern U.S. (15, 18, 23), hosta petiole rot had been reported in the northern U.S. with increasing frequency during the past decade (7).
In the northern U.S., hosta petiole rot appears in mid- to late June or July after extended periods of warm, wet weather (15). *S. rolfsii* and *S. rolfsii var. delphinii* infect hosta at the base of the petiole, causing brown and macerated lesions. Leaves may turn yellow and wilt. White, fan-shaped mycelia and sclerotia can be seen on infected tissue and surrounding soil.

*S. rolfsii* infects more than 600 species of vegetable, grain, and ornamental crops in > 100 families (8). Over 270 host genera have been reported in the U.S. alone (8).

*S. rolfsii var. delphinii* attacks at least 27 herbaceous ornamental species worldwide (8).

While these fungi are similar with regard to life cycle and disease management, *S. rolfsii* is distinct from *S. rolfsii var. delphinii* in morphology of sclerotia cultured in vitro, geographic distribution, and probably in host range (19). Anecdotal information suggests that *S. rolfsii* is more prevalent in relatively warm areas such as the southern U.S., Brazil, and South Africa, whereas *S. rolfsii var. delphinii* has been reported primarily from cooler areas such as the northern and midwestern U.S. (2, 8, 19). The cause of this apparent difference in geographic distribution is not known. It is possible that regional differences in temperature could affect the survival of *S. rolfsii* and *S. rolfsii var. delphinii* and thereby influence their geographic distribution, but no evidence supporting or refuting this hypothesis has been published.

Research on overwinter survival of these fungi has focused primarily on *S. rolfsii* and has shown that *S. rolfsii* survival was influenced by both location and environmental conditions (1, 2, 25). Sclerotia survived in the soil from a few months to several years. Beute and Rodriguez-Kabana (3) reported that 28 to 73% of sclerotia of *S. rolfsii* in soil survived up to 10 months in Alabama and North Carolina. In another study, sclerotia at the soil surface and buried in field soil survived at least 9 months in North Carolina and Georgia (22). In the
United Kingdom, *S. rolfsii* var. *delphinii* was reported to survive in soil for 6 months to 2 years (10, 27). Some studies used sclerotia that were produced on agar media; and, such sclerotia are morphologically and physiologically distinct from sclerotia produced by natural infections (12, 18, 20, 22). Using sclerotia produced under controlled conditions that mimicked natural infections, Edmunds and Gleason (7) found that sclerotia of *S. rolfsii* var. *delphinii* survived 10 months in Iowa, until at least July of a subsequent growing season - long enough to have caused hosta petiole rot in the latter season. It is unclear, however, whether *S. rolfsii* var. *delphinii* can overwinter in the southern U.S. or *S. rolfsii* can overwinter in the northern U.S.

Abiotic and biotic external factors can influence the survival of both fungi (2, 18). Larger size, and consequently smaller surface to volume ratio, has been shown to increase survival of sclerotia of *Sclerotium spp* (1). Although sclerotia of *S. rolfsii* var. *delphinii* are larger than those of *S. rolfsii* in agar plate culture, it is not known whether sclerotia of these fungi differ in size when produced under more natural conditions in the soil or on crop debris. If a size difference exists, it could be related to overwinter survival ability in a manner similar to that of other sclerotial fungi (1).

In this study we tested the overwintering survival of *S. rolfsii* and *S. rolfsii* var. *delphinii* in different latitudes of the United States. This information is critical to test the hypothesis that overwinter survival differentially affects the geographic distribution of these fungi. Furthermore, knowledge on overwinter survival will shed new light on the ecology of both fungi, and could thereby improve our ability to manage hosta petiole rot and other diseases incited by these fungi. The objective of this study was to
compare the survival of sclerotia of *S. rolfsii* and *S. rolfsii* var. *delphinii* in the northern and southeastern U.S.

**MATERIALS AND METHODS**

**Isolates.** Three isolates of *S. rolfsii* var. *delphinii* (Srd 1, obtained from an infected hosta (*Hosta* spp.) plant in Ankeny, IA; Srd 2, obtained from an infected hosta plant in Ames, IA; and Srd 3, isolated from an infected iris (*Iris* spp.) in New Brunswick, Canada) and three isolates of *S. rolfsii* from Georgia (GA0006, designated here as Sr 1, isolated from an infected peanut (*Arachis hypogaea*) plant; GA03001 (Sr 2), isolated from an infected watermelon (*Citrullus lanatus*); and GA0009 (Sr 3), isolated from an infected peanut plant) were used in the study. Cultures were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) in 9-cm-diameter petri dishes under a daily regime of 8 h incandescent light and 16 h dark at room temperature (20 to 23 °C).

**Production of sclerotia.** A single sclerotium of each isolate was placed on PDA in a 9-cm-diameter petri dish and incubated at 25°C under continuous incandescent light for 4 days. In order to mimic natural conditions for sclerotia production as closely as possible, 9-mm-diameter mycelial plugs from the edge of actively growing cultures on PDA were transferred onto cotton strips (30 cm long, 2.5 cm wide, 1 cm thick) that had been placed on a 3-cm-deep layer of sterilized moist sand in plastic crispers (7). Lids were then loosely placed on the crispers, and the crispers were incubated in dew chambers (Percival Inc., Perry, IA) at 27°C and 100% RH with a 12 h light/12h dark regime. After 2 weeks, sclerotia that had formed on the cotton strips were brushed into a glass petri dish and stored in a sealed plastic crisper at room temperature.
Germination of sclerotia was tested initially on 20 Oct 2005. For each isolate of *S. rolfsii* var. *delphinii* (Srd 1 and Srd 2) and *S. rolfsii* (Sr 1 and Sr 2), 100 sclerotia were assessed. Viability of sclerotia was evaluated on Bacto-agar amended with carrot puree, tetracycline hydrochloride (150 mg/liter), and streptomycin sulfate (150 mg/liter) (carrot agar) (7). Carrot puree was prepared 3 weeks in advance by blending twice-autoclaved carrots at high speed for 30 sec, dividing the puree into 200-ml plastic beakers, and freezing them. Carrot agar was prepared 1 day before plating.

**Field experiment.** Bags to contain sclerotia were made from 0.5-mm-mesh nylon screen by sewing together two 15×15 cm squares with polyester thread along three sides. Twenty-five sclerotia of an isolate were placed in each bag and the open end of the bag was then sewed shut. The nylon bags containing sclerotia were subsequently stored in plastic bags in a crisper at 25°C for 1 week before deployment in the field.

In 2005, 192 bags were placed at each site (two fungi × two isolates per fungus [Srd1, Srd2, Sr1, and Sr2] × two soil depths [surface and 20 cm] × six sampling times × four replications per sampling time). Paired bags (surface and 20 cm depth) were deployed approximately 1m apart in a randomized complete block design with 4 replications (block). Bag were placed on the soil surface or buried at 20-cm depth individually. Location and deployment dates were as follows: 24 Oct at the North Dakota State University Agricultural Experiment Station, Fargo, North Dakota (N 46.900°, W 96.819°) (The site had dry edible bean (*Phaseolus vulgaris*) in 2005 and soybean (*Glycine max*) in 2004), 26 Oct at the Iowa State University Horticulture Station, Gilbert, Iowa (N 42.109°, W 93.589°) (The site had no crops planted before), 3 Nov at North Carolina State University, Central Crops Research Station, Clayton, North Carolina (N 35.670°, W 78.493°) (The site had tobacco
(Nicotiana spp.) two years before the experiment was started and was fallow the year before the experiment was started), and 12 Nov at the Dempsey Research Farm, Griffin-Dempsey, Georgia (N 33.251°, W 84.299°) (The site had been planted with daylily (Hemerocallis spp.) for two years prior to the experiment). Bags on the soil surface were secured by landscape staples. The soil types at each location were Silty clay (Fine, smectitic, frigid Typic Epiaquerts) with 40-60% clay, 40-60% clay, and <15% sand, Storden loam (Fine-loamy, mixed, superactive, mesic Typic Eutrudepts) with 18-27% clay, 63-82% silt, and 35-40% sand, Fuquay sand (loamy, siliceous, thermic, arenic plinthic Kandiudult) with 5-18% clay, 80-100% silt, and 55-85% sand], and Cecil sandy clay loam (clay, Kaolinitic, thermic Typic Hapludult) with 25-35% clay, 63-80% silt, and 45-100% sand, respectively.

Assessment of survival. At each sampling time, four bags were retrieved from each depth at each location, placed in insulated chests, and delivered to Iowa State University (ISU). At ISU bags were rinsed under running tap water until they were free of adhering soil. In a laminar-flow hood, bags with sclerotia were surface sterilized in 10% NaOCl for 90 s, in 95% alcohol for 30 s, rinsed for 1 min with sterilized deonized water, rinsed again for 1 min with sterilized deonized water, and then blotted dry between paper towels. After bags were cut open, sclerotia were removed with sterilized forceps and placed in an empty sterile petri dish.

Sclerotia were transferred from the petri dish five at a time, placed equidistant from each other on the surface of a carrot agar plate, and incubated under continuous light at 25°C. A sclerotium was scored as viable if it produced a characteristic white fan-like mycelium in culture after 48 h of incubation. Sclerotia that did not germinate after 48 h were transferred to
a new carrot agar plate, incubated for an additional 48 h, and then re-rated for viability as described above.

**Evaluation of sclerotia size.** One hundred sclerotia of each of three isolates of *S. rolfsii* var. *delphinii* (Srd 1, Srd 2, and Srd 3), and three isolates of *S. rolfsii* from Georgia (Sr 1, Sr 2, and Sr 3) were produced as described above and collected in petri dishes. Sclerotia and an adjacent ruler were photographed under a dissecting microscope. In Photoshop 7.0 (Adobe Inc, CA), diameter of sclerotia in the digital images was measured as pixel size, which was calibrated against pixel size of the ruler image.

**Weather data.** Average daily air temperature (1 to 2 m above the ground) and soil temperature (20-cm depth), based on hourly measurements at automated weather stations, were downloaded from the North Dakota Agricultural Weather Network (Fargo), the Iowa Agriculture Climate Network (ISU Agronomy Farm Station, Ames), the State Climate Office of North Carolina (Central Crops Research Station, Clayton), and the Georgia Automated Environmental Monitoring Network (Griffin) during the entire exposure period for sclerotia. All weather stations were located within 30 km of the field experiment sites.

