EVALUATION OF STRATEGIES FOR MANAGING SOILBORNE PATHOGENS OF ASPARAGUS IN MICHIGAN

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ABSTRACT

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In 2012, Michigan produced 8.8 million kg of asparagus spears for fresh market and processing worth $17.3 million. Asparagus is a perennial crop, with fields remaining profitable for 12 to 15 years. Fusarium crown and root rot (caused by *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum*) and Phytophthora spear and root rot (caused by *Phytophthora asparagi*) contribute to early decline, and decrease the vigor of young stands in fields previously cropped with asparagus. Nine isolates of *P. asparagi* were tested for virulence using asparagus spears of five commercial cultivars and asparagus seedlings of three cultivars. Significant differences in isolate virulence and host susceptibility were observed on spears, but not on seedlings. Seedlings of 18 and 11 cultigens were screened for resistance to *P. asparagi* and *Fusarium* spp., respectively. While all cultigens were susceptible to the pathogens, significant differences in the degree of susceptibility were detected. Pathogen virulence varied, and the interaction between pathogen and cultigen significantly affected root rot severity. Fungicides and biocontrol agents were screened for efficacy as seed treatments against *Fusarium* spp. and *P. asparagi* in vitro. Two fungicides and one biocontrol agent increased germination of seeds inoculated with *P. asparagi*. Disease incidence was reduced when one biocontrol agent limited *P. asparagi* and *Fusarium* spp. growth. This is the first evaluation of modern hybrids and seed treatments for management of both *Fusarium* spp. and *P. asparagi.* Identifying resistant cultivars and effective seed treatments is important for the development of an integrated pest management system to control soilborne pathogens for Michigan asparagus grower

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LITERATURE REVIEW

ASPARAGUS

Asparagus is a dioecious, herbaceous perennial crop. *Asparagus*, formerly included in the family Liliaceae (Drost, 1997), is the only genus of family Asparagaceae (Batchelor and Scott, 2006; Kubitzki and Randall, 1990; The Angiosperm Phylogeny Group, 2009). The number of species is estimated to be 170-300 (Kubitzki and Randall, 1990), but *Asparagus officinalis* Linnaeus is the only species grown as an edible crop. Asparagus cultivation began in Europe by the Romans and Greeks, and was produced for culinary and medicinal purposes (Drost, 1997; Kubitzki and Randall, 1990; Schofield, 1946).

In 2009, the production of asparagus worldwide encompassed 195,819 hectares across 62 countries, a decrease of 30,176 ha since 2005. The major asparagus producing countries of China, the United States, Spain, the Philippines, Australia, and New Zealand, have experienced losses of production areas, while Peru and Mexico have increased production areas (Benson, 2012). Countries in the Southern Hemisphere are able to produce spears 12 months each year. Peru and Mexico export nearly all of their asparagus in order to meet consumption demands in regions such as the U.S. where the harvest season is relatively short (Benson, 2012; Elmer, 2001b).

Michigan ranks third in the U.S. for asparagus production, following California and Washington. In 2012, Michigan produced 8.8 million kg of spears, with a total value of $17.3 million, for fresh market and processing (Anonymous, 2013). Over the last decade, the production area in Michigan has decreased by 2,104 ha (Anonymous, 2013; Kleweno and Matthews, 2002). All production in Michigan is used for domestic consumption, and approximately 72% is utilized as canned or frozen (Benson, 2012).

The asparagus crown consists of a rhizome, adventitious or storage roots, and lateral or feeder roots. Buds grow on the sympoidal rhizome in a bud cluster; individual shoots arise from separate buds and new storage roots originate at the base of actively growing buds (Drost, 1997; Kubitzki and Randall, 1990). Feeder roots grow from young tissue on the storage roots, and are the primary nutrient and water absorbing structures (Drost, 1997). Fleshy storage roots contain reserves of soluble carbohydrates, which are depleted during the harvest season (Drost, 1997; Shelton and Lacy, 1980). Shoots emerge from the soil during the spring as succulent spears, which mature into extensively branched ferns up to 2 m tall (Drost, 1997). A fern stand is comprised of a primary stalk, secondary branches and needle-like cladophylls. Although the entire fern tissue is able to undergo photosynthesis, the cladophylls are the primary photosynthetic structures of asparagus plants.

Open-pollinated cultivars are dioecious and have substantial genetic variability, which can limit the average vigor and yield of a field compared to fields planted with homogenous, high performance cultivars (Stephens and Elmer, 1988; Zandstra et al., 1992). Male plants have higher marketable yield and number of spears, earlier season production, reduced time between emergence of individual spears, longer production duration, and lower mortality rate compared to female plants (Drost, 1997). All-male hybrids from New Jersey (i.e. 'Jersey Giant') have been developed to homogenize and increase average vigor and yield within each cultivar (Ellison and Kinelski, 1985). 'Jersey Giant' and 'Millennium,' a cultivar developed in Canada, are commonly grown in Michigan (Rodriguez Salamanca, 2010).

Asparagus requires well-drained soils; fields that retain standing water for prolonged periods after rain events should be avoided (Sanders, 2001; Zandstra et al., 1992). Although asparagus can tolerate soil with a pH between 5.0 and 7.5 (Zandstra et al., 1992), the optimum

range for healthy growth is 6.2 to 6.8 (Sanders, 2001). Soil pH below 6.0 favors *Fusarium* infection (Zandstra et al., 1992).

Asparagus crowns are produced from seed that is planted in outdoor crown nurseries. To limit soilborne pathogens, the soil in the seedbed should be fumigated prior to planting (Saude et al., 2008). During preparation of the seedbed, fertilizer is applied such that approximately 224 kg each of P_2O_5 and K_2O is available per hectare. Once the seedlings are 15 to 20 cm high, approximately 56 kg N per hectare is broadcast along the rows. In Michigan, asparagus seeds are planted between mid April and mid May, and placed approximately 2.5 to 3.8 cm deep and 5 cm apart in rows that are spaced 61 to 76 cm apart. Approximately 5.6 kg of seed are used per hectare (Zandstra et al., 1992). After seedling emergence, weeds must be controlled by hand (Sandsted et al., 1985). Approximately one year after planting, crowns are dug using a potato digger in the early spring before bud break. The crowns are stored in loose piles or in bulk boxes at 1.7 to 7.2°C for up to 2 months until they can be planted in production fields (Zandstra et al., 1992).

