CHARACTERIZATION OF *SETOPHOMA TERRESTRIS* CAUSING PINK ROOT IN ONION, DISEASE MANAGEMENT, AND AGE-RELATED RESISTANCE

By

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ABSTRACT

CHARACTERIZATION OF *SETOPHOMA TERRESTRIS* CAUSING PINK ROOT IN ONION, DISEASE MANAGEMENT, AND AGE-RELATED RESISTANCE

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Setophoma terrestris infects onion roots causing a characteristic discoloration called 'pink root'. This understudied pathogen negatively impacts onions worldwide as a result of compromised root systems that result in reduced yields. Variability of *S. terrestris* isolates was determined based on genetic, morphology, and virulence. A collection of 98 isolates of *S. terrestris* was identified based on partial DNA sequences encoding the large-subunit ribosomal DNA. High identity (>97%) was revealed when compared to the nucleotide sequences of *S. terrestris* on the NCBI database. Seven inter-simple sequence repeats (ISSR) markers were used to determine population structure of *S. terrestris* across nine sites in Michigan. There was no population structure detected when the isolates were grouped by geographical origins. Variability in morphological characteristics and disease virulence were observed among isolates within the populations rather than among the populations.

Thirty onion cultivars were evaluated for pink root susceptibility in commercial fields with a history of pathogen infestation. Roots were assessed 64, 76, 96, and 110 days after seeding for disease incidence in 2011 and 2012; disease severity was assessed at 110 days in 2013. The cultivars Frontier, Highlander, and Scout were highly susceptible to pink root, while the cultivars Hendrix, Redwing, and Sedona were the least susceptible. Fungicide treatments were evaluated for their efficacy against pink root in a greenhouse trial after they were drenched

either 0 day post inoculation (dpi) or 0 and 14 dpi. The fungicide penthiopyrad resulted in the greatest plant height, fresh weight, bulb circumference, and root density, and was significantly different from other fungicide treatments and the inoculated untreated control.

Age-related resistance to pink root was examined in 'Hendrix' and 'Highlander' onions. Plants were inoculated when 3, 5, 7, and 9 weeks old using millet seeds infested with *S. terrestris* and incubated in the greenhouse for 42 days. Microscopic observation revealed that pathogen colonization could be detected prior to root discoloration and that onion roots of all ages could become infected. The incidence of root colonization increased when older plants were inoculated compared with younger plants. Root density compared among plant ages of the two cultivars was not statistically different following inoculation. Reduction (%) of plant growth parameters (plant height, fresh weight, number of total leaves per plant, and bulb circumference) compared to the uninoculated onions was generally greater when young plants were inoculated compared to older plants regardless of cultivar. The growth of onions when inoculated at 3 weeks was significantly less for 'Highlander' than 'Hendrix'.

The variability among the *S. terrestris* isolates in Michigan may be useful for developing onion cultivars that are less susceptible to pink root. Identification of effective fungicide treatments and efficient application timing may reduce crop losses.

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Introduction

Pink root caused by *Setophoma terrestris* (H.N. Hansen) Gruyter, Aveskamp and Verkley is one of the most devastating diseases of onions grown in warm environmental conditions. The disease occurs worldwide and occurs most severely in subtropical or tropical regions when onions are grown in infested soils (Sumner 1995). *Setophoma terrestris* has a wide host range, including many economically important crops and weeds (Sumner 1995). Among the hosts of *S. terrestris*, onions and tomatoes are among the key vegetables produced in Michigan (Johnson 2011). Plants infected by *S. terrestris* appear stunted as a result of the damaged root system, leading to significant losses of yield and marketable quality of onions (Sumner 1995) and tomatoes (Thornberry and Anderson 1940).

Integrated cultural and chemical management approaches are required to reduce damage caused by *S. terrestris*. The use of resistant onion cultivars was suggested by Jones and Perry (1956). Onion cultivars with varying levels of resistance or tolerance to pink root are available, but the expression of resistance of onions to pink root under field conditions might be influenced from variation of climate conditions and soil types (Coleman et al. 1997). Gorenz et al. (1949), for example, showed that the Yellow Bermuda variety was more resistant than other varieties, but its resistance was suppressed when the temperature rose to 28 °C or greater. Soil fumigation is another strategy for controlling pink root incidence. Fumigants (1,3 dichloropropene, metam sodium, or potassium N-methydithiocarbamate) are registered (Bird et al. 2013); however, fumigation is unlikely to be practiced due to high cost of application and increasing environmental concerns (Nasr Esfahani and Ansari Pour 2008).

Since the genetic structure is defined as the amount and distribution of genetic variation within and among populations, the genetic structure of populations can be affected by the evolutionary potential of pathogen populations (McDonald and Linde 2002). The rate of pathogen evolution is indicated by the genetic variation being maintained within a population, therefore the fungal populations with high levels of genetic diversity are assumed to adapt to diverging environments; such as resistant hosts and fungicide applications, more rapidly than populations with low genetic variation (McDonald and McDermott 1993). Thus, the information of genetic variation might be useful to inform management either to deploy resistant hosts or fungicides to achieve the effective control (Lapchin and Shtienberg 1999). For *S. terrestris*, study of genetic diversity of the populations from South Africa and the United State of America was determined by Ferreira et al. (1991). High variation of the population was shown when isozyme polymorphism was used as the markers. In addition, pathogenicity and cultural characteristics of isolates tested showed variety and differentiation among the isolates (Ferreira et al. 1991).

The etiology and epidemiology of this review provides an overview of *S. terrestris* and its primary host, onion root disease, and also provides management options of the pink root disease.

Onion (*Allium cepa* L.)

Onion production

According to the United Nations Food and Agricultural Organization, onions are grown in at least 175 countries. Each year, it is estimated that 6.7 million acres of onions are grown around the world producing a yield of 105 billion pounds. Leading onion producing countries include China, India, the United States, Turkey, and Pakistan. In 2010, total U.S. onion

production was 7.32 billion pounds and the crop value was $1.5 billion (Anonymous 2011). The value reported by the USDA (Anonymous 2011) for fresh market and storage onions was $531.7 and $923.4 million, respectively. Storage or dry bulb onions account for about 70% of annual onion production in the United States (Huntrods 2011). In Michigan, onions are normally produced in the south central and southwest regions of the lower Peninsula, including Allegan, Barry, Eaton, Ionia, Kent, Ottawa, Newaygo, and Van Buren counties (Datt et al. 2002).

Biology

Onions (*Allium cepa* L.) are cool-season bulb-producing biennial plants varying in color, shape, and taste. Bulbs can be white, yellow, or red and round, flattened, or torpedo-shaped. Some bulbs are sweet while others are pungent (Brewster 1994). Onions can be grown from seeds, sets, or transplants. They grow well in many types of soil: sand, loam, clay, and organic-peat, but produce especially well on muck soil at a pH of 6.0 to 8.4. Moist and cool conditions are best for growth in the early stages, while a combination of hot (21 to 27°C) and dry conditions in combination with correct day length favors the maturity and bulb-forming stages (Masabni and Lillard, n.d.).

Onion bulbs are modified stems with fibrous roots and fleshy leaves. Bulb formation involves the swelling and thickening of leaf sheaths. A perfectly developed onion will have at least 13 leaves and 13 rings of scales around the bulbs. Each leaf correlates with a ring in the bulb. Larger leaves produce larger rings within the bulb (Hynes et al. 2009). Root systems in onions are shallow reaching about 1 foot deep or less. They have low root densities and lack root hairs (Brewster 1994).

Varieties

Cultivars are classified and characterized by foliage (color, length, and erectness) and bulb (shape and skin color) characteristics (Brewster 1994). However, day-length sensitivity, short-day (SD), intermediate-day (ID), long-day (LD), and very long-day (VLD), is commonly used to classify onion cultivars (Bosch Serra and Currah 2002). The LD and VLD types are characterized by long-storing cultivars from the north-eastern USA and northern European (Brewster 2008). Each category differs in the number of hours needed for bulb initiation. SD, ID, and LD cultivars require 8 to 12, 13 to 14, and >15 h of photoperiod, respectively. In Michigan, LD cultivars are common and are grown successfully from seed. SD and ID types can also be grown but production is improved when they are grown from transplants (Datt et al. 2002).

Setophoma terrestris

Setophoma terrestris is a causal agent of onion pink root disease. The fungus is a common inhabitant of soils in many growing regions in the United States and most parts of the world (Gorenz et al. 1949). It is well known for infecting roots of onions and other members in the *Allium* genus, but can also infect other vegetable crops, such as tomato, eggplant, pepper, and carrot, having weakened roots (Sumner 1995).

Taxonomy

Setophoma terrestris (H.N. Hansen) Gruyter, Aveskamp and Verkley is classified as an anamorphic fungus in the phylum Ascomycota, kingdom Fungi (Anonymous 2011). In 1929, Hansen found that the genus *Phoma* was a causal agent of the pink-root disease. He suggested a new species, *Phoma terrestris,* based on the considerable differences in morphological and cultural characteristics from the comparatively few other species of the genus described as root

pathogens. Subsequently, Gorenz et al. (1948) determined that the characteristics of the pycnidia corresponded to the description by Hansen (1929) but the setae were missing in Hansen's characterization. All fruiting bodies of many isolates from various sections of the United States were found to be setose. The fruiting bodies of the pathogen were compared to both *Phoma* and *Pyrenochaeta* as given by Grove (1953). Their descriptions corresponded to the characteristics of the fruiting bodies of *Pyrenochaeta,* and were distinct from the genus *Phoma,* which had no setae on the surface of the fruiting bodies. Therefore, the name *Phoma terrestris* Hans was then transferred to *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker, and Larson by Gorenz et al. (1948). Although the generic name was changed decades ago, *Phoma terrestris* is still being used in some documents and online publications. However, sequencing data from the 18S and 28S nuclear ribosomal DNA (nrDNA) regions combined with morphological and molecular data led to the reclassification of *Pyrenochaeta terrestris* to the newly developed genus *Setophoma terrestris* by Gruyter et al. (2010).

Morphology

Pycnidia are generated by accumulating, swelling, and lateral dividing hyphal cells to assemble masses of dark thick-walled bodies (Hansen 1929). Setae are generally located around the ostiole but may occur over the entire pycnidium. The number of setae varies from few to numerous. Different isolates produce different pycnidia that vary in size, shape, papillation, and length and number of the ostioles. For example, in one study, California isolates had short setae, were fewer in number, and grouped around the ostiole of the pycnidium, whereas the setae of Louisiana isolates were longer, numerous and scattered over the surface of the pycnidium, and had more ostioles than California isolates (Gorenz et al. 1948).

Microsclerotia, a dense aggregate of darkly pigmented, thick-walled hyphal cells, are thought to have the ability to survive for long periods in soil (White and Scott 1973). Microsclerotia produced *in vitro* were found to vary significantly among *S. terrestris* isolates. Isolates from Texas produced a high number of microsclerotia at high temperatures (32°C), whereas New Mexico isolates produced the most microsclerotia at low temperature (15°C) (Biles et al. 1992).

Pycnidia and microscleriotia are capable of generating mycelium to cause infection when susceptible crops are introduced into infested soil (Tjamos et al. 1999). The mycelium of *S. terrestris* usually penetrates the root bases and become established in the bulb plate (Hansen 1929). Under favorable conditions, the pathogen grows in the infected roots resulting in plasmolysis of invaded root cells and distortion of nuclei. In addition, cells adjoining those affected cells lose turgor even though they are not invaded (Kreutzer 1941). Although the pathogen does not appear to invade the basal plate of the bulb, a reddish discoloration can be observed in this area (Hansen 1929). Kreutzer (1941) found that the pathogen constantly attacks the dead outer scale tissue of the bulb.

Setophoma terrestris survives the winter in the soil as pycnidiospores produced in the pycnidia or in plant debris of susceptible crops. Hyphae produced from germinating conidia directly penetrate the onion root tips. Colonies of the fungus form on roots and the fungus proliferates throughout the roots. Affected plants can exhibit symptoms within seven to 21 days of infection. New pycnidia are produced in the epidermal and cortical cells after the onion root die (Babadoost 1990; Sumner 1995).

The optimum temperature for growth of the fungus is between 24 and 28°C (Biles et al. 1992; Kim et al. 2003). Factors influencing growth of the pathogen were determined by

Gunasekaran and Weber (1981). They found that the optimal mycelia growth occurred with a medium pH of 6.5 with sucrose and sodium nitrate as the carbon and nitrogen sources. In addition, the culture incubated under artificial light had a higher mycelia growth when compared to a culture grown in the dark.

Pigments

Pyrenocines A, B, and C produced by *S. terrestris* were believed to have a role in the pink root disease of onions but only pyrenocine A was found to inhibit seedling elongation and to be toxic to onion protoplasts (Sparace and Mudd 1985). As a result of relatively weak phytotoxicity of pyrenocine compounds reported (as cited by Steffens and Robeson 1987), another phytotoxic compound, secalonic acid A, was identified as a potent inhibitor of seedling elongation, and was confirmed to accumulate in *S. terrestris*-infected onion tissues (Steffens and Roberson 1987). Zeng et al. (2001) found that secalonic acid A produced by *S. terrestris* and *Penicillium oxaicum* at concentrations of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} inhibited the onion seedling elongation by 4, 32, 40, 68, and 94%, respectively. Pigment produced by *S. terrestris* was highest under the same conditions required for the optimal mycelia growth (Gunasekaran and Weber 1981). Furthermore, adding tyrosine in the medium enhanced the pigment production (Gunasekaran and Weber 1981). Variability of pigment production in the root was dependent on severity, age of infection (Hansen 1929), isolate, and temperature (Biles et al. 1992).

Host range

Bulb onions (*Allium cepa*) are the major hosts of the pink root pathogen; however, other economically important species of *Allium* such as Welsh onion (*A. fistulosum*), leek (*A. ampeloprasum*), shallot (*A. cepa* var. *aggregatum*), garlic (*A. sativum*), and chive (*A.*

schoenoprasum) are also known to be the hosts of this pathogen (Punithalingram and Holiday 1973). Furthermore, *S. terrestris* is able to attack the roots of other vegetable crops besides *Allium* families including Amaranthaceae (spinach), Apiaceae (carrot), Brassicaceae (cauliflower), Cucurbitaceae (cantaloupe, cucumber, pumpkin, squash, and muskmelon), Fabaceae (cowpea, lima bean, and soybean), and Solanaceae (eggplant, pepper, potato, and tomato) (Hansen 1929; Kreutzer 1941; Sprague 1944; Thornberry and Anderson 1940). In the Poaceae family, both economic crops (barley, corn, sugar cane, oats, and wheat) and weedy grasses (pigweed, crab grass, and crowfoot grass) were reported to be the hosts of *S. terrestris* (Carvajal 1945; Kreutzer 1941).

Geographical distribution of *S. terrestris*

Setophoma terrestris is present worldwide but is most common in subtropical and tropical regions including Argentina, Australia, Brazil, Canada, Egypt, Germany, Hong Kong, Mauritius, New Zealand, Pakistan, Sierra Leone, South Africa, Uganda, UK, Venezuela (Punithalingram and Holiday 1973), Senegal, Sudan, Uganda, Brunei, Israel, Greece, the Netherlands, Poland (Kinsey 2002), Japan (Watanabe and Imamura 1995), Korea (Kim et al. 2003), France (Villeneuve and Maignien 2008), Vietnam (Luong et al. 2008), Canada (LeBoeuf et al. 2010), and Iran (Rabiei-Motlagh et al. 2010).

In the US, pink root was first reported in Texas by Taubenhaus and Johnson (1917). Thereafter, it spread to other regions such as in the west and southwest as a result of increasing of onion industry in California (Hansen 1926). Currently, the distribution of pink root disease in the United States covers 31 states, including Alabama, California, Colorado, Delaware, Florida, Georgia, Hawaii, Idaho, Illinois, Kentucky, Louisiana, Maine, Maryland, Montana, Nebraska, Nevada, New Jersey, New Mexico, New York, North Carolina, North Dakota, Oklahoma,

Oregon, Pennsylvania, South Carolina, Tennessee, Texas, Vermont, Virginia, Washington, and Wisconsin (Anonymous 2011).

Symptoms of pink root

On onions, the typical symptom of affected roots is a characteristic pink color of varying shades. Infection has been found to occur on plants as early as 6 to 8 weeks after seeding (Coleman et al. 1997). After infection has occurred, color of the roots progresses from a rhodonite pink to a spinal red coloration. In early stages, the pink color is the only sign of infection but a water-soaked appearance is common in advanced stages (Hansen 1929). The pathogen does not infect the basal stem plate or fleshy scales of the bulb. However, pink to purple blemishes may be observed on the outer scales of white cultivars when transplants or bulbs are grown in infested soil one to three weeks after transplanting and water-soaked areas appear on the outer scales of yellow or red cultivars (Sumner 1995). Disease development on onion leads to a loss in turgidity and subsequent collapsing of infected-root cells (Hansen 1929). The roots become shriveled, and may eventually disintegrate (Davis and Handerson 1937). New roots become infected, turn pink, and die (Sumner 1995). However, some infected roots observed by Kreutzer (1939) failed to show pink coloration. This phenomenon can be shown by growing the fungus on a standard acid medium such as prune agar. A study of the influence of the H-ion concentration on pigment manifestation was done by Sideris (1929). Agar slices with a pansy purple to violet carmine color and diseased roots with deep pink color were tested in distilled water having different pH. The color markedly changed to yellow to yellow-brown when the pH decreased to pH of 4.5, but turned back to the original pink-root pigment when the pH increased to pH 8.5 (Kreutzer 1939).

Aboveground symptoms of onion seedlings progress from becoming pale green at the tip of the primary leaf, to browning from the tip downward, and finally leaf death. More mature plants may display a grayish discoloration of the older leaves. The leaves remain attached to the stem but the upper half of the leaves droop (Davis and Handerson 1937). In cases of severe infection, affected plants appear to be nutrient deficient or under drought. Leaf number and size are reduced, and bulb development begins earlier in infected plants compared to healthy plants. Surviving plants are stunted, and bulbs shrivel or are not of a marketable size (Sumner 1995). Levy and Gornik (1981) found that the disease shortens the growth period by 4 to 14 days of early and intermediate maturing cultivars, and by 18 to 45 days of late maturing cultivars.

Isolation methods

Gorenz et al. (1948) described a method to isolate *S. terrestris* directly from infected roots. Successful isolation was done on dry roots by disinfecting the roots thoroughly in 1:1000 mercuric chloride solution or BK (chlorine bearing powder) solution, before placing them on the agar medium. Watson (1961) disinfected the roots or portions of the onion stem plate with roots in 20% bleach for 2 to 4 minutes, placed on wheat straw agar, and determined the presence of pink color on the wheat straw agar for a positive test. Awuah and Lorbeer (1989) used cool white fluorescent light (CWFL) for 12 h per day at 20°C as the incubation conditions. Kim et al. (2003) were able to induce the formation of pycnidia and pycnidiospores of the fungus by incubating the culture plates under a combination cycle of 12 h near ultraviolet light and 12 h dark. In 2011, Levic et al. (2011) suggested using carnation leaf agar (CLA) to identify *S. terrestris* because it does not form pycnidia and produces a distinctive red pigment when grown on this media. Sneh et al. (1974) developed a method to isolate *S. terrestris* directly from soil by

using thiabendazole, 2-(4′-thiazolyl) benzimidazole (TBZ) as an indicator of presenting *S. terrestris* in the soil.

Successful isolation was performed after diseased muskmelon and watermelon roots were placed on PDA, V8 agar, and Synthetischer Nahrstoffarmer Agar (SNA). On these media the fungus sporulated within three weeks. Confirmation of *S. terrestris* was based on the disease showing pink symptoms on Watson's wheat straw agar (Bruton et al. 1997). Newby (1997) isolated *S. terrestris* from infected corn roots and infested soil of corn field by using methods described by Watson (1961) and Sneh et al. (1974).

Maintenance of *S. terrestris* cultures

A specific technique for preserving the pink root fungus long-term has not yet been published. However, several methods have been used for fungi that produce spores in stomata. Castellani's method, which preserves the cultures in sterile distilled water, could be used to store *Pyrenochaeta mackinonii, P. ramoroi,* and *Pyrenochaeta* sp. for 1 to 15, 11, and 10 years with viability of 80%, 33.4%, and 50%, respectively (Capriles et al. 1989). According to Shishkoff (1992), *P. lycopersici* was stored as tomato root infested in soil culture tube, and then kept in the freezer. *P. lycopersici* stored under sterile paraffin at laboratory temperature and subcultured every 2 to 3 years remained pathogenic after 10 years (as cited by Shishkoff 1992). Borba and Rodrigues (2000) found that covering *P. romeroi* with 0.2-cm mineral oil could maintain its viability for over 10 years but the culture subsequently lacked the ability to sporulate.

Disease management

Integrated disease management, which combines several control strategies, is likely to be more effective and sustainable rather than using a single method. General recommendations used to control pink root disease include cultural management (rotation with non-susceptible

crops, the use of resistant cultivars, and soil solarization) and chemical management (soil fumigation) (Hartz et al. 1989; Porter et al. 1989; Sumner et al. 1997; Sumner 1995; Thornton and Mohan 1996).

Cultural management

A rotation of crops not associated with onion should be implemented for at least three to four years. This method can not eliminate the pathogen altogether from the soil (Taubenhaus and Mally 1921) but it can reduce disease pressure (Sumner 1995).

In general agricultural production, resistant cultivars with horticulturally acceptable characteristics should be planted. However, the expression of host resistance can be suppressed at 28°C or greater (Sumner 1995). Onions frequently escape the disease when they are transplanted when soil temperatures are less than 24°C and harvested before soil temperature average above 30°C (Sumner 1995).

Evaluation of pink root resistance has been conducted in field and greenhouse conditions. Under field conditions, yellow sweet Spanish onion cultivars and several hybrids were evaluated by Thornton and Mohan (1996). They concluded that hybrid lines were found to have low level of pink root incidence when compared to cultivars of yellow sweet Spanish onion. Coleman et al. (1997) selected commercial cultivars and lines from the USDA breeding program (cultigens) growing in organic soils in New York. They found that the cultigens showed fewer symptoms than the commercial cultivars. Selection of cultivars from fields with high inoculum levels of the pink root pathogen showed that ‘NuMex Snowball’, a late-maturing, intermediate-day, white-colored cultivar (Cramer and Corgan 2001b), and ‘NuMex Chaco’, an early maturing, short-day, yellow cultivar were resistant to pink root (Cramer and Corgan 2001a).

Since field conditions have great discrepancies, such as inconsistent distribution of the fungal pathogen and variations of the environmental factors, results obtained from selection and screening for pink root resistance may be uncertain. Furthermore, field screening can only be conducted once or twice a year (Netzer et al. 1985). In greenhouses, selection and screening for pink root resistance were conducted under controlled conditions, amount of the inoculum, and distribution of the pathogen. Gorenz et al. (1949) tested the resistance of 23 commercial varieties by using a pan, sand-culture technique with a standardized inoculation procedure and controlling environmental condition. They found that yellow Bermuda and Beltsville branching were the most resistant varieties. Levy and Gornik (1981) grew 11 onion cultivars in flats containing soil infested with *S. terrestris*. They found symptoms on the roots of all cultivars including the resistant cultivars (Dessex, Granex, Laredo, and Grano 502); however, they still grew and produced acceptable yields.

Soil solarization or soil heating was developed in Israel to control soil-borne pathogens and weeds. This method is performed by tilling, moistening, and mulching the soil with clear polyethylene plastic for 30 to 40 days during hot, sunny periods. By sufficiently increasing the soil temperature to 45 to 55°C, the pink root pathogen and other fungal pathogens are killed (Katan 1981; Lee et al. 2007; Rabinowitch et al. 1981). By using this method for controlling pink root, Katan et al. (1980) were able to significantly reduce the incidence and severity of the disease by 73 to 100% during six and seven months of plant growth. After 195 days of growth, the study plots still exhibited reduced incidence rates (Katan 1981). Rabinowitch et al. (1981) showed the onion-seedling survival was significantly improved in solarized plots as compared to untreated plots. Furthermore, total yields as well as the marketable yields were significantly increased. However, soil solarization can only be used in areas with intense solar radiation and

high temperatures (as cited by Halloin 1993). In Michigan, soil solarization is not feasible because weather conditions do not fit these criteria (Hallion 1993).

Chemical management

Fungicide treatments were first introduced to minimize the incidence of pink root by Taubenhaus and Mally (1921). Formaldehyde or copper sulphate were found to eradicate *S. terrestris* but caused injury to the green onion sets after treatment. Hartz et al. (1989) examined the efficiency of metam sodium used for soil fumigation. The chemical was shown to reduce pink root incidence as well as weeds, which led to the improvement of plant stands. Metam sodium was compared to biofumigant treatments consisting of 'Idagold' mustard and 'Colonel' oil seed radish for controlling pink root. Metam sodium was better at reducing pink root severity (Geary et al. 2008), but had a lower efficacy when compared to methyl bromide mixed with chloropicrin or chloropicrin alone (Sumner et al. 1997). Maudarbaccus and Beni Madhu (1999) showed that infection was reduced and yield was increased by 57% when dazomet was used to fumigate the soil. In Michigan, the fumigants1,3-dichloropropene/chloropicin, metam sodium, or potassium N-methylidithiocarbamate are registered for controlling pink root (Bird et al. 2011).

Ahmed et al. (1991) evaluated fungicides used to control pink root for both greenhouse and field conditions. In greenhouses, Folicure (25%), Sumisclex W.P. (50%), Sumisclex D.FI. (50%), and Ronilan W.P. (50%) followed by Ronilan dust (5%) and KZ-120 (50%) minimized pink root infection and severity on the susceptible cultivar Giza-20. The most effective treatment to reduce infection and increase bulb production was Folicure (25%), Sumisclex W.P. (50%), and Sumisclex D.FI. (50%), and followed by Ronilan W.P. (50%), Ronilan dust (5%), and KZ-120 (50%). Other studies on closely related species were conducted on *Pyrenochaeta lycopersici* causing corky root rot of tomato. The most effective result obtained by using

azoxystrobin could reduce the severity by 98% and 83% in the greenhouse and in the field, respectively (Bubici et al. 2006). Azoxystrobin, boscalid, and cyprodinil+fludioxonil fungicides have been studied in the field and have repeatedly demonstrated their effectiveness at controlling Phoma basal rot of romaine lettuce caused by *Phoma exigue* (Koike et al. 2007).