**Data analysis.** The percentage of viable sclerotia remaining in the bags at the soil surface and at 20-cm depth was calculated for each location and sampling time. Sclerotium survival was determined by counting the number of viable sclerotia and dividing by 25, the original number of sclerotia buried. Data were analyzed using the SAS PROC Mixed procedure (SAS Institute, Cary, NC). Data for percentage of viable sclerotia were subjected to arcsin transformation in order to meet ANOVA assumptions of normality. Isolates of each fungus were treated as a random factor. Treatment means were compared using the Tukey
studentized range multiple comparison adjustment ($P<0.05$). The apparent daily death rate of sclerotia was calculated as follows:

$$r=(-1/\Delta t)\ln(N_t/N_{t-1})$$

in which $\Delta t$=time increment (days) between two sampling dates; $N_t$=percentage of viable sclerotia at sampling date $t$; and $N_{t-1}$= percentage of viable sclerotia at sampling date $t-1$ (24). Data for evaluation of sclerotia size were analyzed using the SAS PROC GLM procedure (SAS Institute, Cary, NC).

RESULTS

Some sclerotia of *S. rolfsii* var. *delphinii* survived until at least July in all four states (Fig. 1). In contrast, no *S. rolfsii* sclerotia survived until June in either North Dakota or Iowa, whereas 18.5% survived until August in North Carolina and 10.3% in Georgia.

*S. rolfsii* var. *delphinii* had a significantly ($P<0.05$) higher percentage of survival than *S. rolfsii* in the northern states on most sampling dates, whereas no clear differences between the fungi were observed in the southeastern states.

From May through July, there was no statistical difference in percentage of viable sclerotia of either fungus at the soil surface compared with the 20-cm depth at any location (Fig. 1). In general, sclerotia survival for both species was slightly greater at 20-cm soil depth than at the soil surface.

In North Dakota and Iowa, apparent daily death rate ($r$) of *S. rolfsii* sclerotia increased sharply in the spring, whereas the increase for *S. rolfsii* var. *delphinii* was less pronounced (Fig. 2 A-D). For *S. rolfsii* the maximum values of $r$ were 0.178 (soil surface) and 0.197 (20-cm depth) in North Dakota, and 0.12 (soil surface) and 0.243 (20-cm depth) in Iowa, whereas for *S. rolfsii* var. *delphinii*, the maximum values of $r$ were 0.043 (soil surface) and 0.04 (20-cm depth).
cm depth) in North Dakota, and 0.021 (soil surface) and 0.05 (20-cm depth) in Iowa. In North Carolina and Georgia, apparent daily death rate showed no sharp increase from April to June for either fungus, and there was little difference in rates between the fungi (Fig. 2 E-H).

In North Dakota, average daily air temperature dropped as low as –17 °C and –19 °C in December and March, respectively (Fig. 3 A). Similarly, air temperature in Iowa fell as low as –19 °C in early December and –20 °C in late February, respectively (Fig. 3 C). In contrast, minimum average daily air temperature in North Carolina was –2 °C in the middle of December and always exceeded 0 °C in Georgia (Fig. 3 E and G). In North Dakota and Iowa, average daily soil temperature (20-cm depth) was 0 °C or below throughout the winter and early spring, but seldom fell below 5° C in North Carolina or Georgia (Fig. 3 B, D, F and H).

Average diameter of sclerotia of three isolates of *S. rolfsii* var. *delphinii* (1.52 mm) was 63% larger than that of *S. rolfsii* (0.93 mm) (Table 1).

**DISCUSSION**

The findings are the first evidence that *S. rolfsii* may be unable to overwinter long enough in the northern U.S. to persist as a pathogen of hosta or other crops from year to year. In contrast, survival of *S. rolfsii* var. *delphinii* in the northern U.S. until at least June (long enough for infection and petiole rot to occur) was consistent with findings of Edmunds and Gleason (7). A possible explanation for the apparent predominance of *S. rolfsii* var. *delphinii* as the hosta petiole rot pathogen in the North may be inability of *S. rolfsii* sclerotia to survive
in this region until June, when the onset of warmer temperatures increases the risk of petiole rot.

In North Dakota and Iowa, lower average daily air and soil temperature in winter corresponded with lower survival of *S. rolfsii* during late winter and than in North Carolina and Georgia. The apparent absence of *S. rolfsii* in areas with severe winters may be due to its inability to tolerate low temperatures (2). In contrast, sclerotia of *S. rolfsii* var. *delphini* appeared to tolerate severe winter temperatures based on our results.

We also found that both *S. rolfsii* and *S. rolfsii* var. *delphini* can survive until at least August in the southeastern states. This is the first quantified report of overwinter survival of *S. rolfsii* var. *delphini* in the southern U.S. The apparent absence of *S. rolfsii* var. *delphini* in the southeastern states, however, is not explained by our results. It is possible that other factors may limit distribution and dispersal of *S. rolfsii* var. *delphini* in this region. In addition to differential overwinter survival, the two fungi differ in optimum growth temperature. Punja and Daminai (19) found that *S. rolfsii* had a higher optimum temperature for sclerotial production than *S. rolfsii* var. *delphini*, which may favor prevalence of *S. rolfsii* in warmer regions. It is also possible that *S. rolfsii* var. *delphini* occurs in the South but has been misidentified as *S. rolfsii*. The fact that disease symptoms and signs of the two pathogens (mycelia and sclerotia) *in vivo* may be indistinguishable to the naked eye make this a plausible possibility.

Colonization of sclerotia by *Fusarium oxysporum*, *F. tricinctum*, *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp. and other fungi, as well as empty rinds of sclerotia, were common in May or June at all locations (Xu, unpublished data). For both fungi, the coincidence of increasing apparent daily death rate of sclerotia in the spring or
early summer with rising temperatures suggests that mortality was associated with an upsurge in colonization by soil microbiota (18).

Overwinter survival of *S. rolfsii* sclerotia in North Carolina and Georgia was consistent with results of previous studies in these states (3, 22). In contrast to our findings, however, a growth chamber study (3) and a field study (22) indicated that survival of sclerotia was greater at the soil surface than when buried at 5- to 15-cm depth. In Iowa, Edmunds and Gleason (7) found that survival of *S. rolfsii* var. *delphinii* was not significantly affected by depth of burial until June and July and that the magnitude of this difference varied among locations. In general, we found that sclerotial survival at the surface was slightly lower, but not statistically different, than at 20-cm depth throughout the experiment. Variability in the results suggests either that the effectiveness of burial in preventing overwinter survival of sclerotia may depend on site- or region-specific factors (1).

Alexander and Stewart (1) found that sclerotia of *S. rolfsii* survived longer than those of *Sclerotinia minor* and *Sclerotium cepivorum* and attributed the greater survival to larger sclerotia, which may contain more nutrients in the cortex and be more resistant to unfavorable conditions (1, 4). In the two cold-winter sites in the present study, *S. rolfsii* var. *delphinii* survived better than *S. rolfsii*, which may indicate that *S. rolfsii* var. *delphinii* sclerotia are more resistant to very low temperatures. A larger sclerotium possesses a smaller surface to volume ratio, which means that the cortex is less exposed to the environment (21). Larger size thus confers a protective effect by shielding the cortex from temperature extremes and microbial attack. Further experimentation is needed to confirm or refute this hypothesis. In Georgia and North Carolina, however, survival of *S. rolfsii* var. *delphinii* generally exceeded that of *S. rolfsii*, suggesting that
*S. rolfsii* var. *delphinii* sclerotia may have superior resistance to other, unidentified environmental factors in addition to temperature.

Soil texture may also affect survival of sclerotia (13). Mitchell and Wheeler (14) and Alexander and Stewart (1) showed more rapid sclerotial degradation and lower sclerotia survival in higher clay content soil. Alexander and Stewart (1) argued that this could have resulted from higher water retention and higher levels of microbial activity in clay soil. The fact that survival of both fungi were generally greater in Iowa (Storden loam) than in North Dakota (Silty clay) and survival in North Carolina (Fuquay sand) exceeded that in Georgia (Cecil clay sandy loam) may be related to different clay content in each location. The extent to which soil characteristics at the four sites may have influenced sclerotial survival is unknown. However, greater similarity of survival trends within regions than within soil type suggests that temperature-related influences were stronger.

In previous studies, sclerotia of *S. rolfsii* were found to survive from 2 months to 3 years in field soil (2) and *S. rolfsii* var *delphinii* survived 6 months to 2 years (5, 10, 11, 26, 27). Some of those studies used sclerotia that were either produced on culture media or air dried before burial; it is likely, however, that conditions under which sclerotia are produced can impact their durability in survival trials. Sclerotia produced from natural infections differ physiologically and structurally from those produced on culture media (3, 12, 18, 20, 22). Linderman and Gilbert (12) suggested that naturally produced sclerotia are more uniform in size and should be used in experiments. Sclerotia produced on rich media also have a thicker cortex than those produced under natural conditions. Sclerotia formed under natural conditions are likely to be more adversely affected by environment in the soil and to have a lower rate of survival than those formed on agar plates (1, 6). Our method of producing
sclerotia represented a compromise between in vivo and in vitro conditions that facilitated rapid, consistent production of large numbers of similar sclerotia under simulated natural conditions. Morphology of sclerotia produced by our technique closely resembled that of sclerotia produced in field infections of hosta petiole rot (Xu, unpublished data).

The results of this study supported the hypothesis that overwinter survival can impact the biogeography of S. rolfsii and S. rolfsii var. delphinii. Additional studies measuring multiple environmental factors will be required to achieve a clearer understanding of overwintering survival in these fungi. Improved diagnostic procedures, combining PCR assays (9, 16, 17) and examination of sclerotial size and morphology in culture, would be helpful in identifying these pathogens, rather than assuming identity based solely on field signs.

ACKNOWLEDGEMENTS

We thank Cody D. Chesrown, Kala Parker, John D. Youmans, Mike Priebe, Adam Sisson and Jon Massman for assistance with sample collection, Zamir K. Punja for providing isolates of S. rolfsii, Jean C. Batzer, Miralba Agudelo, Emily Schaefer, and Danielle Wynthein for assistance with producing sclerotia and making nylon bags, and Dave Volkers for maintaining the dew chamber.

