ABSTRACT

KOEHLER, ALYSSA MARIE. Etiology and Management of Stem Rot Diseases of Stevia and Brown Patch of Tall Fescue. (Under the direction of Dr. David Shew).

Stevia (*Stevia rebaundiana*) is an herbaceous perennial that is an emerging crop in the US. Stevia leaves contain multiple glycosides that are extracted for use as a nonnutritive sweetener, and following USDA approval of stevia as a non-nutritive sweetener in 2008, many companies expanded their use of this low calorie sweetener. Stevia plantings in North Carolina began in 2011. Wilting and death of plants in first and second year commercial plantings were observed in NC in 2012 and 2013, and Koch's postulates were performed to verify that *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* were pathogens of stevia. There are currently no disease-control products labeled for use on stevia in the US. Field trials were conducted to determine optimal application rates and timing for fungicide and biological control products reported to be effective against *S. rolfsii*. A trial was planted in May 2014 to investigate the efficacy of multiple biological control agents and fungicides on suppression of stem rot caused by *S. rolfsii* in a field with a history of the disease on stevia. Two biocontrol products, clontri+strepse and veramin (Sacom, Larino, Italy) and two fungicides tebuconazole (Folicur, Bayer Crop Science), and flutolanil (Convoy, Nichino America, Inc.) suppressed disease compared to the control. A second trial was planted in July 2014 to determine the effects of application of fungicides at or before transplanting on stem rot. Azoxystrobin (Abound, Syngenta Crop Protection, Inc.), applied as a spray treatment one week prior to planting and as a foliar spray at planting offered the highest disease suppression, followed by tebuconazole applied as a spray treatment applied one week prior to planting.

Brown patch, caused by multiple species of *Rhizoctonia* and *Rhizoctonia*-like fungi, is the most severe summer disease of tall fescue in home lawns across the southeastern US. Home lawns were surveyed in May 2014 and during July and August of 2013 and 2014 to determine the organisms present during a brown patch epidemic. Pathogens were isolated from individual lesions present on tall fescue leaves in lawns managed by lawn-care companies, with or without fungicides applied for brown patch control. Early season isolates were obtained from 56 home lawns across central NC in May 2014. Isolates were identified to species and anastomosis group based on ITS rDNA sequence analysis. Of 108 isolates collected in May 2014, 77% were *Ceratobasidium* sp., 11% were *R. solani* anastomosis group (AG) 2-2IIIB, 9% from *R. solani* AG 1-1B, and 3% were *R. zeae*. Of the 79 late season isolates collected from 57 home lawns in 2013 and 2014, 2.5% were *Ceratobasidium* sp., 44% were *R. solani* AG 2-2-IIIB, 37% were *R. solani* AG 1-1B, and 14% were *R. zeae*. In highly managed lawns typically three applications of fungicide are applied each summer for brown patch control. A total of 124 isolates, including six historic isolates from untreated yards, 20 isolates from fungicide-treated lawns collected in 2003, and 108 from May 2014, were assayed for sensitivity to the fungicides azoxystrobin, flutolanil, fluxapyroxad, and propiconazole which are used to treat lawns. Mean EC_{50} values varied across fungicides and species, but no fungicide resistance was observed. After at least 10 seasons of fungicide use, no loss of efficacy was observed to any of the fungicides among home-lawn isolates of brown patch pathogens. The shift in pathogens that cause disease during a brown patch epidemic and the range of fungicide sensitivities observed for those pathogens, indicate that optimal management strategies in home-lawn tall fescue may vary during the season, but there is no strong selection pressure for selection of fungicide insensitivity

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Etiology and Management of Stem Rot Diseases of Stevia and Brown Patch of Tall Fescue

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DEDICATION

To my wonderful family.

BIOGRAPHY

Alyssa Marie Koehler was born on June 29, 1990 in Rochester, NH. In 1993, her family moved to Asheboro, NC were she would grow up able to spend many hours outdoors and develop a strong interest in plants. As a high school student, this interest was further developed through participation in the National FFA Organization and working at Toms Creek Nursery and Landscaping.

Alyssa attended North Carolina State University beginning in biological and agricultural engineering in August 2008. Alyssa sought to incorporate plants into her engineering curriculum, and in the spring of 2009 a fortuitous web search resulted in her discovering the field of plant pathology and becoming a nematology lab assistant under the direction of Dr. Rick Davis. As an undergraduate, Alyssa would go on to complete the NCSU Kelman Scholar summer research program, work as an intern for the Animal Plant Health Inspection Service (APHIS) modeling pest risk, complete a summer internship working with pineapple diseases in Costa Rica, and transition her degree, graduating with a B.S. in Plant Biology in May 2012. These experiences led her to pursue a Master's degree in plant pathology under the guidance of Dr. David Shew. Her research focused on soilborne fungal pathogens including *Phytophthora nicotianae* on tobacco, *Rhizoctonia solani* on tall fescue, and *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* on stevia.

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The Genus *Stevia*

Discovery and Early Uses of Stevia

Stevia is a New World genus with species occurring in the southern United States, Central America, and South America. The genus contains about 230 species, and includes a diverse array of herbs and shrubs that grow along mountain slopes, and in forests and grasslands (King and Robinson, 1987, Kinghorn, 2003). Of the known species, 34 have been documented with ethnobotanical uses, but the most well-known and widely studied species is *Stevia rebaudiana* (Bertoni) Bertoni. *S. rebaudiana* is a perennial herb native to the highlands of Paraguay in the valley of the Rio Monday (Katayama et al., 1976; Brandle et al., 1998), where it grows in grassland communities or along marsh edges in acidic, infertile, sandy soils with shallow water tables at altitudes of 200-700m (Shock, 1982; Brandle et al., 1998). In this semi-humid subtropical climate, temperatures range from -6 to 43°C. In commercial systems, where stevia is often planted outside of its native environmental conditions, the plant does not survive cold winters, and must be grown as an annual (Yadav et al., 2010). In its native environment, *S. rebaudiana* tends to have a slender stem 30-50 cm tall with little branching. Commercial plantings of stevia are harvested by combining, which encourages lateral branching and secondary shoots that give a dense appearance. Cultivated plants may reach up to 65 cm in height (Brandle et al., 1998; Kinghorn, 2002; Ramesh et al., 2006).

S. rebaudiana was introduced to scientific research through the Italian-Swiss botanist-naturalist Moises Santiago de Bertoni who immigrated to Paraguay in 1882. As he was beginning to explore northeastern Paraguay in 1887, he learned of the existence of a sweet herb from the native Indians of Mondaih. The plant, called caá-êhê or "sweet herb", had been used for generations by the Guarani Indians of the Paraguayan highlands as a medicinal, a general sweeting agent, and to sweeten maté, a bitter caffeinated beverage (Kinghorn, 2003; Soejarto et al., 1983). Bertoni first came in contact with this plant years later when he received a dried specimen from a customs officer in Asunción, the capital city of Paraguay. Based on the dried fragments, he identified the plant as belonging to Tribe Eupatorieae in the family Compositae (Asteraceae), and in 1899 Bertoni communicated the name *Eupatorium rebaudianum*. In 1904, Bertoni received his first live specimen and determined the correct genus was *Stevia*, publishing the name as *Stevia rebaudiana* Bertoni in 1905 (Kinghorn, 2003).

The sweetness of stevia comes from multiple diterpene glycosides; 11 glycosides have been identified to date in *S. rebaudiana* (Prakash et al., 2014). The most notable glycosides are stevioside and several rebaudiosides (Kinghorn, 2003). Steviol glycosides can be up to 300 times sweeter than sucrose, but since glycosides are not metabolized, they are an acceptable non-caloric sweetener safe for diabetic users. Commercial use of stevia first began in Japan in 1971, but use in the US has developed over many decades. In 1991, the FDA cited stevia as "an unapproved food additive" and banned its sale in the US. Following the Health Freedom Act of 1995, stevia was designated as a dietary supplement. Import of stevia was resumed, but as a dietary supplement, teas or packaged foods remained banned. In

December 2008, the USDA approved stevia for use as a non-nutritive sweetener (FDA GRAS Notice GRN 000253 and GRN 000252). Since that time, numerous stevia products have appeared in US markets, and interest in stevia continues to increase.

Plant Morphology

Leaves. *S. rebaudiana* has simple leaves 2-3 cm long in opposite arrangement (Kinghorn, 2003). Leaves are often inconsistent in size and shape with various combinations of oblong, lanceolate, elliptical, and ovate leaf shapes occurring. Leaf margins are crenate to serrate on the upper portion of the leaf, while the lower half of the leaf has an entire margin (Figure 1.1). Short white trichomes surround the branches and leaves of *S. rebaudiana*. Trichomes can be either large, 4-5 µm, or small, 2.5µm (Yadav et al., 2010). Leaves are sessile, subsessile, or have a petiole 3-4mm in length (Kinghorn, 2003). The diterpene glycosides of *S. rebaudiana* are concentrated in the leaves, making them the focus of commercial extraction and the desired economic component of this crop (Dacome et al., 2005; Ramesh et al., 2006).

Figure 1.1. Leaves of *S. rebaudiana*

Roots. *S. rebaudiana* has a filiform root system and perennial rhizomes that allow it to overwinter in areas where tops are killed back by cool temperatures (Figure 1.2). Fine roots spread just under the soil surface while thicker roots develop deeper in the soil profile (Taiariol, 2004). Steviol glycosides do not accumulate in the root system and account for less than 0.1% of the dry root mass (Bondarev et al., 2003).

Figure 1.2. *S. rebaudiana* root system showing fibrous surface roots and thick roots that grow deeper into the soil profile

Glycoside identification and extraction

Since its original description, the glycosides present in *S. rebaudiana* have been the primary interest and the focus of extensive research. In his 1905 publication, Bertoni reported preliminary findings on sweet tasting constituents. The specific epithet *rebaudianum*, which would later be renamed to *rebaudiana*, was given in honor of Ovidio Rebaudi, a Paraguayan chemist admired by Bertoni, who received samples from Bertoni to perform the first chemical study on stevia (Kinghorn, 2003). The first glycoside identified was stevioside, which occurs in the highest concentrations in wild plants. Stevioside was isolated in an impure form by Bridel and Lavielle in 1931, but the final structure was not

identified until the work of Mosettig et al. (1963). Stevioside is approximately 250 times sweeter than sugar, but can leave a bitter after taste for many people (Yadav et al., 2010). In 1976, Kohda et al. isolated a second major glycoside, rebaudioside A (RebA). The flavor profile of RebA is more pleasing to users, and maintaining a proper stevioside/RebA ratio has become a key parameter in measuring stevia quality (Pal et al., 2014). In addition to these two major glycosides, minor glycosides rebaudiosides B-E, steviolbioside, and dulcoside A were identified during the 70s and early 80s (Kohda et al., 1976; Yamasaki et al., 1976; Kobayashi et al., 1977; Tanaka, 1982). RebF was discovered in 2002, and the most recent discovery was the minor glycoside RebM in 2013 (Starratt et al., 2002; Prakash et al., 2014). At present, eleven diterpene glycosides have been identified (Table 1.1) (Carakostas et al., 2011; Prakash et al., 2014).

There are many techniques employed to obtain glycosides from stevia leaves. Categories of extraction patents include solvent extraction (Bondarev et al., 2001; Morita, Fujita, and Iwamura, 1978), chromatographic adsorption (Ahmed, 1982; Kolb, 2001), ion exchange (Fuh, 1990; Giovanetto, 1990), selective precipitation (Kumar, 1986), membrane processes (Fuh, 1990; Giovanetto 1990), and superficial fluids (Kienle, 1992). Rebaudioside A has higher water solubility than stevioside, so hot water is generally the preferred medium for extraction. Some patents use other solvents like ethanol, methanol/chloroform, glycerin, sorbitol, or propylene glycol (Lemus-Mondaca et al., 2012). Brandle and Rosa (1992) found that leaf yield, leaf:stem ratio and stevioside concentration are all heritable traits, showing the potential for improvement through selection. It has also been identified that stevioside is the substrate for the synthesis of rebaudioside A, indicating that plants with higher RebA levels

will likely have lower stevioside levels (Shibata et al., 1991). Breeding work will continue to focus on increasing levels of desirable glycosides while reducing levels of stevioside.

Floral Biology

S. rebaudiana typically begins to flower in September in the northern hemisphere, but variability in photoperiod sensitivity ranges from 8 to 14 hours. Flowering can begin any time following the formation of the first four true leaves. Once flowering has begun, it takes a plant over one month to complete development of flower stages and overall flowering (Ramesh et al., 2006). The small white flowers (15-17 mm) have pale purple throat corollas, and are arranged in an irregular cyme (Brandle et al., 1998; Pande and Gupta, 2013) (Figure 1.3). Florets are perfect, having male and female organs, in small corymbs with two to six florets (Yadav et al., 2010), but insect pollination is important because plants are self-incompatible. In seed production fields, introduction of three to four beehives per hectare are recommended to achieve high levels of pollination and seed production (Oddone, 1999). A complete diallel cross with eight parents found that selfing ranged from 0-0.5%, while outcrossing ranged from 0.7-68.7%, indicating the presence of a self-incompatibility system (cited from Katayama 1976 in Brandle et al., 1998).

Figure 1.3. Flowers of *S. rebaudiana*

Seeds are formed in slender achenes 3mm in length. Each achene has around twenty pappus bristles; these, in combination with low endosperm levels, allow the seeds to be dispersed in the wind (Ramesh et al., 2006). Fertile seeds are dark in color, while pale or "clear" seeds are infertile (Goettemoeller and Ching, 1999). Overall, seed viability tends to be poor and can be highly variable. Seeds should be stored at 0°C, but even at low temperatures germination will decline fifty percent within three years. Seeds of *S. rebaudiana* are very small; one thousand seeds weigh only 0.15-0.3g (Brandle et al., 1998). When used commercially, seeds are de-bearded and sometimes pelletized to make them easier to work with in mechanical seeding operations (Figure 1.3).

Figure 1.4. Pelletized, de-bearded, and raw seed of *S. rebaudiana*

Propagation of Stevia

S. rebaudiana can be propagated from seed and stem cuttings, and is amenable to callous formation and regeneration via tissue culture. Seed germination rates are often poor and seedlings are slow to develop. Due to this, seeds typically are started in a greenhouse and seedlings are transplanted in the field. Direct seeding is not a viable option for establishing stands of stevia (Yadav et al., 2010). Once germinated in the greenhouse, plants are grown for six to ten weeks and are transplanted to the field after danger of frost has passed. In addition to germination challenges, seeds are not homogenous, so there can be high variability among plants, including glycoside level and composition (Alhady, 2011).

Another method of propagation is through stem cuttings. When cuttings are taken from the leaf axils, 98-100% rooting can be attained (Gvasaliya et al., 1990). Clonal propagation can be used for small-scale production, but due to labor costs, it is generally not economically viable for large-scale production (Brandle at al., 1998). With the high labor

costs associated with stem cuttings, low germination and variability among seeds, and the development of new germplasm, tissue culture was also investigated for large-scale propagation of stevia (Alhady, 2011; Pande and Gupta, 2013). Under current conditions, however, stem cuttings and tissue culture practices are not amenable to large-scale production. Research is needed to develop varieties with consistently high seed germination rates, optimized glycoside content, and perhaps adaptability to direct seeding under field conditions.

