MANAGEMENT AND CHARACTERIZATION OF *ALTERNARIA* SPP. AND SEEDBORNE PATHOGENS ASSOCIATED WITH AMERICAN GINSENG (*PANAX QUINQUEFOLIUS*) GARDENS IN WISCONSIN

By

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ABSTRACT

MANAGEMENT AND CHARACTERIZATION OF *ALTERNARIA* SPP. AND SEEDBORNE PATHOGENS ASSOCIATED WITH AMERICAN GINSENG (*PANAX QUINQUEFOLIUS*) GARDENS IN WISCONSIN

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Leaf blight, caused by *Alternaria panax,* is a yearly challenge for those who cultivate ginseng, requiring frequent fungicide applications to protect valuable yield. In this study, the incidence and pathogenicity of *Alternaria* spp. on American ginseng in Wisconsin and effective leaf blight-controlling strategies were assessed. Five hundred and ninety-two isolates of *Alternaria* spp. were obtained from leaves (491), drupes (88) and seeds (10) of ginseng between 2011 and 2015. Resistance to azoxystrobin was determined using an amended agar plate assay and a molecular assay that detected a common mutation responsible for strobilurin resistance in fungi. The majority (74.5%) of the isolates were identified as small-spored *Alternaria* spp. and their pathogenicity was confirmed using a detached leaf assay. Wounding increased the occurrence of infection by small-spored *Alternaria* spp. although infection occasionally (<6%) occurred without wounding. *Alternaria panax* was consistently pathogenic; lesion area resulting from *A. panax* and small-spored *Alternaria* spp. infection ranged from 4.34 to 28.88 mm^2 and from 0.04 to 27.72 mm^2, respectively. Results from the plate assay show 65.2% of *A. panax* isolates and 82.6% of small-spored *Alternaria* spp. isolates were classified as resistant to azoxystrobin at an EC50 of greater than 4.00.

Leaf blight-control strategy field trials were conducted in commercial ginseng gardens over two years. One trial compared fourteen fungicides (boscalid, chlorothalonil, cyprodinil+fludioxonil, famoxadone+cymoxanil, penthiopyrad, fluazinam, pyraclostrobin,

difenoconazole+azoxystrobin, pyrimethanil, fluxapyroxad+pyraclostrobin, difenoconazole, extract of giant knotweed, mancozeb, and azoxystrobin) and a non-treated control. In a second trial, a disease forecaster program, TOM-CAST, with spray thresholds of 10 or 15 disease severity values (DSVs) was tested. Penthiopyrad, difenoconazole, fluazinam, difenoconazole+azoxystrobin and pyraclostrobin limited disease incidence compared to the control ($P < 0.05$). Applications of pyraclostrobin and mancozeb resulted in a higher seedhead yield than the control, the highest number of healthy drupes and the lowest number of diseased drupes ($P < 0.05$). In the forecasting trial, the disease severity rating of TOM-CAST 10 DSV plots was similar to those treated every 10 days, but higher than for plots treated every 7 days. The TOM-CAST 15 DSV plots were severely diseased. Yields were similar among plots treated every 7 days and according to TOM-CAST 10 DSV.

The establishment costs of a ginseng planting are high and seed health is an important consideration. Because of this, fungal presence on drupes, green seed coats, and endosperms was surveyed using a plate assay. A field trial to assess seed treatments in commercial ginseng gardens included stratified seed treated with the fungicides penthiopyrad, oxathiapiprolin, fluopicolide, ethaboxam, captan, mefenoxam + fludioxonil, or azoxystrobin. *Alternaria* spp. were commonly isolated from drupes and green seed coat and *Fusarium* spp. were recovered from all seed parts, including the endosperm. *Cylindrocarpon destructans* was only recovered in low numbers on green seed coats. Although none of the treatments significantly increased final plant stand compared to the control, oxathiapiprolin and azoxystrobin resulted in the highest plant stands throughout the season in year one ($P < 0.05$). In year two the control and fluopicolide resulted in the highest final plant stands ($P < 0.05$).

To Mozy,
for being a constant throughout this process
and providing unending love and warmth.

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INTRODUCTION

The genus *Panax,* in the family Araliaceae, contains eleven species of plants referred to as ginseng because they contain ginsenosides, a medically important compound used most commonly in traditional Chinese medicine (Yang et al 2014). Of these species, two are important commodities: *Panax ginseng* (Korean ginseng) and *P. quinquefolius* (American ginseng). American ginseng (the focus of this dissertation and hereafter referred to simply as 'ginseng') is native to North America—mainly Canada and the Eastern United States—and is grown commercially as a specialty crop (Proctor and Bailey 1987). Ginseng prices fluctuate annually, based on the market, but most recently averaged between $66 to $110/kg of dried root and $55 to $66/kg of green seed (Ginseng Herb Co-op, *personal communication*). Due to its profitability, ginseng was over-harvested and is now rarely found wild (U.S. Fish & Wildlife 1999), resulting in restrictive legislation for wild harvest in most states (U.S. Fish & Wildlife 2015). To meet demand for ginseng, commercial cultivation has gained popularity since 1975 when the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) came into effect (U.S. Fish & Wildlife 1999). In the United States, Wisconsin produces approximately 90% of the country's cultivated (mainly under shade panels, discussed below) ginseng (Drilias 2002). Since the beginning of commercial ginseng cultivation in the 1880s, production has grown substantially (Proctor and Bailey 1987). In Wisconsin, 670 hectares are cultivated by roughly 150 growers. Production yields between 560 to 2,200 kg/ha, and Wisconsin-grown ginseng makes up 10% of the global ginseng supply (Proctor and Bailey 1987; Drilias 2002). In 1995, Michigan producers began growing woods-grown ginseng (under a natural forest canopy, discussed below) in the Upper Peninsula region of the state (Hausbeck

2011). Woods-grown ginseng, sometimes referred to as wild-simulated, is valued as more than ten times that of artificial shade-grown ginseng (Ginseng Herb Co-op, *personal communication*). Michigan's ginseng industry is estimated at $50 million annually (Hausbeck 2011). Many other states cultivate ginseng for export including West Virginia (Scott et al. 1995), North Carolina (Davis 1997), Oregon (Putnam and du Toit 2003) and Washington (Brun 1999). In addition, the Atlantic provinces of Canada cultivate about 3,200 hectares of artificial shade-grown ginseng (Anonymous 2005).

Ginseng is a perennial herb found as an understory plant in hardwood forests, requiring reduced light—approximately 30% of full sunlight. Its leaves are palmately compounded, consisting of three leaflets, which are ovate and saw-toothed. Ginseng plants are determinate and grow to full height by July of each year at which point no further leaves are produced. The aboveground portion of the plant dies back each fall and sprouts again from the crown the following spring. Seedlings have only one leaf and each subsequent year, an additional leaf grows continuing until maturity when the plants have 4 to 6 leaves in a whorl, reaching 30 to 40 cm in height (Proctor and Bailey 1987).

At the center of a mature ginseng plant's leaf whorl, an umbelliferous inflorescence of up to 50 white, perfect, self-fertilizing flowers (Proctor and Bailey 1987). The fertilized flowers become bright red drupes in late summer. Each drupe contains 1-3 seeds but most commonly contains two 5 - 6 mm long seeds that are 4 - 5 mm wide. Each plant might have 30 - 40 drupes. The seeds are recalcitrant and contain an immature embryo that is roughly 0.4 - 0.5 mm at drupe ripening and grows to be 5 - 6 mm at maturity (Baskin et al. 2001). The seed must undergo a relatively long stratification period to break its deep, simple morphophysiological dormancy, requiring a sequence of warm and cold conditions (Baskin et al. 2001). Traditionally, seed is

either buried in a seedbox located outside or in a controlled-temperature environment for a period of 18 to 22 months in a mixture of moist sand and kept at a 10% w/w moisture level to reach seed maturity. (Proctor et al. 2001). This process is referred to as stratification. While morphophysical is the most common, Stoltz and Snyder (1985) found that there are three types of dormancy in ginseng seed: physiological (chemical change) and morphological (physical change) dormancy that are broken by 360 days at 20ºC at which point it is possible to break a second physiological dormancy at 5ºC for 150 days. The fluctuating temperatures help the embryo mature and the endocarp will crack roughly one month before germination. Research shows that controlled-temperature aboveground stratification works equally well as burying the seed underground in a seedbox and may help avoid exposure to soil-borne pathogens present in runoff (Proctor et al. 2001).

Ginseng has a fibrous, fleshy taproot that narrows toward the bottom and apically towards the crown (Proctor 1996). This crown is where the buds for subsequent years' stems originate. The root has a golden-brown exterior, with a white or yellowish interior. The dried root, only about 30% of its fresh weight, is the economically most important part of the plant as it contains the beneficial chemicals contributing to its medicinal properties: ginsenosides and glyosides (Proctor and Bailey 1987).

As ginseng is an understory plant that tolerates only 30% of full sunlight, it requires shielding from direct sunlight (Proctor 1996). Ginseng is typically grown in two ways: 1) woods-grown or wild-simulated: under a natural forest canopy after clearing all of the existing understory plants; or 2) artificial shade-grown—underneath plastic shade cloth or wooden lathe suspended 3 m above a cleared agricultural field in raised beds 9 to 12 inches high. In either case, ginseng is broadcast seeded at a rate of 100-112 kg/ha into rows 1 m wide. A 15-cm layer

of mulch is used to help with weed control, temperature control, and moisture retention. Straw mulch (wheat, barley, rye or oat) is common, but natural leaf litter and sawdust also are sometimes utilized (Proctor 1996). Little is known about the effects of different mulches on production, disease and insect problems in ginseng gardens and this is an area that warrants future investigation.

Loamy, well-drained soil provides the most favorable growing conditions for ginseng and between 0.5 and 1.5 m of water is required per year (Pritts 1995). After stratification, seeds are planted in the fall to germinate the following spring. Prior to the stratification process, seeds are sometimes treated with sodium hydroxide to initiate splitting and stimulate germination (Lee et al. 1983). Germination times vary between 8 and 20 months after planting depending on the success of the stratification method (Proctor et al. 2001). In subsequent years, to break bud dormancy, a minimum of 60 days at or below 9° C is required (Hovius et al. 1995).

Cultivating ginseng requires a relatively large initial financial investment for custom equipment, land, labor, seed and, if the artificial shade-grown method is used, shade materials. Ginseng takes 3-5 years to reach maturity for harvest in artificial shade-grown gardens, and 6-10 years for harvest from woods-grown plots (Proctor and Bailey 1987). Harvest is accomplished is the fall with a modified potato harvester or by hand, and roots are initially stored at 15-25ºC for a few days prior to being washed and dried on wire mesh in a well-ventilated room (Harrison et al. 2000). Drying takes 3-6 weeks during which period roots are frequently turned and the temperature is gradually increased to 30ºC to avoid sudden overheating and adversely affecting the color and texture. To preserve quality, the roots are stored in a dry, well-ventilated, rodent-proof container just above freezing until distribution (Ginseng Herb Co-op, *personal communication)*.

Root rots are a primary concern for ginseng growers because the root is the marketable part of the plant. Root pathogens that affect ginseng include *Fusarium* spp., *Pythium* spp., *Phytophthora cactorum, Cylindrocarpon destructans,* and *Rhizoctonia solani* AG 2-1 (Chang et al. 1997; 1998; Punja et al. 2008; Reeleder and Brammall 1994). Pre-emergence damping-off and seedling root rot caused by these pathogens are also a major problem in ginseng gardens, especially for 1- and 2-year-old plants (Reeleder and Brammall 1994). Low germination rates are sometimes attributed to the attack by these pathogens before radicles emerge from the soil. Disappearing root rot is a disease caused by *Cylindrocarpon destructans.* This pathogen may cause discoloration of the root, making it unmarketable, or it may cause the root to disintegrate and disappear, reducing yield and eventually leading to plant death (Hausbeck 2011). Rusty root, another disease that causes discoloration of the root and reduced value, has been identified as a complex of pathogens including *C. destructans* as well as *Fusarium* spp. and *Rhexocercosporidium panacis* sp. *nov.* (Reeleder et al. 2006). Many of these pathogens are present in soils but have also been isolated from ginseng seed leading to speculation that seedborne pathogens are a major factor in low and variable germination rates within a seedlot (Ziezold et al. 1998; Reeleder et al. 2002; Punja et al. 2008).

ALTERNARIA LEAF BLIGHT

In addition to root rots, growers must also contend with foliar diseases. The microclimate created by shade cloth or wood lathe panels increases humidity and prolongs leaf wetness (Hausbeck 2011), which promotes foliar diseases that are a problem in temperate regions like Wisconsin (Parke and Shotwell 1989) and Michigan (Hausbeck 2011). Alternaria leaf blight is the most common foliar disease known to ginseng growers worldwide (Hausbeck 2011). Typical foliar symptoms are necrotic lesions with dark brown margins and yellow-green halos (Hausbeck

2011). Brown lesions may form on the stem just above the soil line and cause girdling and plant tipover (Brammall 1994). Infection of the root by *Alternaria* spp. is rare, but infection of the stems and leaves can lead to reduced root weight or increased winterkill, resulting in yield losses of 50% to 100% if uncontrolled (Drilias 2002).

Alternaria panax Whetzel is widely accepted as the main causal agent of Alternaria leaf blight (Simmons 2007). *Alternaria panax* infects *P. quinquefolius*, as well as species of *Meryta, Pseudopanax, Aralia, Dizygotheca, Polyscias, Tupidanthus* and has been reported on other species in the Araliaceae family such as *Schefflera actinophylla* and *S. elegantissima* (Garibaldi et al. 2004; Simmons 2007; Uchida 2003). However, molecular characterization and pathogenicity tests suggest that the *Alternaria* sp. associated with non-*Panax* genera is a separate species (Deng et al. 2012). In addition to *A. panax*, Chinese and Korean journals (Lee et al. 2011; Kim et al. 2008), *A. alternata* is sometimes documented as the causal agent of foliar blight on *Panax ginseng*. *Alternaria alternata* is a widespread and cosmopolitan opportunistic saprotroph reported on numerous plant materials (Rotem 1994). Documentation of this species causing infections on *P. quinquefolius* is rare, but one study in Canada isolated *A. alternata* repeatedly from lesions of ginseng leaves and subsequently showed in pathogenicity studies that these isolates caused new lesions that were significantly larger than those caused by *A. panax* (Punja 1997). In Wisconsin, researchers repeatedly found *A. alternata* in spore traps placed in ginseng gardens (Hill and Hausbeck 2009). Conidia also were isolated from lesions, asymptomatic tissue, and seed in Wisconsin, but pathogenicity tests were not conducted (Hill and Hausbeck 2008).

The genus *Alternaria* contains species that are pathogenic as well as many that are saprobic or endophytic (Rotem 1994). *Alternaria* species clades do not always correlate with

species-group based on morphological characteristics and this therefore makes classifying some species challenging, especially those with conidia 22-65µm in length, so called "small-spored species" like *A. alternata* (Woudenberg et al. 2013). The color, size and shape of conidia of *Alternaria* spp. can be affected by differences in maturity, light, temperature, and humidity (Rotem 1994; Simmons and Roberts 1993). Production of conidia varies under different environmental conditions and nutrient availability (Rotem 1994). Therefore, identification based on conidial characteristics alone can be unreliable unless strictly consistent growth conditions are provided following accepted taxonomic literature (Simmons 2007). Host range, virulence, and molecular characters (GAPDH, RPB2 and TEF1, plasma membrane ATPase, and calmodulin loci) are more useful identification tools (Simmons and Roberts 1993; Lawrence et al. 2013; Woudenberg et al. 2013).

Alternaria panax colonies can be dense, velvety, and dark brown, or appressed, shiny, and dark grey to green on PDA, PCA and dilute V8 agar (Quayyum et al. 2005). On V8 agar, after 5-7d, colonies are approximately 6.0 cm in diameter with strong concentric zonation and many individual chains on medium golden-brown conidiophores (Simmons 2007). Conidia range from 60.9 to 87.2 µm in length in culture and are elongated, elliptical to obclavate, and brown. The conidia found on natural substrates are larger, ranging 150-160 µm long and 12-20 µm wide. The conidia may have multiple transverse and/or longitudinal septa (Simmons 2007). Regardless of conidial differences, widely distributed isolates of *A. panax* were all equally virulent, shared the same infection process, and had matching ITS1 and ITS2 rDNA and β-tubulin nucleotide sequences (Quayyum et al. 2005). For some aging natural colonies of this fungus, most conidia are considerably smaller with chains of two to several conidia (Simmons 2007).

In culture on potato carrot agar (PCA), after 5-7d, *A. alternata* colonies are approximately 4 cm in diameter with concentric rings of growth (Simmons 2007). The conidia are small (22-65µm in length), produced in chains with a relatively short conidiophores and between 4 and 125 conidia, branching simply or geniculate. Conidia on the chain range in shape from long-elliptical to ovoid, ellipsoid or subsphaeroid (Simmons 2007). Researchers often misclassify small-spored species and disagree on species designation; other small-spored *Alternaria* spp. that are often mistaken for *A. alternata* include *A. infectoria* or *A. tenuissima* (Rotem 1994). Simmons (2007) insists that a morphological distinction of *A. alternata* from other similar species is the shape of the initial conidia (narrowly elliptical, straight or slightly inequilateral, densely, obscuringly minutely ornamented, and up to 40 x 10 µm with 3-5 transepta when it initiates an apical secondary conidiophore) and the nature of the spore wall ornamentation (progressively coarser and more striking as conidia age). For *A. tenuissima*, Simmons (2007) acknowledges this species is commonly misidentified as *A. alternata*. The chain pattern reported for representative *A. tenuissima* isolates is the defining characteristic: the initial 1-2 conidia of a chain usually have only transverse septa, and only one or two mature conidia in a chain have median, subconstricting transverse septum. The emphasis on utilizing morphology for determining taxonomic nomenclature for small-spored *Alternaria* spp. has led to issues in disease reporting (Rotem 1994), and researchers have proposed *Alternaria* sections and species-groups based on molecular data (Lawrence et al 2013; Woudenberg et al 2013).

