FUSARIUM SPP. ASSOCIATED WITH PERENNIAL SPECIALTY CROPS

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ABSTRACT

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The genus *Fusarium* comprises an array of soilborne plant pathogens capable of causing crop significant loss of quality and yield. For perennial crops with significant start-up costs, infection by pathogenic *Fusarium* spp. can also reduce plant longevity which can be especially costly. Our objective was to determine the incidence and pathogenicity of *Fusarium* spp. associated with asparagus crowns and ginseng seed and roots. Fusarium causes root rot on asparagus decreasing the crop yield and plant longevity. Root lesions on ginseng root from Fusarium decreases the crop's quality and value. Species identification was achieved via DNA extraction, PCR using EF-1 and EF-2 primers, and Sanger sequencing. Asparagus crown (491) samples were collected from commercially popular cultivars and included 'Jersey Supreme' (100), 'Guelph Millennium' (258), and 'Guelph Eclipse' (133). 'Guelph Millennium' crowns included those grown in soils fumigated pre-plant with metam sodium (131) or not fumigated (127). Seven Fusarium species were identified from asparagus crowns; *F. oxysporum* (92%) was most abundant. *Fusarium* spp. were isolated from 'Jersey Supreme' (39%), 'Guelph Eclipse' (47%), and 'Guelph Millennium' (30%). *Fusarium* spp. were isolated from 36% and 25% of the crowns grown in non-fumigated or fumigated soils, respectively. Pathogenicity of *Fusarium oxysporum* isolates (51) differed signficantly. Ginseng seed (1,216) and roots (222) were obtained from 11 growers. *Fusarium* spp. were rarely recovered from seed (5%) or roots (2%). Eight *Fusarium* species were isolated including *F. avenaceum* (62%), *F. solani* (11%), and *F. oxysporum* (10%). Pathogenicity of select isolates was tested on seedlings and fresh roots and significant differences were noted. Results will be used to develop strategies to limit *Fusarium* spp. affecting these perennial crops.

“When you feel that what you are doing is just a drop in the ocean, remember that the ocean would be less because of that missing drop” -Saint Mother Teresa

To my mama and in memory of my dad who support me through everything, (no matter how crazy), who taught me that faith can get you through anything, and who instilled in me my passion for fungi, all without which I would not be where I am today.

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INTRODUCTION

Michigan is the nation's largest producer of Asparagus (*Asparagus officinalis*, L.), a perennial specialty crop (USDA, 2018). Michigan asparagus growers produce spears for the processing and fresh markets. While the major asparagus production areas are located in Oceana and Mason counties, the crop is also widely grown in small quantities for road side markets throughout the state. The cost of crop establishment is relatively high at more than $4,000/acre with returns dependant on whether it is marketed as fresh or for processing (Talley and Werling, 2016). A minimum of three years is required before a full season's harvest can be taken (Talley and Werling, 2016).

American ginseng (*Panax quinquefolius*, L.), another perennial specialty crop is grown in the Great Lakes region of Ontario, Wisconsin, Michigan, and the northeastern U.S. It is prized for its root which is used in traditional Chinese medicine (Park, 2012; Burkhart and Jacobson, 2017; Westerveld, 2019). In Michigan, ginseng is produced on small parcels in the northern lower and upper peninsulas. Most of the conventional ginseng production in the U.S. is located in central Wisconsin (Burkhart and Jacobson, 2017). Overhead expenses for establishment and production are approximately $73,000/acre, with the value of root and seed ranging from $13-$48/lb. and $150-$200/lb., respectively (Westerveld, 2018). Ginseng requires a minimum of four years to reach full maturity, and may be marketed as a fresh or dried root (Hausbeck, 2017).

Fusarium is an important pathogen in many agricultural systems causing root rot and vascular wilt diseases (Leslie and Summerell, 2008). If unmanaged, the pathogen can increase rapidly in soils, leading to serious crop losses. Perennial cropping systems require a significant investment for crop establishment. Thus, determing the *Fusarium* spp. associated with these

perennial crops and their pathogenicity is needed to assess current *Fusarium* control measures and determine whether new mitigation strategies are needed.

FUSARIUM

First described in 1809 by Johann Heinrich Friedrich Link, the *Fusarium* genus contains plant pathogens (Leslie and Summerell, 2008) that cause crop destruction whereas others are used in food products (Geiser et al., 2013). *Fusarium* spp. are ubiquitous and survive in a wide range of climates. Although many are parasitic, some are endophytes (Summerell et al., 2003). Some Fusarium spp. are human pathogens and infect the eye or nail (Nucci and Anaissie, 2007).

Diseases incited by *Fusarium* spp. have caused crop losses worldwide throughout history (Leslie and Summerell, 2008). The host range of *Fusarium* spp. is extensive and includes major crops such as banana (Leslie and Summerell, 2008; Magdama et al., 2019), mango (Leslie and Summerell, 2008), corn (Leslie and Summerell, 2008), asparagus (Elmer, 2001), pine (Leslie and Summerell, 2008), tomato (McGovern, 2015), potato (Aktaruzzaman et al. 2014), and celery (Epstein et al., 2017). Plant disease caused by *Fusarium* spp. can significantly reduce yield and increase in severity over time (Elmer, 2014). *Fusarium* disease has damaged the banana industry in Central and South America; tropical race 4 has resulted in losses totaling millions of dollars (Ploetz, 2015). Gibberella ear rot of corn and head blight of wheat are also caused by *Fusarium* spp. causing pink mold of corn ears or pink discoloration of wheat stalks, respectively (Goswami, 2004). Mycotoxins including deoxynivalenol, nivalenol, zearalenone, T-2 toxin, and fumonisins may be produced as a result of these infections and ingestion by humans or animals can lead to feed refusal, decrease in fertility, weight loss, and vomiting (Schmale and Munkvold, 2009). *Fusarium* is classified under the kingdom Fungi**,** phylum Ascomycota**,** subphylum

Pezizomycotina**,** class Sordariomycetes, order Hypocreales**,** family Necriaceae, and genus *Fusarium* (Wollenweber and Reinking, 1935). The species were initially identified primarily by their banana-shaped conidia and secondarily by host plant. However, other fungi were found to produce similar looking spores (Leslie and Summerell, 2008). In 1935, Wollenweber and Reinking published *Die Fusarien,* and redefined *Fusarium* spp. taxonomy (Wollenweber and Reinking, 1935) based on morphology across media types and techniques; 65 species of *Fusarium* and 77 sub-specific forms were described. This updated system used morphological characters including macroconidia, color, and overall growth pattern on media (Leslie and Summerell, 2008). Following *Die Fusarien,* Snyder and Hanson proposed nine species of *Fusarium* from 1940 to 1945 (Nelson et al., 1994). Thereafter, researchers worked on various concepts for *Fusarium* taxonomy in Canada (Gordon, 1952), Britain (Booth, 1971), Germany (Gerlach and Nirenberg, 1982), and the U.S. (Nelson et al., 1994) to combine or further delineate the species recorded within the genus (Nelson et al., 1994). Currently, as a result of sequence-based taxonomy and molecular methods, over 300 species are attributed to and described for *Fusarium* (O'Donnell et al., 2015). Previously, *Fusarium* referred only to the anamorph or asexual stage, but now refers to the fungus as a whole (Geiser et al., 2013). *Fusarium* spp. frequently occur in their asexual stage (Summerell et al. 2010). Geiser et al. (2004) developed a website to assist in *Fusarium* spp. classification by providing sequence data. Geiser et al. (2004) compiled these data using the partial translation elongation factor 1-alpha (TEF) sequences from identified *Fusarium* species, against which unknowns may be compared.

All *Fusarium* spp. produce characteristic asexual, banana-shaped macroconidia that are commonly septate and vary in size, shape, and degree of curvature. The basal and apical cells may be notched, elongated, or hooked. The microconidia are categorized based on the number of

cells, overall size and whether they are produced on monophialides or polyphialides. Some species form micro conidial chains (ex: *F. verticillioides, F. proliferatum*) or chlamydospores, which are thick-walled, often pigmented, spherical resting structures produced among the hyphae. Presence of chlamydospores and hyphal coils are characteristic of certain species and can be used in diagnostics (Agrios, 2005; Leslie and Summerell, 2008).

Observation of macroscopic growth is needed for *Fusarium* identification and requires that the cultures be grown for 7 to 10 days at 20 to 25° C with light (Summerell et al., 2003). The resulting mycelia may appear yellow, pink, and/or purple and flat or fluffy, depending on the species. Pigmentation, overall appearance, and growth rates define macroscopic characters for *Fusarium* spp. Aroma and growth rate of an isolate may also be observed (Summerell et al, 2003). The range of textures and pigments often depend on the type of culture media (Leslie and Summerell, 2008). Carnation leaf-piece Agar (CLA) favors chlamydospores, and macro- and microconidia (Leslie and Summerell, 2008). Other media commonly used include Spezieller-Nahrstoffarmer agar (SNA) and potato dextrose agar (PDA). SNA is made with sucrose and glucose added to water agar and sterile filter paper strips are applied to the medium surface as it cools. PDA is used to observe overall growth rates and pigmentation of the mycelia (Leslie and Summerell, 2008). These characters are used in conjunction with microscopic characters and molecular techniques (Summerell et al., 2003).

Some *Fusarium* spp. can be further delineated and assigned a *formae specialis* which designates a particular host plant for specific strains (e.g. *F. oxysporum*). Since pathogenicity tests are time consuming, vegetative compatibility group (VCG) studies can be an effective alternative for those species containing few VCGs based on prior studies. Exceptions include *F. oxysporum f. sp. asparagi*, which includes many VCGs (Summerell et al. 2003).

Some *Fusarium* spp. may infect plant roots and block the vascular system, resulting in wilting, chlorosis, and death (Beckman, 1987; Elmer, 2001; Yadeta and Thomma, 2013). In the soil, *Fusarium* grows toward concentrated nutrients associated with potential hosts (Yadeta and Thomma, 2013). Following infection, the fungus grows intercellularly and into the xylem tissue (Yadeta and Thomma, 2013). Root colonization may prompt the formation of tyloses that limit the pathogen's movement in the vascular system. Inside the xylem vessels, conidiophores bearing conidia are produced and move in the vascular system along with xylem fluids, contributing to vessel clogging (Yadeta and Thomma, 2013). *Fusarium* uses cell wall degrading enzymes to penetrate adjacent cells and move through the plant (Yadeta and Thomma, 2013; Di Pietro et al., 2003). Horizontal gene transfer may occur with certain species such as *Fusarium oxysporum* to ensure traits are shared among strains and other fungi, increasing virulence (Laurence et al., 2015).

Fusarium crown and root rot (FCRR) of asparagus is a destructive disease, causing wilt, fern chlorosis, vascular discoloration, root rot, and crown death (Elmer, 2001). FCRR can reduce yields by nearly 50% and was a significant factor in asparagus decline in the 1950s (Elmer, 2001). Fusarium root rot of ginseng may result in poor quality roots with symptoms including red-brown lesions or 'russetting' on the root. A dry rot of the root is characteristic (Punja et al., 2007, Yadeta and Thomma, 2013; Jiao et al., 2015).

Fusarium spp. prefer poorly-drained soils and humid climates (Leslie and Summerell, 2008). The pathogen may be disseminated by wind, rain splash, and soil movement and is resistant to harsh conditions, surviving on plant residues and in the soil (Leslie and Summerell, 2008) for 20 years or more (Gordon, 2017). *F. oxysporoum* from bananas was recovered 20 years following the last banana crop (Rishbethm, 1955).

Strategies to limit *Fusarium* diseases in the field include sanitation such as removing infected crop residues from fields and cleaning machinery between fields to prevent dissemination of contaminated soil (Elmer, 2014). Managing weed hosts (Punja et al. 2007; Elmer, 2014), minimizing abiotic plant stress (Nigh, 1990; Orr and Nelson, 2018), and crop rotation (Tillmann et al., 2017) are suggested. *Fusarium* is seedborne in some crops including asparagus so disinfesting the seed is important (Damicone et al., 1981). Replanting asparagus, on a site previously planted to the crop is not recommended (Elmer, 2014).

ASPARAGUS

Asparagus (*Asparagus officinalis* L.) is a perennial vegetable belonging to the Asparagaceae family; the sprouts/spears are edible (Cornell, 2017). Asparagus is thought to have originated in the Middle East and was introduced to Europe (Elmer, 2001). The crop increased in popularity and interest in its cultivation, growth and medicinal uses flourished between 160 BC and 57 AD. Although interest declined during the middle ages, Louis IV of France revived it during his reign (Elmer, 2001). During the early 20th century, California, Washington, and Michigan were leaders in asparagus production. Demand and interest declined in the mid- 20th century. Although the popularity of asparagus has increased, U.S. growers compete with imports from Central and South America. Currently, Michigan is the largest asparagus producer in the U.S. (USDA, 2018) with production concentrated in Oceana and Mason counties (Talley and Werling, 2016). Michigan asparagus is grown for the fresh market (60%) and processing (40%) (Farm News Media, 2018).

Asparagus grows best in well-drained, sandy soils with a high organic matter (OM) content and a neutral pH, but may tolerate alkaline soils (Cornell, 2017; Zandstra et al., 1992).