Production of Stevia

S. rebaudiana is adaptable to a wide array of climates. During sugar shortages of World War II, stevia seeds were sent to England, but the establishment was unsuccessful. The first documented commercial cultivation began in Paraguay in 1964 (Katayama 1976; Brandle et al., 1998). In the 1960's, strict regulation concerning the use of artificial sweeteners was beginning in Japan, and saccharin use was banned in 1970. Sumida (1968) led efforts to establish stevia as a Japanese crop. Stevia was introduced in 1970, and the stevia market got its start. Many of these early products had a "licorice off-taste" and a lingering sweet aftertaste that limited the expansion of commercial products (Carakosta et al., 2008). With improved formulations and increasing regulatory acceptance, areas of cultivation have expanded to include Argentina, Brazil, Canada, China, Indonesia, India, Korea, Mexico, Russia, Tanzania, Thailand, and USA (Pal et al., 2014; Brandle et al., 1998).

As a perennial, and due to the high cost of establishing a crop, stevia is grown for three to five seasons prior to rotation or crop reestablishment. After the first season, two to

four harvests per year are possible depending on the climate and the level of fertilization. The above ground plant parts have a critical low temperature tolerance of -3°C and will die back during winter months (Ramesh et al., 2006). The temperature sensitivity of overwintering stevia crowns is unknown across soil types in the southeastern US, but if they survive, will give rise to new stems as soil temperatures rise in spring (Figure 1.5).

Figure 1.5. *S. rebaudiana* emerging from overwintering root system

Production in the United States is currently located on the west coast and in the southeast, particularly in North Carolina and Georgia. The favorable climate, natural rainfall, and existing infrastructure found in these states have made them good candidates for

production. Climate and rainfall are important because stevia has a poor tolerance of water stress and frequent shallow irrigation or precipitation is needed. Stevia production in NC is being marketed to tobacco growers as a replacement crop or as a rotation crop in traditional and organic systems. Production of stevia is an adaptable system that can share much of the equipment utilized in tobacco production. Stevia is started in a greenhouse float-tray system, can be planted with a tobacco transplanter, managed with tobacco field equipment, and dried in tobacco barns or peanut trailers. The only difference is how the plant is harvested, where stevia is cut with a combine, and allowed to regrow.

Stevia was planted for the first time in NC in one field in 2011, and by 2015 expanded to 350-400 acres in production. Currently, stevia is seeded in a greenhouse float system and grown for 6-10 weeks prior to transplanting seedlings into the field after danger of frost. First year crops typically are harvested only once, in August or September. At frost, the foliage dies and plants are trimmed back for the winter. New shoots emerge from the perennial rhizome the following spring (Figure 1.5). In second year crops there are typically two harvests, the first in June or July and the second in August or September. It is best to harvest prior to flowering, when the plant has its maximum level of glycoside content. After harvest, the plant is dried for 2-3 days at 55°C. After drying, leaves are separated from the stem because stems have very low glycoside levels and their removal lowers processing costs (Brandle et al., 1998). Dried leaf material is baled and shipped to extraction facilities to separate the desired glycosides.

Challenges Associated with Stevia Production

As a new crop, there are many unknowns that can impede successful commercial production of stevia. As mentioned previously, poor seed viability, and expenses associated with vegetative cuttings hamper the establishment of plant material, and further research is needed to optimize transplant production. Nutrient requirements are thought to be low since this plant is native to a habitat with poor growing conditions. Murayama et al. (1980) showed that fertilizer application increased dry leaf yield and growth rate in Japanese plantings, but studies are needed to develop the optimal fertilization regime for commercial cultivation of stevia in NC. Thomas (2000) noted the possibility of infestation by aphids, white flies, mealy bugs, or red spider mites, but insects have not been a major problem in production of stevia. Greenhouse-grown plants may become infested with thrips. Slugs also have been documented as a problem on plants reemerging in the spring (Shock, 1982). By far, the biggest challenges in stevia production are weed management and disease control.

Weed Management

S. rebaudiana is slow to establish following transplant. During the first month after planting, this slow growth can make weeds the principle challenge to successful establishment (Ramesh et al., 2006), especially until herbicides become available. In the US, glyphosate is labeled for use on fields prior to planting of stevia, but there are currently no herbicides labeled for use on stevia once it is in the field. Cultural methods of weed control have been utilized to manage weed populations, but also significantly increase the costs

associated with production. Multiple herbicides are being investigated for use in stevia and may become available in 2015.

Pathogens and diseases

Fungal diseases reported in *S. rebaudiana* include diseases caused by *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, and *Septoria steviae* (Maiti, 2007; Chang, Howard, and Gaudiel, 1997; Kamalakannan et al., 2007; Ishiba et al., 1982). *A. alternata* was reported in India in 2007. Symptoms are documented as light brown small circular spots that turn dark brown to grey and are circular to irregular in shape with concentric rings. Spots may coalesce forming large areas of necrosis (Maiti, 2007). In 1997, *S. sclerotiorum* was reported on stevia in Canada, and the first report in the US was published in 2014 (Chang, Howard, and Gaudiel, 1997; Koehler and Shew, 2014). Symptoms of *S. sclerotiorum* include wilting, chlorotic leaves, necrotic leaves at the base of the stem, and bleached stems. Symptomatic plants often have tufts of white hyphae present on stems and large, irregularly shaped 2 to 8mm black sclerotia on the base of the stem. The first report of *S. rolfsii* was in India in 2007, and the first report in the US was in 2014 (Kamalakannan et al., 2007, Koehler and Shew, 2014). Symptoms of *S. rolfsii* include yellowing leaves that wilt, bleached stems, and eventual plant necrosis. White cord-like mycelia growth is visible at the base of stems, especially early in the morning. Mycelium is accompanied by the formation of brown sclerotia 0.5-2mm in diameter. *Septoria steviae* was reported in Japan in 1982 and in Canada in 1996 (Reeleder, 1999; Ishiba et al., 1982). *Septoria steviae* symptoms include shiny olive-gray foliar lesions that are depressed and angular. Lesions often have a

chlorotic halo and rapidly coalesce, turn necrotic, and fall from the plant. Up to 50% of the foliage can become necrotic in severe cases. The only virus reported on stevia has been tomato spotted wilt virus (TSWV), with symptoms of chlorotic and necrotic rings on the leaves, and occasional dwarfing or general chlorosis. TSWV was first reported in Greece in 2007 (Chatzivassiliou et al., 2007).

Summary

Stevia rebaudiana (Bertoni) Bertoni is a perennial herb of the Asteraceae family that is gaining worldwide recognition for its sweet leaves that contain diterpene steviol glycosides. Stevia is indigenous to Paraguay, where the natives of the Paraguayan highlands have used dried leaves as a natural sweetener for centuries. Production and commercialization of stevia began in the 1960s (Brandle et al., 1998). However, the USDA did not approve the use of stevia as a non-nutritive sweetener until December 2008. Since that time numerous stevia products have appeared in the US market, and interest in stevia is rapidly growing. Increased commercial use has encouraged companies to investigate and try to establish stevia production areas in the US. Production is currently focused in the southeast states North Carolina and Georgia. Favorable climate, natural rainfall, and existing infrastructure found in these states have made them good candidates for production. Stevia is grown as a perennial crop, up to 5 years, that is harvested 1-2 times annually. Factors that limit the successful establishment of this crop include establishment of plant material, and weed and disease management. Continued research is needed to develop germplasm containing optimized glycoside levels with higher germination rates and seed viability.

Research is also needed to develop good agricultural practices to manage early season weed populations, and potential diseases.

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Table 1.1. Currently known steviol glycosides and sweetness potency (sucrose =1)

Compound	Sweetness Potency
Dulcoside A	30
Rebaudioside A	200-300
Rebaudioside B	150
Rebaudioside C	30
Rebaudioside D	221
Rebaudioside E	174
Rebaudioside F	200
Rebaudioside M	250
Rubusoside	114
Steviolbioside	90
Stevioside	150-250

Source: Prakash et al., 2014; Carakostas et al., 2011

CHAPTER II.

Etiology and Management of Stem Rot Caused by *Sclerotium rolfsii* in Stevia

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ABSTRACT

Koehler, A. M., and Shew, H. D. 2015. Etiology and management of stem rot caused by *Sclerotium rolfsii* in stevia. Plant Dis. 99:000-000.

Stevia (*Stevia rebaundiana*) is an herbaceous perennial that is an emerging crop in the US. Stevia leaves contain multiple glycosides that are extracted for use as a nonnutritive sweetener. Stevia plantings in North Carolina began in 2011. Wilting and death of plants in first and second year commercial plantings were observed in NC in 2012 and 2013, and Koch's postulates were performed to verify that *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* were pathogens of stevia. There are currently no disease-control products labeled for use on stevia in the US, so fungicide and biological control field experiments were conducted to determine optimal application rates and timing for products reported to be effective against *S. rolfsii* on other crops. A trial was planted in May 2014 to investigate the efficacy of multiple biological control agents and fungicides on suppression of stem rot caused by *S. rolfsii* in a field with a history of the disease on stevia. Two biological control products, clontri+strepse and veramin (Sacom, Larino, Italy) and two fungicides tebuconazole (Folicur, Bayer Crop Science, Research Triangle Park, NC), and flutolanil (Convoy, Nichino America, Inc., Wilmington DE) suppressed disease compared to the

control. A second trial was planted in July 2014 to determine the effects of application of fungicides at or before transplanting. Azoxystrobin (Abound, Syngenta Crop Protection, Inc., Greensboro, NC), applied as a spray treatment one week prior to planting and as a foliar spray at planting had the lowest disease incidence, followed by tebuconazole applied as a spray treatment applied one week prior to planting.

INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni is an herbaceous perennial that is an emerging crop in the United States. *S. rebaudiana* is a member of the Asteraceae family native to Paraguay (Kinghorn, 2003). The Guarani Indians of Paraguay have used stevia or kaá-éhé, "sweet herb", for centuries to sweeten mate, a bitter herbal beverage (Soejarto et al., 1983). *S. rebaudiana* was introduced to the world at the beginning of the twentieth century by the Italian-Swiss botanist-naturalist Moises San-tiago de Bertoni, and has been the most studied species of *Stevia*.

Stevia leaves contain multiple diterpene glycosides that are used as a natural non-caloric sweetener. To date, there are eleven known glycosides with sweetness potencies ranging from 30-300 times as sweet as sucrose (Prakash et al., 2014). Stevioside is the most abundant glycoside, but is associated with a bitter after taste, so it is not the most desirable leaf component. Rebaudioside A is the second most abundant glycoside and has been judged to have the most favorable sensory attributes (Dacome et al., 2005). Glycosides from stevia are not metabolized by the body, and provide a safe alternative to sugar for diabetics.

Commercial production of stevia first began in Paraguay in 1964, with the first commercial interest in Japanese markets occurring in the 1970s (Carakosta et al., 2008; Katayama, 1976). Since that time, stevia cultivation has expanded to Argentina, Brazil, Canada, China, Indonesia, India, Korea, Mexico, Russia, Tanzania, Thailand, and the USA (Pal et al., 2014; Brandle et al., 1998). The USDA approved stevia for use as a non-nutritive sweetener in December 2008 (FDA GRAS Notice GRN 000253 and GRN 000252). As companies have expanded low calorie sweetening options, the stevia market has rapidly

grown, and by 2011, the United States accounted for 44% of all new stevia products launched (Datamonitor, 2011). In order to meet the demand of increasing commercial products using stevia glycosides, stevia cultivation is expanding rapidly in the US.

Due to the presence of a favorable climate, natural rainfall, and existing infrastructure, stevia is being investigated as a new crop in North Carolina. Stevia plantings in North Carolina began at one location in 2011 and expanded to >300 acres in production in 2013. Stevia is typically grown for 3-5 years before reestablishment is necessary, and harvested twice per growing season once established. Stevia is being investigated for use as a replacement crop for tobacco, a rotation crop in traditional and organic tobacco systems, and as an organic transition crop. Important tobacco diseases in NC include black shank (caused by *Phytophthora nicotianae*), Granville wilt (caused by *Ralstonia solanacearum*), root knot nematodes (*Meloidogyne incognita*) and Tomato Spotted Wilt caused by tomato spotted wilt virus (TSWV). No disease was observed in greenhouse inoculations or in infested field plantings with high levels of *P. nicotianae* and *R. solanacearum*; root knot and TSWV testing are in progress (Koehler and Mendoza-Moran, unpublished).

Wilting and death of plants in first and second year commercial plantings were observed in NC in 2012 and 2013. *Sclerotium rolfsii* and *Sclerotinia sclerotiorum* were observed on diseased plants and Koch's postulates were performed to verify these organisms as pathogens of stevia (Koehler and Shew, 2014a; Koehler and Shew, 2014b). Many aspects of the disease cycle of these two pathogens on stevia have not been fully described and require further investigation. Additionally, since there are no fungicides currently labeled for stevia in the US, identification of efficacious and economically feasible approaches to

disease management are needed. Both of these pathogens produce abundant sclerotia capable of surviving long periods in soil, so inoculum accumulation over the 3 to 5 year production cycle is possible. High inoculum levels may result in these pathogens becoming a limiting factor to the expansion of commercial production of stevia in NC as well as to other crops grown in rotation with stevia if they are not managed. The objectives of this project were to identify fungicide and biological control products that have potential to be labeled for control of *S. rolfsii* in stevia, and to determine optimal application rates and timing.

MATERIALS AND METHODS

Fungicide and Biocontrol Field Experiment

An experiment was established in May 2014 at the Caswell Research Farm in Kinston, NC. *Stevia rebaudiana* was originally planted at this site in 2012 and high levels of disease caused by *S. rolfsii* were observed in first and second year plants. Stem rot caused by *S. sclerotiorum* was also observed in second year plants at this location in the spring of 2013. The stevia crop was removed in May 2013 and soybeans were grown. One month prior to transplanting stevia, the 0.15 ha plot was amended with 235 kg K ha^{-1}, and two days prior to transplanting a mixture of urea and ammonium were applied at 280 L N ha^{-1}. Twelve weeks after planting, soil was amended with 392 kg N ha^{-1}. Due to high inoculum levels of *S. rolfsii* naturally present in the soil, no additional inoculum of the pathogen was added to the test site.

Rooted seedlings of the commercial line G7 (Sweet Green Fields) of *S. rebaudiana* were used in the trial. Seedlings were planted at a density of 87,000 plants ha^{-1} with a row

space of 0.76 m. Treatments were arranged in a randomized complete block design with four 7.62 m rows in each of five replications per treatment. Treatments included five biological control treatments, four fungicide treatments, and one untreated control (Table 2.1).