LITERATURE CITED


Table 1. Comparison of diameter of sclerotia of *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*

<table>
<thead>
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<th>Fungi</th>
<th>Isolate</th>
<th>Diameter (mm)</th>
<th>Standard deviation</th>
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</thead>
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<tr>
<td><em>S. rolfsii</em></td>
<td>Sr 1</td>
<td>0.93</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>Sr 2</td>
<td>0.94</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>Sr 3</td>
<td>0.92</td>
<td>0.117</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.93</td>
<td>0.125</td>
</tr>
<tr>
<td><em>S. rolfsii</em> var. <em>delphinii</em></td>
<td>Srd 1</td>
<td>1.48</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>Srd 2</td>
<td>1.48</td>
<td>0.384</td>
</tr>
<tr>
<td></td>
<td>Srd 3</td>
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<tr>
<td></td>
<td>Mean</td>
<td>1.52</td>
<td>0.532</td>
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</table>
Figures

Figure 1. Mean percent survival of sclerotia of *Sclerotium rolfsii* isolates (Sr 1 and Sr 2) and *S. rolfsii* var. *delphinii* isolates (Srd 1 and Srd 2) at the soil surface and at 20-cm soil depth in Fargo, North Dakota (A and B, respectively), Gilbert, Iowa (C and D), Clayton, North Carolina (E and F), and Griffin, Georgia (G and H) from October 2005 to August 2006. Significant differences ($P<0.05$) in survival between *S. rolfsii* and *S. rolfsii* var. *delphinii* on sampling dates (marked with asterisks): Nov., Dec., Apr. and May for both soil surface and 20-cm in the soil in North Dakota; Nov., Jan., Apr., May and Jun. for soil surface, and Jan., Apr., May, Jun. and Jul. for 20-cm in the soil in Iowa; Jun. for soil surface in Georgia according to the Tukey test.

Figure 2. Apparent daily death rate at the surface and at 20-cm depth in the soil for North Dakota (A and B, respectively), Iowa (C and D), North Carolina (E and F), and Georgia (G and H).

Figure 3. Average daily air temperature and 20-cm soil temperature for North Dakota (A and B, respectively), Iowa (C and D), North Carolina (E Q and F R), and Georgia (G and H). Dates having snow cover on the ground were: 15-17 Nov and 25 Nov 2005 –29 Mar 2006 for Moorhead, MN (total numbers of snow cover days: 128); and 15-17 and 29-30 Nov, 1-31 Dec 2005, 1-2 and 20-22 Jan, 8-12 and 16-19 Feb, 21-24 Mar 2006 for ISU Agronomy Farm (total numbers of snow cover days: 54). Both weather stations were within 30 km of the experiment.
Fig. 1

Surface

Soil (20-cm depth)
Fig. 2

Apparent Daily Death Rate (r)

Surface

Soil (20-cm depth)

S. rolfsii

S. rolfsii var. delphinii
Fig. 3

Average Daily Temperature (°C)

Air

Soil (20-cm depth)
CHAPTER 3. DEVELOPMENT OF A RAPID METHOD TO ASSESS RESISTANCE AMONG HOSTA CULTIVARS TO PETIOLE ROT CAUSED BY SCLEROTIUM ROLFSII VAR. DELPHINII

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ABSTRACT

A rapid assay was developed to assess hosta cultivars for resistance to petiole rot caused by S. rolfsii var. delphini. Leaves of greenhouse-grown hosta (Hosta kikutii and H. spp. cultivars Lemon Lime, Munchkin, Tardiflora, Pearl Lake, Zounds, Honeybells, Gold Drop, and Halcyon) were treated with 20 µl of oxalic acid (50 mM) on a cotton swab, then incubated at 100% relative humidity and 27 °C. After 4 days, incidence of leaves with lesions was evaluated. Cultivars Lemon Lime, Munchkin, and Tardiflora had a relatively high incidence of leaves with lesions, whereas Halcyon and Gold Drop had much lower incidence. These results were generally consistent with those of field and greenhouse cultivar screening tests in which whole plants were inoculated with the pathogen and rated for disease incidence. Additional screening methods, including spray application of either oxalic acid or mycelial fragments of S. rolfsii var. delphini, were not as repeatable or simple to conduct as
the cotton swab assay. This method showed potential to accelerate identification of highly
resistant hosta cultivars, and thereby aid efforts to breed resistance to petiole rot.

Additional keywords: herbaceous perennials, plant breeding, ornamental plants

INTRODUCTION

Petiole rot, incited by *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, causes
substantial losses in hosta plantings in the U.S (Fig. 1). Previously known only from the
southern U.S. (11,13, 15), hosta petiole rot was reported in the midwestern U.S. with
increasing frequency during the 1990s (5). Petiole rot is difficult and expensive to control in
nurseries and landscapes because the causal fungi can survive in the soil for many years, and
can spread from hosta to hundreds of annual and perennial plant species (5). Available
information suggests that *S. rolfsii* var. *delphinii* is the predominant petiole rot pathogen in
the northern U.S., whereas *S. rolfsii* is predominant in southern states (2, 17).

Using resistant cultivars can be an effective method of managing diseases incited by
these fungi (11). Identifying hosta cultivars and species with high levels of resistance to
petiole rot would benefit breeders, growers, marketers, and hosta enthusiasts by providing a
durable, cost-effective management option. Information on levels of genetic resistance to
*S. rolfsii* and *S. rolfsii* var. *delphinii* among the more than 4,000 named cultivars of hosta is
scant. In a greenhouse evaluation of 18 hosta cultivars, Edmunds et al. (6) inoculated plants
by placing a carrot disk infested with mycelium of *S. rolfsii* var. *delphinii* at the base of each
plant. The cultivars Halcyon and Zounds displayed a high level of resistance, whereas Lemon
Lime, Munchkin, Nakaiana, Platinum Tiara, and Tardiflora developed severe symptoms.
However, this assay was impractical for screening large numbers of hosta cultivars because it was time-consuming (about 3.5 months), expensive, and labor-intensive.

Techniques used to identify resistance to *Sclerotinia sclerotiorum*, the fungus that causes white mold in numerous crops, may have potential for adaptation to screening for petiole rot resistance. Like *S. rolfsii* and *S. rolfsii* var. *delphinii*, *Sclerotinia sclerotiorum* releases oxalic acid, which overcomes host defenses during the infection process. Screening techniques for *S. sclerotiorum* include detached leaf inoculation, an oxalic acid resistance test, and stem inoculation of intact seedlings in controlled environments (8, 9, 10, 12, 16, 18). Compared to resistance evaluations on intact plants in the field or greenhouse, a detached tissue assay has several potential advantages. For example, a detached tissue assay could yield results in a relatively short time – a few days compared to months for intact plants – and requires much less space than those using intact plants. An additional benefit is that rare breeding stock and expensive plants could be conserved rather than destroyed by the assay.

Oxalic acid can combine with calcium in plant tissues, removing it from association with pectic compounds in plant cell walls, lowering cell wall pH, and thereby facilitating activity of endopolygalacturonase and cellulase (4, 7). Mechanisms of resistance to *S. rolfsii* or *S. rolfsii* var. *delphinii* are unknown, but breaking down or excluding oxalic acid could reduce the pathogenicity of *S. rolfsii* (13). Wegulo et al. (16) developed an oxalic acid assay to screen soybean resistance to stem rot caused by *S. sclerotiorum* in which the excised ends of soybean petioles were placed in test tubes containing 40 mM oxalic acid in a growth chamber. Kolkman and Kelly (9) found that oxalic acid was useful for assessing resistance of common bean to *S. sclerotiorum*. An assay using oxalic acid rather than inoculum possesses
practical advantages because it could be conducted more rapidly and cheaply, and require less equipment and expertise.

The objective of the present study was to develop an assay to screen hosta cultivars for resistance to petiole rot fungi that is simpler and faster than whole-plant inoculation methods.

**MATERIALS, METHODS AND RESULTS**

**Isolate**

Cultures of *S. rolfsii var. delphinii* isolate Srd1, obtained from a symptomatic hosta in Ames, Iowa, were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) in 9-cm-diameter petri dishes under intermittent light at room temperature (22 to 25 °C).

**Cultivar selection**

Hosta cultivars or species used in the present trials - Nakaiana, Munchkin, Lemon Lime, Tardiflora, Pearl Lake, Zounds, Gold Drop, Honeybells, Halcyon, and *H. kikutii*-exhibited a range of resistance to petiole rot in previous greenhouse inoculation trials (6). Halcyon was highly resistant, whereas the other cultivars ranged from very susceptible to moderately resistant. Single-eye crowns were obtained from Wade and Gatton Nursery (Bellville, OH), and tissue cultured plants were donated by Hans Hansen (Shady Oaks Nursery, LLC, Waseca, MN). Because of unavailability of some cultivars for the trial using tissue-cultured plants, sports of these cultivars were substituted. Natural sports arise from mutations that impart new patterns of variegation in leaf color but are unlikely to affect the susceptibility or resistance to a particular pathogen (1, 14, Kevin Hurd, personal...
communication, Walters Gardens, Inc., Zeeland, MI). Tissue-cultured plants used in the spray-inoculation trial were Masquerade (sport of Munchkin), Twist of Lime (sport of Lemon Lime), Zounds, Plantaginea (sport of Honeybells) and Halcyon.

**Plant maintenance**

Single-eye plants for each cultivar were planted in 15-cm-diameter plastic pots in a potting mix (50% peat : 30% perlite : 20% steamed soil) in a greenhouse at Iowa State University. Plants were grown at 23 to 25 °C under high-intensity sodium vapor lamps with a 14-h light/10-h dark regime. Plants were watered weekly and fertilized (N20-P4.4-K16.6) monthly. Flower stalks were removed as soon as they appeared.

**Assessment of resistance in the field**

In order to validate results of the Edmunds et al. (6) greenhouse screening trial, seven hosta cultivars (Lemon Lime, Munchkin, Nakaiana, Snow Mound, Honeybells, Zounds and Halcyon) used in that study were tested outdoors for resistance to petiole rot from 13 May to 7 August 2005 and 23 May to 20 August 2006.