Soils that are higher in pH may limit *Fusarium* spp, that prefer acidic soil (Myers, 2013; Egel et al., 2018). Asparagus is also tolerant of soils that have relatively high salinity (Cornell, 2017). The once popular open-pollinated varieties Mary Washington, Martha Washington, and Walthom Washington are no longer used in Michigan's commercial plantings. The high yielding, all-male hybrids from Rutgers University ('Jersey Giant', 'Jersey Supreme', and 'Jersey Gem') and the University of Guelph ('Guelph Millennium' and 'Guelph Eclipse') are well-suited for Michigan (Myers, 2013) and are widely grown in the state.

The cost of establishing asparagus is relatively high ($4,082/A in 2015) (Talley and Werling, 2016) and includes the cost of crowns, herbicides, fertilizer, manure, cover crops, and labor. Seed is planted directly into a crown nursery and grown for one year to produce crowns that are dug and then planted into production fields; approximately 15,000 crowns are planted per acre (Talley and Werling, 2016). Following planting into a production field, a reduced number of harvests may be taken the second year following establishment and a full number of harvests taken the third year (Penn State Extension, 2018). In Michigan, up to 20 harvests are taken over a two-month period from May through June in the spring (Elmer, 2001; Egel et al, 2018) after which the spears are allowed to develop into fern (Cornell, 2017; Orzolek et al, 2014). A healthy asparagus stand may produce for 20 or more years (Elmer, 2014). However, Talley and Werling (2016) determined that the average life expectancy of an asparagus stand is 13 years with 3-5,000 lbs. asparagus/A per year.

The perennial nature of the asparagus cropping system poses challenges in managing FCRR. Plant stress can arise from nutrient deficiency, incorrect pH, and/or disease (Nigh, 1990; Orr and Nelson, 2018). Nigh (1990) and Hodupp (1983) determined that abiotic stress influences FCRR disease incidence and severity. Crowns from previous plantings emit allelopathic

compounds that prevent establishment of new transplants (Elmer, 2014). In the greenhouse environment, earthworm incorporation assisted in the growth of asparagus crowns by improving the soil microbial community (Elmer, 2014).

Fusarium is a persistent and pervasive soil plant pathogen in Michigan's asparagus growing region (Hartung et al., 1990), making management difficult (Hartung and Stephens, 1983; Morrison et al., 2014). FCRR symptoms were first noted in 1908 and is a major disease of asparagus (Elmer et al., 1996; Elmer, 2001). *F. proliferatum* and *F. oxysporum* f.sp. *asparagi* are commonly associated with FCRR (Damicone and Manning, 1985; Arriola et al., 2000; Elmer, 2001). *F. moniliforme* (Hartung and Stephens, 1983; Damicone and Manning, 1985; Hartung, et al., 1990), reclassified as *F. verticillioides* (Seifert, et al., 2003), has occasionally been detected. Hartung et al. (1990) observed a higher number of *F. moniliforme* isolates recovered from plant debris versus the soil. *Fusarium* spp. were concentrated within the asparagus rows compared to between rows.

FCRR is seed-borne (Elmer, 2001) and both *F. oxysporum* and *F. moniliforme* were found on seed in Washington (Inglis, 1980). Grove (1976) suggested that seed infection is internal but Inglis (1980) did not detect the pathogen within the seed or berries; hyphae were observed on the seed surface. The fungicide Benomyl, in solution with acetone (25,000 ppm), effectively disinfests seed contaminated by *F. moniliforme* and *F. oxysporum*; reduced acetone concentrations are effective against *F. moniliforme*, but not *F. oxysporum*. Washing the seed after the treatment, prevents toxicity to the seedlings (Damicone et al., 1981; Counts and Hausbeck, 2007).

Host resistance may not eliminate *Fusarium* spp. as some remain endophytic (Damicone and Manning, 1985). Damicone and Manning (1985) evaluated 576 FCC susceptible ('Mary

Washington') and resistant ('Jersey Centennial') asparagus crowns to determine the associated *Fusarium* species. Despite treatment before planting with Benomyl and soil fumigation with Vorlex, *F. oxysporum* and *F. moniliforme* were isolated from the crowns. Regardless of cultivar, *Fusarium* spp. were isolated from every crown.

Management may include the biocontrols *Trichoderma harzianum* and the arbuscular mycorrhizal fungus *Glomus intraradices* which have been shown to decrease root rot on asparagus seedlings caused by *F. oxysporum* f.sp. *asparagi* (Arriola et al., 2000).

GINSENG

Ginseng originates from the Chinese name "Jen-Shen" and means "man root", due to the shape of the taproot which when split, resembles human legs (BCMAF, 1999). While the species name is Chinese, the genus name, *Panax,* originates from the Greek language and translates to "all-healing" (Barrett, 2018). There are three *Panax* species of ginseng recognized and sold worldwide including American ginseng (*Panax quinquefolius),* Asian ginseng (*Panax ginseng*), and Notoginseng (*Panax notoginseng*). Asian ginseng is grown in China, Korea, and Japan and considered by some to be the most authentic, while American ginseng is grown in the Midwest and the northeastern U.S. (Burkhardt and Jacobson, 2017) and Ontario, Canada (Westerveld, 2019). Siberian ginseng (*Elutherococcus senticsus)* is marketed as ginseng but does not contain the same ginsenoside compounds as *Panax* spp. Ginseng is a member of the Araliaceae family, along with a number of other perennial vines and herbs. Ginseng may be sold as either white or red in reference to the root's color following a specific preparation (BCMAF, 1999). Cultivar distinctions do not exist (Brun, 1999).

Ginseng may be commercially cultivated as wild simulated, woods cultivated, and field cultivated (Burkhart and Jacobson, 2017). Wild-simulated cultivation commands the highest price and is grown in the forest's understory. Seed is broadcast and natural shade is provided by the tree canopy (Hankins, 2009). Woods cultivated ginseng is planted and maintained similarly to methods used for field cultivation but uses the natural shade provided by the forest canopy (Burkhart and Jacobson, 2017). Field cultivation via "gardens" includes construction of raised plant beds (23cm to 30cm high and 1.5m wide) covered with straw mulch under an artificial shade canopy (Hausbeck, 2011).

Field cultivated American ginseng dominates production in Canada, Wisconsin, and Michigan (Hausbeck, 2017). Approximately 90% of the U.S. cultivated ginseng is produced in central Wisconsin, primarily in Marathon County. Wisconsin production accounts for 10% of the world's supply of ginseng (Hausbeck, 2011). In Wisconsin, cultivated ginseng sales yield $75-$100 million, annually (Hausbeck, 2017). Ginseng prices fluctuate yearly. In 2009, the price for wild ginseng was $425/lb. and field-grown ginseng was $10/lb. (Hankins, 2009). Wild ginseng may sell for as much as $500/lb. In 2017, wild and cultivated ginseng was exported to Asia for $250/lb. and $20-$40/lb., respectively (Burkhart and Jacobson, 2017).

The largest producer of ginseng in the world is China, followed by South Korea, Canada, and the U.S. China produces nearly 50% of the world's ginseng. The majority of North American ginseng is exported to Asian countries, primarily Hong Kong (Hankins, 2009). Canada is the leading exporter with $66 million in 2009. The U.S. ranks fourth, accounting for $37 million in 2009 exports. Hong Kong is the largest importer of ginseng from North America with exports valued at $107 million in 2009 (Baeg and So, 2013).

The ginseng plant has compound palmate leaves, each consisting of five leaflets with serrated edges. In the fall, red berries develop in clusters, each containing a single seed (BCMAF, 1999). As it ages, the plant produces additional compound leaves and at each stage is referred to as a 1-prong, 2-prong, etc. (Burkhart Jacobson, 2017). In field cultivation, a minimum of three years is required to produce a marketable root; large roots command the highest prices (BCMAF, 1999). Ginseng prefers a cool climate, shade, well-drained soils with considerable organic matter content, and a pH not to exceed 6.0 (Burkhart and Jacobson, 2017). Wild ginseng may require up to 10 years before it can be harvested (Burkhart and Jacobson, 2017).

Ginseng is direct seeded following stratification (Burkhart and Jacobson, 2017). After the drupe is harvested, it is depulped, the seed mixed with moist sand (Li et al., 2000), subjected to cold temperature in controlled storage, and planted the following year (Burkhart and Jacobson, 2017). Ginseng is planted in the fall and covered the next spring by lath or shade cloth hung on wooden supports to achieve 80% shade, mimicking forest conditions. Ginseng roots are mechanically dug from September to November. The plant beds are hand weeded and the ginseng drupes harvested by hand (Hausbeck, 2011). After harvest, roots are initially dried at 60°-80° F. The roots are then dried at 90º F for 3 to 6 weeks, and subsequently kept in a cool environment (Hausbeck, 2011). In Ontario, annual production costs for cultivated ginseng were estimated at $11,400, not including land rental or specialized machinery (Westerveld, 2018). Seed may command $150-$200/lb. (Ha, et al., 2017;Westerveld, 2018) depending on demand. Pesticides are costly but critical for field cultivation (Burkhart and Jacobson, 2017). Total revenue during the four-year timeframe was estimated at $70,000 in 2018, which includes dried root and stratified seed sold in years three and four (Westerveld, 2018).

After the ginseng roots are dried, they may be packaged as a whole root, ground into powder, made into capsules, teas, sweets, toothpaste or other products (Harrison et al., 2000). The health benefits of ginseng are associated with ginsenoside compounds in the plant's roots. Ginsenosides are phyto-chemicals that act as antioxidants, stimulants, anti-cancer compounds, and provide other benefits (Leung and Wong, 2010). The medicinal use of ginseng in Asian and North American cultures dates back nearly 4,500 years (Baeg and So, 2013). Ginseng was widely used by native Asian and American cultures; European settlers discovered it in the early 18th century in Canada. Explorers, and those who followed, exploited the wild ginseng populations to near extinction. Harvesting and/or selling wild ginseng in Canada is prohibited and only the cultivated root can be exported in quantity (Environment Canada, 2014). In the U.S., ginseng is regulated by the Convention on International Trade in Endangered Species of Fauna and Flora (CITES). This treaty dictates that individual states have specific requirements including harvesting within a specific timeframe to ensure a mature plant, licensing, and restrictions based on plant age. Many areas require that ginseng be at least five years old before harvest to ensure that seed has dispersed to repopulate the area (Burkhart and Jacobson, 2011).

Field cultivated ginseng is susceptible to *Fusarium* spp. causing seedling damping off and root rot or russetting of the roots (Cheng et al., 1997; Cheng et al., 1998; Hudelson, 2004-1; Hudelson, 2004-2). Russetting of the root may also be caused by *Cylindrocarpon destructans* (Rahman and Punja, 2005), but *Fusarium* spp. is considered the predominant causal agent (Reeleder, et al., 2006). *Fusarium* in the soil can lead to vascular wilt, preventing the flow of water and nutrients and leading to discolored xylem tissue (Yadeta and Thomma, 2013). Goswami and Punja (2008) found that lignified cells develop near the site of infection in a hypersensitive response to limit pathogen colonization resulting in rusty orange-colored lesions

on the root, rendering it unmarketable. The genes responsible for the production of phenolic compound and detoxification are upregulated in ginseng plants infected with *Fusarium* spp (Hammerschmidt, 1999). Goswami and Punja (2008) proposed that the reddening of the ginseng roots was due to these phenolic compounds.

Limiting Fusarium disease in ginseng is challenging due to the pathogen's ability to survive as a saprophyte in the soil and on plant debris including the straw mulch (Punja, 2011; Howard et al., 1994) providing inoculum for subsequent years. Chemical control is also difficult, as products are limited (Hausbeck, 2011), however, the number of registered fungicides is increasing (Hausbeck, 2017; Drilias, 2002). Azoxystrobin (Quadris/Quadris Top, Syngenta) is registered for use on ginseng and can be used to limit *Fusarium* spp. and other pathogens. Chloropicrin (Telone C-17/Telone C-35, Corteva Agriscience) is a preplant soil fumigant that can be used against soil-borne pathogens (Hausbeck, 2017). Other products registered for use on ginseng, include fludioxonil (Cannonball 50WP/Cannonball WG, Syngenta), fluopyram/pyrimethanil (Luna Tranquility, Bayer CropScience), fluopyram/trifloxystorbin (Luna Sensation, Bayer CropScience), and penthiopyrad (Fontelis, Corteva Agriscience); penthiopyrad was highly effective in greenhouse trials (Hausbeck, 2017).

CONCLUSION

Managing *Fusarium* spp. on asparagus and ginseng crops challenge producers. The overall objective of this study is to identify the *Fusarium* species associated with these crops and determine their ability to cause disease in order to develop effective control strategies.

