Chemical and Biological Control of Silvery Threadmoss (*Bryum argenteum* Hedw.) on Creeping Bentgrass Putting Greens

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ABSTRACT

Silvery threadmoss is a problematic weed of golf putting greens, growing interspersed with turf, decreasing aesthetic quality and playability. Moss is typically controlled postemergence and currently only one herbicide, carfentrazone, is registered for silvery threadmoss control on greens. Carfentrazone controls moss up to 75% applied at a three week interval throughout the growing season. Alternatives providing longer residual or more effective control are desirable. Studies were conducted to examine the growth of moss gametophytes from spores and bulbils and to evaluate turf protection products for pre and postemergence moss control. Moss gametophytes develop best from spores at 30°C and from bulbils at 23°C. Products which control moss equivalent to carfentrazone (>70%) both pre and postemergent include sulfentrazone, saflufenacil, flumioxazin, oxadiazon, and oxyfluorfen. Fosamine and fosetyl-Al alone controlled moss equivalent to carfentrazone post-, but not preemergent. ^{14}C glyphosate absorption and translocation through moss colonies was examined from 12 to 192 hours after treatment (HAT) to understand how herbicides are absorbed by silvery threadmoss. It appears that ^{14}C reaches equilibrium by 24 HAT in capillary water of the moss colony and inside moss tissues. Subsequently, ^{14}C is lost to the system presumably through microorganism degradation of ^{14}C glyphosate in capillary water. The final objective of this work was to identify and evaluate two fungal organisms observed to cause disease of

silvery threadmoss on putting greens in efforts to develop a biological control. The organisms were identified by morphology and *ITS* sequence as *Alternaria* sp. and *Sclerotium rolfsii*. *Alternaria* sp. causes a leaf disease of silvery threadmoss and *Sclerotium rolfsii* causes Southern blight of silvery threadmoss. Host specificity testing demonstrated moderate pathogenicity of *S. rolfsii* to annual bluegrass but not to 'Penn A4' creeping bentgrass. Both organisms have potential to be effective biological controls for silvery threadmoss; however, host specificity indicates *Alternaria* sp. may be a better choice. Data from these experiments suggest herbicides in two chemical classes control mosses both pre and postemergence, and sulfentrazone, fosetyl-Al, and *Alternaria* sp. may be new alternatives to carfentrazone for use on golf putting greens.

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Chapter 1. Literature Review

Golf course putting greens are an intensively managed recreational fine turf with a unique suite of weed problems. Silvery threadmoss is currently one of the most troublesome weeds on putting greens (Happ 1998; Hummel 1986). Several researchers have attributed the increasing moss problem to the loss of mercury-based pesticides (Hummel 1986; Radko 1985; Vargas 1994). Beginning in 1976, the U. S. Environmental Protection Agency (EPA) banned mercury-based pesticides, granting only limited uses in turfgrass for some fungicides such as phenyl mercuric acetate (PMAS, Cleary Chemicals, LLC, Dayton, NJ 08810) through 1994 (United Nations 2002). Other heavy metal-based pesticides containing cadmium and arsenic were also banned by EPA within the same time frame (EPA 1998). These products were mainly used as fungicides in turfgrass until 1994 (Latin 2011; Shurtleff et al. 1987; Vargas 1994). Though previous studies have not addressed silvery threadmoss control with heavy-metal pesticides, environmental studies have shown several moss species are negatively affected by heavy metals, particularly mercury (Panda 2003; Simola 1977). Kaur and Kumar (2010) reported Hg concentrations as low as 0.05 ppm prevented spore germination for seven different moss species, though they did not report what form of mercury was utilized. No research has reported an LD_{50} of Hg specific to silvery threadmoss; however, exposure to 20 ppm mercury is sufficient to kill *Sphagnum* species which is a lower threshold than in higher plants (Simola 1977).

Some pesticides may enter mosses by exchanging with cations bound to cell surfaces at cation binding sites. Brown and Wells (1990) found that mosses will “leak”

cations such as potassium, calcium and magnesium into surrounding substrate in the presence of heavy metals such as cadmium. Richardson (1981) reported that as much as half of the Mg+ present in bryophyte cells may be exchangeable for other cations. The release of exchangeable cations in favor of heavy metals may explain why silvery threadmoss was once easily controlled with heavy-metal based pesticides and why it is now an increasingly problematic weed on putting greens.

Another factor contributing to the increase in silvery threadmoss on putting greens is mowing height. To meet golfer demand for firmer and faster playing surfaces superintendents have decreased mowing heights recently (Turgeon 2005). In the state of Virginia, and throughout much of the Southeastern U.S., most courses are mowing greens between 3 and 4 mm (GCSAA 2012) requiring increased passes of equipment over the green which can fragment moss and spread the propagules. More passes with equipment along with decreased nutrient inputs and an open turf canopy all contribute to moss encroachment on golf course putting greens (Hummel 1994; Radko 1985).

The most common species of moss infesting golf greens is silvery threadmoss (*Bryum argenteum* Hedw.) (Borst et al. 2008). However, silvery threadmoss is not the only weedy moss affecting golf greens. Several other species have been reported in the mid-Atlantic U.S. including *Amblystegium serpens*, *Amblystegium trichopodium*, *Brachythecium* sp., *Bryum lisae*, and *Entodon seductrix* (Borst et al. 2008). Silvery thread and other moss species tend to encroach into weak turf and bare areas and proliferate where competition is low. There are few products available to golf course superintendents that can help manage moss and, of those products, none provide complete control.

A lack of labeled products for moss control has driven turf managers to experiment with many off-label substances including peroxides, baking soda, lime, and Ultra Dawn dish detergent and fatty acid soaps. Data describing efficacy of these off-label products routinely appear in trade magazine articles (Cook et al. 2002; Happ 1998; Hummel Jr. 1994; Landschoot et al. 2004; Nelson 2007). These products have activity against moss but can also be damaging to turfgrass at rates needed to effectively control moss (Kennelly et al. 2010; Thompson et al. 2011). They are all desiccants which act against the moss by dehydrating it, and severe turf injury can result if care is not taken during application, not to mention application of these off-label products is illegal (Kennelly et al. 2010; Thompson et al. 2011). Efficacy testing for many of these products has had inconsistent results for control of silvery threadmoss, and all researchers reporting effective control with sodium bicarbonate and potassium bicarbonate also report unacceptable injury to desired turfgrass (Kennelly et al. 2010; Thompson et al. 2011).

Several fungicides are reportedly effective at controlling silvery threadmoss. The premix mancozeb + copper hydroxide (Junction, SePRO Corporation, Carmel, IN) is labeled for moss control on putting greens; however, several researchers have reported poor control (<15%) (Borst et al. 2010; McCalla et al. 2002). Many studies report silvery thread moss control with chlorothalonil greater than 70% (Burnell et al. 2004; Gelernter & Stowell 1999; Kennelly et al. 2012; McCalla et al. 2002), sometimes with effective residual control of moss up to eight weeks after treatment (McCalla et al. 2002). However, other studies have found no moss control with chlorothalonil (Cook et al. 2002; Fausey et al. 2003).

Fertilizer products, especially those containing heavy or transition metals, have also been effective at controlling silvery threadmoss in some situations. In the past, Burnell et al. (2004) found that fertilizers containing iron could be used to reduce silvery thread moss population densities on bentgrass putting greens though McCalla et al. (2002) reported no control of moss with ferrous ammonium sulfate ($FeNH_4SO_4$) at 317 ml 100 m^{-2}. These varying results are plausible based on work by Vukojevic et al. (2004) showing that ferric(III)citrate an organic chelated form of Fe is more bioavailable to silvery threadmoss than potassium hexacyanoferrate(III). Bioavailability based on organic matter content, pH, and other soil conditions may alter the effectiveness of fertilizer treatments for control of silvery threadmoss. Fausey et al. (2003) reported short residual control of silvery threadmoss with copper sulfate at 195 kg ha^{-1} lasting only 7 to 14 days. Boesch and Mitkowski (2005) reported excellent moss control with silver nitrate; however, there are concerns over potential toxicity and the environmental fate of this product. Most of the heavy and transition metal containing fertilizers, while having the potential to control silvery threadmoss, also need to be applied at high rates which can build up to toxic levels in soils and cause phytotoxicity to desirable turf (Borkert et al. 1998).

Only a few herbicides have been reported in peer-reviewed literature to effectively control silvery threadmoss. In containerized ornamentals, flumioxazin, oxyfluorfen, pelargonic acid, and oxadiazon controlled silvery thread moss acceptably (>80%) (Fausey 2003); however, only oxadiazon is labeled for use on fine turf (Anonymous 2007), and many of the others would likely cause significant injury to bentgrass putting greens. Endothal has also been used in a long-term experiment where

repeated yearly applications controlled silvery threadmoss in the fourth and fifth years of the study (Brauen et al. 1986). The current industry standard for silvery threadmoss control on golf putting greens is carfentrazone (Quicksilver, FMC Corporation, Philadelphia, PA). It is effective at suppressing silvery threadmoss but moss shoots quickly recover and sequential applications are required for acceptable control (Borst et al. 2007; Anonymous 2005), increasing the pesticide load on greens and increasing the cost to the grower. Borst et al. (2010) also showed increased control using sequential applications of carfentrazone plus nitrogen or topdressing or both, controlling moss up to 78% though 16 weeks after initial treatment (WAIT) (Borst et al. 2010). However, applied alone at the labeled rate twice at a two-week interval, the best control was only 77% at 3 weeks after initial treatment, dropping to 43 and 36% control at 5 and 11 WAIT, respectively. In 2008, Borst et al. reported control up to 86% 3 WAIT with carfentrazone applied in the same way; however, control dropped to only 39% by 4 WAIT.

It is clear that control with carfentrazone requires repeat applications throughout the season to remain effective against moss. However, several superintendents across the Commonwealth and researchers at Virginia Tech have found that even repeat applications of carfentrazone throughout the growing season do not always control silvery threadmoss (Post et al. unpublished data). In addition, legally, carfentrazone may only be applied four times per year at the rate required for moss control. However, it is likely that many golf course superintendants exceed this rate with 6 or more applications per year when moss is a major problem on their course. With limited peer-reviewed information available on effective chemical control, and the increasingly common

occurrence of moss as a problematic weed of golf putting greens, the evaluation of proper moss management is highly desirable.

Though there are no peer-reviewed studies examining the fate of radiolabeled herbicides in mosses, there are several studies examining the activity of certain herbicides against various moss species. Wacker et al. (1988) noted the microtubule inhibitor oryzalin reduced growth and interfered with polar migration of microtubules within protonematal cells of the moss *Funaria hygrometrica*. Asulam, a herbicide thought to interfere with microtubule assembly and function and inhibit folic acid biosynthesis (Hoffman and Vaughn 1994; Stephen et al. 1980; Veerasekaran et al. 1981), inhibited moss gametophyte elongation in 21 moss species in sterile culture (Rowntree and Sheffield 2005; Rowntree et al. 2003). In the field, Newmaster et al. (1999) demonstrated some mosses and lichens are susceptible to glyphosate and triclopyr citing decreases in abundance and species diversity as a result of glyphosate or triclopyr applications over a period of two years. But other studies with glyphosate have found little to no activity against moss species (Narvaez Parra et al. 2005; Ronoprawiro 1975). Based on the literature, it is clear that mosses, like higher plants, vary in susceptibility to herbicides depending on the species.

No research to date has addressed the susceptibility of silvery threadmoss to glyphosate, an amino acid synthesis inhibitor, which inhibits the EPSP synthase enzyme in the shikimic acid pathway responsible for manufacture of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Amrhein et al. 1980; Jaworski 1972). We have observed silvery threadmoss tolerance to glyphosate though the mechanism of glyphosate tolerance is unclear. One hypothesis for mosses in general is that some herbicides simply

do not get absorbed into the plant due to lack of a true vascular system. An evaluation of whether herbicides can move into moss tissues and how they move across and within a colony would better inform management decisions about herbicide use for silvery threadmoss control.

Biology. Silvery threadmoss is an endohydric acrocarpous moss species with leaves but no stomata and no leaf buds at the base of each leaf. Leaf lamellae allow for gas exchange through the leaf surface. Each plant in a colony has a well-developed central strand of internally conducting tissue, and the ability to conduct water over their outer surface through capillary action (Glime 2007). Silvery threadmoss has been reported to survive both desiccation and dark conditions of herbaria for a period of two years resuming normal protein synthesis upon rehydration (Malta 1921; Richardson 1981). Many mosses also produce cuticular waxes to prevent desiccation (Richardson 1981), and silvery threadmoss gets its name for the thick layer of cuticular wax on its leaves. Mature shoots also have the capability to adjust the architectural structure of their leaves in the field to avoid desiccation (Richardson 1981). Some mosses can maintain net photosynthesis at water potentials -5.5 to -20 bars depending on the species (Busby and Whitfield 1978; Dilks and Proctor 1979; Richardson 1891). In comparison, most land plants experience negative photosynthesis at -12 to -15 bars (Richardson 1981). Moss cell walls have a high polyphenol content which also makes them resistant to decomposition (Davey & Currah 2006).

The species is dioecious with a female-biased field sex ratio and a primary sex ratio of 1:1 (Stark et al. 2010). Horsley et al. (2011) found that both sexes are equal in their ability to produce protonemal biomass, shoot biomass, and axillary bulbils.

However, males produce 24 times more inflorescences than females (Horsley et al. 2011). Mature sporophytes are produced infrequently in silvery threadmoss, which may explain the female-biased sex ratio. Rydgren et al. (2010) described a similar phenomenon for *Hylocomium slendens,* which also exhibits a strongly female-biased field sex ratio. On golf putting greens, mature sporophytes are produced even less frequently than in nature due to daily mowing which inhibits the development of sporophytes which are borne on a 1-2 cm stalk (Crum and Anderson 1981). However, spores are likely still present in this system as they are wind and water dispersed and can be moved over long distances (Miles and Longton 1992). Most reproduction on putting greens likely results from the two types of moss vegetative propagules, bulbils and plant fragments (Best 1904). Silvery threadmoss produces asexual vegetative structures termed protonemal and axillary bulbils (Best 1904) which can easily detach from the mother plant and quickly generate a moss plant at a new site. It is unclear or undescribed in the literature what causes silvery threadmoss to initiate these structures, but in research plots at Virginia Tech, Blacksburg VA, they are observed in the spring and fall of the year when seasonal temperature changes begin and also shortly after core aerification, verticutting, and/or vigorous topdressing. A single moss shoot will produce several bulbils during each event, having the potential to increase a moss population several-fold (Smith 1999). Axillary bulbils detach from the mother plant through schizolysis and may be dislodged by water or mechanical means. Bulbils can presumably be moved from green to green on golf shoes and maintenance equipment. They also float and can move around the green in flowing water from heavy rain events or even irrigation (Rudolph 1970; Smith 1999). Common cultural practices used in the maintenance of golf greens

can also enhance moss propagation. Based on the biology of silvery threadmoss, frequent mowing, core aerification and verticutting of greens all have the potential to create and move moss fragments across the green allowing new colonies to form. Alternatives to these disruptive cultural practices would be alternating mowing events with rolling to potentially reduce the fragmentation of moss colonies decreasing the spread of moss across the green, while maintaining the same ball roll distance (Dernoeden 2000). Only a small fragment of healthy tissue is required for a moss plant to fully regenerate (Best 1904; Smith 1999). Though the hypothesis was not directly tested, studies at Virginia Tech indicate fragments as small as 0.5 mm long can regenerate into a new shoot.

Mosses have the capability to bioaccumulate or hyperaccumulate metals from their immediate environment and many moss species have been used for bioremediation or as bioindicators of environmental contaminants especially when those pollutants contain metals (Brown and Wells 1990; Bull et al. 1977; Cameron and Nickless 1977; Little and Martin 1974; Richardson 1981; Richardson et al. 1980; Winner et al. 1978). Several moss species, including silvery threadmoss, bioaccumulate both heavy and transition metals (Brown and Wells 1990; Kaur et al. 2010; Martensson and Berggru 1954). Many angiosperms when exposed to the strong natural selection pressure of toxic levels of metal concentrations in the environment quickly adapt to tolerate high levels of toxic metals sometimes within a few generations (Antonovics et al. 1971; Walley et al. 1974; Woolhouse 1983). However, this type of adaptation is usually metal-specific (Shaw 1993). Contrastingly, many moss species have wide tolerance levels to many metals even when they have not been recently exposed to heavy selection pressure in

their immediate environment (Gullvag 1974; Shaw and Albright 1990). Silvery threadmoss likely falls into this category with evidence from Shaw et al. (1988; 1990), who examined seven different populations from varyingly contaminated sites and found no differences in response to media supplemented with lead, copper, nickel, or zinc.

Moss pathogens. Compared to land plants where hundreds of pathogens have been described, only a few pathogens have been reported for bryophytes, which some researchers attribute to a lack of investigation rather than the lack of existence of such organisms (Bradner et al. 2000; Davey and Currah 2006; Longton 1973; Mitkowski and Chaves 2013; Morita 2009; Wilson 1951). The earliest report of a potential moss pathogen was from the Oxford University Expedition to Jan Mayen Island in 1947 when Wilson (1951) described concentric "fairy rings" in moss colonies of *Rhacomitrium canescens* var. *ericoides*. A recent mini-review by Davey and Currah (2006) examined the current state of knowledge for moss-fungus interactions, and indicated it is likely that all pathogenic interactions which occur in higher plants also occur in bryophytes, but these processes are not well understood in bryophytes. There are reported obligate, facultative and opportunistic pathogens, as well as mycorrhizae (Rabatin 1980) associated with mosses. Most suggest pathogens of mosses penetrate the tissues using enzymes to digest portions of the cell wall to gain entry (Redhead 1981; Untiedt and Muller 1985; Simon 1987; During and van Tooren 1990). Mosses have cell walls composed of polyphenolic compounds and cellulose (Popper and Fry 2003; Lee et al. 2005) though it is generally accepted that most mosses lack the ability to produce true lignin (Basile et al. 1999; Goffinet et al. 2009; Shoefield and Hebant 1984; Umezawa 2003; Weng and Chapple 2010). A lack of lignin may allow for easier enzymatic

degradation of the cell walls and other structural components of the cells (Davey and Currah 2006).