Single-eye hosta plants were established in a naturally shaded field plot at the Iowa State University Horticulture Farm. Composted hardwood bark mulch was used to maintain soil moisture. In each year, the study was set up as a randomized complete block design with six replications. Within each replication, there were three plants per cultivar; petiole bases of two plants of each cultivar were inoculated with carrot disks infested with *S. rolfsii* var. *delphinii* on 4 July and 11 July in 2005 and 2006, respectively. Moistened sterile cotton balls covered the carrot disks to prevent inoculum from drying out. Infested
carrot disks and cotton balls were pinned to the soil in order to prevent inoculum from losing contact with the host plants. Control plants had non-infested carrot disks covered by cotton balls that were treated as described above.

Disease severity was assessed as percent symptomatic petioles. Beginning 4 days after inoculation, plants were rated every 5 days until 7 August 2005 and 20 August 2006. Area under the disease progress curve (AUDPC) was calculated (3). The Tukey-Kramer test was used to compare means of AUDPC. Spearman’s coefficients of rank correlation were used to measure the correlation between ranks of cultivars response in greenhouse trial by Edmunds et al (6) and ranks of cultivars in field trials.

In field trials, cultivars Lemon Lime, Munchkin, and Nakaiana were most susceptible to petiole rot, Zounds showed moderate resistance, and Snow Mound, Halcyon and Honeybells were the most resistant (Table 1). Spearman’s coefficients of rank correlation between the greenhouse trial by Edmunds et al (6) and field trials were 0.57 (nonsignificant, \( p=0.18 \)) for the 2005 trial and 0.83 (significant, \( p<0.05 \)) for the 2006 trial, indicating that results from the field trials were generally consistent with those of greenhouse inoculation trials (6).

**Spray-applied inoculum assay**

Spray-applied inoculum was produced from 10 1-week-old cultures of *S. rolfsii* var. *delphinii* that were grown on PDA in 9-cm-diameter petri dishes under intermittent light at room temperature (22 to 25 °C), blended in a Waring™ blender for 100 sec at high speed in 1 L of distilled water, and filtered through two layers of cheesecloth.
Concentration of the inoculum suspension was adjusted to $2 \times 10^5$ mycelial fragments/ml by using a hematocytometer. Cultivars of tissue-cultured plants (ranging from 8 to 18cm in height) were Masquerade, Twist of Lime, Plantaginea and Halcyon. After approximately 5 ml of inoculum were sprayed on each inoculated plantlet on 8 July 2006, plantlets were placed in a dew chamber (100% relative humidity) at 27 °C. Two flats of plants per cultivar were inoculated (12 plants per flat); each flat constituted one replication. One flat containing non-inoculated control plants of each cultivar, sprayed with approximately 5 ml of sterile distilled water (SDW), provided comparison to inoculated plants. Symptoms were observed daily for 2 weeks. Time from inoculation until symptoms appeared was recorded. The Tukey-Kramer test was used to compare means of time until symptom appearance.

Spearman’s coefficient of rank correlation was used to measure the correlation between ranks of cultivars response in greenhouse trial by Edmunds et al (6) and the spray-applied inoculum trial.

Symptoms appeared on all hosta cultivars within 6 days after spray inoculation and significant differences were found among cultivars (Table 1). Cultivars Masquerade and Twist of Lime were highly susceptible to *S. rolfsii* var. *delphinii*. In contrast, cultivars Halcyon and Zounds were moderately resistant and Plantaginea was highly resistant (Table 1).

Spearman’s coefficient of rank correlation between the greenhouse trial by Edmunds et al (6) and the spray-applied inoculum trial was -0.67 (nonsignificant, $p=0.22$).

**Spray-applied oxalic acid assay**
In separate trials, on 24 October and 21 November 2007, 6- and 10-week-old hosta leaves, respectively, of *H. kikutii* and *Hosta* spp. cultivars Lemon Lime, Munchkin, Tardiflora, Pearl Lake, Zounds, Honeybells, Gold Drop, and Halcyon were arbitrarily chosen, excised from intact greenhouse-grown plants with sterile scissors, and placed on hardware cloth. The excised base of each petiole was covered with cling-type plastic wrap in order to prevent oxalic acid from coming into contact with it. Fifty ml of oxalic acid (50 mM) (Fisher Scientific, Fair Lawn, NJ) per replication were sprayed evenly on excised leaves using a hand-trigger sprayer, and non-treated control petioles were sprayed with SDW. Sprayed leaves were immediately placed in a dew chamber (100% relative humidity) at 27 °C. The trials were set up as a randomized complete block design with three replications. Each replication consisted of four petioles for each of three replications of each cultivar. Lesions were noted daily for 1 week. The incidence (%) of leaves with lesions, calculated by counting the number of leaves with lesions and dividing by the total number tested (×100), was determined 4 days after treatment. The Tukey-Kramer test was used to compare means of incidence of leaves with lesions among cultivars. Spearman’s coefficient of rank correlation was used to measure the correlation between ranks of cultivars and species response in greenhouse trial by Edmunds et al (6) and the spray-applied oxalic acid trial.

Lesions appeared on most cultivars within 2 days after treatment of 6-week-old petioles and leaves. Cultivar resistance could not be differentiated based on the incidence of leaves with lesions (Xu, unpublished data). When 10-week-old tissues were used, however, there were significant differences (*p*<0.05) among cultivars and species in incidence of leaves with lesions 4 days after treatment (Table 1). Cultivars Halcyon and Gold Drop had
the lowest incidence of leaves with lesions, whereas Lemon Lime and Munchkin had the highest incidence.

Spearman’s coefficient of rank correlation between the greenhouse trial by Edmunds et al (6) and the spray-applied oxalic acid trial was 0.77 (significant, \( p<0.05 \)).

**Cotton swab oxalic acid assay**

In separate trials on 5 December 2007 and 11 January 2008, 12- and 17-week-old hosta leaves, respectively, of *H. kikutii* and cultivars Lemon Lime, Munchkin, Tardiflora, Pearl Lake, Zounds, Honeybells, Gold Drop, and Halcyon were arbitrarily chosen, excised with sterile scissors at the base of the crown, and placed on hardware cloth. The tip of a cotton swab was placed at the leaf-petiole junction. Twenty \( \mu l \) of oxalic acid (50 mM) was dispensed onto the tip of the swab with a pipette (Fig. 2). Non-treated control petioles received 20 \( \mu l \) of SDW on swab tips. The trials were set up as a randomized complete block design with four replications. Immediately after treatment, excised tissues and hardware cloth were placed in a dew chamber at 100% relative humidity and 27 °C. Lesions were observed daily for 1 week. In each trial, incidence (%) of leaves with lesions was determined 4 and 5 days after oxalic acid treatment. Analysis of variance was conducted using the SAS PROC Mixed procedure (SAS Institute, Cary, NC). The Tukey-Kramer test was used to compare means of incidence of leaves with lesions among cultivars. Spearman’s coefficient of rank correlation was used to measure the correlation between ranks of cultivars and species response in the earlier greenhouse trial (6) and the cotton swab oxalic acid trial.
In both trials, lesions appeared on leaves within 5 days, beginning where the tip of the cotton swab was located. Although results from two runs of the experiment were significantly different ($p<0.05$), the $F$ value for interaction between cultivars and experiments was not significant, so results from both experiments were combined. In both trials, cultivars Lemon Lime, Munchkin, and Tardiflora were highly susceptible to oxalic acid, whereas Halcyon and Gold Drop were highly resistant (Table 1; Fig. 3).

Spearman’s coefficient of rank correlation between the greenhouse trial by Edmunds et al (6) and cotton swab oxalic acid trial based on average performance was 0.79 (significant, $p<0.05$).

DISCUSSIONS AND CONCLUSIONS

Results from the oxalic acid trials suggest that this approach can dramatically speed up identifying hosta cultivars that are highly resistant to petiole rot. These results are also encouraging evidence that screening methods under controlled conditions can accurately represent cultivar resistance to petiole rot in the “real world” – but much more efficiently. Cultivar resistance determined by either spray or cotton swab application of oxalic acid on excised leaves was generally similar to that determined by whole-plant inoculation trials of Edmunds et al. (6) based on Spearman’s coefficients, but required only a few days compared to at least 3.5 months for greenhouse trials using the whole-plant inoculation method.

Results of field trials generally validated the cultivar resistance ratings obtained in greenhouse trials by Edmunds et al. (6), in which Halcyon was the most resistant cultivar and Zounds, Snow Mound and Honeybells were moderately resistant. The result indicated that the greenhouse screening trials method of Edmunds et al. (6) was a valid predictor of cultivar
resistance in the field. Greenhouse inoculation assays have an advantage over field trials in that greenhouse temperature and humidity can be controlled to insure conditions favorable for petiole rot development, whereas disease occurrence in the field is more erratic.

Preliminary results in the spray-applied oxalic acid trial suggested that plant age could affect the speed with which leaves developed symptoms. Based on observations in this study, discrimination of cultivar resistance may be more effective with 10-week-old than with 6-week-old petioles or leaves.

When *S. rolfsii* var. *delphinii* inoculum was spray-applied on plantlets, cultivar resistance ratings corresponded to those from greenhouse (6) and field trials, although disease symptoms appeared predominantly on foliage rather than at the base of petiole as in field infections. An advantage of the sprayed-inoculum assay over field or greenhouse whole-plant inoculation trial was that the spray trials required only 2 weeks compared to 3.5 months for whole-plant trials. A substantial disadvantage of the spray-inoculation method compared to oxalic acid methods is that the former requires a laboratory, special equipment, and trained technicians to prepare inoculum of the pathogen. In contrast, the oxalic acid assays are simple enough to be carried out without specialized laboratory equipment or expertise.

Several precautions are advisable when using the oxalic acid assays, however. A dual-cartridge respirator with face shield should be used for the spray application of oxalic acid, since inhaling the vapor can be harmful. No respirator is needed for the cotton-swab assay, but latex gloves should be worn because oxalic acid is a severe skin irritant.