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CHAPTER I

FUSARIUM SPECIES ASSOCIATED WITH MICHIGAN ASPARAGUS CROWNS

ABSTRACT

Fusarium crown and root rot (FCRR) of asparagus reduces yield and crop longevity. The objective of this research was to determine the *Fusarium* spp. currently associated with asparagus crowns. Asparagus crowns (491 total) were sampled from three Michigan growers and included 'Jersey Supreme' (100), 'Guelph Millennium' (258), and 'Guelph Eclipse' (133). Samples from 'Guelph Millennium' included those grown in soils fumigated pre-plant with metam sodium (131) or not fumigated (127). Tissue from the rhizome and storage roots was plated onto a *Fusarium*-selective medium. Species identification was achieved via DNA extraction from pure cultures, PCR using EF-1 and EF-2 primers, and Sanger sequencing. *Fusarium* spp. were isolated from 'Jersey Supreme' (39%), 'Guelph Eclipse' (47%), and 'Guelph Millennium' (30%). *Fusarium* spp. were isolated from 36% and 25% of the crowns grown in non-fumigated and fumigated soils, respectively. *Fusarium* spp. were isolated more frequently from the storage roots (45%) than from the rhizomes (28%). Seven species were identified, with *F. oxysporum* occurring most frequently (92%). A subset (51) of *F. oxysporum* isolates from 'Guelph Millennium' crowns grown in fumigated or non-fumigated soil were tested for pathogenicity. There were significant differences among isolates; the majority of the isolates were pathogenic. Results indicate that FCRR disease control measures could be focused on seedlings while in the crown nursery in an effort to mitigate disease in production fields.

INTRODUCTION

Asparagus (*Asparagus officinalis*, L.) is a perennial vegetable belonging to the Asparagaceae family (Cornell, 2017). Michigan leads the nation in asparagus production with the state's acreage concentrated in Oceana and Mason counties (Talley and Werling, 2016). Asparagus is a dual-market vegetable that is grown for either the processing or fresh market. The underground rhizome gives rise to bud clusters from which the edible spears (stems) develop. The root system is comprised of adventitious storage roots and lateral feeder roots (Weaver and Bruner, 1927; Blasberg, 1932; Mullendore, 1935; Drost, 1997). These below-ground structures are collectively called the crown (Drost, 1997). In Michigan, asparagus seed is planted into a seedbed in a field nursery. A year later, the resulting crown is dug, and transplanted into production fields (Talley and Werling, 2016). Asparagus plantings three years or older are harvested up to 20 times over a two-month period in the spring (Elmer, 2001), after which the spears are allowed to develop into fern (Cornell, 2017). A healthy asparagus planting may be productive for 20 or more years (Elmer, 2014).

Fusarium crown and root rot (FCRR) is a major disease of asparagus (Elmer, 2001). Symptoms of FCRR were first noted in 1908 and include chlorosis of the fern and discoloration of the vascular system (Elmer, 2001). *F. proliferatum* (Matsush.) and *F. oxysporum* f.sp. *asparagi* (Schlecht.) are commonly associated with FCRR (Damicone and Manning, 1985; Arriola et al., 2000; Elmer, 2001). In Michigan, a survey of *Fusarium* species associated with FCRR was conducted nearly 30 years ago by Hartung et al. (1990) who sampled 28 fields in Oceana and Van Buren counties. *F. oxysporum* was always recovered from the soil and 38% of the isolates were pathogenic. *F. moniliforme* isolates were also recovered, and all

were shown to be pathogenic. The name *F. moniliforme*, however, is no longer used and has been replaced largely by *F. verticillioides* (Seifert, et al. 2003).

F. oxysporum may be found on asparagus seed, affecting seedlings and mature plants (Elmer et al., 1996; Elmer, 2001). The pathogen may also be disseminated to production fields by crowns infected while in the seedbed (Elmer et al., 1996; Elmer, 2001). *Fusarium* spp. are persistent and pervasive soil-borne plant pathogens making management difficult (Hartung and Stephens, 1983). *Fusarium* spp. survive in the soil and on plant debris (Elmer, 2014). Fungicides may be used to disinfest seed prior to planting. Some chemical controls have shown promise in the first year or two of application or under low pathogen pressure, however, many of them are largely ineffective in this perennial system. (Counts and Hausbeck, 2007; Elmer, 2014). The objective of this work was to determine the *Fusarium* spp. associated with asparagus crowns and their pathogenicity.

MATERIALS AND METHODS

Sampling. One-year-old asparagus crowns in storage (491) (Fig. 1.1A) were sampled from three Michigan growers located in two counties. 'Jersey Supreme' crowns (100) were obtained from one grower in Berrien County (hereafter designated as Berrien). Grower 1 located in Oceana County (hereafter designated as Oceana 1) provided 'Guelph Millennium' crowns (127 total). Grower 2, also located in Oceana County (hereafter designated as Oceana 2), provided 'Guelph Millennium' (131) and 'Guelph Eclipse' (133) crowns. All crowns were grown in soil fumigated preplant with metam sodium (Sectagon-K54, Vapam HL), with the

exception of those from Oceana 1 which were grown in a non-fumigated field not previously cropped to asparagus.

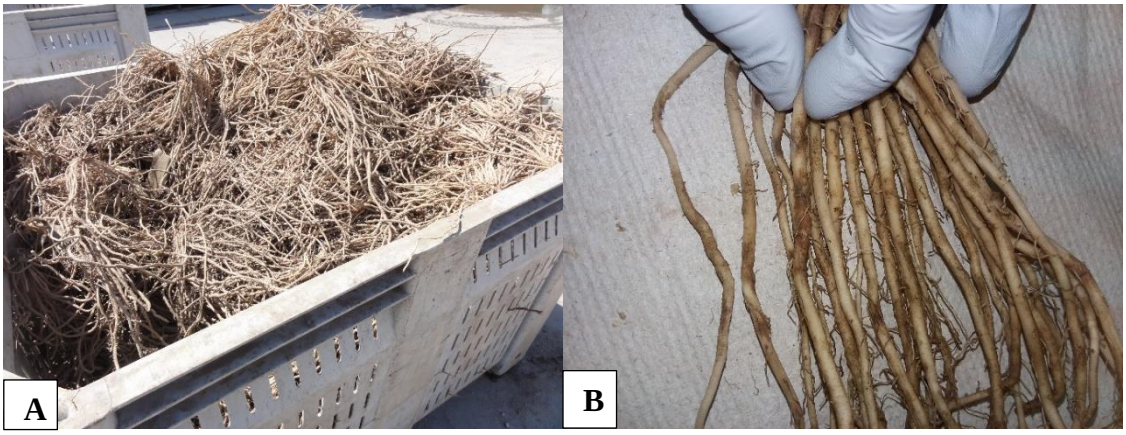

Figure 1.1: A) One-year-old asparagus crowns in bulk boxes. B) Storage roots exhibiting brown lesions characteristic of Fusarium crown and root rot.

***Fusarium* isolation.** Crowns were rinsed with tap water and six sub-samples were excised from each crown; three samples each were obtained from the rhizome and storage roots. Tissue samples were taken from both symptomatic (Fig. 1.1B) and asymptomatic roots. Tissue samples were surface sterilized with 0.825% sodium hypochlorite for one minute, rinsed in sterile water for thirty seconds, and rinsed briefly a second time in sterile water to remove residual sodium hypochlorite. Tissue pieces were allowed to air dry on sterile paper towel. Samples were placed, three per culture plate, onto Nash-Snyder agar (Nash and Snyder 1962; Toussoun and Nelson 1976; Leslie and Summerell, 2008) in a triangular configuration. Isolates were subcultured onto water agar (Neogen, Lansing, MI, USA) and then transferred to 50% strength potato dextrose agar (PDA) (Neogen, Lansing, MI, USA) using the hyphal tipping method described by Leslie and Summerell (2008). All isolates were identified by morphology and growth characters to genus, and species where possible, on a culture plate. Riddell mounts

(Riddell, 1950) were also prepared with select isolates in order to view defining characteristics (Fig. 1.2A-B) including microconidial chains, chlamydospores, and macroconidia.

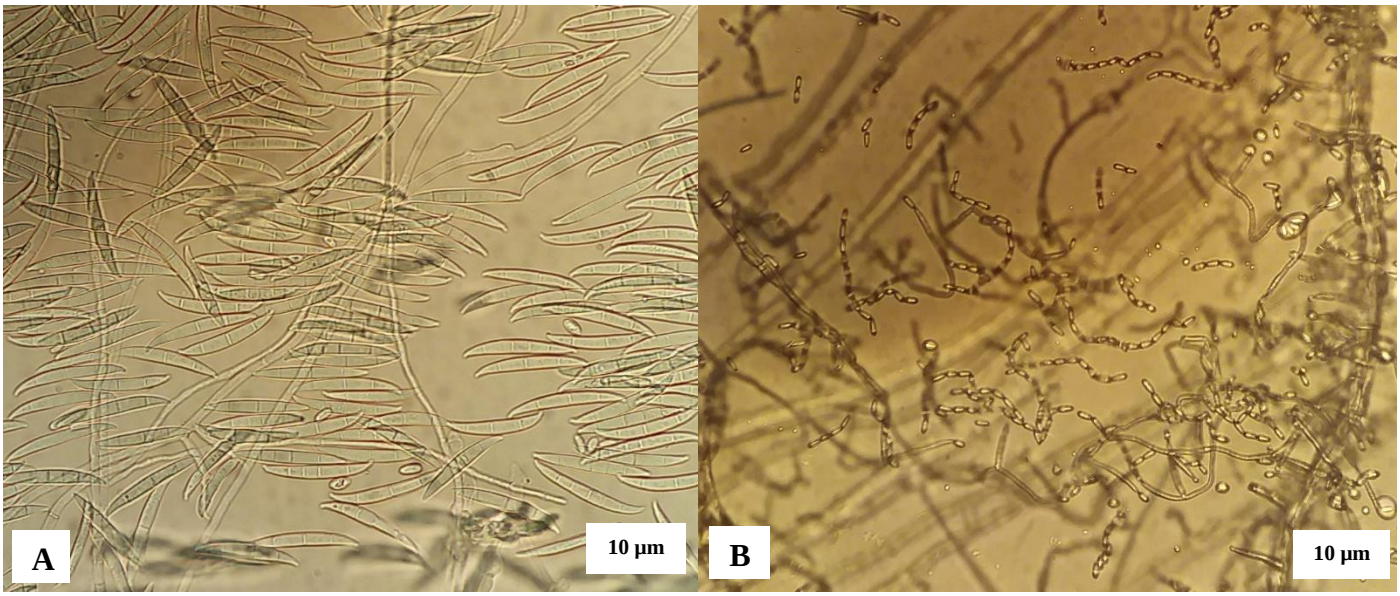

Figure 1.2: Macroconidia of *Fusarium oxysporum* (A) and microconidial chains of *Fusarium proliferatum* (B).

Isolate Storage. Skim milk was prepared using 10 g Difco skim milk powder (BD Biosciences, Franklin Lakes, NJ, USA) per 100 mL of distilled water and autoclaved for 15 minutes. Sterile skim milk was stored in the refrigerator and kept on ice when in use. Screw cap glass scintillation culture tubes (20 mL capacity) were filled three quarters of the way with silica gel (Beantown Chemical, Hudson, NH, USA). The vials were covered with aluminum foil, sterilized with dry heat at 121°C for 90 minutes, and then stored in the refrigerator. Vials were kept on ice during use. Seven to 10-day-old PDA culture plates were flooded with sterile skim milk and gently agitated with a pipette tip to create a spore suspension. The milk suspension (200 µL) was pipetted evenly onto the silica gel. The opening of the vial was flame sterilized, capped, and sealed with Parafilm®. Vials were vortexed briefly to evenly distribute the suspension and stored at 6°C. Two vials per isolate were prepared.

DNA Extraction. Individual isolates were removed from the refrigerator and placed on ice while in use. Silica gel crystals were shaken onto the surface of water agar media, then pressed into the media using the flat side of a sterile scalpel. Cultures were allowed to grow under constant light conditions and at room temperature (23°C) for 3 to 4 days before being subcultured onto full-strength PDA (39g agar and 1000mL distilled water). Cultures were allowed to grow for 7 to 10 days before DNA was extracted.

All sample DNA was extracted either manually using a Qiagen DNeasy Plant Mini kit (Qiagen, Hilden, Germany) or with the KingFisher Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA, USA) and the Omega 1130 Mag-Bind DNA extraction kit (M1130, Omega Bio-Tek, Norcross, GA, USA) with the following modifications. Manual extraction used 90 to 100 mg of mycelium (fresh weight) frozen in liquid nitrogen with five 3-mm glass beads (Merck, Darmstadt, Germany) and lysed in a TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for three minutes. The lysis buffer (600 µl, Buffer AP1) and 6 µl of RNase A stock solution ((100 mg/ml) Roche Diagnostics, Bale, Switzerland) were added and incubated for 30 minutes at 65ºC. Final elution was performed with 30 µl of buffer. For automated extractions, 50 to 70 mg of mycelium were lyophilized (Genesis Pilot Lyophilizer (SP Scientific, Warminster, PA, USA)) in 1.1 mL polypropylene 8-strip collection tubes (Thomas Scientific, Swedesboro, NJ, USA) and lysed with a single 4 mm stainless-steel ball bearing (Grainger, Lake Forest, IL, USA) as described above. Initial incubation time for lysis was increased to 60 min at 56ºC and mixed every 10 min. The following reagent volumes were modified: RNase-A (6 µl), lysate (450 µl), RBB buffer (450 µl). Incubation time with RNase was increased to 30 min at room temperature (23°C). All DNA extracts were quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at -20 ºC.