Epidemics of Alternaria leaf blight can quickly develop depending on several factors, including weather, spray programs, and inoculum level (Hausbeck 2011). The fungal causal agents can overwinter, can be spread by wind, splashing and equipment, and within 5 to 7 days of infection symptoms begin to show (Uchida 2003). The necrotic lesions (166.0±34.0 mm^2

after five days) on the leaves are caused by a protein toxin called AP-toxin, which is exuded from the conidial germination fluids of *A. panax* (Quayyum et al. 2003). The conidiophores covering these dead areas produce additional infective conidia throughout the season (Parke and Shotwell 1989). This can be particularly devastating to young plants that have not had time to store energy into the root and since the fungus is able to overwinter in plant tissue, infection can continue in subsequent years. As ginseng is a determinate plant that does not produce new leaves once it reaches maximum growth mid-season, the loss of any photosynthetic capability of the leaves in a year can be detrimental. Mycelia can overwinter in plant debris and produce next year's inoculum to spread to newly emerged plants in the spring via rain, splashing water onto the leaves or stem (Hausbeck 2011). Ideal temperatures for *A. panax* conidial production are 18 to 25ºC (Brammal 1994; Hill and Hausbeck 2009) and 24 to 27ºC for mycelial production (David 1988).

Formation of germ tubes with bulbous appressoria can be observed in *A. panax* on detached ginseng leaves as soon as 6 h post-inoculation (Quayyum et al. 2003). These authors also found that appressoria penetrate leaflets directly through epidermal cells or cell junctions and indirectly through stomatal openings. Twelve hours after inoculation, swollen chloroplasts and disintegration of organelle membranes were observed.

Creating an unfavorable environment for the pathogen could reduce disease development. Unfortunately, the humid microclimate created by shade cloths and wood lathes in artificial shade-grown ginseng gardens provides favorable conditions for foliar diseases. Increasing plant spacing, planting smaller gardens, and orienting gardens in the direction of prevailing winds help to improve airflow and reduce humidity (Hausbeck 2011). Removal of plant debris and replacing infested mulch can reduce inoculum (Davis and Shoemaker 1999). These practices are

rather impractical and do not solve the issue of conidia arriving on air currents; therefore, control of foliar pathogens of ginseng often requires chemical control.

The fungicide iprodione (Rovral) once provided excellent control for Alternaria leaf blight; however, in 1987 resistance was detected throughout Wisconsin ginseng gardens and no longer works (Hausbeck 2011). In 1988, a copper hydroxide fungicide (Kocide) was made available for mixing with iprodione to help control Alternaria leaf blight; however, the combination did not provide adequate control throughout the season, allowed inoculum buildup and reduced seed yield (Hausbeck 2011). New fungicides registered on ginseng for Alternaria leaf blight control include pyraclostrobin (Cabrio, BASF Ag Products, FRAC code 11), trifloxystrobin (Flint, Gem, Bayer CropScience, FRAC code 11), and azoxystrobin (Quadris, Syngenta Crop Protection Inc; Satori, Loveland Products, Inc., FRAC code 11), chlorothalonil (Bravo WeatherStik, or Chloronil, Syngenta Crop Protection, Inc; Echo, Sipcam Agro, USA Inc; Equus, MANA, Inc., FRAC code M05), boscalid (Endura, BASF Ag Products. FRAC code 7), fluazinam (Omega, Syngenta Crop Protection, Inc, FRAC code 29), cyprodinil/fludioxonil (Switch, Syngenta Crop Protection, Inc., FRAC code 9/12), pyrimethanil (Scala, Bayer Cropscience LP, Research Triangle Park, NC, FRAC code 9) and pyraclostrobin/fluxapyroxad (Merivon, BASF Ag Products, FRAC code 11/7). These fungicides increased management options for Alternaria leaf blight control. Nonetheless, the risk of exceeding the allowable fungicide residue on the root, precluding exportation, continues to be a concern, because many destination countries have yet to set maximum residue limits (MRLs) for these compounds. When an MRL has not been set, the product is often rejected if the compound is found at any level (Bryant Christie Inc. 2018). Many of these newer compounds also have single-site targets and medium to high resistance risk (FRAC 2018). To reduce residue potential, as well as for

fungicide resistance management, growers could benefit from a disease forecasting program to help adequately time fungicides sprays for weather conducive to Alternaria leaf blight for sufficient control.

The use of disease forecasting systems is a common practice to manage pathogens while also reducing cost, fungicide residues and resistance development (Bourke 1970; Berger 1969; Bounds and Hausbeck 2007; Byrne et al. 1997; Dorman et al. 2009; Gillespie and Sutton 1979; Meyer et al. 2000; Zadocks 1984). These systems use weather variables in conjunction with information about the biology and epidemiology of the pathogen to predict when conditions are favorable for infection and disease (Krause and Massie 1975). TOM-CAST is a disease forecasting system originally developed for tomato diseases that calculates daily disease severity values (DSVs) based on the temperature during leaf wetness (LW) periods and their duration. When a predetermined cumulative DSV threshold is reached, a fungicide is applied and the cumulative DSV is reset to zero (Pitblado 1992). This program was evaluated in 2008 for the control of *Alternaria* spp. on ginseng (Hill and Hausbeck 2008). The study compared the use of TOM-CAST to limit fungicide applications to when weather conditions were most conducive to disease versus regular calendar-based applications. Using the fungicides available in 2008 lead to inconsistent results and reduced seed yield when following the TOM-CAST regimen that was developed for tomato that was adapted for *A. panax* (Hill and Hausbeck 2008). Considering the risk of fungicides resistance and the strict regulations on fungicide residues, continued efforts to reduce sprays through forecasting systems are desirable.

Strobilurins are a class of fungicides discovered in 1997 and originally derived from the fungus *Strobilurus tenacellus* (Gullino et al. 2000), however, most current fungicides in this class were synthetically developed. These fungicides target the complex III: cytochrome bc1

(ubiquinol oxidase) at the Qo site (cyt b gene) and belong to the FRAC group 11 (FRAC 2018). After repeated special exemptions for control of Alternaria leaf blight, the strobilurin fungicides azoxystrobin (Quadris, Syngenta Crop Protection Inc; Satori, Loveland Products, Inc.), pyraclostrobin (Cabrio, BASF Ag Products), trifloxystrobin (Flint or Gem, Bayer CropScience) and a tank-mixed product of pyraclostrobin/fluxapyroxad (Merivon, BASF Ag Products) were registered for use on ginseng in Wisconsin (Hausbeck 2011). Strobilurins are registered for use on 84 different crops in 72 countries, for over 400 crop/disease systems (Bartlett 2002). These compounds target the respiratory function of the pathogen's mitochondria by binding to the cytochrome b complex, in other words shutting off the major pathway by which the pathogen generates usable energy in the form of adenosine triphosphate (ATP) (Bartlett 2002). Since this pathway is controlled by only a few amino acids coded for in the pathogen's mitochondrial DNA, a single point mutation in this gene sequence can result in resistance (Koller et al. 2001). Resistance is most commonly acquired through the replacement of glycine with alanine in position 143 in the gene coding for the cytochrome b target site, as demonstrated in multiple organisms showing resistance to strobilurins (Heaney et al. 2000; Koller et al. 2001; McCartney et al. 2007), including *Alternaria* spp. (Karaoglanidis et al. 2011; Luo et al. 2007; Ma et al. 2003; 2004). This mutation has not resulted in any apparent fitness penalty and therefore there is a strong selection for the resistant population (Karaoglanidis et al. 2011). Another mutation shown to be involved in this resistance is F129L—a mutation from phenylalanine to leucine at position 129 (Vincelli and Dixon 2002; Kim et al. 2003; Pasche et al. 2005; 2008). There have been conflicting results regarding the fitness of the F129L mutants (Genet et al. 2006; Heaney et al 2000). A third mutation suspected to be involved is G137R (Thind 2012). However, isolates exhibiting the F129L or G137R mutations tend to show only partial resistance.

Reduced sensitivity to strobilurins was first reported on wheat powdery mildew in Northern Germany in 1998 (Sierotzki et al. 2000). Since then, resistance to azoxystrobin has been reported for more than 36 plant pathogens, including the *Alternaria* spp. responsible for blight on pistachio (Ma et al. 2003; Ma and Michailides 2004), potato (Pasche et al. 2002), and apple (Lu et al. 2003), as well as several other fungal pathogens of crops including soybean, wheat, barley, and sugar beet (Blixt et al. 2009; Bolton et al. 2012; Delgado et al. 2012; Foerster et al. 2009; FRAC 2013; Ishii et al. 2001; Kim et al. 2003; Langston 2002; Semar et al. 2007; Sierotzki et al. 2000a; 2000b; 2005; 2006; Steinfield et al. 2006; Vincelli and Dixon 2002; Walker et al. 2009). The Fungicide Resistance Action Committee (FRAC) indicates, "strobilurins should not exceed 30-50% of the total fungicide sprays made to the crop per season" to reduce risk of resistance development (FRAC 2009).

To reduce the risk of pathogens acquiring full resistance, it is recommended that growers rotate the use of strobilurin fungicides with those that utilize different mechanisms for control, or to reduce number of applications within a season. For example, with strobilurin application to ginseng in conjunction with rainfall events (Hill and Hausbeck 2009), rotating strobilurins with a protectant fungicide prior to disease development or use of a biopesticide when disease pressure is low is recommended (Hausbeck 2011). Use of a forecasting system to assess disease potential could help reduce fungicide applications by as many as ten per year—an average of 20 spray applications per year is common (Hill and Hausbeck 2009).

SEED PATHOLOGY

Pathogens often can survive on seeds and infect the young plant tissue upon emergence (Agarwal and Sinclair 1997). This makes disease management difficult because the plants can be impacted before they reach the surface (Biddle 2009). Ginseng is largely propagated by seed.

As seeds are stored in moist chambers for an 18-22 month-stratification period, opportunity for fungal colonization of seeds is high and seedborne diseases is a major concern (Proctor 1996). In Ontario, Canada, pathogenic fungi isolated from stratified seed embryos included *Fusarium* spp., *Alternaria* spp., and *Cylindrocarpon destructans*, which are genera with known pathogenicity to ginseng (Ziezold et al. 1998). Another study in Seoul, Korea, identified *Fusarium* spp., *Botrytis* spp., *Rhizoctonia* spp. and *Alternaria* spp. on the endocarp of dehiscent ginseng seed (Lee et al. 1983). These studies indicate tat an official protocol for seed pathology or viability testing of ginseng seeds could be beneficial.

According to McGee (1981) there are four basic perspectives of seed pathology: 1) pathogens for which the seed is the main source of inoculum, therefore when seed infection is controlled, disease is controlled; 2) the seedborne phase is of minor significance as inoculum source; 3) organisms never shown to cause disease as a result of presence on seed; and 4) pathogens that infect seed in the field or in storage that reduce yield and quality (McGee 1981). It is important to delineate which occurs in ginseng. In crops like ginseng, in which the seed is harvested by growers and used for new plantings rather than involving any regulated commercial seed propagation facilities (*personal communication)*, knowledge of reduction of pathogen presence on seed is important.

Tianyu and Weiqun (1992) recovered *A. panax* from tender green drupes and *C. destructans* in pedicels during the red drupe stage, suggesting these pathogens enter the seed early in the development process. Several studies showed *Fusarium* spp. were abundant on seeds and damped-off ginseng seedlings (Ziezold et al. 1998; Reeleder et al. 2002; Punja et al. 2008). However, little is known about which species are virulent, how important the inoculum on seed is for development of disease on the plant or whether they may even play beneficial or

endophytic. Reeleder et al. (2002) found that two species of *Fusarium—F. graminearum* and *F. solani*—as well as *Cylindrocarpon destructans* were the cause of visibly rotted seeds, but their results did not show these species to be significantly virulent on seedlings. Another study evaluated the diversity of *Fusarium* spp. originating from diseased ginseng roots and found that 90% of damaged roots showed the presence of *F. equiseti, F. sporotrichioides, F. avenaceum*, and *F. culmorum* (Punja et al 2008).

A widely used method of seedborne pathogen management is seed treatment with fungicides prior to planting (McGee 1981). For many crops, such as corn and soybean, seeds are routinely treated with a fungicide to protect them from fungal infection (Agarwal and Sinclair 1997). Seed treatments can significantly reduce disease incidence in seedlings and reduce the need for fungicide sprays or soil fumigation that is both expensive and dangerous to workers and non-target organisms (Biddle 2009; Mastouri et al. 2010; Schoeny and Lucas 1999). Some commonly used seed treatment active ingredients on corn are captan and fludioxinil (broad-spectrum contact fungicides), and metalaxyl and mefenoxam (narrow-spectrum systemic oomyticides) (Thomson 1997). Fludioxonil was found in the coleoptiles of germinating corn seeds, indicating that this treatment, at least, persists into the germination stage of the seeds (Mueller et al. 1998).

Seed treatments can affect seedling emergence and germination (Agarwal and Sinclair 1997). This was demonstrated when ginseng seeds were treated with captan before stratification, which subsequently delayed maturation of the embryo and suppressed dehiscence (Lee et al. 1983). The proposed explanation for this is that captan reduces microflora that soften the endocarp so that germination may take place. Similarly, treatment of seed after stratification with thiram, benomyl, and tebuconazole all significantly reduced emergence compared to non

treated controls (Ziezold et al 1998). Thus, care needs to be taken in use of seed treatments. Further investigation into effective seed treatments options on American ginseng is needed.

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CHAPTER I: CHARACTERIZATION OF *ALTERNARIA* SPP. ISOLATES ON AMERICAN GINSENG AND THEIR AZOXYSTROBIN SENSITIVITY

ABSTRACT

American ginseng (*Panax quinquefolius)* is commercially cultivated under shade cloth for three or more years; the root is harvested, and the majority is exported to Asia for use as a traditional medicine. *Alternaria panax* is the primary causal agent of leaf blight, the most common fungal disease in ginseng plantings with losses up to 100% when left untreated (Hausbeck 2011); *A. alternata* also has been reported causing a foliar disease of ginseng. The objective of this study was to determine the incidence, pathogenicity and virulence of *A. alternata* on American ginseng in Wisconsin. The sensitivity of *A. panax* and *A. alternata* to the strobilurin fungicide azoxystrobin, a common management tool, also was of interest for disease management. Isolates (592) of *Alternaria* spp. were obtained from leaves (491), drupes (88) and seeds (10) of ginseng between 2011 and 2015. Pathogenicity was determined using a detached leaf assay. Resistance to azoxystrobin was determined using an amended agar plate assay and a molecular assay to detect a common mutation responsible for strobilurin resistance in other fungi. The majority (74.5%) of the isolates were identified as *A. alternata* and their pathogenicity was confirmed. Wounding increased the occurrence of infection by *A. alternata* although infection occasionally occurred without wounding. *Alternaria panax* was consistently pathogenic; lesion area (necrosis) resulting from *A. panax* and *A. alternata* infection ranged from 4.34 to 28.88 mm^2 and from 0.04 to 27.72 mm^2, respectively. Results from the plate assay show 65.2% of *A. panax* isolates and 82.6% of *A. alternata* isolates were resistant to azoxystrobin at an

EC50 of greater than 4.00. This raises concerns about continued ability to manage *Alternaria* spp. on ginseng.

INTRODUCTION

American ginseng *(Panax quinquefolius)* is native to North America and grows naturally under the shade of mature forest canopies where it may survive for 25 years or longer (Proctor 1996). Ginseng can be commercially cultivated under an artificial shade canopy where it reaches maturity after a minimum of three years (Hausbeck 2011). More than 90% of the U.S. crop is grown in central Wisconsin (Hausbeck 2011). The dried root is valued as a traditional medicine in Asia with a value of $66 to $110 per kilogram (Ginseng Herb Co-op 2018, *personal communication*).

Alternaria leaf blight is the most common disease occurring on cultivated American ginseng (Brammall 1994; Hill and Hausbeck 2009; Li and Choi 2009; Quayyum et al, 2005). The disease is characterized by dark brown lesions with yellow halos on the leaves and stem blighting (Punja 1997). Foliar diseases are detrimental for ginseng, a determinate plant that does not continually grow new leaves throughout the season. *Alternaria* spp. have been found on stems, drupes and seeds in addition to the leaves (Proctor and Bailey 1987). *Alternaria panax* Whetzel is widely recognized as the causal agent of leaf blight; however, in 1997, *A. alternata* was documented to cause leaf blight symptoms in Canada (Punja 1997). In China and Korea, *A. alternata* often is reported as a causal agent of leaf blight symptoms on Korean ginseng (*P. ginseng)* (Kim et al. 2008; Lee and Yu 2011). Researchers isolated *A. alternata* from lesions on ginseng in Wisconsin and frequently observed conidia in spore traps (Hill and Hausbeck 2008; 2009). The morphology within *Alternaria* spp., particularly those deemed "small-spored," is diverse and the color, size, production rate and shape of conidia vary with maturity, light,

temperature, nutrient availability and humidity (Rotem 1994; Simmons and Roberts 1993). Thus, identification based on conidial characteristics alone can be unreliable. Virulence and molecular characters are considered more useful taxonomic tools (Rotem 1994, Lawrence et al. 2013, Woudenberg et al. 2013), while morphological characters are most reliable using a standardized set of growing conditions (Simmons 2007).