Three of the treatments contained Clonotri and Strepse of the Greenpower line (Sacom, Larino, Italy). Clonotri is a liquid formulation containing *Glomus sp*. and conidia of *Trichoderma sp.* and *Clonostachys sp.* Strepse is a liquid formulation that contains *Glomus sp.*, *Streptomyces sp*., and *Pseudomonas sp*. The other biological control products were Veramin (Sacom Larino, Italy), a bio-stimulant made from aloe vera extract and vegetable amino acids and Terraklin (Tegucigalpa, Honduras) is composed of liquidambar, marigold, and ageratum extracts. Clonotri and Strepse were applied using three application methods. In the first treatment, 288 cell trays were submerged in a container that included a mixture of 100 ml Clonotri and Strepse in 50L H_20. Trays were allowed to soak for 30 min, removed, and seedlings were pulled for transplanting. The second application method was an in-furrow irrigation of Clonotri and Strepse applied at 30.02 L ha^{-1}. The third application method consisted of monthly foliar sprays applied at 1.02 L ha^{-1}. Terraklin was applied using in-furrow irrigation at a rate of 46.77 L ha^{-1}. Veramin was applied as a monthly foliar spray at 0.77 L ha^{-1}. Foliar sprays were banded over rows using a TeeJet TP8006 flat fan nozzle on a CO_2 sprayer calibrated to deliver product at a rate of 280.6 L H_2O ha^{-1}. Monthly applications of products were completed in June, July, and August 2014 (Table 2.1).

Fungicide treatments included monthly spray applications with 0.53 L ha^{-1} tebuconazole (Folicur, Bayer Crop Science, Research Triangle Park, NC), one application of 1.02 L ha^{-1} followed by two applications of 1.53 L ha^{-1} azoxystrobin (Abound, Syngenta

Crop Protection, Inc., Greensboro, NC), monthly applications of 2.34 L ha^{-1} flutolanil (Convoy, Nichino America, Inc., Wilmington DE), and a rotation of 2.34 L ha^{-1} flutolanil and 1.53 L ha^{-1} azoxystrobin. Foliar sprays were applied as described above. Monthly applications were completed in June, July, and August 2014 (Table 2.1). Dual magnum (Syngenta Crop Protection, Inc., Greensboro, NC), Basagran (Arysta LifeScience North America, LLC, Cary, NC), and Select max (Valent, Walnut Creek, CA) were used to control weed populations.

Since no other diseases were present in the field, disease progress was monitored by counting the numbers of healthy plants periodically between May-August 2014. Harvest was done in September and consisted of removing all above ground plant tissue in sections 2.44 m in length from the center two rows of each plot. Harvested samples were placed into cloth bags and dried at 55°C for two days, weighed and converted to kg ha^{-1}.

Mid-Season fungicide trial

A second experiment was established in July 2014 at the Caswell Research Farm in Kinston, NC in the same field as described above. The 0.07 ha plot was treated with 260 kg K ha^{-1} and 390 L N ha^{-1} as urea and ammonium three months prior to planting. One week prior to planting, 624 kg N ha^{-1} and 673 kg K ha^{-1} were applied. Rooted seedlings of the commercial line G7 of *S. rebaudiana* were planted in all plots. Seedlings were planted at a density of 43,000 plants ha^{-1} with a row spacing of 0.76 m.

Treatments were arranged in a randomized complete block design with two 7.62 m rows in each of five replicates per treatment. Treatments included three fungicides, azoxystrobin (Abound, Syngenta Crop Protection, Inc., Greensboro, NC), flutolanil (Convoy,

Nichino America, Inc., Wilmington DE), and tebuconazole (Folicur, Bayer Crop Science, Research Triangle Park, NC) applied with three methods, and an untreated control (Table 2.4). Treatment methods included a foliar application one week prior to planting, incorporation in the transplant water, and a foliar spray directly after planting.

Pre-plant treatments consisted of foliar sprays of 1.53 L ha^{-1} azoxystrobin, 2.34 L ha^{-1} flutolanil, or 0.53 L ha^{-1} tebuconazole applied to the seedlings in the greenhouse one week prior to planting. Treated plants remained in the greenhouse until the time of planting. Transplant water treatments consisted of 1.53 L ha^{-1} azoxystrobin, 2.34 L ha^{-1} flutolanil, or 0.53 L ha^{-1} tebuconazole. The tank was rinsed between each fungicide application. Foliar spray treatments included spraying plants with 1.53 L ha^{-1} azoxystrobin, 2.34 L ha^{-1} flutolanil, or 0.53 L ha^{-1} tebuconazole immediately after transplant as described above. All of the treatments, excluding the control, received a foliar spray of their corresponding fungicide at rates of 1.53 L ha^{-1} azoxystrobin, 2.34 L ha^{-1} flutolanil, or 0.53 L ha^{-1} tebuconazole three weeks after planting using the CO_2 sprayer. Dual magnum (Syngenta Crop Protection, Inc., Greensboro, NC), Basagran (Arysta LifeScience North America, LLC, Cary, NC), and Select max (Valent, Walnut Creek, CA) were used to help control weed populations. Stand counts of healthy plants were taken through September 2014 to monitor disease progression. No harvest was conducted on these plots.

Data Collection and Analysis

For each of the field trials, disease was measured by taking stand counts of healthy plants throughout the growing season. Stand counts were compared to the number of plants set at the date of planting to obtain mean percent disease. All data was subjected to analysis

of variance using the PROC GLM procedure in SAS 9.4 and means were separated using Fishers least significance difference (alpha =0.05).

RESULTS

Disease development

Stem rot was observed soon after transplanting and was active throughout the growing season in the fungicide and biological control experiment (Figures 2.1 and 2.2), with a final incidence over 50% in control plots. Eighteen days after planting (DAP), disease incidence in the control treatments was 20% (Table 2.2). Disease incidence did not increase between 19 and 28 DAP, but this was followed by a rapid increase in disease between 29 and 42 DAP, with disease nearly doubling for all treatments, including those receiving fungicide and biocontrol sprays. After 42 DAP, disease increased slowly in the control and veramin treatments over the next 41 days, but remained steady for other treatments throughout the remainder of the season. Signs of the pathogen, white hyphae and masses of sclerotia, were commonly observed around the base of stems of infected plants from shortly after transplant until harvest in September.

In the mid-season trial, disease was observed soon after transplanting, but progressed slower than the trial planted in May (Figure 2.6). At 20 DAP disease incidence in the control treatment was 3%, and at 41 DAP disease incidence in the control was 25% (Table 2.5). Disease incidence increased from 20 to 29 days, but as observed in the first trial, the largest increase occurred between 29 and 41 DAP (Table 2.5). Stem rot caused by *S. rolfsii* was the only disease observed in both experiments.

Fungicide and Biocontrol Field Trial

This experiment contained five biological control, and four fungicide treatments. There were significant treatment effects (*P >0.005*, 0.05). Among the nine treatments, four significantly suppressed disease compared to the control (Figure 2.3). Products that significantly reduced disease incidence included tebuconazole, flutolanil, clonotri+strepse, and veramin, all applied multiple times as a foliar spray.

Three biological control treatments contained the products clonotri and strepse applied in combination with varying methods of application. The transplant dip and in furrow treatments had similar levels of disease and were not different than the control (Figure 2.3). The multiple applications of the foliar spray had significantly less disease, and was the top performing biological control treatment with a final disease incidence of 35%. The second best biological control treatment was foliar application of veramin, which ended with 39% disease. At 61%, terraklin had a higher mean percent disease incidence than the control. Tebuconazole had significantly lower disease than the control and was the best performing product overall, with a final disease incidence of 32%. Flutolanil applications were also significantly better than the control at 35% disease incidence. Azoxystrobin and azoxystrobin rotated with flutolanil had mean disease of 41% and 42% respectively.

Across treatments, yields ranged from 2186 kg ha^{-1} to 4447 kg ha^{-1} (Table 2.3). Due to variation in disease among replications, no treatment effects on yield were observed (Figure 2.4). However, when disease incidence was plotted against mean yield for each treatment, there was a significant linear relationship (R^2 of 0.83) (Figure 2.5). Tebuconazole

had the lowest disease incidence and also the highest yield, while terraklin had the highest disease incidence and lowest yield (Figure 2.3, 2.4, 2.5).

Fungicide Timing Trial

In this trial, three fungicide products were tested using three timing-of-application methods. There were significant treatment effects (*P >0.01*, 0.05). Among the nine treatments evaluated, azoxystrobin as a pre-plant spray and foliar spray, and tebuconazole as a pre plant spray significantly reduced disease compared to the control (Figure 2.7). Symptomatic plants were visible within one week of planting and reached 25% by 41 DAP (Figure 2.6). Disease was evenly distributed across the field and there were no differences in total mean disease among replications.

Of the three fungicides tested, azoxystrobin provided the best control (Table 2.5). Azoxystrobin applied one week prior to planting had a final disease incidence of 7% and when applied as a foliar spray had a final incidence of 13%. Azoxystrobin did not work as well when applied in transplant water (Table 2.5). Application method did not alter the efficacy of flutolanil, with all methods having a final disease incidence 17 to 22%. The pre-plant application of tebuconazole also gave good control with 15% disease incidence and was more effective than the tebuconazole transplant water and foliar spray treatments (Table 2.5).

DISCUSSION

Based on scouting of stevia fields in NC over several years, infection by *S. rolfsii* occurs soon after planting in first year crops, or soon after emergence of stems from overwintering crowns. The optimal temperature for growth of *S. rolfsii* has been reported as

27-30°C, but disease can develop at or just below 20°C (Aycock, 1966), which is closer to soil temperatures at the time of early season planting. In this study, planting occurred in late May and again in mid-July, and soil and air temperatures were favorable for disease development. In both trials, temperatures were favorable and disease began shortly after seedlings were planted, but disease progressed much quicker in the May planting than the July planting. In May, between planting and the first foliar treatment applications at 19 DAP, disease developed rapidly and mean incidence averaged 19% across all treatments. The average daily air temperature during this time was 30.1°C with a night average of 17.6°C, and there was 8.7 cm of rain recorded from four rain events. Between 19 and 27 DAP, disease incidence remained almost unchanged in all treatments, including the control, even though environmental conditions were favorable for disease development. The daily temperature during this time was 32.7 °C, with a night temperature of 21.1°C and 11.8 cm precipitation from three rain events. Between 28 and 41 DAP, disease levels nearly doubled in all treatments. The mean daily temperatures were similar to the previous weeks, averaging 31.8°C in the day with a night temperature of 19.8°, but during this time there was approximately 23.1 cm of rain in four rain events, which may have contributed to the large increase in disease.

Disease progressed more steadily in the mid-season trial, but overall incidence was lower. For example, at 40 DAP, disease incidence was 25% in the control in the mid-season trial and 44% in the early season trial. Disease was observed at low levels during the first week after planting. Environment was again favorable for disease, daily temperatures in August averaged around 31°C, with night temperatures averaging 20°C. As in the early

season trial, the largest increase in disease occurred 29 to 41 DAP. During this time daily temperature was 31.4°C with night temperatures of 21.7°C and 11.7 cm of precipitation from four rain events in a row following 18 days without rain. This marked the highest amount of rainfall since planting, and is similar to the rapid disease development trends observed in the early season planting.

Once dried, the sclerotia of *S. rolfsii* have been reported to eruptively germinate following exposure to volatile compounds produced from plant tissue. In peanuts, Beute and Rodriguez-Kabana (1979) observed that dried and remoistened peanut tissues emitted volatiles that stimulated sclerotia germination. Volatiles from alfalfa hay, methanol, isopropyl, and butyl alcohols have also been shown to cause eruptive germination of sclerotia (Linderman and Gilbert, 1969; Rodriguez-Kabana et al., 1980; Punja et al., 1984). Due to the rapid development of *S. rolfsii* on stevia soon after transplanting or emergence, it is possible there is a stimulant within stevia that promotes germination of sclerotia. However, this needs to be investigated further.

In peanuts, high moisture levels have been associated with an increase in disease incidence (Shew and Beute, 1983). The greatest increase in stem rot of stevia occurred between 4 and 6 wks after planting, during periods of high rainfall and favorable temperatures. In addition, by this time, each plant had multiple stems and a dense canopy, creating a favorable microclimate for disease. It is unknown why disease did not increase after week six, except very slowly in the control. If this occurs in future tests, late season applications of fungicides may not be necessary. The early season development of disease

and the steep increase up to 42 DAP, indicate that early season treatments may be the most important for managing stem rot on stevia.

Several biological control agents and fungicides were effective in suppressing disease. Clonotri+strepse and veramin applied as monthly foliar sprays were the top performing biocontrol products and were similar to the best fungicide treatments. Terraklin, the worst performing biocontrol, is a boifumigant and resulted in stunting of seedlings when applied to the transplant water. By 19 DAP, 36% of the plants in the Terraklin treatment were diseased and this treatment ended the season with the highest mean disease rating. Tebuconazole was the most effective fungicide in this trial. Based on observations in the mid-season trial, it is possible that azoxystrobin would have performed better if applied prior to disease onset. Tebuconazole, flutolanil, and azoxystrobin have been documented for suppression of *S. rolfsii* in other systems (Mehan et al., 1994; Rideout et al., 2002).

In the mid-season trial, the objective was to determine if pre-plant and at-plant applications of fungicides would prevent or suppress the high level of disease observed in the early season experiment. We obtained much better disease suppression with several of the fungicides when they were applied before planting. For example, in the early season trial, when the first application was three weeks following planting, azoxystrobin did not offer significant suppression of disease. Azoxystrobin appears to have higher efficacy if it is on the plant prior to disease onset. Additional trials are needed to confirm that azoxystrobin treatments have higher efficacy in early season applications, and that differences were not due to differences in planting date. Tebuconazole has a recommended spray interval of two weeks. In this trial, the second application was made three weeks after planting, and the final

stand count was taken three weeks after the second application, which may have caused treatments with this product to have higher disease incidence. Disease levels in the flutolanil treatments did not differ.

Inoculum and disease were high in the field, but disease incidence was not uniform in the field. The highest mean level of disease was observed in rep 3, 51% across treatments, but ironically this replicate also contained plots on one side of the field that had very low levels of disease. This non-uniform occurrence of disease may explain why there were not significant differences in yield among treatments, even though several of the treatments significantly suppressed disease. In the third replication, the control was on the right edge of the field and had less disease pressure than the center treatments. In this replication, the control has the highest yield of all treatments, 5653 kg ha $^{-1}$. The average of the other control plots was 3021 kg ha $^{-1}$. When mean percent disease was plotted versus mean yield for each treatment, a linear trend was observed (Figure 2.5). Stem rot and death of symptomatic plants support that higher disease levels would result in lower yields.

S. rolfsii is an important disease of stevia that causes wilting and death of whole plants. Since stevia is grown as a perennial crop, inoculum buildup of sclerotia in the soil could be a limiting factor to the expansion of stevia production in North Carolina. Based on the results of these two trials, *S. rolfsii* is best controlled when products are applied prior to disease establishment. Continued research is needed to optimize product timing and application rates. Two biological control products clonotri+strepse and vermin, along with two fungicides, tebuconazole and azoxystrobin, merit additional field testing. At this time, these products offer the best potential to be labeled for use on stevia.