PCR, sequencing, and phylogenetic analysis. The Translation Elongation Factor 1-α (TEF) coding region was amplified using EF-1 (ATGGGTAAGGA(A/G)GACAAGAC) and EF-2 (GGA(G/A)GTACCAGT(G/C)ATCATGTT) primers. Reactions were run with a master mix comprising 12 µl water, 3.6 µl buffer 5X Green GoTaq® Reaction Buffer (Promega), 0.5 µl (2.5 mM) dNTPs (Promega), 3 µl (25mM) magnesium chloride (Promega, Madison, WI, USA), 0.5 µl (10 µg/µl) BSA (New England BioLabs, Ipswich, MA, USA), 0.30 µl GoTaq® polymerase (Promega, Madison, WI, USA), and 0.5 µl each of the (5 µM) EF-1 and EF-2 primers (Integrated DNA Technologies, Coralville, IA, USA), resulting in 20.9 µl master mix per well and followed by 2 µl of the template DNA in each well of a 96-well plate. Samples were run in a Vapo.Protect Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY, USA) using the following program; 1 h at 94°C; 34 cycles of 30 min at 94°C, 45 min at 59°C, 2 hrs at 72°C. Sample quality was verified by gel electrophoresis using the PowerPac Basic (BioRad, Hercules, CA, USA). Samples were subsequently sent to Macrogen (Macrogen, Rockville, MD, USA) for forward and reverse Sanger sequencing of the TEF region, using primers EF-1 and EF-2 respectively. Sequences were analyzed with Geneious® (Biomatters Ltd, Auckland, New Zealand) to align, assemble, and trim forward and reverse reads to achieve consensus sequences for each isolate. Sequences were then identified to species complex or species, where possible, through a BLAST search of the TEF region in the National Center for Biotechnology Information GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and cross referenced with the FUSARIUM-ID database (http://isolate.fusariumdb.org/blast.php). Sequences were aligned using MUSCLE in Mesquite version 3.51. A maximum likelihood estimate using CIPRES version 3.3 (https://www.phylo.org/) was conducted. Bootstrap analysis using 1,000 replications was run and the resulting tree viewed in FigTree version 1.4.4.

Pathogenicity testing. To determine the pathogenicity of select isolates, an asparagus seedling bioassay was used. 'Guelph Millennium' seed was surface disinfested following the protocol described in Damicone et al. (1981). Seed was placed into a suspension of 2.5 g benomyl and 100 mL of 99.9% acetone stirred on a stir plate with a magnetic stir bar for 24 hours. Seed was rinsed three times in 99.9% acetone and then three times in sterile distilled water. After rinsing in acetone, the seed was again stirred on a stir plate in 1.65% sodium hypochlorite. After one hr, seed was rinsed five times in sterile distilled water and allowed to dry on a sterile paper towel in a laminar flow hood. Water agar plates were prepared (16 g agar and 1000 mL distilled water) and five seeds per plate were embedded into the agar and incubated in the dark for 7 to 14 days until germination. Hoagland's media (Hoagland and Snyder, 1933) was prepared and heated on a stir plate until the agar was dissolved. The medium (12 mL) was pipetted into 25 x 150 mm test tubes, autoclaved, and allowed to solidify (Stephens and Elmer, 1988). Once the hypocotyls emerged, seedlings were transferred to the test tubes containing Hoagland's media and maintained in a growth chamber (16-hr photoperiod and 25º/20º C day/night temperatures) for approximately two weeks, until seedlings reached an approximate height of 11 cm (Woods and Hausbeck, 2018).

Fusarium oxysporum isolates (51) were chosen for pathogenicity testing. Isolates were chosen based on grower/cultivar and culture morphology. Conidial suspensions of each *Fusarium* isolate were made by flooding a 10-day-old culture plate with sterile distilled water and agitating with a sterile pipette tip. Suspensions were measured using a hemacytometer and diluted to 10^6 spores/mL (Stephens and Elmer, 1988). Each plant was inoculated with 500 µl of the spore suspension and then incubated for 5 days (16-hour photoperiod at 25/20°C day/night) (Fig. 1.3). Following the incubation period and every 7 to 10 days thereafter, disease severity

was rated on a scale from 0 to 4 (0=10-20%, 2=20-50%, 3=50-80%, and 4=>80%) based on the root area covered with lesions (%) (Fig. 1.5) and presence or absence of rot symptoms on the feeder roots.

After approximately four weeks, seedlings were removed from the test tubes, the roots were washed to remove the agar medium from the roots (Fig. 1.4), and symptomatic tissue sampled from each seedling to fulfill Koch's postulates.

Figure 1.3: Test tubes containing asparagus seedlings inoculated with a *Fusarium oxysporum* spore suspension (500 µl), arranged in a completely randomized design in test tube racks (40 tubes each), and incubated in a growth chamber.

Figure 1.4: Asparagus seedlings grown in test tubes, inoculated with *Fusarium* spp., and removed from the culture media to isolate the pathogen and fullfill Koch's postulates.

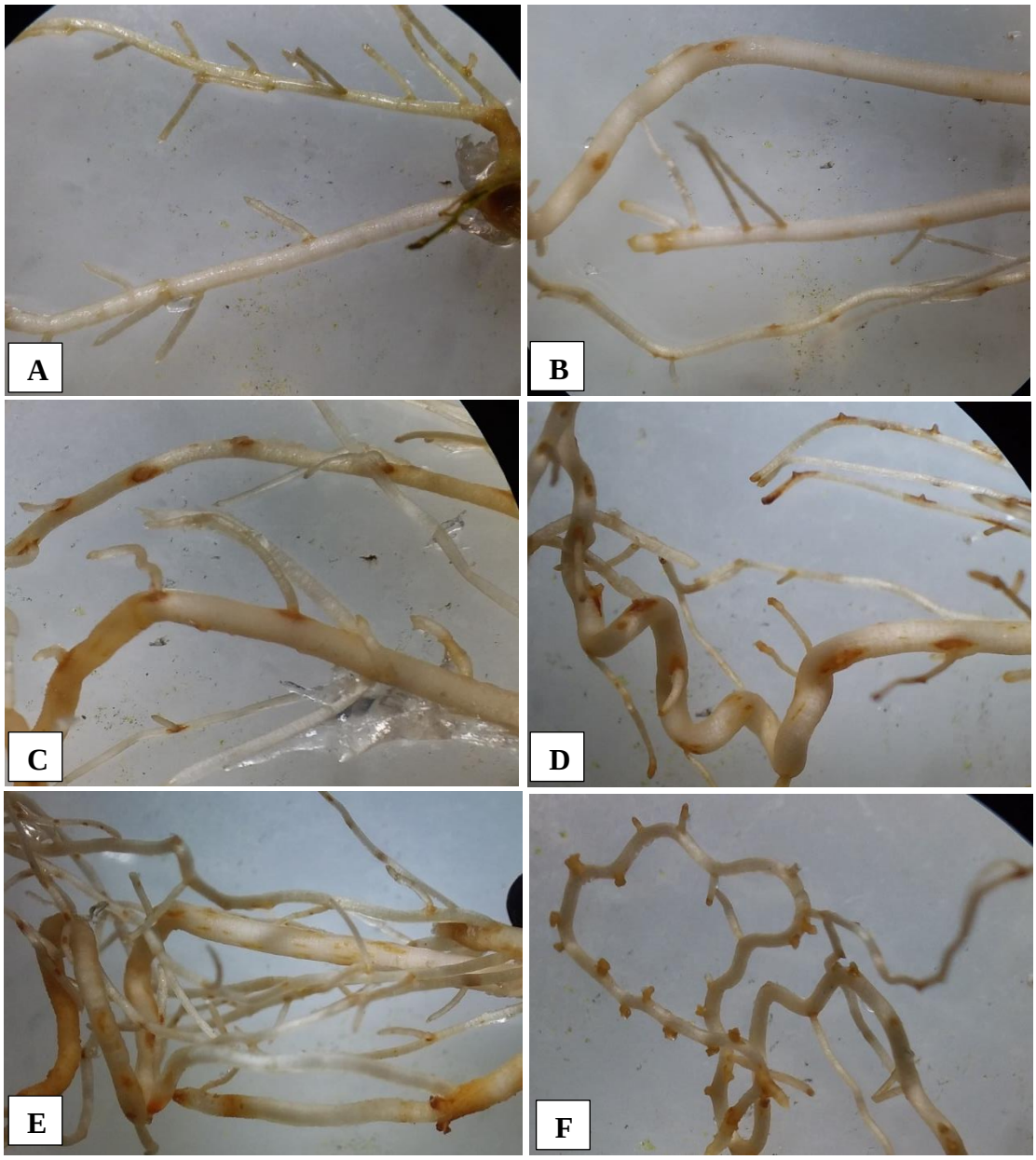

Figure 1.5: Disease symptoms on asparagus seedling roots when grown in Hoagland's media in test tubes, inoculated with *Fusarium oxysporum*, and rated: A) 0: little to no lesions, healthy feeder roots. B) 1: Few, small, yellow-brown lesions. C) 2: Large, dark-brown lesions. D) 3: Large, dark brown lesions, covering 50-80% of the surface area. E and F) 4: Dark-brown lesions extending from the root tip along the root length with rotted feeder roots.

Data Analysis. Disease severity data over the 4-wk period were used to calculate the area under the disease progress curve (AUDPC). These data were subsequently analyzed using the

PROC glimmix procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC). A one-way Analysis of Variance (ANOVA) test using Satterthwaite's adjustment was conducted in order to account for the unequal variances found in the dataset. Prior to this analysis, various transformations of the dataset were explored, however, it was determined that Satterthwaite's adjustment would be the most effective in addressing the unequal variances. Following the ANOVA test, a Tukey HSD test with the Satterthwaite's adjustment was conducted.

RESULTS

***Fusarium* isolation:** *Fusarium* spp. were recovered from storage root and rhizome tissue regardless of cultivar or pre-plant fumigation (Table 1.1). Seven *Fusarium* spp. were recovered, including *F. oxysporum* (92%), *F. proliferatum* (2%), *F. solani* (5%), *F. acuminatum* (1%), *F. avenaceum* (<1%), *F. graminearum* (<1%), and *F. incarnatum-equiseti (*<1%) (Table 1.2). *Fusarium oxysporum* was the most prevalent species isolated from crowns across growers, cultivars, and tissue type (Table 1.3). *F. solani* (5%) was the second most prevalent species and was isolated from both storage root and rhizome tissue. *F. proliferatum* was only recovered from rhizome tissue (Table 1.2). *Phytophthora asparagi* was also recovered from 3% of storage roots sampled.

Table 1.1: Number of *Fusarium* spp. isolates obtained from asparagus rhizomes and storage roots and identified based on culture morphology.

Sample location		*Fusarium* spp. isolates	
(#crowns)	Cultivar	Rhizome	Storage root
Berrien (100)	Jersey Supreme	142	90
Oceana 1 (127)*	Guelph Millennium	48	144
Oceana 2 (131)	Guelph Millennium	95	190
Oceana 2 (133)	Guelph Eclipse	131	245

*****Crowns were grown in a non-fumigated seedbed.

Table 1.2: *Fusarium* spp. isolated from asparagus rhizome and storage root tissue of 1-year-old crowns obtained from Michigan growers and identified by sequence analysis.

County (# of isolates)	**Tissue Sampled**	***F. oxysporum*** (% of total)	***F. proliferatum*** (% of total)	***F. solani*** (% of total)	***F. acuminatum*** (% of total)	***F. avenaceum*** (% of total)	***F. graminearum*** (% of total)	***F. incarnatum -equiseti*** (% of total)
Berrien (49)	Rhizome	**38** (77%)	**1** (2%)	**5** (10%)	**3** (6%)	**1** (2%)	**0**	**1** (2%)
(45)	Root	**41** (91%)	**0**	**4** (9%)	**0**	**0**	**0**	**0**
Oceana 1 (34)	Rhizome	**31** (91%)	**1** (3%)	**2** (6%)	**0**	**0**	**0**	**0**
(75)	Root	**69** (92%)	**0**	**6** (8%)	**0**	**0**	**0**	**0**
Oceana 2 (100)	Rhizome	**89** (89%)	**8** (8%)	**2** (2%)	**0**	**0**	**1** (1%)	**0**
(166)	Root	**164** (99%)	**0**	**1** (<1%)	**1** (<1%)	**0**	**0**	**0**
Totals	**469**	**432 (92%)**	**10 (2%)**	**20 (5%)**	**4 (1%)**	**1 (<1%)**	**1 (<1%)**	**1 (<1%)**

Pathogenicity testing: All selected *F. oxysporum* isolates produced disease symptoms on inoculated asparagus seedlings; the non-inoculated control remained asymptomatic (Table 1.3). However, according to the AUDPC data, not all isolates caused a level of disease symptoms that was significantly different from the non-inoculated control. Three isolates were similar to the non-inoculated control for both experiments including OP19, OP87 and OK18. According to the AUDPC data, 12 and 7 additional *F. oxysporum* isolates resulted in disease symptoms similar to to the non-inculated control for Experiments 1 and 2, respectively. Twenty-nine isolates were pathogenic across both experiments. AUDPC values indicated differences among those isolates determined to be virulent in each experiment. In Experiment 1, OP104 and OK106 were significantly more virulent than 17 isolates whereas in Experiment 2, OP61 was more virulent than 16 other isolates. There did not appear to be a relationship between pathogenicity of the isolates and pre-plant fumigation (data not presented).