An in *vitro* study was evaluated by Ahmed et al. (1991). They found that Bavistin (50%), Benlate (50%), and Folicure (25%) at rate of 3, 5, and 10 ppm, respectively, could prevent the growth and sporulation of *S. terrestris*. Fungicides that belong to dimethylation inhibitor group including prochloraz, tebuconazole, difenoconazole, and cyproconazole greatly reduced the mycelial growth of *Phoma ligulicola* compared to untreated controls (Pethybridge et al. 2005). Thomidis et al. (2011) tested the sensitivity of *P. glomerata* to thiophanate-methy, carbendazim, and tebuconazole. They found that all fungicides inhibited the development and conidial germination of the fungus.

One of alternative method is the use of natural products and antagonist microorganism to suppress the disease. Biesiada et al. (2004) examined fresh garlic extracts in concentrations of 1% and 2% and *Trichoderma viridae* strain B35 used as treatments and 0.1% Topsin 70 WP (thiophanate-methyl) or 2% Rovral FLO 255 SC used as control. Results suggested that pink root incidence was reduced and yields were increased compared to the fungicide application by combining the application of root dipping at planting time and plant drenching after planting with 2% garlic extract.

Summary

Management of this disease needs to focus on determining of resistant varieties and fungicide products for onion crops, and studying of variability of *S. terrestris* populations. The objectives of this research are to characterize the populations of *S. terrestris* based on genetic and

morphological diversity, and virulence variation among the *S. terrestris* populations, to evaluate the resistance of onion varieties suitable for growing in Michigan and determine the efficacy of non-fumigant fungicides at controlling pink root under greenhouse conditions, and to determine the effect of plant age in resistance to pink root infection.

CHAPTER 1: CHARACTERIZATION OF *SETOPHOMA TERRESTRIS* POPULATIONS IN MICHIGAN ONION FIELDS

ABSTRACT

Setophoma terrestris (H.N. Hansen) Gruyter, Aveskamp & Verkley is a soil-borne fungal pathogen that has a worldwide distribution. The pathogen infects numerous crops but it is primarily on dry bulb onion. The objective of this study was to use morphological, molecular, and virulence data to determine variability of *S. terrestris* within Michigan. During the growing seasons of 2011 and 2012, 98 isolates of *S. terrestris* were obtained from the roots of onions collected around nine Michigan cities including Byron Center, Charlotte, Grant, Hudsonville, Lansing, Martin, Plainwell, Portland, and Stockbridge. Morphological characteristics (colony formation, chlamydospore production, and pycnidia abundunce) were variable within populations; however, there was little between population variability. Disease virulence among populations did not differ statistically when evaluated using green bunching onions, however, there was a tendency for the Charlotte population to be the least virulent while the Grant population was the most virulent. The distribution of genetic variability within and among six populations of *S. terrestris* was assessed using seven inter-simple sequence repeats (ISSR) markers. The percentage of polymorphic bands, Nei's gene diversity and Shannon's information index at the population and species level were 76.4%, 0.2603, 0.3910 and 96.2%, 0.2843, 0.4336, respectively, suggesting high levels of genetic diversity of the pathogen. The population differentiation was moderate (G_{ST} = 0.0888), indicating a substantial proportion of genetic variability was found within populations (96%). Pearson's correlation coefficients indicated

significant positive relationship between the colony diameter and disease virulence. In this study, there was no geographic pattern across the populations of *S. terrestris* in Michigan.

INTRODUCTION

Setophoma terrestris (H.N. Hansen) Gruyter, Aveskamp & Verkley, a soil-borne pathogenic fungus, is a limiting factor for many Michigan onion producers who have grown the crop in short rotations with celery and other vegetables in specific muck fields for decades. Weakened or dead root system limits normal development of the onions leading to undersized bulbs (Taubenhaus and Johnson 1917; Tims 1953). The pathogen has a relatively wide host range, including carrot, corn, cucumber, eggplant, onion, pepper, tomato (Kreutzer 1941), and board-leaf weeds (Sprague 1944); onions, garlic, and shallots are highly susceptible (Porter and Jones 1933).

Disease symptoms include pink discoloration of the affected roots developing initially as a light pink coloration then darkening to red and purple, shriveling, and eventually disintegrating (Hansen 1929). New roots of severely diseased plants become infected as early as they are formed, resulting in permanent stunting (Taubenhaus and Mally 1921). Foliage of affected plants exhibit symptoms of drought stress or nutrient deficiency with chlorosis, wilting, and dieback from the leaf tip, that mimics symptoms commonly caused by other diseases or unfavorable environmental conditions (Hansen 1929).

Current geographical distribution of *S. terrestris* includes Africa, North and South America, Asia, Australia, Europe, and New Zealand (Kim et al. 2003; Kinsey 2002; LeBoeuf et al. 2010; Luong et al. 2008; Punithalingram and Holiday 1973; Rabiei-Motlagh et al. 2010; Villeneuve and Maignien 2008; Watanabe and Imamura 1995). The disease is especially severe in tropical and subtropical climates (Sumner 1995). In the United States, pink root was first

reported in 1917 in Texas where it was observed in fields with onions grown for two years or more in succession (Taubenhaus and Johnson 1917). Subsequently, the disease has been documented in many other states (Carvajal 1945; Davis and Handerson 1937; Hansen 1929; Kreutzer 1941; Marlett and McKittrick 1958; Sprague 1944; Taubenhaus and Mally 1921; Tims 1943). Affected onions may exhibit differing amounts of root damage with the potential for a yield reduction up to 50% (Davis and Handerson 1937; Porter and Jones 1933; Taubenhaus and Johnson 1917).

The disease cycle begins as hyaline, one-celled conidia produced in a pycnidium that develops into hyphae that can penetrate young roots, and grow through the cortical tissue. Disease symptoms may be evident within 7 to 21 days following infection (Sumner 1995). The pathogen produces microscopic, black, subglobose structures in the epidermal and cortical cells. After the onion roots die, pycnidia bearing pycnidiospores are formed. The infection cycle occurs repeatedly throughout the growing season (Babadoost 1990).

S. terrestris is variable in size, shape, and papillation of pycnidia, and the number and position of setae (Gorenz et al. 1948). In *vitro* study showed that chlamydospore production and pigment synthesis varied significantly, depending on the isolate (Biles et al. 1992). Isolates from South Africa and the United States exhibited a high degree of genetic variation based on isozyme analysis and were highly diverse based on virulence evaluation even though some isolates originated from the same field (Ferreira et al. 1991).

Population genetics can provide information for developing management strategies by deploying resistance genes or implementing fungicide programs (McDonald and McDermott 1993). Inter-simple sequence repeat (ISSR) analysis can be used to study population genetics of *S. terrestris* as they have been used to identify genetic variation in a wide range of organisms

(Zietkiewicz et al. 1994) including several *Fusarium* spp. (Arif et al. 2008; Dinolfo et al. 2010; Miedaner et al. 2001), *Monilinia fructicola* (Villarino et al. 2012), *Plurotus eryngii* var. *tuoliensis* (Zhao et al. 2013), *Pseudocercospore griseola* (Abadio et al. 2012), and *Rosellinia necatrix* (Armengol et al. 2010). ISSR-PCR employs a single primer which is 16 to 20 bases in length based on di-, tri-, tetra- or penta-nucleotide microsatellite repeats in amplification reactions (Gupta et al. 1994; Zietkiewicz et al. 1994). The advantages of using ISSR analysis for studying genetic diversity of organisms are that oligonucleotide primers can be designed without requiring nucleotide sequence information (Zietkiewicz et al. 1994). This method can detect polymorphism better than restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) analyses (Godwin et al. 1997). The objective of this study was to characterize populations of *S. terrestris* in Michigan using morphology, molecular, and virulence.

MATERIALS AND METHODS

Sampling, isolation, and identification. Fifty onion bulbs were collected randomly from each field from June to September in 2011 and 2012 (Table 1.1). Plants were put in plastic bags and kept at 4°C to preserve the turgidity of the roots. Isolates were obtained by excising 1.0 to 1.5 cm in length of an individual root with pink discoloration, followed by soaking in 0.5% sodium hypochlorite for 10 minutes, rinsing three times with sterile distilled water, and blotting dry with paper toweling. Five disinfected roots were placed on water agar and incubated under darkness for 7 days at 26 ± 1°C before being placed under a 13-h photoperiod and 11-h darkness for another 7 days at the same temperature. Dark brown setose pycnidia bearing hyaline, one-celled, ellipsoidal conidia that formed on the culture medium were preliminarily identified as *Setophoma terrestris*.

Table 1.1. Collecting sites of onions showing symptoms of pink root

Population	Number of samples	Locality (City, County)	Year	Latitude (°N)	Longitude (°E)
Charlotte	7	Charlotte, Eaton	2011	42.481529	-84.775726
	7		2012	42.481529	-84.775726
Grant	14	Grant, Newaygo	2011	43.356327	-85.709882
	6		2012	43.350897	-85.738767
Hudsonville	7	Hudsonville, Ottawa	2011	42.856790	-85.899453
	7		2012	42.881871	-85.865101
Lansing	4	Lansing, Ingham	2011	42.799357	-84.497085
	7		2012	42.793574	-84.496729
Portland	7	Portland, Ionia	2011	42.830918	-85.033637
	7		2012	42.847826	-85.018687
Other sites	1	Byron Center, Kent	2011	42.779575	-85.763977
	7		2012	42.779575	-85.763977
	7	Martin, Allegan	2012	42.552243	-85.580539
	7	Plainwell, Allegan	2011	42.527476	-85.554609
	3	Stockbridge, Ingham	2011	42.435522	-84.194158
Total	98				

Single spore isolation. A pycnidium was collected using a sterile scalpel blade and placed into a 1.7-mL microcentrifuge tube containing 1 mL sterile distilled water. The microcentrifuge tube was shaken vigorously to release spores, a micropipette was used to transfer 200 μL of the spore suspension onto 2% water agar, and the spore suspension was spread using a sterile glass rod. The Petri dishes were incubated for 12 to 16 hours at room temperature. Spore germination was examined using a compound light microscope (200x); thereafter, a square block of agar containing a single spore with germ tube was excised and placed onto fresh cornmeal agar using a sterile scalpel blade.

Culture preservation. For short-term culture preservation, a square block of agar with an actively growing colony from an individual isolate was transferred to Petri dishes (60 mm diameter x 15 mm height) containing carnation leaf agar and incubated under conditions described previously for two weeks or until pycnidia formed. Plates were wrapped with Parafilm

to limit dehydration and were kept at 4°C. For long-term storage, multiple pycnidia produced on carnation leaf agar medium were collected using a sterile scalpel blade and were placed into a 2-mL cryogenic vial containing 1.5 mL of 30% glycerol. The vial was shaken vigorously to release conidia. All cultures were pre-cooled at 4°C for one hour followed by freezing at -20°C. Duplicates of each isolate were prepared.

Morphological characterization. According to the methodologies described in Q-bank webpage (Anonymous 2013) and in the studies of Borema et al. (2004), colony diameter was measured on malt extract agar (MEA), and colony form and colony margin was determined on oatmeal agar (OA). Each agar plate was transferred with a 0.5 mm disc obtained from the growing margin of a single-spore culture of *S. terrestris* that was grown on cornmeal agar at 26 ± 1°C for 7 days. All MEA plates were incubated at 26 ± 1°C for 14 days under dark conditions, and the OA plates were incubated at the same temperature under darkness for 7 days and then removed to a combined 13-h photoperiod and 11-h darkness conditions for 7 days. Radial growth was measured perpendicularly along two axes for each plate and averaged from three plates. Colony form and margin were recorded 14 days after incubation.

Microscopic structures (chlamydospores, pycnidia) were examined on cornmeal agar (CA). The presence of chlamydospores, appearing as thick-walled, dark-colored, spherical, and multicellular, was determined using a light compound microscope (200x) 14 to 28 days after the culture was transferred. The abundance of setose pycnidia (dark brown fruiting bodies produced on the surface of agar medium) was visually observed to be absent (no pycnidia observed on an agar medium), low (<25% of pycnidia covered on a surface of agar medium), moderate (≥25% to 50% of pycnidia cover on a surface of agar medium), or high (>50% of pycnidia covered on a surface of agar medium) after 28 days of incubation (Figure 1.1).

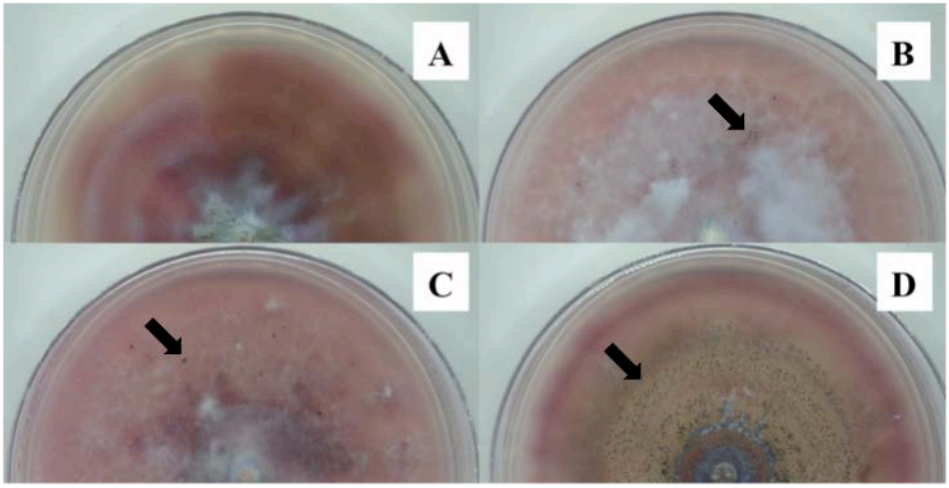


Figure 1.1. Production of pycnidia of *S. terrestris* (dark brown fruiting bodies produced on the surface of agar medium) on cornmeal agar: A= no pycnidia, B = low (<25% of pycnidia covered on a surface of agar medium), C = moderate (≥25% to 50% of pycnidia cover on a surface of agar medium), and D = high (>50% of pycnidia covered on a surface of agar medium) amount of pycnidia. The cultures were incubated at 26 ± 1°C under darkness for 7 days and removed to place in 13-h photoperiod and 11-h darkness for 21 days.

Virulence testing. A subset of 98 *S. terrestris* isolates, chosen based on collecting sites and year, was evaluated for virulence on green bunching onions. Pathogen inoculum was prepared by culturing each isolate on cornmeal agar and incubating at 26 ± 1°C under darkness for 7 days. A mycelium disc (15-mm in diameter) was taken from the edge of the advancing colony and placed on top of a sterile moist cotton ball in a sterile test tube (150 mm in length x 24 mm outer diameter). The green bunching onions purchased from a local grocery store were disinfected with 0.5% sodium hypochlorite for 5 minutes, rinsed three times with distilled water, and blotted dry on paper toweling. Roots of each plant were trimmed to 1 cm in length. A disinfected green onion was placed on top of the colonized agar plug in the test tube. A disc of

sterile cornmeal agar was used as the uninoculated control. A plastic cap was placed on each test tube and sealed with parafilm to maintain a high relative humidity. The test tubes were incubated at 26 ± 1°C and a 16-h photoperiod. Each isolate was inoculated on three green onions. The experimental unit was performed as a completely randomized design with two replicates. Disease virulence was visually assessed 10 days post inoculation (dpi) using a scale from 0 to 3; where 0 = healthy roots, no disease symptoms, 1 = <10% of the roots with significant discoloration, 2 = ≥10% to 50% of the roots with significant discoloration, and 3 = >50% of the roots with significant discoloration (Figure 1.2).

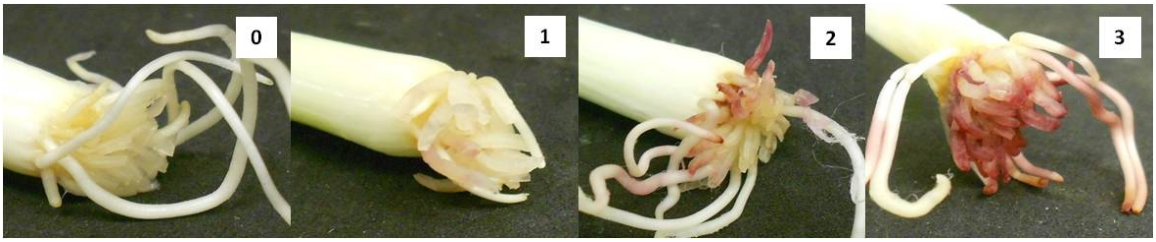


Figure 1.2. Scale used to rate disease virulence on green bunching onion in growth chamber evaluation; where 0 = healthy roots, no disease symptoms, 1 = <10% of the roots with significant discoloration, 2 = ≥10% to 50% of the roots with significant discoloration, and 3 = >50% of the roots with significant discoloration.

Genomic DNA extraction. A 250-mL Erlenmeyer flask containing 50 mL of half strength of potato dextrose broth was transferred with 1 mL of spore suspension of *S. terrestris* (10^6 spores/mL) and incubated on a rotary shaker (100 rpm) at 26 ± 1°C under darkness for 5 days. Mycelia were harvested, vacuum-filtered through a 0.45 μm-pore-size filter (Whatman), and frozen at -20°C until use (modified from Rodriguez-Salamanca 2013). Genomic DNA was isolated from 40 to 80 mg of lyophilized mycelium using the Wizard Genomic DNA Purification

kit (Promega Corp., Leiden, The Netherland) by following the yeast protocol and modification method. Genomic DNA extraction was performed according to DNA extraction manual by adding 600 µL of nuclei lysis solution in the ground mycelium, shaken vigorously using a vortex for 3 seconds to wet the mycelium, 200 µL of protein precipitation solution was added and then shaken on the vortex at high speed for 20 seconds before placing the sample on ice for 5 minutes. The sample was centrifuged for 20 minutes at top speed (15,000 rpm) in a centrifuge (Centrifuge 5424, Eppendorf) after the addition of 200 µL of chloroform (This step was modified according to DuTeau and Leslie n.d.). The top aqueous layer was pipetted into a new 1.7-mL microcentrifuge tube then precipitated the DNA by following the steps in the protocol. DNA quality was determined on 1% agarose gel and DNA quantity was measured using Nanodrop 1000 spectrophotometer (Thermo Scientific Wilmington, DE).

Confirmation of the pathogen species. Partial DNA sequences encoding the large-subunit (LSU) ribosomal DNA (rDNA) were amplified with primers LR0R (5'-GTACCCGCTGAACTTAAGC - 3') (Rehner and Samuels 1994) and LR7 (5' - TACTACCACCAAGATCT - 3') (Vilgalys and Hester 1990). A total volume of 25 µl of PCR mixture contained 1x PCR buffer, 2mM $MgCl_2$, 40 µM dNTPs, 0.2 µM primer, and 0.5 U Taq polymerase (Promega, Madison, WI). The PCR reactions were conducted in a Mastercycler Pro thermocycler (eppendrof, Hauppauge, NY) with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 48°C for 45 sec, and extension at 72°C for 2 min. The final extension step was performed at 72°C for 7 min. DNA amplicons were separated on 1% agarose gel, dissolved in 1x Tris-borate-EDTA (TBE) buffer. The amplified DNA products were sequenced with a single PCR primer by submitting to Macrogen Corp. (Macrogen USA, Rockville, MD). The nucleotide sequences were compared to

the nucleotide collection in NCBI using a BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast) analysis.

Inter-simple sequence repeat (ISSR) amplification and data analysis. Seven ISSR primers (Table 1.4) were used in the PCR amplification. The reaction mixture contained 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 2 μM primer (Sigma-Aldrich, The Woodlands, TX), and 0.09 U Taq polymerase (Promega, Madison, WI) in a total volume of 12.5 μl. DNA amplifications were performed in a Mastercycler Pro thermocycler (eppendrof, Hauppauge, NY) under the following conditions; initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 92°C for 1 min, annealing at the proper temperature (Table 1.4) for 45 sec, extension at 72°C for 2 min, and the final extension step was conducted at 72°C for 7 min. The amplified products were determined using electrophoresis on 2% agarose gel in 1X TBE and ethidium bromide staining (10 mg ml^{-1}). A 1-Kb-plus DNA ladder (Invitrogen, Grand Island, NY) was used as molecular weight markers to estimate the size of the amplified DNA fragments. ISSR amplification products were visualized under ultraviolet light using a Gel Doc^{TM} XR^{+} (Bio-Rad, Berkeley, CA), and pictures of the gel were taken using a gel analyzer software; Image Lab 3.0 (Bio-Rad, Berkeley, CA). A reaction without DNA was performed to determine if contaminant DNA was present. The experiment was repeated twice, and only reproducible bands were scored. The ISSR data were transformed into a binary data set; present or absent bands were scored as 1 or 0, respectively.

To determine genetic diversity across the 98 individuals, the populations with number of isolates less than 10 were pooled and analyzed as other site population. Number of polymorphic bands (NP) with the frequency less than 99% of the most common bands, percentage of polymorphic bands (P), observed number of alleles per locus (N_A), effective number of alleles

per locus (N_E), Nei's gene diversity (H), and Shannon information index (I_S) were calculated using the software program POPGENE ver. 1.32 (Yae et al. 1999). The diversity within population (H_S), total gene diversity (H_T), and coefficient of genetic differentiation (G_{ST}) were calculated on the basis of Nei's method using the same software. The estimate of gene flow (N_M) from G_{ST} were calculated as $N_M = 0.5(1-G_{ST})/ G_{ST}$. To investigate the relationships among populations, unweighted pair group method with arithmetic average (UPGMA) was generated from a matrix of Nei's genetic distance between individuals using POPGENE ver. 1.32. Analyses of Molecular Variance (AMOVA) evaluated for genetic variability within and among populations and genetic differentiation among individuals (PhiPT) were performed using program GenALex ver. 6.5 (Peakall and Smouse 2012). The principal coordinates analysis (PCoA) of the individuals by binary genetic distance matrix used for AMOVA was performed using the standardized distance matrix method in GenALex. Mantel test was performed for testing the correlation between Nei's genetic distance and geographical distance of the populations, where the other site population was not included in the analysis because the isolates were collected from different locations. The geographical distances were estimated based on the GPS coordinates using Google Earth Pro software. Pearson's correlation coefficients used to determine the relationship of morphological characteristics, disease virulence, or genetic diversity were calculated using PROC CORR of SAS version 9.3 (SAS Institute Inc., Cary, NC).

Statistical analysis. Means of the colony diameter and standard error (SE) were subjected to statistically calculate using the excel program. Experimental replicates of virulence were combined for statistical analysis. Means of disease virulence of each individual and population were analyzed according to the Kruskall-Wallis multiple comparison tests at $P = 0.05$ using the PROC NPAR1WAY of SAS version 9.3 (SAS Institute Inc., Cary, NC).

RESULTS

Sampling, isolation, and identification. Onions with pink colored roots were sampled from fields located near nine Michigan cities (Table 1.1) during the growing seasons of 2011 and 2012. Varying stages of disease development were observed with various shades of pink discoloration. The root density of severely affected plants was sparse. Many diseased roots were shriveled and appeared to be disintegrated. Isolation was made successfully on water agar incubated at 26 ± 1°C for approximately two weeks. The presence of dark brown setose pycnidia, bearing hyaline, one-celled, ellipsoidal conidia were used as the principal structure for pathogen identification (Figure 1.3A and 1.3B).

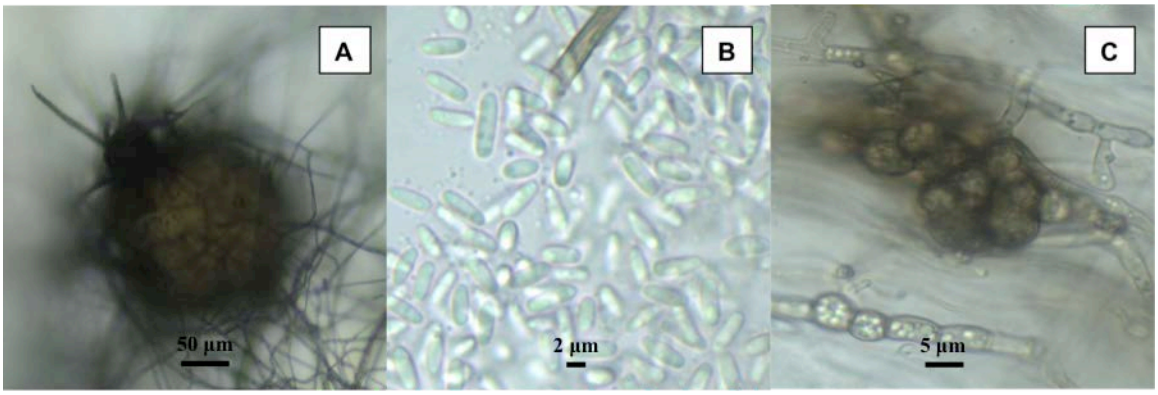


Figure 1.3. Dark brown, globose, papillate ostiolate setose pycnidium (A), hyaline, aseptate, elliptical conidia with several distinct guttules (B), and globose to subglobose, intercalary, and aggregated chlamydospores (C). Bars: A = 50 µm; B = 2 µm; C = 5 µm.

Morphological characterization. Morphological characteristics of the *S. terrestris* isolates were defined based on colony form and margin, percentage of chlamydospore and pycnidia production, and colony diameter (Table 1.2). Overall, every population displayed nearly the full range of variation possible. The colony forms were circular and irregular types,

and the colony margins of most populations were entire, lobate, and undulate. However, the colony margins of the Hudsonville population were lobate and undulate (Table 1.3). Chlamydospores (Figure 1.3C) were observed in some isolates. The percentage of isolates producing chlamydospores ranged from 7.1%% (Portland population) to 64.0% (Charlotte population) (Table 1.3). The abundance of pycnidia production varied among the populations. The Grant population had the greatest percentage of isolates with a high level of pycnidia production on cornmeal agar at 28 days, while the Hudsonville population had the greatest percentage if isolates with a low level. Colony diameters compared among the populations did not differ statistically ($P = 0.8026$) and ranged from 34.52 ± 1.70 mm (Hudsonville population) to 41.42 ± 1.34 mm (Lansing population) (Table 1.3).