Production fields are prepared the year prior to planting crowns by applying lime to achieve a pH of 6.8 (Sanders, 2001; Zandstra et al., 1992), using herbicides to kill weeds, and planting cover crops (i.e. sudangrass or clover). The cover crops are plowed into the soil in the fall, and winter wheat or rye is planted. In the spring, prior to planting the crowns, fertilizer may be applied so that approximately 280 kg per hectare each of P_2O_5 and K_2O is available in the soil. The fertilizer is typically spread over the cover crop and plowed down 30 cm, to ensure that the nutrients will be in the soil below the crowns. Up to 78 kg P_2O_5 per hectare is applied in the furrows at the time of planting (Zandstra et al., 1992). Furrows are 20.3 to 25.4 cm deep with middle-buster plows, with 1.2 to 1.5 m between rows. Crowns are spaced 30.5 cm apart and

covered with 2.5 to 5.0 cm of soil. During the first season of growth, soil is gradually cultivated into the furrow until the soil is level (Zandstra et al., 1992).

Harvesting of spears typically begins the third year after planting the crowns (Drost, 1997; Elmer, 2001b; Zandstra et al., 1992). In Michigan, harvesting typically occurs from early to mid May through mid to late June, depending on the weather and geographical location (Benson, 2012; Zandstra et al., 1992). Shoots grow rapidly when temperatures reach 27°C and can be harvested daily. Under cooler temperatures (< 21°C), the spear growth rate may be slow enough to harvest every 2 to 3 days (Zandstra et al., 1992). In Michigan, spears are hand harvested by snapping at the base once they have grown 18 to 22 cm in length (Elmer, 2001b; Zandstra et al., 1992). Commercial recommendations suggest harvesting for 2 weeks during the third year and then for 6 weeks during the fourth and subsequent years. Spear diameter decreases as crown carbohydrate supplies are depleted over the harvest period and it is recommended that harvesting conclude when the spears are < 0.95 cm in diameter (Zandstra et al., 1992). Overharvesting newly established fields can decrease marketable yield up to 15% in the following season (Shelton and Lacy, 1980).

When the harvest season ends, the shoots are allowed to grow and mature into fern. As the fern photosynthetic surface area increases, carbohydrate levels in the storage roots increase rapidly, peaking in mid to late August (Shelton and Lacy, 1980). The fern growth period is critical for replenishment of carbohydrate reserves and fern vigor and impacts the size and number of spears the following spring (Drost, 1997). As Michigan temperatures cool in mid to late September, the fern senesces and eventually becomes defoliated. The dead fern is left standing until the following spring, when it is mowed down with a rotary chopper (Zandstra et al., 1992).

Asparagus plantings are a long-term investment and can remain profitable for up to 20 years if properly maintained (Elmer et al., 1996; Zandstra et al., 1992; Zandstra et al., 2013). Therefore, it is important for growers to promote plant vigor and minimize factors that negatively affect stand health. Asparagus is considered to be a drought-tolerant crop, since over-irrigation causes reduced yield and provides favorable conditions for pathogens (Drost, 1997). However, adequate irrigation during dry years is needed to maintain yield and stand vigor (Drost, 1997). Up to 84.1 kg N per hectare should be reapplied every year and potassium levels should be replenished at least every 3 years according to soil test recommendations (Zandstra et al., 1992).

Weeds and volunteer asparagus seedlings compete for sunlight, nutrients, and water, and can reduce plant vigor and yield. Common perennial weeds in asparagus fields include quackgrass (*Elytrigia repens*), milkweed (*Asclepias syriaca*), bindweed (*Convolvulus arvensis*), horsenettle (*Conyza canadensis*), and Canada thistle (*Cirsium arvense*) (Zandstra et al., 1992; Zandstra et al., 2013). Michigan growers have adopted a no-tillage system (Zandstra et al., 1992) to prevent crown damage, which can reduce stand vigor and marketable yield (Wilcox-Lee and Drost, 1991) and lead to infection by soilborne pathogens. The no-till practice is effective against volunteer asparagus, since most seeds do not germinate on the soil surface. Herbicides are applied to the rows in the spring before spear emergence and in the summer after harvest (Zandstra et al., 1992). Caution must be used in choosing preemergence herbicides, since herbicide damage to asparagus stands can cause significant yield losses (Zandstra et al., 2013).

Insects can cause spear and fern damage, leading to yield loss and opening the asparagus stands to pathogen infection. Pests include cutworms (*Peridroma saucia* and *Euxoa messoria*), common and spotted asparagus beetles (*Crioceris asparagi* and *Crioceris duodecimpunctata*),

asparagus aphids (*Brachycorynella asparagi*), and asparagus miners (Zandstra et al., 1992). The asparagus miner, *Ophiomyia simplex*, causes severe damage to fern stems and is believed to transmit *Fusarium* spp. between plants (Morrison et al., 2011).

With proper maintenance, a healthy asparagus field will remain profitable for 12 to 15 years or more (Elmer et al., 1996; Sanders, 2001; Zandstra et al., 1992). However, asparagus early decline can shorten field lifespan by 5 to 10 years (Elmer et al., 1996). As plant vigor decreases gradually over time, the size and number of marketable spears and fern shoots are reduced (Keulder, 1999). The associated "replant problem" occurs when new stands fail to become successfully established in a field previously cropped with asparagus (Elmer et al., 1996; Falloon et al., 1991; Keulder, 1999; Morrison et al., 2011).

Asparagus decline can be exacerbated by many factors, including environmental stress, allelopathic residues, and damage from tillage, herbicides, and insects (Keulder, 1999; Morrison et al., 2011; Zandstra et al., 2013). However, several diseases are considered to be the primary contributors of premature decline and replant failure (Elmer et al., 1996). Soilborne pathogens, *Fusarium* spp. and *Phytophthora* spp., infect the roots and crowns, leading to direct loss of yield during the season of infection as well as long term decline in stand vigor. Michigan fields recently planted have productive lifespans approximately one-half as long as the lifespan of fields planted in the 1950s (Morrison et al., 2011). Although crown damage from soilborne diseases often appears to progress slowly, it is irreversible. In plots infested with *Phytophthora* spp. that had not been treated for 2 years, adding metalaxyl to the soil in third season did not increase yield (Falloon et al., 1985).