Several researchers have described fungal presence in moribund colonies of mosses. In 1999, Hoshino et al. reported *Pythium ultimum* Trow var. *ultimum* on the moribund moss *Sanionia uncinata* (Hedw.) Loeske in the northern islands of Norway. The earliest reports come from Longton (1973) who described fungal infections on *Drepanocladus uncinatus* colonies caused by *Thyronectra antarctica* var. *hyperantarctica.* Overall, only a few pathogens have ever been reported for mosses. Longton (1973) was also the first to report an apparent disease in a *Bryum* species. On the arctic Ellesmere Island, *Bryum cryophyllum* was infected with an unknown fungal pathogen having septate hyphae without clamp connections. The organism was not further described except to indicate that it was killing the moss in single rings up to 10 cm in diameter (Longton 1973).

More recently, *Waitea circinata* Warcup and Talbot was reported as a fungal pathogen causing disease on silvery threadmoss in Colorado (Mitkowski and Chaves 2013). Waitea patch is a common disease of annual bluegrass (*Poa annua* L.), and less commonly creeping bentgrass (*Agrostis stolonifera* L.), causing rings of yellowing necrosis on infected turf (Wong and Kaminski 2007). This particular pathogen would not be a good candidate for selective biological control of silvery threadmoss on creeping bentgrass putting greens because it can discolor moss to almost white and has potential for injury to desirable turf. While selective control of moss is desirable, an aesthetically pleasing green color is generally more desirable in golf turf. Waitea patch is one of three organisms reported in the literature specifically associated with silvery threadmoss. In

2008, a Japanese scientist isolated and identified *Sclerotium rolfsii* from diseased silvery threadmoss tissues and named the disease southern blight of *Bryum argenteum* (Morita 2009). Bradner et al. (2000) described a previously unknown *Embellisia* sp. associated with silvery threadmoss in the Antarctic. *Embellisia* species have often been found in association with some higher plants, sometimes as endophytes but more often as pathogens (Lee et al. 2002). It was unclear whether the species described by Bradner et al. (2000) was endophytic or pathogenic on silvery threadmoss.

In this research, two naturally occurring fungi which have been observed effectively killing silvery threadmoss in a fine turf setting will be evaluated for their potential as biological control agents. There are currently no biocontrol agents available on the market to control moss in turfgrass. The development of a biological control product suitable for use in fine turfgrass, particularly golf course putting greens, has the potential to increase recreational value. The last Virginia turfgrass survey was performed by USDA-NASS (2006), at which time the state boasted 345 public, private and semi-private golf courses with 36,900 acres of maintained turfgrass. A majority of reporting golf courses (53%) indicated weeds were a major management problem on their course (USDA 2006). Moss is surely present as part of this 53% and across the state golf courses spent 2.8 million on weed control products alone.

All Virginia courses taken together received 8.65 million rounds of play in 2004 which encompasses a large portion of Virginia recreational tourism dollars (USDA 2006). If a single golfer plays a hypothetical average of 20 rounds per year, this means golf courses around the state are seeing 432,500 golfers walk across their greens. Putting greens free of moss encroachment increase the ease of play for the golfer and provide an

overall better experience. If golf courses are able to provide an exceptional experience for their golfers by providing a higher quality product, they are more likely to attract more and repeat golfers, meaning more revenue for the year.

At the same time a decrease in the use of chemical pesticides in favor of an effective biological control organism would provide increased benefits to the natural environment as well. Many golf courses are situated near waterways where pesticide runoff could become a problem at point-source locations and downstream. Any decrease in pesticide use on those properties is considered a positive for managed and natural landscapes.

Objectives

The objectives of this research were to 1) evaluate and characterize two fungal pathogens proving pathogenicity against silvery threadmoss and safety to common putting green turf species, 2) evaluate a suite of preemergence and postemergence herbicides, and fungicides for efficacy against moss, and 3) to begin to understand absorption, translocation, and metabolism of herbicides within moss plants using ^{14}C radio-labeled glyphosate.

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Chapter 2. Preemergence Control of Silvery Threadmoss Grown from Spores and Bulbils in Axenic Culture

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Silvery threadmoss (*Bryum argenteum* Hedw.) naturally reproduces through spore and bulbil production, both of which have potential to be controlled prior to establishment. Studies have not evaluated effects of turf protection products on moss protonema or gametophyte growth from spores or bulbils, and most moss is controlled postemergence on putting greens. Initial studies were performed to determine the optimal growth temperature for spores and bulbils in sterile culture. Protonemata from spores grew optimally at 29.5°C and gametophytes from bulbils grew optimally at 22.5°C. Three subsequent in vitro studies were conducted to evaluate effects of turf protection products on moss development from spores or bulbils in axenic culture at a constant 24°C. Carfentrazone, which effectively controls mature moss gametophytes postemergence, also reduced green cover of moss protonemata and gametophyte production from spores and bulbils. All combinations with carfentrazone reduced area under the progress curve (AUPC) for green cover of moss for both spores and bulbils by 80% or more by three weeks after treatment. Sulfentrazone, oxyfluorfen, oxadiazon, saflufenacil, flumioxazin, and pyraflufen-ethyl reduced AUPC of moss equivalent to carfentrazone for both propagule types. The two fosetyl-Al products, phosphite, and mineral oil caused an increase in moss cover between 22 and 113% of the nontreated for spores; however, only

methiozolin positively influenced AUPC (90.2%) compared to the nontreated for bulbils. Though silvery threadmoss is typically targeted postemergence on putting greens, there are products labeled for moss control that can provide preemergence control, including the industry standard of carfentrazone. These data suggest that differences may occur between turf protection products in their ability to suppress moss establishment from spores or bulbils.

Nomenclature: carfentrazone; flumioxazin; oxadiazon; oxyfluorfen; pyraflufen-ethyl; saflufenacil; sulfentrazone; silvery threadmoss; *Bryum argenteum* Hedw.; creeping bentgrass; *Agrostis stolonifera* L.

Keywords: moss control; putting green; silver moss.

Silvery threadmoss (*Bryum argenteum* Hedw.) is a major weed of golf course putting green turf capable of producing three propagules types including spores, bulbils and fragments with varying longevity (Best 1904; Miles and Longton 1992). Silvery threadmoss is typically managed postemergence and there are no herbicides labeled for preemergence control. During sexual reproduction spores are produced by the sporophyte. Sporulation rarely occurs on putting greens since the spore capsule is borne on a stalk that is approximately 2-cm long and mowing would remove it before maturity (Crum and Anderson 1981). In the state of Virginia, and throughout much of the Southeast, most greens are mowed between 3 and 4 mm so it is unlikely that sporulation could occur (GCSAA 2012). However, spores may be produced by colonies nearby a green in higher heights of cut, and spores are spread by wind and water so they may come from long distances to infest a putting green (Miles and Longton 1992). The most common way silvery threadmoss spreads around a green is through the two types of vegetative propagules, fragments and bulbils (Best 1904). Moss fragments are created by mowers and other equipment which are used daily on most putting greens. Each healthy moss fragment, as small as only a few cells, can regenerate an entire plant (Best 1904; Smith 1999). Bulbils are a vegetative reproductive structure formed by silvery threadmoss which consist of a few leaves attached to a central stem with no rhizoid. It is unclear what physiological or environmental conditions trigger the development of bulbils, but in research plots at Virginia Tech in Blacksburg, VA, they have been observed to form on silvery threadmoss in early spring and after periods of stress such as aerification, topdressing, and verticutting. The bulbils are easily released mechanically by splashing water, movement of equipment, or foot traffic and they can presumably be

moved from green to green by each of these means. They float and can move in water as well (Rudolph 1970; Smith 1999).

Though many other weeds, especially those with small propagules, are commonly controlled with preemergence herbicides, no products are labeled for preemergence moss control and superintendents have no choice but to look to postemergence measures. Preemergence control options would be valuable to the golf industry. The physiology of bryophytes is different from that of vascular plants and many products used for vascular plant control may not affect moss growth. Most studies that have evaluated pesticides or fertilizers for moss control have been limited in scope, testing only a few products (Burnell et al. 2004; Fausey 2003; Kennelly et al. 2010). A broader evaluation of herbicide, fungicide, plant growth regulator and fertilizers on moss growth and development is needed.

The first step in screening large numbers of products on moss or to evaluate preemergence control is to develop simple methods for culture of moss in growth chambers, greenhouse, and laboratory settings. Establishing axenic cultures from surface-sterilized spore capsules is the most commonly used method to obtain sterile bryophyte cultures, and methods have been described for many species mostly for the purposes of conservation and restoration (Basile and Basile 1988; Duckett et al. 2004; Rowntree 2006; Sabovljevic 2003; Sargent 1988). Murashige and Skoog (MS) medium (Murashige and Skoog 1962) is the most common medium used and it can be adjusted with many additives to meet the specific needs of each species. Though Sabovljevic et al. (2002) described axenic methods for culturing silvery threadmoss from spore capsules and gametophyte shoots, it was a short communication and did not fully describe

comprehensive methods for culturing this moss for the intentions of research. Jones and Rosentreter (2006) described methods for growing three moss species including silvery threadmoss on artificial substrates and native soils for the purposes of restoration. But these methods lacked important details such as optimum temperature for growth. Liang et al. (2010) also described methods for culturing silvery threadmoss in liquid media in order to produce moss gametophores for commercial applications; however, liquid suspensions of moss would not accurately simulate field conditions.

Therefore, the first objectives of this work were to determine the optimal temperature for sterile culture of silvery threadmoss from spores and bulbils. The primary objective was to evaluate herbicide, fungicide and combination treatments for preemergence control of bulbils and spores, both propagules which are naturally formed by silvery threadmoss.

Materials and Methods

Bulbil Preparation for Axenic Culture. Silvery threadmoss 'bulbils' were collected in March and December 2011 and placed in cold storage at 4°C until trials were initiated in summer 2012. In a laminar flow hood, bulbils were surface sterilized with 10% bleach for two minutes and then rinsed twice in sterile water for two minutes each. Bulbils were then rinsed into a 100-mm polystyrene Petri dish (Fisher Scientific, Pittsburg, PA) of sterile water where they retain the ability to float even after sterilization. Approximately 50 bulbils were removed from the surface of the water and rinsed onto 60 mm Petri plates with a pipette stream of 400 μL sterile water. Plates were swirled on the benchtop to

evenly distribute the water and bulbils over the surface. Each 60 mm plate contained 4 mm depth MS medium plus Gamborg vitamins (MP Biomedicals LLC, Solon, OH) in a 0.7% Phytagel (Sigma Chemical Co., St. Louis MO). Plates were sealed with Parafilm to prevent moisture loss.

Spore Preparation for Axenic Culture. Silvery threadmoss spore capsules were collected in March 2011 and March 2012 and placed in cold storage at 4°C until experiments were initiated in summer 2012. In a laminar flow hood sporophytes were surface-sterilized in a 10% bleach solution for two minutes. Capsules were then rinsed twice in 1 mL of sterile water for one minute each. Twenty sterile capsules were placed in 5 mL sterile water and crushed with a sterile pipette tip. Approximately 3000 spores were present in 100 μL of the resulting suspension, estimated by counting 10μL under the light microscope. One-hundred μL of the spore suspension was plated into the center of 100-mm Petri plates and 600 μL of sterile water was added to allow more even spreading of spores on the plate. Plates were swirled to spread the suspension evenly on the plate. MS medium + Gamborg vitamins (MP Biomedicals LLC, Solon, OH) were used with the following preliminary medium amendments: none, + 1.5% sucrose, + 1.5% mannose, + 1.5% glucose, and + 1.5% mannitol. Only the unamended medium stimulated spore germination. Subsequent studies were performed with MS medium + Gamborg vitamins only and all media were solidified using 0.7% Phytagel (Sigma Chemical Co., St. Louis MO).

Temperature Response. One experiment was run for spores and one for bulbils. Experiments were arranged as a split plot with three replications. Ten temperature main plots were created in a thermal gradient table similar to that described by Hensley et al.

(1982) set to range from 5°C to 35°C. Plates were randomly assigned to a main plot. Actual temperatures in each lane were: 8.4, 15.9, 23.6, 28.7, 33.1, 33.7, 34.8, 34.9, 35.0, and 35.7°C and deviated 0.001% based on temperatures logged every second throughout the experiment.

Three Pioneer VI Grow lights (Sunleaves Garden Products, Bloomington, IN) suspended at a height of 46 cm above the gradient table emitted approximately 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) for a 16-hour photoperiod. Plates were photographed at initiation and every 7 days for 28 days for digital image analysis to determine germinability and average growth at each temperature. Digital images were taken with a Canon EOS Mark II (Canon Inc., Ohta-ku, Tokyo, Japan) on the following settings: F16, ISO100, two-second shutter speed. Images were analyzed for green color using a program written for Sigma Scan Pro 5.2 which optimizes green pixel detection (Karcher and Richardson 2003). Temperature responses were subjected to regression analysis where appropriate.

Preemergence Testing. Medium consisted of 4 mm Murashige and Skoog (MS) medium plus Gamborg vitamins (MP Biomedical) solidified with 0.7% Phytagel (Sigma Chemical Co., St. Louis MO). Medium was amended with treatments 1-20 (Table 1). Bulbils and spores were surface sterilized and added to amended plates as described in methods for axenic culture (above). All plates were sealed with Parafilm to prevent moisture loss over time. The experiments were arranged as randomized complete block designs with a 2 x 20 factorial treatment arrangement. Factor one was propagule, spore or bulbil, and factor two was chemical treatment (Table 1). The study was repeated three times in space with each trial in a separate growth chamber. Growth chambers were set to a

constant temperature of 24°C. A 16/8h day/night cycle was used for these experiments and growth chambers received light from one 8-bulb Sun System Tek Light (Sunlight Supply, Inc. Vancouver WA).

Digital images of each plate were taken for analysis at 0, 7, 14, and 21 days after treatment as described above. The macro was optimized to evaluate the specific green coloration of silvery threadmoss protonemal and gametophyte growth resulting in settings for a hue range of 26-100 and a saturation of 0-75. Pixel counts at Day 0 were considered 0% cover for a particular plate. Green pixel counts at day 7, 14, and 21were converted to % cover and analyzed as increase in % cover over time. Pixel counts over time were also summarized over dates to calculate area under the progress curve (AUPC) using the following equation as other authors have done for increases in moss severity over time (Kennelly et al. 2010; Thompson et al. 2011):

$$\partial = \sum_{i=1}^{ni-1} \left(\frac{(y_i + y_{(i-1)})}{2} (t_{(i-1)} - t_{(i)}) \right). \quad [1]$$

where ∂ represents AUPC, *i* is ordered sampling date, *ni* is the number of sampling dates, *y* is the proportion of green pixels detected, and *t* is time in days.

Data calculated using Equation 1 were then expressed as a percentage reduction in AUPC compared to the nontreated check as follows:

$$\%\, reduction = \left(1 - \left(\frac{\partial_r}{\partial_c}\right)\right) \text{x } 100 \quad [2]$$

where ∂ represents AUPC, *c* represents the nontreated check, and r represents the treatment. Final data were transformed to arcsine square root to improve normality based on the Shapiro-Wilk statistic using the NORMAL option in PROC UNIVARIATE

in SAS 9.2 (SAS Institute Inc, Cary NC). Transformed data were subjected to ANOVA using the GLM procedure in SAS 9.2 with sums of squares partitioned to reflect the herbicide by propagule factorial design and trials, which were considered random. Main effects and interactions were tested using mean square error associated with each effect's interaction with trial (McIntosh 1983). Means were separated with Fisher's Protected LSD test at p=0.05.

Results and Discussion

Temperature Influence on Propagules. Trial interactions were not significant (p>0.05) so data were pooled across trials. Protonemal development from spores started around 20°C and declined by 35°C. Attempts to fit both Gaussian and Lorentzian functions in proc NLIN in SAS 9.2 failed to converge for least squares determination, so a simple third order polynomial was fitted. Growth from spores began around 20°C and continued to increase through 29.5°C with little to no growth at temperatures below 20 and above 34°C. The calculated optimum, based on the fitted curve, was 29.5°C, although actual data suggested it may have been a little higher (Figure 1). Skewed optimum curves have been reported (Figures 1 & 2). Duckett et al. (2004) reported most bryophytes can germinate and grow *in vitro* in a range of 5 to 25°C. Other studies suggest silvery threadmoss cultures will grow in sterile and non-sterile conditions at temperatures between 20 and 25°C, though none of these studies directly examined effects of temperature or indicated how the temperature was chosen (Buelovic et al. 2004; Horsley et al. 2011; Sabovljevic et al. 2010; Shaw and Albright 1990).

Gametophyte growth from bulbils started as low as 10 to 15°C and peaked at approximately 22.5°C when plotted as a quadratic curve (Figure 2). However, the quadratic curve did not appropriately estimate bulbil biological response to temperature (R^2=0.50) and the optimum growth temperature may be slightly higher than 22.5°C (Figure 2). There is little literature examining moss bulbil or other vegetative propagule growth responses to temperature. Based on data analyzed from this temperature study, the propagule by treatment factorial preemergence trials were executed at 24°C between the two optimal temperatures for spore and bulbil growth.

Preemergence Screening. Trial interactions were not significant ($p>0.05$) so data from all trials were pooled. For percent reduction in area under the progress curve (AUPC) over time, there was a treatment by propagule interaction ($p=0.013$) and data are presented by propagule (Table 1). The main effect of treatment was significant ($p<0.0001$). The treatment by propagules interaction is likely due to a significant difference between bulbil and spore response for five treatments: carfentrazone alone, carfentrazone + fosetyl-Al, chlorothalonil Zn, mancozeb + CuOH, and methiozolin. For these five treatments, percent reduction in AUPC was greater when spores were treated than when bulbils were treated (Table 1). Methiozolin increased gametophyte growth from bulbils significantly compared to the nontreated (data not shown) and positively influenced AUPC for green cover over time (Table 1). No other treatment increased bulbil growth; however, several treatments increased AUPC for cover on spore plates, while methiozolin decreased AUPC for spore growth by 17% (Table 1). The products that increased protonemal growth from spores include both formulations of fosetyl-Al

alone, phosphite, and mineral oil. It is unclear why these treatments significantly increased growth from spores.