Alternaria leaf blight control strategies in Wisconsin include cultural practices to minimize moisture in the leaf canopy and frequent fungicide applications throughout the growing season of May to September (Hausbeck 2011). The strobilurin fungicides, which were discovered in 1997, are registered for use on 84 different crops, in 72 countries, for over 400 crop/disease systems (Bartlett et al. 2002). According to the EPA, the strobilurin azoxystrobin has been registered for ginseng since 2012. While highly effective and widely used, resistance to azoxystrobin is reported for more than 36 plant pathogens, including the *Alternaria* spp. responsible for blight on pistachio (Ma et al. 2003; Ma and Michailides 2004), potato (Pasche et al. 2002), and apple (Lu et al. 2003), as well as fungal pathogens of soybean, wheat, barley, and sugar beet (Blixt et al. 2009; Bolton et al. 2012; Delgado et al. 2012; Foerster et al. 2009; FRAC 2013; Ishii et al. 2001; Kim et al. 2003; Langston 2002; Semar et al. 2007; Sierotzki et al. 2000a; 2000b; 2005; 2006; Steinfield et al. 2006; Vincelli and Dixon 2002; Walker et al. 2009). The most common mechanism by which fungi acquire strobilurin resistance is an alteration in the target protein, cytochrome b: the substitution of glycine by alanine in position 143 (G143A) (Heaney et al. 2000; Koller et al. 2001; Ma et al. 2003; McCartney et al. 2007). With no apparent fitness penalty as a result of this mutation, when strobilurin fungicides are used, there is strong selection pressure for the resistant population (Karaoglanidis et al. 2011).

The goal of this study was to improve Alternaria leaf blight management on cultivated American ginseng through the following objectives: i) Determine the *Alternaria* spp. that cause leaf blight in Wisconsin gardens, and ii) Evaluate the sensitivity of these *Alternaria* spp. to azoxystrobin to assess the potential continued utility of this fungicide in disease management.

MATERIALS AND METHODS

Sample collection. *Alternaria* spp. isolates were obtained from commercial gardens of cultivated ginseng in Marathon County, WI from 2011 to 2015. In 2014, the sampling range also included a Michigan State University (MSU) research ginseng garden in Jackson, MI and commercial ginseng gardens in Lincoln, Waupaca, and Monroe counties, WI. Gardens in Marathon County, WI in 2014 were sampled beginning at the first sign of Alternaria leaf blight (5 June) until leaves began to senesce and turn red (26 August). These 2014 isolates were grouped into three categories: early (June), mid- (July) and late (August) season. All these gardens had been treated with azoxystrobin.

Symptomatic leaves, drupes or seeds were disinfested with 10% bleach, rinsed with sterile ddH_2O and plated onto 1.5 % water agar (WA) (Difco, Detroit, MI, USA). The hyphal tip of the growing mycelium was transferred to dilute V8 agar (2 ml V8 [Campbell Soup Company, Camden, NJ, USA], 20 g agar, in 1-liter ddH_2O) (Miller 1955) in Petri plates sealed with paraffin film (Parafilm, Bemis Company, Inc., Neenah, WI, USA) to maintain high humidity. Cultures were grown under fluorescent light in the laboratory for 7 to 10 days or until the onset of visible sporulation. If sporulation was not observed after 10 days, cultures were transferred to the dark and the Parafilm removed which helped induce sporulation (Marsh 1959). Cultures were flooded with sterile distilled water and rubbed gently with a sterile pipette tip to dislodge conidia. Immediately, 0.5 ml of the suspension was transferred to 60 x 15 mm water agar plates and

incubated under ambient light. After 16-24 h, these plates were examined under a dissecting microscope. Using a sterile scalpel, one germinating conidium was excised from the agar and transferred to a plate containing potato dextrose agar (PDA, Difco, MI, USA). Single-spored cultures were grown for 7 days in the dark at 23.5-24.4°C. Isolates were subsequently suspended in sterile skim milk (Difco, MI, USA) and placed into long-term storage in glass vials on silica crystals and stored at 4°C to preserve their genetic status for subsequent experiments (Perkins 1962).

Morphological measurements. To compare isolates within our collection, and identify *Alternaria* spp., the diameter of the mycelial growth from single-spore-transfer PDA plates was measured after seven days of incubation in the dark. The colony color, margin and texture were noted (Pryor and Michailides 2002). For conidial observations, single-spore-transfer PDA plates were incubated in a growth chamber at constant temperature of 25ºC (day and night) with 14 h of cool fluorescent light at 300 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light intensity. The colonies were flooded with sterile distilled water, rubbed gently with a pipette tip, and the conidial suspension was transferred onto a microscope slide. The average length and width of 50 randomly chosen conidia were measured under a light microscope with a calibrated eyepiece micrometer (Quayyum et al. 2005).

Conidial chain characteristics were observed by growing isolates in Riddell mounts (Riddell 1950) using V8 agar media. The isolate grew onto the slide cover and the sporulation chain pattern was observed under a light microscope within seven days. One Riddell mount was prepared for each isolate. The average number of conidia per chain and branching pattern (sympodial branched or unbranched) were recorded for each isolate by counting ten random conidial chains per Riddell mount. *Alternaria* spp. were identified using morphological

characteristics according to current taxonomic literature (Simmons 2007; Woudenberg et al. 2013).

Molecular Species Identification. DNA sequencing of the RNA polymerase II subunit (rpb1) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) was performed for small-spored (conidia <40 µm in length) *Alternaria* spp. Thirty-six representative isolates of small-spored *Alternaria* spp. and eight large-spored (conidia >40 µm in length) isolates were randomly selected and grown on dilute V8 agar for 7 to 10 days. A small section of the growing edge of the mycelia was transferred to a 150 ml Erlenmeyer flask containing 100 ml of yeast broth (2 g yeast extract, 10 g glucose, 1-liter distilled water) and capped with sterile aluminum foil. The flasks were incubated on a shaker at room temperature for 10 days to allow ample mycelial growth. Mycelium was collected by vacuum filtration, frozen overnight at -20°C and lyophilized. The lyophilized tissue was ground in a mixer mill (Retsch, Haan, Germany). DNA was extracted with a plant DNA kit (Mag Bind Omega Biotek, Inc., Norcross, GA, USA) following the manufacturer's instructions and the tissue was processed in a magnetic particle processor (KingFisher™ Flex, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for DNA extraction.

The GAPDH region was amplified with primers gpd1 and gpd2 (Berbee et al. 1999) and the RPB2 region with RPB2–5F2 (Sung et al. 2007) and fRPB2–7cR (Liu et al. 1999). Amplifications were performed following the protocol by Woudenberg et al. (2013) on a thermocycler (Mastercycler, Eppendorf, Hamburg, Germany). The PCR mixtures were prepared in 50 µl volumes. For GADPH, the mixture consisted of 10 ng genomic DNA, 1´ buffer (GoTaq®, Promega, Madison, WI, USA), 1 µM $MgCl_2$, 40 µM of each dNTP, 0.2 µM of each primer and 0.25 Unit taq DNA polymerase (GoTaq®, Promega). The RPB2 PCR mixture differed from the previous mixture by containing 2 µM $MgCl_2$ and 0.5 taq DNA polymerase.

Conditions for PCR amplification for GADPH were an initial denaturation step for 5 min at 94ºC followed by 40 cycles of 30 s at 94ºC, 30 s at 57ºC and 30 s at 72ºC with a final extension of 7 min at 72ºC. The partial RPB2 gene was obtained by using a touchdown PCR protocol of 5 cycles of 45 s at 94ºC, 45 s at 60ºC and 2 min at 72ºC, followed by 5 cycles with a 58ºC annealing temperature and 30 cycles with a 54ºC annealing temperature. The PCR products were sequenced in both directions by standard sequencing methods at Macrogen USA (Rockville, MD, USA). To avoid misidentification, sequences were aligned with accession numbers from trusted taxonomic sources (Lawrence et al. 2013; Woudenberg et al. 2013 and 2015), in the NCBI reference database (www.ncbi.nlm.nih.gov/BLAST/) using CLC Main Workbench (CLCbio, Aarhus, Denmark).

Pathogenicity and virulence assay. To satisfy Koch's postulates for the small-spored isolates from American ginseng, 54 randomly selected representative *Alternaria* isolates were tested for virulence on detached ginseng leaves in the laboratory. Five *A. panax* isolates were included for comparison. Isolates were grown on PDA as previously described. Leaves from 3-year-old cultivated ginseng gardens that had not been treated with fungicides were harvested in Marathon County, WI or Jackson, MI in June, one month after plant emergence, and kept at 4ºC until use. Leaves were surface disinfested by dipping in 10% bleach solution for 30 s, rinsed twice in sterile distilled water and air dried in a laminar flow hood. Leaves were placed on a sterile glass slide in a Petri dish containing sterile water-moistened filter paper. Half of the total number of leaves were wounded with a sterile pipette tip by firmly pressing into the leaf to create a small hole the size of the pipette tip, approximately 1 cm from the center vein and the leaf edge.

Conidial suspensions of each isolate were prepared in sterile distilled water and adjusted to approximately 1 x 10^5 spores/ml using a hemocytometer. A 100 µl droplet of the suspension was transferred either to the wound or to the corresponding region of the unwounded leaves. Control leaves were inoculated with sterile water droplets. The plates were wrapped with Parafilm to ensure high relative humidity and incubated under fluorescent light at 23.5-24.4°C.

The presence or absence of lesions and their length and width (cm) was determined by a caliper five, eight, and fifteen days post inoculation (DPI). Tissue from the lesions was excised as above and cultured on PDA to confirm the causal organism, identified by morphological characteristics, as described previously. The experiment included three replicates and was repeated twice.

Azoxystrobin resistance. For the plate assay, 161 isolates (Table 1) representing all years and locations were screened for their sensitivity to azoxystrobin. Although the label rate of azoxystrobin for ginseng results in a concentration between 470 and 1200 ppm, based on a preliminary spiral plating assay (Förster et al. 2004), the measurable effect of the fungicide was observed at the concentrations 1, 10, and 100 ppm with 0 ppm serving as the control. Azoxystrobin-amended media was prepared by adding volume:volume suspensions of azoxystrobin (Quadris, Syngenta, Greensboro, NC, USA) in 100 ml sterile distilled water directly to 900 ml WA cooled to 50ºC and amended with 100 mg/liter of salicylhydroxamic acid (SHAM) (Sigma-Aldrich, St. Louis, MO, USA). SHAM eliminates the alternative respiration pathway and is one way that a fungus can demonstrate insensitivity to strobilurins, but this only typically happens *in vitro*. Plant secondary metabolites take up active oxygen species and therefore this does not generally happen *in situ* (Koller et al. 2001).

Isolates retrieved from long-term storage on silica crystals were cultured on dilute V8 agar for 7 to 10 days under ambient light and then flooded with sterile distilled water and gently scraped with the edge of a sterile microscope slide to dislodge the conidia. The conidial suspension of each isolate was adjusted to a concentration of 1 x 10^5 using a hemocytometer and 100 µl of the suspension was transferred to the amended WA plates. Each isolate was replicated three times per fungicide concentration. After 24 h of incubation under fluorescent light at 23.5-24.4°C, 100 random conidia were observed for germination. Conidia were considered germinated if the germ tube was greater than the length of the conidia. EC_{50} values were derived from the response curves based on a Probit analysis of the percentages of inhibition at each concentration of azoxystrobin (Olson and Benson 2011). The EC_{50} value represents the concentration required to inhibit germination to at least 50% of that observed in the control plates without azoxystrobin (Olson and Benson 2011). The replicates were averaged and the EC_{50} was used to compare the varying degree of azoxystrobin sensitivity for each isolate.

DNA extraction was performed as described above for 79 of the isolates screened in the plate assay. To determine whether isolates exhibited a change at codon 143 from guanine to alanine (G143A), one of the most common mutations associated with strobilurin insensitivity and known to occur in *Alternaria* spp. (Heaney et al. 2000; Koller et al. 2001; Ma et al. 2003; McCartney et al. 2007), two allele-specific primers were used (Karaoglanidis et al. 2011; Ma and Michailides 2004). The first set, ARF4 (5'-ATG AGA GAT GTA AAT AAT GGG TGAT-3') and ARR4 (5'-AAG GTT AGT AAT AAC TGT TGC AG-3'), amplified a 246-bp product from resistant isolates only. The second set, ARF4 and ARS4 (5'-AAG GTT AGT AAT AAC TGT TGC AC-3'), amplified a product of the same size from sensitive isolates only. The amplified DNA was analyzed on 1% agarose gels in tris borate EDTA buffer stained with ethidium

bromide and visualized on Quantity One Software (Biorad, Hercules, CA). Presence of bands from ARF/ARS and ARF/ARR signified either sensitivity or resistance to azoxystrobin, respectively. To determine resistance thresholds, the EC_{50} values were compared with results from the genetic analysis. To examine trends in the development of resistant populations of *Alternaria* spp. in Wisconsin ginseng gardens, the data were split by year, plant age, and county (Table 1).

RESULTS

Isolations. Over the five-year collection period, 592 isolates were obtained and identified based on morphology. Of these, 74.5% were small-spored and later identified as either *A. tenuissima* or *A. alternata* (Simmons 2007) or what current taxonomic literature refers to as an alternarioid hyphomycete belonging to Section *Alternaria* (Lawrence et al. 2013); the other 25.5% were the large-spored species *A. panax*. In 2011, 50.7% of the 213 isolates were small-spored. In 2014, 76.2% of the 305 isolates were small-spored. Season-long collecting in 2014 revealed that the relative distribution of *Alternaria* species may not be consistent throughout the year. Early (May-June), mid-(July) and late (August) season isolates collected from Marathon Co, WI were 35 (9), 51(61) and 94 (31) % small-spored, respectively. In that same year, sampling late in the season from Lincoln (12), Waupaca (28) and Monroe (24) counties yielded 100% small-spored isolates.

Conidial characteristics. The size of *A. panax* conidia ranged from 42.0 to 95.0 µm in length and 16.0 to 39.0 µm in width. They were elongated, obclavate, with long beaks and brown with several transverse and longitudinal septa. Conidia were produced singularly or in straight, unbranched chains of two to three conidia (Figure 1.1). Colonies were mainly white to cream and

appressed, but occasionally dark gray to green. The colonies grew to an average of 3.8 cm diameter after seven days with even margins.

The size of the small-spored isolate's conidia was less uniform and ranged from 12.8 to 44.8 µm in length and 6.2 to 21.0 µm in width. They were obclavate, smooth and brown with tapered beaks and several transverse, but few longitudinal, septa. Conidia were produced in chains ranging from 2 to 15 conidia in straight and sympodial chains (Figure 1.1). In general, the small-spored isolates produced more conidia than *A. panax*. Colonies were diverse in color, ranging from light green to dark brown, with an occasional pinkish tinge visible in the mycelia. The textures of the colonies were also variable and were observed to be appressed, velvety or having dense aerial mycelia. The margins of the colonies were mainly wavy, sometimes irregular. Crystals occasionally formed in the agar. The colonies grew to an average of 5.3 cm diameter after seven days in the dark.

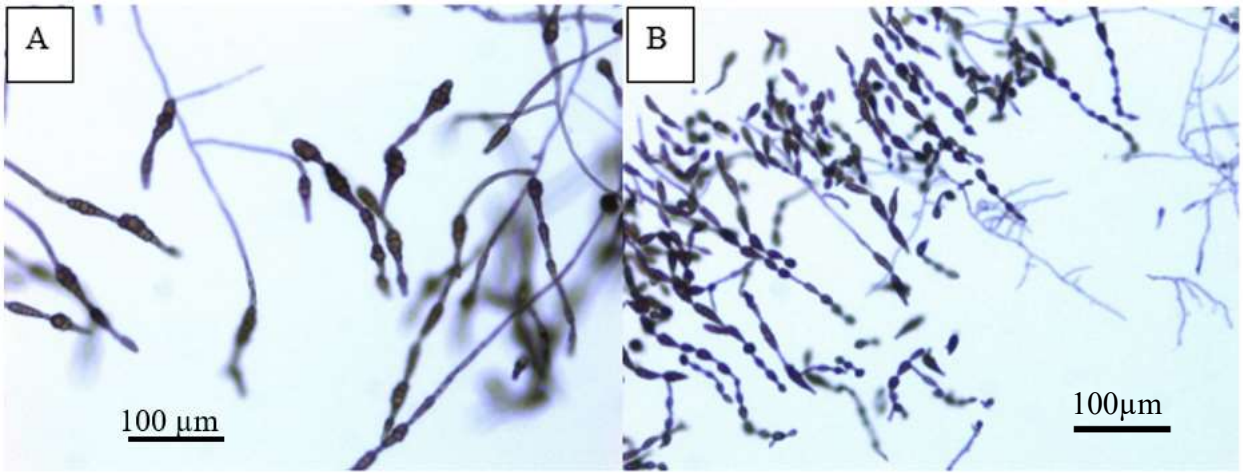

Figure 1.1. Typical appearance of conidial chains of **A,** *Alternaria panax* and **B,** small-spored *Alternaria* spp. isolates.

The sequenced regions of both rpb1 and GADPH genes confirmed the identification of the eight representative *A. panax* isolates. The sequenced regions from all 36 small-spored

isolates showed 99% similarity with the reference sequences for *A. alternata/A. tenuissima* (Woudenberg et al. 2015).