The foliar diseases rust (causal agent *Puccinia asparagi* De Candolle) and purple spot (causal agent *Stemphylium vesicarium* Wallr.) affect the main stem, secondary branches, and

cladophylls. Severe infections can result in premature defoliation, reducing the ability of the fern to replenish carbohydrate supplies in the crown. Smaller and fewer spears are produced the spring following severe foliar disease, and crown health is compromised. If severe disease pressure continues for more than one season, the losses increase each year (Hausbeck et al., 1999; Johnson and Lunden, 1992).

Three viruses: asparagus virus I (AVI), asparagus virus II (AVII), and tobacco mosaic virus (TMV) are known to infect asparagus in the U.S. AVII causes the most damage and is seedborne. AVI and TMV are transmitted by aphids and thrips, but are only harmful to stands when AVII is also present, and the effect is greater than AVII infection alone. Although the viruses themselves do not cause symptoms, viral infection reduces stand vigor and increases susceptibility to Fusarium crown and root rot (Elmer, 2001b; Elmer et al., 1996).

FUSARIUM CROWN AND ROOT ROT

The genus *Fusarium* belongs to the order Hypocreales and includes saprophytic and parasitic members that have adapted to a wide range of hosts and environmental conditions. Most pathogenic species are strictly soilborne, although others have modes of air dispersal and can colonize aerial tissues of hosts. Crescent-shaped macroconidia are produced on sporodochia and microconidia are produced in chains or clusters on phialides. Some species also produce chlamydospores (Agrios, 2005; Burgess, 1981). *Fusarium* species are distinguished through morphological, biological, and phylogenetic characteristics (Leslie et al., 2001). Morphological species concepts consider shape and size of the macroconidia, microconidia, and chlamydospores. Physiological characters such as growth rates, mycotoxins, and secondary metabolites are useful for morphological taxonomy. The biological species concept is based on

reproductive isolation, although problems can arise with mating type allele frequencies, homothallic species, asexual reproduction, or outcrossing. Amplified fragment length polymorphism (AFLP) markers aid in identifying isolates belonging to the same biological species. Phylogenetic species concepts utilize DNA sequences such as ribosomal internally transcribed spacer (ITS) sequences. The "coalescent approach" combines the phylogenetic and biological species concepts by using molecular markers and reproductive barriers to confirm conspecific isolates (Leslie et al., 2001).

Fusarium crown and root rot symptoms on asparagus begin as reddish brown discoloration in the rhizome, shoot, and storage root vascular tissues (Zandstra et al., 1992). As the disease progresses, the feeder roots completely rot away, the storage roots become hollow, and the fern turns yellow and senesces (Elmer et al., 1996; Zandstra et al., 1992).

Several *Fusarium* spp., including *F. redolens* Wollenw., *F. solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans., and *F. culmorum* (W. G. Smith) Sacc. (Schreuder et al., 1995; Wong and Jeffries, 2006) have been reported to infect asparagus in various growing regions in the world. However, *F. oxysporum* Schlectend.:Fr. f. sp. *asparagi* Cohen & Heald and *F. proliferatum* (Matushima) Niremburg are the primary causal agents of Fusarium crown and root rot, which is considered to be the most economically limiting disease to asparagus crops worldwide (Elmer, 2001a). Since it was first documented in 1908 (Stone and Chapman, 1908), Fusarium crown and root rot has been reported in North, Central, and South America, Australia, Japan, New Zealand, Taiwan (Elmer, 2001b), South Africa (Schreuder et al., 1995), the Netherlands (Van Bakel and Kerstens, 1970), Spain (Wong and Jeffries, 2006), and the United Kingdom (Wong and Jeffries, 2006).

Fusarium oxysporum produces abundant chlamydospores but can also survive as hyphae on plant residues in and on the soil. There is no known teleomorph of *F. oxysporum* (Burgess, 1981). Forty-three vegetative compatibility groups (VCGs) have been reported in *F. oxysporum* f. sp. *asparagi* (Elmer et al., 1996) and it is believed that each VCG is a clonal lineage (Correll, 1991). Populations of the *F. oxysporum* species complex are also separated into formae speciales based on host pathogenicity. Historically, VCGs were not expected to cross over formae speciales (Correll, 1991; Puhalla, 1985). The pathogenicity of *F. oxysporum* on asparagus does not appear to correspond with vegetative compatibility, which indicates that pathogenicity on asparagus may be a common trait in *F. oxysporum* (Elmer, 2001a; Elmer et al., 1996). Although pathogenic *forme* in the *F. oxysporum* complex are typically limited to one host genotype, isolates of *F. oxysporum* pathogenic to *A. officinalis* have also been reported to infect the roots of other *Asparagus* spp. as well as corn, pea, celery, gladiolus, lupine, and onion (Dan and Stephens, 1995; Damicone et al., 1988; Elmer, 2001a; Stephens and Elmer, 1988). *F. oxysporum* f. sp. *asparagi* usually infects young plants in nursery fields or newly established production fields (Elmer et al., 1996; Keulder, 1999).

F. proliferatum (Teleomorph *G. fujikuroi* var. *intermedia* Kuhlman) is mating population "D" of the *Gibberella fujikuroi* complex (Elmer, 1995; Samuels et al., 2001), although perithecia have not been found in nature (Elmer, 2001a). The anamorph has not been found to produce chlamydospores and it overwinters as mycelia on plant debris. *F. proliferatum* can colonize asparagus stems, flowers, and berries as well as crowns (Burgess, 1981; Elmer, 2001a). It is commonly found on mature and senescing plants (Keulder, 1999).

Fusarium spp., both nonpathogenic and pathogenic to asparagus, are ubiquitous in fields in the major asparagus producing regions (Elmer, 2001a; Hartung et al., 1990). In a survey of

fields in major asparagus producing counties in Michigan, Hartung et al. (1990) found that 38% of *F. oxysporum* isolates and all isolates of *F. proliferatum* sampled from both asparagus fields and fields without any asparagus history were pathogenic. Both species are soilborne, seedborne, transported on infested crowns, and vectored by insects (Elmer, 2001a; Elmer et al., 1996; Morrison et al., 2011). *F. proliferatum* is also windborne (Elmer, 2001a). Plant stress (Keulder, 1999; Zandstra et al., 1992), allelopathic plant residues (Hartung and Stephens, 1983; Keulder, 1999), viral infection (Elmer, 2001b), and damage from tillage (Shelton and Lacy, 1980), insects (Morrison et al., 2011), and herbicides (Zandstra et al., 2013) increase crop susceptibility to *Fusarium* infection (Elmer, 2001a).