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Table 2.1. Treatments, timing of treatment application, and application rates for fungicide and biocontrol products applied on stevia for control of stem rot caused by *Sclerotium rolfsii*

	Dates of Applications/Rates of Products Applied			
Treatment	**Date/Rate[a]**	**Date/Rate**	**Date/Rate**	**Date/Rate**
Clonotri+Strepse Transplant dip[b]	5/29/14			
Clonotri+Strepse In furrow irrigation	5/29/14 30.02			
Clonotri+Strepse Foliar spray		6/17/14 1.02	7/18/14 1.02	8/8/14 1.02
Veramin Foliar Spray		6/17/14 0.77	7/18/14 0.77	8/8/14 0.77
TerraKlin In furrow irrigation	5/29/14 46.77			
Azoxystrobin Foliar spray		6/17/14 1.02	7/18/14 1.53	8/6/14 1.53
Flutolanil Foliar spray		6/17/14 2.34	7/18/14 2.34	8/6/14 2.34
Tebuconazole Foliar spray	6/10/14 0.53	6/26/14 0.53	7/18/14 0.53	8/6/14 0.53
Flutolanil and Azoxystrobin rotation Foliar spray[c]		6/17/14 (F) 2.34	7/18/14 (A) 1.53	8/6/14 (F) 2.34
Untreated				

[a] Rates are listed in Lha^{-1}, with the exception of the Clonotri+Strepse transplant dip

[b] A solution of 100ml of product in 50L of H20 was used to dip seedling trays for 30 minutes prior to transplant

[c] (F) indicates application of flutolanil, (A) indicates application with Azoxystrobin

Table 2.2. Effects of application method and frequency of biological control agents and fungicides on disease progress for stem rot of stevia caused by *Sclerotium rolfsii*

	Mean % Disease [a]					
	6/17 **19 days**	**6/26** **28 days**	**7/10** **42 days**	**7/29** **61 days**	**8/6** **69 days**	**8/20** **83 days**
Clonotri+Strepse[b] Transplant dip[c]	23 B[d]	23.0 B	40 BC	39 BC	38 BC	43 BC
Clonotri+Strepse In furrow irrigation	22 BC	22 BC	39 BC	37 C	38 BC	41 BC
Clonotri+Strepse Foliar spray	15 CDE	16 CDE	31 C	31 C	32 C	35 C
Veramin Foliar Spray	13 DE	13 DE	31 C	35 C	33 C	39 C
TerraKlin In furrow irrigation	36 A	36 A	59 A	59 A	59 A	61 A
Azoxystrobin Foliar spray	16 BCDE	16 BCDE	36 BC	35 C	37 BC	41 BC
Flutolanil Foliar spray	16 BCDE	16 BCDE	32 BC	34 C	32 C	35 C
Tebuconazole Foliar spray	16 BCDE	16 BCDE	29 C	30 C	30 C	32 C
Flutolanil and Azoxystrobin Foliar spray rotation	10 E	10 E	35 BC	39 C	39 BC	42 BC
None	20 BCD	20 BCD	44 B	50 AB	47 AB	51 AB

[a] Percent disease for each date and treatment was determined by assessing plant stands and comparing to the original stand count for that plot. Stem rot was the only disease observed in the field during the growing season.

[b] Treatments included biocontrols: clonotri+strepse, veramin, and terraklin and fungicides: azoxystrobin, flutolanil, and tebuconazole.

[c] Application methods included a transplant dip, in furrow irrigation, or repeat applications of foliar sprays

[d] Mean % disease from each collection date was analyzed using PROC GLM in SAS (9.4). Letters within each column indicate least significance difference groups (alpha=0.05)

Table 2.3. Yield for fungicide and biological control treatments applied to stevia for control of stem rot caused by *Sclerotium rolfsii*

Treatments	**Yield [a]**
Clonotri+Strepse[b] Transplant dip[c]	3407 AB[d]
Clonotri+Strepse In furrow irrigation	3738 A
Clonotri+Strepse Foliar spray	4236 A
Veramin Foliar Spray	3814 A
TerraKlin In furrow irrigation	2186 B
Azoxystrobin Foliar spray	4236 A
Flutolanil Foliar spray	4417 A
Tebuconazole Foliar spray	4447 A
Flutolanil and Azoxystrobin rotation Foliar spray	4266 A
Untreated	3527 AB

[a] Yield was calculated by harvesting 2.44 m sections from the center two rows of each plot. Harvested samples were placed into cloth bags and dried at 55°C for two days, then weighed and converted to kg ha^{-1}

[b] Treatments included biological control agents: clonotri+strepse, veramin, and terraklin and fungicides: azoxystrobin, flutolanil, and tebuconazole.

[c] Application methods included a transplant dip, in furrow irrigation, or repeat applications of foliar sprays

[d] Yield means were analyzed using PROC GLM in SAS (9.4). Letters indicate least significance difference groups (alpha=0.05)

Table 2.4. Treatments, timing of treatment application, and application rates for fungicide products applied to stevia for control of stem rot caused by *Sclerotium rolfsii*

	Dates of Applications/Rates of Products Applied		
Treatments	**Date/Rate [a]**	**Date/Rate**	**Date/Rate**
Azoxystrobin Pre-plant	7/22/14 1.53		8/20/14 1.53
Flutolanil Pre-plant	7/22/14 2.34		8/20/14 2.34
Tebuconazole Pre-plant	7/22/14 0.53		8/20/14 0.53
Azoxystrobin Foliar Spray		7/29/14 1.53	8/20/14 1.53
Flutolanil Foliar Spray		7/29/14 1.17	8/20/14 2.34
Tebuconazole Foliar spray		7/29/14 0.53	8/20/14 0.53
Azoxystrobin Transplant water		7/29/14 1.53	8/20/14 1.53
Flutolanil Transplant water		7/29/14 1.17	8/20/14 2.34
Tebuconazole Transplant water		7/29/14 0.53	8/20/14 0.53
Untreated			

[a] Rate is listed in L ha^{-1} of product applied in 280.6 L ha^{-1}

Table 2.5. Effects of fungicide products and application method on disease progress for stem rot of stevia caused by *Sclerotium rolfsii*

	Mean % Disease [a]			
	8/6 6 days	**8/20 20 days**	**8/29 29 days**	**9/10 41 days**
Azoxystrobin[b] Pre-plant[c]	0.5 B[d]	0.5 C	4 B	7 D
Flutolanil Pre-plant	5 A	6 A	10 A	22 ABC
Tebuconazole Pre-plant	1.5 B	4 AB	11 A	15 CD
Azoxystrobin Foliar Spray	0 B	3 BC	6 AB	13 CD
Flutolanil Foliar Spray	1 B	3 ABC	10 A	21 ABC
Tebuconazole Foliar spray	1 B	3 ABC	10 A	27 A
Azoxystrobin Transplant water	1 B	3 BC	7 AB	19 ABC
Flutolanil Transplant water	1 B	3 ABC	10 A	17 BCD
Tebuconazole Transplant water	1 B	3 BC	11 A	21 ABC
Untreated	0.5 B	3 ABC	10 A	25 AB

[a] Percent disease for each date and treatment was determined by taking stand counts and comparing to the original stand count for that plot. Stem rot was the only disease observed in the field during the growing season.

[b] Treatments included fungicides: azoxystrobin, flutolanil, and tebuconazole.

[c] Application methods included a pre-plant foliar spray 1 week prior to planting, foliar spray application, and transplant water treatment. All treatments received an additional foliar spray 20 days after planting

[d] Mean % disease from each collection date was analyzed using PROC GLM in SAS (9.4). Letters within each column indicate least significance difference groups (alpha=0.05)

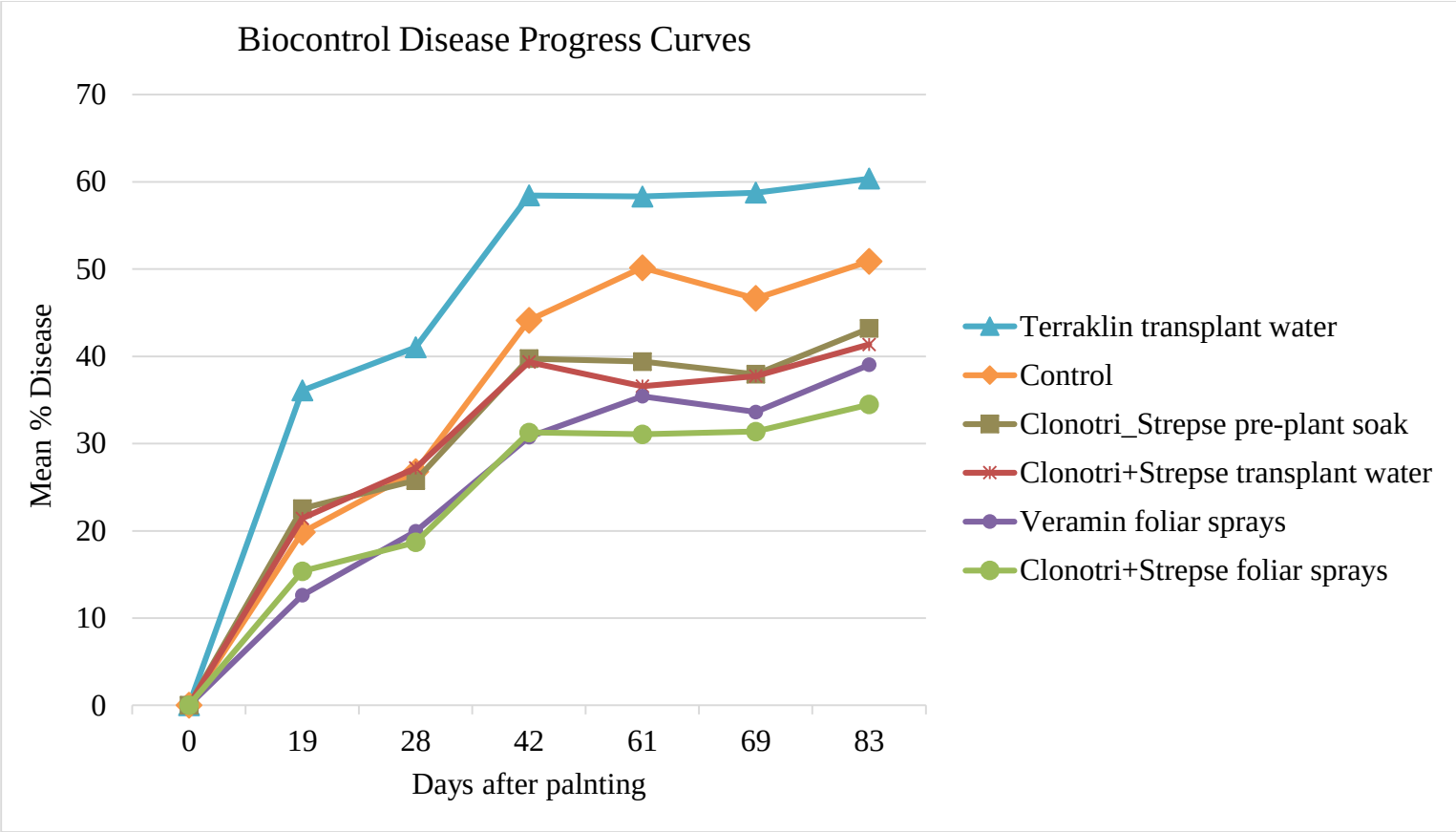

Figure 2.1. Effect of biological control products on disease progress caused by *S. rolfsii*. Percent disease incidence for each date and treatment was determined by taking stand counts and comparing to the original stand count for the plot.

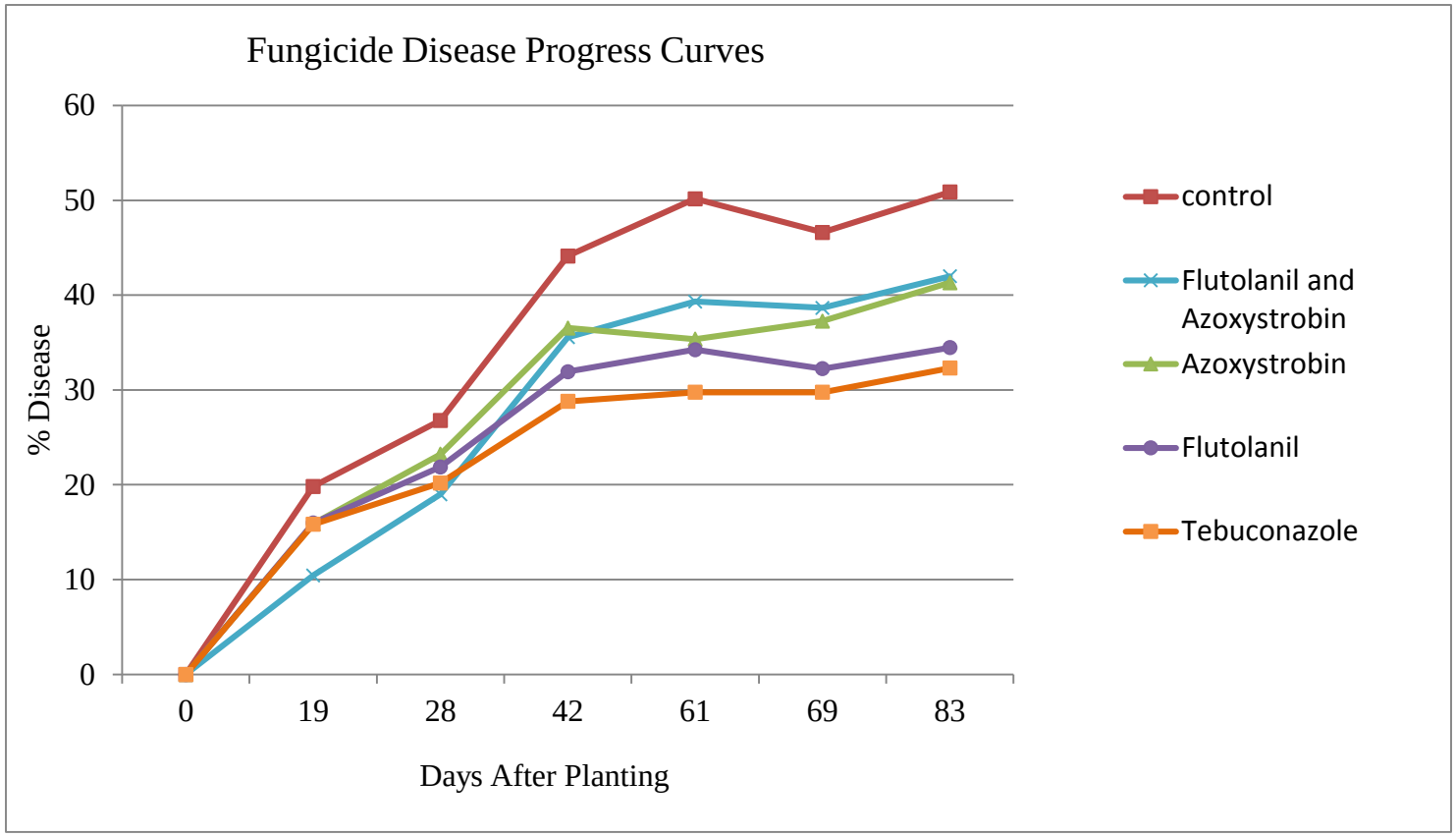

Figure 2.2. Effect of fungicides on disease progress caused by *S. rolfsii.* Percent disease incidence for each date and treatment was determined by taking stand counts and comparing to the original stand count for the plot.