Pathogenicity. Sixty-four percent of 54 small-spored isolates caused lesions on wounded leaves, but only three consistently caused lesions on both wounded and unwounded leaf tissue. All *A. panax* isolates consistently caused lesions on both wounded and unwounded tissue. The small-spored *Alternaria* lesions on wounded tissues varied in area from 4.00-25.20 (μ = 9.99) mm^2 and on unwounded tissue 2.00-27.72 (μ = 10.83) mm^2. The lesion area caused by *A. panax* were generally larger, ranging from 4.76-28.88 (μ = 17.54) mm^2 on wounded tissue and 4.34-29.40 (μ = 17.22) mm^2 on unwounded tissue (Figure 1.2). Twenty-four (44.4%) small-spored isolates produced symptoms in as few as 5 DPI on leaves. Other isolates did not produce symptoms at all (1.5%) or showed a delay in symptoms. Control leaves did not develop symptoms. Isolations from symptomatic leaves inoculated with small-spored yielded similar, small-spored *Alternaria* isolates fulfilling Koch's postulates.

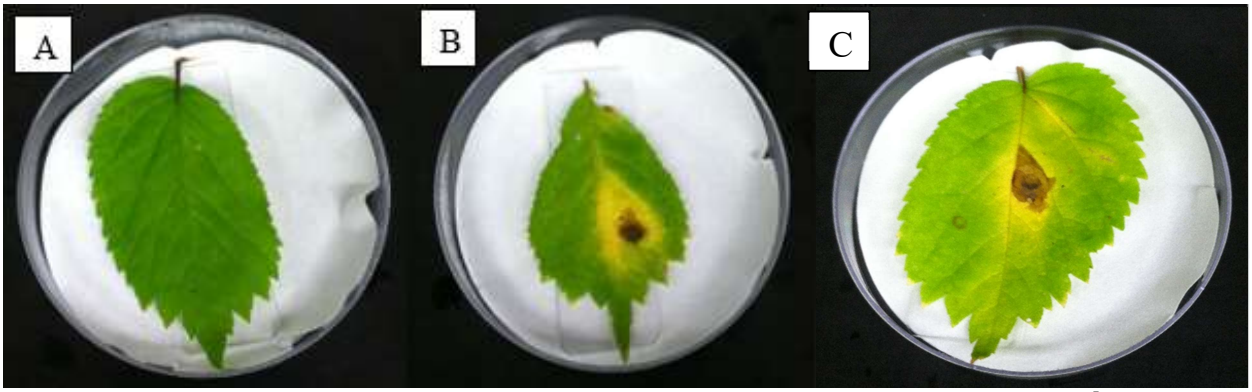

Figure 1.2. Typical appearance of unwounded ginseng leaves inoculated with 1 x 10^5 conidial suspensions from a detached leaf virulence assay 8 DPI: **A,** control, **B,** small-spored *Alternaria* spp. isolates, and **C,** *Alternaria panax*.

Azoxystrobin resistance. A wide range of EC_{50} values (0.63 to 1358.83) were found among the isolates tested. Isolates that lacked the G143A mutation and were amplified by the ARF4/ARS4 primer combination, had EC_{50} values <4.00 among the small-spored isolates, and

<1.88 among *A. panax* isolates. All isolates with EC_{50} values greater than this were amplified by the ARF4/ARR4 primer combination. Fifteen (65.2%) of the 23 *A. panax* isolates had an EC_{50} value greater than the resistance threshold EC_{50} of 1.88 (Table 1.1). Of 132 small-spored isolates assayed, 114 (82.6%) had EC_{50} values less than the resistance threshold of 4.00 (Table 1.1). This equates to 80.1% of the total *Alternaria* spp. isolates exhibiting resistance to strobilurin fungicides based on the plate assay. To support this data, the allele-specific primers showed that 84.8 % of the 79 isolates tested carried the G143 cytochrome b genotype associated with strobilurin resistance. Of the 73 small-spored isolates tested, 63 (84.8%) showed the G143A mutation, whereas of the six *A. panax* isolates tested, only four (66.7%) had this mutation.

DISCUSSION

The genus *Alternaria* has suffered from taxonomic flux because conidial characteristics were historically the basis for taxonomic status, but these characteristics vary greatly under environmental conditions (Rotem 1994). With the advance in molecular tools for phylogenetic studies of this genus, researchers proposed grouping small-spored *Alternaria* spp. that cannot be distinguished based on a multi-gene phylogeny, including, but not limited to, *A. alternata*, *A. tenuissima and A. arborescens* into a category Section *Alternaria* (Lawrence et al. 2013; Woudenberg et al. 2015). Two gene regions, rpb1 and GADPH, are considered adequate for distinguishing small-spored species thought to be separate from Section *Alternaria* (Woudenberg et al. 2013; 2015). The molecular variation within this section is low, but includes a broad descriptive morphology as follows: “grown on [potato carrot agar] PCA, have primary conidiophores that are straight or curved, simple or branched, short to very long. Conidial chains are moderately long or long, simple or branched. Young conidia are short ovoid, ellipsoid or obclavate. Mature conidia are obclavate, long ellipsoid or ellipsoid, small or moderate, septate,

Table 1.1. *Alternaria* isolates from American ginseng sorted by county of origin, year isolated, the age of the garden of origin, tissue of origin, resistance status according to plate assay EC_{50}[x] (Resistant = *Alternaria alternata* isolates with EC_{50} > 4.00, *Alternaria panax* EC_{50} >1.88), which of the cytochrome b genotypes[y] were observed using allele-specific primers.

		No.[z] of isolates	No. resistant (plate)	No. sensitive (plate)	No. G143	No. A143	No. small-spored *Alternaria* spp. isolates	No. isolates *Alternaria panax*
Species	Small-spored *Alternaria* spp.	441	114	24	63	10	-	-
	Alternaria panax	151	15	8	4	2	-	-
	Total	592	129	32	67	12	-	-
County	Marathon, WI	523	105	31	46	10	372	151
	Lincoln, WI	12	5	0	6	1	12	0
	Monroe, WI	24	5	1	3	1	24	0
	Waupaca, WI	28	14	0	12	0	28	0
	Jackson, MI	5	-	-	-	-	5	0
	Total	592	129	32	67	12	441	151
Year	2011	213	46	12	19	6	109	104
	2012	9	7	0	-	-	8	1
	2013	41	9	10	-	-	39	2
	2014	303	67	10	48	6	259	44
	2015	26	-	-	-	-	26	0
	Total	592	129	32	67	12	441	151
Garden Age (Years)	1	33	8	7	7	1	33	0
	2	110	29	6	22	2	92	18
	3	197	35	5	16	2	168	29
	4	218	41	10	17	4	123	95
	5	34	16	4	5	3	25	9
	Total	592	129	32	67	12	441	151
Tissue Origin	Leaf	491	122	32	66	12	341	150
	Drupe	88	6	0	1	0	88	0
	Seed	10	1	0	-	-	10	0
	Stem	3	-	-	-	-	2	1
	Total	592	129	32	67	12	441	151

[x]EC_{50} represents the concentration required to inhibit germination of at least 50% of that observed in the control plates

[y]Select isolates were tested for G143A mutation: A143=sensitive, G143=resistant

[z]No.=number with given characteristic

with a few longitudinal septa. Conidia are slightly constricted near some septa. Most conidia narrow gradually into a tapered beak or secondary conidiophore. Beak is no longer than conidial body" (Lawrence et al. 2013). The media used in the current study was PDA, not PCA as recommended by Lawrence et al. (2013) and Simmons (2007); however, it was used following the procedures of Pryor and Michailides (2002) and Quayyum et al. (2005) wherein they suggest that the high carbohydrate content of the media promotes vegetative growth of these species. In addition, PDA is commercially available, providing greater carbohydrate content consistency compared to variation in locally procured produce used to formulate PCA (Pryor and Michailides 2002).

Our results indicate that both *A. panax* and isolates from Section *Alternaria* were present and pathogenic in Wisconsin ginseng gardens; isolates identified as Section *Alternaria* were detected in the first year of the study (2011), and airborne conidial concentrations of *A. alternata* were noted in ginseng gardens as early as 2005 during spore trapping studies (Hill and Hausbeck 2009). None of the small-spored *Alternaria* isolates, by our methods, showed distinct morphological, molecular, virulence or strobilurin sensitivity differences that would justify suspicion that they are a distinct species, but recommended media to detect these was not used. Of the three small-spored *Alternaria* isolates that most consistently caused lesions on both wounded and unwounded tissue, two showed resistance to azoxystrobin and conidial and colony characteristics were variable but fell within descriptions of *A. alternata* (Pryor and Michailides 2002).

Species that fall in the Section *Alternaria* are commonly saprobic (Rotem 1994, Lawrence et al. 2016). When Koch's postulates were carried out for isolates from WI on detached American ginseng leaves, however, findings support the conclusion of Punja (1997)

that *A. alternata* may be a causal agent of leaf blight on ginseng. This does not rule out the possibility that leaf age and temperature play a role in pathogenicity of these fungi. In our study, the highest percentage (46.9%) of small-spored *Alternaria* isolates were isolated from ginseng tissue late in the growing season (August), when plant senescence begins, but the temperature remains high. In our virulence studies, small-spored *Alternaria* isolates rarely infected intact leaf tissue. Both observations imply that leaf senescence or damaged tissue plays a role in American ginseng's susceptibility to *A. alternata* or alternarioid hyphomycetes in Section *Alternaria.* Similarly, reports on other crops showed that older leaves, tissues that are not intact or tissue under increased stress were more susceptible to infection by *A. alternata* (Jia et al. 2010; Pleysier et al. 2006; Stavely and Slana 1971). The role of temperature on virulence of these isolates was difficult to assess from our study, as sampling ceased each year by the end of August. It was also difficult to separate the variables of senescence and temperature in the field. Symptoms of Alternaria leaf blight typically arise in mid-May or June, indicating that either plants may not be susceptible during the cooler spring temperatures, or simply that inoculum is too low to illicit disease. Studies in a controlled-temperature environment may elucidate whether cold temperatures affect the virulence of these small-spored isolates on ginseng as observed in other plant-pathogen interactions (Pleysier et al. 2006).

Rotem (1994) references *A. alternaria* producing a host-specific toxin (HST) that aids the fungus in infecting a particular crop. These HSTs are associated with the presence of small supernumerary chromosomes, termed conditionally dispensable chromosomes (CDCs) (Ajiro et al. 2010; Akagi et al. 2009; Akamatsu et al. 1997, 1999; Hatta et al. 2002; Ito et al. 2004; Johnson et al. 2000; Masunaka et al. 2005; Miyamoto et al. 2009, 2010; Tanaka et al. 1999). Quayyum et al. (2003) found that *A. panax* produces a ginseng-specific toxin, and perhaps a

similar study with the small-spored *Alternaria* isolates found in ginseng gardens would reveal whether they confounded the same or other HST.

Alternaria leaf blight is an annual problem in cultivated ginseng, managed by regular fungicide applications (Hausbeck 2011). Strobilurin-resistant isolates of *Alternaria* spp. although below field rates, may indicate the need for adequate fungicide rotations or new modes of action. Small-spored *Alternaria* isolates showed higher EC_{50} values than *A. panax* isolates, which may mean that the population of small-spored *Alternaria* is being exposed to strobilurin fungicides on other crops, as these species have a wide host range and can often be found as saprobes (Rotem 1994).

The G143A mutation was observed in the current study, but there are other mutations suspected to be involved in this resistance to strobilurin fungicides such as F129L, a substitution of phenylalanine by leucine at position 129 (Pasche and Gudmestad 2008; Pasche et al. 2005; Vincelli and Dixon 2002) and G137R, a substitution of glycine to arginine at position 137 (Sierotzki 2006). Our study did not include any assays to detect these mutations, however, there is a possibility that some of the intermediate EC_{50} values in our isolates reflect resistance due to the F129L or G137R mutation, as other studies have shown G143A conferring complete resistance with EC_{50} values greater than those found in our study (Genet et al. 2006; Heaney et al. 2000). Further research to determine if these mutations play a role in strobilurin-resistance of *Alternaria* spp. pathogenic to ginseng is needed.

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CHAPTER II: ALTERNARIA LEAF BLIGHT MANAGEMENT STRATEGIES FOR AMERICAN GINSENG

ABSTRACT

Cultivated American ginseng (*Panax quinquefolius*) is a perennial herb valued as a traditional Chinese medicine. The crop is commercially produced under polypropylene shade cloth, which creates a climate favorable for foliar fungal pathogens. Leaf blight caused by *Alternaria panax* and other *Alternaria* spp. is a yearly challenge for those who cultivate ginseng, requiring frequent fungicide applications to protect valuable yield of roots and drupes. The objective of the current study was to identify effective leaf blight-control strategies. Two field trials were conducted in commercial ginseng gardens over two years. The first trial compared fourteen fungicides (boscalid, chlorothalonil, cyprodinil+fludioxonil, famoxadone+cymoxanil, penthiopyrad, fluazinam, pyraclostrobin, difenoconazole+azoxystrobin, pyrimethanil, fluxapyroxad+pyraclostrobin, difenoconazole, extract of giant knotweed, mancozeb, and azoxystrobin) and a non-treated control. In a second trial, a disease forecaster program, TOM-CAST, with spray thresholds of 10 or 15 disease severity values (DSVs) were tested. In the first trial, penthiopyrad, difenoconazole, fluazinam, difenoconazole+azoxystrobin and pyraclostrobin limited disease incidence compared to the control. Applications of pyraclostrobin and mancozeb resulted in a higher seedhead yield than the control, the highest number of healthy drupes and the lowest number of diseased drupes. In the forecasting trial, the disease severity rating of TOM-CAST 10 DSV plots was not significantly different than those treated every 10 days, but higher than for plots treated every 7 days. The TOM-CAST 15 DSV plots were severely diseased. Yields were similar among plots treated every 7 days and according to TOM-CAST 10 DSV.

INTRODUCTION

American ginseng (*Panax quinquefolius*), native to North America, is commercially cultivated in the United States and Canada (Proctor and Bailey 1987). This valuable perennial root crop is used as a traditional Chinese medicine and as an herbal supplement (Pritts 1995). Growers collect ginseng seed for future plantings or to sell (Hausbeck 2011). Cultivated ginseng is produced by mimicking its natural habitat either under a natural forest canopy cleared of understory plants or in cleared agricultural fields on raised plant beds under artificial black polypropylene shade cloth or wood lath (Pritts 1995). The shade material creates a microclimate that favors disease development by reducing air movement, increasing humidity and prolonging leaf wetness (Hausbeck 2011; Parke and Shotwell 1989). The roots reach a minimum marketable size after three years of growth (Harrison et al. 2000) and buildup of disease pressure leads most growers to harvest ginseng in the third or fourth year after planting (Hausbeck 2011).

Alternaria leaf blight of ginseng is an annual problem for growers in Michigan and Wisconsin (Hausbeck 2011). *Alternaria panax* Whetzel is widely accepted as the main causal agent of this disease (Garibaldi et al. 2004; Uchida, 2003), resulting in premature defoliation of ginseng plants if uncontrolled (Hausbeck and Harlan 2013a; 2013b). Ginseng plants are determinate; when mature, plants have 4 to 6 leaves in a whorl, reaching 30 to 40 cm in height, and no leaves are produced for the remainder of the growing season (Proctor 1996). Typical leaf blight symptoms include necrotic lesions with dark brown margins and yellow-green halos (Hausbeck 2011). Lesions may form on the stem just above the soil line and cause girdling (Brammall 1994). Lesions have been observed on the stems of older plants, well above the soil line, resulting in tipover (M. Hausbeck, *personal observation*). *Alternaria panax* infection may

cause abortion of developing berries (Brammal 1994), negatively impacting seed yield. The pathogen has been detected on stratified ginseng seed (Hill and Hausbeck 2008).

The frequent and costly fungicide applications that growers rely on to protect their crop can have several negative impacts including residues and fungicide resistance (Hausbeck 2011). Disease forecasters may limit disease while minimizing fungicide sprays (Berger 1969; Gillespie and Sutton 1979; Zadocks 1984). TOM-CAST was adapted from FAST, a program designed to time fungicide sprays for *A. solani* on tomato (Pitblado 1992) and it has proven beneficial in other systems (Byrne et al. 1997; Meyer et al. 2000; Bounds and Hausbeck 2007; Dorman et al. 2009). TOM-CAST calculates daily disease severity values (DSVs) ranging from zero (unfavorable for disease development) to four (highly favorable for disease development) based on the duration of the leaf wetness period and the temperature during the leaf wetness period. DSVs are accumulated until a predetermined threshold is reached at which time a fungicide is applied, and the cumulative DSV is reset to zero (Pitblado 1992). Hill and Hausbeck (2008) evaluated TOM-CAST (10 or 15 DSVs) for control of Alternaria leaf blight on ginseng with the fungicides chlorothalonil, pyraclostrobin, and copper hydroxide. Although the TOM-CAST program reduced the number of sprays, foliar blight was not limited, and seed yield was reduced compared to sprays applied every 7 days (Hill and Hausbeck 2008). Since 2008, additional fungicides were registered for use on ginseng for leaf blight (Hausbeck 2007; 2011). These newer fungicides represent different modes of action (Anonymous 2017) and may provide improved disease control suitable for use in conjunction with TOM-CAST.

The overall goal of this study was to advance the disease control strategies for Alternaria leaf blight of American ginseng, with the following objectives: 1) determine the effect of new

fungicide options on seed yield, and 2) compare a TOM-CAST based program with a spray threshold of 10 or 15 DSVs to 7- or 10-day calendar-based programs using selected fungicides.