PHYTOPHTHORA SPEAR AND ROOT ROT

Members of the family Pythiaceae, including *Phytophthora* spp., are characterized by coencytic mycelia with few to no septae (Agrios, 2005; Erwin and Ribeiro, 1996). When conditions are favorable, sporangia (also called zoosporangia) form on sporangiophores or on chains of internally proliferating sporangia. Obpyriform sporangia may or may not have an apical bud called a papilla. Biflagellate, haploid zoospores are produced inside sporangia, and then released. Asexual chlamydospores are spherical to oval, and form a protective cell wall that enables survival in harsh conditions. Diploid oospores are formed when antheridia fuse with and fertilize oogonia. Oospores have a thick cell wall, and can survive in soil for many years. Heterothallic species are able to reproduce only when two mating types are present, resulting in genetic recombination. Homothallic species can self-fertilize and have the potential to produce large clonal populations (Erwin and Ribeiro, 1996). Morphological characteristics such as colony growth and spore production on selective medium and at maximum temperatures are used

to identify *Phytophthora* species. DNA fingerprinting techniques including single-strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), and AFLP analysis of ITS and mitochondrial DNA can distinguish among species which are morphologically similar (Erwin and Ribeiro, 1996; Gallegly and Hong, 2008).

The *P. megasperma* complex was first described by Dreschler (1931) on *Althaea rosea*. The sporangia are internally proliferating and nonpapillate. Paragynous antheridia and oogonia produce oospores in abundance in homothallic cultures. Tompkins et al. (1936) and Hildebrand (1959) expanded the criteria for oospore size ($\geq$ 30 μm diameter) and host range to include similar pathogens in what has been called the broadened species concept. In some cases, species within the "*Phytophthora megasperma* complex" have been identified as a separate species based on morphology or host specificity (Kuan and Erwin, 1980; Waterhouse, 1963). Mitochondrial and chromosomal DNA RFLPs have defined nine distinct groups in the *P. megasperma* complex, five of which are host-specific and the other four are broad host range. Group E belongs to *Phytophthora* spp. that infects *A. officinalis*. The subgroups of E are each from separate geographical locations, and suggest European origin for this genetic lineage (Förster and Coffey, 1993).

Several species including *P. megasperma* Drechsler, *P. megasperma* var. *sojae* Hildebrand, *P. cryptogea* Penthybridge & Lafferty, *P. cactorum* (Leb. & Cohn) Schröeter, and *P. richardiae* Buisman infect asparagus crops worldwide (Falloon, 1990). *P. megasperma* var. *sojae* is the most common cause of Phytophthora rot in New Zealand (Falloon, 1982; Falloon and Grogan, 1991), but *P. cryptogea* is also present in California (Falloon and Grogan, 1991) and the Netherlands (Falloon, 1990). Asparagus root rot has also been confirmed as a result of infection from *P. megasperma* var. *sojae* in France (Baudry, 1995), *P. nicotianae* in Peru

(Aragon-Caballero et al., 2008), and *P. megasperma* in Canada (Vujanovic et al., 2003). *Phytophthora asparagi* Saude & Hausbeck is the only known species to infect Michigan asparagus crops (Saude et al., 2008). Results from the 99 isolates from Michigan tested with AFLP fingerprinting indicated that all were from the same clonal lineage and could be due to the homothallic sexual phase that is self-fertilizing (Saude et al., 2008).

On asparagus spears, *Phytophthora* infection often appears as soft, water-soaked, slightly sunken lesions slightly above or below the soil line. Sometimes the lesions may also appear to be brown or necrotic (Aragon-Caballero et al., 2008; Falloon et al., 1983). Infected spears may "crook" or curl over as they wilt. Infected roots first show white or slightly transparent lesions, then as the disease progresses lesions turn brown to reddish brown with hollow tissue (Falloon et al., 1983). Water-soaked lesions and shriveling can occur on crowns in cold storage, but the tissue can remain firm. Internal crown tissues sometimes turn yellow-brown, similar to Fusarium wilt symptoms. Saude et al. (2008) observed that in drier conditions, lesions can become necrotic on storage roots, and spears may show crooking and wilting but the tissue may not appear to be water-soaked.

It has commonly been noted that *Phytophthora* rot on asparagus is favored during conditions including cool temperatures and saturated soils (Baudry, 1995; Falloon and Grogan, 1991; Saude et al., 2008). Greenhouse trials confirmed that increasing the duration and frequency of flooding along with cooling temperatures increased disease severity (Falloon and Grogan, 1991). In newly established fields, increased flooding frequency (1X, 3X, and 4X) increased spear emergence time and decreased survival by 20% to 43% during the first year (Falloon, 1991). In established fields, wet and cool weather early in the harvest season increased

yield losses (Falloon et al., 1985). Overhead irrigation can also encourage disease (Falloon and Fraser, 1991).

On AR - (150 ppm ampicillin and 30 ppm rifampicin) amended V8 agar (840 ml distilled water, approximately 163 ml unclarified V8 juice, 16 agar, 3 g $CaCO_3$) agar plates, *P. asparagi* grows outward in a stellate rosaceous pattern. High oospore counts and low numbers of sporangia are produced. On dilute V8 (1000 ml distilled water, approximately 40 ml unclarified V8 juice, 16 agar, 3 g $CaCO_3$), many sporangia are produced, and oospores are still present. Sporangia are ovoid, obpyriform, nonpapillate, noncaducous, 42 to 47μm long x 23 to 40 μm wide, produced on simply- or sparingly-branched sporangiophores. Internal proliferation may occur after releasing zoospores. Oospores are amphigynous and 25 to 30 μm in diameter. Chlamydospores have not been observed in this species. The minimum, optimum, and maximum temperatures for growth on AR-V8 agar and spore production were found to be 10, 25, and > 30°C, respectively (Saude et al., 2008). When fresh 'Millennium' spears were inoculated with *P. asparagi* isolates, larger lesions developed at 20°C than at 15°C and 25°C (Rodriguez Salamanca, 2010).