Table 1.3: Pathogenicity test of 51 *Fusarium oxysporum* isolates from asparagus crowns in Experiments 1 and 2.

Experiment 1			Experiment 2		
Isolate	AUDPC	Tukey HSD***	Isolate	AUDPC	Tukey HSD***
OP*104.1S**	54.75	A	OP*104.1S**	39.5	A-M
OK106.2R	54.37	A	OK106.2R	42.37	A-L
OK120.1S	52.5	AB	OK120.1S	42.25	A-L
OP38.3S	50.5	A-C	OP38.3S	32.37	D-M
OK80.3S	47.37	A-D	OK80.3S	25.75	F-N
OK33.1R	46.63	A-E	OK33.1R	40.5	A-L
OP88.1S	45.25	A-F	OP88.1S	15.25	MN
OK47.1S	44.63	A-F	OK47.1S	48.37	A-I
OP61.1S	44.37	A-F	OP61.1S	64	A
OP47.2R	43.25	A-G	OP47.2R	30.63	D-M
OP102.2S	42.63	A-H	OP102.2S	24.25	H-N
OK74.2S	40.12	A-I	OK74.2S	58.62	A-C
OP11.1R	40	A-I	OP11.1R	38.5	B-M
OK64.1R	40	A-I	OK64.1R	20.63	J-N
OP12.2R	37.12	A-I	OP12.2R	34.25	C-M
OP107.1R	36.75	A-I	OP107.1R	23.12	I-N
OP57.2S	35.12	A-K	OP57.2S	18.87	L-N
OP26.1R	34.62	A-K	OP26.1R	60.37	AB
OP65.2S	33.75	A-K	OP65.2S	47.63	A-I
OK53.1S	32.37	B-L	OK53.1S	34.12	C-M
OP112.1S	32.37	B-L	OP112.1S	45.75	A-J
OP8.2S	32	B-L	OP8.2S	45	A-J
OK82.1R	31.75	B-L	OK82.1R	19.63	K-N
OK31.2S	30.62	B-L	OK31.2S	41.12	A-L
OP6.1R	29.87	C-L	OP6.1R	52.37	A-D
OK95.1S	29.75	C-L	OK95.1S	33.87	C-M
OP36.3S	29	C-L	OP36.3S	31.25	D-M
OK56.3S	28.75	C-L	OK56.3S	32.5	D-M
OK18.2R	28.63	C-L	OK18.2S	31.37	D-M
OP22.2S	28.25	D-L	OP22.2S	49.25	A-H
OK125.2R	27.75	D-L	OK125.2R	44.13	A-K
OK117.2R	27.37	D-L	OK117.2R	29.37	D-M
OK79.1R	26.62	D-L	OK79.1R	47.63	A-I
OP19.1S	26.37	D-L	OP19.1R	34.12	C-M
OP126.1R	25.63	D-L	OP126.1R	45	A-J
OK128.1R	24.75	E-L	OK128.1R	50.5	A-F
OK126.2R	24.37	F-M	OK126.2R	32.25	D-M
OK72.1S	23.87	F-M	OK72.1S	49.37	A-H
OP19.1R	23.87	F-M	OP19.1S	25.25	G-N
OP87.1R	23.63	F-M	OP87.1R	26	E-N
OP62.1S	23.37	F-M	OP62.1S	58.37	A-C
OP106.2R	21.63	G-M	OP106.2R	20	B-M

Table 1.3: (cont'd).

OP15.1R	20.75	H-M	OP15.1R	52.37	A-D
OP16.1R	20	I-M	OP16.1R	37.87	B-M
OK47.1R	19.87	I-M	OK47.1R	49.63	A-G
OK52.1S	18.5	I-M	OK52.1S	34.12	C-M
OK49.2S	18.5	I-M	OK49.2S	33.87	C-M
OK39.1R	18.5	I-M	OK39.1R	43.87	A-L
OK21.2S	15	J-M	OK21.2S	40.37	A-M
OK18.2S	13.25	K-M	OK18.2R	18.87	L-N
OK122.1S	10.87	LM	OK122.1S	51	A-E
Control	0	M	Control	0	N

*The designation 'OP' refers to those samples which were collected from soil which was not fumigated. 'OK' refers to those samples collected from fields which were fumigated preplant with metam sodium.

**The designation 'S' indicates that the isolate was recovered from the storage roots. 'R' indicates that isolates were recovered from the rhizome tissue.

***Plant ratings (0-4) were taken every 7-10 days for four weeks. AUDPC values were calculated and used to run a one-way analysis of variance, followed by a Tukey HSD test for significance ($P=0.005$).

DISCUSSION

It has been more than 30 years since the *Fusarium* spp. associated with asparagus plantings in Michigan have been identified (Hartung et al., 1990). In the early 1980s, Hartung et al. (1990) determined that *F. oxysporum* was most prevalent among soil samples collected from 28 Michigan asparagus fields across two counties; 38% of the isolates were pathogenic. They also recovered *F. verticillioides* (*F. moniliforme*) and all isolates were pathogenic. In our study, seven *Fusarium* species were recovered from storage roots or rhizome tissue. *F. oxysporum* was most prevalent (92%) with all others occurring at $\leq$ 5%. Previously, *F. proliferatum, F. oxysporum,* and *F. verticillioides* (*F. moniliforme*) were associated with FCRR in Michigan (Hartung and Stephens, 1983; Damicone and Manning, 1985; Hartung, et al., 1990; Arriola et al., 2000; Elmer, 2001). Although *F. verticillioides* was previously recovered from asparagus in

Michigan (Hartung et al., 1990), it was not found in our study. *F. solani* was not reported in the 1990 survey, nor commonly reported in other studies (Hartung and Stephens, 1983; Damicone and Manning, 1985; Hartung, et al., 1990; Arriola et al., 2000; Elmer, 2001) but we identified *F. solani* at a low incidence (5%). *F. proliferatum* was also found at a low incidence (2%).

The pathogenicity experiments indicated that all *F. oxysporum* isolates tested caused some level of discernable disease symptoms but not all were significantly virulent compared to the non-inoculated control. Other *Fusarium* spp. were not tested due to the low incidence.

'Guelph Millennium' and 'Guelph Eclipse' are considered by Michigan growers to be vigorous and potentially tolerant to FCRR (M. Hausbeck, personal communication). In our study, *Fusarium* spp. were recovered consistently from these cultivars and 'Jersey Supreme'. Damicone and Manning (1985) sampled 'Mary Washington' and 'Jersey Centennial' crowns and recovered *Fusarium* spp. from both varieties. Seven *Fusarium* spp. were recovered, most were part of the *F. oxysporum* species complex (Damicone and Manning, 1985), similar to the results of our study. These current findings suggest that the asparagus cultivars currently grown in Michigan may become infected by *Fusarium* spp.

Our results and those of others (Hartung et al., 1990; Stephens and Elmer, 1988) indicate that asparagus crowns can become infected with *Fusarium* spp. while in the nursery. Pathogenic *F. oxysporum* isolates may affect the storage roots and the rhizome (Elmer, 2001). Metam sodium is used as a soil fumigant primarily for weed control and may suppress other soil-borne plant pests (Carlock and Dotson, 2010). In our study, *F. acuminatum, F. avenaceum,* and *F. graminearum* were only isolated from crowns grown in fumigated soil and have not previously been reported on asparagus. Pathogenicity of these isolates was not evaluated in this study as the

overall incidence was ≤ 1%. Soil samples collected by Hartung et al. (1990) indicated that *Fusarium* spp. infestations in Michigan's soils occurred regardless of prior asparagus cultivation.

Fusarium spp. including *F. verticillioides* and *F. oxysporum* may be seedborne in asparagus (Inglis, 1980). Disinfestation of the seed, while effective, is not practical for commercial growers whose farms are of significant size (Damicone et al., 1981; Inglis, 1980). The nursery bed may be fumigated prior to planting. The crowns are dug after one year and may be dipped into a fungicide solution before planting in a production field. Damicone and Manning's (1985) research on one-year-old crowns indicated that fumigation and an integrated pest management approach could be viable options for control of FCRR. Counts and Hausbeck (2007) looked at the efficacy of seven different treatments including fungicides and biorationals but there were no significant differences among treatments. Elmer et al. (1996) also noted fumigation of soils as a possible control tactic. Focusing disease management efforts at the crown nursery could be an effective means of targeting pathogenic *Fusarium* at early plant stage.

Results from this study provide information regarding the *Fusarium* spp. associated with crowns dug from nursery beds treated or not treated preplant with metam sodium. *Phytophthora asparagi* was also recovered from crowns and is considered a major limiting factor for asparagus production in the state (Saude et al., 2008). Although the pathogen was recovered at a relatively low incidence, it's presence indicates that mitigation efforts that begin in the crown nursery would result in benefits beyond Fusarium control.

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LITERATURE CITED

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CHAPTER II

FUSARIUM SPECIES ASSOCIATED WITH FIELD-CULTIVATED WISCONSIN GINSENG

ABSTRACT

The roots of cultivated American ginseng (*Panax quinquefolius*, L.) are valued as a traditional Chinese medicine. Lesions on the roots decrease their value and may render them unmarketable. Seeds (1,216) and roots (222) were sampled from eleven commercial growers. Tissue was excised from seeds and symptomatic and asymptomatic roots and cultured. Species identification was achieved first by morphology and subsequently via DNA extraction, PCR using EF-1 and EF-2 primers, and Sanger sequencing. *Fusarium* spp. were recovered from seed (5%) and roots (2%). Eight unique species were isolated including *F. avenaceum* (62%), *F. solani* (11%), and *F. oxysporum* (10%). The pathogenicity of 40 isolates was tested in two experiments using ginseng seedlings. Additionally, the pathogenicity of 82 isolates was also evaluated on fresh roots. Significant differences of pathogenicity were noted among the isolates; five isolates were consistently pathogenic. Results indicate that *Fusarium* spp. may not pose a significant threat to this perennial crop.

INTRODUCTION

American ginseng (*Panax quinquefolius*, L.) is native to North America and has long been valued for its medicinal properties (Harrison et al., 2000). The genus, *Panax,* originates from the Greek language and means “all-healing” (Barrett, 2018). The health benefits of ginseng are associated with ginsenoside compounds, phyto-chemicals acting as antioxidants, stimulants and anti-cancer compounds (Leung and Wong, 2010). Ginseng roots are usually dried prior to marketing and may be processed and packaged as a whole root, ground into powder, made into capsules, teas, sweets, toothpaste and other products (Harrison et al., 2000).

Ginseng is commercially field cultivated in “gardens” consisting of raised plant beds (23-30 cm high and 1.5 m wide) that are covered with straw mulch with a shade canopy of cedar lath or polypropylene woven fabric (Hausbeck, 2011). Field-cultivated ginseng dominates production in Canada, Wisconsin, and Michigan (Hausbeck, 2017). Approximately 90% of the U.S. field-cultivated ginseng is produced in Wisconsin, primarily in Marathon Co. Production in the state accounts for 10% of the world’s supply (Hausbeck, 2017). In Wisconsin, field-cultivated ginseng sales yield $75-100 million, annually (Hausbeck, 2017). In 2017, field-cultivated ginseng was exported to Asia for $20-$40/lb. (Burkhart, 2017). Hong Kong is the largest importer of ginseng (Baeg and So, 2013).

Field-cultivated ginseng is susceptible to plant pathogens including *Alternaria panax* (Parke and Shotwell, 1989; Hill and Hausbeck, 2008), *Cylindrocarpon destructans* (Reeleder and Brammal, 1994; Punja, 1997; Reeleder et al., 1999; Rahman and Punja, 2005), *Pythium* spp. (Reeleder and Brammal, 1994), *Phytophthora cactorum* (Lee, et al., 2015), *Botrytis cinerea* (Punja, 1997; Kim et al., 2009; Lu et al., 2015), *Fusarium* spp. (Reeleder et al., 1999), and *Rhizoctonia solani* (Reeleder and Brammal, 1994). *Fusarium* spp. may cause seedling damping off and root rot or russetting (Punja, et al., 2007). Russetting of the root may also be caused by *C. destructans*, but *Fusarium* spp. are considered the predominant causal agent (Reeleder, et al., 2006). Few fungicides are registered for use on ginseng against *Fusarium* spp. (Hausbeck, 2017).