MATERIALS AND METHODS

Experimental sites and disease assessment. All experiments were conducted on well-drained sandy loam field sites (gardens) in collaboration with commercial growers of cultivated ginseng in Marathon County, Wisconsin. Gardens were covered with black polypropylene shade material supported by wooden posts spaced every 3.6 m. Stratified seed was broadcast onto raised plant beds 1.2 m wide with 0.3 m between plant beds at a rate of approximately 112.1 kg/hectare in September. Plant beds were mulched with shredded straw immediately following seeding according to standard practice. Fertilizer and weed control measures were applied by the commercial growers according to their standard practice. Experimental plots were 13.2 m wide and 18.6 m long portions of gardens in a randomized complete block design with treatment plots consisting of 3-m row sections with 0.6-m buffers. The fungicide efficacy and seedhead yield studies were conducted during 2014 and 2015; treatments were applied to three-year-old plants (the first year seedheads are abundantly produced (Proctor and Bailey 1987)) at two sites each year designated as A and B (2014) and C and D (2015) with four replicates per site. The disease forecaster trial plots were established in two seedling (one-year-old) gardens (Sites 1 and 2) and two two-year-old gardens (Sites 3 and 4) in 2013 with six replicates per site. These same experimental plots were treated and analyzed in 2014 when plants were one year older.

The number of infected plants per plot (incidence) and disease severity (visual rating scale 1-10: 1≈0-10% of the foliage in the plot with lesions, 2≈11-20%, 3≈21-30%, 4≈31-40%, 5≈41-50%, 6≈51-60%, 7≈61-70%, 8≈71-80%, 9≈81-90%, 10≈91-100%) were recorded every two weeks. Disease incidence was used to calculate area under the disease progress curve

(AUDPC) values as described by Shaner and Finney (1977) as a measure of the number of symptomatic plants throughout the growing season. Data were analyzed using analysis of variance (ANOVA), and treatment efficacy differences were compared using a Fisher's least significant difference test ($p = 0.05$) (Statistical Analysis Software [SAS] 9.3; SAS Institute, Inc., Cary, NC).

Fungicide trial. Fifteen fungicide treatments (Table 2.1) were assessed for their efficacy against leaf blight each year: boscalid (Endura 70WG, 315.2 g/ha; BASF Ag Products, Research Triangle Park, NC, FRAC code 7), chlorothalonil (Bravo Weather Stik 6SC,2.34 l/ha; Syngenta Crop Protection, Inc., Greensboro, NC, FRAC code M05), cyprodinil+fludioxonil (Switch 62.5 WG, 980.7 g/ha; Syngenta Crop Protection, Inc. , FRAC code 9/12), famoxadone+cymoxanil (Tanos 50DF, 540.4 g/ha; DuPont Crop Protection, Wilmington, DE, FRAC code 11/27), penthiopyrad (Fontelis SC, 1.17 l/ha; DuPont Crop Protection, FRAC code 7), fluazinam (Omega 500F, 1.75 l/ha; Syngenta Crop Protection, FRAC code 29), pyraclostrobin (Cabrio EG, 840.6 g/ha; BASF Ag Products, FRAC code 11), difenoconazole+azoxystrobin (Quadris Top SC, 1.02 l/ha; Syngenta Crop Protection, Inc. , FRAC code 3/11), pyrimethanil (Scala SC, 1.31 l/ha; Bayer Cropscience LP, Research Triangle Park, NC, FRAC code 9), fluxapyroxad+pyraclostrobin (Merivon SC, 438.5 ml/ha; BASF Ag Products, FRAC code 7/11), difenoconazole (Inspire EC, 511.5 ml/ha; Syngenta Crop Protection, Inc., FRAC code 3), *Reynoutria sachalinensis* (giant knotweed) extract (Regalia, 9.35 l/ha; Marrone Bio Innovations, Davis, CA, FRAC code P05), mancozeb (Roper DF Rainshield, 2.24 kg/ha; Loveland Products, Loveland, CO, FRAC code M03), and azoxystrobin (Quadris SC, 1.13 l/ha; Syngenta Crop Protection, FRAC code 11). Treatments were applied with a CO_2 backpack boom sprayer equipped with four 8003VS nozzles (TeeJet, Spraying Systems Co., Wheaton, IL) spaced 45.7

cm apart, operating at 275.8 kPa, and delivering 7 L/ha. All treatments were applied at 7-day intervals beginning at full plant emergence (29 May 2014 and 15 May 2015) and ending when foliage began to senesce in late summer (12 August 2014 and 21 August 2015) for a total of 11 sprays in 2014 and 14 sprays in 2015.

Table 2.1. Fungicides field tested for efficacy against *Alternaria* leaf blight on American ginseng 2014 and 2015.

Treatment	Active ingredient	Company	Rate/ha
Non-treated	-	-	-
Endura 70WG	boscalid	BASF	315.2 g
Bravo WeatherStik 6SC	chlorothalonil	Syngenta	2.34 l
Switch 62.5 WG	cyprodinil + fludioxonil	Syngenta	980.7 g
Tanos 50DF	famoxadone + cymoxanil	DuPont	540.4 g
Fontelis SC	penthiopyrad	DuPont	1.17 l
Omega 500F	fluazinam	Syngenta	1.75 l
Cabrio EG	pyraclostrobin	BASF	840.6 g
Quadris Top SC	difenoconazole + azoxystrobin	Syngenta	1.02 l
Scala SC	pyrimethanil	Bayer	1.31 l
Merivon SC	fluxapyroxad + pyraclostrobin	BASF	438.5 ml
Inspire EC	difenoconazole	Syngenta	511.5 ml
Regalia	*Reynoutria sachalinensis* extract	Marrone	9.35 l
Roper DF Rainshield	mancozeb	Loveland	2.24 kg
Quadris SC	azoxystrobin	Syngenta	1.13 l

In 2014, a severe leaf blight outbreak occurred at Site A such that incidence could not be meaningfully assessed after June 26 due to defoliation, plant death, and tissue breakdown. Therefore, only disease severity was assessed for the remainder of the season. Seedhead yield was determined and quality was visually assessed by the methods described below. In the center of each plot, all seedheads in a 1-m^2 area were hand-harvested by cutting the peduncle 7.5 cm below the seedhead. In 2014, seedheads were not produced at Site A due to severe disease pressure, but were collected from Site B. In 2015, seedheads from both sites (C and D) were collected from only three of the four replicates from each site due to time constraints. Seedheads

were weighed immediately and the drupes visually assessed for color, maturity, and quality. A categorical scale from 1-5 was used: 1=seedheads intact and drupes ripened, 2=seedheads intact but drupes green and/or undersized, 3=seedheads appear healthy, drupes readily detach and appear underdeveloped, 4=discolored, small, underdeveloped drupes, 5=drupes absent or shriveled, discolored with fungal growth occasionally observed (Figure 2.1). Seed yield and categorical data were analyzed using analysis of variance (ANOVA) and treatment differences were compared using a Fisher's least significant difference test (p = 0.05) (Statistical Analysis Software [SAS] 9.3).

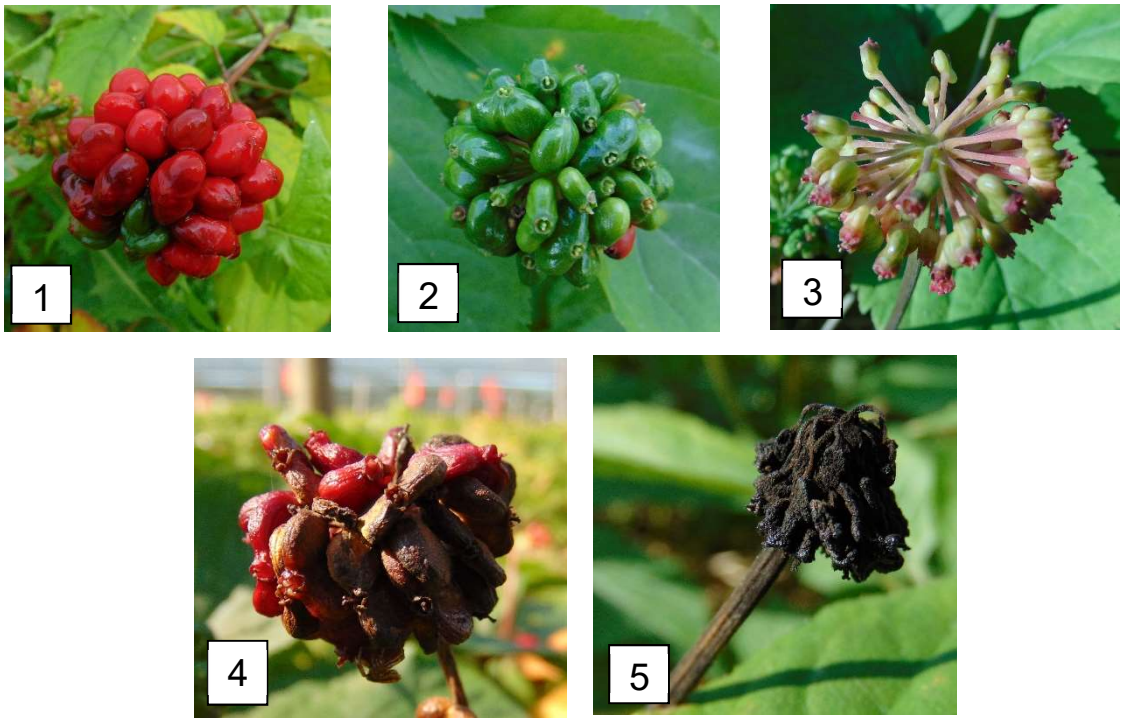

Figure 2.1. Category ratings of American ginseng seedhead quality. 1, seedheads intact and ripened, 2, seedheads intact but some still green or small, 3, seedheads appear disease free but crumble when picked, underdeveloped drupes, aborted, 4, some visible sign of disease, 5, seedhead completely diseased.

Twenty-five representative drupes from each category for each treatment were selected from one replication and plated onto 2% water agar (WA) and monitored for mycelial growth for 10 days. When mycelia were present, single hyphal tips were transferred to dilute V8 agar (2 mL

V8 [Campbell Soup, Inc.], 20 g agar, in 1 L ddH_2O) (Miller 1955) to induce sporulation for morphological identification.

TOM-CAST trial. Air temperature and leaf wetness were recorded hourly using a WatchDog 1000 Series weather station with an external leaf wetness sensor (SpecWare Software, Spectrum Technologies, Inc., Aurora, IL) fixed on a stake located at the center of each research plot. The leaf wetness sensor was sloped at a 45-degree angle facing north and placed within the upper 25% of the plant canopy, approximately 33 cm high. Five treatments included a non-treated control, fungicides applied every 7 or 10 days, or according to TOM-CAST 10 or 15 DSVs. Daily DSVs were calculated using mean temperature and hours of leaf wetness per day to generate a value ranging from 0 to 4, where 0 was unfavorable for disease development and 4 was highly favorable, for disease development (Pitblado 1992) (Table 2.2). Daily DSVs accumulated until either the 10 or 15 DSV threshold was reached, at which point, a fungicide spray was applied and the cumulative DSV for the respective TOM-CAST program was reset to zero.

Table 2.2. Disease severity values (DSV) for a 24-hour period given hours of leaf wetness per day and mean temperature.

DSV calculation[x]					
Mean temp (°C)	**Hours of leaf wetness per day**				
<17	0-6	7-15	16-20	21+	
18-20	0-3	4-8	9-15	16-22	23+
21-25	0-2	3-5	6-12	13-20	21+
>26	0-3	4-8	9-15	16-22	23+
Daily DSV	0	1	2	3	4

[x] Pitblado, 1992.

Fungicide programs included the following products in alternation: chlorothalonil (Bravo Weatherstik SC, 2.34 l/hectare; Syngenta Crop Protection, Inc.), boscalid (Endura 70 WG, 315.2 g/hectare; BASF Ag Products), azoxystrobin (Quadris F, 1.13 1/hectare; Syngenta Crop

Protection, Inc.), fluazinam (Omega 500 F, 1.75 l/hectare; Syngenta Crop Protection, Inc.), cyprodinil+fludioxonil (Switch 62.5 WDG, 980.7 g/hectare; Syngenta Crop Protection, Inc.) (Table 2.3). Fungicides were applied with a CO_2 backpack boom sprayer equipped with four 8003VS nozzles (TeeJet, Spraying Systems Co., Wheaton, IL) spaced 45.7 cm apart, operating at 275.8 kPa, and delivering 7 liter/hectare according to the treatment schedule (Table 2.3) beginning at emergence (29 May 2013 and 15 May 2014) and ending when plants began to senesce in the fall (12 August 2013 and 21 August 2014). In 2013, the 7-day spray schedule resulted in 14 to 15 applications and TOM-CAST 10 DSV treatments received 10 to 11 applications. In 2014, the 7-day spray schedule and TOM-CAST 10 DSV treatments received 12 and 8 applications, respectively.

Table 2.3. Fungicide program for the calendar-based and TOM-CAST forecasting model treatments in 2013 and 2014 on American ginseng to manage Alternaria leaf blight.

Treatment	Active ingredient	Rate/hectare
Bravo Weatherstik SC	chlorothalonil	2.34 l
alt.[x] Endura 70 WG	boscalid	315.2 g
alt. Quadris F	azoxystrobin	1.13 l
alt. Omega 500 F	fluazinam	1.75 l
alt. Switch 62.5 WG	cyprodinil/fludioxonil	980.7 g

[x] *alt*. = alternated with.

In 2014, three-year-old roots were harvested from Sites 3 and 4 on 18 October. Thirty roots from each plot were randomly selected, washed, air-dried, weighed (fresh weight), oven-dried for 48 hours at 43°C, and weighed again (dried weight).

RESULTS

Fungicide trial. In 2014, lesions were first observed on 4 June and disease incidence reached 100% in the control plots by 6 August. At Site A, plants were defoliated and collapsed making it difficult to assess disease incidence after 2 July so only disease severity was assessed for the remainder of the growing season. In 2014, pathogen pressure was relatively low at Site B

and all fungicides effectively controlled disease severity, except *R. sachalinensis* extract (Table 2.4). Pathogen pressure was higher at Site A and only difenoconazole+azoxystrobin limited leaf blight to a level considered acceptable to growers. In 2015, leaf blight developed on 23 July and 50% of control plants were diseased by 28 August (*data not shown*). Chlorothalonil, fluazinam, and mancozeb were effective in 2015, controlling leaf blight to an acceptable level (Table 4). Overall, pyraclostrobin and difenoconazole+azoxystrobin were most effective at controlling leaf blight (Table 2.4). *Reynoutria sachalinensis* extract was not significantly different from the non-treated control.

When combined over two years, only pyraclostrobin and mancozeb resulted in higher seedhead yield than the control (Figure 2.2). Treatments of mancozeb and pyraclostrobin resulted in 35% and 30.6% of high quality (category 1, seedheads intact and ripened) seedheads, respectively, with only 3.3% that were unhealthy (category 5) (Table 2.5). Conversely, non-treated control plots had few high quality seedheads (2.2% in category 1) and 37.8% seedheads in category 5. Treatments with extract of *R. sachalinensis* and famoxadone+cymoxanil produced poor quality (category 5) seedheads that were similar to the control. Azoxystrobin, mancozeb, difenoconazole+azoxystrobin, and penthiopyrad all had a significantly greater percentage of category 2 (seedheads intact but drupes green and/or undersized) seedheads than the control. The chlorothalonil treatment had significantly more seedheads (46.7%) in category 3 (seedheads appear healthy, drupes readily detach and appear underdeveloped) than the control (19.4%). The control, boscalid, and azoxystrobin treatments had similar percentages of seedheads in category 4 (discolored, small, underdeveloped drupes), while the mancozeb treatment had the fewest (6.7%).

Table 2.4. Disease severity of Alternaria leaf blight and the area under the disease progress curve (AUDPC) values of cultivated ginseng when treated with fungicides and a biocontrol at four field sites in 2014 and 2015.

Treatment	2014[x]			2015			
	AUDPC Incidence	Severity		AUDPC Incidence		Severity	
	(Site B)	(Site A)	(Site B)	(Site C)	(Site D)	(Site C)	(Site D)
non-treated	10,046.6 a[y]	10.0 a	9.0 a	506.6 ab	1,233.8 ab	6.8 a	3.5 ab
cyprodinil + fludioxonil	174.6 f	8.0 bc	2.3 d	587.3 a	937.1 a-d	6.8 a	4.0 a
difenoconazole + azoxystrobin	328.8 ef	6.3 d	2.0 d	184.9 cd	170.6 de	4.0 cd	2.3 de
pyraclostrobin	343.8 ef	6.7 cd	2.0 d	82.1 d	84.0 de	3.0 de	2.0 e
fluxapyroxad + pyraclostrobin	461.8 ef	7.0 cd	2.0 d	178.9 cd	477.8 b-e	5.0 bc	2.3 de
pyrimethanil	517.5 ef	9.3 ab	2.3 d	284.6 bcd	910.9 a-e	6.8 a	2.8 cd
mancozeb	659.4 def	10.0 a	2.3 d	46.9 d	44.6 e	2.0 e	2.0 e
azoxystrobin	836.0 c-f	7.7 cd	2.3 d	238.1 bcd	887.3 a-e	6.0 ab	2.5 de
fluazinam	858.3 c-f	9.3 ab	2.0 d	85.5 d	84.0 de	2.3 e	2.0 e
boscalid	1,113.5 c-f	10.0 a	2.8 cd	392.6 abc	1,141.9 abc	6.5 ab	3.3 bc
chlorothalonil	1,571.0 c-f	10.0 a	2.8 cd	69.4 d	254.6 cde	3.3 de	2.0 e
difenoconazole	1,901.0 cde	9.7 a	2.8 cd	80.3 d	249.4 de	4.0 cd	2.0 e
penthiopyrad	2,139.6 cd	10.0 a	3.3 cd	116.6 cd	223.1 de	2.8 de	2.3 de
famoxadone + cymoxanil	2,317.5 c	10.0 a	3.8 c	645.0 cd	1,323.0 ab	6.8 a	3.8 ab
Reynoutria sachalinensis extract	5,937.9 b	10.0 a	6.5 b	583.1 a	1,606.5 a	7.0 a	3.5 ab

[x] No incidence data was recorded for Site A in 2014 due to severe disease pressure.
[y] Column means with a letter in common are not significantly different (Fisher's LSD; P=0.05).