INTEGRATED PEST MANAGEMENT

Effective management of soilborne diseases requires an integrated approach. Because many factors affect plant susceptibility and pathogen infection, direct as well as indirect strategies are needed to minimize crown damage. Cultivar selection, cultural practices, soil fumigation, fungicides, and biocontrol agents may be used to limit *Fusarium* and *Phytophthora* rots. Managing foliar pathogens, viruses, insects, and weeds and selecting safe herbicides are also important to maintain crop health and reduce susceptibility to Fusarium crown and root rot.

Currently, all *A. officinalis* cultivars are susceptible to *F. oxysporum* f. sp. *asparagi*, *F. proliferatum*, and *P. asparagi*. All-male hybrids have higher plant vigor than open-pollinated cultivars. Because good vigor reduces plant susceptibility to *Fusarium* pathogens, all-male hybrids offer some protection against disease (Ellison and Kinelski, 1985). In vitro screens have identified tolerance to *Fusarium* spp. in the ornamentals *A. densiflorus* 'Sprengeri' and 'Myersii' (Dan and Stephens, 1995; Stephens and Elmer, 1988; Stephens et al., 1989). Sexual incompatibility between *A. officinalis* and *A. densiflorus* has prevented hybrids from being developed (Stephens et al., 1989). Using recurrent selection, Falloon et al. (2002) have identified experimental hybrids with significantly higher survival than susceptible 'UC 157' against *P. megasperma* var. *sojae* and *P. cryptogea* isolates from New Zealand, France, Switzerland, and California. Seven hybrids also resulted in higher yield and quality equal to or better than industry standards 'UC 157' and 'JWC1' (Falloon et al., 2002). As resistant cultivars are identified and developed, cultivar selection will become a useful management tool for growers.

Using cultural practices to avoid and prevent infection is recommended for *Fusarium* and *Phytophthora* management. Fields with a history of Fusarium crown and root rot or Phytophthora spear and root rot should be avoided if possible. Saturated soils create conditions that are unfavorable for asparagus crown growth and increase plant susceptibility while creating a favorable environment for pathogen development (Saude et al., 2008). Sand and sandy loam soils can provide good drainage and irrigation should be applied as needed to prevent water-logging and water-stress (Drost, 1997; Elmer, 2001a). Lime and fertilizer applications to maintain soil pH above 6.0 and nutrient levels are important to maintain stand health and prevent *Fusarium* infection (Zandstra et al., 1992). Falloon and Fraser (1991) suggested transplanting

crowns in late spring to avoid cool, wet conditions that are favorable to Phytophthora spear and root rot. Using greenhouse-grown transplants has been recommended to avoid crown damage at planting, but this practice has not been adopted by the Michigan industry because it represents a significant change from current practices (M. Ngouajio, personal communication, 2014). Overharvesting depletes the crown of nutrients and reduces stand vigor, and should therefore be avoided (Shelton and Lacy, 1980). Michigan growers have adopted the no-till system, which prevents crown damage and openings to infection (Wilcox-Lee and Drost, 1991; Zandstra et al., 1992).

Crop rotation is a recommended method of reducing pathogen populations, but growers should be careful in choosing a rotation crop. Although *P. asparagi* is the only known species to cause asparagus spear and root rot in Michigan, Rodriguez Salamanca (2010) found that at 20 and 25°C *P. nicotianae* and *P. capsici* caused lesions on small 'Jersey Knight' spears comparable with *P. asparagi* when incubated at 15 and 20°C. Although the asparagus harvest season would normally end before soil temperatures reach 20 to 25°C, unusually warm springs with heavy rainfall could create favorable conditions for infection by *P. nicotianae* and *P. capsici.* Saude et al. (2008) tested the ability of *P. asparagi* to infect crops that are economically important to Michigan. *P. asparagi* did not cause infection on soybean, alfalfa, or clover seedlings, but was reisolated from roots of all seedlings. *P. asparagi* caused infection of cucurbit fruits when the flesh tissue was wounded. Symptoms appeared as water-soaked lesions on zucchini, water-soaked and necrotic lesions on yellow squash, and water-soaked lesions with mycelial growth on slicing and pickling cucumbers (Saude et al., 2008).

Historically, sodium chloride was used on small parcels within the U.S. to manage weeds and increase yields. When growers began to use herbicides, the number of reports of Fusarium

crown and root rot increased, so it was believed that sodium chloride could be used to control the disease (Elmer, 2001a). Sodium chloride can improve yields in fields with severe Fusarium crown and root rot symptoms, but studies in greenhouses and fields with less severe symptoms have not resulted in consistent disease control (Elmer, 2004; Reid et al., 2001). Alternative forms of chloride salt (calcium, manganese, and ammonium) were found to either have no effect or to increase root rot symptoms (Reid et al., 2001). The mechanism of control and the environmental impact of sodium chloride are unknown and the effect on environment as well as future crops is not well understood (Elmer, 2001b; Elmer et al., 1996; Keulder, 1999).

During cool, wet periods, fungicides may be helpful to control Phytophthora spear and root rot. When the fungicide metalaxyl was applied to production fields, marketable spear yield increased by 22% to 43% (Falloon et al., 1983). In a newly established field, when the same soil treatments were applied immediately after transplant they decreased the emergence time and increased the stand survival in flooded fields, although the fungicide efficacy was decreased with increased flooding frequency (Falloon, 1991). A 4-year-old field treated with 1.12 kg/ha applications showed decreased rot and increased yield (38% and 118%) as well as increased large spear counts (Falloon et al., 1985). Falloon and Fraser (1991) noted that mefenoxam applied as 300 mg/liter crown soak was more effective than soil applications. The fungicide soak allowed direct uptake, but soil applications can only reach the crowns after the water moves the fungicides through the soil to the roots and crown. This can leave crowns vulnerable to infection. However, crown soaks applied at rates higher than 150 ppm or 300 mg active ingredient/liter have been phytotoxic to asparagus plants (Falloon and Fraser, 1991; Green et al., 2008). Mefenoxam is registered for use on asparagus and can be applied before the harvest

begins. Saude et al. (2008) confirmed that 131 isolates of *P. asparagi* isolates from Michigan were sensitive to 100 ppm of mefenoxam.