Treatments that reduced AUPC for gametophyte growth from bulbils greater than 70% included the postemergence industry standard of carfentrazone alone or in any combination, sulfentrazone, oxyfluorfen, oxadiazon, saflufenacil, flumioxazin, and pyraflufen-ethyl (Table 1). Oxadiazon, oxyfluorfen and flumioxazin have all been reported in the literature for effective (>70%) postemergence and preemergence moss control in containerized nursery ornamentals (Fausey 2003). Only oxadiazon has been reported as a postemergence moss control on putting greens reducing moss populations by as much as 50% 10 WAT (Burnell et al. 2004). The aforementioned products that reduced AUPC for gametophyte growth from bulbils by at least 70% also reduced protonemal growth from spores at similar or greater levels. Two additional products also reduced AUPC of spore protonemal growth by over 70% and they were: chlorothalonil Zn and mancozeb + copper hydroxide (Table 1). An important result from this study is that the postemergence industry standard, carfentrazone, is also an effective preemergence control product for silvery threadmoss spores and bulbils, controlling them 92.7 and 83.8%, respectively (Table 1).

The phosphite products are labeled for use on greens for their fungicidal properties. Use of these products on silvery threadmoss-infested greens may exacerbate the problem, particularly when germinating from spores. However, programs utilizing carfentrazone as a tank-mixing partner may alleviate this trend. Methiozolin is a new product registered on creeping bentgrass putting greens in Japan and Korea for the control of annual bluegrass (*Poa annua* L.). Methiozolin is currently being evaluated

under an experimental use permit for registration in the United States. Due to high annual bluegrass pressure on creeping bentgrass greens, methiozolin is projected to be used by the majority of golf courses in the Northern U.S. Based on these data, methiozolin has the potential to increase moss pressure, both through opening the turf canopy by selectively removing annual bluegrass, and significantly increasing moss growth from bulbils. In previous studies on two Virginia golf courses, moss cover in methiozolin treated plots did not significantly differ from nontreated plots after two years of treatment (Askew, unpublished data); however, superintendents at these courses were actively treating for silvery threadmoss.

In the case of both phosphite fungicides and methiozolin, increases in both creeping bentgrass growth and vigor due to pest control may lead to a reduction in moss due to turf competition. Future research should evaluate the impact of these products on moss populations on putting greens. It is currently unknown how much moss population growth on putting greens may be due to existing colony expansion versus introduced propagules establishment. These data represent a first step at better understanding these processes and developing a more integrated approach to moss control.

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Table 1. Percent reduction in area under the progress curve for green pixels from bulbil and spore development over time in growth chamber experiments.

Treatment	Rate	Rate	Bulbils	Spores
	g ai ha^{-1}	ppm	% Reduction	
carf.+ chloro.+ fos.[1]	111+7940+10200	159+32+24	89.2	91.7
sulfentrazone	140	95	87.4	92.4
oxyfluorfen	538	19	87.0	86.3
oxadiazon	1700	9	86.4	92.2
saflufenacil	25	0.15	86.0	83.1
flumioxazin	224	0.88	85.9	91.8
carf. + fosetyl-Al	111+10200	159+24	83.9*	92.9*
carfentrazone	111	159	83.8*	92.7*
carf. + chloro.	111+7940	159+32	83.4	91.0
pyraflufen-ethyl	6	0.58	83.2	73.7
chlorothalonil Zn	7940	32	60.1*	92.8*
fosetyl-Al+ prop.[1]	10200	24+6	50.8	17.9
mancozeb+CuOH	1910	25	41.3*	90.9*
fosamine	5820	28	34.5	23.4
phosphite	4950	38	29.7	-96.7
fosetyl-Al(generic)	10200	24	23.5	-113
mineral oil	52900	108	16.1	-65.2
fosetyl-Al(Sig.)	10200	24	3.50	-21.9
methiozolin	1500	28	-90.2*	17.3*
LSD			32.8	103

*Significant difference between spore and bulbil response for indicated treatment.

[1]Abbreviations: carf.=carfentrazone; chloro.=chlorothalonil Zn; fos.=fosetyl-Al; prop.=propiconazole; and Sig.=Signature.

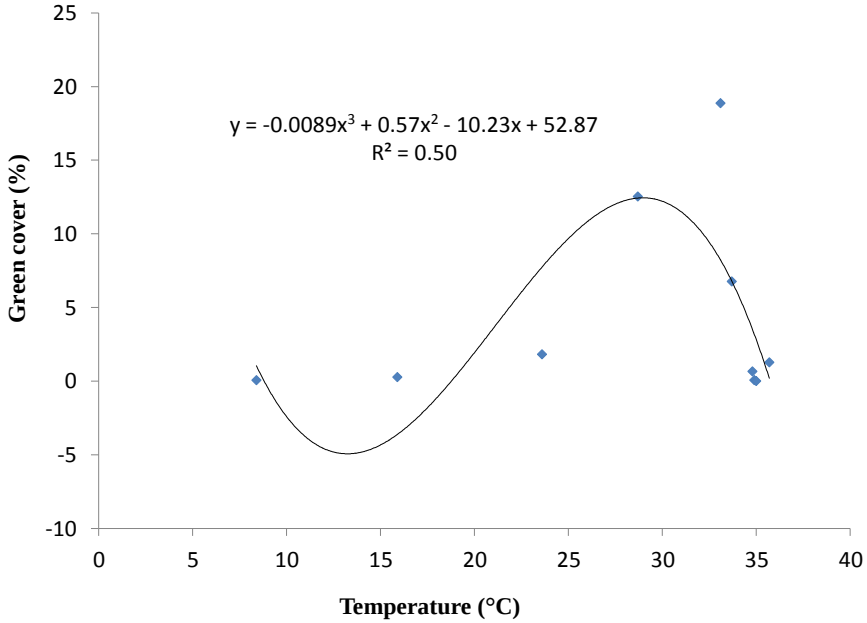

Figure 1. Temperature influence on moss protonemal cover from spores.

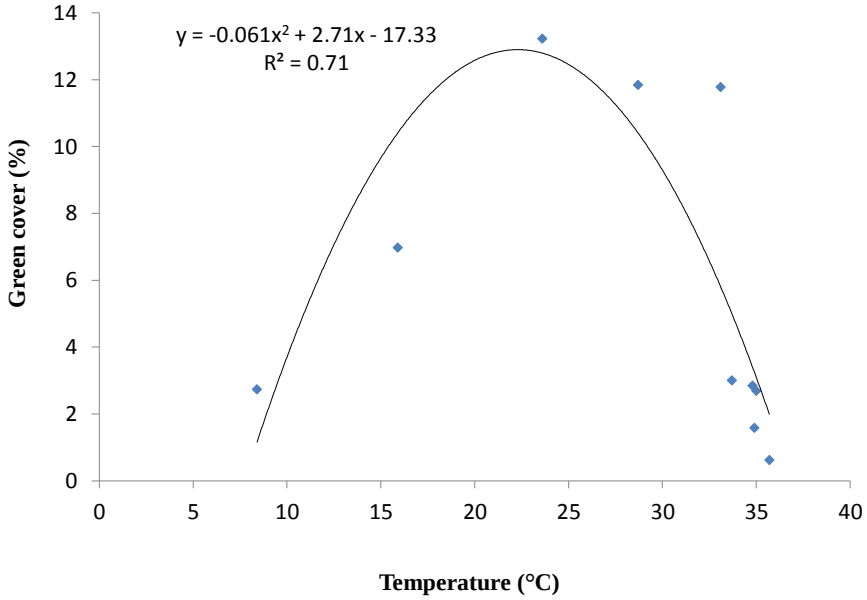

Figure 2. Temperature influence on moss gametophyte growth from bulbils.

Chapter 3. Postemergence Control of Silvery Threadmoss with Herbicide and Fungicide programs

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Silvery threadmoss is a problematic weed of golf course putting greens. It grows interspersed within the turf or as solid colonies, and has the potential to interrupt ball roll during play. Only one herbicide, carfentrazone, is currently registered for moss control on creeping bentgrass putting greens. Initial experiments were conducted to examine the potential of 49 turf protection products for silvery threadmoss control in vitro in a growth chamber. Several treatments from these initial studies and additional herbicides and fungicides reported effective from the literature were tested in the field for moss control on three varieties of creeping bentgrass, 'L93', 'Penn A4' and 'Tyee'. Treatments were applied either once at initiation, or four times at three week intervals according to label directions. Carfentrazone controlled moss 57% four weeks after treatment (WAT) and 95% 12 WAT. Several products were equivalent to carfentrazone including all combinations with carfentrazone, fosamine, fosetyl-Al, flumioxazin, oxadiazon, oxyfluorfen, saflufenacil, and sulfentrazone. Products which controlled moss >70% and were safe to creeping bentgrass (less than 15% injury) included fosetyl-Al, oxadiazon, sulfentrazone, and all combinations with carfentrazone. Though these products are not all labeled for use on putting greens, this work is an important step in evaluating alternatives to carfentrazone for silvery threadmoss control.

Nomenclature: carfentrazone; chlorothalonil Zn; fosamine; fosetyl-Al; flumioxazin; oxadiazon; oxyfluorfen; saflufenacil; sulfentrazone; silvery threadmoss; *Bryum argenteum* Hedw.; creeping bentgrass; *Agrostis stolonifera* L.

Keywords: moss control; postemergence; putting green; silver moss.

Moss has become an increasingly problematic weed of golf courses, particularly since the loss of mercury and other heavy-metal-based pesticides for turfgrass uses in 1994 (EPA 1998; United Nations 2002; Vargas 1994). Though not labeled for moss control, heavy-metal-based pesticides were used extensively on golf course putting greens as fungicides (Latin 2011; Shurtleff et al. 1987; Vargas 1994) and at the same time controlled moss. To meet golfer demand for firmer and faster playing surfaces superintendents have decreased mowing heights (Turgeon 2005). In the state of Virginia, and throughout much of the Southeastern U.S., most courses are mowing greens between 3 and 4 mm (GCSAA 2012) requiring increased passes of equipment over the green which can fragment moss and spread the propagules. Increased passes of equipment along with decreased nutrient inputs and an open turf canopy all contribute to moss encroachment of golf course putting greens (Hummel 1994; Radko 1985).

A lack of labeled products for moss control has driven turf managers to experiment with many off-label substances including peroxides, baking soda, lime, and dish detergent and fatty acid soaps (Cook et al. 2002; Happ 1998; Hummel Jr. 1994; Landschoot et al. 2004; Nelson 2007). Data describing efficacy of these off-label products routinely appear in trade magazine articles. These products are all desiccants which act against the moss by dehydrating it, and severe turf injury can result if care is not taken during application, not to mention application of these off-label products is illegal (Kennelly et al. 2010; Thompson et al. 2011). Efficacy testing for many of these products has produced inconsistent results for control of silvery threadmoss and all researchers reporting control better than 70% with sodium bicarbonate and potassium

bicarbonate also report greater than 25% injury to desired turfgrass (Kennelly et al. 2010; Thompson et al. 2011).

Several fungicides are reportedly effective at controlling silvery threadmoss. The premix mancozeb + copper hydroxide (Junction, SePRO Corporation, Carmel, IN) is labeled for moss control on putting greens; however, several researchers have reported control less than 15% (Borst 2010; McCalla et al. 2002). Many studies report silvery thread moss control with chlorothalonil greater than 70% (Burnell et al. 2004; Gelernter & Stowell 1999; Kennelly et al. 2012; McCalla et al. 2002), sometimes with effective residual moss control up to eight weeks after treatment (McCalla et al. 2002). However, other studies have found no moss control with chlorothalonil (Cook et al. 2002; Fausey et al. 2003).

Fertilizer products, especially those containing heavy or transition metals, also control silvery threadmoss in some situations. Burnell et al. (2004) found that fertilizers containing iron could be used to reduce silvery thread moss population densities on bentgrass putting greens, though McCalla et al. (2002) reported no control of moss with ferrous ammonium sulfate ($FeNH_4SO_4$) at 317 mL 100 m^{-2}. Research by Vukojevic et al. (2004) suggests that ferric(III) citrate, an organic chelated form of Fe, is more bioavailable to silvery threadmoss than potassium hexacyanoferrate(III). Bioavailability based on organic matter content, pH, and other soil conditions may alter the effectiveness of fertilizer treatments for silvery threadmoss control. Fausey et al. (2003) reported residual control of silvery threadmoss with copper sulfate at 195 kg ha^{-1} lasting only 7 to 14 days. Boesch and Mitkowski (2005) reduced moss cover completely with two summer applications of silver nitrate; a potentially toxic and environmentally persistent

product. Most of the heavy and transition metal-containing fertilizers, while having the potential to control silvery threadmoss, also need to be applied at high rates which can accumulate in soils and cause phytotoxicity to desirable species (Borkert et al. 1998).

Only a few herbicides have been reported in peer-reviewed literature to effectively control silvery threadmoss. In containerized ornamentals, flumioxazin, oxyfluorfen, pelargonic acid, and oxadiazon controlled silvery thread moss greater than 80% (Fausey 2003). Endothal has also been used in a long-term experiment on bentgrass putting greens where repeated yearly applications completely controlled silvery threadmoss in the fourth and fifth years of the study (Brauen et al. 1986). The current industry standard for silvery threadmoss control on golf putting greens is carfentrazone (Quicksilver, FMC Corporation, Philadelphia, PA). It is effective at suppressing silvery threadmoss but moss shoots quickly recover and sequential applications are required for control greater than 70% (Borst et al. 2007; Anonymous 2005). Borst (2010) reported increased moss control using sequential applications of carfentrazone plus nitrogen or topdressing or both, controlling moss up to 78% until 16 weeks after initial treatment (WAIT) (Borst 2010). However, applied alone at the labeled rate twice at a two-week interval, the best control was only 77% at 3 weeks after initial treatment dropping to 43 and 36% control at 5 and 11 WAIT, respectively.

It is clear that moss control with carfentrazone requires repeat applications throughout the season. However, carfentrazone may only be applied four times per year at the rate required for moss control (Anonymous 2005). With limited peer-reviewed information available on effective chemical control, and the increasingly common occurrence of moss as a problematic weed of golf putting greens, the evaluation of proper

moss management is highly desirable. Therefore, the objective of this research was to broadly evaluate herbicides and fungicides applied alone or in combination for postemergence silvery threadmoss control on creeping bentgrass golf course putting greens.

Materials and Methods

Growth Chamber Screening. Silvery threadmoss plugs were collected from a moss-infested creeping bentgrass putting green in Blacksburg, VA August 2010 and August 2011. Moss plugs were removed from the green with a 12-mm diameter cork borer to a depth of 25 mm. Plugs were allowed to desiccate on the laboratory bench until bentgrass stolons died. Plugs were re-hydrated and creeping bentgrass carcasses were removed from the plugs with tweezers. Growth chambers were retrofitted with a custom sub-irrigation system. Autoclaved USGA-specification (USGA 2004) topdressing sand was placed ~1-cm deep in a plastic irrigation tray. Moss plugs were placed on the sand, top-dressed as they would be on a green, and allowed to acclimate in the chamber with 24°C constant temperature and 16-hour photoperiod. Moss was sub-irrigated by applying water to the sand base. Plugs were acclimated for 10 to 14 days prior to treatment. Acclimation was defined as development of new leafy tissue and no visual evidence of fungal or algal contamination. Two experiments were initiated as completely randomized designs with 10 replications and a 49 by 2 factorial treatment design (Table 1), factor one being treatment and factor two, rate. Acclimated moss plugs were removed from the growth chambers and organized into ten uniform replicates. Replicates were randomized

into 24-well cell culture plates (Fisher Scientific, Pittsburg, PA) which were altered to have a 3-mm hole in the bottom center of each well allowing for sub-irrigation. Initial plate images were taken before treatment and each replicate of ten plugs was removed from the randomization, treated in a spray chamber calibrated to deliver 815 L ha^{-1} and replaced in the same randomization. Digital photographs of each plate were taken for analysis at 0, 3, 7, 10, 14, 21, and 28 days after treatment with a Canon EOS Mark II (Canon Inc., Ohta-ku, Tokyo, Japan). Photographs were partitioned into 24 images containing one moss plug each for detection of green pixels. Each cropped image was analyzed in Sigma Scan Pro 5.2 with a macro developed at University of Arkansas (Karcher and Richardson. 2003). The macro was optimized for this purpose to evaluate each image for green pixels in a hue range of 38-100 and a saturation of 0-100. Pixel counts at Day 0 were considered as 100% green cover for a particular plug and pixel counts on subsequent days were standardized based on Day 0 counts using the following equation

$$\frac{\omega_n - \omega_0}{\omega t} \times 100 \quad [1]$$

where ω_0=Day zero pixel count and ω_n= Day *n* pixel count and ω_t =total pixels in image. Pixel counts over time were converted to area under the progress curve (AUPC) using the following equation as other authors have done for increases in moss severity over time (Thompson et al. 2011; Kennelly et al. 2010):

$$\partial = \sum_{i=1}^{ni-1} \left(\frac{(y_i + y_{(i-1)})}{2} (t_{(i-1)} - t_{(i)}) \right). \quad [2]$$

where ∂ represents AUPC, *i* is ordered sampling date, *ni* is the number of sampling dates, *y* is the moss pixel cover, and *t* is time in days. Data calculated using Equation 2 were

then expressed as a percentage reduction in AUPC compared to the nontreated check. Final data were determined to be normal using the NORMAL option in proc UNIVARIATE and Shapiro-Wilk statistic. Data were subjected to ANOVA in SAS 9.2 (SAS Institute Inc, Cary NC) with sums of squares partitioned to reflect the herbicide by rate factorial design and trials, which were considered random. Main effects and interactions were tested using mean square error associated with the random variable interaction (McIntosh 1983). Appropriate means were separated with Fisher's Protected LSD test at p=0.05.