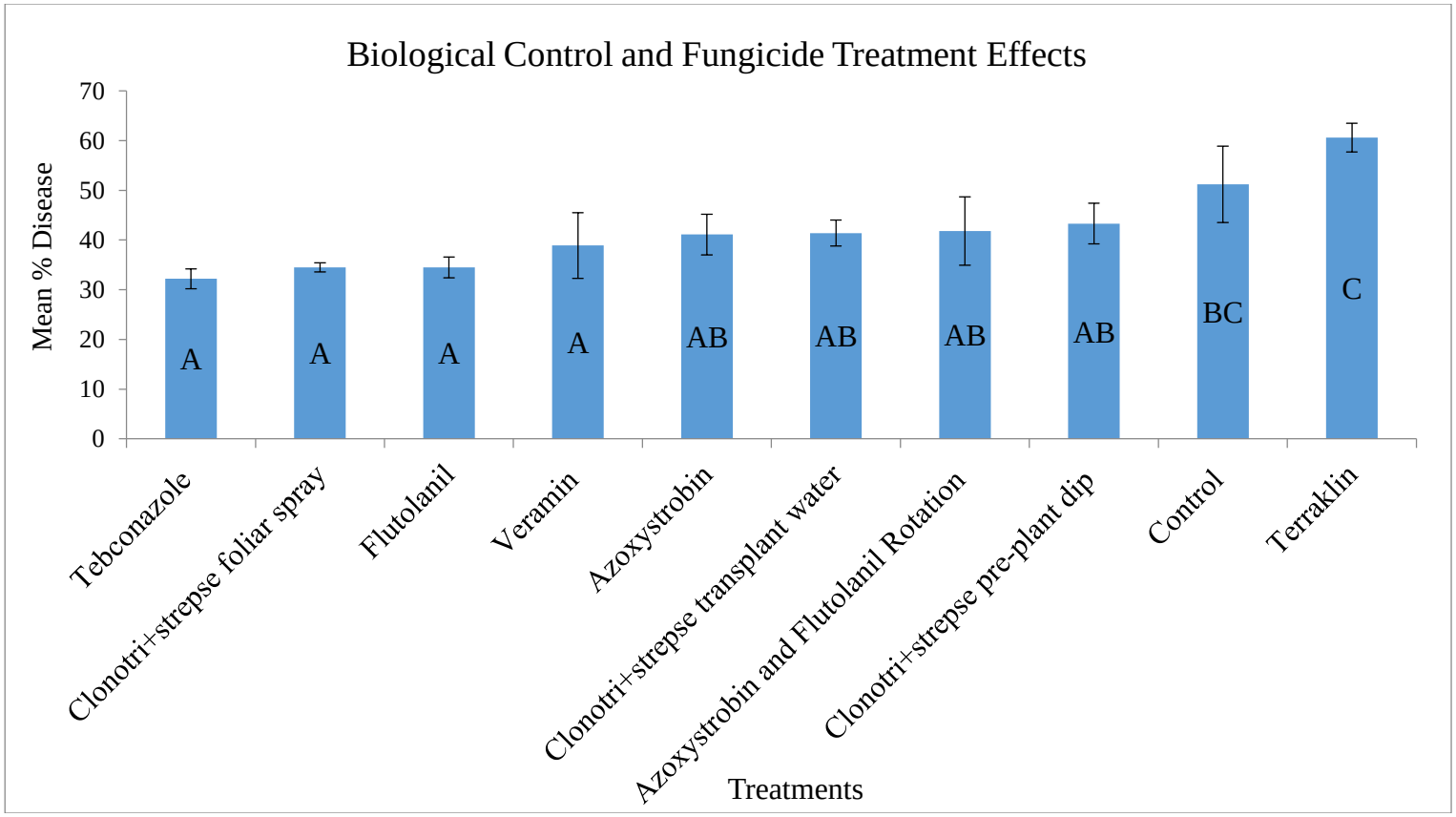

Figure 2.3. Effects of fungicides and biological control products on mean % disease caused by *S. rolfsii* on stevia at the final stand count, 83 days after planting. Treatment effects were analyzed using PROC GLM in SAS (9.4). Letters within each bar indicate least significance difference groups (alpha=0.05). Error bars represent standard error of the mean.

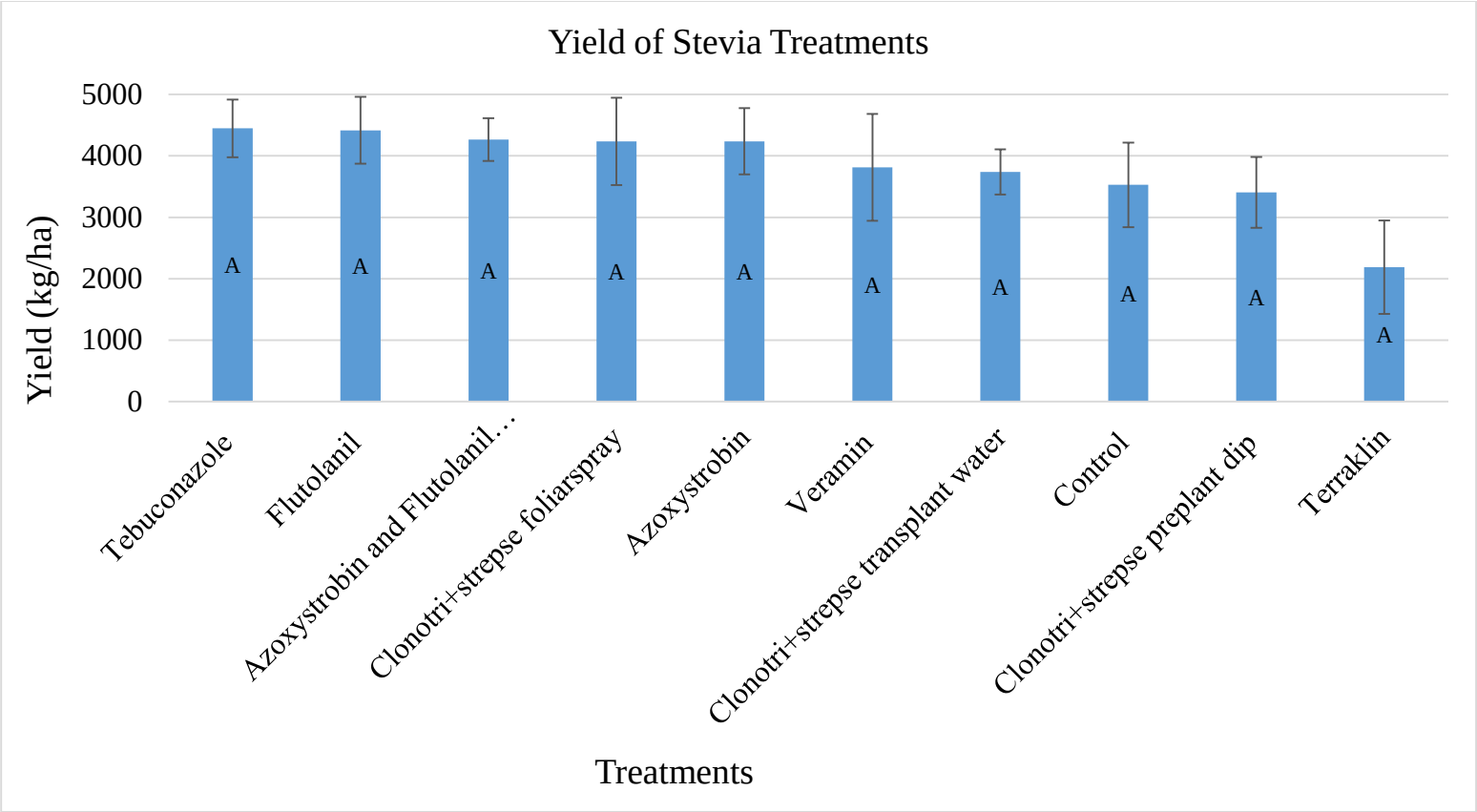

Figure 2.4. Yield in kg ha^{-1} for fungicide and biological control treatments used to control stem rot caused by *S. rolfsii* on stevia. Effects of treatments on yield were analyzed using PROC GLM in SAS (9.4). Letters within each bar indicate least significance difference groups (alpha=0.05). Error bars represent standard error of the mean.

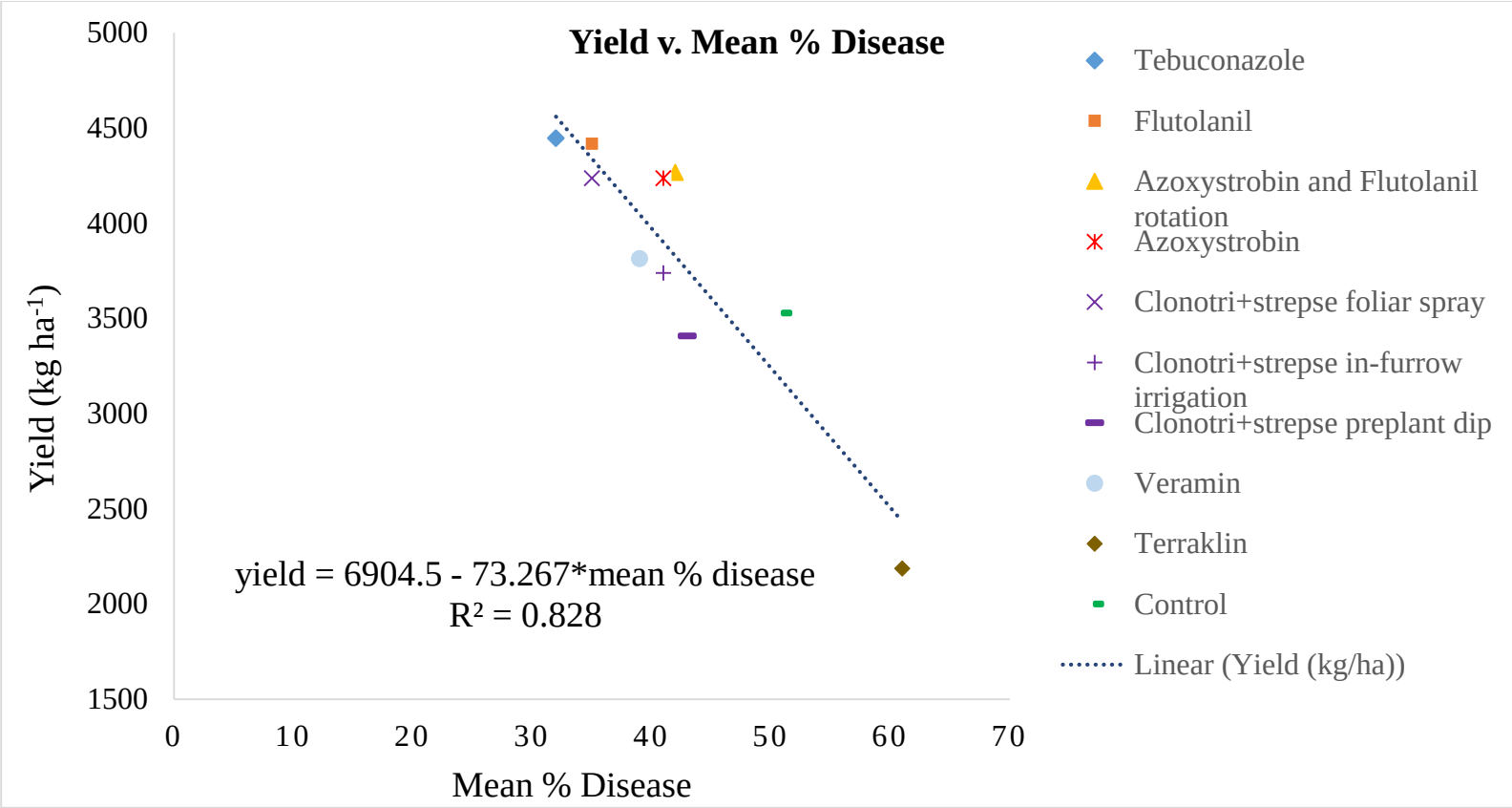

Figure 2.5. Effect of mean % disease from stem rot caused by *S. rolfsii* on yield of stevia in kg ha-1 for fungicide and biological control treatments

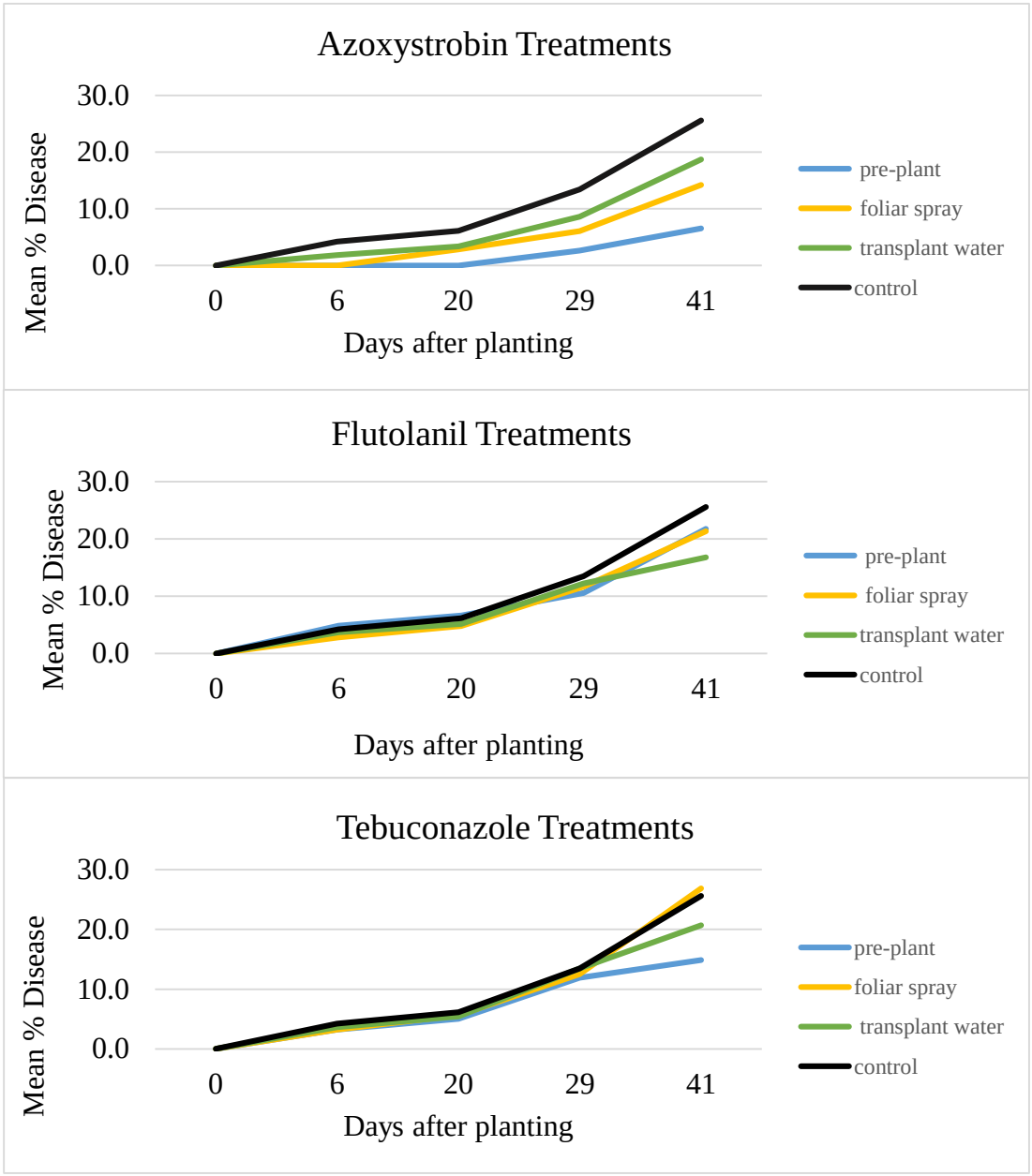

Figure 2.6. Effect of fungicide and application methods on disease progress caused by *S. rolfsii*. Percent disease incidence for each date and treatment was determined by taking stand counts and comparing to the original stand count for the plot.