Virulence testing. Inoculated green onions initially exhibited pink coloration 3 dpi. All new roots of the inoculated plants became infected. Control plants inoculated with sterile agar disc did not show disease symptoms during the 10-day observation period. All isolates of *S. terrestris* used in this study were pathogenic to green bunching onions (Table 1.2). Disease virulence of the isolates ranged from 1.5 to 3.0 with an average of 2.48 (Table 1.2) and of each population ranged from 2.36 to 2.61 (Table 1.4). Among the *S. terrestris* isolates tested, 11NYE18, 11MOR4, 11VAN4, 12FOX10, 12MAR5, 12MAR6, and 12POR6 were the most virulent while 12NYE8 was the least virulent (Table 1.2). Pathogen was statistically different among the isolates ($\lambda^2 = 131.8587$, df = 92, $P = 0.0041$; Table 1.2). In contrast, populations did not differ from each other statistically ($\lambda^2 = 10.8373$, df = 5, $P = 0.0547$), but there was a slight trend where the Grant population was the most virulent whereas the Charlotte population was the least virulent (Table 1.4).

Confirmation of the pathogen species. The partial sequences of large-subunit (LSU) ribosomal DNA (rDNA) were successfully amplified with primers LR0R and LR7. The PCR products were approximately 1,300 bp. There was no amplified product in the negative control. The nucleotide sequences of LSU region had 97% to 100% homology with those of nucleotide sequence data of *S. terrestris* species that are available in the NCBI database.

ISSR analysis. Initially, 20 ISSR primers were screened against genomic DNA of *S. terrestris* for their ability to amplify DNA fragments. Of the 20 primers, seven primers, UBC807, UBC808, UBC820, UBC 835, UBC848, UBC868, and UBC889, produced multiband patterns of the 98 isolates, which represented nine populations, of *S. terrestris*. The number of bands amplified was between 11 and 19 (Table 1.5). No contaminant DNA band was detected in any negative control amplification (Figure 1.4).

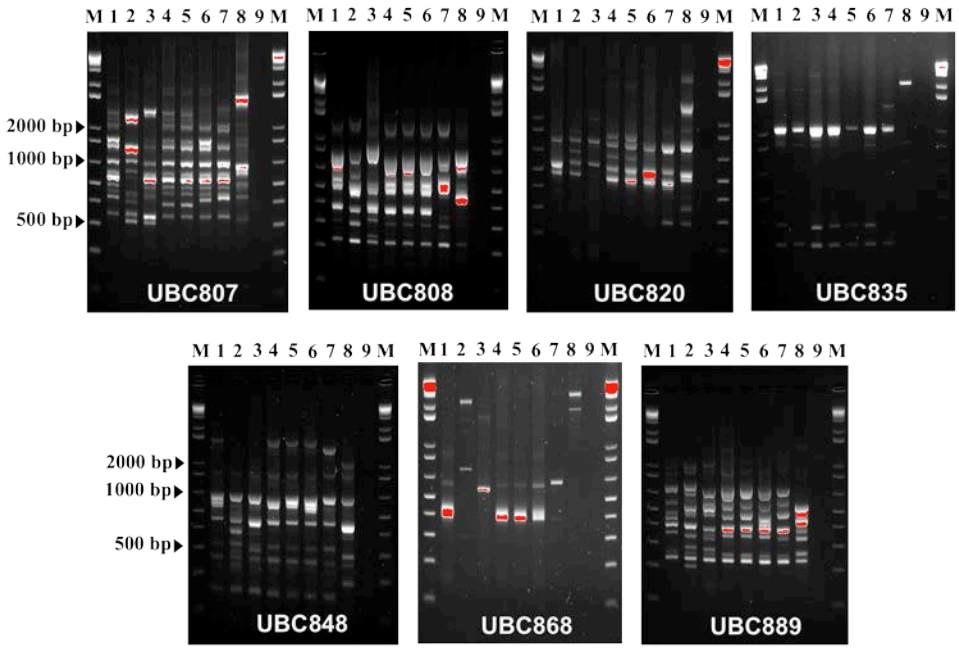


Figure 1.4. Polymorphic DNA patterns of *S. terrestris* isolates amplified using the ISSR markers, consisting of UBC807, UBC808, UBC820, UBC835, UBC848, UBC868, and UBC889. Legend: 1 Kb plus DNA ladder (M), 12FOX10 (1), 12FOX28 (2), 12SPR9 (3), 12SPR20 (4), 12VEU5 (5), 12VEU18 (6), 12FRE1 (7), 12FRE6 (8), and negative control (9).

Table 1.2. Colony characteristics, chlamydospore production, abundance of pycnidia production, colony diameter, and mean disease virulence of 98 isolates of *S. terrestris* from Michigan onion fields collected during the growing seasons of 2011 and 2012.

No.	Isolate ID	City	Population	Year	Colony		Chlamydospore production	Abundance of pycnidia on CA	Diameter ± SE[y]	Means of disease virulence[z]	
					form	margin					
1	11BC1	Byron Center	Other site	2011	circular	undulate	absent	intermediate	38.0 ± 1.13	2.50	bcd
2	12BYC4	Byron Center	Other site	2012	circular-irregular	undulate	absent	low	32.3 ± 0.92	2.50	bcd
3	12BYC7	Byron Center	Other site	2012	circular-irregular	undulate	absent	low	33.2 ± 0.60	2.50	bcd
4	12BYC11	Byron Center	Other site	2012	circular-irregular	undulate	present	high	31.2 ± 1.11	2.50	bcd
5	12BYC15	Byron Center	Other site	2012	circular-irregular	undulate	absent	intermediate	31.3 ± 0.95	2.33	cde
6	12BYC18	Byron Center	Other site	2012	circular-irregular	undulate	absent	intermediate	38.2 ± 4.16	2.17	de
7	12BYC1	Byron Center	Other site	2012	irregular	undulate	absent	intermediate	36.8 ± 0.31	2.33	cde
8	12BYC32	Byron Center	Other site	2012	irregular	lobate	absent	low	29.3 ± 0.42	2.00	e
9	11GN2	Charlotte	Charlotte	2011	irregular	undulate	present	low	35.3 ± 0.42	2.17	de
10	11GN7	Charlotte	Charlotte	2011	irregular	undulate	absent	low	36.0 ± 2.90	2.33	cde
11	11GN11	Charlotte	Charlotte	2011	circular	undulate	absent	absent	35.7 ± 0.33	2.17	de
12	11NYE1	Charlotte	Charlotte	2011	circular	undulate	absent	high	65.7 ± 0.33	2.17	de
13	11NYE12	Charlotte	Charlotte	2011	irregular	undulate	absent	absent	22.0 ± 0.52	2.50	bcd
14	11NYE18	Charlotte	Charlotte	2011	irregular	undulate	absent	absent	24.0 ± 0.37	3.00	a
15	11NYE21	Charlotte	Charlotte	2011	circular-irregular	undulate	present	intermediate	40.2 ± 2.01	2.50	bcd
16	12NYE1	Charlotte	Charlotte	2012	circular-irregular	undulate	absent	absent	25.3 ± 0.33	2.50	bcd
17	12NYE5	Charlotte	Charlotte	2012	circular-irregular	undulate	absent	absent	35.0 ± 0.93	2.33	cde
18	12NYE8	Charlotte	Charlotte	2012	circular	entire-undulate	absent	low	25.8 ± 0.79	1.50	f
19	12NYE14	Charlotte	Charlotte	2012	irregular	undulate	absent	high	25.5 ± 0.89	2.17	de
20	12NYE22	Charlotte	Charlotte	2012	circular	entire-undulate	absent	high	43.2 ± 0.60	2.67	abc
21	12NYE25	Charlotte	Charlotte	2012	circular-irregular	entire-lobate	absent	low	34.5 ± 1.18	2.50	bcd
22	12NYE27	Charlotte	Charlotte	2012	circular	entire	absent	intermediate	37.2 ± 0.40	2.50	bcd
23	11CEN1	Grant	Grant	2011	circular-irregular	undulate	absent	intermediate	28.3 ± 0.71	2.67	abc
24	11CEN9	Grant	Grant	2011	irregular	undulate	absent	high	35.3 ± 0.88	2.33	cde
25	11MOR4	Grant	Grant	2011	circular	undulate	present	low	36.3 ± 0.88	3.00	a

Table 1.2. (cont'd)

No.	Isolate ID	City	Population	Year	Colony		Chlamydospores	Abundance of pycnidia on CA	Diameter ± SE	Means of disease virulence	
					form	margin					
26	11MOR3	Grant	Grant	2011	irregular	undulate	present	low	31.5 ± 0.43	2.50	bcd
27	11MOR6	Grant	Grant	2011	irregular	undulate	absent	low	34.2 ± 3.11	2.50	bcd
28	11NEW2	Grant	Grant	2011	circular-irregular	undulate	absent	high	29.5 ± 0.72	2.33	cde
29	11NEW11	Grant	Grant	2011	circular-irregular	undulate	present	high	35.3 ± 1.09	2.50	bcd
30	11VAN4	Grant	Grant	2011	circular	undulate	absent	intermediate	28.2 ± 0.70	3.00	a
31	11VAN1	Grant	Grant	2011	irregular	undulate	absent	absent	33.5 ± 1.54	2.00	e
32	11VS2310	Grant	Grant	2011	circular	undulate	absent	high	41.2 ± 5.98	2.50	bcd
33	11VS2317	Grant	Grant	2011	circular-irregular	undulate	absent	high	43.7 ± 3.12	2.83	ab
34	11VWY6	Grant	Grant	2011	irregular	undulate-lobate	absent	absent	44.0 ± 0.37	2.83	ab
35	11VWY12	Grant	Grant	2011	irregular	entire	absent	low	30.2 ± 1.22	2.67	abc
36	11VWY15	Grant	Grant	2011	irregular	undulate-lobate	absent	low	29.8 ± 0.31	2.67	abc
37	12FOX10	Grant	Grant	2012	irregular	undulate	present	high	46.3 ± 1.99	3.00	a
38	12FOX28	Grant	Grant	2012	irregular	undulate-lobate	absent	low	33.2 ± 1.11	2.33	cde
39	12SPR9	Grant	Grant	2012	circular-irregular	undulate	absent	intermediate	44.8 ± 2.01	2.67	abc
40	12SPR20	Grant	Grant	2012	circular-irregular	undulate-lobate	present	high	48.0 ± 2.35	2.67	abc
41	12VEU5	Grant	Grant	2012	circular-irregular	undulate	absent	high	48.7 ± 1.63	2.67	abc
42	12VEU18	Grant	Grant	2012	circular-irregular	undulate-lobate	absent	intermediate	37.0 ± 0.86	2.50	bcd
43	11HV1	Hudsonville	Hudsonville	2011	circular	undulate-lobate	absent	low	42.7 ± 6.44	2.00	e
44	11HV2	Hudsonville	Hudsonville	2011	circular	undulate	absent	high	32.3 ± 0.61	2.00	e
45	11HV3	Hudsonville	Hudsonville	2011	circular-irregular	undulate	absent	absent	26.2 ± 0.75	2.33	cde
46	11HV4	Hudsonville	Hudsonville	2011	irregular	undulate	present	low	33.2 ± 1.42	2.33	cde
47	11HV5	Hudsonville	Hudsonville	2011	circular-irregular	undulate	absent	low	22.2 ± 0.31	2.33	cde
48	11HV6	Hudsonville	Hudsonville	2011	irregular	undulate	absent	low	39.5 ± 1.52	2.67	abc
49	11HV8	Hudsonville	Hudsonville	2011	irregular	undulate	absent	low	37.3 ± 1.91	2.50	bcd
50	12HUD4	Hudsonville	Hudsonville	2012	irregular	undulate-lobate	absent	low	22.2 ± 2.47	2.00	e
51	12HUD10	Hudsonville	Hudsonville	2012	circular-irregular	undulate	present	intermediate	44.5 ± 0.56	2.50	bcd
52	12HUD13	Hudsonville	Hudsonville	2012	circular-irregular	undulate	absent	high	42.8 ± 0.79	2.67	abc

Table 1.2. (cont'd)

No.	Isolate ID	City	Population	Year	Colony		Chlamydospores	Abundance of pycnidia on CA	Diameter ± SE	Means of disease virulence	
					form	margin					
53	12HUD17	Hudsonville	Hudsonville	2012	circular	undulate	absent	intermediate	35.0 ± 0.58	2.67	abc
54	12HUD21	Hudsonville	Hudsonville	2012	circular-irregular	undulate	present	low	32.3 ± 1.86	2.67	abc
55	12HUD26	Hudsonville	Hudsonville	2012	circular-irregular	lobate	absent	low	46.8 ± 3.68	2.67	abc
56	12HUD29	Hudsonville	Hudsonville	2012	irregular	undulate-lobate	absent	absent	26.3 ± 0.95	2.67	abc
57	11LAN207	Lansing	Lansing	2011	circular-irregular	undulate-lobate	absent	intermediate	25.5 ± 0.34	2.17	de
58	11LAN113	Lansing	Lansing	2011	irregular	undulate	present	high	26.2 ± 0.40	2.33	cde
59	11LAN304	Lansing	Lansing	2011	irregular	undulate	absent	low	26.0 ± 0.58	2.83	ab
60	11LAN414	Lansing	Lansing	2011	circular-irregular	undulate	absent	intermediate	33.3 ± 0.49	2.83	ab
61	12KEI2	Lansing	Lansing	2012	irregular	lobate	absent	high	38.0 ± 0.45	2.50	bcd
62	12KEI5	Lansing	Lansing	2012	circular	entire	absent	low	49.5 ± 2.22	2.50	bcd
63	12KEI8	Lansing	Lansing	2012	circular	entire	absent	high	44.3 ± 5.65	2.83	ab
64	12KEI11	Lansing	Lansing	2012	circular	entire	absent	absent	65.3 ± 1.28	2.00	e
65	12KEI12	Lansing	Lansing	2012	irregular	undulate-lobate	absent	intermediate	32.3 ± 2.36	2.33	cde
66	12KEI14	Lansing	Lansing	2012	circular	entire	absent	low	68.7 ± 0.71	2.50	bcd
67	12KEI16	Lansing	Lansing	2012	circular-irregular	entire	absent	high	46.5 ± 0.22	2.83	ab
68	12MAR5	Martin	Other site	2012	circular-irregular	lobate	present	high	22.7 ± 0.84	3.00	a
69	12MAR2	Martin	Other site	2012	circular-irregular	entire-undulate	present	high	25.0 ± 1.10	2.83	ab
70	12MAR7	Martin	Other site	2012	irregular	undulate	absent	high	29.2 ± 0.40	2.17	de
71	12MAR4	Martin	Other site	2012	irregular	undulate	absent	low	40.0 ± 0.86	2.00	e
72	12MAR6	Martin	Other site	2012	irregular	lobate	present	high	45.0 ± 1.13	3.00	a
73	12MAR9	Martin	Other site	2012	circular-irregular	entire-undulate	present	low	30.7 ± 1.09	2.00	e
74	12MAR10	Martin	Other site	2012	circular-irregular	undulate	absent	intermediate	36.8 ± 1.22	2.33	cde
75	11PW1	Plainwell	Other site	2011	circular	entire-undulate	absent	low	37.2 ± 0.65	2.33	cde
76	11PW6	Plainwell	Other site	2011	circular-irregular	undulate-lobate	present	high	28.3 ± 1.09	2.83	ab
77	11PW9	Plainwell	Other site	2011	circular-irregular	entire-undulate	present	high	52.3 ± 1.87	2.67	abc
78	11PW10	Plainwell	Other site	2011	circular-irregular	undulate	absent	low	35.8 ± 0.60	2.67	abc
79	11PW12	Plainwell	Other site	2011	circular	entire	absent	high	45.5 ± 0.34	2.67	abc
80	11PW15	Plainwell	Other site	2011	circular-irregular	undulate-lobate	absent	low	44.0 ± 2.22	2.83	ab

Table 1.2. (cont'd)

No.	Isolate ID	City	Population	Year	Colony		Chlamydospores	Abundance of pycnidia on CA	Diameter ± SE	Means of disease virulence	
					form	margin					
81	11PW21	Plainwell	Other site	2011	circular	undulate	present	high	59.8 ± 3.29	2.67	abc
82	11PL1	Portland	Portland	2011	irregular	undulate	absent	absent	23.8 ± 0.17	2.17	de
83	11PL3	Portland	Portland	2011	circular-irregular	undulate	absent	high	32.7 ± 1.15	2.50	bcd
84	11PL6	Portland	Portland	2011	circular-irregular	undulate	absent	intermediate	28.7 ± 3.05	2.17	de
85	11PL8	Portland	Portland	2011	circular-irregular	undulate	absent	absent	30.5 ± 0.22	2.33	cde
86	11PL9	Portland	Portland	2011	irregular	undulate	absent	low	32.5 ± 1.89	2.50	bcd
87	11PL11	Portland	Portland	2011	circular-irregular	undulate	present	high	32.0 ± 0.73	2.67	abc
88	11PL13	Portland	Portland	2011	irregular	undulate	absent	absent	32.0 ± 0.68	2.67	abc
89	12POR1	Portland	Portland	2012	circular-irregular	lobate	absent	low	51.7 ± 1.28	2.00	e
90	12POR4	Portland	Portland	2012	irregular	undulate-lobate	absent	intermediate	31.2 ± 1.28	2.67	abc
91	12POR6	Portland	Portland	2012	circular	entire	absent	intermediate	45.7 ± 0.76	3.00	a
92	12POR9	Portland	Portland	2012	circular-irregular	entire	absent	low	52.0 ± 1.32	2.33	cde
93	12POR13	Portland	Portland	2012	circular-irregular	lobate	absent	low	38.8 ± 1.74	2.17	de
94	12POR15	Portland	Portland	2012	circular-irregular	undulate	absent	high	45.5 ± 0.96	3.00	a
95	12POR17	Portland	Portland	2012	circular	undulate	absent	intermediate	44.5 ± 0.22	2.33	cde
96	11ST1	Stockbridge	Other site	2011	circular	undulate	absent	high	30.7 ± 1.67	2.17	de
97	11ST4	Stockbridge	Other site	2011	circular-irregular	undulate	absent	intermediate	36.3 ± 0.49	2.00	e
98	11ST7	Stockbridge	Other site	2011	irregular	undulate	absent	intermediate	31.7 ± 0.61	2.33	cde

[y]Means of colony diameter (mm.) ± standard error (SE). Measurements were taken on MEA plate 14 days after inoculation.

[z]Means with a common letter do not differ significantly based on Kruskall-Wallis multiple comparison tests ($\alpha = 0.05$). Virulence was rated based on a scale of 0 to 3; where 0 = healthy roots, no disease symptoms, 1 = <10% of the roots with significant discoloration, 2 = ≥10% to 50% of the roots with significant discoloration, and 3 = >50% of the roots with significant discoloration.

Table 1.3. Colony characteristics, percentages of isolates within populations that produced chlamydospore and pycnidia, colony diameter, and means of disease virulence of nine populations of *S. terrestris* from Michigan onion fields collected during the growing seasons of 2011 and 2012.

Population	Sample size	Colony formation[x]		% Isolates with chlamydospores[y]		% Isolates with pycnidia [z]				Colony diameter (mm)
		Form	Margin	Absent	Present	Absent	Low	Intermediate	High	± SE
Charlotte	14	cir, irr	ent, lob, und	85.7	14.3	35.7	28.6	14.3	21.4	34.67 ± 0.86
Grant	20	cir, irr	ent, lob, und	75.0	25.0	10.0	30.0	20.0	40.0	36.95 ± 1.55
Hudsonville	14	cir, irr	lob, und	78.6	21.4	14.3	57.1	14.3	13.3	34.52 ± 1.70
Lansing	11	cir, irr	ent, lob, und	90.9	9.1	9.1	27.3	27.3	36.3	41.42 ± 1.34
Portland	14	cir, irr	ent, lob, und	92.9	7.1	21.4	28.6	28.6	21.4	37.25 ± 1.10
Other site[w]	25	cir, irr	ent, lob, und	36.0	64.0	0.0	32.0	28.0	10.0	36.05 ± 1.16
									Mean	36.81 ± 1.29

[w]Populations with number of isolates less than 10 were pooled and analyzed as other site population.

[x]cir = circular; irr = irregular; ent = entire; lob = lobate; and und = undulate.

[y]Percentage of isolates that showed absence or presence of chlamydospore production calculated by population.

[z]Percentage of isolates that were absent, or had low, intermediate, and high pycnidia production calculated by population.

Table 1.4. Means of disease virulence of six populations of *S. terrestris* from Michigan onion fields collected from 2011 through 2012.

Population	Sample size	Mean[z]
Charlotte	14	2.36
Grant	20	2.61
Hudsonville	14	2.43
Lansing	11	2.52
Portland	14	2.46
Other site[y]	25	2.45

[y]Populations with number of isolates less than 10 were pooled and analyzed as other site population.

[z] Means do not differ statistically. Disease virulence was rated based on a scale of 0 to 3; where 0 = healthy roots, 1 = slight pink discoloration of most of the roots and only a few (<10%) roots with dark pink coloration; 2 = 10 to 50% of the roots with strong pink coloration, 3 = >50% of the roots completely pink.

Table 1.5. ISSR primers with sequences, and annealing temperatures used in this study, and number of bands amplified.

Primer	Sequence (5'- 3')	Annealing temperature (°C)	No. of bands amplified
UBC 807	$(AG)_8T$	46	16
UBC 808	$(AG)_8C$	49	14
UBC 820	$(GT)_8C$	50	12
UBC 848	$(CA)_8RG$	55	15
UBC 835	$DBD(AC)_7$	51	19
UBC 868	$(GAA)_6$	55	11
UBC 889	$DBD(ACA)_5$	52	18

[z]B = C/G/T; D = A/G/T; R = A/T

Genetic diversity of the 98 *S. terrestris* isolates was analyzed based on polymorphic bands amplified using the ISSR markers. The genetic diversity varied among populations with the percentage of polymorphic bands values ranging from 67.62% (Lansing population) to

80.95% (Charlotte population) with an average of 76.35% at the population level. The mean of observed number of alleles (N_A) ranged from 1.6762 ± 0.4702 in the Lansing population to a maximum of 1.8095 ± 0.3946 in the Charlotte population. Means of effective number of alleles (N_E) were lower than those of the N_A, ranging from 1.4120 ± 0.3814 (Lansing population) to 1.4870 ± 0.3774 (Grant population). The effective number of alleles estimated for the reciprocal homozygosity was less than the actual number, suggesting that there was no evidence of mutation existed in the populations. The means of Nei's gene diversity ranged from 0.2393 ± 0.1987 (Lansing population) to 0.2801 ± 0.1912 (Grant population). Shannon information indices (I_S) were lowest at 0.3577 ± 0.2794 in the Lansing population and highest at 0.4170 ± 0.2627 in the Grant population. The gene diversity index (H) for all populations was 0.2843 ± 0.1724, the Shannon information index was 0.4336 ± 0.2246, and the number of polymorphic bands was 101, estimating to be 96.19% of the polymorphic bands (Table 1.6).

Among the six populations, total gene diversity was high (H_T = 0.2856) and gene diversity within populations was relatively moderate (H_S = 0.2603). G_{ST} that describes the average amount of genetic diversity attributed to genetic variability being subdivided among populations relative to the total level of genetic diversity was moderate (0.0888) for over all loci, indicating that the genetic variation in *S. terrestris* appeared mainly among individuals within populations rather than among populations. The average number of migrants per generation (N_M) among population was 5.1316, indicating genotype flow occurred among populations (Table 1.7). The result was consistent with the AMOVA analysis that 96% of the genetic variation occurred within populations while 4% occurred among populations. The total genetic differentiation among individuals within populations was significant (PhiPT = 0.044, *P* = 0.001 (Table 1.8).

Pairwise comparisons of Nei's genetic similarity and genetic distance among six populations demonstrated that the Grant and Portland population pair had highest genetic similarity (0.9851) while the Charlotte and Portland population pair had the lowest (0.9609) (Table 1.9). Genetic distance was low between Grant and Portland (0.0150), but major difference in genetic differentiation occurred between Charlotte and Portland populations (0.0399) (Table 1.9).

Table 1.6. Genetic diversity within different populations of *Setophoma terrestris* from Michigan onion fields during the growing seasons of 2011 and 2012 using ISSR analysis.

Population	Sample size	N_A	N_E	H	I_S	NP	%P
Charlotte	14	1.8095 ± 0.3946*	1.4605 ± 0.3544	0.2729 ± 0.1795	0.4123 ± 0.2468	85	80.95
Grant	20	1.7905 ± 0.4089	1.4870 ± 0.3774	0.2801 ± 0.1912	0.4170 ± 0.2627	83	79.05
Hudsonville	14	1.7619 ± 0.4280	1.4398 ± 0.3859	0.2551 ± 0.1925	0.3844 ± 0.2641	80	76.19
Lansing	11	1.6762 ± 0.4702	1.4120 ± 0.3814	0.2393 ± 0.1987	0.3577 ± 0.2729	71	67.62
Portland	14	1.7619 ± 0.4280	1.4725 ± 0.3654	0.2751 ± 0.1891	0.4099 ± 0.2638	80	76.19
Other site[z]	25	1.7810 ± 0.4156	1.4036 ± 0.3664	0.2391 ± 0.1898	0.3644 ± 0.2609	82	78.10
Population level	6	1.7635 ± 0.4242	1.4459 ± 0.3718	0.2603 ± 0.1901	0.3910 ± 0.2619	80	76.35
Species level	98	1.9619 ± 0.1923	1.4802 ± 0.3531	0.2843 ± 0.1724	0.4336 ± 0.2246	101	96.19

N_A = observed number of alleles; N_E = effective number of alleles; H = Nei's gene diversity; I_S = Shannon's information index; NP = number of polymorphic bands; %P = percentage of polymorphic bands; * indicates standard deviation.