Field Testing. Treatments controlling moss more than 70% in the herbicide screening along with additional fungicide treatments and tank-mixtures reported from the literature were included in this study to assess efficacy in the field. Three sites were established in Blacksburg, VA in 2012 on three different varieties of creeping bentgrass: 'Tyee', 'L93', and 'Penn A4'. All three greens were built to USGA-specifications (USGA 2004). All greens received between 146 and 196 kg N ha^{-1} annually. The 'Tyee' putting green turf was less than 12 months old when the study was established and the green itself was built in 2005. The green was maintained at 4 mm and had 0.8% organic matter (OM) content and a pH of 6.3. The 'Tyee' also had low levels of calcium, magnesium, and potassium. The 'L93'and 'Penn A4' putting greens were built in 2001 and 2008 and each had a pH 6.4. The 'Penn A4' was maintained at 4.0 mm and the 'L93' was maintained at 3.2 mm. Only the 'L93' site was topdressed during the course of this trial. Standard fungicide programs were suspended for the duration of this experiment to determine treatment effects from fungicides included in the studies. However, chlorothalonil was mistakenly applied twice early in the season to the 'L93' site.

Each experiment was arranged as a randomized complete block design with 26 treatments (Table 2). The 'Tyee' and 'Penn A4' sites had three replications and the 'L93' site had four replications. Plots were 0.91m x 0.91m. Percent moss and turf cover and percent turf injury were assessed visually at 0, 14, and 28 days after initial treatment and monthly thereafter, and NDVI was recorded at each rating. Normality was tested as in the previous study and, where needed, percentage data were arcsine square root transformed to stabilize variance. Data were subjected to ANOVA in SAS 9.2 (SAS Institute, Cary NC). Main effects and interactions were tested using mean square error associated with each effect interaction with trial (McIntosh 1983). Appropriate means were separated with Fisher's Protected LSD test at $p=0.05$.

Results and Discussion

Preliminary Herbicide Screening. Trial interactions were not significant ($p>0.05$) so data were pooled across trials. Herbicide rate was not significant ($p>0.05$) so data were also pooled across rate. The main effect of herbicide was significant ($p<0.0001$) for percent reduction in AUPC (Table 1). Products equivalent to the industry standard, carfentrazone, at 28 days after treatment (DAT) reduced moss cover over time 35-61% (Table 1) and were selected for additional screening in the field. These products included pelargonic acid, flumioxazin, carfentrazone, sulfentrazone, saflufenacil, and glufosinate. Glufosinate was excluded due to the potential for severe injury to creeping bentgrass. Pelargonic acid was included as a spot treatment because of its fast activity, reducing moss cover to zero within hours of application with subsequent moss recovery after two

weeks. Additional fungicide and herbicide products reported in the literature were also included in field testing.

Several products tested in the preliminary screening increased moss cover over time including, dicamba, bentazon, 2,4-D amine, amicarbazone, MCPA ester, and quinclorac. For the hormone herbicides dicamba, 2,4-D amine, and quinclorac, this was likely an artifact of the way data were collected through pixel counts. Hormone herbicides cause epinasty, which for moss actually opens the canopy and causes leaves to be held at wider angles to the stem, resulting in higher pixel counts even where injury is present.

Field Testing. A trial by treatment interaction occurred for creeping bentgrass injury and silvery threadmoss control ($p<0.0001$), so data are given for each site separately at 2, 4, 8, and 12 weeks after treatment (Tables 3 and 4). Only a treatment main effect ($p<0.0001$) occurred for percent reduction in moss cover, so data were pooled across sites (Table 5). For injury, data are shown only for treatments which caused at least 5% injury on at least one rating date; other treatments were deleted to stabilize variance (Table 3). Flumioxazin and oxyfluorfen severely injured all three cultivars of creeping bentgrass (Table 3). Fosamine injured 'Penn A4' 67% at two WAT which took 12 weeks to recover (Table 3). Fosamine also injured 'Tyee' as much as 48% by 8 WAT; however, no injury was noted on the 'L93' site. Oxadiazon injured 'L93' and 'Penn A4' creeping bentgrass less than 10%, but injured the 'Tyee' 62% at 2 WAT (Table 3). Sulfentrazone also injured creeping bentgrass at the 'L93' and 'Tyee' sites 19, and 13% respectively 2 WAT; however, no injury was noted on the 'Penn A4' (Table 3). More instances of injury were observed on the 'Tyee' than any other site. The 'Tyee' creeping bentgrass

was less than 12 months old at the time of trial initiation. Less mature bentgrass along with high sand content, low organic matter, and fertility issues over the course of the season caused thin, stressed bentgrass turf, and could have caused more susceptibility to herbicide injury.

Moss control is provided for all treatments controlling silvery threadmoss greater than 60% for at least one rating date (Table 4). Carfentrazone and all combinations with carfentrazone controlled silvery threadmoss 93 to 95% on the 'L93', 78 to 98% on the 'Penn A4', and 68 to 98% on the 'Tyee' at 12 WAT (Table 4). Flumioxazin, oxyfluorfen, and fosamine also controlled silvery threadmoss greater than 70%, but caused severe injury to all three cultivars tested (Table 3). Fausey (2003) reported similar moss control from flumioxazin and oxyfluorfen 100 and 90%, respectively four WAT. Oxadiazon severely injured the 'Tyee' but was safe on the other two cultivars and controlled moss 70 to 73%, and equivalent to carfentrazone alone up to 5 WAT on all three cultivars (Table 4). Other researchers have reported up to 26% reduction in moss 10 WAT with oxadiazon (Burnell et al. 2004), which is similar to the 33% reduction 12 WAT reported here (Table 5). Fausey (2003) also reported 95% control, 5 WAT with oxadiazon. Fosetyl-Al only controlled moss 45 to 78% after a single application, but after three applications control increased to 70 to 87% and remained equivalent to carfentrazone through 12 WAT (Table 4). Sulfentrazone, applied in a single application (Table 2), controlled silvery threadmoss 63 to 88%, equivalent to carfentrazone alone up to 8 WAT at all sites (Table 4). Sulfentrazone and fosetyl-Al have not been reported previously in the literature for silvery threadmoss control.

Percent reduction in moss cover was pooled over trials within treatment (Table 5). Single application products which reduced moss cover by 50% or more through 12 WAT were flumioxazin, fosamine, fosetyl-Al, oxyfluorfen, and sulfentrazone (Table 5). However, only sulfentrazone and fosetyl-Al were safe to use on creeping bentgrass cultivars tested in this study. Carfentrazone alone and all combinations with carfentrazone significantly reduced silvery threadmoss cover at 8 and 12 WAT (Table 5); however, these products were all applied four times at three-week intervals.

The importance of these comparisons for superintendents is cost. The carfentrazone label allows four applications of the maximum use rate (0.11 kg ai/ha) for moss control on creeping bentgrass putting greens in a given year (Anonymous 2005). Superintendents with continual moss problems will apply at least that amount at a cost of \$292.53 ha^{-1} $application^{-1}$ (Table 6). A single application of sulfentrazone, as it is currently formulated, would cost a fraction of that, at \$55.75 ha^{-1} $application^{-1}$; however, it is not currently labeled for putting green use (Table 6). Fosetyl-Al was equally effective based on results of this study, but does not offer a significant cost savings as an alternative moss control product at \$721.77 ha^{-1} $application^{-1}$. However, fosetyl-Al also offers control of some fungal diseases (Anonymous 2011). While labeling of these products for moss control is not on the horizon, this work is an important step toward providing golf course superintendents with alternatives to carfentrazone for silvery threadmoss control.

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Table 1. Percent reduction in area under the progress curve (AUPC) for green pixels detected at 0, 3, 7, 10, 14, 21, and 28 days after treatment (DAT). Given rate is the 1X rate. Each treatment was also applied at a 2X rate. Responses were similar so data are shown pooled across all rates and trials.

Treatment	Rate	Reduction in AUPC
	g ai ha^{-1}	——————%——————
pelargonic acid	5% v/v[1]	61*
flumioxazin	224	47*
carfentrazone	111	46*
sulfentrazone	140	44*
saflufenacil	25	36*
glufosinate	2190	35*
diquat	421	28
fosamine	5820	27
lactofen	219	26
ethofumesate	859	23
chlorimuron	14	21
metolachlor	2780	21
dithiopyr	561	20
prodiamine	365	20
paraquat	280	19
bispyribac sodium	74	18
sulfosulfuron	69	18
thifensulfuron	27	17
tribenuron	13	16
oxadiazon	1700	16
metsulfuron	9.5	16
fenoxaprop	18	16
oxyfluorfen	538	15
dimethenamid-p	1680	15
EPTC	2940	14
glyphosate	2840	14
pyraflufen-ethyl	6	13
penoxulam	20	11
isoxaben	555	11
ethephon	3820	11
bromoxynil	280	11
pendimethalin	1650	10
halosulfuron	70	10
imazapic	70	10
MSMA	2240	9

pyrithiobac sodium	112	9
cumyluron	39	8
methiozolin	1500	7
topramezone	36	7
pyrazosulfuron-ethyl	112	7
fluazifop	70	2
paclobutrazol	280	1
fluroxypyr	421	1
dicamba	280	-4
bentazon	841	-5
2,4-D amine	185	-6
amicarbazone	103	-7
MCPA ester	1037	-7
quinclorac	210	-15
LSD	-------	15.6

* Statistically equivalent to the industry standard carfentrazone.

Table 2. Treatments applied, rates, and application intervals for all field study sites: L-93', 'Penn A4', and 'Tyee'.

Treatment	Rate	Application Interval
	g ai ha^{-1}	
fosamine	5820	Once
flumioxazin	224	Once
carf.+chloro.+fos.[1]	111+7940+10200	3 Weeks[4]
carf.+fos.	111+10200	3 Weeks
fosetyl-Al	10200	3 Weeks
carf.+chloro.*	111+7940	3 Weeks
sulfentrazone	140	Once
oxyfluorfen	538	Once
fenoxaprop	18	Once
dicamba fb[2] carf.	280+111	Once
2,4-D amine	185	Once
Pelargonic acid	5% v/v[3]	Once
2,4-D amine fb carf.	185+111	Once
mancozeb+CuOH	1910	3 Weeks
oxadiazon	1700	Once
mancozeb	15300	3 Weeks
bensulide	14300	Once
chlorothalonil Zn	7940	3 Weeks
amicarbazone	103	Once
saflufenacil	25	Once
pyraflufen-ethyl	6	3 Weeks
carfentrazone-ethyl	111	3 Weeks
nontreated check	NA	NA
methiozolin	1500	Once
MSMA	2240	Once
chlorothalonil	8250	3 Weeks

[1] Abbreviations: carf.=carfentrazone; chloro.=chlorothalonil Zn; and fos.=fosetyl-Al.

[2]Dicamba and 2,4-D were applied once at initiation of the study followed by carfentrazone as a single application 10 days after initial.

[3] Pelargonic acid was a spot application applied with a sponge-dabber which has an output of 972L ha^{-1}. Application rate for this product was 120.2 kg ai ha^{-1}.

[4]All applications made at three-week intervals were applied 4 times

Table 3. Percent creeping bentgrass injury (0-100) from treatments that caused at least 5% at any date.

	L 93 Site				Penn A4 Site				Tyee Site			
Treatment	2 WAT	4WAT	8WAT	12WAT	2 WAT	4WAT	8WAT	12WAT	2 WAT	4WAT	8WAT	12WAT
	% Injury											
amicarbazone	0	0	0	0	0	0	0	0	16.7	5.00	0	0
flumioxazin	100	98	90	84	93	92	53	25.0	95	83	75	50
fosamine	0	0	0	0	67	70	68	13	12	23	48	25
methiozolin	0	0	0	0	0	0	0	0	12	5	0	0
oxadiazon	9	3	0	0	10	5	0	0	62	23	0	0
oxyfluorfen	43	73	50	30	67	25	0	0	68	38	18	0
sulfentrazone	19	0	0	0	0	0	0	0	13	0	0	0
LSD	13.9	3.23	11.3	15.8	5.4	4.1	8.6	3.1	18.5	14.1	13.6	2.6

Table 4. Treatments which controlled silvery threadmoss greater than 60% at any rating date.

	L 93 Site				Penn A4 Site				Tyee Site			
Treatment	2 WAT	4WAT	8WAT	12WAT	2 WAT	4WAT	8WAT	12WAT	2 WAT	4WAT	8WAT	12WAT
	%Control											
saflufenacil	73	78	60	0	67	43	0	0	63	60	60	0
carfentrazone	65	65	78	95	47	60	62	78	62	57	63	77
carf. + chloro. [1]	65	69	74	89	67	70	65	83	57	83	87	90
carf.+chloro.+fos.	71	68	81	88	73	67	97	83	63	67	80	98
carf. + fos.	65	84	86	93	90	78	98	98	77	60	63	68
flumioxazin	88	99	80	70	100	100	100	100	100	100	100	95
fosamine	60	75	75	68	95	100	100	93	77	97	100	100
fosetyl-Al	45	75	78	70	78	97	83	87	43	50	65	70
oxadiazon	71	73	63	50	80	70	60	43	80	70	57	67
oxyfluorfen	83	89	70	43	97	90	60	43	85	73	62	75
sulfentrazone	85	75	65	50	75	63	72	63	80	83	87	88
LSD	25.4	19.3	18.8	16	24	22.1	25.1	29.0	25.4	24.3	26.1	32.2

[1]Abbreviations: carf.=carfentrazone; chloro.=chlorothalonil Zn; and fos.=fosetyl-Al.

Table 5. Percent reduction in visually estimated moss cover pooled over trial at 8 and 12 weeks after treatment compared to initial moss cover.

Treatment	Rate	8WAT	12WAT
	g ai ha^{-1}	———% Reduction———	
fosamine	5820	95	72
flumioxazin	224	90	83
carf.+chloro.+fos.[1]	111+7940+10200	84	88
carf.+fos.	111+10200	66	88
fosetyl-Al	10200	58	68
carf +chloro.	111+7940	57	86
sulfentrazone	140	51	57
carfentrazone-ethyl	111	45	70
oxyfluorfen	538	25	57
fenoxaprop	18	24	21
dicamba+carf.	280+111	23	20
2,4-D amine	185	22	14
Pelargonic acid[2]	5% v/v	20	31
2,4-D amine+carf.	185+111	15	14
mancozeb+CuOH	1910	14	42
oxadiazon	1700	12	32
mancozeb	15300	11	2
bensulide	14300	4	7
chlorothalonil Zn	7940	1	42
amicarbazone	103	1	-13
saflufenacil	25	-1	14
pyraflufen-ethyl	6	-11	17
nontreated check	NA	-11	-14
methiozolin	1500	-15	10
MSMA	2240	-23	-4
chlorothalonil	8250	-70	28.8
LSD	-----	41.5	40.1

[1]Abbreviations: carf.=carfentrazone; chloro.=chlorothalonil Zn; and fos.=fosetyl-Al.

[2] Pelargonic acid was a spot application applied with a sponge-dabber which has an output of 972L ha^{-1}. Application rate for this product was 120.2 kg ai ha^{-1}.

Table 6. Per hectare cost of products used in programs effective at controlling moss equivalent to carfentrazone alone as the industry standard, and not injuring bentgrass more than 20% at any rating.

Treatment	Rate	Manufacturer	Cost	Cost	Applications
	product ha^{-1}		$ $unit^{-1}$	$ ha^{-1} $app.^{-1}$	$year^{-1}$
carfentrazone	0.490L	FMC	143.28/ 240mL	292.53	4
chlorothalonil Zn	15.45L	Quali-Pro	139.95/9.5L	227.60	4
fosetyl-Al	12.36kg	Quali-Pro	145.99/ 2.5kg	721.77	4
carf.+chloro. [1]	-----	Tank-mix	-----	520.13	4
carf.+chloro.+fos.	-----	Tank-mix	-----	1241.90	4
carf.+fos.	-----	Tank-mix	-----	1014.30	4
sulfentrazone	0.284L	FMC	69.69/355mL	55.75	1 to 2

[1]Abbreviations: carf.=carfentrazone; chloro.=chlorothalonil Zn; and fos.=fosetyl-Al.

Chapter 4. Silvery Threadmoss Absorption, Translocation, and Metabolism of ^{14}C Glyphosate

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Studies were conducted to evaluate absorption, translocation and metabolism of ^{14}C glyphosate by silvery threadmoss (*Bryum argenteum* Hedw.). No studies have examined the mechanism of herbicide absorption in bryophytes, which have no true vascular system and lack many protections, such as cuticle wax and trichomes, cited as providing herbicide tolerance in some higher plants. Glyphosate is not known to control silvery threadmoss, but the mechanism of tolerance is unknown for this species. Foliar applied ^{14}C glyphosate was quickly absorbed into moss shoots and rhizoids, reaching equilibrium within the plant by 24 hours after treatment (HAT). Total ^{14}C recovery from all parts and sections was 70-88% 24 HAT but decreased significantly with time, to 40 to 55% recovered at 48 HAT and only 35% recovered by 192 HAT. The water wash had the most significant decrease in total recovered radioactivity over time; however, no complimentary increase was observed inside moss tissues or the methanol wash over time. Thus, ^{14}C glyphosate was lost, presumably to the capillary water in the moss colony, where it is possible for microorganisms to degrade ^{14}C glyphosate rapidly into $^{14}CO_2$.

Nomenclature: glyphosate; (*Bryum argenteum* Hedw.).

Keywords: moss; radiolabeled herbicide.