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CHAPTER I

VIRULENCE OF *PHYTOPHTHORA ASPARAGI*, SUSCEPTIBILITY OF ASPARAGUS CULTIGENS TO SPEAR AND ROOT ROT, AND THE EFFICACY OF SEED TREATMENTS

ABSTRACT

Phytophthora asparagi, causal agent of Phytophthora spear and root rot of asparagus, is an important contributor to crop decline and replant problem in Michigan. Management strategies are limited, and identifying resistant cultigens and seed treatments will provide valuable strategies for the asparagus industry. Nine isolates of *P. asparagi* were tested for virulence using asparagus spears of five commercial cultivars and asparagus seedlings of three cultivars. On asparagus spears, differences in isolate virulence and host susceptibility were observed with 'Jersey Knight' being the least susceptible and 'Jersey Supreme' the most susceptible. No significant differences were detected among cultivars or isolates in the seedling root rot experiment. Two virulent isolates were selected to inoculate asparagus seedlings of 18 cultigens. Isolate SP317 was significantly more virulent than SP316, and 'Jersey Giant' and 'UG009' were the least susceptible cultigens. The interaction between isolate virulence and cultigen susceptibility significantly affected root rot severity. 'Pacific 2000' and 'Jersey Giant' were the least susceptible cultigens for isolates SP316 and SP317, respectively. Seven seed treatments, including experimental fungicides and biocontrol agents, were screened for efficacy against two isolates of *P. asparagi* on 'Millennium' in vitro. Seeds treated with mefenoxam and *Bacillus subtilis* had the highest germination overall and on plates inoculated with SP326. When seeds were inoculated with SP316, mefenoxam, DPX-QGU42, and *B. subtilis* treatments germinated at the highest rates. Antagonism of mycelial growth was observed in the *B. subtilis* treatment, which resulted in significantly decreased disease incidence compared to all other treatments. This is the first reported evaluation of cultigen susceptibility to *P. asparagi*-induced rot in asparagus seedling roots. These results are consistent with previous research suggesting

that virulence of isolates of *Phytophthora* spp. is a combination of host and pathogen genetics and that some asparagus cultigens are less susceptible than others to *P. asparagi*.

INTRODUCTION

Michigan ranks third in the United States for asparagus production, following California and Washington. In 2012, Michigan produced 8.8 million kg of spears for the fresh market and processing, with a total value of $17.3 million, (Anonymous, 2013). With proper maintenance, a healthy asparagus field will remain profitable for 12 to15 years or more (Elmer et al., 1996; Sanders, 2001; Zandstra et al., 1992). However, asparagus early decline can shorten a field's lifespan by 5 to 10 years (Elmer et al, 1996). As plant vigor decreases gradually over time, the size and number of marketable spears and fern shoots are reduced (Keulder, 1999). The replant problem occurs when new stands fail to become successfully established in a field previously cropped with asparagus (Elmer et al., 1996; Falloon and Fraser, 1991; Morrison et al., 2011; Keulder, 1999). Historically, Fusarium crown and root rot was considered to be the primary soil-borne disease affecting crop decline in Michigan, but recently Phytophthora spear and root rot (caused by *P. asparagi* Saude & Hausbeck) has been reported as an important contributor as well (Saude et al., 2008).

Phytophthora root rot infections begin underground, causing water-soaked lesions, shriveling, and red-brown discoloration in crown and root tissue. As the disease progresses, lesions spread longitudinally down the roots, which may become hollow. On crowns, affected buds will fail to produce spears, and spears that grow from weakened crowns are usually smaller than those from healthy crowns. Affected fields appear to have dead patches, especially in low areas where water accumulates. Spear rot occurs when soil infested with zoospores splashes

onto shoots near the soil level. Symptoms appear as soft, water-soaked, slightly sunken lesions slightly above or below soil line. Sometimes the lesions may also show necrosis (Falloon et al., 1983; Aragon-Caballero et al., 2008).

P. asparagi is a homothallic, or self-fertilizing, species. DNA fingerprinting tests indicated that isolates collected from asparagus fields in three Michigan counties were from one clonal lineage, which suggests low genetic variability in Michigan populations (Saude et al., 2008). Other *Phytophthora* spp., such as *P. capsici*, have significant genetic diversity and varying levels of virulence within populations. Levels of disease severity depend on the interaction between isolate and host genotypes (Glosier et al., 2008; Granke et al., 2012; Kim and Hwang, 1992). Saude et al. (2008) found no differences in virulence among 115 isolates on spears of 'Jersey Giant', an all-male hybrid with little genetic variability. Testing multiple isolates across several cultigens will increase differences between host genotypes, and may increase differences in disease severity between the isolates.

Cultural practices to manage the disease are limited. Falloon and Fraser (1991) suggested transplanting crowns in late spring to avoid cool/wet conditions. Crop rotation may be useful, but growers are advised to be careful in choosing a rotation crop. Cultivar selection is a cultural practice often used in vegetable crops, but host resistance has not been identified against *P. asparagi*. Using recurrent selection, Falloon et al. (2002) identified experimental hybrids with significantly higher survival than the susceptible 'UC 157' against *Phytophthora* spp. from New Zealand, France, Switzerland, and California. Identifying cultivars with resistance or tolerance to *P. asparagi* would be useful for the Michigan asparagus industry.

Traditionally, Michigan growers have not fumigated the soil of their crown nurseries, though pathogens may be present. Saude et al. (2008) sampled crowns stored in bulk boxes from

a commercial producer and found that 40% of the samples were infected with *P. asparagi*. To prevent *P. asparagi* inoculum from reaching production fields, growers should use only certified clean material raised in nursery beds that are fumigated. However, fumigation can create a biological vacuum, allowing new pathogens to colonize the soil (Keulder, 1999). There are currently no fungicides labeled to protect young seedlings in nursery beds. Young plants are especially susceptible to Phytophthora root rot (Falloon and Grogan, 1991) and effective seedling treatments would be a valuable management tool.

The objectives of this study were to: *i*) Test the virulence of nine *P. asparagi* isolates on asparagus spears and seedling roots of commercial cultivars, *ii*) Compare the relative susceptibility of asparagus cultivars and breeding lines to Phytophthora spear and root rot, and *iii*) Screen fungicides and biocontrol agents for efficacy against *P. asparagi*.