Alternaria alternata was isolated from all drupes from categories 1, 2, and 3. *Fusarium* spp. were identified on 44.0% and 48.0% of the drupes from categories 4 and 5, respectively. *Alternaria alternata* was found on 44.0% of category 4 drupes and 24.0% of category 5 drupes, while *A. panax* was found on 4.0% of category 4 drupes and 12% of category 5 drupes (Table 2.5).

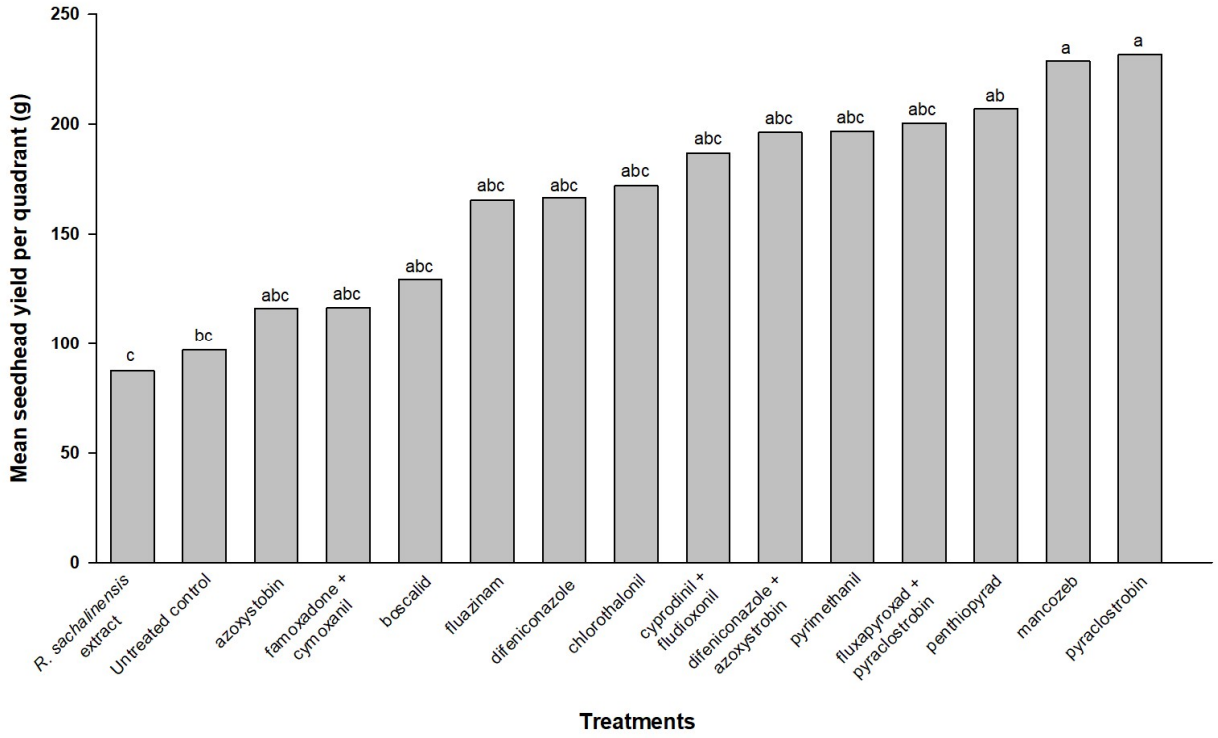

Figure 2.2. Mean American ginseng seedhead yields from three replicates of one-square-meter quadrants within a ginseng garden treated with various fungicides in 2014 and 2015. Column means with a letter in common are not significantly different (Fisher's LSD; *P*=0.05).

Table 2.5. Average percentage of American ginseng seedheads from each fungicide treatment that were assessed for quality and placed into each category.

Treatment	Seedhead health category (% of total seedhead yield)[x]				
	1	2	3	4	5
non-treated	2.2 d[y]	0.0 c	19.4 b	40.6 a	37.8 ab
mancozeb	35.0 a	24.4 a	30.6 ab	6.7 c	3.3 c
pyraclostrobin	30.6 ab	15.6 abc	36.1 ab	14.4 bc	3.3 c
fluxapyroxad + pyraclostrobin	28.9 abc	11.1 abc	27.8 ab	15.6 bc	16.7 bc
difenoconazole + azoxystrobin	21.7 a-d	21.7 ab	32.8 ab	17.2 bc	7.8 c
difenoconazole	18.9 a-d	14.4 abc	30.6 ab	13.9 bc	22.2 bc
fluazinam	17.8 a-d	18.9 abc	43.9 ab	10.6 bc	9.4 c
penthiopyrad	16.7 a-d	21.1 ab	40.0 ab	16.1 bc	6.1 c
cyprodinil + fludioxonil	14.4 bcd	18.9 abc	39.4 ab	12.8 bc	14.4 bc
pyrimethanil	12.2 bcd	16.1 abc	36.1 ab	18.3 bc	17.2 bc
chlorothalonil	10.0 cd	12.2 abc	46.7 a	18.3 bc	12.8 bc
boscalid	6.1 d	18.9 abc	34.4 ab	26.1 ab	14.4 bc
Reynoutria sachalinensis extract	5.0 d	2.8 bc	25.0 ab	17.8 bc	49.4 a
azoxystrobin	3.3 d	26.1 a	27.2 ab	23.3 abc	20.0 bc
famoxadone + cymoxanil	1.7 d	10.0 abc	31.1 ab	21.1 bc	36.1 ab

[x]Seedheads were rated using the following scale: 1, seedheads intact and ripened, 2, seedheads intact but some still green or small, 3, seedheads appear disease free but crumble when picked, underdeveloped drupes, aborted, 4, some visible sign of disease, 5, seedhead completely diseased.

[y]Column means with a letter in common are not significantly different (Fisher's LSD; P=0.05).

TOM-CAST trial. In 2013, leaf blight disease incidence was observed in moderate levels at seedling site 1, and two-year-old sites 3 and 4 (Figure 2.3) with >50% of the foliage in control plots diseased by 21 August; disease symptoms were not observed at seedling site 2. In 2014, leaf blight developed at all four sites (Figure 2.4); plants in control plots were completely defoliated by 1 July at sites 1, 2 and 3. Site 4 exhibited moderately high disease levels and plants in the control plots were defoliated by 22 July (data not shown). All treatments limited disease severity compared to the control in 2013 and 2014 (Table 2.6). In 2014 (sites 3 and 4), the 7-day application program provided the greatest level of control compared to the other treatments ($P > 0.05$). Treating according to TOM-CAST 15 DSV in 2013 resulted in disease severity similar to the 7-day program for sites 1 and 4. However, disease severity was greatest for TOM-CAST 15

DSV in 2013 at site 3 and in 2014 at sites 1, 2, and 4 when compared with the other fungicide programs. A one-way ANOVA was conducted to compare the effect of garden age on treatment effectiveness, but no significant effect was found.

According to the AUDPC data, all treatments limited disease compared to the non-treated control in 2013 and treatments were similar to each other (Table 2.6). The AUDPC data from 2014 showed that the 7-day treatment had fewer diseased plants than the control at each of the four sites; the TOM-CAST 10 and 15 DSV treatments limited disease compared to the control at sites 1, 2, and 4 (Table 2.6). The TOM-CAST 10 DSV resulted in a reduced AUDPC compared to the 15 DSV for sites 1 and 2. For sites 2 and 4, the TOM-CAST 10 DSV resulted in AUDPC values not significantly different for the 7-day treatment; for the other sites the 7- or 10-day treatments had reduced AUDPC values compared to the TOM-CAST 10 or 15 DSV treatments.

Fresh and dried root yield was significantly reduced in the control plots compared to the 7- and 10-day spray schedules at both sites (Table 2.6). The TOM-CAST 10 DSV treatment yielded similarly to the 7-day treatment schedule at each site for both fresh and dried root; the TOM-CAST 10 and 15 DSV treatment yields were not significantly different for dried root at both sites.

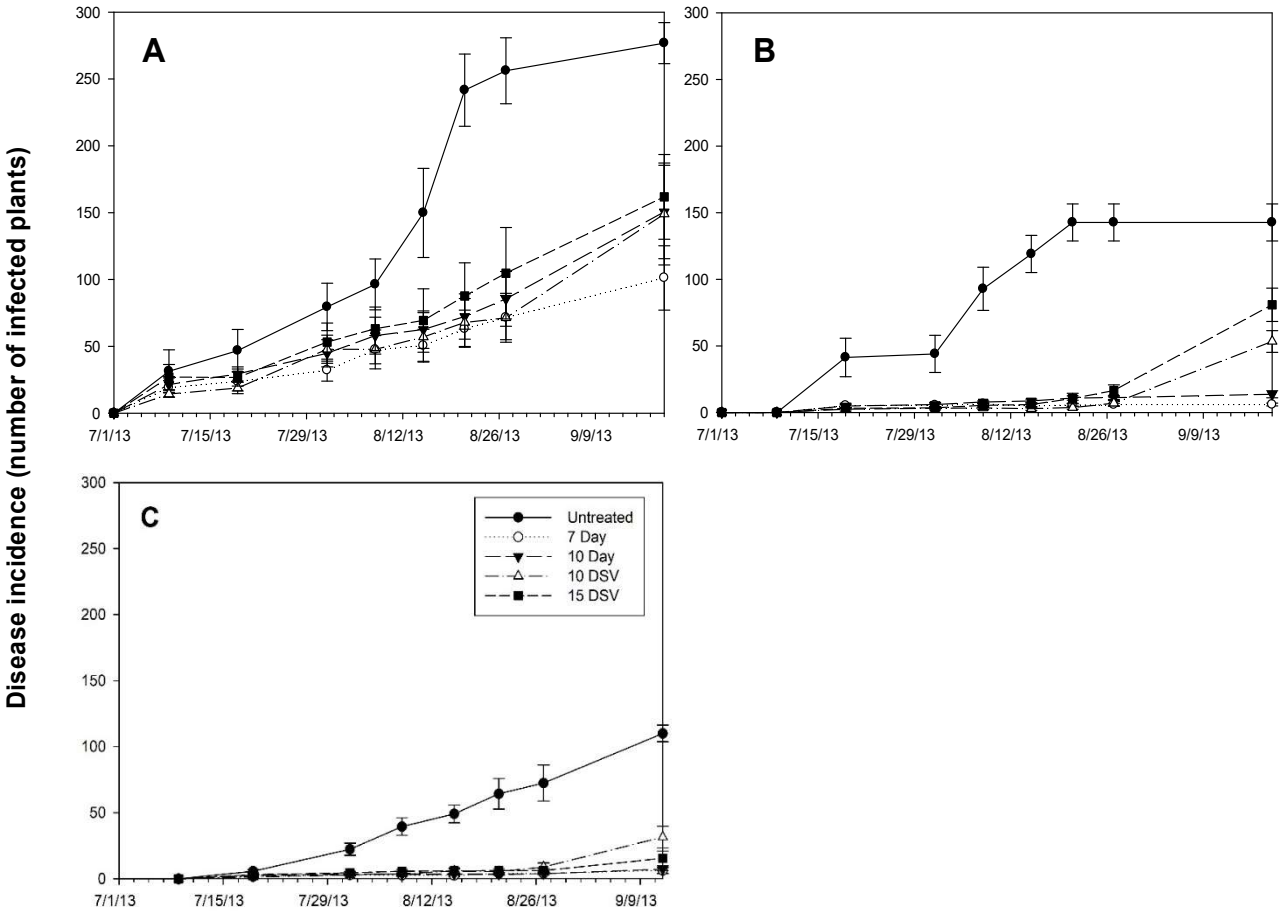

Figure 2.3. Disease progress curves for Alternaria leaf blight infection on American ginseng of **A,** Site 1, **B**, Site 3, and **C**, Site 4 in 2013 not treated or treated with fungicides every 7 or 10 days, or according to the TOM-CAST disease forecaster using disease severity values (DSVs) of 10 or 15. Site 2 not pictured because no disease occurred in 2013. Error bars represent the standard error of the mean.

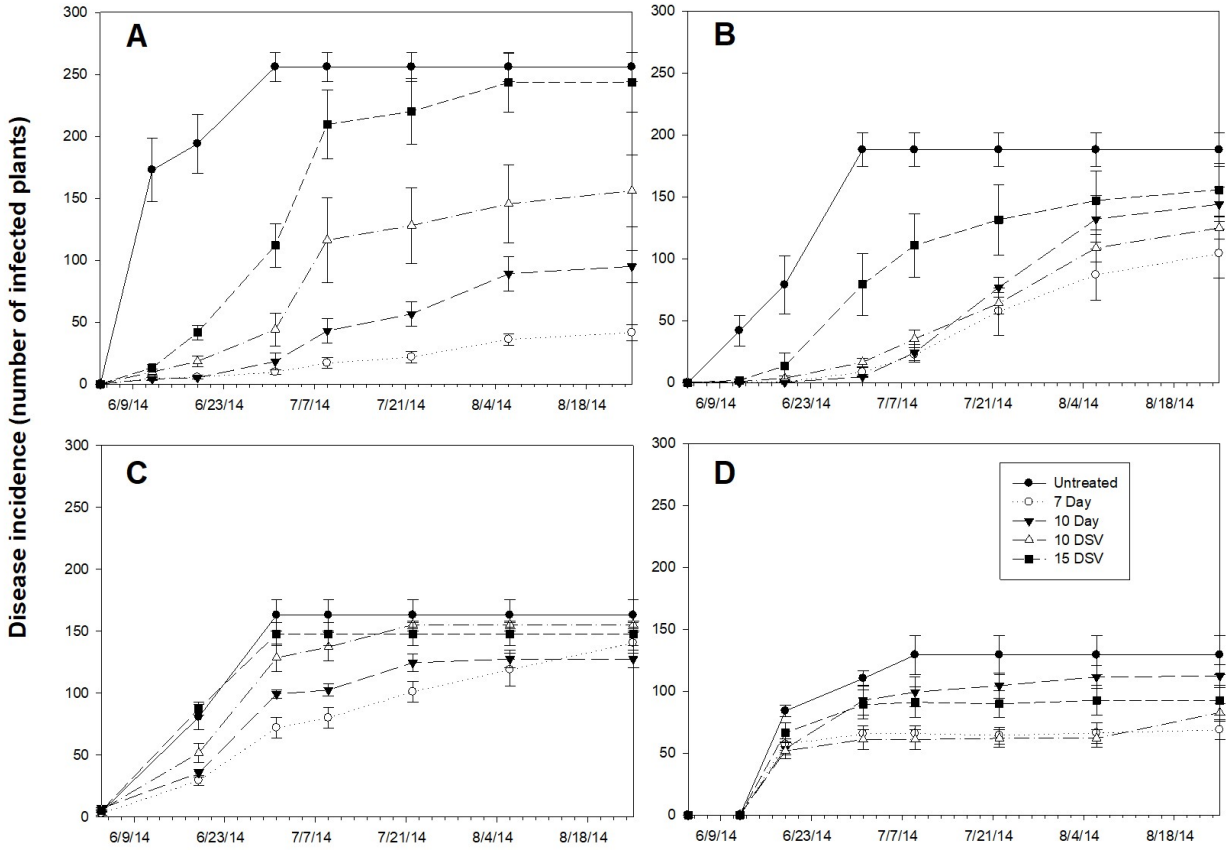

Figure 2.4. Disease progress curves for Alternaria leaf blight infection on American ginseng for **A**, Site 1, **B**, Site 2, **C**, Site 3, and **D**, Site 4 in 2014 not treated or treated with fungicides every 7 or 10 days, or according to the TOM-CAST disease forecaster using disease severity values (DSVs) of 10 or 15. Error bars represent the standard error of the mean.