Though there are no peer-reviewed studies examining the fate of radiolabeled herbicides in mosses, there are several studies examining the activity of certain herbicides against various moss species. Wacker et al. (1988) noted the microtubule inhibitor oryzalin reduced growth and interfered with polar migration of microtubules within protonematal cells of the moss *Funaria hygrometrica.* Asulam, a herbicide thought to interfere with microtubule assembly and function and inhibit folic acid biosynthesis (Hoffman and Vaughn 1994; Stephen et al. 1980; Veerasekaran et al. 1981), inhibited moss gametophyte elongation in 21 moss species in sterile culture (Rowntree and Sheffield 2005; Rowntree et al. 2003). Karunan et al. (1976) reported 67% reduction in germination of *Polystichum commune* when exposed in sterile media to 200 ppm EPTC, a fatty acid synthesis inhibitor (Wilkinson and Smith 1974; 1975). In the field, Newmaster et al. (1999) demonstrated some mosses and lichens are susceptible to glyphosate and triclopyr citing decreases in abundance and species diversity as a result of glyphosate or triclopyr applications over a period of two years. But other studies with glyphosate have found little to no activity against moss species (Narvaez Parra et al. 2005; Ronoprawiro 1975). Based on the literature, it is clear that mosses, like higher plants, vary in susceptibility to herbicides depending on the species.

No research to date has addressed the susceptibility of silvery threadmoss to glyphosate, an amino acid synthesis inhibitor, which inhibits the EPSP synthase enzyme in the shikimic acid pathway responsible for manufacture of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Amrhein et al. 1980; Jaworski 1972). These amino acids are required for the formation of lignins and many other complex proteins (Tohge et al. 2013; Weng and Chapple 2010). We have observed silvery threadmoss

tolerance to glyphosate though the mechanism of glyphosate tolerance is unclear. One hypothesis for mosses in general is that some herbicides simply do not get absorbed into the plant due to lack of a true vascular system. Higher plants have specialized conducting tissues which can actively move glyphosate and other herbicides through the plant, while mosses have a passive water retention system consisting of a central strand of conducting tissue, which moves water and other materials by capillary action over an osmotic potential (Goffinet and Shaw 2009). It is also possible that epicuticular waxes play a role in herbicide uptake by silvery threadmoss, which is so-named for the reflectance of its cuticle waxes (Knoche and Bukovac 1993; Wyrill and Burside 1976). Epicuticular waxes may inhibit shoot absorption by silvery threadmoss. The objectives of this research were to evaluate absorption and translocation of ^{14}C-glyphosate in silvery threadmoss.

Materials and Methods

Silvery threadmoss was collected from golf course putting greens and stored dry at room temperature for several weeks until creeping bentgrass stolons died. Plugs were rehydrated with sterile water and creeping bentgrass stolons were removed to create pure moss stands. Colonies were trimmed to 3 x 12 mm for treatment. Experiments were arranged as randomized complete block split-split plot designs with three replications. Main plots consisted of a 2 by 6 factorial treatment arrangement with two surfactant treatments and six times of harvest. Moss colony harvest timings were 12, 24, 48, 72, 96 and 192 hours after treatment. Subplots consisted of four sections of the moss colony, each 3 mm by 3 mm, to test for spatial movement of herbicide through the moss colony.

Radiolabeled glyphosate (glyphosate [phosphonomethyl-^{14}C], Figure 1) was diluted in sterile water and applied as one 3-µl drop of solution containing 38.4 kBq glyphosate with 1% v/v non-ionic surfactant (Helena Chemical Co., Collierville, TN) on the left side of a 3 x 12 mm colony approximately 1 mm from each edge. Experiments were conducted at ambient temperature in the laboratory with supplemental lighting provided by one 8-bulb Sun System Tek Light (Sunlight Supply, Inc. Vancouver WA) set to provide a 12 hour photoperiod.

Plugs were harvested by cutting each with a sterile razor into four equal 3 mm by 3 mm sections. Each section was vortexed 10 s in 3 ml sterile deionized (DI) water, followed by 30 s in 3 ml methanol to strip the cuticle and any embedded herbicide. The remaining plant tissue was divided further into shoot and rhizoid parts and each part was placed in a combustocone (Fisher Scientific, Pittsburg, PA) for biological oxidation. Samples were dried at 70°C, weighed, and burned in a Biological Oxidizer OX500 (R. J. Harvey Instrument Corporation, Tappan, NY) for a 2-minute oxidation cycle. Samples were counted on a LS 6500 multipurpose scintillation counter (Beckman Coulter Inc, Indianapolis, IN) to determine total recovered radioactivity. Data were converted to percent of applied ^{14}C and subjected to ANOVA in SAS 9.2 (SAS Institute, Inc. Cary, NC) with sums of squares partitioned to reflect a factorial spit-split plot treatment structure and trial effects. The six harvest timings were considered main plots, the four sections were subplots and the four parts of each section (shoot, rhizoid, water wash, and methanol wash) were considered sub-subplots. Data were analyzed separately by part. Significant effects were explained using regressions of the response variables over time or space. Means were separated using Fisher's Protected LSD at p=0.05.

Results and Discussion

Absorption and Translocation. A trial by time by treatment interaction occurred for shoots ($p=0.048$), rhizoids ($p=0.0047$) and the methanol wash ($p=0.0041$). The main effect of time was significant for water and methanol washes ($p<0.0001$) and section main effect was significant for shoots only ($p=0.046$).

Total percent of applied ^{14}C recovered from all parts of all sections for trial one averaged 89% 12 hours after treatment (HAT), but declined to 38% by 192 HAT (Figure 2). With a similar trend in the second trial, ^{14}C recovery declined from an average of 70% 12 HAT to 40% 192 HAT (Figure 2). Average percent of applied ^{14}C recovered from shoots and rhizoids remained steady over time (Figure 3) and recovery of bound material in the methanol wash was also steady (1%) throughout the experiment (Figure 4). However, there was a steep logarithmic decline in recovery of applied ^{14}C from the water wash over time. At 12 HAT average percent of applied recovery was 11%. By 48 HAT recovery declined by more than half, and by 192 HAT, recovery was only 2% (Figure 4). In previous experiments with plants, declining recovery from the water wash would generally indicate more ^{14}C is being absorbed by the plants or bound in the cuticle layer over time. However, when radiolabeled glyphosate is applied to silvery threadmoss colonies, ^{14}C appears to be lost to some other mechanism. One hypothesis is that the [$_{14}$C-phosphonomethyl] glyphosate (Figure 1) is being metabolized by microorganisms in the water matrix and released in the form of $^{14}CO_2$ (Figure 9).

Again, this evidence suggests that since ^{14}C was not moving back into the shoot tissues or found in the water or methanol washes over space, it was presumably lost through metabolism by microorganisms. While sterile materials were used where possible in the experimental setup, it was not a completely sterile system. Moss colonies were collected from the field along with their water matrix and any microorganisms that may have been present. The degradation pathway for glyphosate is given in Figure 9. Glyphosate is stable over a wide range of water pHs and is highly soluble in water. It does not break down easily through hydrolytic decomposition (Bronstad and Freistad 1985) and has a hydrolysis half-life greater than 35 days (Kollman and Segawa 1995). Ghassemi et al. (1981) reported that breakdown in water is slower due to fewer numbers of microorganisms in water compared to soil. However, the water matrix held within a moss colony closely resemble the microclimate in a soil than in an aquatic environment. There are air spaces to support aerobic microorganisms and saturated areas where anaerobic organisms would thrive. Both types of microorganisms have pathways to break down glyphosate into its metabolites, and degradation by microorganisms is the main route of environmental degradation of glyphosate (Franz et al. 1997). In a four week study, Rueppel et al. (1977) found that as much as 55% of radiolabeled carbon from ^{14}C labeled glyphosate could be released as $^{14}CO_2$ in non-sterile soil conditions. Results of this study suggest as much as 60% of applied ^{14}C from ^{14}C glyphosate is being lost over a period of only eight days. It will be important for future research to examine the role of the non-sterile water matrix of moss in the loss of ^{14}C from application of ^{14}C glyphosate.

Spatially, percent of applied ^{14}C recovered from shoots was 4% in the treated section and decreased to 2.5% in all other sections of the moss colony when averaged over

trials and surfactant use (Figure 6). ^{14}C recovered from rhizoid tissues however, tended to decrease as distance from treated section increased from 0 to 12 mm (Figure 7). The average percent of applied ^{14}C recovered from rhizoids in the treated section was between 5 and 6%, while recovery from the 9 to 12 mm section was 3 to 5% (Figure 7).

The interaction of trial, surfactant, and section were significant for radioactivity recovered from moss rhizoids ($P<0.05$). The response of surfactant was consistent in the first trial but not in the second, and probably caused the interaction. In trial 1, percent of applied radioactivity over space was curvilinear but equivalent amounts were recovered at the site of treatment and the most distal point in the colony 12 mm away (Figure 7). In trial 2, a trend similar to trial 1 occurred when surfactant was not added to ^{14}C glyphosate but a linear decrease in detected radioactivity over space occurred when surfactant was added to the treated solution. Overall, data show some small spatial differences but fundamentally suggest that when ^{14}C glyphosate is applied to a moss colony, radioactivity can rapidly disperse throughout the colony. The lack of a time interaction fits the data in that few spatial differences were observed over time and, as shown earlier, radioactivity in shoots and rhizoids was essentially static over time (Figure 3). These data do not, however, indicate the structure of the radioactive compound absorbed into shoots or rhizoids or the mechanism by which radioactivity was able to move throughout the colony. Some clues to the translocation mechanism may be found by comparing shoot and rhizoid data to water wash data.

A trial by surfactant by section interaction was significant for radioactivity recovered from the rinse water ($P<0.05$). The trial interaction was probably due to differences in surfactant effects as occurred with radioactivity recovered from rhizoids

(Figure 7). In the case of the water rinse, all sections had equivalent amounts of radioactivity except the treated section that did not contain surfactant in trial 1, which had slightly over 7% of applied radioactivity compared to approximately 5 to 6% from all others (Figure 8). Increased radioactivity in the water rinse when surfactant was excluded would suggest that addition of surfactant may slightly improve absorption into moss shoots or rhizoids but actual recovered radioactivity from shoots and rhizoids do not necessarily support that suggestion (Figures 6 and 7). Although more radioactivity was recovered from shoot and rhizoids at the site of action, the differences were small and either did not differ by surfactant (shoots) or differed slightly (rhizoids) (Figures 6 and 7). Surfactant has increased glyphosate absorption in plants (Knoche and Bukovac 1993; Nalewaja et al. 1996) but no data are available to compare herbicide absorption in bryophytes. It is more noteworthy that spatial differences in water rinse did not exceed 2% and indicate that ^{14}C can move throughout the capillary water held by moss colonies and quickly reach equilibrium, as evidenced by the lack of a time by section interaction ($P>0.05$). Since little to no spatial differences were noted in moss shoots (Figure 5) and rhizoids (Figure 7) and the same was true for the water rinse (Figure 8), these data suggest absorption of radioactivity following application of labeled glyphosate occurs at or near the site of detection rather than by systemic translocation. Evidence for this assumption may be found in translocation studies conducted on plants where translocation spatially within the plant usually occurs as an interaction with time (Koger and Reddy 2005). Further evidence exists in anatomical differences between silvery threadmoss and plants. Mosses do not have a true vascular system and are limited to a narrow "central strand" of cells putatively associated with water conduction and structural support (Goffinett and

Shaw 2009). Higher plants have more specialized conducting tissues yet the paucity of herbicide detected in foliage other than the treated leaf and temporal trends associated with glyphosate mobility in plants (Koger and Reddy 2005) suggest that translocation of glyphosate out of the treated leaf and over distances of just 12 mm to other plant parts occurs in small quantities over extended periods of time. Most of a moss stem is made of large parenchyma cells surrounded by a thin epidermis (Goffinet and Shaw 2009). Silvery threadmoss is named for the reflectance of cuticle waxes, which may inhibit shoot absorption but does not have trichomes, which reduce herbicide absorption into some plant species (Knoche and Bukovac 1993; Wyrill and Burnside 1976). Moss also does not have stomata, which have been cited as significant sources of herbicide entry in plants (Goffinet and Shaw 2009). The available information on moss anatomy suggests silvery threadmoss may be able to absorb herbicides similar to higher plants but should be much slower at systemic translocation of absorbed herbicide.

These data suggest that 14C glyphosate may move throughout silvery threadmoss colonies in capillary water and that decreases in recovered ^{14}C from moss over time is presumably being lost in capillary water, potentially due to microorganism degradation. Glyphosate or its metabolites readily move into moss tissues and establish equilibrium across the colony. Silvery threadmoss appears to readily absorb ^{14}C glyphosate; however, moss is not controlled by glyphosate suggesting possible metabolism or an altered state of the EPSP-synthase enzyme. Future research should attempt to characterize the extent to which ^{14}C recovered from moss tissue is the parent herbicide, or its metabolites being absorbed from capillary water after degradation.

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Figure 1. Structure of [14C-labeled phosphonomethyl] glyphosate. * denotes position of radiolabeled carbon.

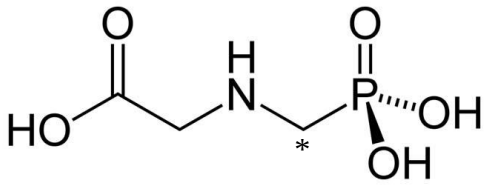


Figure 2. Total percent of applied ^{14}C recovered from all parts of all sections over time

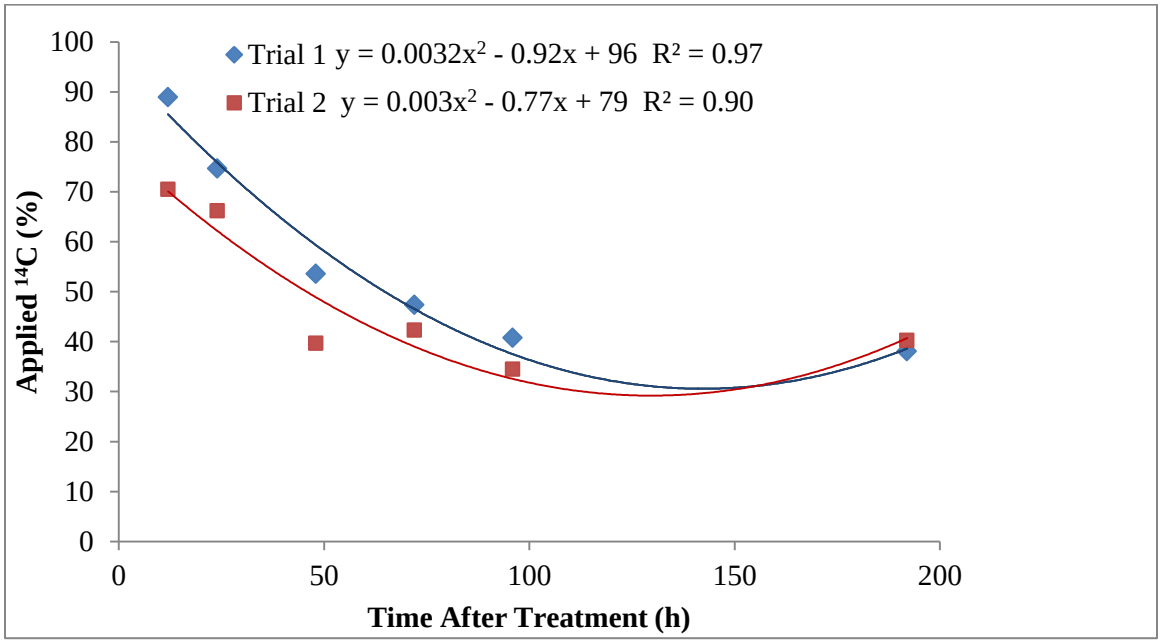


Figure 3. Percent of applied ^{14}C recovered from shoots and rhizoids over time.

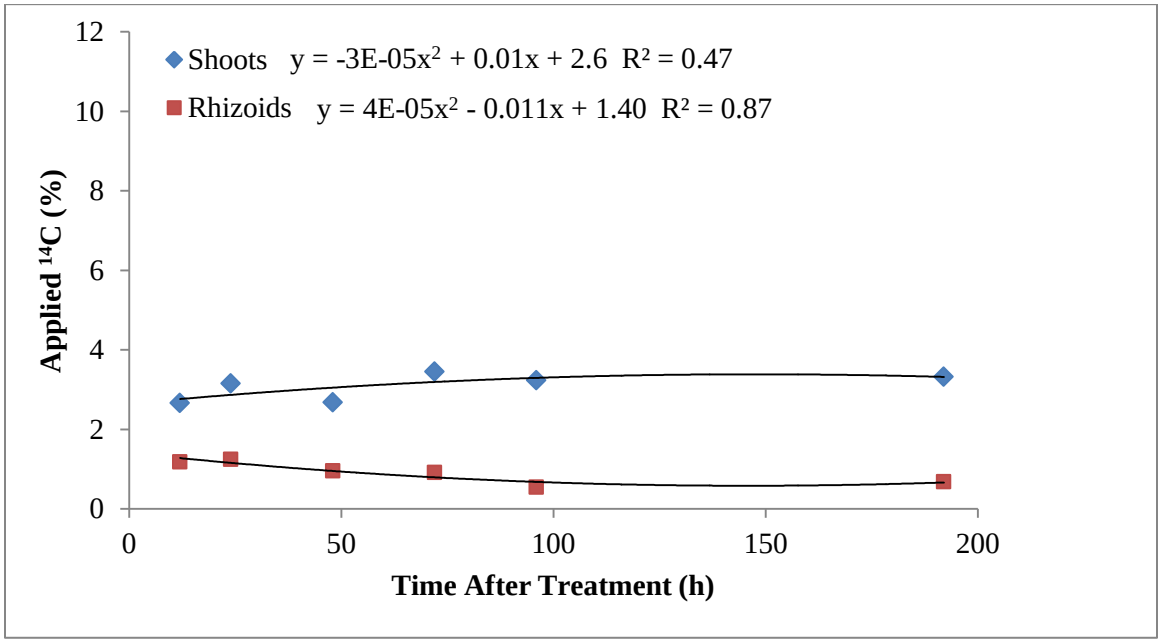


Figure 4. Percent of applied ^{14}C recovered from water and methanol washes wash over time.