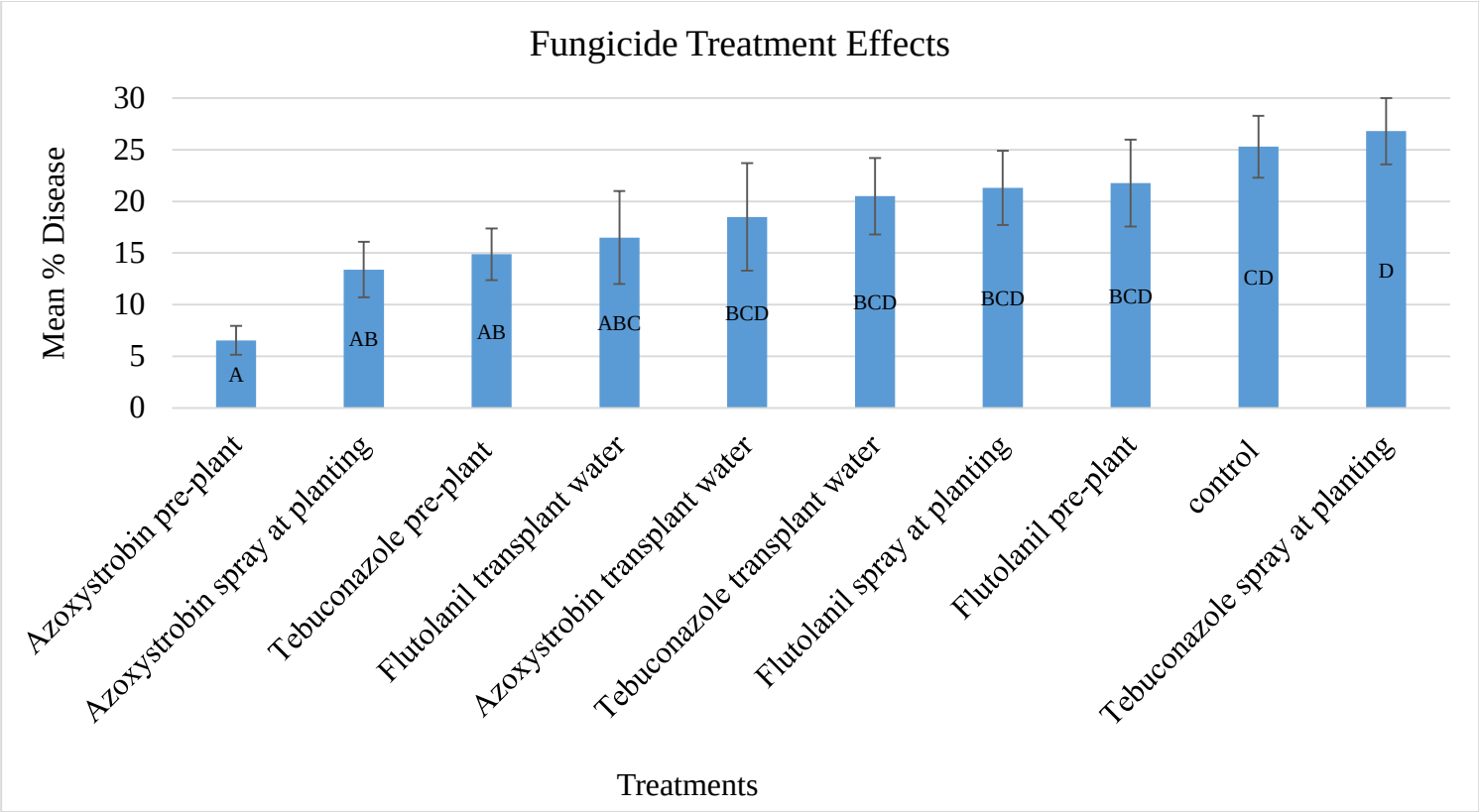

Figure 2.7. Effect of fungicide treatments on mean % disease caused by *S. rolfsii* on stevia at the final stand count, 41 days after planting. Treatment effects were analyzed using PROC GLM in SAS (9.4). Letters within each bar indicate least significance difference groups (alpha=0.05). Error bars represent standard error of the mean.

CHAPTER III.

Seasonal dynamics and the impacts of fungicide use on the diversity of *Rhizoctonia* species associated with brown patch of tall fescue

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ABSTRACT

Koehler, A. M., and Shew, H. D. 2015. Seasonal dynamics and the impacts of fungicide use on the diversity of *Rhizoctonia* species associated with brown patch of tall fescue. Plant Dis. 99:000-000.

Brown patch, caused by multiple species of *Rhizoctonia* and *Rhizoctonia*-like fungi, is the most severe summer disease of tall fescue in home lawns across the southeastern US. In highly managed lawns typically three applications of fungicide are applied each summer for brown patch control. Home lawns were surveyed in May 2014 and during July and August 2013 and 2014 to determine the organisms present during a typical epidemic of brown patch in tall fescue. *Rhizoctonia* and *Rhizoctonia*-like organisms were isolated from individual brown patch lesions present on tall fescue turf in lawns managed by lawn-care companies, with or without fungicides applied for brown patch control. Early season isolates were obtained from 56 home lawns across central NC in May 2014. Isolates were identified to species and anastomosis group by ITS rDNA sequence analysis. Of 108 isolates collected in May 2014, 77% were *Ceratobasidium* sp., 11% were *R. solani* anastomosis group (AG) 2-

2IIIB, 9% from *R. solani* AG 1-1B, and 3% were *R. zeae*. Of the 79 late season isolates collected from 57 home lawns in 2013 and 2014, only 2.5% were *Ceratobasidium sp*., 44% *R. solani* AG 2-2-IIIB, 37% *R. solani* AG 1-1B, and 14% *R. zeae*. One hundred twenty four isolates, including six historical isolates from untreated yards, 20 isolates from fungicide treated lawns in 2003, and 108 from May 2014, were selected and assayed for sensitivity to multiple concentrations of the fungicides azoxystrobin, flutolanil, fluxapyroxad, and propiconazole. Mean EC_{50} values varied across fungicides and species, but no resistance was observed. *R. zeae* was least sensitive to flutolanil and *R. solani* AG 1-1B was least sensitive to propiconazole. EC_{50} values for azoxystrobin with or without the addition of SHAM remained at >10µg ml^{-1}. After at least 10 seasons of fungicide use, no loss of efficacy was observed to any of the fungicides tested with the home-lawn isolates. The seasonal shift in pathogens causing symptoms and the range of fungicide sensitivity observed for those populations, indicate that optimal management strategies in home lawn tall fescue may vary during the season, but that there is no strong selection pressure for selection for resistant isolates.

INTRODUCTION

Multiple species of *Rhizoctonia* and *Rhizoctonia*-like binucleate fungi have been associated with leaf blight and brown patch on tall fescue (*Festuca arundinacea*) (Burpee, 1980; Martin and Lucas, 1984; Burpee and Martin, 1992; Amaradasa et al., 2013). North Carolina is located in an area known as the transition zone, where the range of adaptation for warm-season and cool-season turfgrasses intersects. Tall fescue is a popular turf for home lawns because it is a cool-season grass that is able to tolerate high temperatures in the summer and remains green in the winter. *Rhizoctonia* blight, or brown patch, is the most limiting disease to the successful growth and maintenance of tall fescue stands in the southeastern US (Burpee and Martin, 1992; Couch, 1995), and is the only disease that many home owners routinely attempt to manage with the application of fungicides.

Before 1970, *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) was the only organism associated with the etiology of brown patch in turfgrasses (Couch, 1973). Since that time, pathologists have identified *R. solani* to be a species complex having multiple anastomosis groups (AG) (Cubeta and Vilgalys, 1997). To date, six anastomosis groups of *R. solani* have been isolated from turfgrasses including AG 1, AG 2-2, AG 3, AG 4, AG 5, and AG 6. AG 1-IB and AG 2-2-IIIB are the dominant causal agents recovered from brown patch on tall fescue (Zhang and Dernoeden, 1995; Amaradasa et al., 2014). In addition to the *R. solani* species complex, *R. zeae*, *R. cerealis*, and species of *Ceratobasidium* have been identified as pathogens on tall fescue (Burpee, 1980; Martin and Lucas, 1984; Burpee and Martin, 1992). *Ceratobasidium* sp. have binucleate hyphal cells unlike the multinucleate cells of *R. solani* (Parameter et al., 1963). *Ceratobasidium* sp. are

distinguished by sorting into anastomosis groups (CAG) (Burpee et al., 1980). While symptoms of brown patch vary based on environmental conditions, pathogen, and mowing height, diseased turfgrasses typically have circular areas (patches) of blighted brown leaves (Smiley et al., 1992). The similarity in symptoms caused by a diverse array of organisms eliminates the use of simple visual diagnosis as a tool to determine the causal pathogen. *Rhizoctonia* sp. and *Rhizoctonia*-like organisms have different optimal temperatures and also may respond to fungicides differently (Martin et al., 1984; Carling et al., 1990). Accurate identification of the causal species and/or anastomosis group (AG or CAG) is important to optimize disease management recommendations.

Historically, characterization of *Rhizoctonia* has been based on morphological characteristics and hyphal anastomosis with known tester isolates. The most efficient method of determining AG group is to pair isolates on a thin layer of water agar and observe for the type of interaction that occurs between the hyphae (Zhang and Dernoeden, 1995). Despite attempts to improve efficiency, anastomosis testing can be cumbersome, time consuming, and often inconclusive. Beginning in the 1990s, many studies began to incorporate primers specific to the ribosomal DNA internal transcribed spacer (rDNA-ITS) to identify isolates (Boysen et al., 1996; Kuninaga et al., 1997;; Gonzalez et al., 2001). Recent research also has focused on using UP-PCR analysis (Amaradasa et al., 2013; Amaradasa et al., 2014).

Martin and Lucas (1984) first characterized species of *Rhizoctonia* in tall fescue in NC. In that study *R. solani*, *R. zeae*, *R. cerealis*, and binucleate *Rhizoctonia*-like fungi other than *R. cerealis* were found to be pathogenic on turfgrass. Little was done to further

characterize population structure and dynamics of *Rhizoctonia* in tall fescue until the work of Lee et al. (2003). Lee collected leaves with distinct brown patch lesions from managed tall fescue lawns in central North Carolina. The isolates they collected were predominately associated with two major groups, *R. solani* AG 1 and binucleate *Ceratobasidium sp.* CAG1. They concluded that further work on the role of CAG 1 in brown patch severity was needed. Amaradasa (2013) completed a diversity study of brown patch isolates in tall fescue and bentgrass in Virginia and Maryland and identified *R. solani* AG 2-2 IIIB and AG 1-IB as the most common causal agents on tall fescue, with binucleate *Rhizoctonia* accounting for a very small proportion of isolates collected. There have been no studies conducted to attempt to resolve these different observations and findings. The objectives of this current study were to identify the species and groups of *Rhizoctonia* present in tall fescue home lawns during brown patch epidemics, to determine if the fungi responsible for brown patch in NC have changed following continued use of strobilurin fungicides, and to characterize isolate sensitivity to fungicides currently used in brown patch management compared to isolates collected in prior epidemics.

MATERIALS AND METHODS

Isolate collection and identification

Isolates of *Rhizoctonia* were collected from tall fescue turf in lawns maintained by commercial lawn-care companies across the piedmont of North Carolina during the summers of 2013 and 2014. Early epidemic isolates were collected at disease onset, in mid to late

May 2014 and mid to late epidemic isolates were collected in July of 2013 and 2014. The May isolates were obtained immediately prior to the first fungicide application of the season, and the July isolates were obtained prior to the third and final application of fungicide during the peak of the brown patch epidemic.

Leaf blades with distinct brown patch lesions were collected from up to three patches at each location. Individual leaf lesions were excised, surface disinfested in 10% Clorox® for 30s, rinsed in sterile deionized H2O, and placed onto 2% Bacto water agar (Difco, Detroit, MI) in Petri plates. Petri plates were incubated at room temperature (21°C) for 48 h, and then actively growing hyphae with characteristics of *Rhizoctonia* were transferred to new Petri plates containing potato dextrose agar (PDA, Difco) amended with streptomycin sulfate (100 µg ml^{-1}) and penicillin G (100 µg ml^{-1}). Long-term storage of isolates consisted of colonized filter paper discs stored at -80°C. A colonized hyphal plug was transferred to a 9 cm diam Petri plate containing PDA and four pieces of sterile 2.5 cm diam filter paper were placed around the plug (Fisher Scientific). Once the isolates had fully colonized the discs, they were removed, placed in coin envelopes, and stored at -80°C.

Each isolate and location was photographed to keep a record of site location and isolate morphology. Isolates were separated into preliminary groups based on morphological characteristics. Putative isolates of *R. zeae* were identified based on the formation of salmon pink to orange sclerotia that formed within the medium. Species of *Ceratobasidium* were pale white to yellow in color while species of *R. solani* were buff to brown (Burpee and Martin, 1992; Burpee, 1980) (Figure 3.1).

To obtain tissue for DNA extraction, three 6.75 mm plugs of each isolate were transferred into 20 ml of potato dextrose broth (Difco). Isolates were grown for 7 days at ~21°C and the mycelial suspension was aspirated, transferred into 1.5 microcentrifuge tubes and lyophilized. Genomic DNA was extracted by grinding tissue with Qiagen cell lysis solution and 3 µL Proteinase K and incubating at 55°C for 1 h. Samples were then treated with RNase (3 µL) and incubated for 30 min at 37°C before adding Qiagen protein precipitation solution. Each sample was added to isopropanol to precipitate DNA and washed with 70% ethanol. Samples were eluted with 50µl dH_20, analyzed by spectrophotometry, and standardized to a concentration of 20 ng ml^{-1}.

For each isolate, the rDNA internal transcribed spacer (ITS) regions ITS 1, 5.8S rRNA, and ITS2 were amplified by polymerase chain reaction (PCR) using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990). PCR reactions were 50 µl in volume and consisted of 10 mM Tris-HCL(pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTPs, 4µM of each primer, 1.25 U of Taq polymerase (Applied Biosystems, Branchburg, New Jersey), and 80 ng of genomic DNA. Thermal cycling parameters included an initial denaturation step at 95°C for 3 min, followed by 34 cycles of DNA denaturation at 95°C for 30s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 1 min. A final extension step of 72°C for 10 min completed the cycling parameters. Amplified products were cleaned using ExoSap-IT (Affymetrix®). Samples were sent for sequencing to Eton Bioscience Inc. (Research Triangle Park, NC). Sequences were identified through a BLAST search of the GenBank

database (National Center for Biotechnology Information). Analysis of sequence data was completed using CLC Main Workbench 7.0 (CLC Bio a Qiagen Company).