Fusarium spp. may survive as a saprophyte in the soil or on plant debris including the straw mulch (Howard, 1994). *Fusarium* spp. have also been associated with ginseng seed (Hausbeck, unpublished data). Punja et al. (2007) conducted a survey of American ginseng roots, straw mulch, and soil in British Columbia and recovered *Fusarium spp.* from all. Species associated with ginseng root included *F. equiseti, F. acuminatum, F. avenaceum, F.*

sporotrichioides, F. culmorum, F. oxysporum, F. solani, and *F. tricinctum.* They concluded via in vitro pathogenicity assays that *F. equiseti, F. avenaceum, F. sporotrichioides,* and *F. culmorum* were the most pathogenic of the species recovered (Punja et al., 2007). The objective of our study was to identify and determine the incidence and pathogenicity of *Fusarium* spp. associated with Wisconsin field-cultivated ginseng seed and roots.

MATERIALS AND METHODS

Sampling. Stratified ginseng seed (1,216) and root (222) samples (Fig. 2.1, 2.2) were obtained from eleven growers located in Marathon County, Wisconsin (Table 2.1). Three growers supplied both seed and root samples (Table 2.1).

Table 2.1: Ginseng seed and root samples collected from commercial growers in Marathon County, Wisconsin.

		Root samples (no.) and age (year)			
Grower	**Seed (no.)**	1	2	3	4
1	116	-	30	30	-
2	100	40	62	-	40
3	100	-	30	-	-
4	0	-	-	33	-

***Fusarium* isolation.** Seed was rinsed with tap water and surface disinfested by soaking seeds in 0.825% sodium hypochlorite for one min. Seed was rinsed twice with sterile water for a minimum of 30 sec and allowed to dry on sterile paper towel in a laminar flow hood. Each seed was divided into three pieces comprising the two halves of the seed coat and the endosperm and plated in a triangular configuration onto Nash Snyder agar (1 seed/plate) (Leslie and Summerell, 2008). Once isolates had sufficiently grown from the seed pieces, they were sub-cultured onto

water agar (16 g agar and 1000 mL distilled water). After approximately five days, the cultures were hyphal tipped under a dissecting microscope from the water agar plate onto 50% strength potato dextrose agar (16 g potato dextrose agar (PDA) and 1000 mL distilled water) (Leslie and Summerell, 2008).

Ginseng roots were not surface sterilized, as preliminary studies' results indicated that this significantly inhibited all fungal growth. Roots were washed with tap water. Three tissue pieces were excised from symptomatic or asymptomatic tissue (Fig. 2.1) from each root and plated directly onto water agar amended with 2 mL rifampicin/1000 mL agar and 2 mL ampicillin/1000 mL agar. After mycelia were observed to have grown out of the root tissue, they were subcultured onto water agar. Water agar plates were allowed to grow for approximately three to five days, depending on the growth rate of each isolate. Isolates were hyphal tipped as described above. All isolates were morphologically identified to genus, and species where possible, on a culture plate. Riddell mounts (Riddell, 1950) were also prepared with select isolates in order to view many defining characteristics, including microconidial chains, chlamydospores, and macroconidia (Fig. 2.2).

Figure 2.1: Ginseng roots exhibiting red-brown lesions and discoloration (top). Ginseng seeds stored in moist sand to satisfy dormancy requirements (bottom).

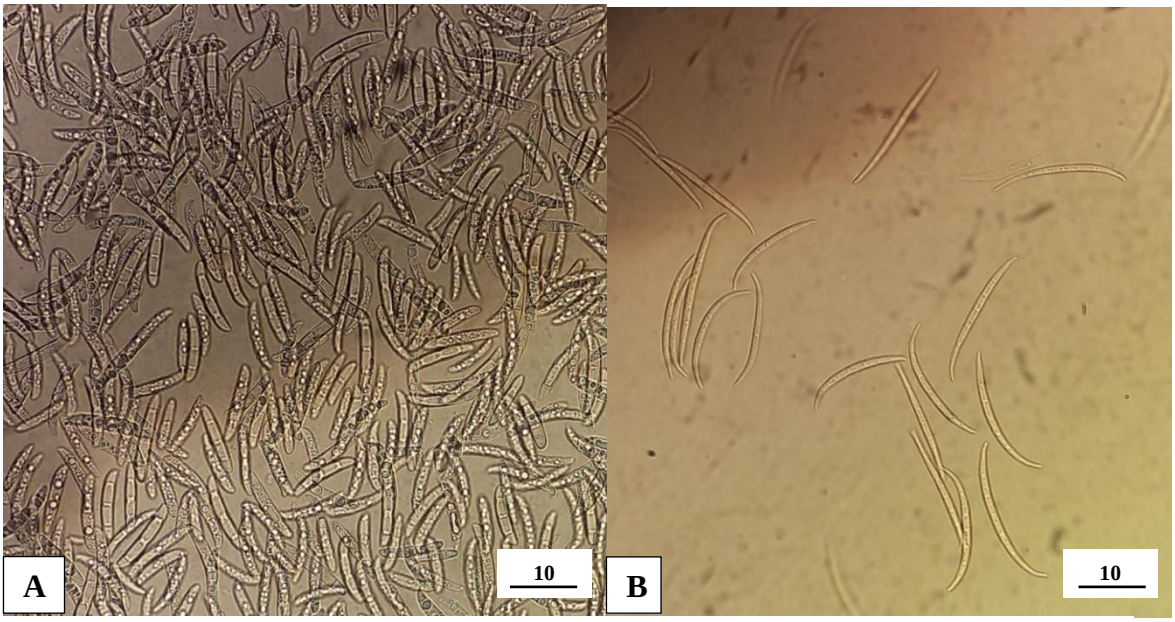

Figure 2.2: Macroconidia of *Fusarium solani* (A) and *Fusarium avenaceum* (B) from cultures obtained from ginseng roots.

Isolate storage. Skim milk was prepared using 10 g Difco skim milk powder (BD Biosciences, Franklin Lakes, NJ) per 100 mL of distilled water and autoclaved for 15 min.

Sterile skim milk was stored in the refrigerator and kept on ice when in use (adapted from Leslie and Summerell, 2008). Screw cap culture tubes (glass scintillation vial. 20 ml capacity) were filled three quarters full with silica gel (Beantown Chemical, Hudson, NH). Vials were covered with aluminum foil, sterilized with dry heat at 121°C for 90 min, and stored in the refrigerator. Vials were kept on ice during use (adapted from Leslie and Summerell, 2008). Seven- to 10-day-old PDA culture plates were flooded with sterile skim milk and gently agitated with a pipette tip to create a spore suspension. The milk suspension (200 µL) was pipetted evenly onto the silica gel crystals. The mouth of the vial was flame sterilized, capped, and sealed with parafilm®. Vials were vortexed briefly to evenly distribute the suspension and stored at 6°C. Two vials per isolate were prepared (adapted from Leslie and Summerell, 2008).

DNA extraction. Individual isolates were removed from the refrigerator and placed on ice while in use. Silica gel crystals were shaken onto the surface of water agar media and pressed into the media using the flat side of a sterile scalpel. Cultures were allowed to grow under constant light conditions and at room temperature (23°C) for 3 to 4 days before being subcultured onto PDA (39 g PDA agar and 1000 mL distilled water). DNA was extracted from 7- to 10-day old cultures grown on PDA

All sample DNA was extracted either manually using a Qiagen DNeasy Plant Mini kit (Qiagen, Hilden, Germany) or with the KingFisher Flex Magnetic Particle Processor (Thermo Scientific, Waltham, MA, USA) and the Omega 1130 Mag-Bind DNA extraction kit (M1130, Omega Bio-Tek, Norcross, GA, USA) with the following modifications. Manual extraction used 90 to 100 mg of mycelium (fresh weight) frozen in liquid nitrogen with five 3-mm glass beads (Merck, Darmstadt, Germany) and lysed in a TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for three minutes. The lysis buffer (600 µl, Buffer AP1) and 6 µl of RNase A stock solution

((100mg/ml) Roche Diagnostics, Bale, Switzerland) were added and incubated for 30 minutes at 65ºC. Final elution was performed with 30 µl of buffer. For automated extractions 50 to 70 mg of mycelium were lyophilized (Genesis Pilot Lyophilizer (SP Scientific, Warminster, PA, USA)) in 1.1 mL polypropylene 8-strip collection tubes (Thomas Scientific, Swedesboro, NJ, USA) and lysed with a single 4 mm stainless-steel ball bearing (Grainger, Lake Forest, IL, USA) as described above. Initial incubation time for lysis was increased to 60 min at 56ºC and mixed every 10 min. The following reagent volumes were modified: RNase-A (6 µl), lysate (450 µl), RBB buffer (450 µl). Incubation time with RNase was increased to 30 min at room temperature (23°C). All DNA extracts were quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and stored at -20 ºC.

PCR, sequencing, and phylogenetic analysis. PCR amplification was run for the Translation Elongation Factor 1-α (TEF) coding region and employed the EF-1 (ATGGGTAAGGA(A/G)GACAAGAC) and EF-2 (GGA(G/A)GTACCAGT(G/C)ATCATGTT) forward and reverse primers, respectively (Geiser et al., 2004). Reactions were set up with a master mix comprising 12 µl water, 2.6 µl buffer 5X Green GoTaq® Reaction Buffer (Promega, Madison, WI), 0.5 µl (2.5mM) dNTPs (Promega, Madison, WI), 3 µl (25mM) magnesium chloride (Promega, Madison, WI), 0.5 µl (10 µg/µl) BSA (New England BioLabs, Ipswich, MA), 0.30 µl GoTaq® polymerase (Promega, Madison, WI), and 0.5 µl each of the EF-1 and EF-2 primers (Integrated DNA Technologies, Coralville, IA) at a 5 µM concentration. Resulting in a total of 20.9 µl master mix plus 2 µl of template DNA per well of a 96-well plate. Samples were run in a Vapo.Protect Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) using the following program; 1 h at 94°C; 34 cycles of 30 min at 94°C, 45 min at 59°C, 2 hrs at 72°C (modified from the Promega protocol).

Once the PCR was completed, sample quality was verified by gel electrophoresis using the PowerPac Basic (BioRad, Hercules, CA). Samples were subsequently sent to Macrogen (Macrogen, Rockville, MD) for forward and reverse Sanger sequencing of the TEF region, using primers EF-1 and EF-2 respectively. Returned sequences were analyzed using Geneious® version 2019.1.1 (Biomatters Ltd, Auckland, New Zealand) to align, assemble, and trim forward and reverse reads to achieve a consensus sequence for each isolate. Sequences were then identified to species complex or species, where possible, through a BLAST search of the ID region in the National Center for Biotechnology Information GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and cross referenced with the FUSARIUM-ID database (http://isolate.fusariumdb.org/blast.php). Sequences were aligned using MUSCLE in Mesquite version 3.51. A maximum likelihood estimate using CIPRES version 3.3 (https://www.phylo.org/) was conducted. Bootstrap analysis using 1,000 replications was run and the resulting tree viewed in FigTree version 1.4.4.

Pathogenicity testing. *Seedlings:* Following stratification in moist sand, germinated seed was grown in 72-cell flats. Vermiculite was placed in the bottom of each cell which was then filled to the top with fine sand (Quikrete® companies). Sand was moistened with water and individual germinated seeds were planted into each cell and grown for approximately three months. *Fusarium* spp. isolates (40) representing each growers' seed and root samples were chosen for pathogenicity testing. Isolates were grown for 7 to 10 days on PDA, macerated in a blender, and combined with sterile water to achieve a final volume of 125 mL. The spore concentration in the slurry was measured using a hemocytometer. Two (Experiment 1) to three (Experiment 2) month-old seedlings were individually inoculated with *Fusarium* spp. by dipping their roots into the inoculum slurry. Inoculated seedlings were planted into 3-in pots. Four

replications per isolate plus non-inoculated control were used for Experiment 1. Three replicates per isolate plus a non-inoculated control were included for Experiment 2. All plants were arranged in a completely randomized design on a greenhouse bench as in Fig. 2.3. Temperatures were maintained at 21º to 25º C with 90% relative humidity for the duration of the experiment. Disease severity was assessed on a scale from 0 to 4 (0=healthy, 4=dead) every 3 to 4 days (Fig. 2.4).

Figure 2.3: Ginseng seedlings inoculated with *Fusarium* spp. and arranged in a completely randomized design on a bench in a research greenhouse.

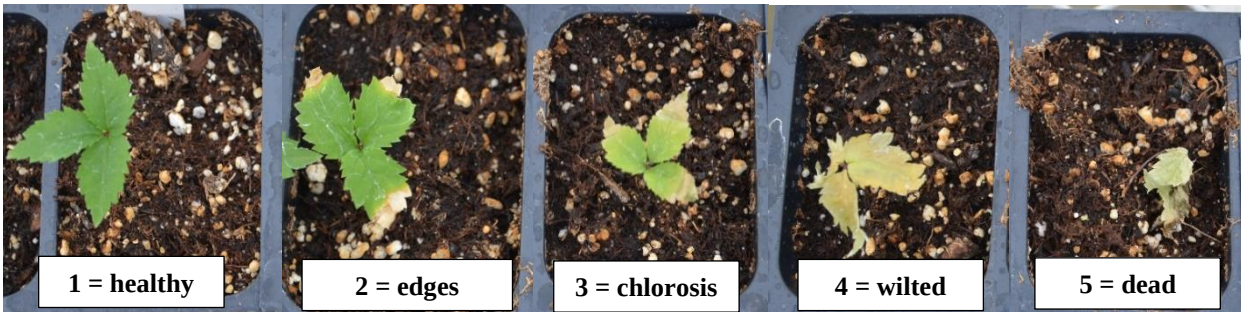

Figure 2.4: Symptoms of disease on ginseng seedlings inoculated with select *Fusarium* spp. isolates were rated using the following scale: 1= Leaves dark green; no chlorosis or necrosis, 2=Leaves chlorotic and/or leaf tips necrotic, 3= Leaves chlorotic and necrosis of the leaf tips has expanded, 4= Entire plant is chlorotic and wilted, 5=Seedling is necrotic

Figure 2.5: Ginseng roots (20) inoculated with *Fusarium* spp. isolates were arranged in a clear plastic box with an opaque white lid.