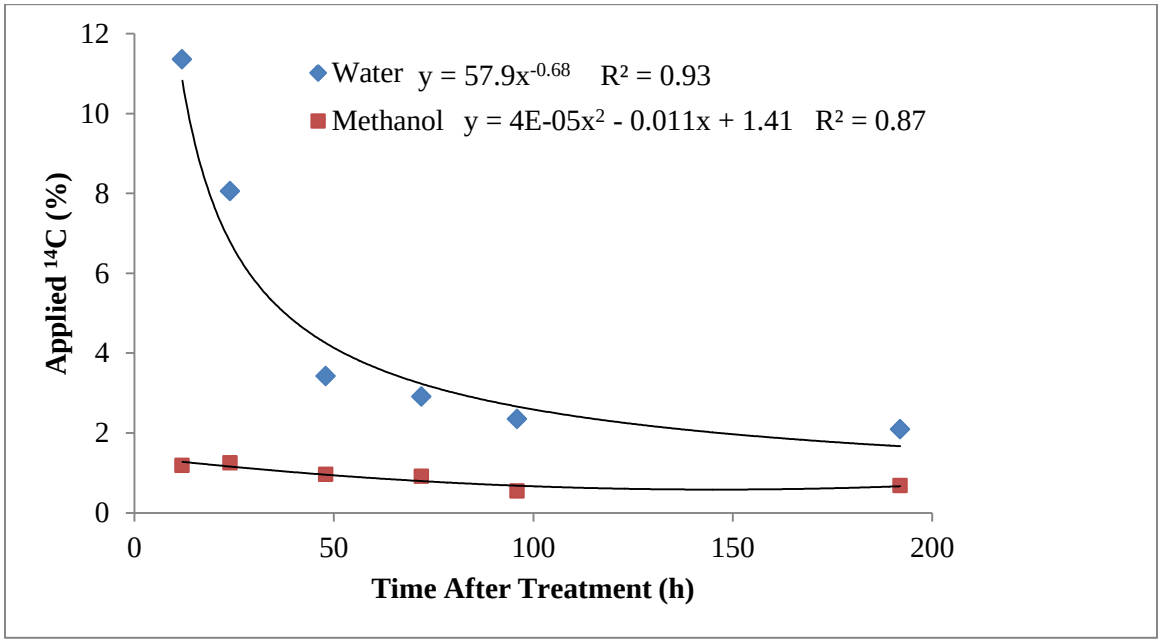


Figure 5. Percent of applied ^{14}C recovered from all parts of each section over time.

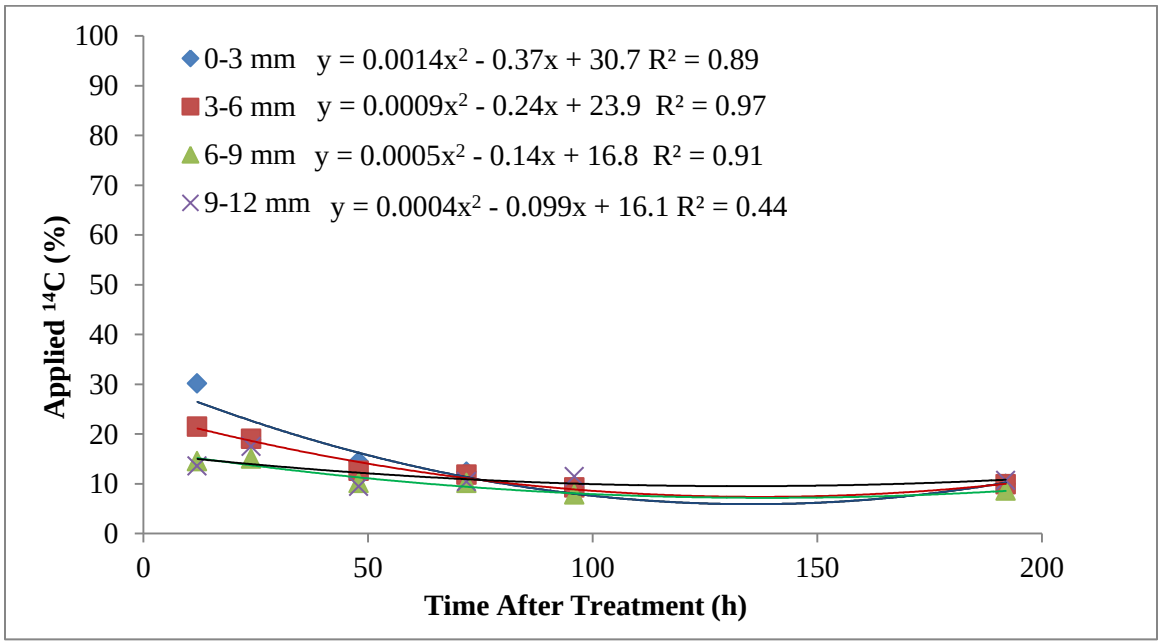


Figure 6. Percent of applied ^{14}C recovered from shoots over space.

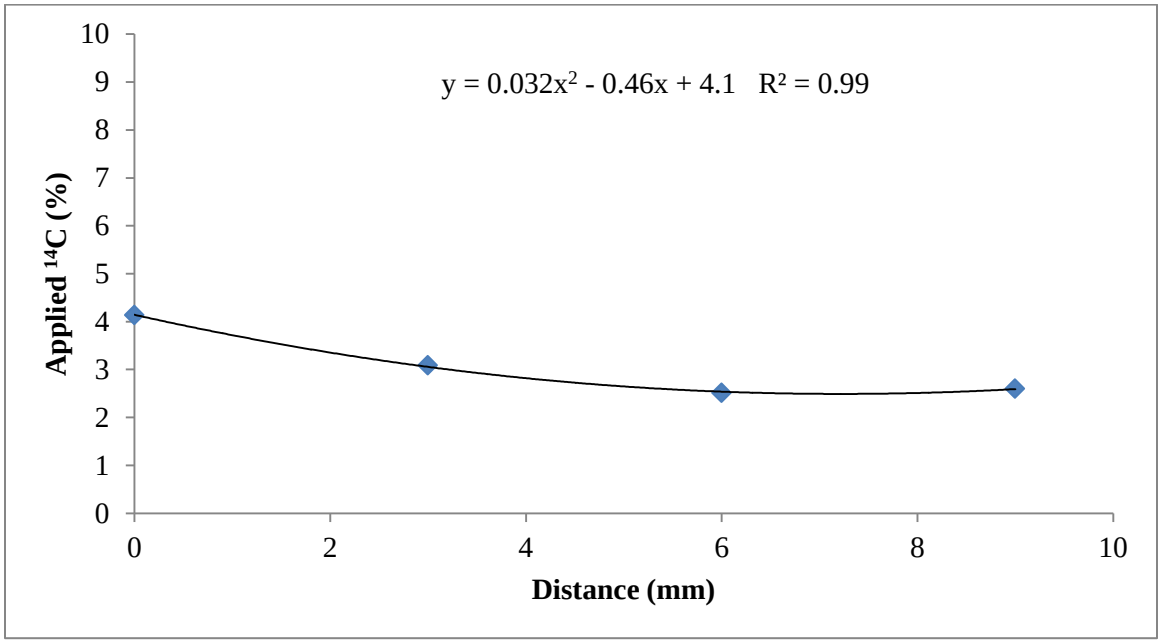


Figure 7. Percent of applied ^{14}C recovered from rhizoids over space.

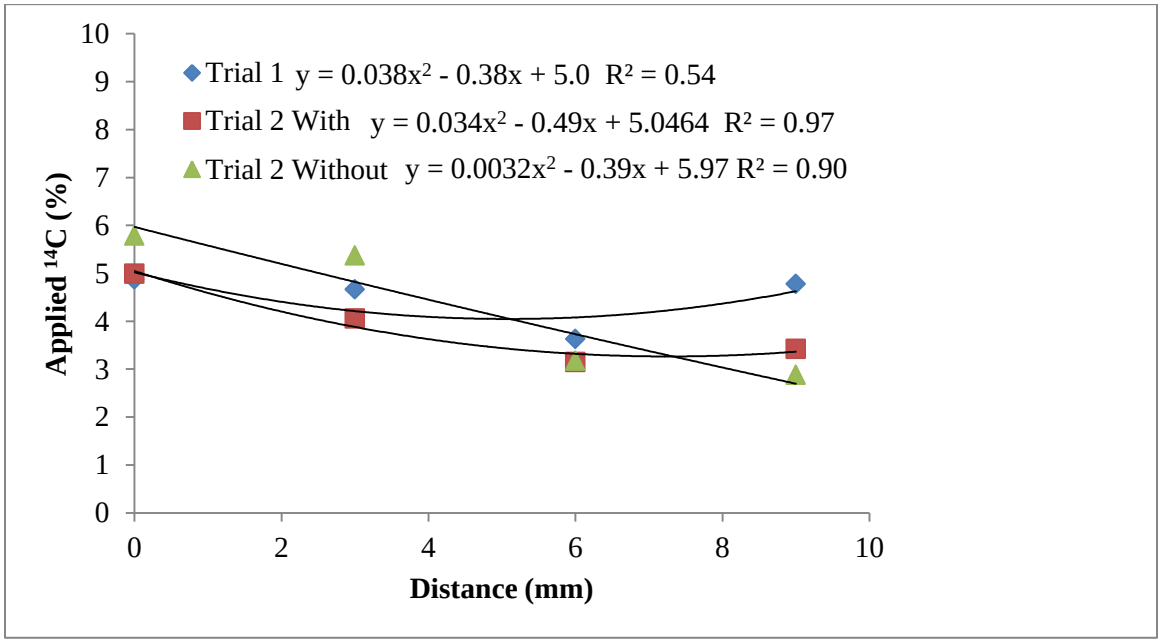


Figure 8. Percent of applied ^{14}C recovered from water or methanol wash over space.

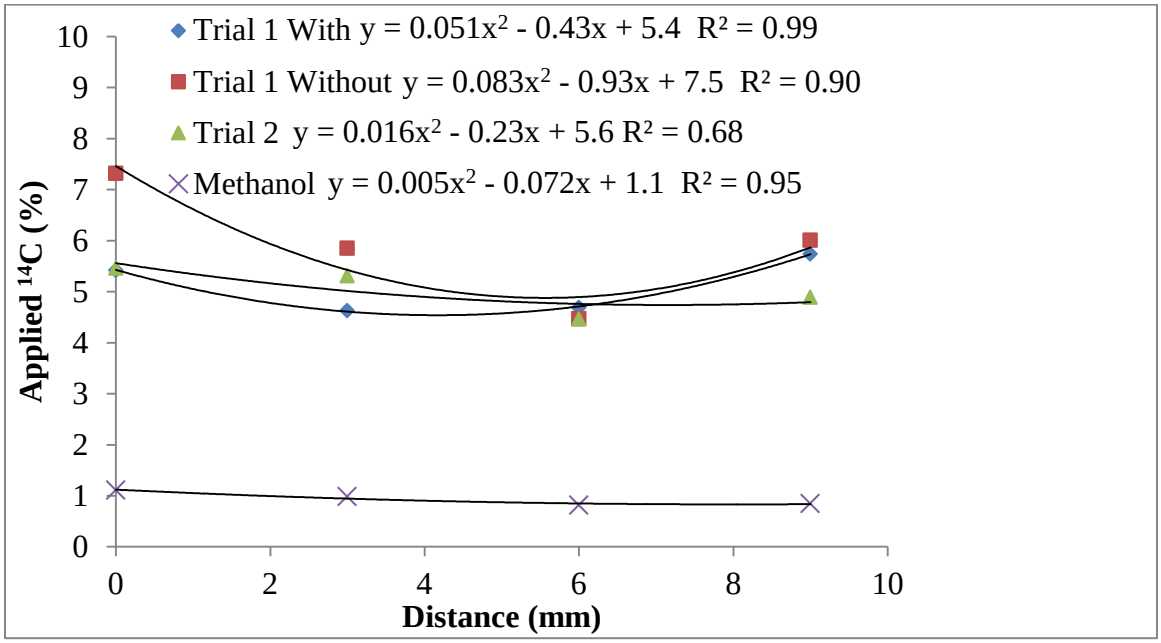
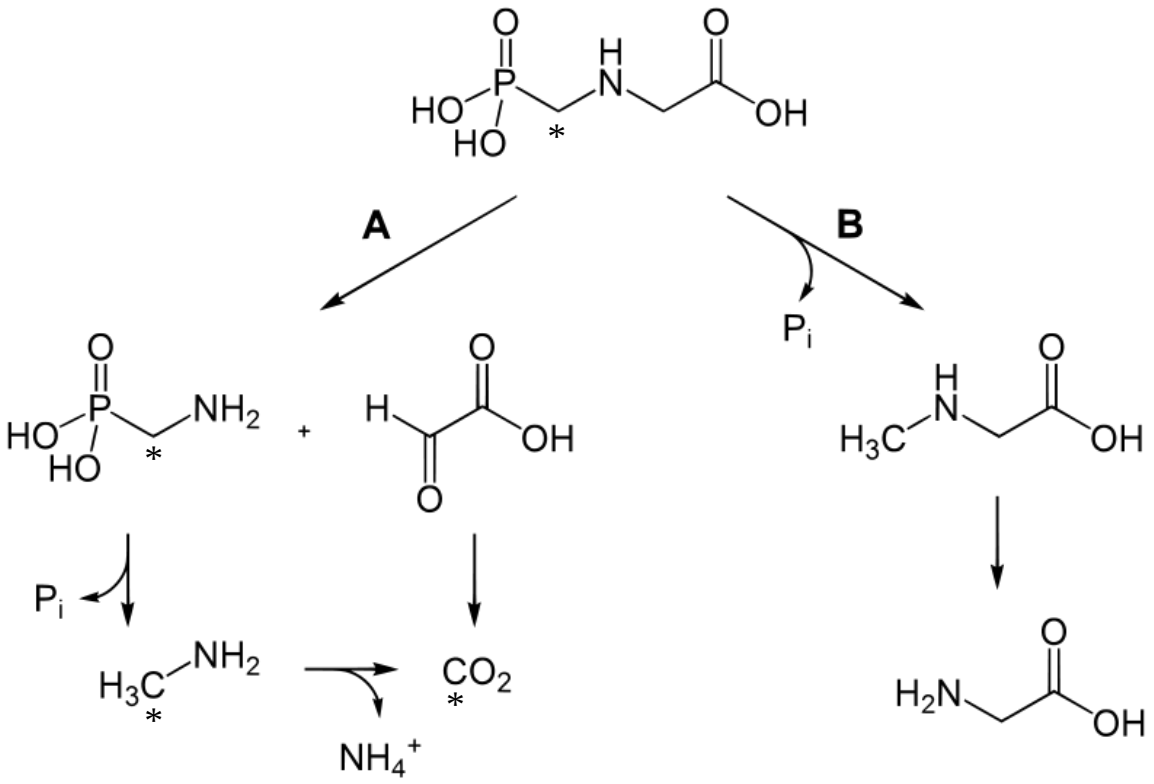


Figure 9. Glyphosate [phosphonomethyl ^{14}C] degradation pathway. * denoted radiolabeled carbon.



Chapter 5. Characterization of Biological Control Organisms for Silvery Threadmoss (*Bryum argenteum* Hedw.).—Identification, Descriptions, Koch's Postulates, and Safety to Putting Green Turf Creeping Bentgrass (*Agrostis stolonifera* L.) and Annual Bluegrass (*Poa annua* L)

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Silvery threadmoss (*Bryum argenteum* Hedw.) is a weed problem on golf course putting greens. Only one postemergence product, carfentrazone, is labeled to control moss on putting greens, and no preemergence products are labeled. Recently, two fungal organisms were observed causing disease symptoms in silvery threadmoss on golf course putting greens in Blacksburg, VA, and Knoxville, TN. These studies examined the two organisms for their potential use as a biological control for silvery threadmoss. Koch's postulates were fulfilled and the organisms were identified through morphological examination and ITS sequence as *Alternaria* sp., and *Sclerotium rolfsii*. Two isolates of *Alternaria* sp. and one of *S. rolfsii* were used in inoculation studies to examine host specificity and pathogenicity. All three isolates reduced moss green cover to 20% or less 14 days after inoculation (DAI). *Alternaria* sp. isolates were safe to both creeping bentgrass (*Agrostis stolonifera* L.) and annual bluegrass (*Poa annua* L.). The *S. rolfsii* isolate also reduced moss green cover to less than 20%; however, it was moderately pathogenic to annual bluegrass reducing green cover by 20% of the nontreated 14 DAI. These results suggest *Alternaria* sp. isolates would be the best choice for further

evaluation as a biological control agent for silvery threadmoss on creeping bentgrass and annual bluegrass putting greens.

Only a few pathogens have ever been reported to infect mosses. *Waitea circinata* Warcup and Talbot was recently reported as a fungal pathogen causing disease on silvery threadmoss (*Bryum argenteum* Hedw.) in Colorado (Mitkowski and Chaves 2013). Waitea patch is a common disease of annual bluegrass (*Poa annua* L.), and less commonly creeping bentgrass (*Agrostis stolonifera* L.), causing rings of yellowing necrosis on infected turf. This particular pathogen would not be a good candidate for selective biological control of silvery threadmoss on creeping bentgrass putting greens because it can discolor moss to almost white and has potential for injury to desirable turf. While selective control of moss is desirable, an aesthetically pleasing green color is generally more desirable in golf turf. Annual bluegrass, though not a desired turf for putting greens, is often managed as putting green turf along with creeping bentgrass due to its prevalence, especially in the transition zone where it comprises over half of the putting green turf (Askew et al. 2012).

Bradner et al. (2000) found a previously unknown *Embellisia* sp. associated with silvery threadmoss in the Antarctic. *Embellisia* species have often been found in association with higher plants, sometimes as endophytes but more often as pathogens (Lee et al. 2002). It was unclear whether the species described by Bradner et al. (2000) was endophytic or pathogenic on silvery threadmoss. A recent mini-review by Davey and Currah (2006) examined the current state of knowledge for moss-fungus interactions. It is likely that all pathogenic interactions that occur in higher plants also occur in bryophytes, but these processes are not well understood in bryophytes. There are reported obligate, facultative and opportunistic pathogens, as well as mycorrhizae associated with mosses (Rabatin 2008). Most described pathogens of mosses penetrate the tissues using enzymes

to digest portions of the cell wall to gain entry (During and van Tooren 1990; Redhead 1981; Simon 1987; Untiedt and Muller 1985). Mosses have cell walls composed of polyphenolic compounds and cellulose (Lee et al. 2005; Popper and Fry 2003) though it is generally accepted that most mosses lack the ability to produce true lignin (Basile et al. 1999; Goffinet et al. 2009; Shoefield and Hebant 1984; Umezawa 2003; Weng and Chapple 2010). A lack of lignin may allow for easier enzymatic degradation of the cell walls and other structural components of the cells by fungi.