Table 2.6. Yield, disease severity caused by Alternaria leaf blight on American ginseng, and the area under the disease progress curve (AUDPC) values of cultivated ginseng when treated with fungicides according to a calendar-based (7 or 10 days) or TOM-CAST-based (10 or 15 DSV) fungicide program during 2013 and 2014

		No. of Applications		Disease Severity [z]		AUDPC [x]		Yield (g) [y]	
Site[w]	Treatments	2013	2014	2013	2014	2013	2014	Fresh	Dry
1	Non-treated	-	-	7.2 a [u]	9.8 a	9824.7 a	18752.4 a	- -	- -
	7 day	14	12	2.3 b	2.0 d	3449.8 b	1662.9 d	- -	- -
	10 day	10	8	3.0 b	3.3 d	4421.8 b	3913.8 d	- -	- -
	10 DSV [v]	7	7	3.0 b	5.5 c	3947.6 b	7645.6 c	- -	- -
	15 DSV	5	4	3.0 b	7.3 b	5013.7 b	13341.3 b	- -	- -
2	Non-treated	-	-	0.0 a	10.0 a	0.0 a	12520.5 a	- -	- -
	7 day	14	12	0.0 a	3.2 c	0.0 a	3586.8 c	- -	- -
	10 day	10	8	0.0 a	4.3 c	0.0 a	4989.7 c	- -	- -
	10 DSV	6	7	0.0 a	4.5 c	0.0 a	4501.3 c	- -	- -
	15 DSV	4	5	0.0 a	6.7 b	0.0 a	7912.9 b	- -	- -
3	Non-treated	-	-	10.0 a	10.0 a	5905.3 a	10753.2 a	7.47 a	1.74 a
	7 day	15	12	1.5 d	7.2 b	337.5 b	6718.6 b	14.20 c	3.85 d
	10 day	11	8	2.5 d	9.0 a	523.9 b	7656.8 b	13.27 bc	3.51 cd
	10 DSV	7	6	4.2 c	9.8 a	627.8 b	9570.3 a	11.80 bc	2.87 bc
	15 DSV	5	4	5.3 b	10.0 a	1038.9 b	9899.3 a	10.40 ab	2.41 ab
4	Non-treated	-	-	9.3 a	10.0 a	2948.1 a	8487.2 a	4.64 a	1.41 a
	7 day	15	12	1.8 c	4.5 d	202.8 b	4561.5 c	15.20 c	4.67 c
	10 day	11	8	2.0 c	8.7 b	232.2 b	6893.4 ab	10.42 b	2.99 b
	10 DSV	6	8	3.0 b	6.3 c	489.6 b	4440.1 c	14.70 c	4.44 c
	15 DSV	4	4	2.2 c	7.8 b	388.9 b	6183.8 bc	12.02 b	3.39 b

[z] Disease severity was rated using a rating scale 1-10: 1=0-10% foliage in plot covered in lesions, 2=11-20%, 3=21-30%, 4=31-40%, 5=41-50%, 6=51-60%, 7=61-70%, 8=71-80%, 9=81-90%, 10=91-100%.

[x] Area under the disease progress curve (AUDPC) is calculated from the number of diseased plants per plot over the course of a growing season.

[y] Average weight per root.

[w] Sites 2, 3 and 4 were 2 yrs old in 2013 and 3 yrs old in 2014. Site 1 was 1 yr old in 2013 and 2 yrs old in 2014.

[v] Disease severity value (DSV).

[u] Values followed by the same letter in a column/site are not significantly different (LSD, p = 0.05).

DISCUSSION

The root and seed of American ginseng are economically important for growers. Alternaria leaf blight, which can impact root and seed yield, occurs annually in cultivated ginseng and effective, resilient strategies are needed to limit outbreaks, protect yield and quality, and prevent winterkill of this valuable perennial root crop (Hausbeck 2011). The high cost of establishing cultivated ginseng, along with the relatively high farm-gate value of the root, necessitates the development of a consistently effective fungicide program that both reduces the risk of leaf blight and the development of fungicide resistance.

Differences among fungicides in limiting leaf blight were observed similar to what has been reported for *Alternaria* in other systems. Pyraclostrobin and difenoconazole+azoxystrobin limited leaf blight in both years of the study. According to the EPA website (epa.gov/pesticide-registration/reduced-risk-and-organophosphate-alternative-decisions last accessed June 23, 2018), Quadris Top (difenoconazole+azoxystrobin) was registered for use in ginseng in December 2015, shortly after this research was conducted and Cabrio (pyraclostrobin) was registered for use in ginseng since 2006 (National Pesticide Information Retrieval System 2018). Pyraclostrobin and azoxystrobin are strobilurin fungicides and belong to the quinone outside inhibitor (QoI) group (FRAC group 11) as they bind to the Qo site of cytochrome *b* (Bartlett 2002). Due to a single site mode of action, the potential for development of resistance within the pathogen population is high (Vincelli 2002). Resistance to azoxystrobin has been reported for more than 36 plant pathogens, including *Alternaria* spp. responsible for blight on pistachio, potato, tomato, and apple, and several other fungal pathogens of crops including soybean, wheat, barley, and sugar beet (FRAC 2013).

Resistance also is a concern with other fungicides. For example, resistance to the iprodione fungicides was detected in an *A. panax* population from Wisconsin after only three years of use (Rahimian 1987). Malandrakis et al. (2015) found a positive cross-resistance relationship between iprodione and fludioxonil, indicating that the mutations responsible for resistance to iprodione could lead to resistance to fludioxonil. Iprodione is not regularly used for leaf blight control in ginseng but fludioxonil is used (*personal communication*). Although applications of cyprodinil+fludioxonil resulted in significantly less disease than the control in 2014, efficacy was not observed in 2015 as the disease levels were not significantly different between this fungicide treatment and the control at both locations. While this could be a result of increased pathogen pressure observed in 2015, it might indicate that resistance to this fungicide may be developing in the pathogen population, similar to what was previously reported by Rahimian (1987). This warrants further testing.

Other *Alternaria* spp. have demonstrated resistance to fungicides that are commonly used to control *A. panax*. For example, *A. alternata* and *A. solani* have shown resistance to azoxystrobin (Tymon and Johnson 2014) and boscalid (Tymon and Johnson 2014; Landschoot et al. 2017). *A. alternata* has also been found to be resistant to fludioxonil, cyprodinil and pyraclostrobin (Avenot and Michailides 2015). Pyraclostrobin and difenoconazole+azoxystrobin should be alternated with effective fungicides from different FRAC groups, such as mancozeb (FRAC group M03), which was the most effective treatment in 2015, and fluazinam (FRAC group 29), which provided a similar level of disease control as pyraclostrobin and difenoconazole+azoxystrobin. In addition, tank mixing fungicides that include two modes of action (i.e. contact and systemic) may improve long-term control and reduce the risk of pathogen resistance (Skylakakis et al. 1981).

Fungicide studies previously conducted in 2011 with Wisconsin ginseng growers indicated boscalid was highly effective against leaf blight (Hausbeck and Harlan 2013a; 2013b). In the current study, boscalid limited disease in 2014 but was not significantly different from the control in 2015. Boscalid belongs to the succinate dehydrogenase inhibitor fungicides and differs from strobilurins in its target site and mode of action, although it also targets a single site and, therefore, may be at risk for pathogen resistance development (Anonymous 2013; Avenot and Michailides 2007). In some instances, strobilurin-resistant isolates showed increased sensitivity to boscalid, as reported by Markoglou et al. (2006) in *Botrytis cinerea*. Wisconsin ginseng growers began to use boscalid in 2008 (Hausbeck 2011). Strobilurin fungicides have been widely used to protect ginseng in Wisconsin for over 10 years (*personal communication)*. While difenoconazole+azoxystrobin was highly effective, azoxystrobin alone provided limited protection in the current study. In prior testing, azoxystrobin was effective compared to the control in 2011 but resulted in a relatively high disease level similar to the contact fungicide, chlorothalonil (Hausbeck and Harlan 2013b).

Growers collect ginseng seed for future plantings or to sell. In our study, seedhead yield and drupe quality was correlated to fungicide treatments. Applications of mancozeb and pyraclostrobin resulted in increased seedhead yields compared to the control (Figure2.2). Fungi were recovered from drupes. *Alternaria alternata* was isolated from all drupes in categories 1 (seedheads intact and drupes ripened) through 3 (seedheads appear healthy, drupes readily detach and appear underdeveloped). *Alternaria alternata* was reported as a foliar pathogen of ginseng (Punja, 1997). *Fusarium* spp. also were isolated from poor quality drupes and have been reported to cause root (Vea and Palmer 2016) and seed rot (Reeleder et al. 2002) in American ginseng as well as several other crops.

Growers desire to optimize the timing of fungicide applications to reduce production costs, the potential for pesticide residue on the dried root, and the development of fungicide resistance in the pathogen. The fungicides used in the TOM-CAST study included alternating applications of fungicides representing six FRAC groups expected to delay development of fungicide resistance. Chlorothalonil and fluazinam are contact fungicides, whereas boscalid, azoxystrobin, cyprodinil, and fludioxonil all have translaminar or systemic activity (Anonymous 2017). The amount of leaf blighting that can be tolerated is low due to the determinate nature of the ginseng plant whereby new foliage is not produced beyond that which emerges in the spring. In our study, the TOM-CAST 10 DSV program consistently provided disease control in 2013 that was similar to the 10-day calendar spray program for all sites according to the AUDPC data. The results from 2014 differed; only one site showed similar AUDPC data between TOM-CAST 10 DSV and the 10-day spray program. Yield data support our finding that TOM-CAST 10 DSV could be used to time fungicide sprays for leaf blight in ginseng without sacrificing yield. For both sites, neither yields of fresh or dried roots from the 10 DSV treatment differed significantly from the 7-day treatment; meanwhile, the number of fungicide applications was reduced by 4 to 6 sprays. Even when the number of fungicide applications was the same as the 10-day treatment, as seen in Site 4 in 2014, yield was higher in the 10 DSV treatment, suggesting that timing of fungicide sprays is more important than frequency of fungicide applications, which fits with what has been found for *Alternaria* in other systems.

A 15 DSV threshold with TOM-CAST is adequate to control disease on tomato (Keinath et al. 1996), carrot (Bounds et al. 2007), and asparagus (Meyer et al. 2000). In each of these crops, some disease symptoms are tolerable. Conversely for celery, no foliar disease can be tolerated as the petiole is marketed; a conservative threshold of TOM-CAST 10 DSV is needed

(Bounds and Hausbeck 2007). In the current study, using a threshold of 15 DSV was inadequate to control Alternaria leaf blight damage in American ginseng production systems. The more conservative threshold of 10 DSV may provide a balance between reducing fungicide applications while protecting valuable yields. Equal attention should also be paid to the fungicides used with the forecaster to ensure that pathogen resistance is not playing a significant role.

While our study demonstrates a potential for TOM-CAST to decrease the fungicide applications needed to protect cultivated ginseng from leaf blight, further studies are warranted to optimize the fungicides used and their order of application in conjunction with the program to ensure consistency across crop ages, sites, and growing seasons.

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CHAPTER III: IDENTIFICATION OF FUNGI ASSOCIATED WITH CULTIVATED AMERICAN GINSENG SEED AND EFFECT OF SEED TREATMENTS ON PLANT STAND IN A COMMERCIAL SETTING

ABSTRACT

Cultivated American ginseng is a perennial herb valued as a traditional Chinese medicine. This perennial crop is direct seeded under artificial shade provided by wooden lathe structures or polypropylene shade canopies. The seed must be stratified, requiring a sequence of cold-warm-cold conditions, over an 18- to 22-month period. The establishment costs of a ginseng planting are high and seed health is an important consideration. The objectives of this research were to determine the fungi associated with ginseng drupes, green seed, and the endosperm, and assess the impact of seed treatment on plant stand in the field. A field experiment was conducted in each of two years in a commercial ginseng planting and included stratified seed treated with the fungicides penthiopyrad, oxathiapiprolin, fluopicolide, ethaboxam, captan, mefenoxam + fludioxonil, and azoxystrobin. *Alternaria* spp. were commonly isolated from drupes and green seed coat and *Fusarium* spp. were recovered from all seed parts, including the endosperm. *Cylindrocarpon destructans* was only recovered in low numbers on green seed coats. Although none of the treatments significantly increased final plant stand compared to the control, oxathiapiprolin resulted in the highest plant stands compared to other treatments on the first rating date, 8 June, and azoxystrobin resulted in the highest plant stands compared to other treatments on 7 July. There were no significant differences among treatments and control on all other rating dates. In year two, all the treatments resulted in plant stands similar to the non-

treated control, except for fludioxonil + mefenoxam which had a significantly reduced plant stand compared to the control on the first and last rating dates.

INTRODUCTION

American ginseng (*Panax quinquefolius*) is a perennial herb native to woodlots in the eastern U.S. and Canada (Proctor and Bailey 1987). The crop is commercially cultivated under artificial shade provided by wooden lathe structures or polypropylene shade canopies to simulate the forest understory (Proctor and Bailey 1987). Roots, three years or older, are harvested and dried for use in traditional Chinese medicine (Hu 1976) and other products (Proctor 1996).

Ginseng growers rely on seed produced from their own plantings to establish new plantings (Proctor and Bailey 1987). A mature ginseng plant produces umbelliferous inflorescences of up to 50 white, perfect, self-fertilizing flowers (Proctor 1987). Following fertilization, one red drupe per flower develops in late summer containing 1 to 3 seeds. Each plant may have 30 to 40 drupes (Schluter and Punja 2000). The seed contains a rudimentary embryo that is approximately 0.4 - 0.5 mm in length at ripening, growing to 5 - 6 mm in length at maturity. The seed must undergo stratification to break dormancy, requiring a sequence of cold-warm-cold temperatures (Baskin and Baskin 1998). Stoltz and Snyder (1985) described a physiological and morphological dormancy that is broken by 360 days at 20ºC at which point a second physiological dormancy is overcome when the seed is exposed to 5ºC for 150 days. The fluctuating temperatures enable the embryo to mature and the endocarp may split approximately one month before germination (Stoltz and Snyder 1985). Historically, stratification was achieved using a sand mixture in a seed box buried in the ground. However, stratification can be effectively achieved using a seed and sand mix placed in barrels that are maintained in

controlled-temperature ware-house type conditions, thus avoiding exposure to soil-borne pathogens (Proctor 2001).

Growers in Michigan and Wisconsin harvest drupes from August to September, depulp them, mix the seed in moist sand, and store them under temperature-controlled conditions for approximately one year (*personal communication)*. The moist conditions during stratification may favor infection by plant pathogens (Tianyu and Weiqun 1992; Proctor 1996; Punja et al 2008a; 2008b). Following stratification, the seed is broadcast on the soil between July and October and covered with a thick layer of straw mulch where it is subjected to a final cold period during the winter. The seed germinates the following spring and the seedling is susceptible to damping-off (Lee et al. 1981; Proctor 1996; Ziezold et al. 1998; Punja et al. 2008a; 2008b). Typically, damping-off is caused by soil-borne or seed-borne pathogens either preventing germination, seedling emergence after germination, or result in the rotting and collapse of seedlings at the soil level (Lamichhane et al. 2017).

The objectives of this study included 1) identifying the fungi present on drupes and non-stratified (green) seed and endosperm, and 2) testing seed treatments for effect on plant stand when applied to stratified seed and planted in a field trial.

MATERIALS AND METHODS

Survey of drupes and green seed. Drupes and non-stratified (green) seed were obtained in September from Heil Ginseng Enterprises in Marathon County, Wisconsin. Drupes and seed were stored at 4ºC for approximately one week before samples were cultured. Two types of media were used to culture the drupe and seed tissue: 2% water agar (WA) and dichloran rose bengal choloramphenicol agar (DRBCA: 10g glucose, 5g peptone, 1g KH_2PO_4, 0.5g $MgSO_4$, 25 mg Rose Bengal, 2 mg dichloran, 0.1 g chloramphenicol, 15g agar per 1L distilled water)

(Agarwal and Sinclair 1997). WA was used for general fungal isolations and DRBCA was used to limit the rapidly growing fungi from the culture plates to allow the isolation of important fungi (Agarwal and Sinclair 1997).

Drupes and green seed were surface disinfested in 10% chlorine bleach for five minutes, rinsed twice in sterile distilled water, and dried in a laminar flow hood. Sixty drupes were placed onto WA and 60 drupes were placed onto DRBCA. Using aseptic technique, seeds were sliced in half and the embryo and seed coat halves were plated separately (Figure 3.1). Fifteen of these separated seeds were plated onto WA and 15 were plated onto DRBCA. WA and DRBCA plates were incubated under fluorescent light in the laboratory at ambient temperature and observed daily for 30 days. Once mycelia were observed, a hyphal tip was transferred to dilute V8 agar (200 mL V8 juice (Campbell Soup Company, Camden, New Jersey, USA), 2 g $CaCO_3$, 15 g agar in 800mL distilled water) (Miller 1955) to promote sporulation and incubated as described above prior to identification. Morphological characteristics were observed, and representative keys were used to identify fungi to genus or species (Simmons 2007; Barnett and Hunter 1972).

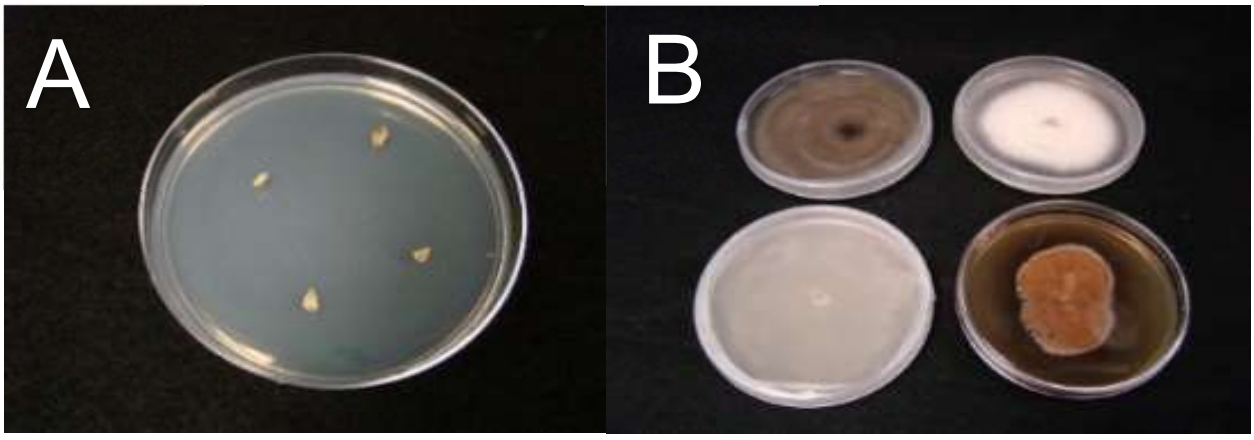

Figure 3.1. Experimental technique for isolating fungi from American ginseng seed. **A,** Endosperm of green seed cut into four parts and plated on water agar, **B,** fungal isolates transferred to PDA for identification.