Current management techniques for petiole rot include fungicide application and cultural techniques such as sanitation (11). However, labeled fungicides are not consistently
effective and some are human carcinogens (11). Cultural management techniques, such as soil removal and replacement, are labor-intensive and usually impractical. Identifying more cultivars with high levels of resistance will benefit the entire hosta industry by providing a durable, cost-effective option to combat this highly destructive, difficult-to-manage disease. Because *S. rolfsii* and *S. rolfsii* var. *delphinii* also attack hundreds of species of perennial plants, developing an efficient screening technique for hosta is likely to streamline resistance screening methods for these fungi on many other herbaceous perennials.

Further research should include applying oxalic acid methods on excised leaves of field-grown rather than greenhouse-grown plants. This modification would benefit breeders who wished to screen their own collections of field-grown plants. Future studies should also determine whether the oxalic acid test on excised leaves could made even simpler and cheaper by replacing a laboratory dew chamber with much cheaper sealed plastic crispers containing moist paper towels, providing an even more practical and low-cost option for screening hosta cultivars.

**ACKNOWLEDGEMENTS**

This study was funded by grants from the Iowa Nursery and Landscape Association Research Committee, The Perennial Plant Association, and The Fred C. Gloeckner Foundation. We thank Anna Peterson for help with statistical analysis, Anne Dombroski, Alex Carlson, Nick Zdorkowski, Edwin Han, Carolina Arce, Adam Sisson, Nenad Tatalović, Alicia Owen, and Rosalee Coelho for technical assistance, and Dave Volkers for maintaining plants in the greenhouse.
LITERATURE CITED


Table 1. Area under the disease progress curve (AUDPC) of hosta cultivars for field trials in 2005 and 2006, and a previous greenhouse trial (6), time until symptom appearance of hosta cultivars in the spray-applied inoculum trial, incidence of leaves with lesions of hosta cultivars in spray-applied oxalic acid trial, and mean incidence of leaves with lesions of hosta cultivars in cotton swab oxalic acid trials.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Field trials</th>
<th>Greenhouse trials</th>
<th>Spray-applied inoculum assay</th>
<th>Oxalic acid assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUDPC (2005)</td>
<td>AUDPC (2006)</td>
<td>AUDPC</td>
<td>Time until symptoms appeared (days)</td>
</tr>
<tr>
<td></td>
<td>a,b</td>
<td>b</td>
<td></td>
<td>d</td>
</tr>
<tr>
<td>Nakaiana</td>
<td>2607 a</td>
<td>2012 a</td>
<td>1215 a</td>
<td>--</td>
</tr>
<tr>
<td>Munchkin</td>
<td>2490 a</td>
<td>2137 a</td>
<td>1228 a</td>
<td>4.8 ab</td>
</tr>
<tr>
<td>Lemon Lime</td>
<td>2460 a</td>
<td>1833 b</td>
<td>1402 a</td>
<td>4.3 a</td>
</tr>
<tr>
<td>Tardiflora</td>
<td>--</td>
<td>--</td>
<td>1142 ab</td>
<td>--</td>
</tr>
<tr>
<td>H. kikutii</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pearl Lake</td>
<td>--</td>
<td>--</td>
<td>810 bc</td>
<td>--</td>
</tr>
<tr>
<td>Zounds</td>
<td>1520 b</td>
<td>--</td>
<td>412 c</td>
<td>6.0 c</td>
</tr>
<tr>
<td>Gold Drop</td>
<td>--</td>
<td>--</td>
<td>611 c</td>
<td>--</td>
</tr>
<tr>
<td>Halcyon</td>
<td>1020 c</td>
<td>638 c</td>
<td>54 d</td>
<td>5.4 cb</td>
</tr>
<tr>
<td>Snow Mound</td>
<td>972 c</td>
<td>751 c</td>
<td>447 c</td>
<td>--</td>
</tr>
<tr>
<td>Honeybells</td>
<td>795 c</td>
<td>506 cd</td>
<td>416 c</td>
<td>6.0 c</td>
</tr>
</tbody>
</table>

a,b AUDPC of hosta cultivars for field trials. Plants were rated every 5 days, starting 4 days after inoculation, until 7 August 2005 and 20 August 2006.

c Results of Edmunds et al. 2003 (6).

d Days after inoculation

e Percent leaves with lesions 4 days after treatment

f Percent excised leaves with lesions 4 days and 5 days after treatment with oxalic acid in droplets on cotton swab on 9 December 2007 and 15 January 2008; Means shown combine data for both trials.

g Means followed by the same letters are not significantly different at p=0.05 according to the Tukey-Kramer test.

h Means followed by the same letters are not significantly different at p=0.05 according to the least square difference (LSD) test (6).
Sports of cultivars whose names are shown in the table were used in the spray-applied inoculum assay: Masquerade (sport of Munchkin), Twist of Lime (sport of Lemon Lime), and Plantaginea (sport of Honeybells).

\(^{\text{j}}\)Not available.

\(^{\text{k}}\)Species *Hosta kikutii*
Figures

Figure 1. Symptoms and signs of hosta petiole rot.
Figure 2. Excised leaf (cv. Tardiflora) showing cotton swab onto which a droplet of oxalic acid was dispensed.
Figure 3. Excised leaves of a susceptible cultivar (Munchkin, left) and a resistant cultivar (Gold Drop) after treatment with oxalic acid on cotton swabs, followed by 3 days of incubation at 100% relative humidity and 27 °C.
CHAPTER 4. PHYLOGENETIC PLACEMENT OF PLANT PATHOGENIC
SCLEROTIUM SPECIES AMONG TELEOMORPH GENERA

A manuscript in preparation for submission to *Mycologia*

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**ABSTRACT**

Phylogenetic evidence and morphological characteristics were used to assess the
taxonomic placement of eight plant-pathogenic *Sclerotium* species. Sequences of rDNA large
subunit (LSU) and internal transcribed spacer (ITS) regions were determined for isolates of
*Sclerotium cepivorum, S. coffeicola, S. denigrans, S. hydrophilum, Ceratorhiza oryzae-
sativa*, *S. perniciosum, S. rhizodes, S. rolfsii*, and *S. rolfsii var. delphinii*. Parsimony analysis
grouped two species thought to be in the Basidiomycota, *S. denigrans* and *S. perniciosum,*
within the Ascomycota; these species were found to have affinities with the teleomorph
genera *Sclerotinia* and *Stromatinia*. Based on rDNA analysis and morphology the
basidiomycetous *Sclerotium hydrophilum* and *S. rhizodes* were transferred to the genus
Ceratorhiza, the anamorph genus of Ceratobasidium species, and S. coffeicola and S. rolfsii var. delphinii were found to have affinities to the teleomorph genus Athelia.

Additional Keywords: disease management strategies, pathogen

INTRODUCTION

Fungi in the genus Sclerotium form sclerotia and sterile mycelia but no spores (Saccardo 1899). There are more than 40 plant-pathogenic Sclerotium species (Farr 2008). Many Sclerotium species do not or rarely reproduce sexually and are known only from their asexual stage (Punja 1988; Punja and Rahe, 2001; Kohn 2004). The name Sclerotium was first introduced by Tode (1790) to describe eight species. In 1822, Fries sanctioned the genus Sclerotium Tod., and the first-listed species, Sclerotium complanatum Tode, was suggested as the type species by Clements and Shear (1973). S. complanatum may be the anamorph of Typhula phacorrhiza Reichard ex Fries (Remsberg 1940), which produces basidiocarps from sclerotia (Donk 1962). S. rolfsii Sacc., the best known species in this genus, has the teleomorph Athelia rolfsii (Curzi) Tu and Kimbrough, which forms resupinate hymenia of basidia and has hyphal strands coming out of germinating sclerotia (Tu and Kimbrough 1978).

Although no sexual stage is known for S. cepivorum Berk., based on similarity of the sclerotia to those seen in the ascomycete Stromatinia gladioli (Drayt.) Whetzel, Sclerotium cepivorum was transferred to the teleomorph genus Stromatinia Boud. by Whetzel (1945). Analyses of rDNA sequences suggested that Sclerotium cepivorum was closely aligned with Sclerotinia sclerotiorum (Lib) Debary, another important species within the Sclerotiniaceae
(Carbone and Kohn 1993). Species of *Sclerotinia* form apothecia arising from a tuberoid sclerotium, whereas *Stromatinia* species form two kinds of sclerotia, apothecia arising from a sclerotium with a thin, black, subcuticular, effuse covering or mantel, or sclerotia that are small black sphaerules borne free on the mycelium and not giving rise to apothecia (Whetzel 1945). However, *Stromatinia cepivorum* forms only the latter sclerotia.

Some *Sclerotium* species may be related to the anamorph genus *Rhizoctonia* (de Candolle 1815), a basidiomycetous genus forming sclerotia and sterile mycelia with hyphae branching at right angles (Donk 1962). *Rhizoctonia solani* is believed to be the anamorph of *Thanatephorus cucumeris* (Frank) Donk, but most taxonomists agree that *R. solani* is not a single species but rather a species complex, and there are more than 30 synonyms for *R. solani* (Bridge 2002; Carling and Sumner 2001; Gonzalez et al 2001; Parmeter and Whitney 1970; Vilgalys and Cubeta 1994). The most well-known teleomorph genera connected with *Rhizoctonia* anamorphs are *Ceratobasidium* D.P. Rogers and *Thanatephorus* Donk. Some binucleate (two nuclei per hyphal cell) *Rhizoctonia* species often have a *Ceratobasidium* teleomorph, whereas some multinucleate *R. solani* J.G. Kühn have a *Thanatephorus* teleomorph. The two teleomorph genera differ in the shape of their basidia (Staplers 1996), but recent phylogenetic studies suggest that *Ceratobasidium* and *Thanatephorus* are not mutually monophyletic groups (Gonzalez et al 2001; 2006a; 2006b). Some authors (Moore 1987; 1989; Shan et al 2002; Stalpers and Andersen 1996) have classified binucleate *Rhizoctonia* species with dolipore/parenthesome (O_1/P_1-type d/p septa) in the anamorphic genus *Ceratorhiza* Moore. *Ceratorhiza oryzae-sativae* (Saw.) Moore (≡ *Sclerotium oryzae-sativae* Saw.) is the anamorph of *Ceratobasidium oryzae-sativae* P.S. Gunnell & R.K. Webster (Mordue 1974; Moore 1989).
Our study focused on determining phylogenetic placement and identification of putative teleomorphs for important plant-pathogenic species of *Sclerotium* by using parsimony analysis of sequences of the nuclear ribosomal DNA operon, sclerotium morphology, and hyphal morphology.