Host response to pathogen infection and colonization are not well understood in bryophytes. There have been observations of potential hypersensitive response or programmed cell death (Longton 1973), and cell partitioning (Untiedt and Muller 1985) to slow the spread of a pathogen in some moss species; however, no conclusive studies have been performed to confirm these reports. *Physcomitrella patens* (Hedw.) Bruch. has a confirmed suite of resistance genes homologous to those found in vascular plants, but their expression and function have not been fully characterized (Akita and Valkonen 2002). There are at least a few pathogens reported as host specific, only known to infect a single species of moss, including *Discinella schimperi,* which infects *Sphagnum squarrosum* Crome (Redhead and Spicer 1981). There are other bryophyte pathogens reported with much broader host ranges. For example, *Octospora similis* (Kirchstein) Benkert can infect several species of *Bryum* (Benkert 1996) and *Stemphylium botryosum* Sacc., which infects *Leptodictyum riparium* (Hedw.) Warnst. as well as several vascular plant species (Redhead and Spicer 1981).

Two naturally occurring fungi have been observed to negatively affect silvery threadmoss in a fine turf setting. The development of a host-specific biological control

product suitable for use in fine turfgrass, particularly golf course putting greens, has the potential to increase recreational value. The last Virginia turfgrass survey was performed by USDA-NASS (2006), at which time the state boasted 345 public, private and semi-private golf courses with 36,900 acres of maintained turfgrass. The majority of reporting golf courses (53%) indicated weeds were a major management problem on their course (USDA 2006). Moss is undoubtedly included in this 53%, and across the state, golf courses spent $2.8 million on weed control products alone.

All Virginia courses taken together received 8.65 million rounds of play in 2004 which encompasses a large portion of Virginia recreational tourism dollars (USDA 2006). If a single golfer plays a hypothetical average of 20 rounds per year, this means golf courses around the state are seeing 432,500 golfers walk across their greens. Putting greens free of moss encroachment increase the ease of play for the golfer and provide an overall better experience. If golf courses are able to provide an exceptional experience for their golfers by providing a higher quality product, they are more likely to attract more and repeat golfers, meaning more revenue for the year.

At the same time, a decrease in the use of chemical pesticides in favor of an effective biological control organism would provide increased benefits to the natural environment as well. Many golf courses are situated near waterways where pesticide runoff could become a problem at point-source locations and downstream. Any decrease in pesticide use on those properties is a positive for the natural landscape. With these potential positive impacts in mind, the objectives of this study were to 1) prove pathogenicity for each biological control organism on silvery threadmoss; 2) describe each organism; and 3) evaluate the potential for use as biological control organisms against

silvery threadmoss on putting green surfaces consisting of creeping bentgrass and annual bluegrass.

Materials and Methods

Isolation, Culture, and DNA extraction. Initial fungal cultures were isolated from silvery threadmoss collected from the 'Penn-A1' putting green at the Glade Road Research Facility in Blacksburg, VA in 2009, and multiple times from several greens in the Blacksburg, VA area in 2010, 2011, and 2012 (Isolates GR8A and GR8B). A second organism was collected and isolated from silvery threadmoss in Tennessee (Isolate TN1) in July 2011. All fungi were maintained in pure culture at the Glade Road Research Facility in the turfgrass pathology laboratory. Fungi were cultured on 0.25 strength potato dextrose agar (PDA) solidified with an additional 1% v/v agar and transferred every 14 to 30 days to maintain fresh contaminant-free cultures. Pure cultures were obtained by transferring single spore or single hyphal tips onto water agar and subsequently transferring the pure culture back to 0.25 strength PDA. Pure cultures on PDA were grown for 7 to 14 days and tissues were scraped from the plate with a sterile blade for DNA extraction. DNA was extracted using Qiagen Plant Minikit (Qiagen, Hilden, Germany) following the manufacturer's protocol including all optional steps. PCR was run for 35 cycles using Immomix red (Bioline, Taunton, MA) to amplify *ITS* 4 ('TCCTCCGCTTATTGATATGC') and 5 (GGAAGTAAAAGTCGTAACAAGG) described by White et al. (1990). DNA sequencing was performed by University of Chicago-Cancer Institute (Chicago, IL). Clean sequences were blasted in the NCBI

database to determine if exact or close matches exist (Geer 2010). The most closely related sequences were retained for comparison.

Morphological characterization. Fourteen individual transfers were made to full strength PDA solidified with an additional 1% v/v agar for each organism in order to examine the progression of a culture over the course of two weeks, or the approximate time it requires for these cultures to reach the edge of a 100-mm Petri plate. Cultures were maintained in the laboratory at ambient temperature and light. Diameter measurements were taken in two directions several times throughout the study and cultures were examined under the microscope for condition of the hyphae and presence of sexual or asexual reproductive structures. If present, measurements were taken of five of each structure to get an average. In a separate evaluation, five plates of each isolate were maintained at 24°C in a growth chamber receiving 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) from one 8-bulb Sun System Tek Light (Sunlight Supply, Inc. Vancouver WA). Sporulation was still slow to develop; however, some additional observations and structure measurements were obtained during this evaluation. Characteristics did not differ from those found under ambient conditions.

Pathogenicity and host specificity. Two trials were initiated to determine efficacy against moss and safety to commonly managed turfgrass species on putting greens, creeping bentgrass and annual bluegrass. Plugs of annual bluegrass, creeping bentgrass, and silvery threadmoss (1 cm in diameter) were collected from a 'Penn-A1' creeping bentgrass putting green in Blacksburg, VA. One plug of each species was placed together in a covered 10-cm square pot. Each trial was maintained in a separate growth chamber with a constant temperature of 24°C ± 2°C and a 16-hour photoperiod. Plants were

exposed to 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) from one 8-bulb Sun System Tek Light (Sunlight Supply, Inc. Vancouver WA) 46 cm above canopy height. Plants were allowed to acclimate in the chambers for seven days before inoculation. All plugs were maintained at a height of 4 mm by mowing with scissors or a razor three times per week and pots were watered as needed. Each plug in each pot received one inoculum treatment: Isolate TN1, GR8B, or GR8A and a nontreated control. Isolates were grown on full strength potato dextrose agar (PDA) solidified with 11.25 g L^{-1} agar for 14 days. All fungal tissue including sclerotia, mycelium and spores were scraped from the culture and macerated in sterile water to create inoculum slurry. Leaf surfaces of each plug were treated with 25 μL of slurry evenly distributed. The nontreated control received sterile water applied in the same manner. Digital images were taken at 0, 7, and 14 days after inoculation. At each rating, all species were evaluated for presence of disease symptoms, and percentage green cover. Fourteen days after initiation ten leaves were harvested from each grass and ten shoots from each moss plug and surface-sterilized in 5% bleach for two minutes, rinsed twice in sterile deionized water for one minute each, and plated onto full strength PDA solidified with 11.25 g L^{-1} agar. Plates were evaluated daily for seven days. All fungal growth was sub-cultured to obtain pure strains and evaluated based on morphology.

Results and Discussion

Isolation, Culture, and DNA extraction. Three fungal isolates GR8A, GR8A1, and GR8B, collected in Blacksburg, VA were identified as *Alternaria* sp. with a 99% match in NCBI nucleotide BLAST database (Geer 2010). Closest matches were *Alternaria*

tenuissima and nonspecific *Alternaria* sp. Alignment with *Alternaria tenuissima* is given in Figure 1. Only two differences were detected in 585 basepairs (Figure 1) for the sequence returned as *A. tenuissima*. It was deposited and annotated by J. Jiang, X. Cao, and F. Chen, all scientists housed at the State Key Laboratory of Plant Genomics and part of the Chinese Academy of Sciences. However, since sequences deposited in GenBank are annotated by the submitter, there are many reports of misidentifications. De Hoog and Horre (2002) reported approximately 14% of sequences deposited for a particular group of *Alternaria* and *Ulocladum* species were misidentified. *Alternaria tenuissima* is part of a larger group of *Alternaria* species classified as "small-spored *Alternaria*." These cannot be reliably distinguished morphologically or phylogenetically with common genetic loci. *ITS* is a conserved genetic region for *Alternaria* species and has demonstrated little variability, particularly in this small-spored group (Chou and Wu 2002; Kang et al. 2002; Pryor and Gilbertson 2000). For these reasons, we refrain from definitively naming our isolates *A. tenuissima* and leave them as *Alternaria* species until genetic regions capable of distinguishing between these groups can be examined. However, we can place this isolate in the group of small-spored *Alternaria* which includes: *A. tenuissima, A. arborescens*, and *A. alternata* among others (Andrew et al. 2009).

After several attempts clean sequence data could not be obtained from the fourth evaluated isolate, TN1; however, this organism was identified based on morphology as *Sclerotium rolfsii* (see below).

Morphological characterization. Isolates GR8B, GR8A and GR8A1 all have a similar morphology but differing growth rates on 0.25 strength PDA (Figure 2). Since *ITS* sequence data have placed them together and they were isolated from the same host

colonies, they are treated here as one organism. Initial growth rate was 5 to 8 mm per day for 5 days and then growth slowed to 3-4 mm per day (Figure 2). Hyphae are septate, darkly pigmented, and 1-2 μm in diameter. Colonies are dark brown to black in color and the surface appears powdery. The organism begins to pigment the medium 7 to 9 days after transfer. First the medium near the edge of the colony turns pale yellow, and within two weeks the medium is brown throughout. Sporulation of GR8A and B on PDA was slow to develop. Transfers to V8 (Campbell Soup Company, Camden, NJ), cornmeal, water, and sugar agars did not improve sporulation in culture. Therefore, all spore examinations for these isolates were made from host tissues. Conidiophores are simple and un-branched with elliptical conidia having 3 to 4 transverse and 1 to 2 longitudinal septa (Figure 3).

Isolate TN1, identified here as *Sclerotium rolfsii*, grows 15-18 mm in diameter per day in 0.25 strength PDA, filling 100 mm Petri plates to capacity within 4-5 days (Figure 2). Hyphae are white to translucent in sterile culture but once infection has occurred aerial hyphae on moss cultures become pigmented pale-brown to gold. Hyphae are septate with clamp connections and 3 to 4 μm in diameter which is similar to previous descriptions (Smiley et al. 2005). At eight days sclerotia begin to form on colony edges (Figure 4). Initially, sclerotia are white and puffy, perfectly round and exude moisture droplets from their surface. They begin to melanize after 5 to 6 days turning from white to orange to dark brown. Initially smooth, sclerotia surfaces become pitted upon maturity and they range from 0.5 to 2 mm in diameter (Figure 4). Sclerotia separate from hyphae easily and have the ability to float in water. Surface sterilized sclerotia transferred to fresh PDA or to

moss colonies germinate in about three days. No sexual structures were noted during examination of this isolate in culture or after infection of host tissues.

Pathogenicity and Host specificity. Isolates GR8B, GR8A, and GRA1, identified as *Alternaria* sp. caused disease symptoms in all inoculated moss colonies and reduced average percent green cover of moss to 18% by 14 days after inoculation (DAI) (Figure 5). Re-isolations were successful from all colonies where disease symptoms developed. Two to three DAI areas of the moss colony appear water-soaked and dark. Shoot tissue in the water-soaked area begins to die within two days, first turning black and then rotting. Fungal mycelia move radially outward from water-soaked colony lesions to infect surrounding tissues. Hyphae can often be seen with a hand lens crossing from one shoot to another. We were unable to determine how this pathogen is penetrating host tissues but speculate it occurs through wounds in host tissue. Putting greens are mowed six days per week, constantly wounding moss tissues, and infections have been observed radiating from the edge of holes where moss plugs were removed. Preliminary studies in culture could not confirm a wounding requirement for GR8A and B. Scanning electron micrographs of moss, shortly after infection, also provided little evidence for the mechanism of penetration. Many *Alternaria* species produce toxins which can move into host cells ahead of the disease front, injuring tissues and causing cells to leak contents for absorption by fungal hyphae (Laemmlen 2001). Thus, an alternate hypothesis would be that penetration occurs through enzymatic digestion of host tissues. The fact that the host tissues collapse and rot shortly after infection is supportive of this hypothesis.

Alternaria diseases occur worldwide and have been reported for hundreds of host plants (Laemmlen 2001). *Alternaria tenuissima* was the closest species match (99%) to

ITS sequence submitted as a NCBI nucleotide BLAST (Geer 2010) for all three GR8 isolates in this study (Figure 1). It has been reported from 336 hosts in the USDA fungal database (Farr and Rossman 2013) several of which are grasses; however, it has not been reported for annual bluegrass or creeping bentgrass. *Alternaria tenuissima* has been reported in Zambia from bermudagrass (*Cynodon dactylon* (L.) Pers), another common species used as putting green turf in the southern U.S. (Lenne 1990).

Isolate TN1, identified as *Sclerotium rolfsii* causes southern blight of several turfgrass species including annual bluegrass and creeping bentgrass (Punja 1982; Smiley 2005). *Sclerotium rolfsii* caused disease symptoms in 75% of moss colonies inoculated with only sclerotia and 100% of colonies inoculated with macerated fungal tissues. TN1 reduced average percent green cover of moss to 18% 14 DAI (Figure 5). Re-isolations were successful from all colonies where disease symptoms occurred. For sclerotia-only inoculations, disease symptom development was dependent on whether the sclerotia germinated or not. It is possible that some moss colonies were inoculated with immature sclerotia or that 24°C is too low for 100% sclerotia germination. Smiley et al. (2005) reported *S. rolfsii* germinates best at temperatures above 24°C.

Infection occurred within three days after inoculation. A pale brown to gold mycelial mat formed over the moss colony surface. The moss shoots were stunted compared to shoots in nontreated colonies but continued to photosynthesize and appeared healthy. Seven to ten days after inoculation, moss shoot tissues turned straw colored and the entire colony died within three days. After host death, *S. rolfsii* initiated formation of sclerotia which progressed the same way as in sterile culture, maturing from white to orange to dark brown.

These results represent the first efforts to identify and characterize a potential biological control agent for silvery threadmoss. Both *S. rolfsii* and the *Alternaria* species tentatively identified as *A. tenuissima* controlled inoculated moss colonies 100% within three weeks of inoculation. Future research should address the effects other pesticides currently used on greens have on the organism, the most appropriate formulation for delivery of *A. tenuissima,* ideal rates of application in order to ensure control in the field, and further host specificity testing to avoid injury to non-target plant species.

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Figure 1. 565 basepair *ITS* alignment from closest match in NCBI nucleotide BLAST database (Greer 2010) to GR8A and B. Query denotes GR8A or B sequence and subject denotes *Alternaria tenuissima* sequence.

Figure 2. Average growth rate over time of fungal colonies in pure culture on 0.25 strength PDA.

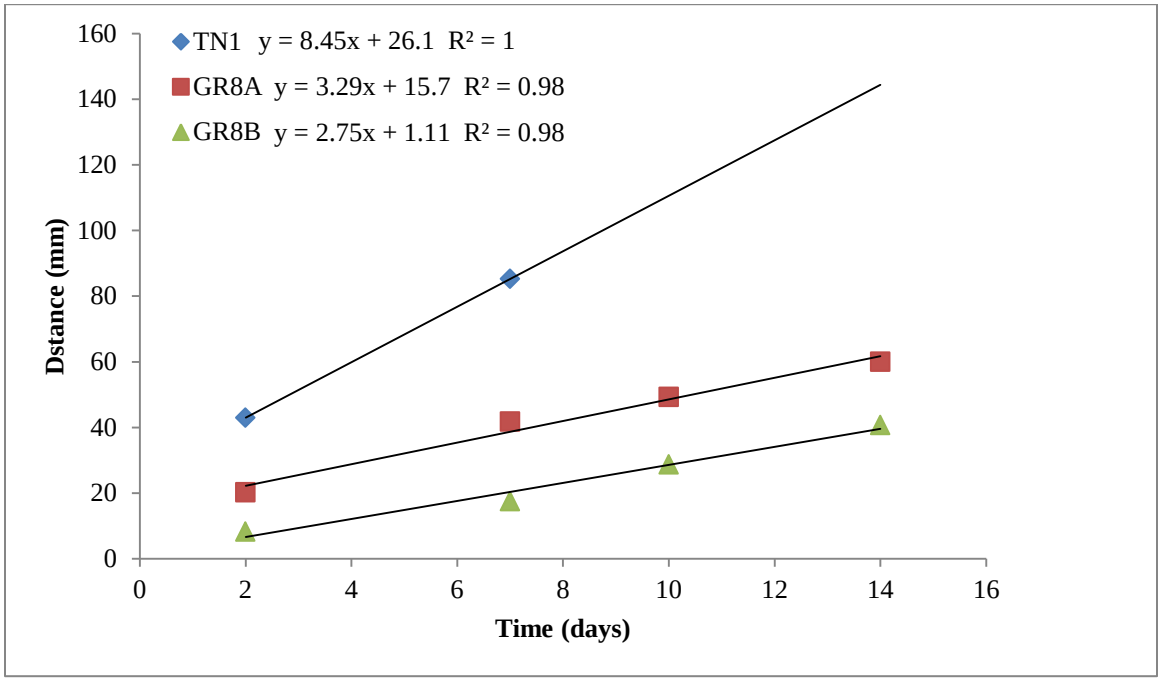


Figure 3. *Alternaria* sp. (Isolates GR8A and B). a) hyphae wrapped around a moss rhizoid; b) spores [Photo by Dr. Anton Baudoin, used with permission]; c) infected moss colony along the disease front; d) diseased moss colony on a golf putting green.