Table 3.1. Seed treatments tested for effect on seedling plant stand of American ginseng in 2014 and 2015.

Treatment	Active Ingredient	Company	Rate/6.8 kg of seed
Fontelis	penthiopyrad	DuPont Crop Protection	4.4 ml
Orondis	oxathiapiprolin	Syngenta Crop Protection	4.4 ml
Presidio	fluopicolide	Valent U.S.A. LLC	4.4 ml
Ethaboxam	ethaboxam	Valent U.S.A. LLC	2.7 ml
Captan	captan	Drexel Chemical Company	14.0 g
Apron MAXX	mefenoxam + fludioxonil	Syngenta Crop Protection	22.2 ml
Quadris	azoxystrobin	Syngenta Crop Protection	4.4 ml

Seed treatment field study. For the field studies, 6.8 kg of stratified seed was treated on 5 August 2014 (Trial 1) or 26 August 2015 (Trial 2) with one of nine treatments or not treated (control). Treatments included penthiopyrad (Fontelis, 4.4 ml/6.8 kg seed; DuPont Crop Protection, Wilmington, DE, FRAC code 7), oxathiapiprolin (Orondis, 4.4 ml/6.8 kg seed; Syngenta Crop Protection, Greensboro, NC, FRAC code 49), fluopicolide (Presidio, 4.4 ml/6.8 kg seed; Valent U.S.A. LLC, Libertyville, IL, FRAC code 43), ethaboxam (Ethaboxam, 2.7 ml/6.8 kg seed; Valent U.S.A. LLC, Libertyville, IL, FRAC code 22), captan (Captan, 14.0 g/6.8 kg seed; Drexel Chemical Company, Philadelphia, PA, FRAC code M04), mefenoxam+fludioxonil (Apron MAXX, 22.2 ml/6.8 kg see; Syngenta Crop Protection, Greensboro, NC, FRAC code 4/12), azoxystrobin (Quadris, 4.4 ml/6.8 kg seed; Syngenta Crop Protection, Greensboro, NC, FRAC code 11) (Table 3.1). Seed was added to a cement mixer that was used to coat the seed. Each product was added along with a colorant to indicate product coverage and water was added to create a thick slurry that coated the seed (Figures 3.2 and 3.3). The treated or non-treated seed was then planted at a rate of 112 kg/ha into raised plant beds using a broadcast seeder. Beds were 1.2 m wide with 0.3 m between beds. Each treatment was planted into a 15.2 m bed with a 0.6 m buffer at each end. Treatments were replicated three

times in a randomized complete block design. Fertilization and pest control were applied per commercial production standards by the cooperating grower. Seedlings emerged the following May under black polypropylene canopies. Assessments were made at 14-day intervals from June 24 to August 18, 2015 (Trial 1) and from June 13 to August 15, 2016 (Trial 2) for a total of six and five ratings, respectively. The number of seedlings in the center of each plot (2.0 m x 1.0 m) were counted (Figure 3.4). Damped-off seedlings were collected in July 2015 to determine the pathogen(s) present.

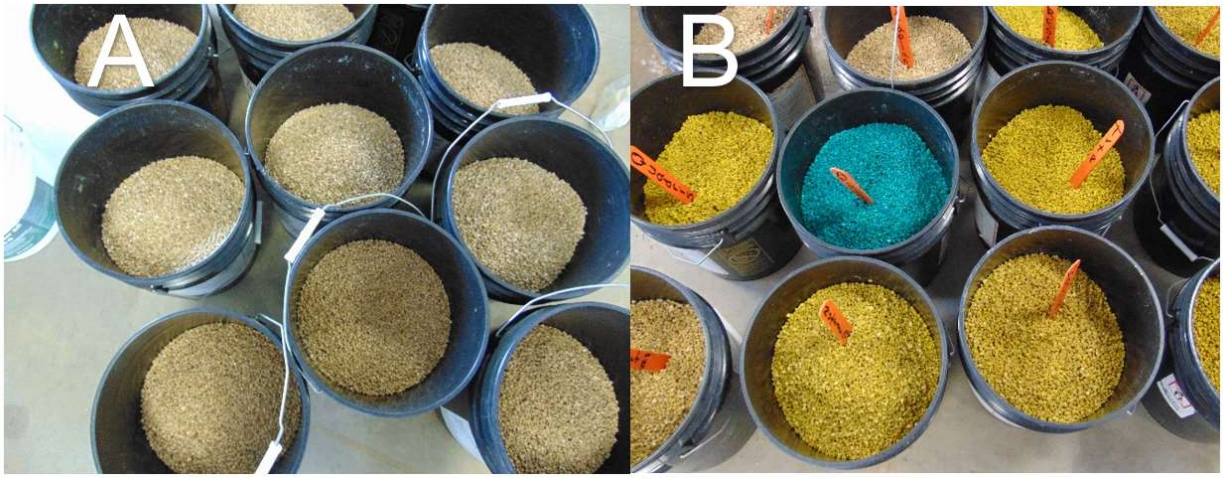

Figure 3.2. **A,** Buckets of non-treated stratified American ginseng seed and **B,** seed after colorant and/or chemical applied.

Figure 3.3. Square-meter plot of American ginseng seedling garden at the center of a 50-ft row per treatment. Orange wooden stakes marked area for entire season. Plant stand was assessed every 14 days.

RESULTS

Survey of drupes and green seed. *Alternaria alternata* and *A. panax* were frequently isolated from drupes (Table 3.2) regardless of the culture media used. *Fusarium* spp. were more frequently isolated on WA (27%) than on DRBCA (6%) (Table 3.2). Other microorganisms isolated from the drupes included *Mucor* spp. (both media) and *Streptomyces* (WA only). In general, microorganisms were more commonly isolated from the seed coat than the endosperm. *Alternaria alternata*, *Penicillium* spp., *Mucor* spp., *Streptomyces* spp., and *Fusarium* spp. were isolated from the seed coat regardless of the media. When DRBCA was used, *A. panax* and *Cylindrocarpon destructans* were recovered from the seed coat. When WA was used as the culture media, *Streptomyces* spp. and *Fusarium* spp. were isolated from the endosperm. When DRBCA media was used, *A. alternata*, *A. panax,* and *Streptomyces* spp. were recovered from the endosperm.

Table 3.2 Incidence of microorganisms isolated from American ginseng drupes and green seed and grown on water agar (WA) or dichloran rose bengal choloramphenicol agar (DRBCA).

			Alternaria alternata	*A. panax*	*Mucor sp.*	*Penicillium sp.*	*Streptomyces sp.*	*Cylindrocarpon destructans*	*Fusarium spp.*
WA[x](%)	Drupes		67	53	13	0	7	0	27
	Green Seed	Seed coat	67	0	27	40	53	0	27
		Endosperm	0	0	0	0	33	0	14
DRBCA[Y](%)	Drupes		80	44	31	0	0	0	6
	Green Seed	Seed coat	20	27	7	7	67	7	14
		Endosperm	7	7	0	0	47	0	0

[x]WA=Water Agar (20g agar per 1L distilled water)
[Y]DRBCA = Dichloran Rose Bengal Choloramphenicol Agar (10g glucose, 5g peptone, 1g KH2PO4, 0.5g MgSO4, 25 mg Rose Bengal, 2 mg dichloran, 0.1 g chloramphenicol, 15g agar per 1L distilled water)

Seed treatment field study. Treatment responses differed significantly between years, so data were analyzed separately by year. In 2015, the stand count in plots established with oxathiapiprolin-treated seed was similar to the non-treated control, but plant stand was significantly higher ($P < 0.05$) compared to all other treatments on the first (8 June) assessment date (Table 3.3). On 7 July, the stand count in plots established with azoxystrobin-treated seed was similar to the non-treated control, but plant stand was significantly higher than all other treatments. Plants stands on 24 June, 22 July, and 5 and 18 August did not differ significantly among treatments.

When the study was repeated in 2016, all the treatments had plant stands similar to the non-treated control with the exception of fludioxonil + mefenoxam, which had a significantly reduced stand count compared to the non-treated control (Table 3.4). The stand count assessments for 27 June and 25 July did not differ among the treatments. On 12 July, the stand counts for ethaboxam- or oxathiapiprolin-treated seed were higher than the plots planted with seed treated with fludioxonil + mefenoxam or azoxystrobin but were similar to the non-treated control. The azoxystrobin-treated seed had a significantly reduced plant stand compared to the non-treated control and the fludioxonil + mefenoxam seed treatment. The last plant stand assessment on 15 August, indicated that the fluopicolide-treated seed was similar to the non-treated control and resulted in a higher plant stand than the azoxystrobin and fludioxonil + mefenoxam seed treatments.

Damping-off of seedlings was occasionally observed. *Pythium* spp., *Fusarium* spp., *Phytophthora* spp., and *Rhizoctonia* spp. were isolated from damped-off seedlings but were not associated with specific treatments.

Table 3.3. Average number of American ginseng seedlings in $2m^2$ plant stand at six dates in 2015 throughout the growing season with various fungicide seed treatments or a control.

	Stand count											
	6/8		6/24		7/7		7/22		8/5		8/18	
non-treated	554.67	bc[x]	552.67	-	555.33	bc	566.33	-	553.67	-	546.00	-
fludioxonil + mefenoxam	569.67	c	590.67	-	582.67	c	589.67	-	585.33	-	533.33	-
captan	593.67	c	609.33	-	605.00	c	617.00	-	633.00	-	572.67	-
penthiopyrad	601.33	c	619.00	-	605.67	c	612.00	-	634.33	-	584.67	-
oxathiapiprolin	672.00	ab	665.33	-	661.67	c	678.33	-	671.33	-	652.00	-
fluopicalide	604.00	c	641.00	-	611.33	c	634.00	-	622.00	-	592.33	-
azoxystrobin	664.67	c	658.33	-	652.00	ab	667.00	-	699.33	-	640.00	-
ethaboxam	601.00	c	622.00	-	597.67	c	610.00	-	606.33	-	578.67	-

[x] Column means with a letter in common are not significantly different (Games-Howell; P=0.05).

Table 3.4. Average number of Amreican ginseng seedlings in a $2m^2$ plant stand at five dates in 2016 throughout the growing season with various fungicide seed treatments or a control.

	Stand count									
Treatment	6/13		6/27		7/12		7/25		8/15	
non-treated	580.33	a[x]	616.67	-	687.33	ab	666.00	-	661.67	a
fludioxonil + mefenoxam	420.00	b	471.00	-	486.33	bc	500.67	-	571.33	c
captan	441.33	ab	519.33	-	532.67	abc	543.33	-	525.30	abc
penthiopyrad	434.00	ab	511.67	-	522.00	abc	545.67	-	518.00	abc
oxathiapiproplin	570.00	ab	610.00	-	664.67	a	667.67	-	601.00	abc
fluopicolide	544.67	ab	606.00	-	602.00	abc	627.33	-	654.33	a
azoxystrobin	428.67	ab	469.67	-	469.33	c	527.00	-	485.67	bc
ethaboxam	541.33	ab	615.00	-	638.00	a	648.67	-	552.67	abc

[x] Column means with a letter in common are not significantly different (Games-Howell; P=0.05).

DISCUSSION

Ginseng drupes, green seed, and endosperm tissue were sampled to survey the fungi present. *Alternaria alternata*, *A. panax*, and *Fusarium* spp. were found on the drupes and are considered ginseng pathogens (Parke and Shotwell 1985; Punja 1997). *Alternaria panax* infection may cause abortion of developing berries (Brammal; 1994), negatively impacting seed yield. *Mucor* spp. were found on drupes and seed coats, which have been reported as a pathogen

of *P. ginseng* (Cho and Shin 2004), but not reported on *P. quinquefolius*. On the seed coat, these fungi also were found, along with *C. destructans*, a known pathogen of ginseng (Hausbeck 2011). Fewer fungi were found on the endosperm which is consistent with the literature and included *A. alternata*, *A. panax*, and *Fusarium* spp. The presence of fungi within the endosperm supports previous research on seedborne pathogens of ginseng that suggests they are present early in seed development (Punja et al 2008a; 2008b) and protection during the 4-week flowering period may limit seed infection (Schluter and Punja 2000). Supporting this, Tianyu and Weiqun (1992) recovered *A. panax* from green drupes and *C. destructans* in pedicels during the red drupe stage, suggesting these pathogens are entering the seed early in development. Although *C. destructans* rarely causes damping-off symptoms (Reeleder and Brammall 1994), it can be a difficult pathogen to control in ginseng gardens, so reducing the introduction of inoculum on seed is important (Reeleder et al. 2002).

In a previous study in Ontario, Canada, pathogenic fungi isolated from stratified seed embryos included *Fusarium* spp., *Alternaria* spp., *C. destructans*, and *Trichoderma* spp. (Ziezold et al. 1998). Other studies showed that *Fusarium* spp. were abundant on all seed stages, including young flowers, and damped-off ginseng seedlings (Ziezold et al. 1998; Reeleder et al. 2002; Punja et al. 2008a; 2008b). Reeleder et al. (2002) found that a type they called *Fusarium roseum* and *Fusarium solani* species complex, in addition to *C. destructans*, were associated with visibly rotted seeds; however, the *Fusarium* species associated with seed were not pathogenic to the roots (Reeleder et al. 2002). Other studies in Canada indicated that *F. equiseti* and *F. oxysporum* could be recovered from all seed stages, but these fungi were most commonly associated with stratified seed and were shown to be virulent on ginseng roots (Punja et al. 2008a; 2008b). *Fusarium* spp. were detected at low numbers on flowers but were found

commonly on endosperm; this suggests that floral tissue could be contaminated, but that the fungus doesn't penetrate the seed until later in seed maturation (Punja et al. 2008b). In this case, ginseng seed treatments would be most beneficial prior to stratification to avoid penetration into the endosperm where damage occurs. *Phytophthora cactorum* (Hill and Hausbeck 2008), *Pythium* spp., and *Rhizoctonia* spp. (Reeleder and Brammall 1994, present study) were recovered from ginseng seedlings showing symptoms of damping-off, however, these microorganisms were not isolated from drupes, green seed coats or endosperm in the present study. This indicates that the main causal agents of damping-off on ginseng may colonize the plant later in the seed stratification process through contaminated tissues, water, or sand, or after planting into soils that are naturally infested. This agrees with what has been found in other systems.

In year one, azoxystrobin on 7 July and oxathiapiprolin on 8 June resulted in a higher plant stand compared to other treatments, but not significantly different from the non-treated control. This was not consistent across the ratings over the season. In year two, plots established with non-treated seeds yielded significantly higher stand counts compared to all other treatments for most of the rating dates. The mefenoxam + fludioxonil and azoxystrobin treatments resulted in a significantly reduced plant stand compared to the non-treated control on 13 June and 15 August. Since some stratified seed may have split coats, phytotoxicity may occur as a result of the fungicide coming into direct contact with the endosperm, as suggested by Ziezold et al. (1998). Fungicidal seed treatments of cereals have also resulted in phytotoxicity on injured seeds (Bateman et al. 1986). Application of seed treatments prior to seed cracking may be preferred to avoid phytotoxicity. However, other research (Lee et al. 1981) has shown that when captan was applied as a pre-stratification seed treatment, delayed maturation of the embryo and

suppressed dehiscence occurred. The researchers suggested this was a result of the captan eradicating fungi that normally soften the endocarp to aid in dehiscence (Lee et al. 1981).

Environmental factors such as soil temperature and moisture may also play a role, not only in efficacy of seed treatments, but also germination and damping-off as observed in soybean seed treatment trials (Bradley 2008). As there are currently no breeding efforts for ginseng (*personal communication)*, genetic diversity in the crop is likely high, and this may affect seedling emergence rates (Schluter and Punja 2000). Stress also can be a factor in seed germination, and varying success rates of seed treatments associated with biotic and abiotic stresses have been shown (Mastouri et al. 2010).

Fungicide-treated seed commonly is used in many cropping systems and can significantly increase emergence, reduce seedling disease, and reduce the need for costly soil fumigation (Agarwal and Sinclair 1997). Chemical seed treatment to control seedborne *A. dauci* (Pryor et al. 1994) and *A. radicina* (Strandberg 1984) on carrot is effective. However, carrots, and most other agricultural crops are planted in early spring and plants emerge within weeks, whereas ginseng germinates approximately nine months after sowing (Proctor 1996).

Prior research on seed treatment efficacy on American ginseng has shown variable results. Ziezold et al. (1998) tested benomyl, thiram, and tebuconazole and found that these fungicides caused a slight to pronounced reduction in seedling emergence but did not significantly affect plant stand six weeks after seeding. Rahman and Punja (2007) tested biological control agents (*Clonostachys rosea* f. *catenulate*, *Trichoderma harzianum*, *Streptomyces griseoviridis*, and *Trichoderma virens*) and fungicides (captan and metalaxyl-m) as seed treatments. Metalaxyl-m, captan and *C. rosea* f. *catenulate* resulted in a significantly increased plant stand compared to the control, however, *C. rosea* f. *catenulate* only resulted in

this higher plant stand when applied as both a seed treatment and a drench to the seedlings (Rahman and Punja 2007). Biological control agents have potential for application prior to stratification where the biological control organisms could potentially colonize the seed and provide pathogen control during stratification (Hoefnagels and Linderman 1999), however this could also have negative effects.

Our results show a potential for success of a seed treatment protocol for American ginseng. Further investigation regarding ginseng seed pathology are warranted to delineate what formulas may be beneficial as a seed treatment. Research comparing the timing of fungicidal seed treatments will also aid in optimizing ginseng seedling success in year one.

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