Fungicide sensitivity

A total of 124 isolates were screened to determine the effective concentration (EC_{50}) values of brown patch isolates to four classes of fungicides used for control of brown patch. Isolates included 108 isolates from the May 2014 collections, 10 isolates from a 2003 collection (Lee, 2003), and six historical isolates sampled from tall fescue with no history of fungicide treatment. The fungicides tested were azoxystrobin (Heritage TL, Syngenta Crop Protection Inc., Greensboro, NC), flutolanil (Prostar 70 WG, Bayer Environmental Sciences, Research Triangle Park, NC), fluxapyroxad (Xzemplar, BASF, Research Triangle Park, NC), and propiconazole (Banner Maxx, Syngenta Crop Protection Inc., Greensboro, NC). PDA was amended with each fungicide to establish fungicide concentrations of 0, 0.01. 0.1. 1.0 or 10.0 µg ml^{-1}. When using azoxystrobin, the medium also was amended with salicylhydroxamic acid (SHAM). The SHAM was dissolved in dimethyl sulfoxide (DMSO) and 100 µg ml^{-1} was added to all concentrations of azoxystrobin meduium (Avile-Adame and Köller, 2002; Steinfeld et al., 2001). An additional control containing SHAM and 0 ug ml^{-1} azoxystrobin was included to observe if pathogen growth was inhibited by SHAM.

Isolates were grown on PDA for two days and a 6.75 mm diam plug was transferred to the center of a 60 x 15 mm Petri plate (USA Scientific, Ocala, FL) containing 8 ml of fungicide-amended PDA. Cultures were incubated at 22°C for 48-72 h, and radial growth of the colony was measured. Each fungicide was tested separately. Each treatment consisted of three replicate plates and each fungicide test was conducted twice. EC_{50} values, identifying

the fungicide concentration required to reduce radial growth by 50%, were calculated using linear regression of the percentage of growth inhibition on $\log_{10}$ transformed fungicide concentration (SAS 9.4., Cary, NC).

RESULTS

Isolate collection

Symptomatic leaf blades of tall fescue were collected from 61 home lawns in mid to late May 2014. Of 139 samples collected, 108 isolates of *Rhizoctonia* fungi were obtained from 56 of the sites. Isolates from more than one collection point per yard were obtained from 32 of the collection sites. Additional isolates were collected from 86 home lawn sites in July of 2013 and 2014. In July, leaf blades were collected prior to the third and final application of fungicide of the year, lesions were difficult to find in lawns, and successful isolation from lesions present was much lower than with early season samples. Of 178 samples collected, *Rhizoctonia* or *Rhizoctonia*-like hyphae were obtained from 79 samples representing 57 collection sites. Twelve of the 57 collection sites yielded isolates from multiple collection points.

Isolate identification

Based on morphology, isolates included species of *Ceratobasidium* with white to pale yellow hyphae, *R. solani* with buff to brown hyphae and *R. zeae* with pink to orange sclerotia (Figure 3.2) (Burpee and Martin, 1992; Burpee, 1980). There was high morphological diversity among the isolates collected (Figure 3.2).

Identification of isolates by sequencing the ITS rDNA region indicated that there were four major groups of organisms present (Table 3.1). Within these groups, *Ceratobasidium* was the dominant pathogen group recovered in the May samples, and *R. solani* was the dominant pathogen group in mid-late season sampling. Only three May isolates and two July isolates could not be identified to species or AG based on ITS sequence, but were similar to *Rhizoctonia* in appearance. These five isolate were eliminated from further tests.

In the May 2014 sample, *Ceratobasidium* sp. accounted for 77% (83/108) of the isolates collected. Isolates also included 11% (12/108) *R. solani* anastomosis group 2-2 IIB, and 10% (10/108) *R. solani* anastomosis group 1-IB. Of the lawns with multiple collection points, 69% (22/32) contained only *Ceratobasidium* sp. and 3% (1/32) contained only *R. solani* AG 2-2 IIIB. The remaining 28% of lawns with multiple collection points had two or more groups recovered including: 13% (4/32) *Ceratobasidium* sp. and *R. solani* AG 2-2 IIIB, 9% (3/32) *Ceratobasidium* sp. and *R. solani* AG 1-IB, 3% (1/32) *Ceratobasidium* sp., *R. solani* AG 2-2 IIIB, and *R. solani* AG 1-IB, and 3% (1/32) *R. solani* AG 2-2 IIIB and *R. solani* AG 1-IB.

In late season sampling, *R. solani* was the dominant group accounting for 81% (64/79) of late season isolates collected, with 44% (34/79) of the isolates identified as *R. solani* AG 2-2-IIIB and 37% (28/79) *R. solani* AG 1-IB. The mid-summer isolates also included 14% (11/79) *R. zeae* and 2.5% (2/79) *Ceratobasidium sp*. In lawns with multiple collection points 17% (2/12) were *R. solani* AG 2-2 IIIB and 17% (2/12) were *R. solani* AG 1-IB. The remaining 66% of lawns included: 42% (5/12) *R. solani* AG 2-2 IIIB *and R. solani*

AG 1-IB, 8% (1/12) *R. solani* AG 2-2 IIIB and *R. zeae*, 8% (1/12) *R. solani* AG 1-IB and R. zeae, and 8% (1/12) *R. solani* AG 2-2 IIIB, *R. solani* AG 1-IB, and R. zeae.

Fungicide Sensitivity

All fungicides were inhibitory to all isolates tested. Trial effects were observed with the fungicides azoxystrobin and propiconazole, and significant isolate effects were recorded for all fungicides except for flutolanil. A trial by isolate interaction was observed for the fungicide azoxystrobin, so trials were analyzed separately. Due to significant isolate effects, single-degree-of-freedom contrast statements (Table 3.2) and PROC means (Table 3.3) were used to compare the response of anastomosis groups within each fungicide. Contrasts indicated that there were significant differences between groups, so each group was analyzed separately to calculate ED_{50} values.

Flutolanil, fluxapyroxad, and propiconazole effectively inhibited mycelial growth of all isolates, but there were differences in efficacy among species and anastomosis groups. Azoxystrobin inhibited fungal growth, but did not eliminate growth at 10 mg L^{-1} in our in vitro assays. Even with the addition of salicylhydroxamic acid (SHAM) to block the alternative growth pathway, EC_{50} values were >10 mg L^{-1} for all isolate groups. In general, species of *Ceratobasidium* had the lowest mean EC_{50} values (Table 3.4). Fluxapyroxad had the lowest EC_{50} value, 0.06 mg L^{-1}, followed by flutolanil with values 0.25 and 0.30 mg L^{-1} in trials 1 and 2 respectively. The highest *Ceratobasidium* sp. EC_{50} values were seen with propiconazole at 0.25 and 0.95 mg L^{-1}. Mean EC_{50} values of *R. solani* AG 2-2 IIIB were 0.45 and 0.49 mg L^{-1} for flutolanil, 0.58 and 0.18 mg L^{-1} for propiconazole, and 0.75 and 1.26 mg L^{-1} for fluxapyroxad. *R. solani* AG 1-IB values were 0.17 and 0.25 mg L^{-1} for flutolanil,

0.19 and 0.40 mg L^{-1} for fluxapyroxad, and 1.64 and 2.93 mg L^{-1} for propiconazole. *R. zeae* EC_{50} values were 0.10 and 0.45 mg L^{-1} for fluxapyroxad, 0.12 and 0.23 mg L^{-1} for propiconazole, and 1.47 and >10 mg L^{-1} for flutolanil. Flutolanil had similar EC_{50} values for *R. solani* AG 2-2 IIIB, 1-IB and *Ceratobasidium* sp., with higher values observed for *R. zeae*. Fluxapyroxad had very low EC_{50} values for *Ceratobasidium* sp., medium values for *R. zeae* and *R. solani* AG 1-IB, with highest EC_{50} observed for *R. solani* AG 2-2IIIB. Propiconazole had lowest EC_{50} values for *R. zeae*, medium values for *Ceratobasidium* sp, and *R. solani* AG 2-2 IIIB and highest EC_{50} values for *R. solani* AG 1-IB.

Isolates were sorted into groups representing year of collection and AG. In this grouping, trial effects were not significant, so trials were combined for analysis. Since the EC_{50} values of azoxystrobin were >10 mg L^{-1} for all isolates over years, it was not included in the analysis. In all other fungicides tested, EC_{50} values were similar across years for isolates within an AG. With the fungicide flutolanil, *R. solani* AG 1-IB was the most sensitive in the untreated historic, 2003, and 2014 collections, and *R. zeae* was the least sensitive. With fluxapyroxad, *Ceratobasidium* was the most sensitive, and *R. solani* AG 2-2 IIIB was the least sensitive in the historical, 2003, and 2014 collections. With propiconazole, *R. zeae* was the most sensitive, and *R. solani* 1-IB was the least sensitive in the historic and 2014 collections.

DISCUSSION

The results of this study support previous findings that multiple species of *Rhizoctonia* are associated with brown patch disease of tall fescue (Amaradasa et al., 2013, and Lee et al., 2003). Our results clearly demonstrate that the organisms present and the frequency of isolation vary based on when samples are taken during a brown patch epidemic. The differences in seasonal occurrence we observed are similar to results from previous studies that have collected organisms from foliar lesions. Lee (2003) collected isolates early in the season and found binucleate species to account for 47% of isolates, while Amaradasa (2013) collected later in the summer and found *R. solani* AG groups to account for 57% of isolates. *R. solani* AG 5, found by Amaradasa et al. (2013), was not detected in our study.

Our results show that *R. solani* AG groups 1-IB and 2-2 IIIB, *R. zeae*, and binucleate *Rhizoctonia*-like *Ceratobasidium* species are the four major groups of organisms associated with brown patch lesions in tall fescue in North Carolina. Species of binucleate *Ceratobasidium* were the dominant group of organisms present in May epidemics. Pathogenicity of this group on tall fescue has been reported but hyphal anastomosis and population dynamics have not been well characterized to date (Burpee and Martin, 1992; Amaradasa, 2013). Binucleate *R. cerealis*, thrives in cool, wet weather, and has been documented to cause "cool-weather brown patch" or "yellow patch" (Burpee, 1980). Binucleate species of *Ceratobasidium* associated with brown patch have lower optimal temperatures than *R. solani* and *R. zeae*, but appear to have higher optimal temperatures than isolated of *R. cerealis* associated with yellow patch (Martin and Lucas, 1984). Further work is needed to better understand binucleate *Ceratobasidium* sp. associated with brown patch of

tall fescue. *R. solani* AG 2-2 IIIB and AG 1-IB were the dominant pathogens in July epidemics which sipport the findings of Amaradasa (2013). *R. zeae* was not observed until July sampling which was expected, as its optimal temperature has been reported as 32°C (Martin and Lucas, 1984).

Prior to ITS sequence analysis, subgroups of *Rhizoctonia* were identified using culture morphology and anastomosis grouping. Zhang and Dernoeden (1995) reported *R. solani* AG 1-IA and AG 2-2 IIIB on tall fescue exhibiting brown patch symptoms based on anastomosis grouping. They found AG 1-IA to have two types, type A and type B based on culture morphology and sclerotia size. Kuninaga et al. (1997) and Hsiang and Dean (2001) utilized DNA sequencing and identified AG 1-IB as the subgroup of AG 1 present in turfgrass. It is possible that studies identifying AG 1-IA would have identified isolates as 1-IB using currently available molecular sequencing techniques (Amaradasa et al., 2013). *R. solani* isolates are reported to have optimal temperatures of 28°C (Martin and Lucas, 1984). Symptoms caused by *R. solani* AG 1-IB and 2-2-IIIB in turf are indistinguishable tan to brown lesions with dark borders, so molecular techniques or anastomosis testing are needed for proper identification of *R. solani* anastomosis groups.

There is a wide array of fungal diseases that are problematic in turf. In commercial settings such as athletic fields or golf courses, fungicides are an important tool to maintain aesthetically pleasing turf. Some diseases, like dollar spot in turf caused by *Sclerotinia homoeocarpa*, require continuous application of fungicide. In these high pressure systems, selection for isolates with reduced fungicide sensitivity results in fungicide resistance (Smiley et. al, 2005). Strobilurin fungicides entered the market in 1996, and have been a

very important class of fungicides on many crops. Resistance due to mutation in the cytochrome b gene was first observed in cereals in 1998, and has extended to include many pathogen/cropping systems (Bartlett et al., 2002). Azoxystrobin is the most commonly used product to manage brown patch in home lawns. To date, there have been no accounts of resistance to strobilurins or any other fungicide used to control brown patch in tall fescue. This is likely due to the fact that brown patch control is a low selection-pressure system. Fungicide applications are typically made only three times in the summer during the peak epidemic. During the rest of the year, *Rhizoctonia* is able to survive as a saprobe and there is no strong selection pressure for isolates with reduced sensitivity to fungicides to predominate within the pathogen population.

Mean EC_{50} values of this trial were consistent with previously reported values for *Rhizoctonia* (Lee et al., 2003). As previously observed, fungicide sensitivity varied among species and subgroups of *Rhizoctonia* (Lee et al., 2003; Carling et al., 1990, Martin et al., 1984a; Martin et al., 1984b), but no resistance was observed. Our results agree with these findings. The sensitivity of *R. zeae* to propiconazole (EC_{50}=0.12 and 0.23 for trial 1 and 2 respectively) was consistent to EC_{50} values reported for *R. zeae* to DMI tebuconazole (0.24 mg L^{-1}) and triadimefon (<0.17 mg L^{-1}) (Lee, 2003; Martin et al., 1984b). Martin (1984b) and Lee (2003) identified *R. solani* as AG 1-IA with EC_{50} values of 2.56 mg L^{-1} and 2.97 mg L^{-1} to tebuconazole and triadimefon respectively. In the present study, *R. solani* AG 1-IB isolates had EC_{50} values 1.64 and 2.93 mg L^{-1} for DMI fungicide propiconazole trials 1 and 2. Fluctuations were observed between the EC_{50} values for flutolanil in Lee (2003) and the current study, but overall trends remained the same. Our results indicate that long term

repeated use of these materials for brown patch control presents a very low chance for resistance development.

Multiple studies have reported a diverse group of pathogens associated with brown patch in tall fescue. With variance in fungicide sensitivity, understanding the populations of pathogens present is an important consideration for the management of this disease. Future research is needed to further understand pathogen succession and distribution within epidemics of brown patch. Repetitive sampling from selected sites throughout the season would aid in understanding these seasonal changes in species occurrence.

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Table 3.1. Frequency of isolation of *Rhizoctonia* species from managed home lawn tall fescue brown patch lesions in 2013-14

Group	May 2014[a]	July 2013-14[b]
R. solani AG 2-2IIIB	12	35
R. solani AG 1-IB	10	29
Ceratobasidium sp.	83	2
R. zeae	0	11
unknown	3	3

[a] 108 isolates were recovered in May 2014
[b] 19 isolates were recovered in July 2013, 60 isolated were recovered in July 2014

Table 3.2. Linear contrasts for effects within fungicide treatment of anastomosis group on percent inhibition of radial colony growth of *Rhizoctonia solani*.