[z]Populations with number of isolates less than 10 were pooled and analyzed as other site population.

Table 1.7. Overall genetic variability across all the populations

Population size	H_T	H_S	G_{ST}	N_M
6	0.2856 ± 0.0296*	0.2603 ± 0.0255	0.0888	5.1316

H_T = total genetic diversity; H_S = genetic diversity within groups; G_{ST} = genetic differentiation among populations; N_M = genotype flow estimated from G_{ST} as $N_M = 0.5(1- G_{ST})/G_{ST}$; * indicates standard deviation.

Table 1.8. Analyses of molecular variance (AMOVA) from six populations of *S. terrestris* collected across nine Michigan cities. PhiPT was calculated based on 999 permutations.

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage of variance	
Among populations	5	126.234	0.668	4	
Within population	92	1336.328	14.525	96	PhiPT = 0.044 (P = 0.001)
Total	97	1462.561	15.193	100	

The UPGMA dendrogram based on Nei's genetic distance revealed that there was no grouping of the isolates according to their geographical origins (Figure 1.5). From the two-dimensional plot, the result also confirmed that the populations were not clearly separated based on their regional origins. The principal coordinates analysis (PCoA), providing spatial representation of the relative genetic distance among isolates, indicated that the first two axes described 26.29% of the total of variance. The coordinates 1 and 2 accounted for 18.19% and 8.10%, respectively (Figure 1.6). Based on the PCoA analysis, the isolates from each population did not clearly distinguish from those of other populations. The Mantel test revealed a non-significant relationship between geographical and genetic distances (P = 0.6950), indicating that isolation-by-distance was not evident.

Pearson's correlation coefficients were calculated for measuring the relationship among morphological characteristics, disease virulence, and number of polymorphic bands (Table 1.10). The result showed that the relationships between disease virulence and colony diameter was positively correlated ($\alpha = 0.05$).

Table 1.9. Nei's (1978) unbiased measures of genetic identity (upper diagonal) and genetic distance (lower diagonal) among six populations of *S. terrestris* collected from Michigan onion fields from 2011 through 2012.

Population	Charlotte	Grant	Hudsonville	Lansing	Portland	Other sites
Charlotte	---	0.9675	0.9636	0.9779	0.9609	0.9613
Grant	0.0330	---	0.9685	0.9716	0.9851	0.9693
Hudsonvill	0.0371	0.0320	---	0.9763	0.9654	0.9755
Lansing	0.0224	0.0288	0.0240	---	0.9663	0.9770
Portland	0.0399	0.0150	0.0352	0.0342	---	0.9734
Other site[z]	0.0395	0.0312	0.0248	0.0233	0.0270	---

[z]Populations with number of isolates less than 10 were pooled and analyzed as other site population.

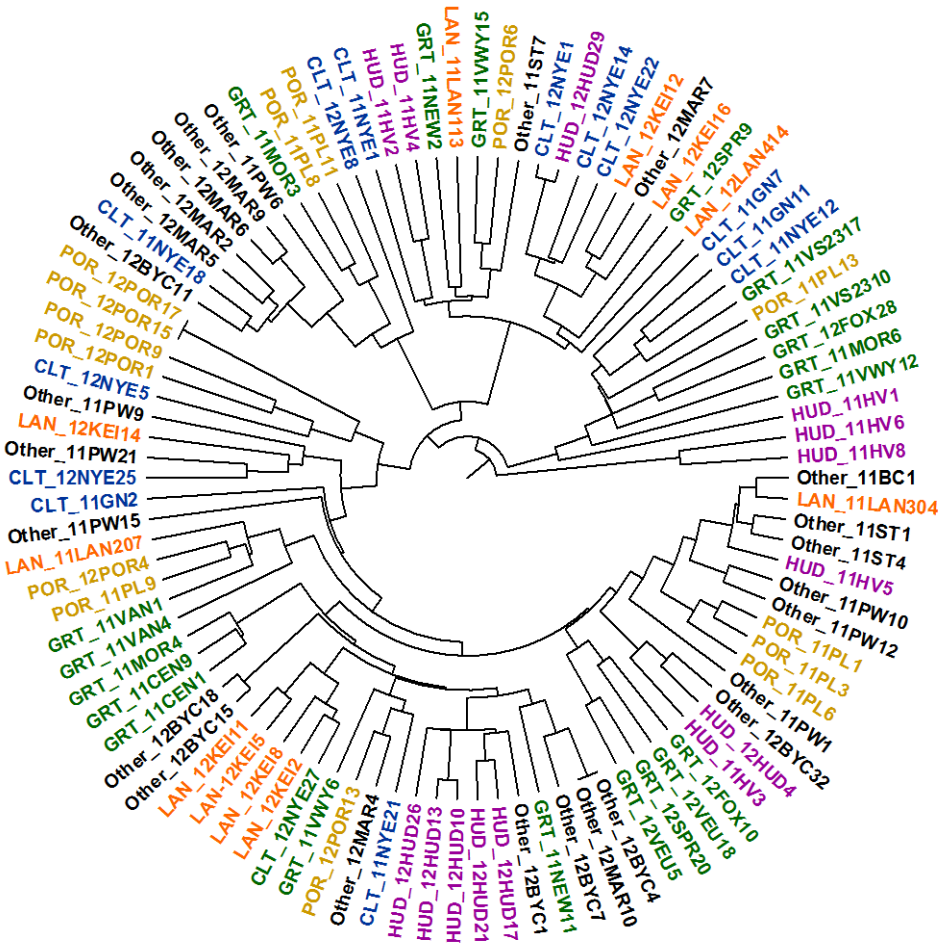


Figure 1.5. Circular dendrogram constructed using Nei's genetic distance of 98 isolated of *S. terrestris* in the ISSR analysis. Color codes are based on the populations: dark blue = Charlotte; green = Grant; violet = Hudsonville; orange = Lansing; cinnamon = Portland; and black = Other site population.

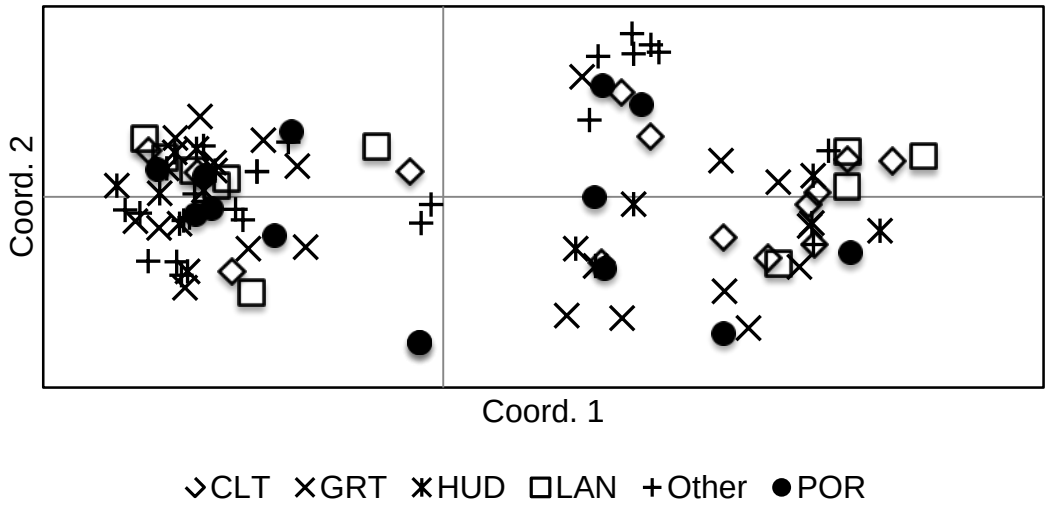


Figure 1.6. A two-dimensional plot of the principal coordinates analysis (PCoA) from the matrix of genetic distances based on 7 ISSR markers of 98 individuals of *S. terrestris*, representing 9 populations in Michigan. Percentage of variance accumulates on the first two axes = 26.29%. Coordinates 1 and 2 accounted for 18.19% and 8.10% of the variation, respectively. CLT = Charlotte, GRT = Grant, HUD = Hudsonville, LAN = Lansing, Other = Other site.

Table 1.10. Correlations among colony diameter, disease virulence, and number of polymorphic bands.

	Pearson correlation coefficient[z]		
Character	Colony diameter	Disease virulence	Number of polymorphic bands
Colony diameter	1.0000	**0.8087**	0.1797
Disease virulence		1.0000	0.4090
Number of polymorphic bands			1.0000

[z]Correlation coefficients were calculated according to the Pearson correlation coefficient.

Values in bold letters indicate that they were significant at $P = 0.05$.

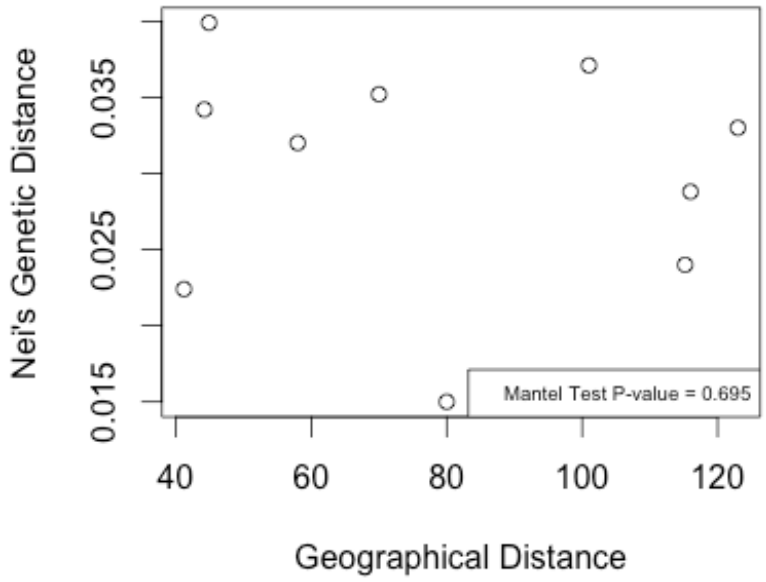


Figure 1.7. Scatter plots of Nei's genetic distance and geographical distance for pairwise population comparisons based on the ISSR analysis.

DISCUSSION

The 98 isolates of *S. terrestris* included in this study were collected from the onion fields located in nine cities in Michigan, and they were identified based on cultural and morphological characteristics and the species were confirmed based on the partial sequence of the LSU rDNA. In our preliminary studies, the two regions, internal transcribed spacer (ITS) and LSU, were amplified, and the nucleotide sequences of both regions were compared to the sequences on GenBank databases. It was found that the amplification of the LSU regions was shown to be more suitable for identifying *S. terrestris* than the ITS regions as they discriminated between *S. terrestris* and other fungi better than the ITS regions.

Variability of morphological characteristics, including colony form, colony margin, the presence or absence of chlamydospores, the abundance of pycnidial production on culture

medium, and colony diameter, existed among isolates of *S. terrestris*; however, there were no differences of these features when compared among the populations. The results were in accordance with Gorenz (1948) who demonstrated that variability in pycnidia morphology of isolated from different geographic areas in the United States were considerably diverse. Study by Ferreira et al (1991) revealed that the isolates obtained from South Africa and the United States differed in their ability to produce pycnidia in cultural medium and had a wide range of colony diameter. Variability in morphology of the pink root pathogen may relate to the dual phenomenon where two genetic elements exist in the same individual (Hansen 1938). Hansen (1938) observed the association between heterokaryotic status and different types of culture of *S. terrestris* where the fungus gave rise to three cultural types, including M (mycelial), C (conidial), and MC (intermediate between M and C) types after single-spore series were made.

Significant differences in disease virulence were observed among isolates but populations did not differ in their level of virulence. The virulence assay showed a moderate-low to high degree of virulence of different isolates at the species level, and a relatively high degree of virulence spectra were observed at the population level. Previous analysis of disease virulence among 30 isolates of *S. terrestris* on three onion breeding lines (resistant, moderate resistant, and susceptible to pink root) demonstrated that the virulence of *S. terrestris* isolates was variable (Ferreira et al. 1991). The relationship between virulence and the size of colony was observed. The more virulent populations are likely to occur by the population of larger size of colony than the populations of smaller colony. It may imply that there was a positive correlation between the growth rate and the amount of pathogenic toxin produced by the pathogen. However, the virulence of the populations did not relate to any other cultural characteristics or the number of polymorphic bands.

Thus far, there was only one study that determined *S. terrestris* isolates at the molecular level using isozyme analysis (Ferreira et al. 1991). In this study, ISSR markers were employed to analyze the population structure of *S. terrestris* isolates collected from Michigan fields as the ISSR technique is able to detect a higher level of polymorphism than detected with restriction fragment length (RFLP) or random amplified polymorphic DNA (RAPD) techniques (Salimath et al. 1995), does not require sequence data for generating primers (Kantety et al. 1995), and produces consistent fingerprints between replicates (Gilbert et al. 1999). In addition, studies have shown that the application of ISSR technique can be used to study genetic diversity of several fungi (Abadio et al. 2012; Archana et al. 2014; Gramaje et al. 2014; Luan et al. 2012; Villarino et al. 2012).

The PCR-ISSR profiles were successfully detected polymorphism among *S. terrestris* isolates. Seven ISSR markers generated reproducible amplified products, which illustrated the genetic diversity of *S. terrestris* isolates. Cluster analysis based on Nei's genetic distance demonstrated that the *S. terrestris* isolates originating from different geographical regions appeared in the same cluster, suggesting that geographical origins did not contribute to the relatedness among the populations. The PCoA was further in agreement with the UPGMA clustering that grouping of isolates was not based on regional origins, as there were multiple populations in both main groups.

According to the AMOVA, the majority of genetic variation was accounted for among isolates within the populations while the minority of diversity was encountered among the populations. It was revealed in the hierarchical analysis of this study that the most variation in population genetics of *S. terrestris* was found within populations. No patterning was found among populations in Michigan (Figure 1.7). The results were in contrast with our expectations

that the pathogen isolates are genetically similar compared among isolates and tend to have low genetic variation according to their clonal reproduction.

In general, collections of pathogen isolates from different geographic locations are considered as separate populations because they commonly have different allele or genotype frequencies (Boeger et al. 1993). Significant level of genotype flow may homogenize the populations originated from two geographical regions into homogeneous genetic groups (Boeger et al. 1993). Moderate degree of genetic differentiation among the populations in this study could be a result of genotype flow or migration of individuals from one geographic region to another. The movement of one migrant per generation is adequate to reduce differentiation between the populations (McDermott and McDonald 1993). Typically, genetic differentiation is not significant when $N_M > 1$ (Wright 1951). In this study, N_M estimated from G_{ST} was 5.1316, indicating that the movement of genotype limited the genetic differentiation among the populations of *S. terrestris* isolated in Michigan. Migration of genotypes or individuals among geographically distant populations could be facilitated through the transportation of conidia by movement of soil or infested plants (McDonald and Linde 2002).

The evidence of genotype flow in the *S. terrestris* species may have a potential risk in disease management to onion industry if virulent mutant alleles are introduced into the field through the movement of the pathogen. However, genotype flow between geographic populations can be prevented by limiting the movement of the pathogen through infected plant materials, soil, or contaminated equipment caused by human-mediated, and by eliminating the susceptible hosts (McDonald and Linde 2002).

In conclusion, this study has demonstrated that genetic uniformity was present among *S. terrestris* populations. However, high genetic heterogeneity was detected within the populations, which could manifest the ability of the pathogen to evolve resistance to fungicides and/or genetic resistance (McDonald and Linde 2002).

CHAPTER 2: RESPONSE OF ONION CULTIVARS AND FUNGICIDES TO *SETOPHOMA TERRESTRIS*

ABSTRACT

Pink root disease incited by *Setophoma terrestris* extensively damages the root system of dry bulb onion (*Allium cepa*) and limits onion production worldwide. In 2011 and 2012, 30 onion cultivars were evaluated for their susceptibility to pink root in naturally infested commercial fields. Onions were destructively sampled 64, 76, 96, and 110 days after seeding to assess diseased roots (%). In both years, 'Highlander' was the most susceptible cultivar and exhibited the highest incidence of root rot symptoms at the final rating; 'Hendrix' and 'Redwing' were less susceptible with less root rot than the other cultivars tested. In 2012, 'Frontier' and 'Scout' were highly susceptible, whereas 'Braddock' was least susceptible to pink root. In 2013, sixteen cultivars were selected based on results from the previous trials and evaluated for disease severity 109 days after planting. Using a disease severity scale, 'Highlander' was the most susceptible cultivar followed by 'Livingston' and 'Safrane'; 'Sedona' was the least susceptible. The cultivars Hendrix and Redwing were statistically similar to 'Sedona' in susceptibility. Nine fungicides were tested in a greenhouse trial. Six-week old 'Highlander' seedlings were transplanted into plastic pots and inoculated with millet seeds infested with *S. terrestris*. Each fungicide was applied as a drench at either 0 day post inoculation (dpi) or at 0 and 14 dpi. The number of total leaves per plant and plant height were assessed at 49 dpi, and plant fresh weight, bulb circumference, and root density, were measured at 55 dpi. Significant differences were observed among fungicide treatments but complete disease control was never achieved. Onion

plants treated with penthiopyrad were larger than the untreated control. Plant injury was observed in response to some treatments.

INTRODUCTION

In 2012, 105.5 million tons of onion (*Allium cepa* L.) bulbs were harvested from 2.1 million hectares worldwide. The vast bulk of bulb onions are produced in China, India, and the United States with 22.6, 16.3, and 3.3 million tons, respectively (Anonymous 2014). In the United States, onions are cultivated on approximately 60,014 hectares with a majority (60%) of the crop comprised of onions for storage (Alston et al. 2013; Anonymous 2014). In Michigan, 41,150 tons of onions were harvested from 1,133 hectares with a value of 11.1 million dollars in 2013 (Anonymous 2014).

Onions are subject to a variety of foliar and soil-borne pathogens that generally lead to a reduction in bulb quality and/or yield (Anonymous 2011). Pink root, caused by the soilborne pathogen, *Setophoma terrestris* (H.N. Hansen) Gruyter, Aveskamp and Verkley, is common in soils (Hansen 1929) and is distributed worldwide (Taubenhaus and Mally 1921). The pathogen may infect numerous crops (Perry et al. 1963) including grains, vegetables (Clark 1940; Ikeda et al. 2012; Kreutzer 1941; Levic et al. 2011; Newby et al. 1997; Sumner 1995; Thornberry and Anderson 1940), and broadleaf weeds (Sprague 1944) and is an important pathogen on onions, garlic, and shallots (Hansen 1929; Kreutzer 1941; Porter and Jones 1933; Tims 1942). Pink root affects the root system causing yield loss (Sumner 1995). However, when optimum growing conditions are maintained, the onion may grow fast enough to produce an average-sized bulb and the disease is minimized (Taubenhaus and Mally 1921). Root disease symptoms include a light pink coloration at an early stage of infection; roots later become dark pink, red, or dark purple, shrivel, and disintegrate (Hansen 1929). Infected plants may become stunted and do not produce

an appropriate bulb size. Severely infected plants may be unable to uptake sufficient nutrients and water (Thronton and Mohan 1996) or may spend energy to produce new roots following infection (Taubenhaus and Mally 1921). The pathogen may stimulate the pre-maturation of bulb development and prevent maximum growth (Levy and Gornik 1981; Rabinowitch et al. 1981).

Onions may become infected by *S. terrestris* at all stages of plant development (Davis and Handerson 1937). Economic losses as a result of seedling collapse can range from 10 to 90% (Davis and Handerson 1937). Yield reduction ranged from 6 to 18% or 25 to 50% when infected onion sets were planted into uninfested or infested soil, respectively (Taubenhaus and Mally 1921). Yield decline ranging from 50 to 96% occurred when onions were grown repeatedly in the same infested fields (Porter and Jones 1933). Pink root may not directly impact onion quality (Taubenhaus and Mally 1921) but approximately 15% of Fusarium bulb rot and 30% of dry rot lesions developed on apparently healthy mature bulbs in storage when onions were grown in soil infested by both pathogens (Davis and Handerson 1937). Similarly, Taubenhaus and Mally (1921) reported that onions grown in fields infested with *S. terrestris* were more likely to be infected by *Aspergillus niger,* causing black mold decay, and *Bacillus caratovorus,* causing soft rot, when compared to those grown in uninfested soils.

The optimum temperature for *S. terrestris* growth and pink root development ranges from 24 to 28°C. Infection is reduced at 20°C and disease development is limited at temperatures lower than 16°C (Sumner 1995). The pathogen presumably survives in soil as chlamydospores, pycnidia, or pycnidiospores or in colonized roots or plant debris of susceptible crops (Sumner 1995). The root tips are directly infected by hyphae and the roots subsequently colonized. Neither the basal stem plate nor fleshly scales of the bulb are infected by the pathogen (Sumner

1995). However, typical reddish discoloration may be observed on the outer dead scale tissue of the bulb, especially of white onions (Kreutzer 1941).

Recommendations to reduce pink root pressure in onion fields include crop rotation, soil solarization, and fumigation (Katan et al. 1980; Rabinowitch et al. 1981; Sumner et al. 1997; Sumner 1995; Taubenhaus and Mally 1921; Vaughan et al. 1971). However, rotation may not be practical, particularly, in onion intensive production areas (Brewster 2008), and it might not be highly beneficial as the pathogen has a broad host range (Coleman et al. 1997). Soil solarization is difficult to accomplish in temperate climates because the growing season for onions occurs during the warmest months of the year. Soil fumigation is costly and detrimental to the environment (Coleman et al. 1997).

Currently, choosing resistant and/or tolerant cultivars is the most effective control measure for pink root (Jones and Perry 1956; Porter and Jones 1933). Onion cultivars may differ in their pink root resistance according to environmental factors, such as day length and temperature (Netzer et al. 1985), and variation in virulence of the pathogen populations (Ferreira et al. 1991). In Idaho, hybrid lines were less prone to pink root infection than the yellow sweet Spanish cultivars tested (Thronton and Mohan 1996). In New York, the cultigens including Sweet Sandwich, Keepsweet II, Spartan Banner 80, and inbred line, MSU5785B, had the fewest disease symptoms and least disease incidence compared to other cultivars and lines tested (Coleman et al. 1997). In New Mexico, cultivars obtained by interbreeding 'NuMex Chaco' and 'NuMex Snowball', were selected for pink root resistance and released (Cramer and Corgan 2001a, 2001b). In Iran, 13 cultivars were determined to be highly resistant to pink root infection (Nasr Esfahani and Ansari Pour 2008). Identification of pink root resistance or tolerance in onion cultivars suitable for Michigan growing conditions would greatly benefit the state's

industry. The objectives of this study were to determine the response of onion cultivars to pink root infection under field conditions and evaluate fungicide treatments for control of pink root disease in a greenhouse trial.

MATERIALS AND METHODS

Cultivar response. The experiment was designed as a randomized complete block with four replicates. Individual plots consisted of double rows, 6 m in length. Plots were spaced 0.9 m apart. Seeds were spaced 2.54 cm apart from each other and 0.3 m apart between the double rows within each plot. Seeds of 30 onion cultivars (Table 2.1) were sown with a precision hand seeder in naturally infested soil in commercial onion fields on May 3rd, 2011 and April 12th, 2012 in Lansing, MI and May 3rd, 2013 in Stockbridge, MI. As 'Highlander' and 'Hendrix' exhibited high and low susceptibility, respectively, to pink root in a field trial in 2011, they were included as standards in a plot with an additional 14 cultivars that were evaluated in 2012. The agricultural soil in both fields was a muck type and was representative of the soils used for growing onion in Michigan. Rotational crops included carrot and potato (Lansing site) and mint and potato (Stockbridge site). Fertilizers and pesticides were applied for crop maintenance according to the commercial standard.

Disease ratings were conducted 64, 76, 96, and 110 days after sowing seeds in 2011 and 2012, and 109 days in 2013. Samples were obtained by carefully digging the bulbs to preserve the root system. Bulbs were then placed in plastic bags to prevent drying of the roots and transported to the laboratory. Bulbs were maintained at 4°C until they were evaluated for disease. In 2011 and 2012, eight bulbs from each plot were collected in order to evaluate the percentage of the root system infected. The total number of roots and the number of roots with pink root symptoms were counted and disease calculated as a percentage. In 2013, twenty-five

bulbs were harvested from the middle 10 ft of each treatment row. Each bulb was examined for roots infected (%) and the incidence of bulbs with pink root was determined. Disease severity on the roots was assessed using a scale from 0 to 8 as follows: 0 = abundant, vigorous and white roots; 1 = < 25% of the bulbs with pink root (each bulb had < 50% symptomatic roots); 2 = 25 to 49% of the bulbs with pink root (each bulb has < 50% symptomatic roots); 3 = 50 to 74% of the bulbs with pink root (each bulb has < 50% symptomatic roots); 4 = $\geq$ 75% of the bulbs with pink root (each bulb has < 50% symptomatic roots); 5 = $\leq$ 25% of the bulbs with pink root (each bulb has $\geq$ 50% symptomatic roots); 6 = 25 to 49% of the bulbs with pink root (each bulb has > 50% symptomatic roots); 7 = 50 to 74% of the bulbs with pink root (each bulb has > 50% symptomatic roots); and 8 = $\geq$ 75% of the bulbs with pink root (each bulb has > 50% symptomatic roots) (Figure 2.1).

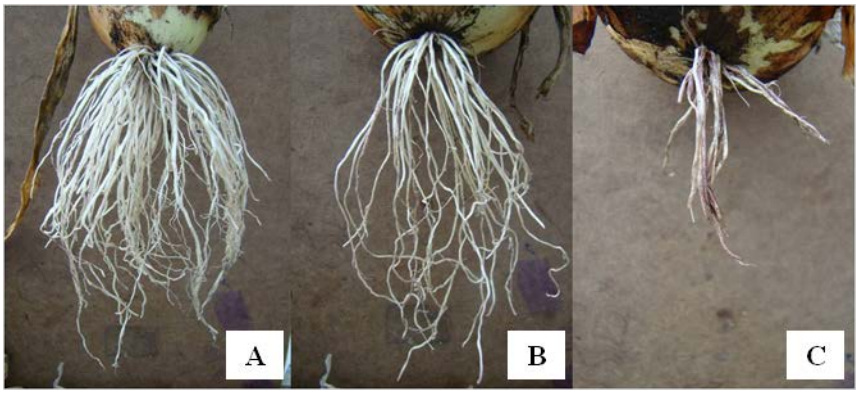


Figure 2.1. Infected root rating used in 2013 for onion cultivars, where A = no symptoms, B = < 50% of the roots symptomatic, and C = > 50% of the roots symptomatic.