MATERIALS AND METHODS

Isolates and inoculum preparation. Michigan *P. asparagi* isolates C013, SP316, SP317, SP318, SP319, SP324, SP325, SP326, and SP3236 from the collection of M. K. Hausbeck were maintained on AR - (150 ppm ampicillin and 30 ppm rifampicin) amended V8 agar (840 ml distilled water, approximately 163 ml unclarified V8 juice, 16 agar, 3 g $CaCO_3$) at room temperature (23 ± 2°C) under continuous fluorescent lighting and transferred every two weeks. Broth cultures were initiated by adding agar plugs from the mycelial margin of actively growing cultures (4 to 9 days) to AR-amended V8 broth (840 ml distilled water, approximately 163 ml clarified V8 juice, 3 g $CaCO_3$). For the spear rot experiments, a 7-mm mycelial plug was added to 4 ml AR-V8 broth and for seedling root rot experiments a 4-mm mycelial plug was

added to 2 ml AR-V8 broth. Broth cultures were incubated under temperature and light conditions as described above for 4 to 7 days before inoculating plant material.

Susceptibility of asparagus cultivars to spear rot. Commercial grade spears (1.5 to 2.0 cm in diameter and ≥ 14 cm tall) of 'Millennium,' 'Tiessen,' 'Jersey Giant,' 'Jersey Knight,' and 'Jersey Supreme' were harvested from fields in Oceana County, MI. In the first and third trials, the spears were kept overnight in a cold room maintained at approximately 4.4°C. In the second trial, the spears were inoculated within 12 hours of harvest.

Autoclaved Benona sandy loam from Oceana County was infested with one of the following *P. asparagi* isolates; C013, SP316, SP317, SP318, SP319, SP324, SP325, SP326, or SP3236 and used to inoculate spears. An AR-V8 broth culture of each *P. asparagi* isolate was prepared as described above. After incubation, 4 ml of the broth culture of each isolate was added to 10 g of autoclaved sandy loam that was placed in test tubes (25 x 150 mm). Sterile AR-V8 broth was used for the uninoculated control treatments.

Asparagus spears were surface disinfested using the method of Saude et al. (2008) that included washing in 5% sodium hypochlorite for 5 seconds, rinsing 3 times in distilled water for 5 seconds each. Spears were air dried on clean paper towels in a laminar flow hood, then trimmed to 12 cm, with the apical tips and below ground tissue removed. Spears were immediately placed into the test tubes with the base in direct contact with the infested soil. Five spears of each cultivar were used for each isolate and for the uninoculated controls. Tubes were arranged in a complete randomized design in test tube racks and incubated for 7 days at room temperature under continuous fluorescent lighting. After incubation, the length and width of each lesion was measured. Because lesions were not uniform in height but were similar to a rectangle shape, area was calculated as A = W*H, where W was width at the spear base edge and

H was the average of 3 separate length measurements. Experiments were conducted three times with 'Millennium,' 'Jersey Giant,' 'Jersey Supeme,' and 'Jersey Knight' spears, and twice with 'Tiessen' spears.

Seedling inoculation and incubation. Asparagus seeds were surface disinfested using the method of Damicone et al. (1981) by agitating in a solution of 25,000 ppm benomyl in 99.9% acetone on a rotary shaker (1000 rpm) for 24 hours, then rinsing 3 times in acetone and 3 times in distilled water. Seeds were then agitated in 1.23% sodium hypochlorite at 1000 rpm for 1 hour and rinsed 3 times in distilled water. After being allowed to air dry for 1 to 2 hours in a laminar flow hood, the seeds were placed on water agar (16 g agar and 1000 ml distilled water) and germinated in the dark at room temperature for 7 to 14 days. Once the hypocotyls emerged, the seeds were planted in 12 ml Hoagland's agar (Stephens and Elmer, 1988) in test tubes (25 x 150 mm) and grown in growth chambers (16-hour photoperiod at 25/20°C day/night).

An AR-V8 broth culture (2 ml) of each *P. asparagi* isolate (see specific isolates in experiments below) was prepared as described previously. When seedlings were approximately 11 cm in height and secondary branches with cladophylls had formed (approximately 2 weeks), the broth culture was placed directly on the agar of each test tube. Following inoculation, seedlings were allowed to grow and the infection to develop for 4 weeks under growth chamber conditions described above. Severity of Phytophthora root rot was visually assessed weekly, starting at 7 days post inoculation (dpi). A disease severity scale developed by Falloon (2002) of 0 to 4 was used (0 = no disease; 4 = water-soaked tissue, rot of > 50% of the roots) (Table 1.1, Figure 1.1). A disease severity index (DSI) where 0 = no disease and 100 = all seedlings with > 50% of their roots water-soaked was calculated for each isolate/cultigen combination (Falloon et al, 2002). For the fourth and final rating, seedling roots were removed from the agar prior to the

Table 1.1. Disease classes of inoculated asparagus seedlings used to calculate disease severity index (DSI*).

Disease class	Symptoms
0	No disease
1	<10%**
2	10-20%
3	20-50%
4	>50%

*DSI=Σ (class x number plants/class) x 100/total plants x 4.
**Indicates area of root system water-soaked.