**MATERIALS AND METHODS**

*Isolates.*—For each of the nine taxa studied, two to eight isolates (TABLE 1) were assessed, for a total of 35 isolates. The number of isolates per species was determined based on availability of isolates from the CBS database (The Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), Dr. Zamir Punja at Simon Fraser University, Canada, and a collection at Iowa State University.

*Morphology of isolates in vitro.*—Cultures were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) in 9-cm-diameter petri dishes under a daily regime of approximately 8 h incandescent light and 16 h darkness at room temperature (21 to 24 °C). Mycelial plugs (7 mm in diameter) were transferred to 9-cm-diameter PDA plates, which were then placed in an incubator in darkness at 20 °C. Color of sclerotia and mycelia was observed, and number of sclerotia per petri dish was counted 1 and 3 weeks after incubation.

Mycelium from the edge of 1- to 2-week-old actively growing cultures of each species was stained with crystal violet, mounted on glass slides, and examined at 40× magnification under a microscope. In order to determine number of nuclei per hyphal cell for isolates of *S. hydrophilum* Sacc., *Ceratorhiza oryzae-sativae* and *S. rhizodes* Auersw., mycelia from 1-week-old culture were stained with DAPI (Polysciences, Inc., PA), and examined under a fluorescence microscope (Olympus BH Series) at 40 magnification.
**Polymerase chain reaction and sequencing.**—A portion of the LSU 28 S region of rDNA were sequenced for each of the 35 isolates. The internal transcribed spacer region (ITS1, 5.8 S rDNA gene, ITS2) was sequenced for isolates of *S. cepivorum*, *S. denigrans* Pape, *S. hydrophilum*, *C. oryzae-sativae*, *S. perniciosum* Slogt. & K.S. Thomas and *S. rhizodes*. Template DNA for polymerase chain reaction (PCR) was extracted directly by scraping mycelium with a pipette tip from 1- to 2-week-old cultures grown on PDA using Prepman Ultra (Applied Biosystems, Foster City, CA). Primer pairs used for amplification of the LSU region were LROR and LR5 and for sequencing of the LSU region were LROR and LR3 (Vilgalys and Hester 1990). The primer pair used for PCR and sequencing of ITS region was ITS-1F (Gardes and Bruns 1993) and ITS4 (White et al 1990). Amplification reactions consisted of 4 mM MgCl₂, 5x Sigma buffer, 200 µM dNTPSs, 0.5 µM of the forward and reverse primers, and 3 units of Taq polymerase (Sigma Chemical Co., St Louis, MO). Cycling conditions (MJ Research Inc. PTC-100 thermocycler, Waltham, MA) for PCR were an initial denaturation at 94 C for 95 s, followed by 35 cycles of denaturation at 94 C for 35 s, annealing at 49 C for LSU and at 52 C for ITS for 60 s, and extension at 72 C for 2 min. The PCR product was purified with a QIAquick DNA Purification Kit (QIAgen, Valencia, California) and quantified on a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, San Francisco, California). Automated sequencing (Applied Biosystems 3730xl DNA Analyzer) was performed at the Iowa State University DNA Sequencing and Synthesis Facility.

**Sequence alignment and phylogenetic analysis.**—To resolve phylogenetic placement for *Sclerotium* species, possible relatives of each of the *Sclerotium* species were determined by subjecting ITS and LSU sequences of representative isolates to BLAST (version 2.2.17,
National Center for Biotechnical Information, National Library of Medicine, National Institutes of Health, Bethesda, MD). Partial sequences of LSU and ITS highly similar to those of *Sclerotium* species were downloaded from Genbank for further phylogenetic analysis (Appendix I). *Saccharomyces cerevisiae* Meyen ex E.C. Hansen was used as the outgroup taxon for the LSU analysis because it is distinct molecularly and morphologically from the rest of the species. Outgroups for two ITS analyses were chosen based on the LSU analysis.

After preliminary alignments were generated using Clustal X with gap opening and gap extension parameters of 50:5 (Thompson et al 1997), aligned sequences were manually adjusted using BioEdit (Hall 1999). For LSU analysis, ambiguously aligned regions (110 characters) were eliminated, resulting in 451 characters including gaps. Alignable gaps were treated as a fifth character. For ITS analysis of the basidiomycete group, alignable gaps were treated as a fifth character and ambiguously aligned regions (211 characters) were eliminated, resulting in 626 characters including gaps. For ITS analysis of the discomycetes group, gaps were treated as a fifth character and ambiguously aligned regions (20 characters) were eliminated, resulting in 534 characters including gaps. Maximum parsimony analysis was performed with PAUP version 4.0b 10 for 32-bit Microsoft Windows. All characters were given equal weight. Heuristic searches were conducted with simple sequence addition and tree bisection-reconnection (TBR) branch swapping algorithms, collapsing zero-length branches. A strict consensus of the most parsimonious trees was generated, as well as a bootstrap analysis of 100 replications for the LSU dataset and 500 replications for the ITS dataset. For ITS analysis of the discomycetes group, initial “MaxTrees” was set at 10000.
RESULTS

Morphology.—Colonies were white for all isolates of all species after 1 week of incubation. Differences in morphological characters among *Sclerotium* species, including colony color, number of sclerotia per petri dish, and *in vitro* sclerotia color were more distinct after 3 weeks of incubation (TABLE II). In general, isolates of *S. cepivorum*, *S. denigrans*, and *S. perniciosum* had darker mycelia colors, and *S. cepivorum* and *S. perniciosum* had darker sclerotia than isolates of the other species (TABLE II).

Clamp connections were exclusively found in isolates of *S. coffeicola* Stahel, *S. rolfsii*, and *S. rolfsii* var. *delphinii* (Welch) Boerema & Hamers. Mycelial branching at right angles was seen in *S. hydrophilum*, *Ceratorhiza oryzae-sativae*, and *S. rhizodes*; for these species, branching hyphae were slightly constricted at the branch origin, and there was often a septum near the branch origin. No clamp connections were found in these species. Isolates of *S. hydrophilum*, *Ceratorhiza oryzae-sativae* and *S. rhizodes* had two nuclei per cell based on fluorescent microscopy.

Phylogenetic placement.—Maximum parsimony analysis of the LSU sequences resulted in 368 equally most parsimonious (MP) trees. A MP tree (FIG. 1) was selected to illustrate phylogenetic placement of the *Sclerotium* species. Results of parsimony analysis indicated that *Sclerotium* species are found in three major clusters, designated S1, S2, and S3. *Sclerotium cepivorum*, *S. denigrans*, and *S. perniciosum* formed cluster S1, which was grouped within the Sclerotiniaceae (Helotiales, Ascomycota). Cluster S2 included *S. hydrophilum*, *S. rhizodes*, and *Ceratorhiza oryzae-sativae*, which was grouped within the Ceratobasidiaceae (Basidiomycota). Cluster S3 consisted of *S. rolfsii*, *S. rolfsii* var. *delphinii*,...
and *S. coffeicola* Stahel, which was grouped within *Athelia* (Atheliales, Basidiomycota) with a bootstrap support of 100%. Both clusters S2 and S3 were grouped with Agaricomycetes.

The LSU analysis grouped all isolates of *S. cepivorum*, *S. denigrans* and *S. perniciosum* with *Sclerotinia sclerotiorum*, *Botryotinia fructicola*, and *Monilinia fructicola* with a bootstrap support of 89% (Fig. 1). Parsimony analysis of ITS sequences grouped five isolates of *S. cepivorum* (CBS189.82, 271.30, 276.93, 320.65, and 321.65) with a strongly supported bootstrap value of 91% (Fig. 2). The closest matches to CBS271.30 in pairwise comparison of ITS sequences using BLAST were sequences from *Sclerotinia sclerotiorum* EU082466 (97% base-pair homology, 455/465 identity) and from *Sclerotinia trifoliorum* EU082465 (97% base-pair homology, 455/465 identity). However, the number of sclerotia on PDA and sclerotia diameter varied among these isolates (Table II). In addition, the ITS sequence of *S. cepivorum* isolate CBS342.47 grouped with three isolates of *S. perniciosum* (CBS268.30, 274.93, and 275.93) with bootstrap support of 55%. These two subclades grouped with bootstrap support of 83%. *Sclerotium cepivorum* isolate CBS276.93 and *S. perniciosum* isolates CBS274.93 and 275.93 were similar in colony color, number of sclerotia per plate, and sclerotia color.

Parsimony analysis of ITS sequences grouped *Sclerotium denigrans* with *Stromatinia rapulum* (Fig. 2) with bootstrap support of 98%. Unlike other *Sclerotium* species that produced sclerotia, *S. denigrans* produced crustose pseudosclerotia on PDA.

Parsimony analysis of the LSU and ITS regions both indicated that *S. perniciosum* isolates CBS268.30, 274.93, and 275.93 were closely related to isolates of *S. cepivorum* (Fig. 1, Fig. 2). Morphological characters of *S. perniciosum* CBS274.93 and *S. cepivorum* CBS276.93 were similar (Table II). However, one of the *S. perniciosum* isolates,
CBS335.47, was in a different clade than other isolates of *S. perniciosum* in LSU and ITS sequences. Colony color and number of sclerotia per plate of isolate CBS335.47 differed from those of the other three *S. perniciosum* isolates, and it may be misidentified. The ITS sequence of CBS335.47 was more similar to that of *Botryotinia fuckeliana* isolates than other *S. perniciosum* isolates (Fig. 2).

All isolates of *S. hydrophilum*, *S. rhizodes*, and *Cerotorhiza oryzae-sativae* were grouped with *Ceratobasidium* sp. and *Thanatephorus cucumeris* with a bootstrap support of 97% (Fig. 1). Morphological characters were similar among isolates of *S. hydrophilum* (CBS201.57 and 385.63). Parsimony analysis of the LSU (Fig. 1) grouped *S. hydrophilum* with *Ceratobasidium* sp. (sequence AF354092), and analysis of the ITS region grouped *S. hydrophilum*, *Ceratobasidium* sp., and *Rhizoctonia* sp. with bootstrap support of 78% (Fig. 3).