Table 2.1. Onion cultivars evaluated for their susceptibility to pink root in naturally infested fields during 2011 through 2013.

Cultivar	Company[y]	Maturity (days)	Bulb color	Year tested
Candy	Seminis	85-90	Yellow	2011, 2012, 2013
Highlander	Am. Takii	85-90	Yellow	2011, 2012, 2013
Sherman	Bejo	94	Yellow	2012, 2013
Frontier	Am. Takii	98	Yellow	2012
Trailblazer	Am. Takii	98-103	Yellow	2012
Pulsar	Nunhems	100-105	Yellow	2011, 2012
Marco	Solar Seeds	100-110	Yellow	2011, 2012
Polo	Solar Seeds	100-110	Yellow	2011, 2012
Madras	Bejo	102	Yellow	2012, 2013
Patterson	Bejo	104	Yellow	2012
Hamlet	Seminis	105	Yellow	2011, 2012, 2013
Prince	Bejo	105	Yellow	2011, 2012, 2013
Infinity	Nunhems	105-100	Yellow	2011, 2012, 2013
Hendrix	Nunhems	105-110	Yellow	2011, 2012, 2013
Milestone	Am. Takii	105-110	Yellow	2011, 2012
Safrane	Bejo	106	Yellow	2012, 2013
Talon	Bejo	106-110	Yellow	2011, 2012
Braddock	Bejo	107	Yellow	2012
Stanley	Solar Seeds	110	Yellow	2011, 2012, 2013
Scorpion	Crookham	110	Yellow	2012
Livingston	Solar Seeds	112	Yellow	2011, 2012, 2013
Vespucci	Siegers	115	Yellow	2011, 2012
Pontiac	Crookham	115	Yellow	2012, 2013
Redwing	Bejo	115-118	Red	2011, 2012, 2013
Delgado	Bejo	116-118	Yellow	2012
Bradley	Bejo	118	Yellow	2011, 2012, 2013
Latigo	Seedway	118	Yellow	2012, 2013
Scout	Crookham	118	Yellow	2012
Sedona	Bejo	120	Yellow	2012, 2013
N7406	Nunhems	N/D[z]	Yellow	2012

[y]Am. Takii = American Takii, Inc., Salinas, CA; Bejo = Bejo Seeds Inc., Oceano, CA; Crookham = Crookham Company, Caldwell, ID; Nunhems = Nunhems USA, Inc., Parma, ID; Seedway = Seedway LLC, Hall, NY; Seminis = Seminis Vegetable Seeds, Inc., St. Louis, MO; Siegers = Siegers Seed Co., Holland, MI; Solar Seeds = Solar Seeds Inc., Eustis, FL.

[z]N/D = no data.

Fungicide evaluation. Two isolates of *S. terrestris* (11VS2310 and 12BYC1) isolated from symptomatic roots of onions grown in Grant in 2011 and Byron Center in 2012 were randomly chosen and used for inoculum. A colonized agar plug of each isolate was transferred onto cornmeal agar, incubated under darkness for one week then moved to an environment with a 13 h light/11 h dark cycle for one week to enhance spore production. All incubation periods were maintained at 26 ± 1°C. The spore suspension was prepared by collecting pycnidia using a sterile scalpel blade and putting them into a 15-mL sterile centrifuge tube containing 5 mL sterile distilled water. The test tube was shaken vigorously to release the conidia. The conidial suspension of each isolate was adjusted to 10^5 spores/mL with sterile distilled water. Ten milliliters of the spore suspension from each of the two isolates were mixed to inoculate sterile millet seeds. Millet seed (600 g) was mixed with water (423 mL) in a clear autoclavable plastic bag, autoclaved for two consecutive cycles at 121°C for 75 minutes, and allowed to cool at room temperature. The sterile millet seed was then inoculated with 20 mL of the conidial suspension and incubated at 26 ± 1°C for 4 weeks.

Nine fungicides were evaluated for their ability to limit pink root disease in a duplicated greenhouse trial conducted on the campus of Michigan State University. The experiment was a completely randomized design with ten replicates. An experimental unit was one 'Highlander' seedling at the three-true-leaf stage (6 weeks old) grown in a 5.5 x 7.5 x 5.0 cm^3 plastic pot containing autoclaved muck soil which was taken from east side of the Plant Pathology farm at Michigan State University. Seedlings were inoculated with 12 grams of millet seeds infested with *S. terrestris*. An equal amount of sterile millet seeds was added to the un-inoculated control.

Fungicides selected for this study (Table 2.2) are commercially available products registered to control foliar diseases of dry bulb onions. The fungicides were applied as a soil drench 0 days post inoculation (dpi) or at 0 and 14 dpi. Fungicide rates were obtained from the manufacturer labels with the exception of penthiopyrad that was not yet labeled at the initiation of these trials. At 49 dpi, the number of leaves per plant was counted. Plant height was measured from the soil line to the tip of the tallest leaf. At 55 dpi, onions were carefully removed from the pots and the roots washed with tap water. The entire bulb with attached foliage was weighed for a fresh weight measurement. Bulb circumference was determined at the widest point of the bulb or stem base. Onion roots were visually assessed for density using a scale from 1 to 5, where 1 = low (0 to 20%), 2 = intermediate-low (>20 to 40%), 3 = intermediate (>40 to 60%), 4 = intermediate-high (>60% to 80%), and 5 = high (>80%) (Figure 2.2).

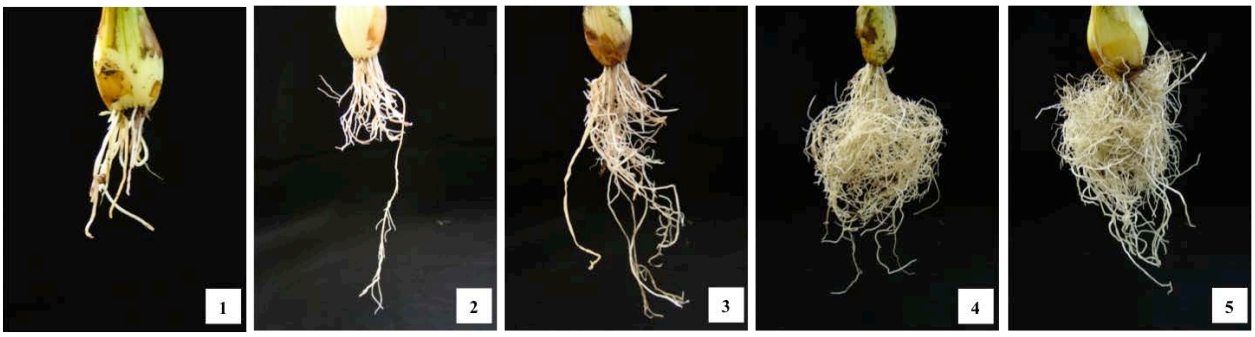


Figure 2.2. Scale used to rate root density on onion following inoculation with *S. terrestris* to evaluate the response to fungicides; 1 = low (0 to 20%), 2 = intermediate-low (>20 to 40%), 3 = intermediate (>40 to 60%), 4 = intermediate-high (>60% to 80%), and 5 = high (>80%).

Table 2.2. Fungicides and application rates evaluated for efficacy against pink root caused by *Setophoma terrestris* on 'Highlander' onion in a greenhouse trial.

Active ingredient (a.i.)	Trade name	FRAC code[z]	Amount of product/liter	Registered as a soil application
fludioxonil	Cannonball[1]	3	1.6 g	Yes
penthiopyrad	Fontelis[2]	7	11.1 mL	Yes
difenoconazole	Inspire[1]	3	1.7 mL	No
cyprodinil + difenoconazole	Inspire Super[1]	9, 3	4.8 mL	No
azoxystrobin	Quadris Flowable[1]	11	4.5 mL	Yes
azoxystrobin + difenoconazole	Quadris Top[1]	11, 3	3.4 mL	No
cyprodinil + fludioxonil	Switch 62.5WG[1]	9, 12	3.2 g	Yes
cyprodinil	Vangard[1]	9	2.3 g	No
Bacillus subtilis	Serenade Soil[3]	--	29.3 mL	Yes

[1]Syngenta = Syngenta Crop Protection, Inc., Greensboro, NC; [2]DuPont = E. I. du Pont Nemours and Co., Wilmington, DE; [3]Bayer = Bayer CropScience LP, Research Triangle Park, NC.

[z]FRAC = Fungicide Resistance Action Committee.

Pathogen reisolation and confirmation. At the conclusion of each experiment after the onions were harvested and evaluated for root rot symptoms and plant growth, three plants were randomly sampled from each replicate from the cultivar and fungicide trials. Root surfaces were disinfected with 0.5% sodium hypochlorite solution for 10 minutes, rinsed three times with sterile distilled water, and blotted dry with paper toweling. Five excised roots were placed on water agar and incubated under darkness for 7 days and then placed under 13-h light/11-h dark for an additional 7 days at 26 ± 1°C. Isolated fungal cultures were identified as *S. terrestris* using morphological characteristics.

Statistical analysis. For the cultivar trials in 2011 and 2012, disease (%) was analyzed with analysis of variance (ANOVA) using the PROC MIXED procedure. Trials and blocks were considered random variables. Cultivars were considered fixed variables. Data were tested for

normality and homogeneity of variance (Levene's test) of the residuals. In 2013, the disease severity was analyzed using the non-parametric procedure by PROC NPAR1WAY procedure of the SAS statistical analysis software version 9.3 (SAS Institute Inc., Cary, NC). For the fungicide trial, means of the number of total leaves per plant, plant height, fresh weight, and bulb circumference were analyzed separately by analysis of variance (ANOVA) using the PROC MIXED procedure. Trials were considered a random variable. Fungicide treatments were considered fixed variables. Data were tested for normality and homogeneity of variance (Levene's test) of the residuals. The root density was compared among treatments by multinomial distribution using the PROC GLIMMIX procedure (SAS Institute Inc., Cary, NC).

Environmental monitoring. For the cultivar trials, daily temperature and precipitation were monitored using the weather-based information system (MSU Enviro-weather website). The East Lansing (HTRC) weather station was used for fields located in Lansing, MI (2011 and 2012) and the Commerce Township station was used for a field located in Stockbridge, MI (2013). For the fungicide trials, the onions were grown in the greenhouse for 55 days at temperatures ranging from 18.2°C to 39.5°C (avg. 27.6°C) and relative humidity ranging from 2.9% to 73.5% (avg. 17.6%) monitored using a WatchDog A-series data logger model A150.

RESULTS

Cultivar evaluation. Pink root rot symptoms were first detected 8 (2011), 6 (2012), and 7 (2013) weeks after seeding and included a light-pink coloration (Figure 2.3A) and shriveling near the root tips; some roots were nearly disintegrated. Disease symptoms on more mature plants included dark-pink colored roots that appeared dried and decayed; newly produced roots were also pink. Dark-brown pycnidia covered with setae were isolated from four symptomatic plants of every cultivar (Figure 2.3B) and identified as *S. terrestris*.

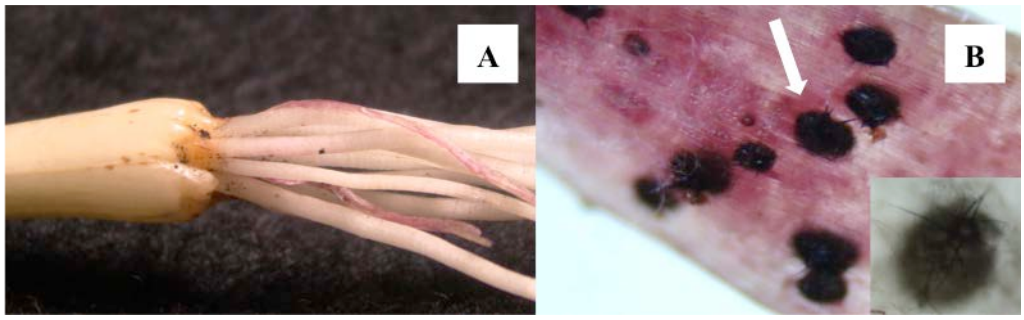


Figure 2.3. Symptoms of pink root rot on onion seedling sampled 57 days after seeding (A), dark-brown setose pycnidia formed on diseased roots after incubation under laboratory conditions (B).

In general, the disease was more severe based on percentages of diseased roots in 2012 than in 2011 except at 76 days; infected root severity (%) was higher in 2011 than in 2012. Significant differences among cultivars, between years, and the cultivar x year interaction were observed (Table 2.3). However, there was no significant difference among cultivars evaluated 64 days after seeding in 2011 when comparisons were made using Tukey's HSD test ($\alpha = 0.05$). According to the final rating (110 days after seeding) in 2011 and 2012, 'Highlander' was highly susceptible, while 'Hendrix' was the least susceptible to pink root (Table 2.4). When additional cultivars were evaluated in a second trial during 2012, 'Highlander' and 'Hendrix' were included as the highly susceptible and less susceptible cultivars, respectively (Table 2.5). The cultivars differed significantly ($P < 0.001$) for each disease rating. At the final rating, twelve cultivars were significantly less susceptible to pink root than 'Highlander'. 'Braddock' had the fewest diseased roots followed by 'Patterson', 'Sedona', and 'Latigo'; they had fewer diseased roots than 'Hendrix' but were statistically similar to 'Hendrix'. There were significant ($P = 0.0009$) differences of disease severity among the 16 cultivars tested in 2013. The severity of pink root

was lowest (4.75) in ‘Sedona’ but highest (8.00) in ‘Highlander’. The cultivar Hendrix was intermediate-low in disease severity (5.25), following the cultivar Sedona and Redwing. Yields of each cultivar were determined based on bulb diameter (i.e. small, medium, large) but the data were not statistically compared among cultivars due to the differences of their genetic inheritance (Table 2.6).

Table 2.3. Effect of interactions between cultivar and year, cultivars, and years on diseased roots (%) evaluated 64, 76, 96, and 110 days after seeding in the field during 2011 through 2012.

Effect	*P* values- type III tests of fixed effects			
	64	76	96	110
Cultivar x year	< 0.0001	0.0121	< 0.0001	0.0198
Cultivar	< 0.0001	< 0.0001	0.0001	< 0.0001
Year	< 0.0001	< 0.0001	0.8005	< 0.0001

Table 2.4. Diseased roots (%) assessed 64, 76, 96, and 110 days after sowing seeds in 2011 and 2102 for a field trial. Means with a common letter do not differ significantly based on Tukey's HSD test at $\alpha = 0.05$.

Cultivar	Sampling (days after seeding)											
	64				76				96				110			
	2011		2012		2011		2012		2011		2012		2011		2012	
Highlander	6.16	a	14.81	a-c	25.14	b	13.83	a-e	28.66	a	25.55	ab	33.96	a	34.05	ab
Hendrix	9.41	a	10.36	cd	25.56	b	14.51	a-e	14.15	de	22.36	a-e	15.01	h	23.64	e
Candy	9.76	a	18.33	ab	33.01	b	16.26	a-e	27.31	ab	25.21	ab	24.92	c-e	36.92	a
Infinity	15.02	a	12.56	a-d	27.90	b	21.34	ab	17.09	cd	25.25	ab	26.04	b-e	34.70	ab
Marco	12.62	a	10.22	cd	23.50	b	13.90	a-e	21.01	c	24.78	ab	28.26	a-d	33.87	ab
Talon	8.56	a	10.56	b-d	34.66	a	18.98	a-c	13.53	de	21.22	b-e	17.08	gh	33.09	ab
Hamlet	4.85	a	8.86	cd	34.00	a	17.70	a-d	21.70	bc	23.74	a-c	26.01	b-e	31.89	a-c
Milestone	11.32	a	20.22	a	23.22	b	22.71	a	20.57	c	24.78	ab	29.39	a-c	31.86	a-c
Vespucci	8.75	a	10.61	b-d	28.01	b	14.25	a-e	18.79	cd	26.59	a	31.78	ab	31.67	a-c
Pulsar	4.99	a	13.65	a-c	30.46	b	18.00	a-d	16.94	cd	23.32	a-d	22.87	c-g	30.92	a-c
Polo	5.19	a	9.31	cd	24.27	b	12.27	b-e	15.93	c-e	19.16	c-e	24.24	c-f	30.63	a-c
Bradley	6.31	a	10.98	b-d	24.47	b	19.12	a-c	9.82	e	20.81	b-e	18.63	f-h	30.32	b-d
Stanley	4.92	a	11.94	b-d	23.41	b	9.06	de	17.40	cd	19.16	c-e	19.70	e-h	26.65	c-e
Prince	10.25	a	8.06	cd	20.24	b	19.14	a-c	15.34	c-e	18.70	de	21.82	d-h	26.48	c-e
Livingston	7.16	a	3.11	e	23.21	b	9.57	c-e	18.31	cd	22.07	a-e	23.70	c-f	24.06	de
Redwing	6.81	a	5.57	de	22.08	b	7.69	e	18.32	cd	18.19	e	17.60	f-h	21.58	e

Table 2.5. Diseased roots (%) assessed 64, 76, 96, and 110 days after sowing seeds in a 2102 field trial. Means with a common letter do not differ significantly based on Tukey's HSD test at $\alpha = 0.05$.

Cultivar	Sampling (days after seeding)			
	64	76	96	110
Highlander	22.50 a-c	13.12 d	25.15 b-f	45.35 a
Hendrix	17.84 a-d	18.80 a-d	22.29 d-g	28.96 d-g
Frontier	13.99 cd	16.15 b-d	26.80 a-d	42.61 ab
Scout	24.29 ab	25.29 a-c	30.72 ab	39.50 a-c
Scorpion	24.34 c	26.52 ab	31.19 a	37.09 bc
Sherman	19.37 a-d	20.52 a-d	28.16 a-c	35.13 cd
Trailblazer	13.49 cd	15.92 cd	27.96 a-d	33.75 c-e
Pontiac	22.78 a-d	20.76 a-d	29.17 a-c	31.21 d-f
Safrane	19.44 a-d	11.85 d	19.72 fg	30.81 d-f
Madras	16.79 a-d	14.68 d	24.36 c-f	30.55 d-f
7406	17.17 a-d	19.17 a-d	27.48 a-d	30.17 d-f
Delgado	20.79 a-d	19.07 a-d	24.40 c-f	27.93 e-g
Latigo	24.05 ab	29.38 d	26.21 a-e	26.84 fg
Sedona	16.28 a-d	17.15 b-d	20.71 e-g	25.05 fg
Patterson	14.22 b-d	14.27 d	20.31 fg	23.34 g
Braddock	12.91 d	17.93 b-d	16.56 g	22.96 g

Table 2.6. Mean pink root disease severity of 16 onion cultivars assessed in 2013. Means with a common letter do not differ significantly based on Kruskal-Wallis test at $\alpha = 0.05$.

Cultivar	Mean of disease severity[y]		Weight (kg)[z]		
			Small	Medium	Total
Highlander	8.00	a	0.50	1.59	2.09
Hendrix	5.25	de	0.36	2.15	2.51
Livingston	7.00	b	0.15	2.75	2.90
Safrane	6.75	b	0.38	2.38	2.76
Sherman	6.50	bc	0.15	2.31	2.46
Madras	6.50	bc	0.37	2.09	2.46
Stanley	6.00	cd	0.80	1.36	2.16
Candy	5.75	cd	0.49	1.90	2.39
Pontiac	5.75	cd	0.40	1.75	2.15
Hamlet	5.75	cd	0.70	1.45	2.15
Prince	5.50	de	0.63	1.75	2.37
Bradley	5.50	de	0.53	1.95	2.48
Latigo	5.50	de	0.12	1.95	2.07
Infinity	5.25	de	0.39	1.79	2.17
Redwing	5.00	de	0.59	1.47	2.07
Sedona	4.75	e	0.54	1.25	1.79

[y]Means of disease severity were evaluated from 25 bulbs /replicate with a total of four replicates.

[z]Weights were taken based on the bulb diameters: small = the bulb diameters are less than 5.08 cm, medium = the bulb diameters are equal or greater than 5.08 cm but less than 7.62 cm, large = the bulb diameters are equal to or greater than 7.62 cm. (Large bulbs were not produced in this trial).

Table 2.7. Meteorological data during onion production in fields located in Lansing in 2011 and 2012 and in Stockbridge in 2013.

Month	Temperature (°C)			Rainfall (mm)		
	2011	2012	2013	2011	2012	2013
April	N/D	1.9 – 14.7* (8.3)	N/D	N/D	44.2** (1.5)	N/D
May	9.7 – 20.3 (15.0)	10.2 – 23.3 (16.8)	8.1 – 22.1 (15.1)	146.3 (2.3)	62.0 (2.0)	76.7 (2.5)
June	14.1 – 25.4 (19.8)	13.7 – 26.9 (20.3)	13.2 – 24.3 (18.7)	40.1 (1.3)	26.7 (1.0)	127.3 (4.3)
July	18.1 – 30.2 (24.2)	17.6 – 31.1 (24.4)	16.1 – 26.7 (21.3)	129.5 (4.1)	36.1 (1.3)	99.8 (3.3)
August	15.1 – 26.8 (20.9)	14.3 – 27.3 (20.8)	14.2 – 25.6 (19.9)	78.2 (2.5)	52.8 (1.8)	64.3 (2.0)
September	10.8 – 21.1 (15.9)	N/D	N/D	67.3 (2.3)	N/D	N/D

N/D = data were not determined; *minimum – maximum temperature; **total rainfall; values in parentheses indicate average temperature and rainfall per day.

Fungicide evaluation. All inoculated plants regardless of treatment developed disease symptoms on the roots, while uninoculated plants remained asymptomatic. *Setophoma terrestris*, producing dark brown setose pycnidia bearing hyaline, one-celled conidia, was consistently isolated from symptomatic roots. According to the parameters measured (i.e. total leaves, plant height, plant fresh weight, bulb circumference, and root density) plants inoculated with *S. terrestis* were smaller than uninoculated controls (Figure 2.4). Significant differences of variances of root density ($F = 19.38$, $P < 0.0001$) and plant height ($F = 10.06$, $P = 0.0016$) were observed between replicates. Therefore, the data of the two trials were analyzed separately; data for the other measurements were pooled for analysis. Each parameter measured for plant growth differed significantly ($P < 0.0001$) among the fungicide treatments (Figure 2.5). Penthiopyrad applied either at 0 dpi or 0 and 14 dpi resulted in increased plant growth compared to other

fungicide treatments and the untreated inoculated control. However, onions treated with penthiopyrad were smaller than the untreated uninoculated controls.

Phytotoxicity was observed on onions drenched with azoxystrobin, azoxystrobin + difenoconazole, cyprodinil, cyprodinil + difenoconazole, and cyprodinil + fludioxonil. Symptoms of phytotoxicity appeared as leaf necrosis with various degrees of damage, including minor, moderate, and severe injury (Figure A1). Two applications (0 and 14 dpi) of these fungicides resulted in an increased percentage of plants with phytotoxicity and increased levels of necrotic leaf tissue compared to symptoms associated with one application (0 dpi). In addition, cyprodinil alone and the mixture cyprodinil + difenoconazol, and cyprodinil + fludioxonil caused more injury on the plants than azoxystrobin or azoxystrobin + difenoconazole (Figure 2.6).

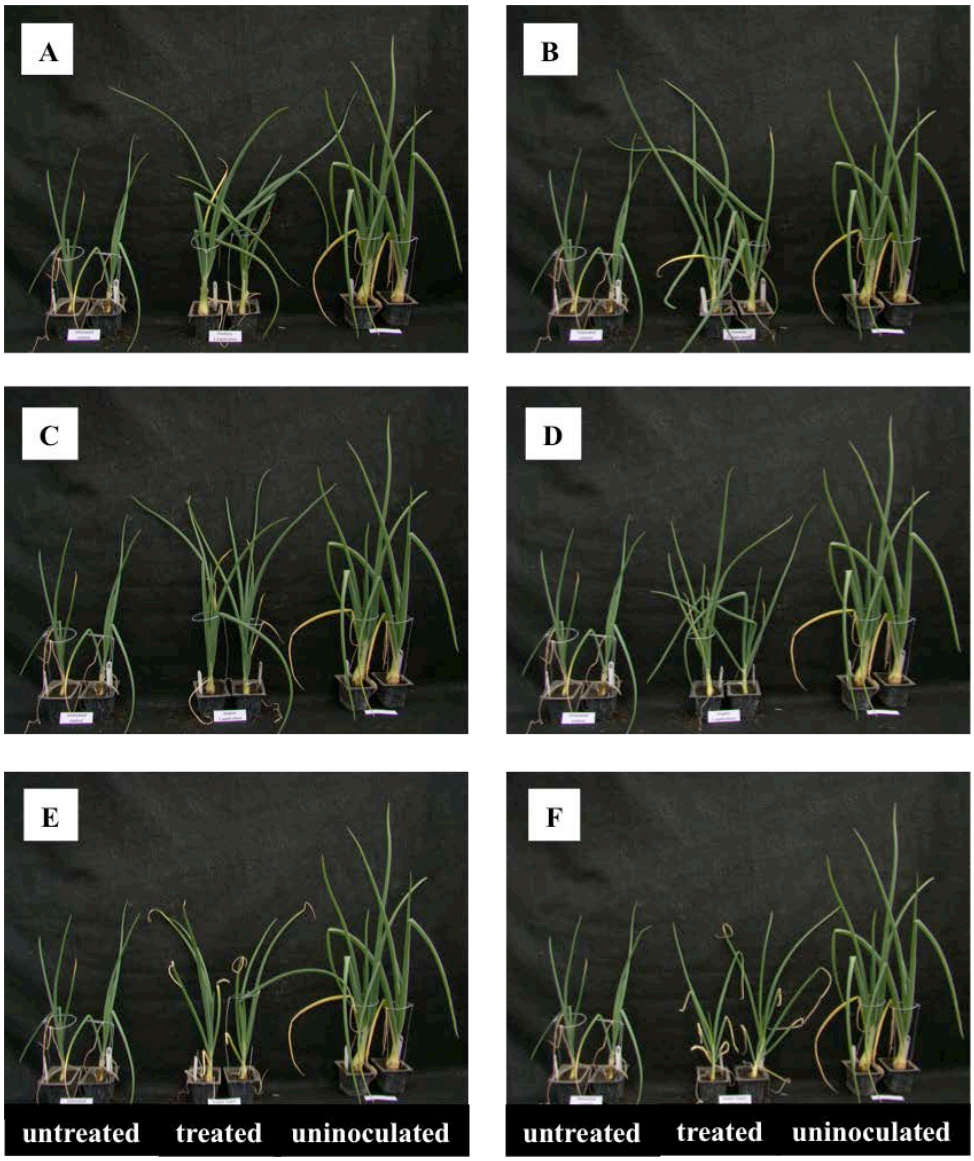


Figure 2.4. Response of onions to fungicides 55 days after inoculation comparing an untreated control (left), fungicide treatment (middle), and uninoculated control (right). Fungicide drenches were applied once (0 dpi) or twice (0 and 14 dpi) and included penthiopyrad (A, B), difenoconazole (C, D), cyprodinil + difenoconazole (E, F), azoxystrobin + difenoconazole (G, H), azoxystrobin (I, J), cyprodinil + fludioxonil (K, L), fludioxonil (M, N), *Bacillus subtilis* (O, P), and cyprodinil (Q, R). Plants treated with one application are shown in pictures A, C, E, G, I, K, M, O, and Q, and two applications are shown in pictures B, D, F, H, J, L, N, P, and R.