Roots: Based on methods described in Punja et al. (2007), four-year-old ginseng roots from the field (80) were washed with tap water, surface sterilized by submerging them for 10 sec in 0.4125 % sodium hypochlorite, rinsed with sterile deionized water, and dried on a sterile paper towel in a laminar flow hood. Roots were arranged on a moist sterile paper towel and divided evenly into four clear-sided plastic boxes with an opaque white lid. Isolates (81), including all of those used in the seedling experiments, were chosen and represented each grower and *Fusarium*

spp. recovered. Additionally, all isolates obtained from root tissue were included. One isolate determined to be virulent in a preliminary seedling study (data not shown) was used as a positive control (DK42.1), while a sterile PDA plug was used as the negative control. Positive and negative controls were placed on each root along with four isolates whose pathogenicity was unknown (Fig. 2.5). Roots were not wounded in the first and second experiments. Roots were wounded in the third experiment. Agar plugs containing mycelium of each isolate to be tested were arranged on each root in a randomized design with four replicates per box (modified from Punja et al., 2007). Pathogenicity was rated two weeks post inoculation based on the presence or absence of sunken lesions associated with each isolate (Punja et al., 2007). Tissue was sampled from the roots and the same *Fusarium* species re-isolated and sequenced to fulfill Koch's postulates. Inoculation with the positive control consistently resulted in a lesion, while the negative control remained asymptomatic.

Data analysis. Seedling disease severity data over the five-week period for Experiments 1 and 2 were used to calculate the Area Under the Disease Progress Curve (AUDPC). These data were subsequently analyzed using the PROC glimmix procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC). A one-way Analysis of Variance (ANOVA) test using Satterthwaite's adjustment was conducted in order to account for the unequal variances found in the dataset. Prior to this analysis, various transformations of the dataset were explored. It was determined that Satterthwaite's adjustment would be most effective in addressing the unequal variances. Following the ANOVA test, a Tukey HSD test with the Satterthwaite's adjustment was carried out. For the root assay, a one-way ANOVA test was run also using the PROC glimmix procedure.

RESULTS

***Fusarium* isolation.** *Fusarium* spp. were frequently recovered from ginseng seed although the number of isolates obtained from the seed samples varied among the growers from which the lots were obtained (Table 2.2). *F. avenaceum* was most commonly recovered from ginseng seed (65%), followed by *F. solani* (10%) and *F. oxysporum* (10%) (Table 2.3, Table 2.4). Other species recovered from seed included *F. fujikuroi* (<1%), *F. graminearum* (8%), *F. incarnatum-equiseti* (3%), *F. proliferatum* (3%), and *F. sporotrichioides* (7%) (Table 2.3). There were no differences among isolates obtained from different seed tissue (data not shown).

Table 2.2: *Fusarium* spp. isolates from seed sampled from commercial growers.

Grower (100 seed)	*Fusarium* spp.
1	25
2	7
3	0
4	1
5	39
6	16
7	24
8	7
9	15
10	10
11	3
Total	147

Table 2.3: *Fusarium* spp. recovered from stratified ginseng seed and roots and identified by sequence analysis.

Fusarium spp. recovered	No. of seed isolates (% of total*)	No. of root isolates (total 11)
F. solani	14 (10%)	3 (27%)
F. avenaceum	95 (65%)	2 (18%)
F. fujikuroi	1 (<1%)	-
F. graminearum	12 (8%)	-
F. incarnatum-equiseti	3 (2%)	-
F oxysporum	10 (7%)	6 (55%)
F proliferatum	3 (2%)	-
F. sporotrichioides	7 (4%)	-

*Total = 145

Table 2.4: *Fusarium* spp. isolated from stratified seed and identified by sequence analysis.

Grower (# of total isolates)	***F. solani* (% of total)**	***F. avenaceum* (% of total)**	***F. fujikuroi* (% of total)**	***F. graminearum* (% of total)**	***F. oxysporum* (% of total)**	***F. proliferatum* (% of total)**	***F. sporotrichioides* (% of total)**	***F. incarnatum -equiseti* (% of total)**
1 (25)	**0**	**21** (84%)	**0**	**0**	**3** (12%)	**1** (4%)	**0**	**0**
2 (7)	**2** (28%)	**4** (57%)	**0**	**0**	**0**	**1** (14%)	**0**	**0**
3 (1)	**0**	**0**	**0**	**0**	**1 (100%)**	**0**	**0**	**0**
5 (39)	**0**	**39** (100%)	**0**	**0**	**0**	**0**	**0**	**0**
6 (16)	**1** (6%)	**12** (75%)	**0**	**0**	**1** (6%)	**0**	**2** (12%)	**0**
7 (24)	**8** (33%)	**2** (8%)	**0**	**5** (21%)	**2** (8%)	**0**	**4** (17%)	**3** (12%)
8 (7)	**0**	**0**	**0**	**7** (100%)	**0**	**0**	**0**	**0**
9 (15)	**1** (7%)	**12** (80%)	**0**	**0**	**2** (13%)	**0**	**0**	**0**
10 (10)	**0**	**5** (50%)	**1** (10%)	**0**	**2** (20%)	**1** (10%)	**1** (10%)	**0**
11 (3)	**2** (70%)	**1** (30%)	**0**	**0**	**0**	**0**	**0**	**0**

Only eleven *Fusarium* spp. isolates were obtained from roots (Table 2.3). Isolates from 2-yr-old roots included *F. avenaceum* (1) and *F. oxysporum* (1). The four isolates from 3-yr-old roots were identified as *F. avenaceum* (1), *F. oxysporum* (2), and *F. solani* (1). Isolates from 4-

yr-old roots included *F. solani* (2) and *F. oxysporum* (3). Other fungi recovered from roots and seed included *Pythium* spp., *Trichoderma* spp., *Chaetomium globosum*, and *Geotrichum* spp. with *Trichoderma* spp. and *Pythium* spp. commonly isolated from root samples (data not shown).

Pathogenicity testing. Eight isolates were pathogenic on seedlings across both experiments and included *F. solani* (DK42.IS), *F. avenaceum* (SG6.IS, VG283.IS, SG6.IS, FR38.2S), *F. graminearum* (MG57.3S), *F. sporotrichioides* (MG88.3S, VG279.IS), and *F. oxysporum* (VG289.3S) (Table 2.5). Five of the 81 isolates tested for pathogenicity on fresh roots (wounded or nonwounded) caused symptoms significantly greater than the control across the three experiments and included *F. sporotrichioides* (SG58.2S, MG86.1S, VG279.1S) *F. avenaceum* (SG2.2S) and *F. solani* (DK42.1S). The negative control remained asymptomatic on both seedlings and fresh roots. The positive control consistently caused disease symptoms in all experiments (Tables 2.5, 2.6). When roots were wounded prior to inoculation, 53 *Fusarium* spp. isolates resulted in significant disease symptoms compared to the 17 or 19 isolates for Experiments 1 and 2, respectively, when roots were non-wounded prior to inoculation (Table 2.5).

Table 2.5: AUDPC values for ginseng seedlings inoculated with *Fusarium* spp.

		Experiment 1		Experiment 2	
Species	Isolate #	AUDPC	Tukey HSD	AUDPC	Tukey HSD
F. solani	DK42.1S	148.75	A	90	C-G
F. avenaceum	MG65.3S	134.25	AB	57.33	D-I
F. avenaceum	FR100.3S	133.25	AB	56.67	D-I
F. avenaceum	SG6.1S	132.75	A-C	185.83	A
F. avenaceum	WI30.1S	125.5	A-D	0	I
F. avenaceum	HJ50.1S	123	A-E	23	F-I
F. oxysporum	BC1.2S	119.75	A-E	42.5	E-I
F. oxysporum	SG7.3S	119.5	A-F	0	I
F. solani	HJ8.1R4	112.38	A-G	1.5	I
F. avenaceum	FR38.2S	110.13	A-H	92.67	C-F

Table 2.5: (cont'd).

F. sporotrichioides	MG88.3S	105.75	A-H	169	AB
F. solani	HJ5.1R4	104.25	A-I	53.83	D-I
F. sporotrichioides	VG279.1S	97.63	A-I	121.50	A-D
F. oxysporum	VG289.3S	97.63	A-I	74.83	C-H
F. graminearum	ZS30.2S	97.38	A-I	23	F-I
F. avenaceum	SG10.1S	91.75	A-J	0	I
F. graminearum	MG57.3S	88.75	A-K	82.5	C-G
F. solani	MG12.1S	88.75	A-K	4	HI
F. avenaceum	HJ1.3R4	81.5	B-L	43	E-I
F. solani	MG61.2S	77.63	B-L	0	I
F. avenaceum	BC12.1R	75.25	B-L	0	I
F. graminearum	ZS10.3S	74.5	B-L	25.83	E-I
F. solani	HJ18.3R	71.88	C-L	0	I
F. oxysporum	BC13.1R	69.5	D-L	0	I
F. avenaceum	VG283.1S	66	D-L	144.33	A-C
F. avenaceum	VG7.1S	64.13	E-L	0	I
F. avenaceum	MG20.2S	58.5	F-M	0	I
F. solani	HJ28.2S	58.25	G-M	47.5	E-I
F. avenaceum	BC75.1S	54.88	G-M	19.67	G-I
F. solani	DK22.1S	54.88	G-M	34.67	E-I
F. avenaceum	HJ86.1S	51.88	G-M	98.67	B-E
F. oxysporum	VG80.2S	50.75	H-M	44.83	E-I
F. oxysporum	BC69.3S	44	I-M	0	I
F. avenaceum	WI20.3S	33.13	J-M	15.83	G-I
F. graminearum	ZS94.3S	32.38	J-M	60	D-I
F. avenaceum	FR57.1S	28.63	K-M	58.67	D-I
F. avenaceum	BC7.1S	27.13	LM	27.67	E-I
F. avenaceum	FR43.3S	26.38	LM	35.83	E-I
F. oxysporum	WI2.1S	26.25	LM	97.17	B-E
F. avenaceum	HJ77.1S	1.63	M	53.83	D-I
Control	Control	0	M	0	I

*AUDPC values are determined using disease severity ratings every 3-4 days post inoculation.

**AUDPC values with the same letter are not significantly different among isolates.

***AUDPC values were used to run a one-way analysis of variance, followed by a Tukey HSD test for significance (*P=0.0001*).

Table 2.6. Fresh ginseng roots, nonwounded or wounded, when inoculated with *Fusarium* spp. and visually assessed as either symptomatic (1) or asymptomatic (0).

		Exp. 1 Nonwounded		Exp. 2 Nonwounded		Exp. 3 Wounded	
Species	Isolate	Rating	Tukey HSD	Rating	Tukey HSD	Rating	Tukey HSD
F. sporotrichioides	SG58.2S	1	A	1	A	1	A
F. sporotrichioides	MG86.1S	1	A	0.5	BC	1	A
F. solani	HJ28.2S	1	A	1	A	0.25	CD
F. solani	DK42.1S	1	A	1	A	1	A
F. sporotrichioides	MG29.1S	0.75	AB	0	D	1	A
F. avenaceum	MG20.2S	0.75	AB	0.25	CD	0.5	BC
F. graminearum	ZS30.2S	0.75	AB	0	D	0.5	BC
F. avenaceum	SG2.2S	0.5	BC	1	A	1	A
F. solani	MG78.3S	0.5	BC	0.75	AB	0	D
F. sporotrichioides	VG279.1S	0.5	BC	0.75	AB	1	A
F. graminearum	ZS10.3S	0.5	BC	0	D	0	D
F. avenaceum	SG6.1S	0.5	BC	0	D	1	A
F. solani	MG61.2S	0.5	BC	0.75	AB	0	D
F. oxysporum	SG7.3S	0.5	BC	0	D	0.75	AB
F avenaceum	FR38.2S	0.5	BC	0	D	0.25	CD
F. graminearum	ZS94.3S	0.5	BC	0	D	0.25	CD
F. avenaceum	HJ50.1S	0.5	BC	0.25	CD	0.5	BC
F. solani	MG48.2S	0.25	CD	0	D	0	D
F. solani	MG22.1S	0.25	CD	0.25	CD	1	A
F. avenaceum	DK90.1S	0.25	CD	0	D	1	A
F. graminearum	ZS10.2S	0.25	CD	0.75	AB	0	D
F. avenaceum	BC80.1S	0.25	CD	0	D	1	A
F. sporotrichioides	SG1.1S	0.25	CD	0.5	BC	1	A
F. graminearum	MG79.2S	0.25	CD	0	D	0.5	BC
F. avenaceum	FR40.2S	0.25	CD	0.5	BC	0.75	AB
F. avenaceum	BC55.2S	0.25	CD	0.25	CD	1	A
F. avenaceum	FR95.1S	0.25	CD	0.25	CD	0.5	BC
F. avenaceum	FR87.2S	0.25	CD	0.5	BC	1	A
F. avenaceum	SG34.3S	0.25	CD	0.25	CD	0.75	AB
F. avenaceum	FR45.3S	0.25	CD	0.25	CD	1	A
F. oxysporum	HJ32.1R4	0.25	CD	0	D	1	A
F. avenaceum	VG283.1S	0.25	CD	0	D	1	A
F. avenaceum	HJ1.3R4	0.25	CD	0	D	0.5	BC
F. solani	MG12.1S	0.25	CD	0.25	CD	0	D
F. sporotrichioides	MG88.3S	0.25	CD	0.5	BC	1	A
F. avenaceum	SG10.1S	0.25	CD	0.25	CD	0.25	CD
F. oxysporum	WI2.1S	0.25	CD	0	D	0.5	BC
F. avenaceum	WI30.1S	0.25	CD	0	D	0.25	CD
F. avenaceum	FR100.3S	0.25	CD	0	D	0.5	BC

Table 2.6: (cont'd).