Fungicide	Contrast between groups[a]	Estimate ± s.e.; $P > \|t\|$	
Azoxystrobin (+ SHAM)	*R. solani* AG 1-IB vs. *Ceratobasidium* sp.	-2.5 ± 0.84	0.0029
	R. solani AG 1-IB vs. *R. zeae*	-7.6 ± 1.9	<0.0001
	Ceratobasidium sp. vs. *R. zeae*	3.0 ± 1.8	0.0904
Flutolanil	*R. solani* AG 2-2 IIIB vs. *R. solani AG 1-1B*	12.5 ± 3.1	<0.0001
	R. solani AG 2-2 IIIB vs. *Ceratobasidium*	2.2 ± 2.3	0.3475
	R. solani AG 2-2 IIIB *vs. R. zeae*	-20.6 ± 5.7	0.0003
	R. solani AG 1-IB vs. *Ceratobasidium* sp.	-4.4 ± 2.5	0.0791
	R. solani AG 1-IB vs. *R. zeae*	-33.1 ± 5.8	<0.0001
	Ceratobasidium sp. vs. *R. zeae*	-7.9 ± 5.4	0.1416
Fluxapyroxad	*R. solani* AG 2-2 IIIB vs. *R. solani AG 1-1B*	13.9 ± 2.4	< 0.0001
	R. solani AG 2-2 IIIB vs. *Ceratobasidium*	6.2 ± 1.8	0.0006
	R. solani AG 2-2 IIIB *vs. R. zeae*	16.7 ± 4.4	0.0001
	R. solani AG 1-IB vs. *Ceratobasidium* sp.	18.5 ± 1.9	< 0.0001
	R. solani AG 1-IB vs. *R. zeae*	2.8 ± 4.4	0.5281
	Ceratobasidium sp. vs. *R. zeae*	-11.4 ± 4.1	< 0.0059
Propiconazole	*R. solani* AG 2-2 IIIB vs. *R. solani AG 1-1B*	-10.1 ± 2.5	<0.0001
	R. solani AG 2-2 IIIB vs. *Ceratobasidium*	-3.4 ± 1.9	0.0685
	R. solani AG 2-2 IIIB *vs. R. zeae*	19.4 ± 4.6	<0.0001
	R. solani AG 1-IB vs. *Ceratobasidium* sp.	15.6 ± 2.0	<0.0001
	R. solani AG 1-IB vs. *R. zeae*	29.5 ± 4.6	<0.0001
	Ceratobasidium sp. vs. *R. zeae*	-3.8 ± 4.3	0.3799

[a] Single-degree-of-freedom linear contrasts between *Rhizoctonia* groups. Trial data was combined and analyzed using PROC GLM in SAS (Version 9.4). Positive estimates indicate that the first group of the contrast had higher mean % inhibition; negative numbers indicate the second group of the contrast had higher mean % inhibition. Mean % inhibition represents radial growth averaged at 0, 0.01, 0.1, 1 and 10 µg ml^{-1}.

Table 3.3. Means of inhibition of *Rhizoctonia* and *Ceratobasidium* sp. to media amended with fungicides at rates of 0, 0.01, 0.1, 1, or 10 µg ml^{-1}

Fungicide	Group	Mean percent inhibition Estimate ± s.e[a]
Azoxystrobin (+ SHAM)	*R. solani* AG 1-IB	31.0 ± 21.1
	R. solani AG 2-2-IIIB	23.3 ± 17.7
	Ceratobasidium sp.	21.2 ± 17.8
	R. zeae	8.2 ± 9.6
Flutolanil	*R. solani* AG 1-IB	56.5 ± 44.8
	R. solani AG 2-2-IIIB	43.9 ± 43.6
	Ceratobasidium sp.	52.1 ± 44.7
	R. zeae	23.3 ± 32.9
Fluxapyroxad	*R. solani* AG 1-IB	51 ± 35.6
	R. solani AG 2-2-IIIB	37.2 ± 33.9
	Ceratobasidium sp.	69.4 ± 34
	R. zeae	53.8 ± 37.6
Propiconazole	*R. solani* AG 1-IB	28.2 ± 34.5
	R. solani AG 2-2-IIIB	38.5 ± 31.86
	Ceratobasidium sp.	43.8 ± 37.8
	R. zeae	57.7 ± 35.0

[a] Potato dextrose agar was amended with each fungicide to establish fungicide concentrations of 0, 0.01, 0.1, 1, or 100 µg ml^{-1} of fungicide. Isolates were transferred to the center of a 60 x15mm Petri dish and incubated at 22°C for 48-72 hours, after which radial growth was measured. Mean inhibition for each group combines radial measurements from all concentrations tested. PROC Means were calculated using SAS 9.4.

Table 3.4. Predicted mean effective concentration (EC50) values of selected fungicides calculated using linear regression models of concentration by inhibition of radial growth

		Fungicides							
		Azoxystrobin (+SHAM)		Flutolanil		Fluxapyroxad		Propiconazole	
		EC_{50} (µg ml^{-1})							
Group	n	Trial 1[a]	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
R. solani (AG 2-2-IIIB)	19[a]	>10[c]	>10	0.45	0.49	0.75	1.26	0.58	0.18
R. solani (AG 1-1B)	16	>10	>10	0.17	0.25	0.19	0.40	1.64	2.93
Ceratobasidium sp.	83	>10	>10	0.25	0.30	0.06	0.06	0.25	0.95
R. zeae	3	>10	>10	1.47	>10	0.10	0.45	0.12	0.23

[a] Potato dexitrose agar was amended with each fungicide to establish fungicide concentrations of 0, 0.01, 0.1, 1, or 100 µg ml^{-1} of fungicide. Isolates were transferred to the center of a 60x15mm Petri dish and incubated at 22°C for 48-72 hours. Each treatment was replicated three times per trial, and trials were conducted twice.

[b] Number of isolates from each group that were tested. A total of 124 isolates were tested, three unidentified isolates were excluded from this table.

[c] EC_{50} values were calculated by measuring isolates following incubation to establish percent inhibition. SAS 9.4 was used to find linear regression models of concentration by inhibition of radial growth

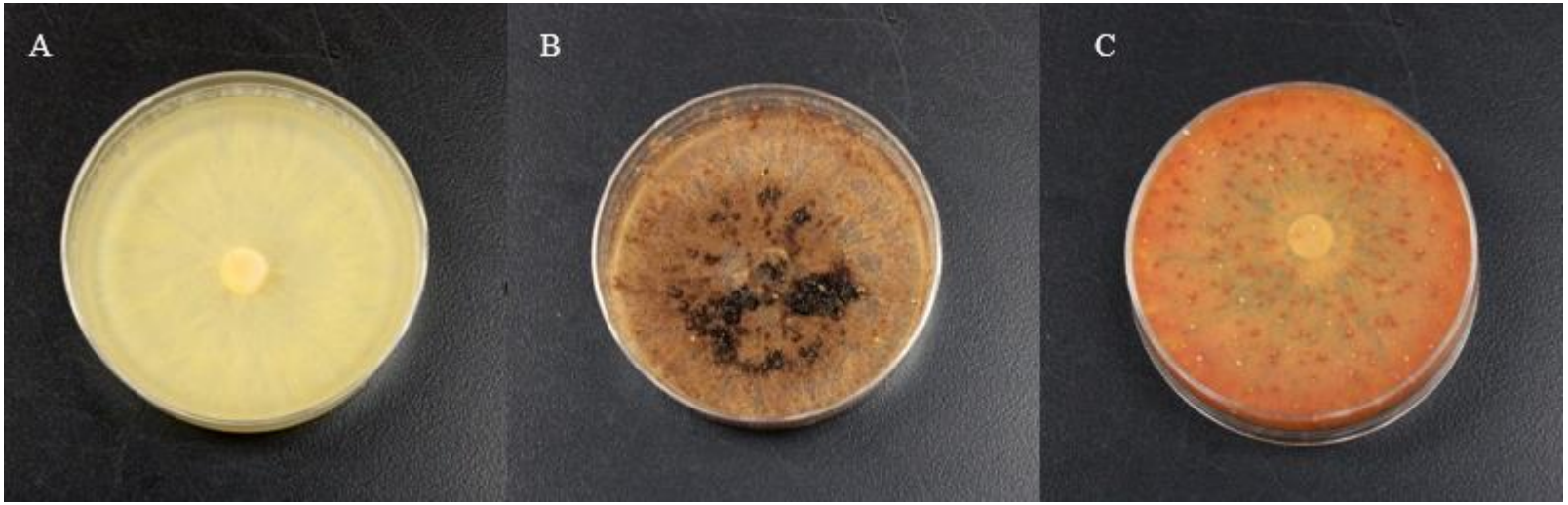

Figure 3.1. Cultures of binucleate *Ceratobasidium* sp. (A), *R. solani* (B) and *R. zeae* (C) grown on potato dextrose agar

Figure 3.2. Diversity among cultures of *Rhizoctonia* fungi isolated from tall fescue during epidemics of brown patch

APPENDICES

Appendix A: *S. rolfsii* Disease Note

First report of stem and root rot of Stevia caused by *Sclerotium rolfsii* in North Carolina

Stevia (*Stevia rebaundia*) is an emerging crop in the US. Once established, the crop is grown for 3 to 5 years and is typically harvested twice per growing season. Stevia leaves contain multiple glycosides that are used as a natural noncaloric sweetener that was approved by the USDA in 2008 as a sugar-substitute. In commercial plantings of Stevia in North Carolina, wilting and death of plants in first and second-year plantings were observed in 2012 and 2013. Diseased plants were observed in multiple counties in the state, with first symptoms observed in May of each year and continuing through the summer months. Prior to Stevia, these fields had been planted primarily in a corn-soybean rotation. Symptoms began as moderate to severe wilting of young shoots and chlorosis of leaves, rapidly followed by death of stems and rotting of roots. White mycelial growth was frequently observed at the base of stem tissue. Theses characteristic hyphae of *Sclerotium rolfsii* were often accompanied by the presence of abundant white-to brown-colored sclerotia. Isolations from infected root and stem tissue were made on potato dextrose agar amended with 50 µg/ml of streptomycin sulfate and penicillin G. Isolations from diseased tissue yielded characteristic white hyphae of *S. rolfsii* (Aycock, 1966). Numerous sclerotia 0.5-2 mm in diameter developed following 4-7 days of mycelial growth. Sclerotia were initially white and melanized turning brown with age. To verify pathogenicity, 10-week-old Stevia seedlings were transplanted in 10-cm diam pots containing sterile 1:1:1 sand, loam, media mix. Inoculum consisted of oat grains infested with one isolate obtained from the field plants. Oats were sterilized on three consecutive days and then inoculated with colonized agar plugs of *S. rolfsii*. Oats were incubated at room temperature to allow the fungus to thoroughly colonize the oats. Three infested oat grains were added to each test pot and plants were then observed over a 3 week period. Symptoms were observed within 5 days on most plants and included chlorotic leaves, bleached stems, wilting, and necrotic roots. White mycelium and abundant sclerotia were found at the base of plants. Uninoculated plants did not develop any symptoms. This is the first report of *S. rolfsii* on Stevia in the US. Kamalakanna et al., (2007) reported a root rot disease of Stevia in India and confirmed *S. rolfsii* as the causal agent.

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Figure 1: Field symptoms of stem rot on stevia in North Carolina: (A) Multiple plants exhibiting wilting and plant death (B) Close up of wilting and necrosis caused by stem rot (C) Abundant sclerotia production at the base of stems (D) Characteristic hyphae of *S. rolfsii* on an infected stem

Appendix B: *S. sclerotiorum* Disease Note

First report of stem rot of stevia caused by *Sclerotinia sclerotiorum* in North Carolina

Stevia (*Stevia rebaundiana* Bertoni) is an emerging perennial crop in the United States. The crop is grown for 3 to 5 years with two harvests per growing season. Stevia contains numerous glycosides that are used as a natural noncaloric sweetener, and in 2008 was approved by the USDA as a sugar-substitute. In commercial plantings of second-year stevia in North Carolina, diseased plants were observed in April and May of 2013. Diseased plants were observed in several counties in the state in fields that had been planted primarily in a corn-soybean rotation prior to stevia planting. Symptoms included wilting, chlorotic leaves, necrotic leaves at the base of the stem, bleached stem lesions, and dead plants. Symptomatic plants often also had tufts of white hyphae present on stems and large, irregularly shaped 2 to 8mm black sclerotia frequently were present on the base of the stem. Isolations from infected stem tissue were made on potato dextrose agar amended with 50 µg/ml of streptomycin sulfate and penicillin G. Based on hyphal and sclerotial characteristics, isolates were tentatively identified as *Sclerotinia sclerotiorum* (Lib.) de Bary (4). Koch's Postulates were confirmed on 10-week-old Stevia plants, *cv. G3*, grown in the greenhouse in 10-cm diameter pots containing a sterile 1:1:1 sand, loam, media mix. Oat grains infested with one isolate obtained from diseased field plants served as the inoculum. Oats were sterilized on three consecutive days, inoculated with colonized agar plugs of *S. sclerotiorum*, and then incubated at room temperature until they were thoroughly colonized. Three infested oat grains were buried 1 cm deep approximately 2 cm from the base of the plant in each of the six test pots and plants were observed over a three-week period for symptoms. Symptoms developed on all plants within 5 days of inoculation. Leaves began to wilt, then turned chlorotic and necrotic, with stem lesions and sclerotia present at the base of the plant. Isolations were taken from infected stem tissue and pure cultures were prepared for molecular identification. Uninoculated control plants did not develop symptoms. Pathogen identification was confirmed using universal primers ITS 4,5 and β-tubulin (2,3). Mycelium from the cultured greenhouse stem isolations were grown in potato dextrose broth. Mycelium samples were aspirated and lyophilized prior to DNA extraction. Extracted DNA was amplified through PCR with ITS and β-tubulin primers and sent for sequencing. Sequences were aligned using CLC Workbench. Sequences from ITS45 had 100% identity to *S. sclerotiorum* GenBank accession number KF859933.1, confirming *S. sclerotiorum* as the causal organism. The β-tubulin sequence was compared against the Broad Institute *S. sclerotiorum* whole genome shotgun sequence and was confirmed to have 100% identity to the beta tubulin chain (5). This is the first report of *S. sclerotiorum* on stevia in the US. Chang et al., (1997) reported a stem rot of stevia in Canada and confirmed *S. sclerotiorum* as the causal organism.

References

(1) Chang K, et. al. *Plant Disease* 81:311, 1997. (2) Freeman, J. et. al. Eur J of Plant Pathol 108:877, 2002. (3) Glass, N.L. and Donaldson, G. C. Appl and Environ Microb 61:1323, 1995. (4) Mordue, J. E. M., and P. Holliday. CMI No. 513, 1976. (5) *Sclerotinia sclerotiorum* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)

Figure 1: Field symptoms of *Sclerotinia sclerotiorum* on Stevia in North Carolina: (A) Plants displaying wilting and necrosis caused by stem rot (B) Necrosis and death of plants (C) Tufts of white hyphae, young sclerotia, and bleaching at the base of the stem (D) Mature sclerotia and stem bleaching