Figure 2.4. (cont'd).

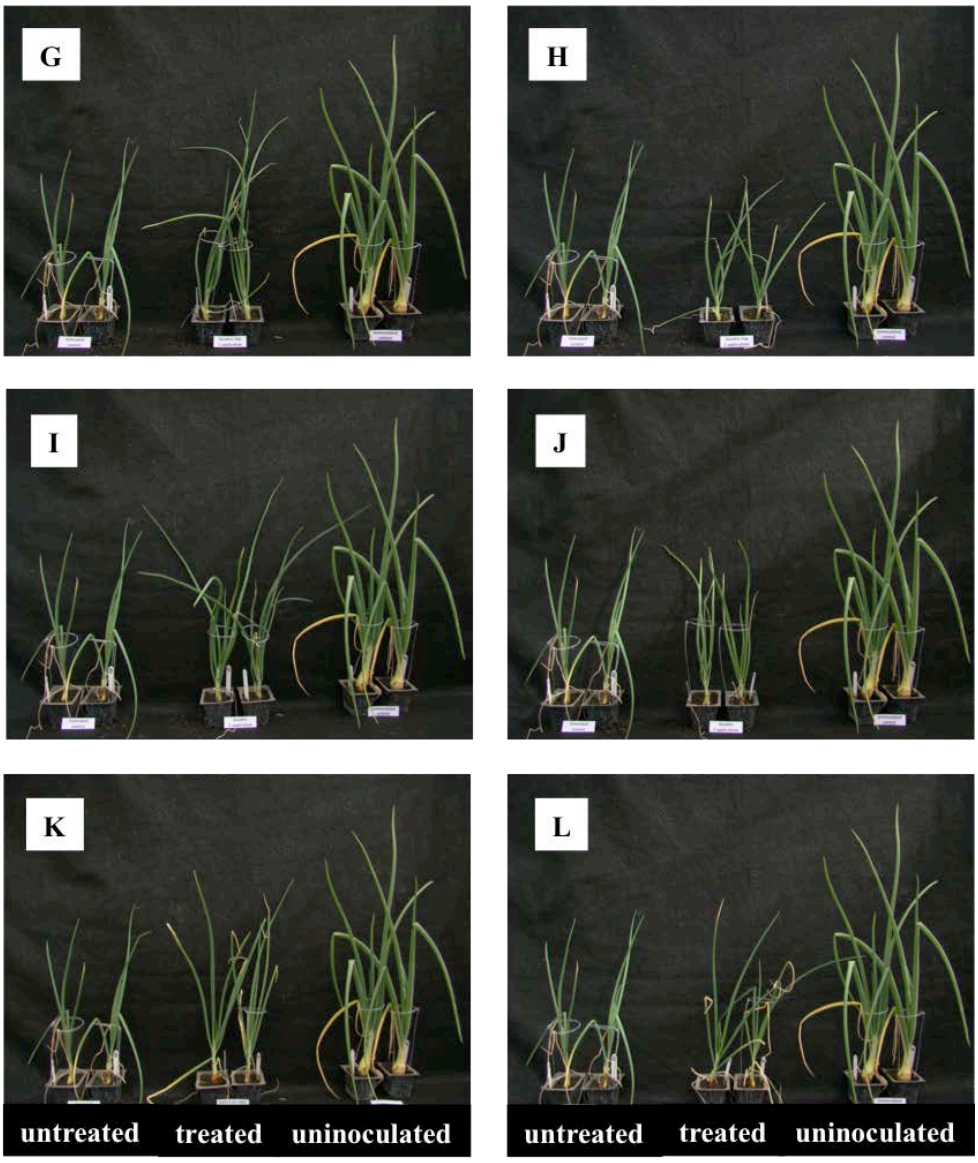
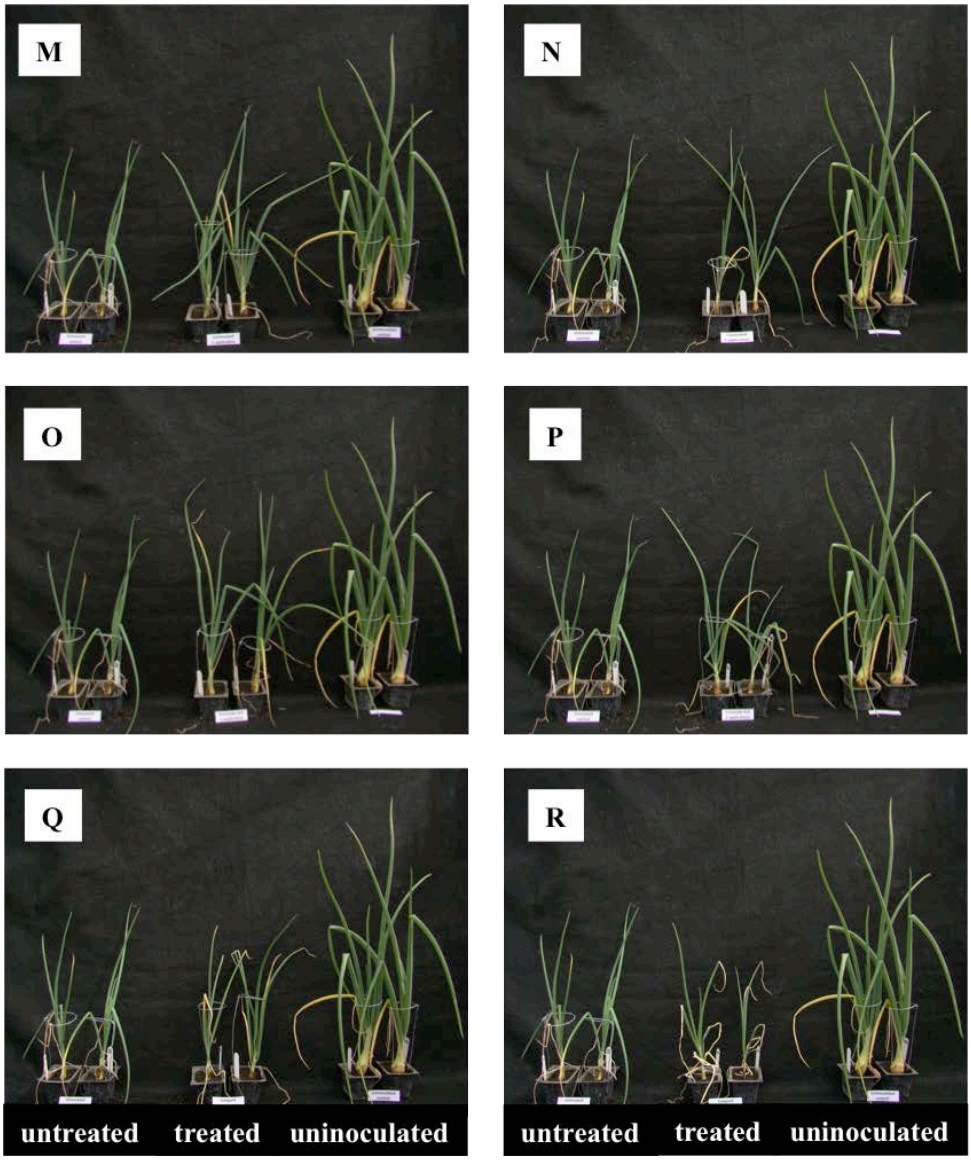


Figure 2.4. (cont'd).



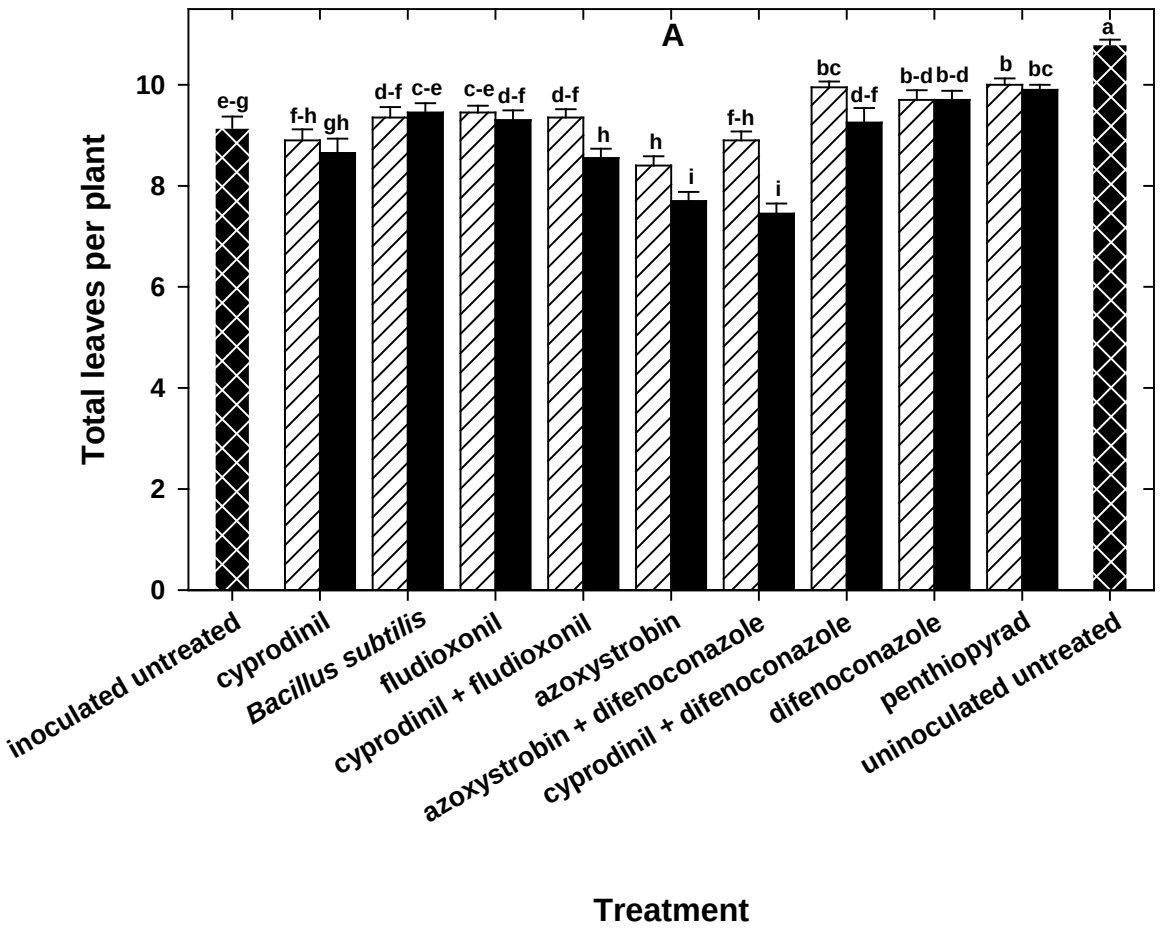
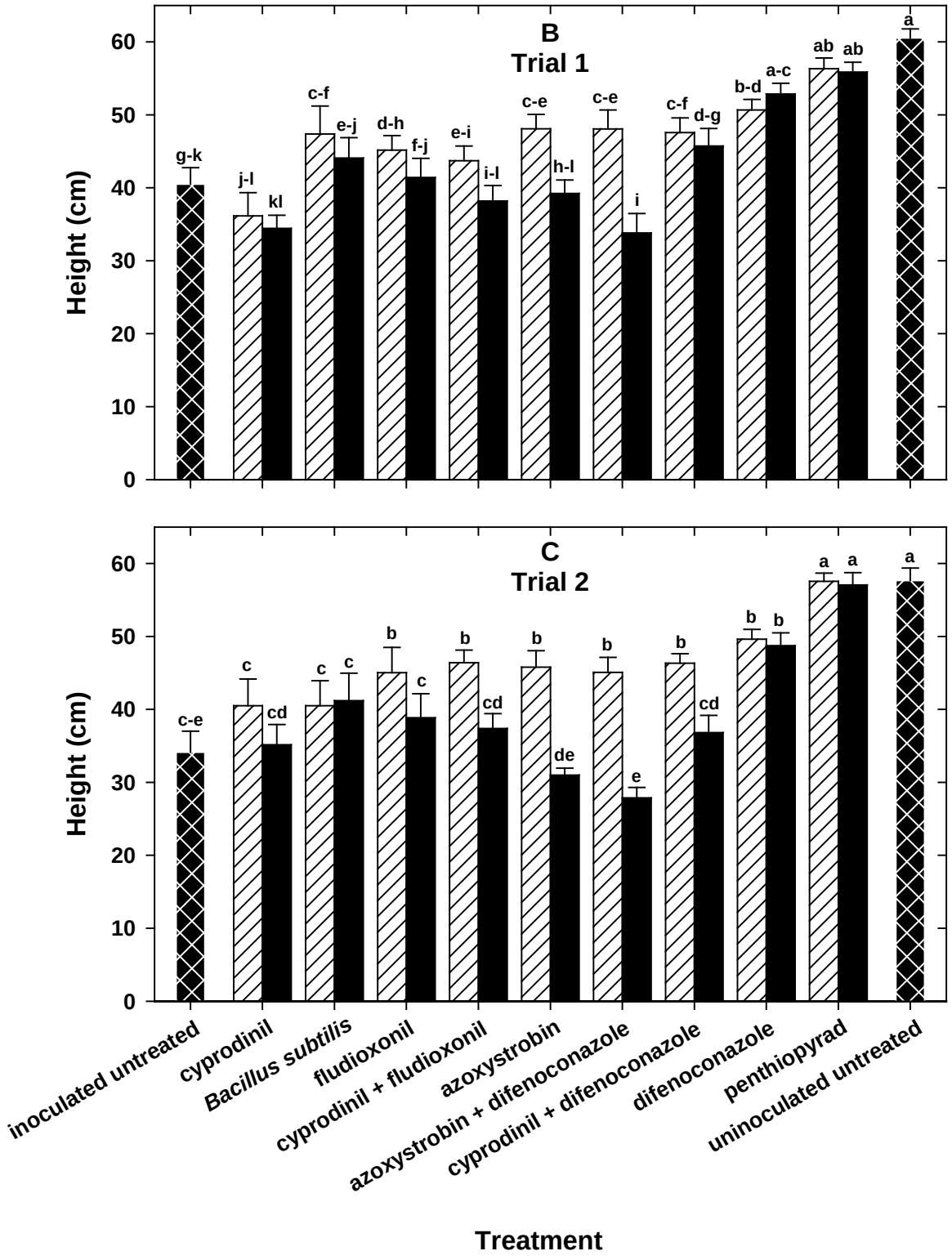


Figure 2.5. Plant development parameters measured in response to fungicide treatment at 0 day post inoculation (dpi) (white bars with diagonal lines) or 0 and 14 dpi (black bars) for control of pink root on onions under greenhouse conditions including: number of leaves per plant (A), plant height (B: trial 1, C: trial 2), plant fresh weight (D), bulb circumference (E), and root density (F: trial 1, G: trial 2). Bars are the means of fungicide treatment and inoculated untreated and uninoculated untreated controls (black bars with crosshatch lines) and ten replicates from two trials. Error bars represent the standard error of the mean. Means with a common letter do not differ significantly ($\alpha = 0.05$).

Figure 2.5. (cont'd).



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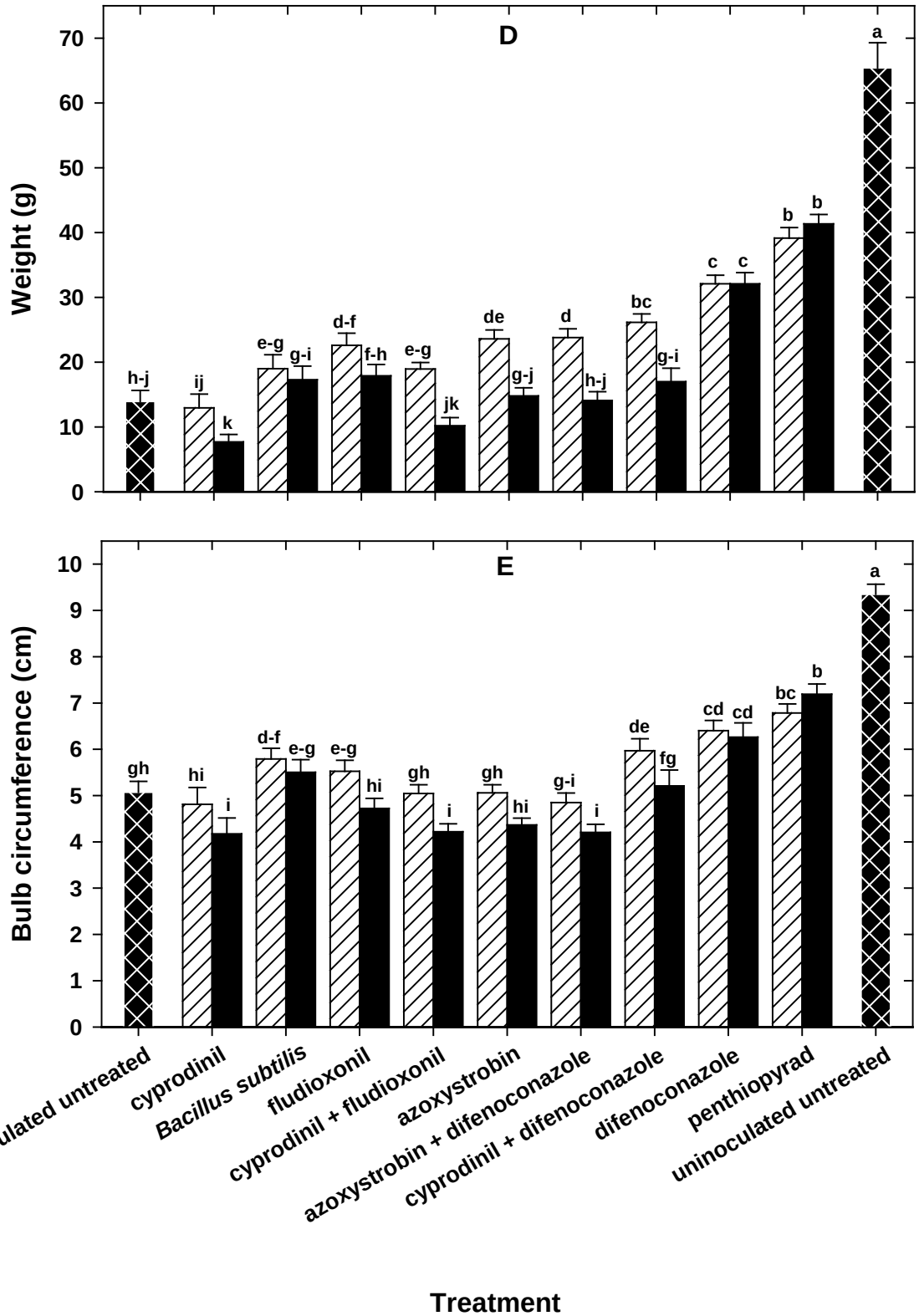

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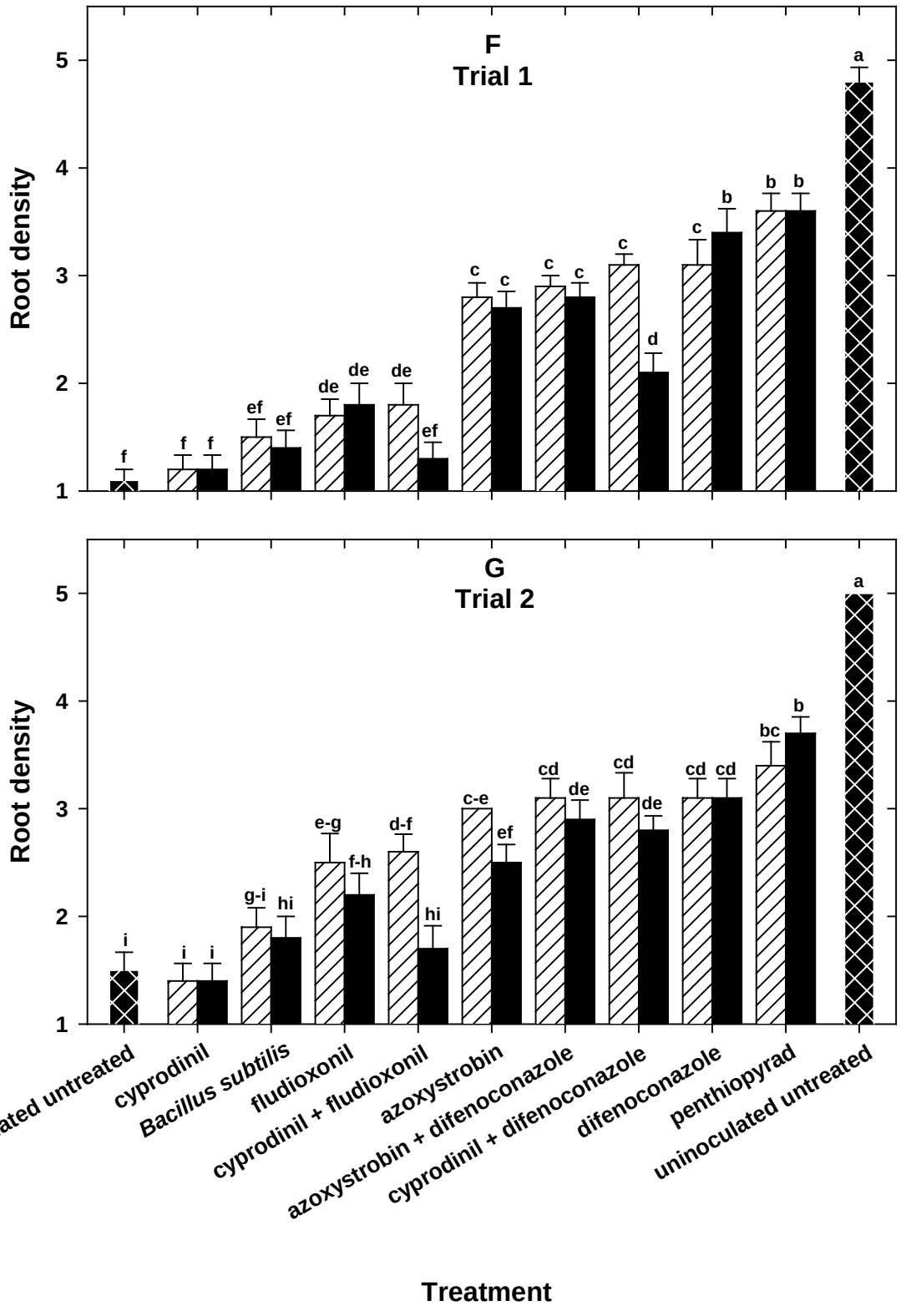

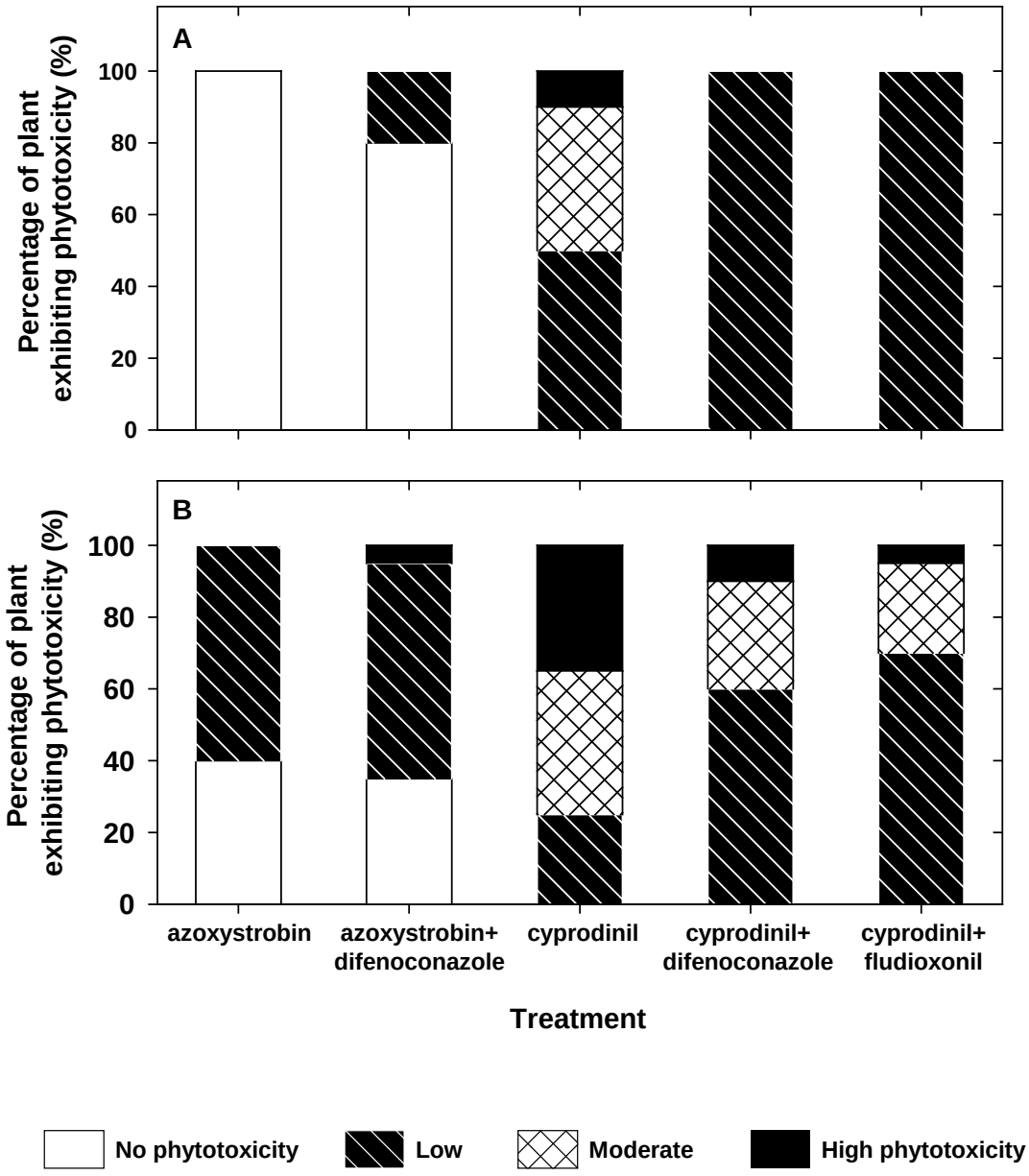

Figure 2.6. Plants (%) showing symptoms of phytotoxicity following drench applications at 0 dpi (A) and applications at 0 and 14 dpi (B). White bars represent plants without phytotoxicity, black bars with diagonal lines represent plants with minor necrotic leaf tissue, white bars with crosshatch lines represent plants with moderate necrosis, and black bars represent plants with severe necrotic leaf tissue.