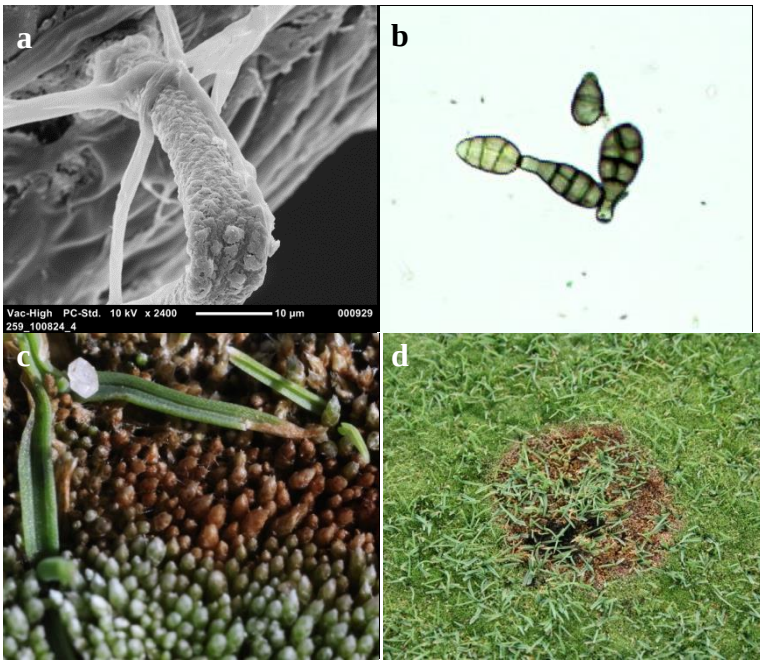


Figure 4. *Sclerotium rolfsii* (TN1). a) hyphae erupting from moss epidermis; b) forming sclerotia; c) mature sclerotia; d) infected moss colony along the disease front.

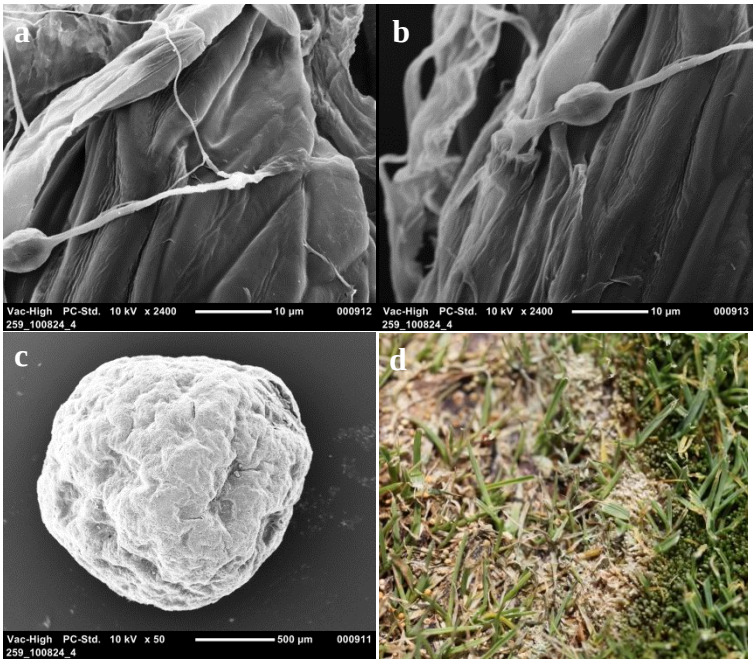
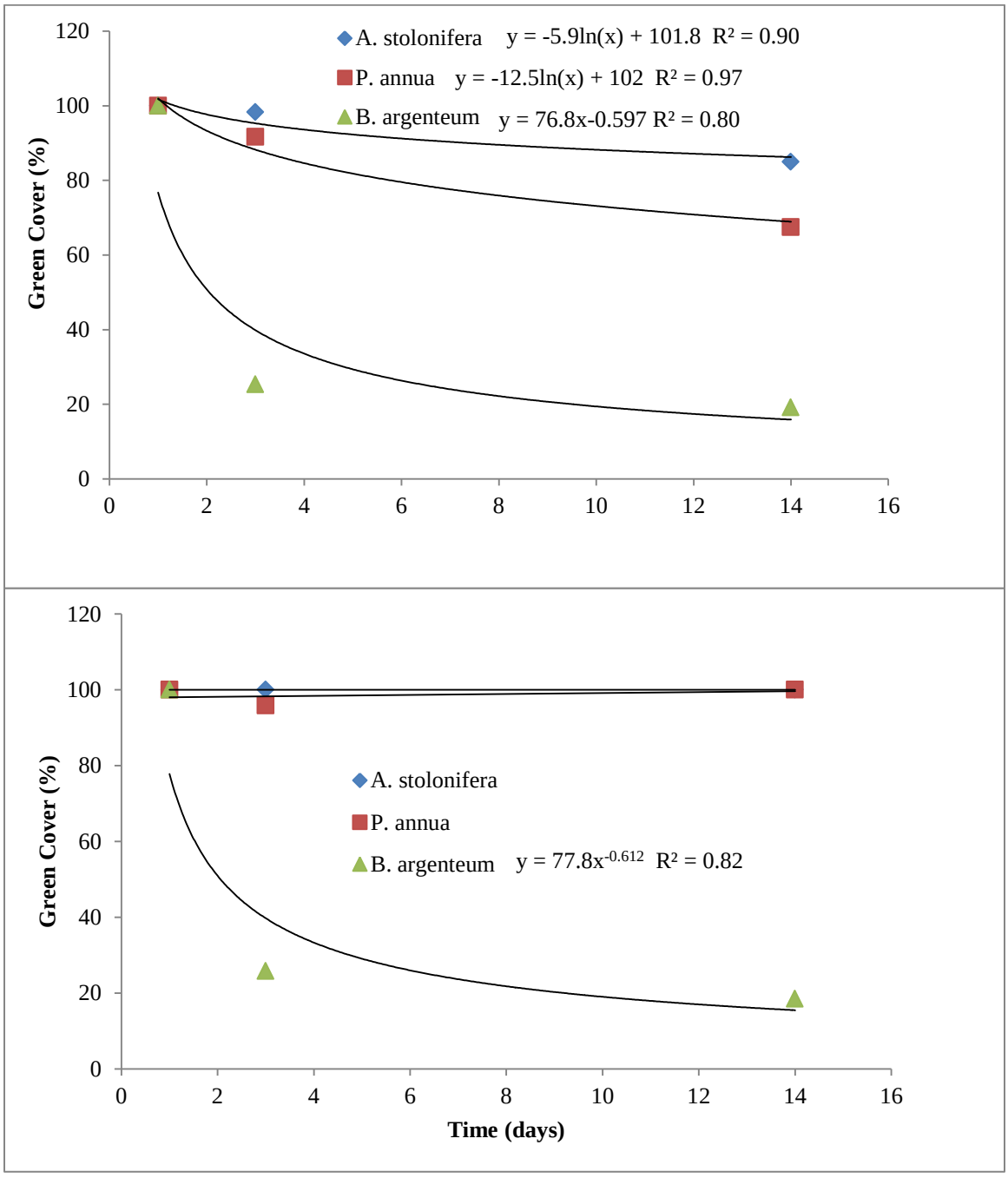


Figure 5. Percent green cover reduction of inoculated *Agrostis stolonifera*, *Poa annua*, and *Bryum argenteum* plugs over time from *Sclerotium rolfsii* (above) and *Alternaria* sp. (below).



APPENDIX A. Supplemental Images.

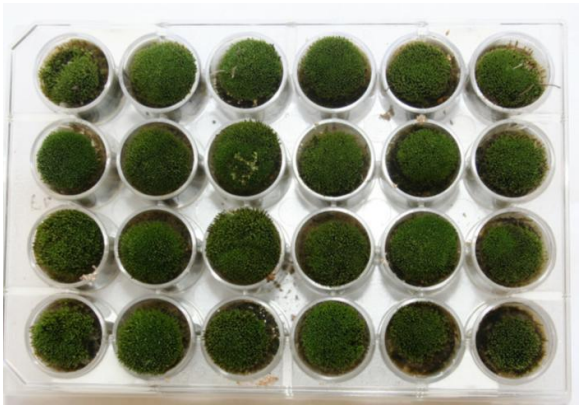


Image 1. *Bryum argenteum* plugs after 21 day acclimation in the growth chamber.

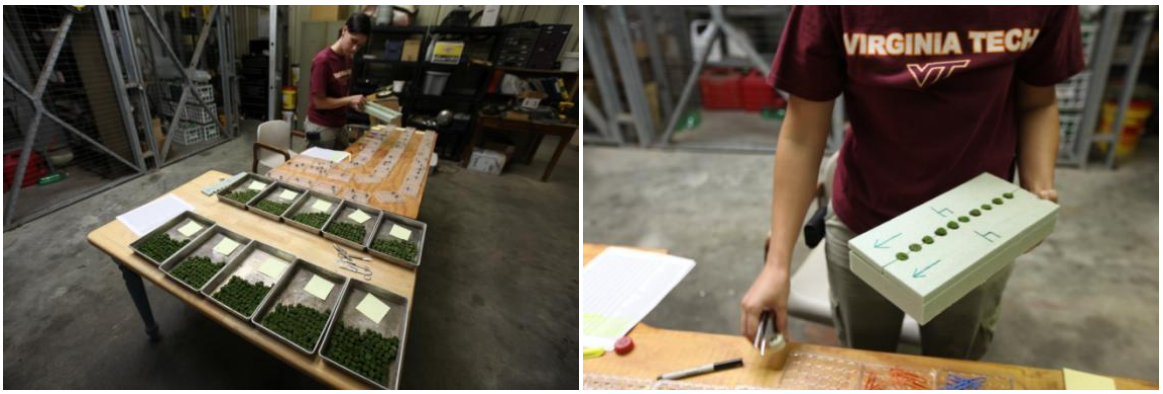


Image 2. Silvery threadmoss plugs sorted by replicate (left); and silvery threadmoss in a jig created to simulate spray coverage on a golf course putting green when sprayed in a spray chamber (right).

Image 3. Moss digital image center designed to take uniform images of all moss plates.

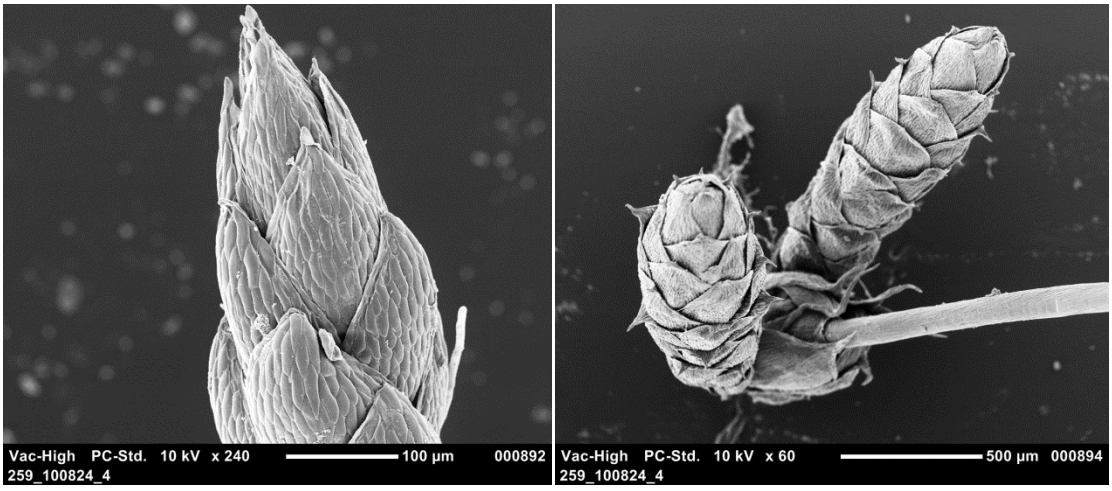


Image 4. Scanning electron micrograph of a healthy silvery threadmoss shoot tip (left); and mature gametophytes (right).

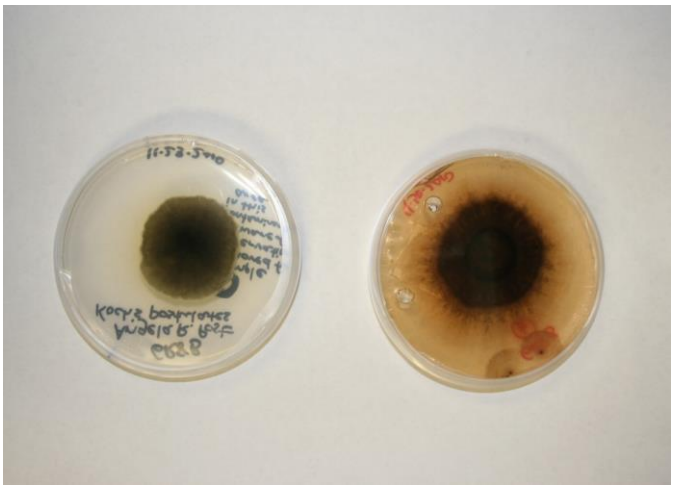


Image 5. *Alternaria* sp. culture 7 days after transfer with pale yellow pigment beginning to form (left); and 3 weeks after transfer with pigment becoming brown in color (right).

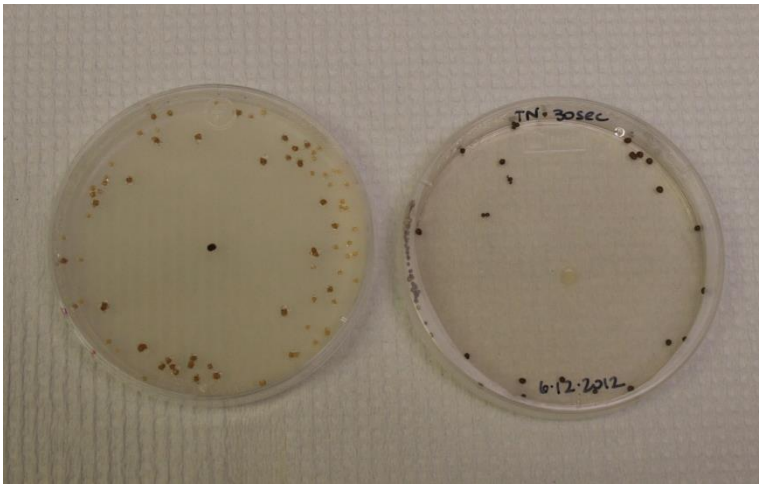


Image 6. *Sclerotium rolfsii* plate 10 days after transfer with orange sclerotia (left) and 3 weeks after transfer with brown sclerotia (right).

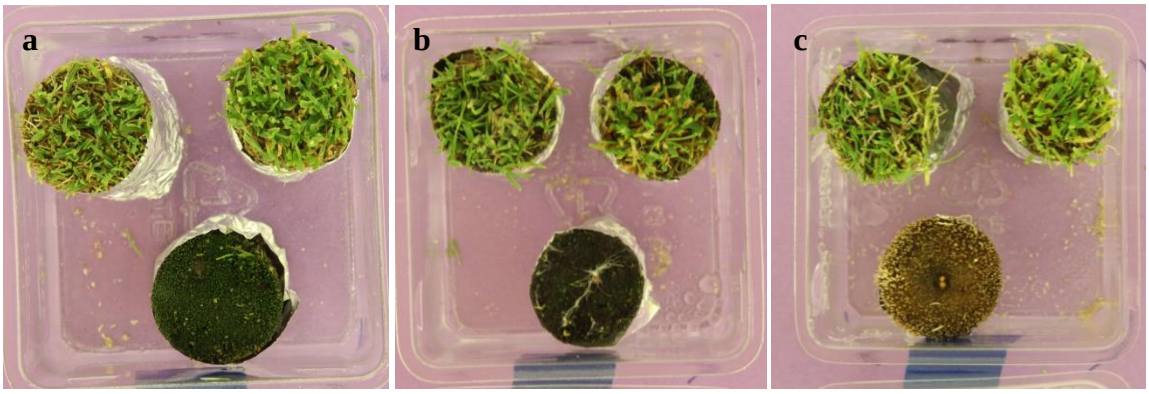


Image. 7. *Sclerotium rolfsii* treated plugs of bentgrass (upper left plug), annual bluegrass (upper right plug) and silvery threadmoss (lower plug) a) nontreated control; b) 7 days after treatment (DAT); c) 14 DAT.

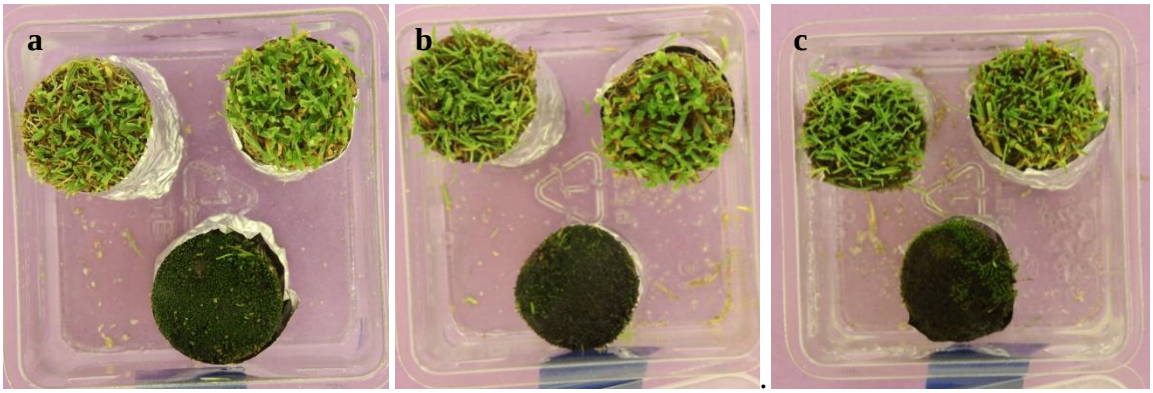


Image. 8. *Alternaria sp.* treated plugs of bentgrass (upper left plug), annual bluegrass (upper right plug) and silvery threadmoss (lower plug). a) nontreated control; b) 7 days after treatment (DAT); c) 14 DAT.