F. avenaceum	HJ86.1S	0.25	CD	0.5	BC	0.75	AB
F. avenaceum	HJ77.1S	0.25	CD	0	D	0.25	CD
F. avenaceum	FR57.1S	0	D	0	D	1	A
F. avenaceum	WI67.2S	0	D	0.25	CD	0.25	CD
F. graminearum	ZS14.1S	0	D	0.5	BC	0.25	CD
F. avenaceum	SG17.3S	0	D	0.5	BC	1	A
F. avenaceum	VG225.1S	0	D	0	D	0.75	AB
F. proliferatum	HJ6.2S	0	D	0	D	0.25	CD
F. avenaceum	WI67.2S	0	D	0	D	0	D
F. avenaceum	FR6.2S	0	D	0	D	1	A
F. avenaceum	FR15.3S	0	D	0	D	1	A
F. fujikuroi	VG208.1S	0	D	0	D	0.75	AB
F. oxysporum	MG75.2S	0	D	0	D	0.25	CD
F. graminearum	ZS75.3S	0	D	0.25	CD	0.25	CD
F. avenaceum	SG57.3S	0	D	0.5	BC	1	A
F. proliferatum	VG216.1S	0	D	0	D	0	D
F. avenaceum	FR41.3S	0	D	0.25	CD	0	D
F. avenaceum	BC33.2S	0	D	0.25	CD	1	A
F. avenaceum	WI99.3S	0	D	0	D	0.75	AB
F. avenaceum	WI14.3S	0	D	0	D	0.75	AB
F. avenaceum	BC28.3S	0	D	0	D	1	A
F. avenaceum	BC32.2S	0	D	0	D	1	A
F. oxysporum	VG80.2S	0	D	0	D	1	A
F. oxysporum	HJ22.3R4	0	D	0.25	CD	0.5	BC
F. oxysporum	HJ11.1R4	0	D	0	D	0.25	CD
F. oxysporum	VG289.3S	0	D	0.25	CD	0.75	AB
F. avenaceum	VG7.1S	0	D	0.25	CD	0.5	BC
F. oxysporum	HJ29.2R4	0	D	0	D	0.75	AB
F. avenaceum	BC12.1R	0	D	0	D	0.25	CD
F. solani	DK22.1S	0	D	0.25	CD	0.5	BC
F. avenaceum	MG65.3S	0	D	0	D	1	A
F. solani	HJ8.1R4	0	D	0	D	0	D
F. solani	HJ5.1R4	0	D	0.25	CD	0	D
F. solani	HJ18.3R4	0	D	0	D	0	D
F. oxysporum	BC69.3S	0	D	0	D	0.75	AB
F. avenaceum	BC75.1S	0	D	0	D	1	A
F. avenaceum	BC7.1S	0	D	0	D	0.75	AB
F. oxysporum	BC13.1R	0	D	0	D	0.75	AB
F. graminearum	MG57.3S	0	D	0.5	BC	0	D
F. oxysporum	BC1.2S	0	D	0.5	BC	1	A
F. avenaceum	WI20.3S	0	D	0	D	0.25	CD
N/A	Control	0	D	0	D	0	D

*Isolates with the same letter are not significantly different between isolates

**Ratings were used to run a one-way analysis of variance, followed by a Tukey HSD test for significance (*P=0.0001*).

DISCUSSION

Infection by *Fusarium* spp. may affect the marketability of the ginseng roots. We conducted a ginseng root bioassay to observe the effect of wounding on infection by *Fusarium*. The number of isolates observed to cause lesions were greater when the roots were wounded (53) versus nonwounded (17-19). These results are consistent with those of Punja et al. (2007) who demonstrated that wounding is necessary for many *Fusarium* species to penetrate older ginseng roots. Natural sources of wounding in ginseng roots may include, small mammal and insect damage, as well as the invasion of other pathogens prior to *Fusarium* inection. Five isolates were pathogenic when tested on fresh roots in all experiments and represented *F. avenaceum, F. sporotrichioides,* and *F. solani*.

The russetting of the root associated with *Fusarium* infection impacts its appearance and decreases its quality. Goswami and Punja (2008) found that lignified cells develop near the site of infection in a hypersensitive response to limit pathogen colonization, resulting in rusty orange-colored lesions that decrease its marketability. In our study, *Fusarium* infection of older ginseng roots (4-yr-old), did not include vascular discoloration, but resulted in lignified, reddened cells on the root's surface. Growers may sell the compromised crop for processing into powders, teas, and other products at a reduced price.

Fusarium spp. were more frequently isolated from ginseng seed (5%) than from roots (2%). Eight species were recovered from seed including *F. avenaceum, F. solani, F. fujikuroi, F. graminearum, F. incarnatum-equiseti, F. oxysporum*, *F. proliferatum,* and *F. sporotrichioides.* Only *F. oxysporum, F. solani,* and *F. avenaceum* were found on roots. Eight isolates were pathogenic on seedlings whereas five were pathogenic on fresh roots across all experiments.

Guan et al. (2019) conducted a survey of ginseng seed in China, identifying over 50 fungi, seven which are known as ginseng pathogens. Their findings suggest, based on a phylogenetic analysis, that the *Fusarium* isolates clustered based on region. Each region uses its own stratification medium, and the authors suggested that the variation in pathogenicity could be attributed to the medium used. In our study, media type and source were not documented but could be of interest in determining the source of *Fusarium* contamination since the number of isolates obtained varied widely among the seed samples collected from the growers and did not appear to cluster. A select number of fungicides are registered for use on ginseng seed and may offer an efficient method to limit crop risk from *Fusarium* spp., ensuring that the pathogen is not introduced into the garden.

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FUTURE WORK

Fusarium crown and root rot (FCRR) of asparagus is a destructive disease, causing wilt, fern chlorosis, vascular discoloration, root rot, and crown death (Elmer, 2001). FCRR can reduce yields by nearly 50% and was a significant factor in the asparagus decline in the 1950s (Elmer, 2001).

In the recent past, growers have been interested in the development of a bioassay to easily test for *Fusarium* in their fields. Gatch and du Toit (2015) tested such an assay on Fusarium Wilt of spinach in Washington where they requested soil samples from growers to be tested on spinach plants which ranged from susceptible to resistant to Fusarium Wilt to develop a gradient against which to test new plants. Following this current asparagus research, a baseline for *Fusarium* has now been developed for Michigan. Future work will be able to use this data and potentially refine the asparagus seedling test tube method described in Stephens and Elmer (1988) to test soil samples from nursery beds for the presence of pathogenic *Fusarium* species in asparagus as well.

All *F. oxysporum* isolates selected for the pathogenicity screening caused disease symptoms, in at least one of the two experiments, in contrast to the control, indicating that all isolates tested were at least moderately pathogenic on this crop. Five of the isolates caused disease in both experiments, indicating a high level of pathogenicity. Additional species isolated from asparagus were not tested at this time due to low recovery rates, as previously noted. These should be followed up on in future work. Furthermore, prior studies reported a significant number of *F. verticillioides* isolates and found them to be consistently pathogenic in this system, whereas this species was not isolated here.

Due to the lack of survey data on newer cultivars such as Guelph Millennium and Guelph Eclipse in Michigan, this work sought to determine their likelihood of harboring *Fusarium*

species (pathogenic and non-pathogenic). While it was anticipated that fewer isolates would be recovered from the Jersey series than from the Guelph series, *Fusarium* spp. were recovered consistently from all three cultivars in this study. In 1985, Damicone and Manning saw similar results in Massachusetts when they sampled Mary Washington and Jersey Centennial crowns and recovered *Fusarium* from them both. Although the researchers recovered seven *Fusarium* spp., most were part of the *F. oxysporum* species complex (Damicone and Manning, 1985) as in this survey. These current findings only suggest that *Fusarium* species may be isolated from asparagus, regardless of cultivar, and do not address resistance or susceptibility of the cultivars to *Fusarium*. This work did not test the isolates collected from the Jersey series for pathogenicity or the Jersey series for susceptibility to *Fusarium* infection, indicating another opportunity for future work.

These results and those of previous studies (Hartung et al., 1990; Stephens and Elmer, 1981), indicate that asparagus crowns can become infected with *Fusarium* spp. while in the nursery bed. Pathogenic *F. oxysporum* isolates may affect the storage roots and the rhizome. Metam sodium (now often replacing methyl bromide) is used as a soil fumigant to provide control primarily for weeds, and secondarily for a number of other soil dwelling plant pests (Carlock and Dotson, 2010). Given the selection pressure provided by chemical management and continual asparagus production, it was expected that a higher level of pathogenic isolates would be recovered from fumigated soils than from the nonfumigated/virgin fields where such selection pressure was not present. However, when evaluating crowns sampled from these fields, differences in isolate pathogenicity were not observed. Further research is required in order to determine whether there is any correlation between soil fumigation and *Fusarium* species diversity. Hartung et al. (1990) noted *Fusarium* infestations in Michigan regardless of prior

asparagus cultivation. Although their study focused on soil samples when surveying for *Fusarium*, this indicates an opportunity for further work on species found in soils to compare results with both studies.

Fusarium is a seedborne pathogen in asparagus and sterilization of the seed, while effective, is intensive and not practical for growers on any significant scale (Damicone et al., 1981; Inglis, 1980). The one-year-old crown stage in the nursery bed is the next possible period for control. Here the soil may be fumigated prior to planting and/or crowns may be dipped into a fungicide solution before planting to the production fields. Incidentally, it is at this stage when asparagus is the easiest to sample and test from for root pathogens, as the root system is still fairly small. This research demonstrates that one-year-old crown control is the most effective means of targeting pathogenic *Fusarium* at the earliest possible plant growth stage. Further research into young crown disease management appears to show the most promise based on this and other studies (Damicone and Manning, 1985; Elmer et al., 1996).

Phytophthora asparagi was also recovered from 3% of collected storage root samples here. As recently as 2016, this pathogen was a major limiting factor for asparagus production in Michigan (Woods and Hausbeck, 2018). It has since declined in recovery frequency, though was collected in this current study, indicating a need for further research and management.

Field cultivated ginseng is susceptible to *Fusarium* spp. causing seedling damping off and root rot or russetting of the roots. Russetting of the root may also be caused by *Cylindrocarpon destructans*, but *Fusarium* spp. is considered the predominant causal agent (Reeleder, et al., 2006). Fusarium root rot of ginseng may result in poor quality roots; russetting and dry rot of the root are characteristic (Punja et al., 2007, Yadeta and Thomma, 2013; Jiao et al., 2015). *Fusarium* in the soil can also lead to vascular wilt, preventing the flow of water and

nutrients and leading to discolored xylem tissue (Yadeta and Thomma, 2013). Goswami and Punja (2008) found that lignified cells develop near the site of infection in a hypersensitive response to limit pathogen colonization resulting in rusty orange-colored lesions on the root, rendering it unmarketable. The genes responsible for the production of phenolic compound and detoxification are upregulated in ginseng plants infected with *Fusarium* spp. Goswami and Punja (2008) proposed that the reddening of the ginseng roots was due to these phenolic compounds.

Limiting Fusarium disease in ginseng is challenging due to the pathogen's ability to survive as a saprophyte in the soil and on plant debris including the straw mulch (Howard et al., 1994) providing inoculum for subsequent years. Chemical control is also difficult, as products are limited (Hausbeck, 2011).

With a better understanding of the *Fusarium* species associated with ginseng and their pathogenicity, future research can now be targeted to specific species. Information suggesting the susceptibility of the younger crop stages can direct efforts at controlling root rot to what appears to be the most vulnerable stages. By establishing a baseline of data for *Fusarium* in Wisconsin ginseng, these studies allow future work to build upon and test those species which are known to be present. These data and the experimental methods described could also be used to develop a bioassay for ginseng in future studies.

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