DISCUSSION

Onion roots can become infected by *S. terrestris* at any stage of plant development (Coleman et al. 1997; Davis and Handerson 1937; Rabinowitch et al. 1981) when environmental conditions are conducive. Coleman et al. (1997) reported that the pink root pathogen infects onion roots early in the season: asymptomatic roots of 6 to 8 week old onions turned pink after laboratory incubation. In our study, symptoms exhibited during routine scouting from 2011 to 2013 for the presence of pink root were observed as early as 6 to 8 weeks, after seeds were planted suggesting that infection could occur earlier than 6 weeks since the symptoms observed at sampling were developed.

None of the 30 cultivars tested were resistant to pink root and disease symptoms were observed in varying degrees on all cultivars. Differences in pink root susceptibility were detected among the cultivars; 'Hendrix', 'Redwing', and 'Sedona' were least susceptible and 'Highlander' was most susceptible to pink root. Less susceptible cultivars may be able to replace infected roots with healthy new roots to compensate for the damage caused by the pathogen (Levy and Gornik 1981).

Results were similar from year to year, even though two disease rating systems were used to assess susceptibility. For example, 'Hendrix' had a relatively low percentage of infected root compared to the other 15 cultivars when diseased roots (%) were calculated in 2011 and 2012; a similar trend was observed in 2013 when a severity rating was applied. Determining the percentage of diseased roots via counting was tedious and time consuming whereas using severity scale provided similar results and required less time.

Results from the 16 cultivars tested in both 2011 and 2012 showed that the cultivar and year significantly influenced disease severity. Generally, the percentage of diseased roots was

higher in 2012 than in 2011. The difference between the two years may have been due to the environmental conditions. Rainfall was lower in 2012 (1.78 mm/d) than in 2011 (3.05 mm/d) potentially resulting in stress for infected onion plants with a root system limited by disease. The average temperature during the same period was higher in 2011 than in 2012; however, the temperatures during both years allowed the pathogen to grow and cause infection. In addition, disease resistance can be affected by different seed lots (Utkhede and Rahe 1984). Nichols et al. (1965) revealed that onion seedlings exhibiting poor top growth produced from low quality seed lots were more susceptible to pink root than high quality seed lots of the same lines.

In the fungicide study, plant growth in the presence of the pathogen was poor in the absence of fungicide treatment. Some fungicide treatments improved plant development when onions were grown in soil infested with high levels of *S. terrestris*. Some fungicides reduced the adverse effects of the pathogen on onion growth compared to the untreated check, even though disease symptoms were not eliminated. Among the fungicides evaluated, penthiopyrad applied either once and twice resulted in significantly increased plant development under high inoculum pressure. In contrast, the plant growth was limited following one application of cyprodinil. When two drenches were applied, azoxystrobin, azoxystrobin + difenoconazole, cyprodinil, cyprodinil + difenoconazole, or cyprodinil + fludioxonil exhibited phytotoxic effects.

Leaf necrosis developed on plants treated with azoxystrobin, azoxystrobin + difenoconazole, cyprodinil, cyprodinil + difenoconazole, or cyprodinil + fludioxonil, but did not occur on the untreated controls. Azoxystrobin and cyprodinil were likely involved in plant injury because neither difenoconazole nor fludioxonil by itself produced necrotic symptoms. The incidence of plants exhibiting phytotoxicity was higher when treated with two applications than one application. For example, 60% of plants showed phytotoxicity after being treated with

two drenches of azoxystrobin, while none of the plants treated with one application of the same fungicide displayed leaf necrosis. The incidence of plants with a higher degree of fungicide injury was observed on plants treated with two applications, such as the cyprodinil treatment. It should be noted that plant injury can be resulted by misuse or misapplication of fungicides (Windham and Windham 2013). In this study, only azoxystrobin, *Bacillus subtilis*, cyprodinil + fludioxonil, fludioxonil, and penthiopyrad are registered for application by soil drench; outside of experimental uses, fungicides may only be applied according to the label.

In this study, treatment with penthiopyrad and cultivar selection improved pink root control and could offer management options for Michigan onion growers. Planting onion cultivars that display reduced pink root susceptibility such as 'Hendrix' and 'Sedona' may limit inoculum build-up in the soil compared to more susceptible cultivars such as 'Highlander'. Even though Michigan producers currently favor 'Highlander' due to its overall suitability to the state's growing conditions, the continued use of this pink root susceptible cultivar could promote pathogen inoculum to increase over time, thereby risking crop failure.

CHAPTER 3: INFLUENCE OF ONION PLANT AGE IN RESISTANCE TO *SETOPHOMA TERRESTRIS*

ABSTRACT

Pink root incited by *S. terrestris* reduces bulb size and yields and is often associated with onion maturity although the susceptibility of a particular plant age to pink root has not been investigated. The cultivars Hendrix (low susceptible) and Highlander (high susceptible) were inoculated when 3, 5, 7, or 9 weeks old and evaluated for disease. Root density, plant height, fresh weight, number of total leaves per plant, and bulb circumference were compared between control and inoculated plants of the same age. Reduction of root density was observed but was not significantly affected by onion age or cultivar ($P = 0.0515$). When 3-week old 'Highlander' was inoculated with *S. terrestris*, plant height, fresh weight, and bulb circumference were significantly limited compared to the control. When 3- or 5-week old 'Hendrix' was inoculated the bulb circumference was reduced compared to the control. Microscopic observation revealed that the frequency of root colonization was low at the young stage but increased with age. Intracellular mycelia were detected in the cells of asymptomatic roots with low frequency. Results suggest that high reduction in size of plants was resulted when the onions were infected at young stage even though pink root susceptibility of the root system increased with age. 'Hendrix' had a reduced frequency of root colonization than 'Highlander'.

INTRODUCTION

Onions are produced primarily in the southwest and south-central regions of Michigan (Hausbeck 2005) and include Allegan, Calhoun, Eaton, Ingham, Ionia, Kent, Livingston, Newaygo, and Ottawa counties. In Michigan the crop is direct seeded in early spring and harvested beginning in August. These cultivars are intermediate- and late-maturing types, requiring at least 13 hours of photoperiod to trigger bulb formation (Goldman et al. 2001), and are characterized as pungent types that can be stored postharvest.

Pink root, incited by the fungus *Setophoma terrestris*, is common in soil and a devastating disease of onions worldwide (Sumner 1995). Infected roots appear light pink in color, become dark red or purple, shrivel, die, and finally detach from the plant. The above-ground symptoms of severely infected plants may resemble drought stress (Hansen 1929). Affected plants commonly survive but are stunted and produce small bulbs (Hansen 1929; Sumner 1995; Taubenhaus and Johnson 1917; Taubenhaus and Mally 1921) due to restriction of the root system (Porter and Jones 1933). Since the root disease symptoms become obvious over time at which time the plant is maturing, Michigan growers have associated the pathogen as a secondary invader that aids in hastening the senescence of the foliage which is desirable near harvest (Hausbeck et al. 2012; Hausbeck et al. 2013). However, Levy and Gornik (1981) revealed that pink root shortens the plant growth period by 4 to 14 days of early- and intermediate-maturing cultivars, and by 18 to 45 days of late-maturing cultivars.

Studies of pink root on onion have illustrated that onions can become infected at many stages of plant development. Seedlings grown in soil inoculated with *S. terrestris* collapsed one month after seeds were sown (Davis and Handerson 1937). *In vitro* development of pink root symptoms occurred at the three-leaf-stage (6 to 8 weeks after seeding), although field symptoms

were not observed (Coleman et al. 1997). Another study showed that pink root disease was observed between 55 and 132 days after transplanting (Rabinowitch et al. 1981).

Plants may become more resistant or tolerant to pathogens as they mature, a phenomenon referred to as age-related or ontogenic resistance (Ficke et al. 2002; Panter and Jones 2002). In soybean, susceptibility to *Fusarium virguliforme* decreased (Gongora-Canul and Leandro 2011) as plants aged. Similar ontogenic resistance was observed in cucurbit fruits (Ando et al. 2009; Krasnow et al. 2014; Meyer and Hausbeck 2013) and potato (Mutty and Hossenkhan 2008). In contrast, resistance to disease may decrease as plants age and has been observed on onions infected by *Alternaria porri* (Everts and Lacy 1996), and barley and wheat infected by *Cochliobolus sativus* (Verma and Spurr 1987).

Currently, use of resistant cultivars is the most efficient strategy to reduce yield loss caused by the pink root (Porter and Jones 1933). Fungicides effective for pink root have not been identified and other practices including crop rotation and soil treatment are not feasible due to limited availability of muck soils and cost and environmental impact of pre-plant soil fumigants. Understanding the stage of onion plant development that is most conducive to infection by the pink root pathogen could be integrated with other approaches (i.e. seed treatment, banded fungicide applications following emergence, etc). The objective of this study was to determine if plant age influences the ability of *S. terrestris* to colonize onion root cells of two onion cultivars representing a high and low level of pink root susceptibility.

MATERIALS AND METHODS

Plant age at inoculation. Two isolates of *S. terrestris* (11VS2310 and 12BYC1) that originated from onion were randomly selected from the culture collection preserved in Hausbeck Lab, Michigan State University. The fungus was grown on carnation leaf agar under darkness at

26 ± 1°C for 7 days and with a 13-h/11-h light/dark period for 7 days. Fresh pycnidial suspension was prepared by collecting 4 to 5 pycnidia of each isolate using a sterile scalpel blade and delivering into a confocal tube containing 5 ml sterile distilled water. The tubes were agitated using a vortex mixer for releasing conidia. The number of conidia for each isolate was estimated using a hemacytometer; the conidial concentration was adjusted to 10^6 conidia/ml with sterile distilled water. Equal volumes (1:1 v/v) of conidial suspension of both isolates were combined and used to inoculate sterile millet seeds. Millet seeds (600 g millet and 432 ml distilled water) were added into an autoclavable plastic bag and sterilized by autoclaving for 45 min at 121°C on two successive days prior to inoculated with 10 ml of mixed pycnidial suspension, mixed thoroughly, and incubated at 26 ± 1°C for 21 days.

Onion cultivars Highlander (high susceptibility) and Hendrix (low susceptibility) were planted over a 9-week period in 72-cell plastic flats containing greenhouse-soilless media (Sure mix, Michigan Grower Product, Inc., Galesburg, MI). Onion plants were maintained in the greenhouse conditions, where temperatures were between 18.2°C and 27.4°C (avg. 26.9°C), and were fertilized daily with 200 ppm of 20-20-20 of Peters water soluble fertilizer (The Scotts Company, Marysville, OH). The experiment was performed and duplicated in a research greenhouse at Michigan State University using a completely randomized design. Each replicate consisted of eight plants per age per cultivar. The onions were transplanted into plastic pots containing autoclaved muck soil and were inoculated with 12 grams of infested millet seeds when 3, 5, 7, or 9 weeks old. Sterile millet was used as the control. At 42 days post inoculation (dpi), plant height was measured from the soil level to the tip of the tallest leaf. The onions were carefully removed from the pots and the roots were washed with tap water. The entire plant with detached roots was weighed individually for fresh weight. Bulb circumference was measured at

the widest point of the stem base or bulb. Roots were evaluated visually for density using a scale from 1 to 5; where 1 = low root density (0 to 20% of the root density compared with the root density of healthy control); 2 = intermediate-low (>20 to 40%); 3 = intermediate (>40 to 60%); 4 = intermediate-high (>60 to 80%); and 5 = high root density (>80%) (Figure 3.1).

Root colonization. Twenty-four onions of each age category (3, 5, 7, or 9 weeks old) were grown in the greenhouse as described above. Twelve plants were inoculated with infested millet seeds and the remaining twelve plants received sterile millet for a non-inoculated control. Six inoculated and non-inoculated plants of each treatment were sampled randomly at 7 and 21 dpi. The roots of each plant were washed using running tap water. From each plant, five asymptomatic roots and another five symptomatic roots were collected and segmented approximately 1.5 cm measured from the root tip. The excised roots were immersed in 10% KOH (w/v), autoclaved at 121°C for 15 minutes, rinsed with distilled water, acidified with 5% HCl for 5 minutes, rinsed again with distilled water, stained with 0.05% Trypan blue in GLW solution (1:1:1 by volume of glycerol, lactic acid, and distilled water) for 2 hours, destained overnight in GLW solution, and finally rinsed with distilled water. Twenty-five roots that had been stained from each treatment were mounted on microscope slides and observed using a light microscope with 200x magnification. One hundred views were counted for frequency of root colonization; 1= presence of intracellular mycelium in the root cells and 0 = absence. Pictures of stained roots were taken using a Leica Digital camera DFC420 (Leica Microsystems, Wetzlar, Germany).

Pathogen reisolation and confirmation. Three plants were randomly selected from each replicate. The entire length of five roots with pink discoloration were obtained from each plant, surface disinfected with 0.5% sodium hypochlorite solution for 10 minutes, rinsed 3X with

sterile distilled water, and blotted dry with paper towel. Disinfected roots were placed on water agar and incubated under darkness at 26 ± 1°C for seven days and then incubated an additional seven days under 13-h light/11-h dark conditions.

Statistical analysis. For the greenhouse trial, analysis of variance (ANOVA) of differences in plant height, number of total leaves, plant fresh weight, and bulb circumference between uninoculated and inoculated treatments were analyzed using the PROC MIXED procedure (SAS Institute, Cary, NC). Data were tested for normality (q-q plot and the Shapiro-Wilk test) and homogeneity of variance (Levene's test) of the residuals. The data were tested for the interaction between onion age at inoculation and cultivar, and significant differences were separated by least squares means (α=0.05). When the interaction was significant, main effects (plant age at inoculation and onion cultivar) were evaluated using the slice option in SAS. The root density of uninoculated and inoculated plants was analyzed separately using PROC GLIMMIX procedure with the multinomial distribution, and the onion age at inoculation and cultivar interaction was analyzed using the contrast option. For the frequency of root colonization, data was analyzed using PROC GLIMMIX with the binomial distribution.

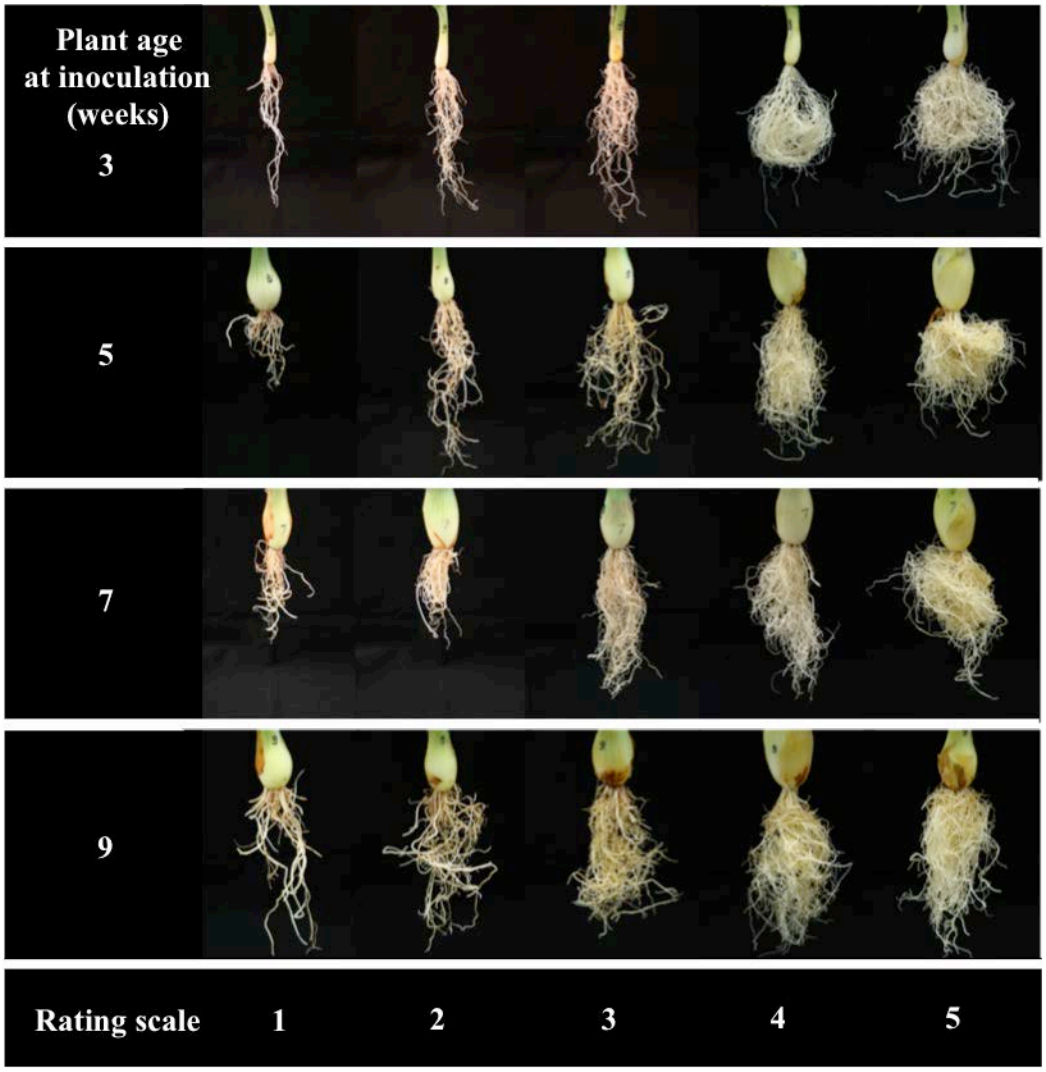

Figure 3.1. Scale used to rate root density on certain plant ages (3, 5, 7, and 9 weeks old at inoculation) after inoculation with *S. terrestris*; where 1 = low root density (0 to 20% of the root density compared with the root density of healthy control); 2 = intermediate-low (>20 to 40%); 3 = intermediate (>40 to 60%); 4 = intermediate-high (>60 to 80%); and 5 = high root density (>80%).

RESULTS

Evaluation of onion plant ages and cultivars. Pink root incidence as determined by pink-colored roots was 100% on the inoculated onions; the control plants remained healthy. The

root density of uninoculated control plants ranged from 4.25 to 4.75 compared to 1.69 to 2.63 for inoculated plants (Figure 3.2). There was no interaction between onion age at inoculation and cultivar when either the root density of the uninoculated (P = 0.3694) or the inoculated plants (P = 0.0515) were compared.

Analysis of variance revealed that the plant height and bulb circumference were affected significantly (P = 0.0399 and 0.0018) by the onion age and cultivar, whereas plant fresh weight and number of total leaves were not significantly different (P = 0.3623 and 0.2705) for age (Table 3.1). Analysis of the main effects in reduction of plant size (%) showed a significant reduction in plant height, number of total leaves per plant, and fresh weight was affected by the cultivar Highlander, and a significant reduction in bulb circumference was influenced by both cultivars (Table 3.2, 3.3 and B1). Reduction in plant height, number of total leaves, and fresh weight were greatest when three weeks old plants of either cultivar were infected by *S. terrestris*, while the bulb size of 3-week old 'Highlander' and 5-week old 'Hendrix' was limited in size after the onions were inoculated. Significant differences in plant height, fresh weight, and bulb circumference between cultivars were observed when onions were inoculated when they were three weeks old (Table 3.4).

Incidence of pathogen associated to the root cells. Intracellular mycelia and the fruiting structures of the pathogen were observed in the roots of inoculated plants; control plants were not infected (Figure 3.5 and 3.6). At 7 dpi, the incidence of root colonization of the cultivars Hendrix and Highlander ranged from 28.0% to 71.0% and 51.3% to 77.5%, respectively. The incidence of root colonization on 7- and 9-week old 'Hendrix' plants was statistically greater than 3- and 5-week old plants. For 'Highlander', the incidence of root colonization for the 5-, 7-, and 9-week old plants differed significantly from the incidence on 3-

week old plants (Figure 3.3). At 21 dpi, the incidence of root colonization of 'Hendrix' ranged from 31.5% to 78.5%; the incidence observed for 'Highlander' was 43.5% to 80.5%. The incidence of root colonization for both cultivars differed significantly among age at inoculation. On both cultivars, the 9-week old plants had the highest incidence of root colonization followed by 7-, 5-, and 3-week old plants; significant differences were observed on onions at 7- and 9-week old onions and the younger ages (Figure 3.3).

It was found that the asymptomatic roots of inoculated plants were colonized by the pathogen, but the incidence was much lower than that of the symptomatic roots. The root colonization observed at 7 dpi showed that the incidence ranged from 1.5% to 5.5% on 'Hendrix' and 2.0% to 8.0% on 'Highlander'. At 21 dpi, the incidence ranged from 1.5% to 8.8% and 5.2% to 10.0% on 'Hendrix' and 'Highlander'. There was no difference ($P > 0.05$) at the same age compared between the two cultivars (Figure 3.4).

Setophoma terrestris was recovered from the roots of the plants from the greenhouse trial and root colonization studied in laboratory, and no growth of the fungus isolated from the plants without inoculation was detected.

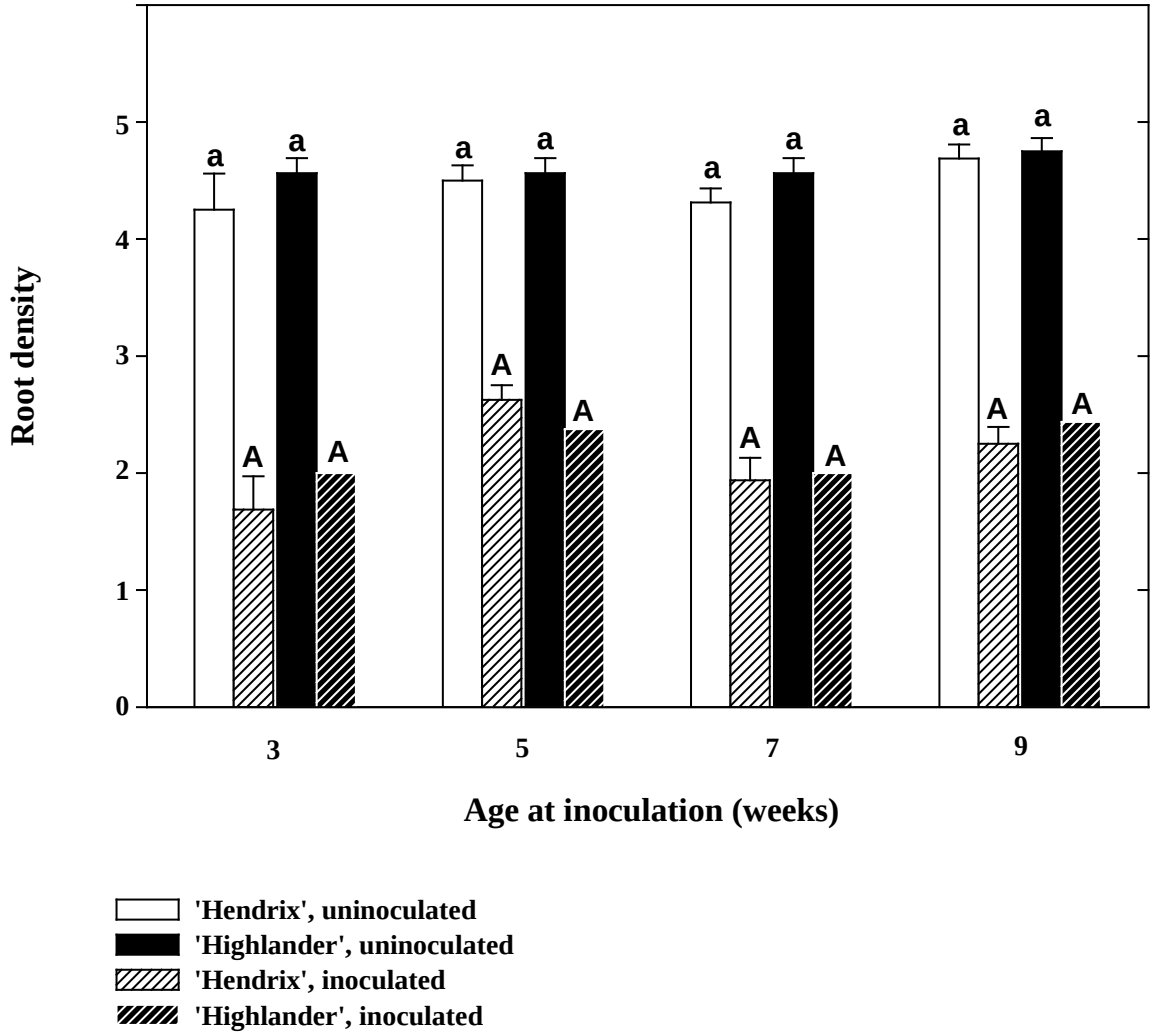

Figure 3.2. Root density of the cultivar Hendrix and Highlander of uninoculated (solid bars) and inoculated (solid bars with diagonal lines) onions when plants were at 3-, 5-, 7-, and 9-week old at inoculation. Bars with a letter in common are not significantly different ($\alpha = 0.05$).