Colony color of *Cerotorhiza oryzae-sativae* isolates CBS 235.91 and 439.80 differed from that of CBS577.81. The isolates also differed in number of sclerotia on PDA (TABLE II). The closest matches to *Cerotorhiza oryzae-sativae* isolates in pairwise comparison of ITS sequences using BLAST were sequences from another isolate of *Cerotorhiza oryzae-sativae*, a *Ceratobasidium* sp., and a *Rhizoctonia* sp. (Fig. 3).

The ITS sequences from *S. rhizodes* isolates CBS126.13, 276.69 and 321.68 differed by only three base pairs (Fig. 3). However, CBS321.68 rarely produced sclerotia on PDA or on cotton (TABLE II). Parsimony analysis of the ITS sequences grouped *S. rhizodes* with isolates of *Ceratobasidium cereale*, *Ceratobasidium* sp. and *Rhizoctonia* sp. with bootstrap support of 100%.
Parsimony analyses of LSU (Fig. 1) and ITS sequences (Xu, unpublished data) both suggested close relationships among *S. rolfsii*, *S. rolfsii* var. *delphinii*, and *S. coffeicola*. They were grouped with *Athelia* species with strong bootstrap support of 100% (Fig. 1). The three taxa had similar colony color, whereas they differed with regard to sclerotia size, sclerotia color and number of sclerotia per plate. *S. rolfsii* and *S. rolfsii* var. *delphinii* could not be differentiated based solely on LSU sequences (Fig. 1). Two isolates identified as *S. rolfsii* var. *delphinii* (1058 and 3092) fell into the *S. rolfsii* group based on LSU analysis, and they were morphologically similar to *S. rolfsii* isolates in culture (TABLE II).

**TAXONOMY**

Anamorphic species of *Sclerotium* were found to be connected to several genera of both basidiomycetes and ascomycetes. Two new combinations in the anamorphic genus *Ceratorhiza* are proposed based on rDNA sequences analyses and morphological characteristics:

**Ceratorhiza hydrophilum** (Sacc.) Xu, Harrington, Gleason, et Batzer, *comb. nov.*


**Ceratorhiza rhizodes** (Auersw.) Xu, Harrington, Gleason, et Batzer, *comb. nov.*


**DISCUSSION**

Sequences of rDNA placed eight phytopathogenic *Sclerotium* species into three teleomorphic genera. Phylogenetic placement confirmed that *S. cepivorum* belongs in the Sclerotiniaceae (Carbone and Kohn 1993) and revealed that *S. denigrans* and *S. perniciosum*
are also in this ascomycotous family. The rDNA data also contradict earlier conclusions (Kirk 2004) that that *S. denigrans* and *S. perniciosum* belong in the Basidiomycota.

**Group (S1).** Based on phylogenies of LSU and ITS regions, there is evidence that *S. cepivorum*, *S. denigrans*, and *S. perniciosum* have affinities to the sclerotial lineage in the family Sclerotiniaceae, which is consistent with the conclusion of Carbone and Kohn (1993) based sequences of ITS1. Whetzel (1945) transferred *S. cepivorum* to *Stromatinia cepivorum* based on morphology of the second type of sclerotia produced by species of *Stromatinia*. *Sclerotium denigrans* produced crustose-like sclerotia in culture that corresponded with the description of manteloid sclerotia produced by *Stromatinia*. Morphology of sclerotia of *S. perniciosum* was similar to that of *S. cepivorum*. Our study based on LSU and ITS sequences also supported the morphology data and suggested that *S. cepivorum*, *S. denigrans* and *S. perniciosum* are closely related to the genera *Sclerotinia* and *Stromatinia*. Sequencing and analysis of more DNA sequences of *Stromatinia* species is needed to further confirm this finding.

**Group (S2).** *Sclerotium* species in this group have similar host ranges (Farr 2006). The fact that the all three species within group S2 have hyphae that branch at right angles suggests that they may be closely related to *Rhizoctonia* species (Tredway and Burpee 2006). Our results supported the conclusion of Johanson et al (1998), who reported that a *R. solani* specific primer pair (GMRS-4 and ITS1) amplified DNA from *S. hydrophilum*. Although septal ultrastructure of isolates of *S. hydrophilum* and *S. rhizodes* was not determined, the binucleate hyphae and high similarity of rDNA sequences to *Ceratorhiza* species and *Ceratobasidium* indicate that *S. hydrophilum* and *S. rhizodes* belong in the anamorph genus *Ceratorhiza*. 
Group (S3). Based on LSU sequence analysis, fungi in this group are closely related to the teleomorph genus *Athelia*. A study of *S. coffeicola*, *S. rolfsii*, and *S. rolfsii* var. *delphinii* (Punja and Damiani, 1996), comparing colony characteristics, sclerotial formation, growth response to different temperature and media, and ability to produce oxalic acid and pectinase enzymes, provided further evidence that the three species are closely related.

*S. rolfsii* and *S. rolfsii* var. *delphinii* are closely related fungi. The first published report of losses due to *S. rolfsii* in the U.S. was made by Rolfs in 1892 on tomato in Florida (Aycock, 1966; Punja, 1988). *S. rolfsii* var. *delphinii* was initially named *S. delphinii* (Welch 1924), but it was renamed *S. rolfsii* var. *delphinii* by Boerema and Hamers (1988). Our sequence and morphological evidence suggested that *S. rolfsii* var. *delphinii* isolates 1058 and 3092 might be misidentified. A further phylogenetic analysis of multiple gene sequences will clarify the taxonomic relationship of these two fungi. (see Appendix II)

Our study provided new insights into phylogenetic relationships of *Sclerotium* species as a whole and phylogenetic placement for each individual species. The information is critical in assessing disease management strategies, such as fungicide application. Some classes of fungicides have modes of action that are specific for either Basidiomycota or Ascomycota (McGrath 2004). For instance, carboxin is active on basidiomycetes, whereas fenhexamid is active only on Ascomycota such as *Botrytis*, *Monilinia*, and *Sclerotinia*. Our results could help growers choose appropriate fungicides, and therefore could improve their ability to manage economically important diseases caused by these phytopathogens.

Results from our study suggested that morphological characters (color of mycelium and sclerotia, and number of and size of sclerotia) alone are insufficient to delimit species of *Sclerotium*. Coupled with phylogenetic data, however, morphological characters are useful
tools in delineating species (Harrington and Rizzo 1999). Future studies might involve detailed comparison of morphology, physiology, and sequence data.

ACKNOWLEDGEMENTS:

We thank Drs. Zamir K. Punja, Timothy B. Brenneman and Kenneth W. Seebold for providing isolates, Dr. Edward Braun for assistance with the fluorescent microscope and Khushboo Hemnani, Edwin Han, Carolina Arce, and Joseph Steimel for technical assistance.

LITERATURE CITED:


Donk, M.A. 1962. The generic names proposed of Hymenomycetes. XII, Deuteromycetes. Taxon. 11:75-104.


[http://www.indexfungorum.org]


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<th>ITS</th>
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<td>FJ231400</td>
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Source:
1Centraalbureau voor Schimmelcultures, The Netherlands
2Timothy B. Brenneman, University of Georgia
3Kenneth W. Seebold, University of Kentucky
4Zamir K. Punja, Simon Fraser University
5Mark L. Gleason, Iowa State University
TABLE II. Colony color, number of sclerotia, sclerotia color, sclerotia diameter, and hyphal type of isolates of *Sclerotium* species

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate number</th>
<th>Colony Color</th>
<th>Number of sclerotia per plate</th>
<th>Sclerotia shape</th>
<th>Sclerotia color</th>
<th>Clamp connection present or absent</th>
<th>Right angle branching present or absent</th>
<th># of Nuclei per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cepivorum</em> CBS 189.82</td>
<td>Center: white to dark olive (1-cm-diameter) Margin: olive buff to citrine drab in</td>
<td>None</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
<td>Olive-buff</td>
<td>Absent</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CBS 271.30</td>
<td>Clove brown</td>
<td>130</td>
<td>Spherical to hemispherical</td>
<td>Dark olive</td>
<td>Absent</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CBS 276.93</td>
<td>Center: pale olive buff (2- to 3-cm-diameter) Margin: olive brown</td>
<td>621</td>
<td>Spherical to hemispherical</td>
<td>Dark olive</td>
<td>Absent</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CBS 320.65</td>
<td>Center: dark olive buff (3-cm-diameter) Margin: olive buff</td>
<td>65</td>
<td>Spherical to hemispherical</td>
<td>Citrine drab</td>
<td>Absent</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CBS 321.65</td>
<td>Center: white to hair brown (3-cm-diameter) Margin: olive buff</td>
<td>95</td>
<td>Spherical to hemispherical</td>
<td>Citrine drab</td>
<td>Absent</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CBS 342.47</td>
<td>Center: white to deep olive (1- to 3-cm-diameter) Margin: buffy brown</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>Absent</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Yellow ocher to buckthorn brown</td>
<td>Present</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>CBS 668.85</td>
<td>White</td>
<td>4</td>
<td>Spherical</td>
<td>Yellow ocher to buckthorn brown</td>
<td>Present</td>
<td>Absent</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>Crustose</td>
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<td>Absent</td>
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<td>80</td>
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<td>Mars brown and white</td>
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<td>Present</td>
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<td>CBS 385.63</td>
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<td>596</td>
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<td>Present</td>
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<td>Absent/Pres</td>
<td>Present/Not</td>
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<td><em>Ceratobhiza</em> oryzae-sativae</td>
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<td>79</td>
<td>Spherical</td>
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<td>Brown</td>
<td>Absent</td>
<td>Absent</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>CBS 274.93</td>
<td>Center: pale olive buff (1.5-cm-diameter)</td>
<td>600</td>
<td>Spherical to hemispherical</td>
<td>Blackish mouse gray</td>
<td>Absent</td>
<td>Absent</td>
<td>NT</td>
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<td></td>
<td>CBS 275.93</td>
<td>Center: white (2 to 3-cm-diameter)</td>
<td>553</td>
<td>Spherical to hemispherical</td>
<td>Blackish mouse gray</td>
<td>Absent</td>
<td>Absent</td>
<td>NT</td>
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<td>CBS 335.47</td>
<td>Pale olive gray</td>
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<td>Spherical to hemispherical</td>
<td>Mouse gray</td>
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<td>Absent</td>
<td>NT</td>
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<td>3089</td>
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<td>Yellow ocher to Present</td>
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**Notes:**

a: Color description was based on: Ridgway, R. 1912. Color Standards and Color Nomenclature. Press of A HOEN and Company, Baltimore, MD. Observation was made 3 weeks after incubation.
b: Average number was based on three PDA plates.
c: Not available
d: Not tested
FIGURES

Fig. 1. One of 128 equally most parsimonious trees of partial sequences of the 28 S large subunit (LSU) region of rDNA from *Sclerotium* species and other Basidiomycota and Ascomycota. The tree is rooted to *Saccharomyces cerevisiae*. 127 parsimony informative characters. Tree length = 385, consistency index (CI) = 0.6182, homoplasy index (HI) = 0.3818, CI excluding uninformative characters = 0.5751, HI excluding uninformative characters = 0.4949, retention index (RI) = 0.9293, rescaled consistency index (RC) = 0.5754. Bootstrap values > 50% are indicated above branches. Branches in bold are supported by strict consensus of the most parsimonious trees.