Table 3.1. Analysis of variance on the effects of onion age at inoculation and cultivar for reduction of plant size (%).

Factors	Reduction of plant size (%)			
	Num DF	Den DF	F	*P* > F
Height				
Age	3	8	10.66	0.0036
Cultivar	1	8	0.05	0.8285
Age x cultivar	3	8	4.80	0.0339
Fresh weight				
Age	3	8	3.88	0.0556
Cultivar	1	8	4.97	0.0563
Age x cultivar	3	8	1.22	0.3623
Number of total leaves				
Age	3	8	7.88	0.0090
Cultivar	1	8	4.65	0.0632
Age x cultivar	3	8	1.67	0.2705
Bulb circumference				
Age	3	8	12.27	0.0023
Cultivar	1	8	1.92	0.2033
Age x cultivar	3	8	13.25	0.0018

Table 3.2. Measurement of 'Hendrix' plant height, plant fresh weight, number of total leaves, and bulb circumference of the unioculated control and plants inoculated when 3-, 5-, 7-, and 9-weeks old.

Age at inoculation (weeks)	Height (cm)		Weight (g)		Number of total leaves		Bulb circumference (cm)	
	Uninoc.*	Inoc.**	Uninoc.	Inoc.	Uninoc.	Inoc.	Uninoc.	Inoc.
3	33.1223	29.8334	7.0179	3.8750	6.0000	5.6250	2.8973	2.2104
5	49.6250	47.4313	31.9375	23.0625	8.6875	8.4375	7.1688	4.4750
7	54.8438	50.5813	40.0625	29.4375	9.4375	9.5625	8.0875	6.1188
9	49.4250	48.7563	46.4375	31.2500	10.5625	10.2500	8.2063	6.4313

*Uninoc. = uninoculated plants; **Inoc = inoculated plants.

Table 3.3. Measurement of 'Highlander' plant height, plant fresh weight, number of total leaves, and bulb circumference of the unioculated control and plants inoculated when 3-, 5-, 7-, and 9-weeks old.

Age at inoculation (weeks)	Height (cm)		Weight (g)		Number of total leaves		Bulb circumference (cm)	
	Uninoc.*	Inoc.**	Uninoc.	Inoc.	Uninoc.	Inoc.	Uninoc.	Inoc.
3	41.0063	30.7688	15.1250	4.5000	6.6875	5.8750	4.2750	2.2375
5	54.1563	54.5688	39.8125	23.9375	9.2500	8.7500	7.9125	5.7063
7	57.3250	58.1625	48.5000	29.9375	9.8750	9.5625	8.9000	7.0813
9	55.0563	55.7438	56.8750	38.7500	10.4375	10.3125	9.3875	7.0363

*Uninoc. = uninoculated plants; **Inoc = inoculated plants.

Table 3.4. Effects of onion age at inoculation and cultivar on reduction (%) in plant height, fresh weight, number of total leaves per plant, and bulb circumference compared to uninoculated control of onions inoculated at 3, 5, 7, and 9 weeks old of the cultivars Hendrix and Highlander.

Plant growth parameter	Age at inoculation (weeks)	Reduction of plant size (%)[z]			
		'Hendrix'		'Highlander'	
Plant height (cm)	3	9.93	a*	24.97	A*
	5	4.42	a	-0.76	B
	7	7.77	a	-1.46	B
	9	1.35	a	-1.25	B
Fresh weight (g)	3	44.78	a*	70.25	A*
	5	27.79	b	39.87	A
	7	26.52	b	38.27	A
	9	32.71	ab	31.87	A
Number of total leaves per plant	3	6.25	a	12.15	A
	5	2.88	a	5.41	A
	7	-1.32	a	3.16	A
	9	2.96	a	1.20	A
Bulb circumference (cm)	3	23.71	b*	47.66	A*
	5	37.58	a	27.88	A
	7	24.34	a	20.43	A
	9	21.63	ab	25.05	A

[z]Means with a letter in common are significantly different when compared among ages within plant growth category of each cultivar.

Arterisk (*) indicates significant difference compared between cultivars within age of each plant growth category.

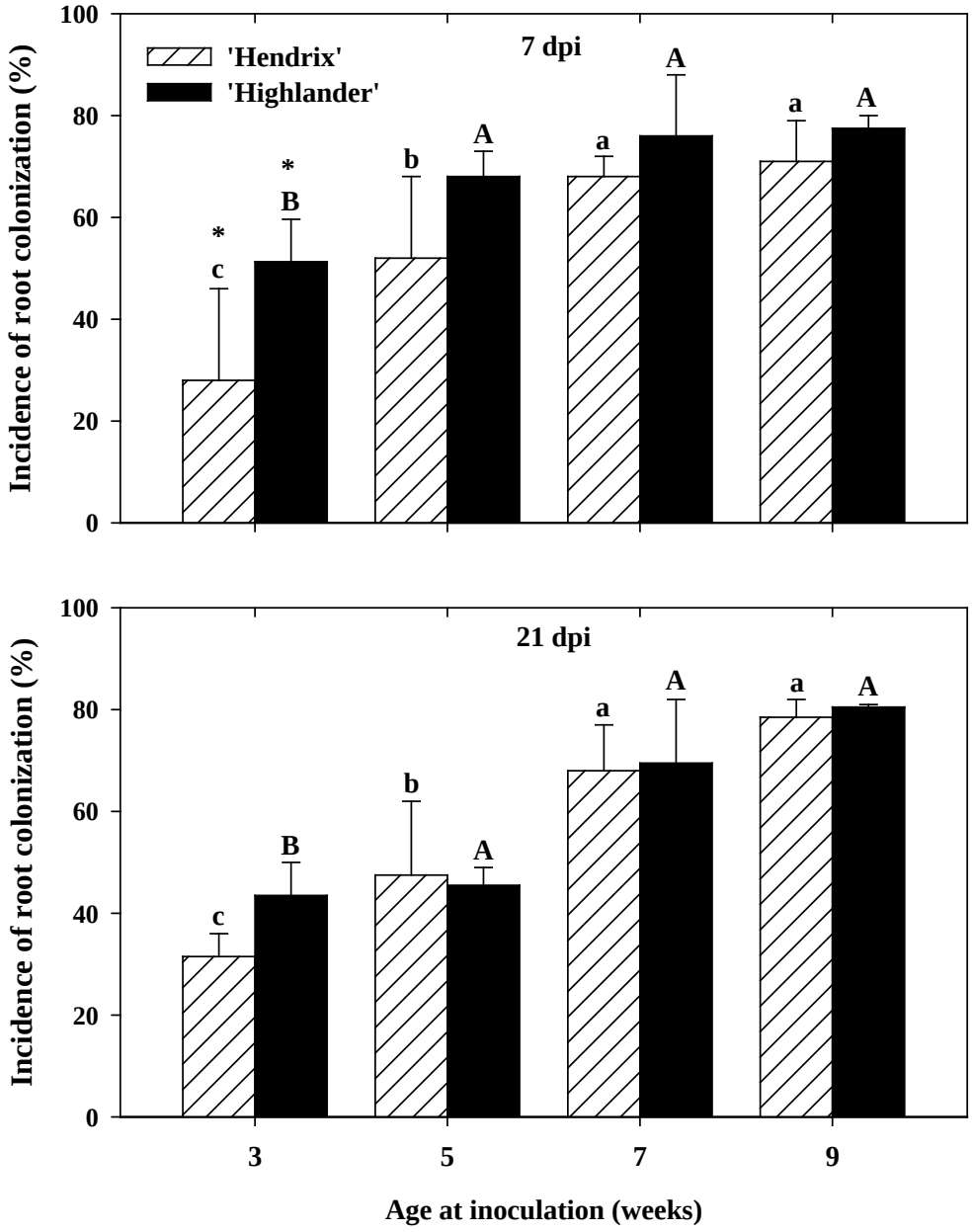

Figure 3.3. Incidence of root colonization (%) by *S. terrestris* determined at 7 dpi (A) and 21 dpi (B) on the symptomatic roots of the cultivars Hendrix (white bars with diagonal lines) and Highlander (black bars) when plants were 3-, 5-, 7-, and 9-week old at inoculation. Bars with a letter in common are not significantly different ($\alpha = 0.05$). Asterisk (*) indicates significant difference between cultivars at the same age.

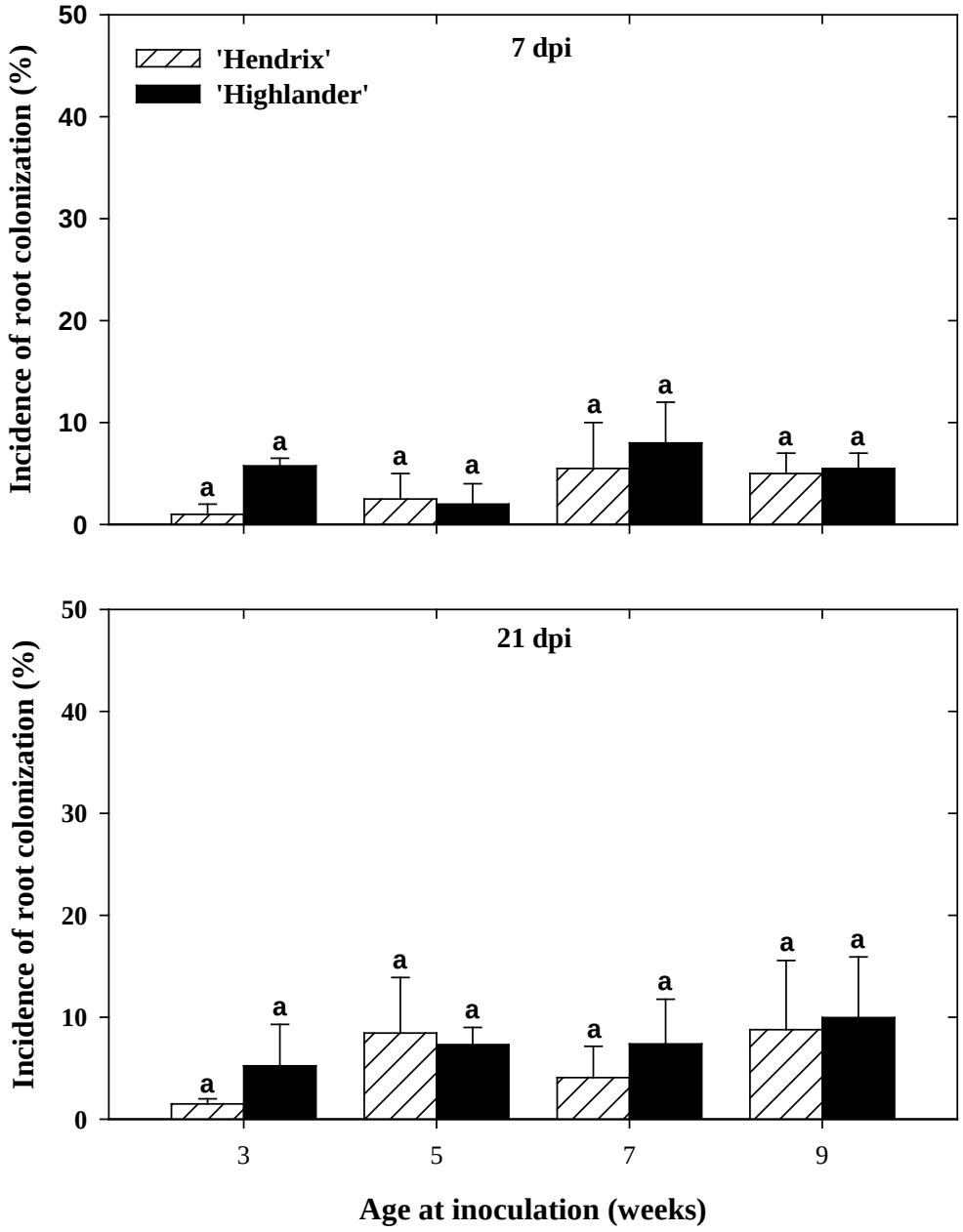

Figure 3.4. Incidence of root colonization (%) by *S. terrestris* determined at 7 dpi (A) and 21 dpi (B) on the asymptomatic roots of the cultivars Hendrix (white bars with diagonal lines) and Highlander (black bars) of 3-, 5-, 7-, and 9-week old at inoculation. Bars with a letter in common are not significantly different ($\alpha = 0.05$).

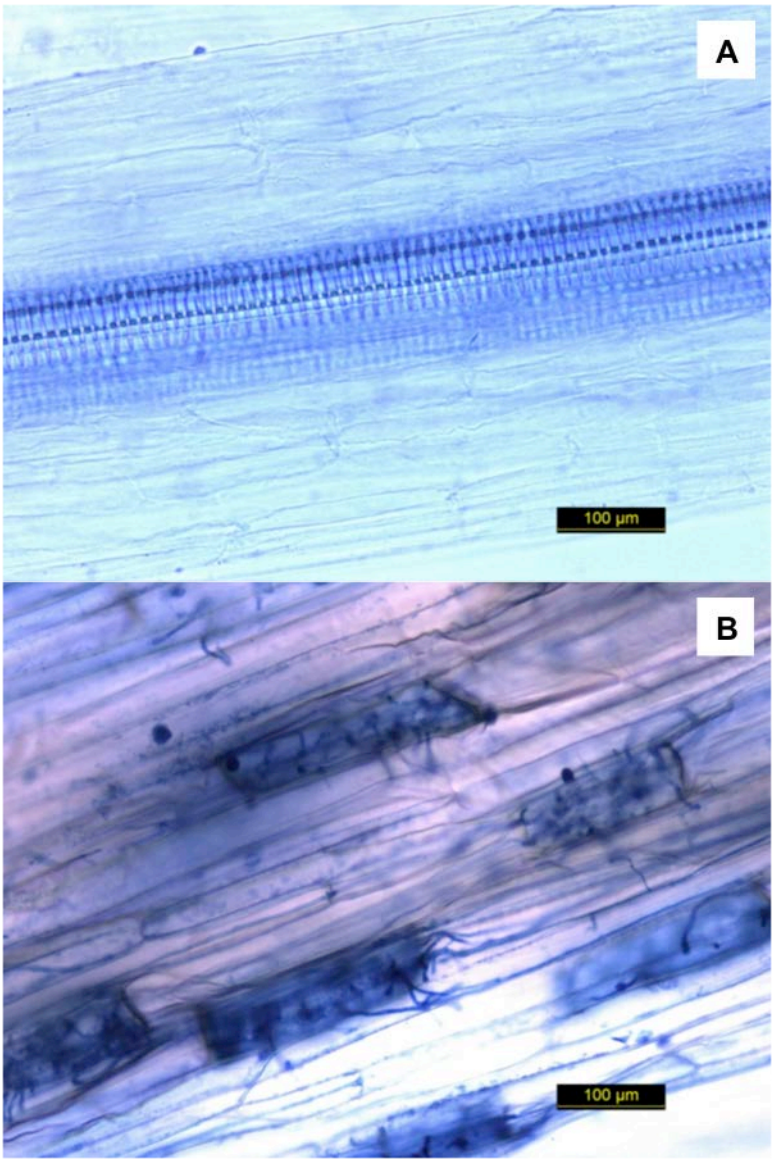

Figure 3.5. Microscopic examination of A) uninfected root cells of uninoculated onions (400x) and B) colonized root cells exhibiting growth of intracellular mycelia of *S. terrestris* (400x). Bars: A and B = 100 µm.

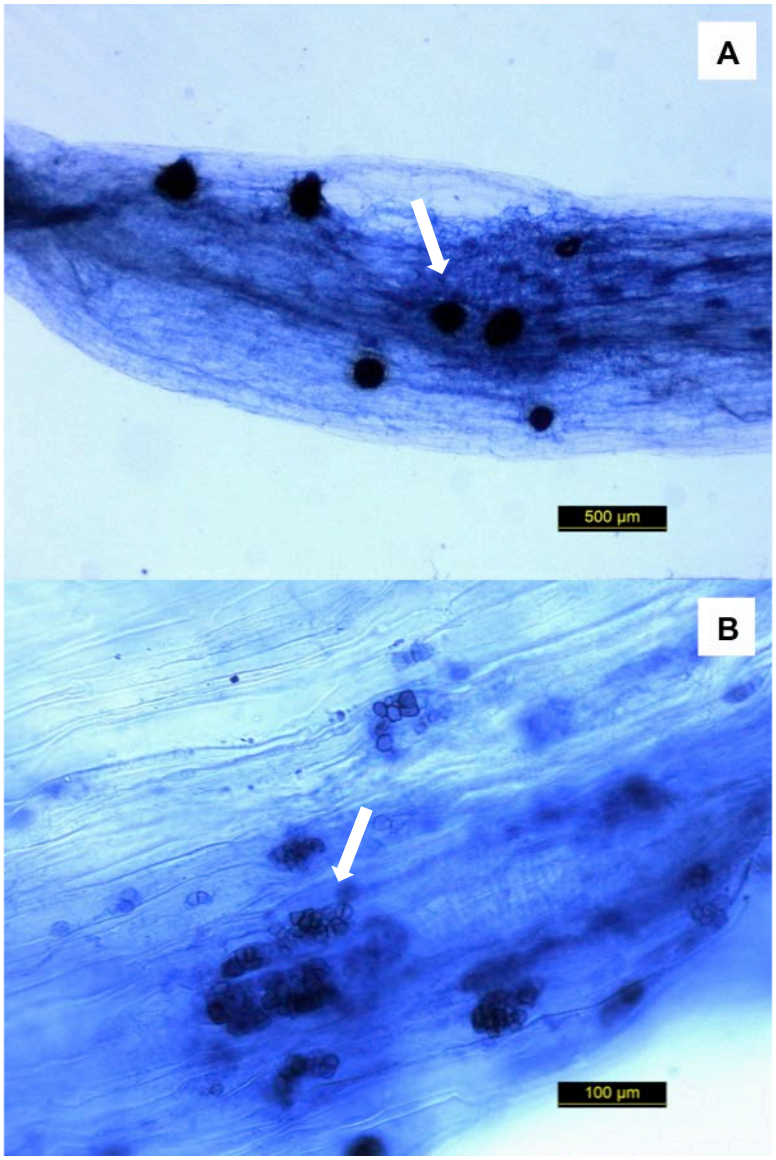

Figure 3.6. Fruiting structures: A) pycnidia (40x) and B) chlamydospores (200x) observed on inoculated onion roots. Pictures were taken 21 days after inoculation. Bars: A = 500 µm; B = 100 µm.

DISCUSSION

Study of age-related resistance to *S. terrestris* on onion was conducted. The inoculation was done on four ages of 'Hendrix' and 'Highlander' cultivars. Overall, plant growth parameters (i.e. root density, plant height, number of total leaves, plant fresh weight, and bulb circumference) of uninoculated control plants were greater than the inoculated plants. All plant ages of both cultivars compared were susceptible to pathogen infection as shown by root decline. There was no significant interaction between onion cultivar and age at time of inoculation in the reduction of root density.

Microscopic study revealed that the incidence of root colonization by *S. terrestris* was lowest on plants inoculated when they were three weeks old and the frequency was likely to increase on older plants where the symptoms of pink root were present. Infection and colonization of the onion roots by the pathogen was detected microscopically when symptoms were absent, but the incidence was low. The incidence of root colonization tended to increase from younger to older plants, and could be related to plant maturity and decreased ability to resist root infection as the onions mature. In general, it was observed that death of most of original onion roots occurred 2.5 months after the onions were grown in soil (Weaver and Bruner 1927). Younger onions have a fewer number of roots than older plants. Once the roots became infected, the root system of the younger plant was decreased more than the older onions. In general, 'Hendrix' was colonized less for both symptomatic and asymptomatic roots than 'Highlander' at the same age. Research has shown that the production of glucanases and chitinases with activity against *S. terrestris* filtrates was greater in the resistant cultivar, *A. fistulosum*, than the susceptible cultivar, *A. cepa* (Zappacosta et al. 2003).

Once the onion roots become damaged, the root system cannot absorb water and nutrient from the soil (Gergon et al. 2002) resulting in stunting and a reduced bulb size. In our study, approximately 50% of the root density became deteriorated regardless of age for both cultivars. The interaction between plant age at inoculation and cultivar showed that a relative difference in plant height occurred for 'Highlander' when 3-week-old onions were inoculated. There was little difference for onions at older ages suggesting that the disease impacted plant height when onions were infected when young but did not impact plant height when plant became older. In contrast, the largest difference of bulb circumference between uninoculated and inoculated onions occurred in 5-week old 'Hendrix' and may indicate that bulb size may be decreased when growth at the vegetative stage is reduced. The analysis of age and cultivar effects was processed separately for plant fresh weight and number of total leaves, as the interactions between the two factors were not significant. The difference between healthy and diseased onions in plant fresh weight and leaf numbers increased with age for each cultivar, suggesting that cultivar has no effect, but age does.

The temperature maintained in the greenhouse was conducive for pathogen growth and infection of the root system (Gorenz 1949; Hansen 1929; Sumner 1995). Taubenhaus and Mally (1921) observed that disease severity on green onions planted during hot summer at high temperature was considerably higher than planted early in the season at low temperature, where they were grown on the same soil. Furthermore, the pink root disease became prevalent throughout the planting season when soil temperature was increased (Taubenhaus and Mally 1921). Hansen (1929) also reported that near 0% onion bulbs were infected at 13°C but the incidence of pink root increased to 100% when the soil temperature increased to 25°C.

Pink root control is critical and preventive action should be taken at an early growth stage. Crop rotation and cultivar selection could decrease the inoculum of pink root and reduce crop loss.

CHAPTER 4: CONCLUSION

Pink root is a destructive fungal root rot disease of onion, which is incited by *Setophoma terrestris.* The disease is a limiting factor to the onion industry in Michigan and many production areas. Once the pink root pathogen is established in the soils, it cannot be easily eliminated. Susceptible onion cultivars are especially impacted. The objectives of this study included the following: 1) Evaluate the variation within *S. terrestris* populations according to their genetics, morphological characteristics, and virulence, 2) Evaluate onion cultivars and fungicide treatments for control of pink root on onions, and 3) Determine the effect of onion age for pink root susceptibility. Genetic variation among the pathogen populations was determined using the inter-simple sequence repeats (ISSR) markers in a collection of 98 *S. terrestris* isolates representing six populations. Differences among pathogen populations representing geographical regions were not significant. Morphological characteristics and virulence among population were also similar. Based on observations from 30 cultivars including those favored by Michigan growers, all of the cultivars evaluated were susceptible to pink root. However, the disease was significantly lower in 'Hendrix', Redwing', and 'Sedona' compared to 'Frontier', Highlander' and 'Scout'. The fungicide penthiopyrad when applied by drench in a greenhouse trial was more effective product than the other fungicides evaluated. Susceptibility to pink root was affected by plant age in 'Hendrix' (less susceptible to pink root) and 'Highlander' (highly susceptible to pink root). Onion plants were most susceptible at a young growth stage.

Identifying cultivars with resistance to pink root is key to improving onion productivity. To date, screening onion cultivars for pink root resistance has been assessed in many U.S. states, but not in Michigan. As a result of this research, growers in Michigan are now aware of

commercially available onion cultivars that are less susceptible to pink root. Also, data from the fungicide study suggests that, currently, only one fungicide registered for use on onions offers promise for the control of pink root. Hence, additional fungicide studies are needed to develop a robust disease management program for pink root on onions.

APPENDICES

APPENDIX A

Response of onion cultivars and fungicides to *Setophoma terrestris*

Figure A1. Necrosis of leaf tissue due to fungicide drenches resulting in minor (A), moderate (B), and severe (C) level of phytotoxicity, and untreated controls (D). Pictures were taken 55 days after inoculation and application of the first fungicide drench.

APPENDIX B

Influence of onion plant age in resistance to *Setophoma terrestris*

Table B1. Analysis of the main effects (onion cultivar and plant age at inoculation) in reduction (%) of plant height, fresh weight, number of total leaves per plant, and bulb circumference compared to the uninoculated control plants.

Effect	Num DF[y]	Den DF[z]	Height		Fresh weight		Number of total leaves per plant		Bulb circumference	
			F	*Pr > F*	*F*	*Pr > F*	*F*	*Pr > F*	*F*	*Pr > F*
Onion cultivar										
‘Hendrix’	3	8	1.18	0.3767	0.58	0.6438	2.91	0.1012	6.38	0.0162
‘Highlander’	3	8	14.28	0.0014	4.52	0.0390	6.64	0.0146	19.14	0.0005
Age at inoculation										
3	1	8	9.39	0.0155	6.72	0.0321	5.08	0.0542	35.12	0.0004
5	1	8	1.19	0.3075	0.87	0.3777	0.97	0.3543	5.61	0.0454
7	1	8	3.58	0.0952	1.06	0.3344	3.12	0.1153	0.91	0.3673
9	1	8	0.28	0.6097	0.01	0.9290	0.48	0.5087	0.03	0.8707

[y]Num DF = Numerator degrees of freedom.

[z]Den DF = Denominator degrees of freedom.

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