Fig. 2. One of 8448 equally most parsimonious trees of partial sequences of the ITS region of rDNA from isolates of *Sclerotium cepivorum*, *S. denigrans* and *S. perniciosum* and other Ascomycota. 29 parsimony informative characters. The tree is rooted to *Chloroscypha enterochrom*. Tree length = 189, consistency index (CI) = 0.9524, homoplasy index (HI) = 0.0476, CI excluding uninformative characters = 0.8163, HI excluding uninformative characters = 0.1837, retention index (RI) = 0.9118, rescaled consistency index (RC) = 0.8683. Bootstrap values > 50% are indicated above branches. Branches in bold are supported by strict consensus of the most parsimonious trees.

Fig. 3. One of 4 equally most parsimonious trees of partial sequences of the ITS region of rDNA from isolates of *Sclerotium hydrophilum*, *Ceratorhiza oryzae-sativae* and *S. rhizodes* and other Basidiomycota. 174 parsimony informative characters. The tree is rooted to *Agaricus bisporus*. Tree length = 706, consistency index (CI) = 0.8003, homoplasy index
(HI) = 0.2962, CI excluding uninformative characters = 0.7038, HI excluding uninformative characters = 0.2962, retention index (RI) = 0.8604, rescaled consistency index (RC) = 0.6886. Bootstrap values > 50% are indicated above branches. Branches in bold are supported by strict consensus of the most parsimonious trees.
10 base pair changes

S3

S1

S2

FIG. 1
- 1 base pair change

FIG. 2.
FIG. 3

- 10 base pair changes

Sclerotium rhizodes CBS321.68
Sclerotium rhizodes CBS276.69
Sclerotium rhizodes CBS126.13
Ceratobasidium sp. AJ302007
Rhizoctonia sp AF222793.
Ceratobasidium sp. DQ278930
Ceratobasidium cereale AJ302009
Ceratobasidium sp AF354086.
Rhizoctonia sp AF063019.

Thanatephorus cucumeris AF354075
Thanatephorus cucumeris AF354076
Ceratobasidium cornigerum AJ302010

ceratobasidium oryzae-sativae CBS439.80
Ceratobasidium oryzae-sativae DQ307249
Ceratobasidium oryzae-sativae CBS235.91
Ceratobasidium sp. AJ000194
Rhizoctonia sp AJ318425

Sclerotium hydrophilum DQ875597
Sclerotium hydrophilum EU152863
Sclerotium hydrophilum EF202574
Sclerotium hydrophilum CBS201.27
Sclerotium hydrophilum CBS385.63

Rhizoctonia sp. AY927346
Ceratobasidium sp. DQ279044
Ceratobasidium sp. DQ102400

Agaricus bisporus DQ404388
CHAPTER 5. GENERAL CONCLUSIONS

Petiole rot, incited by *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*, causes substantial losses in hosta plantings in the U.S. The focus of this dissertation was improving understanding of the ecology and disease management of hosta petiole rot, and taxonomic placement of hosta petiole rot pathogens and other plant-pathogenic *Sclerotium* species. Many questions about this pathosystem remain to be addressed, however.

Published information suggested that *S. rolfsii* is more prevalent in relatively warm regions such as the southern U.S., Brazil, and South Africa, whereas *S. rolfsii* var. *delphinii* has been reported primarily from cooler areas such as the northern and midwestern U.S. The cause of this apparent difference in geographic distribution is unknown. We tested the hypothesis that regional differences in temperature could affect the survival of sclerotia of *S. rolfsii* and *S. rolfsii* var. *delphinii* and thereby influence their geographic distribution. The findings supported the hypothesis and were the first evidence that *S. rolfsii* may be unable to overwinter long enough in the northern U.S. to persist as a pathogen of hosta from year to year. A possible explanation for the apparent predominance of *S. rolfsii* var. *delphinii* as the hosta petiole rot pathogen in the North may be inability of *S. rolfsii* sclerotia to survive in this region until June, when the onset of warmer temperatures increases the risk of petiole rot. To further validate this preliminary conclusion, future research should expand the range of experimental sites and probably the number of isolates for both fungi.

Developing a more rapid screening technique would make it feasible to rate hundreds of hosta cultivars for petiole rot resistance. Results from our study suggest that screening method using oxalic acid on a cotton swab can dramatically speed up identifying hosta
cultivars that are highly resistant or susceptible to petiole rot. Our results were encouraging evidence that screening under controlled conditions could accurately represent cultivar resistance to petiole rot in the real world, but much more rapidly and efficiently. Most commercial hosta breeders maintain collection of field-grown cultivars and do not have access to laboratory equipment. Therefore, the next logical steps making our assay practical for hosta breeders should be i) applying oxalic acid methods on excised leaves of field-grown plants and ii) attempting to further simplify the screening process by avoiding the need for specialized laboratory equipment and expertise.

Taxonomy of species in the genus *Sclerotium* has been questioned for decades. Some anamorphic species of *Sclerotium* have been found to connect to genera of Basidiomycetes, such as *Typhula*, *Athelia*, and *Ceratobasidium*, but others to Discomycetes, such as *Stromatinia*. Our study clarified phylogenetic placement of eight *Sclerotium* species. Two new combinations are proposed based on rDNA sequences analyses and morphological characteristics:

*Ceratorhiza hydrophilum* (Sacc.) Xu, Harrington, Gleason, et Batzer, *comb. nov.*

*Ceratorhiza rhizodes* (Auersw.) Xu, Harrington, Gleason, et Batzer, *comb. nov.*

The results from the Appendix of the taxonomic study (Chapter 4) suggested that *S. rolfsii* and *S. rolfsii* var. *delphinii* should be two different species. We are currently in the process of examining all three regions of ITS, beta-tubulin and elongation factor-alpha and then doing phylogenetic analysis.

To confirm the taxonomic status of *Sclerotium* species, subsequent research should include attempts to produce teleomorph states, assessment of ecological and physiological
characters, and even more genetic evidence for *Sclerotium* species. Researchers should also access and test more plant-pathogenic *Sclerotium* species that were not included in our study.

This research provides basic information on the ecology, resistance screening and genetics of hosta petiole rot pathogens and other *Sclerotium* species. These studies may be valuable to people affected by and interested in diseases caused by *S. rolfsii*, *S. rolfsii* var. *delphinii*, and other *Sclerotium* species.

The results of the survival study may inform decision making on disease management strategies for growers in different areas in the U.S. Along with information on phylogenetic relationship of *S. rolfsii* and *S. rolfsii* var. *delphinii* provided in the Appendix II and further phylogenetic analysis, our results would contribute to the clarification of taxonomic status for *Sclerotium* species. Clarifying phylogenetic placement of plant-pathogenic *Sclerotium* species could help growers choose appropriate fungicides, and therefore could improve the effectiveness of strategies used to manage the diseases caused by these phytopathogens. Identifying cultivars with high levels of resistance would benefit the entire hosta industry by providing a durable, cost-effective option to combat this highly destructive, difficult-to-manage disease.
### APPENDIX I. REPRESENTATIVE ASCOMYCETES AND BASIDIOMYCETES USED IN PHYLOGENETIC ANALYSIS OF *SCLEROTIUM* SPECIES IN CHAPTER 4

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1. All Genbank accession numbers are for **ITS rDNA** unless otherwise specified.
Partial LSU and ITS sequences of taxa with high homology to *Sclerotium* species sequences, and additional ITS and LSU sequences of representative basidiomycota and ascomycota taxa were downloaded for phylogenetic analysis.
APPENDIX II. PHYLOGENETIC RELATIONSHIP OF SCLEROTIUM ROLFSII AND S. ROLFSII VAR. DELPHINII (TABLE AND FIGURE)

Table. Isolates of Sclerotium rolfsii and S. rolfsii var. delphinii included in the study

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<sup>1</sup>Timothy B. Brenneman, University of Georgia  
<sup>2</sup>Zamir K. Punja, Simon Fraser University  
<sup>3</sup>Mark L. Gleason, Iowa State University  
<sup>4</sup>Centraalbureau voor Schimmelcultures, The Netherlands
Fig.

One of 8 equally most parsimonious trees of combined sequences of the β-tubulin gene, translation elongation factor 1-α gene and ITS region of rDNA from isolates of *Sclerotium rolfsii* and *S. rolfsii* var. *delphinii*. The tree is rooted to *Coniophora puteana*. Gaps were treated as “missing data”. A bootstrap analysis of 500 replications was generated. Tree length = 1338, Consistency index (CI) = 0.7668, Homoplasy index (HI) = 0.2332, CI excluding uninformative characters = 0.6268, HI excluding uninformative characters = 0.3732, Retention index (RI) = 0.8323, Rescaled consistency index (RC) = 0.6383. 379 parsimony informative characters. Bootstrap values > 50% are indicated above branches. Branches in bold are supported by strict consensus of the most parsimonious trees.
Fig. 10 base pair changes
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Thank you to Mark L. Gleason, my major professor, for giving me confidence, support and care throughout my Ph.D. studies. Thank you for providing a stimulating and fun environment in which to learn and grow. Your have had a remarkable influence on my entire career in the field of plant pathology research.

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