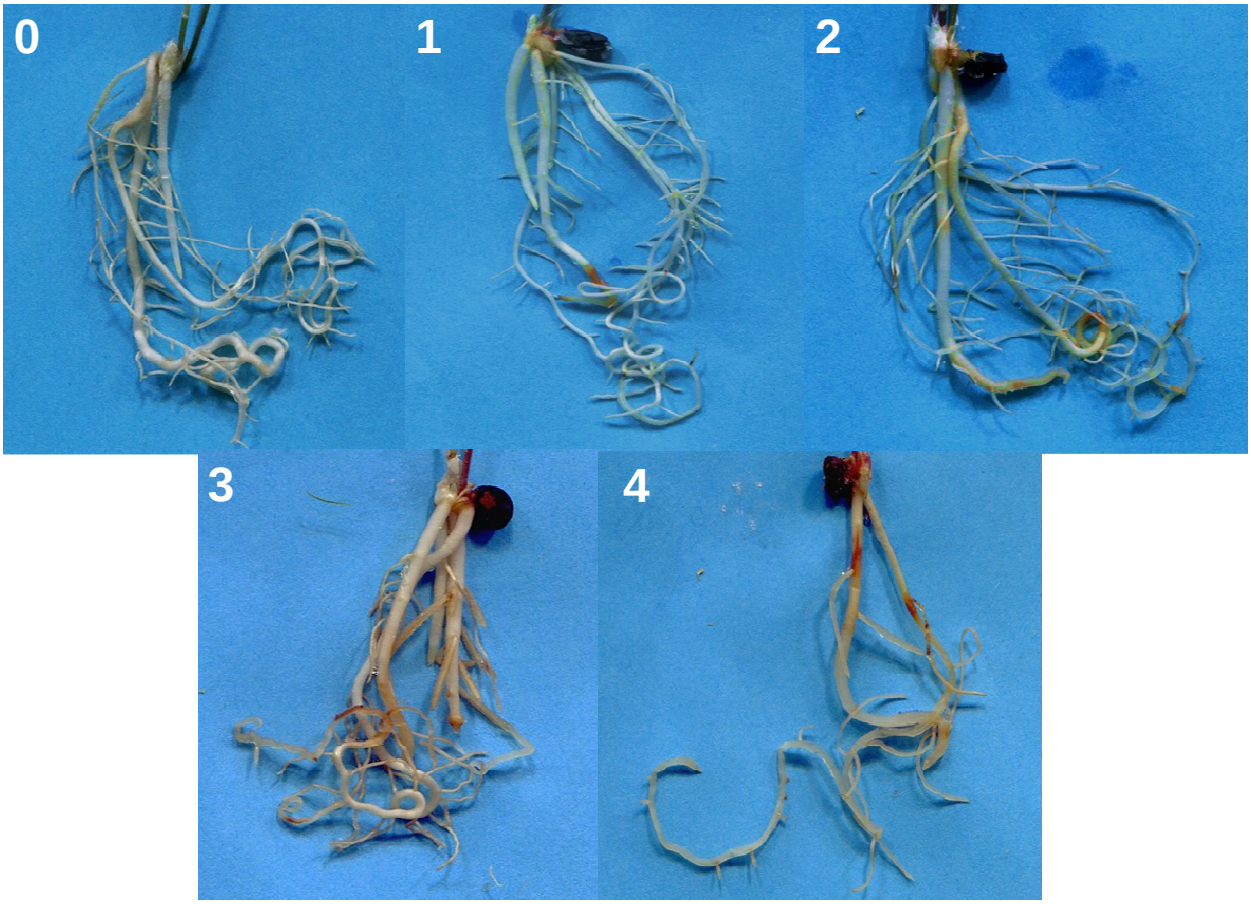

Figure 1.1. The *Phytophthora* symptoms observed on inoculated asparagus seedling root systems receiving the rating of a 0 to a 4.

visual assessment. The area under the disease progress curve (AUDPC) was calculated (Madden et al., 2007) to obtain the cumulative DSI throughout the experiments.

Virulence of isolates on seedlings roots. The virulence of nine *P. asparagi* isolates (C013, SP316, SP317, SP318, SP319, SP324, SP325, SP326, and SP3236) were compared on the seedling root systems of two-week-old 'Millennium,' 'Mary Washington.' and 'Jersey Giant' seedlings. Sterile AR-V8 broth (2 ml) was used for the negative controls. Five seedlings were used for each cultivar/isolate combination and the controls in the first trial and 6 seedlings were used in the second trial. Tubes were arranged in a complete randomized design in test tube racks and incubated in growth chamber conditions previously described. Experiments were conducted twice.

Susceptibility of cultivars and breeding lines to seedling root rot. Asparagus seedlings of 18 cultivars and breeding lines (Table 1.2) were selected to evaluate differences in susceptibility to *P. asparagi* isolates SP316 and SP317. Sterile AR-V8 (2 ml) was used as the negative control. Six seedlings were used for each cultigen/isolate combination and the controls. Seedlings were arranged in a complete randomized design in test tube racks and incubated in the growth chamber conditions as described above.

Efficacy of fungicides and biocontrol agents as seed treatments. The methods used by Broders et al. (2007) were adapted to screen five fungicides and two biocontrol agents (Table 1.3) for efficacy as seed treatments in vitro. A mycelial plug (7-mm in diameter) of SP316 or SP326 was placed in the center of 15-cm-diameter petri plate containing 50 ml of V8 agar. The isolates were allowed to colonize the plates for 3 days. Seeds of 'Millennium' were surface disinfested as previously described and allowed to air dry in a laminar flow hood for approximately 1 hour. Treatments were applied to seed lots (0.33 g) at the rates listed in

Table 1.2. Asparagus cultivars and breeding lines tested for susceptibility to *Phytophthora asparagi*.

Cultigen	Source
Millennium	University of Guelph
UG5	University of Guelph
UG9	University of Guelph
UG10	University of Guelph
UG20	University of Guelph
Jersey Giant	Walker Brothers
NJ 938	Rutgers University
NJ 941	Rutgers University
NJ 1191	Rutgers University
Jersey Supreme*	Vilmorin
Mondeo*	Vilmorin
Pacific Challenger 1	Aspara Pacific
Pacific Challenger 2	Aspara Pacific
Pacific 2000	Aspara Pacific
74 x 22	Aspara Pacific
3 x Phy 99	Aspara Pacific
Cumulus (2735)	Bejo
Bacchus (2827)*	Bejo

*Packaged seeds were pretreated with thiram.

Table 1.3. Fungicides and biocontrol agents tested for efficacy against *Phytophthora asparagi*.

Treatment	Active ingredient	Rate/100 lb seed	Rate/20 seeds (0.48 g)
Untreated	sterile deionized water	--	600.0 µl
Apron	mefenoxam	14.2 ml	0.15 µl
Presidio	fluopicolide	29.6 ml	0.31 µl
Micora	mandipropamid	237.0 ml	2.51 µl
V-10208	experimental	17.7 ml	0.19 µl
DPX-QGU42	experimental	59.1 ml	0.63 µl
Actinovate	*Streptomyces lydicus*[*]	340.2 g	3.60 mg
Serenade Soil	*Bacillus subtilis*[*]	813.2 ml	8.61 µl

[*] Biological control agent

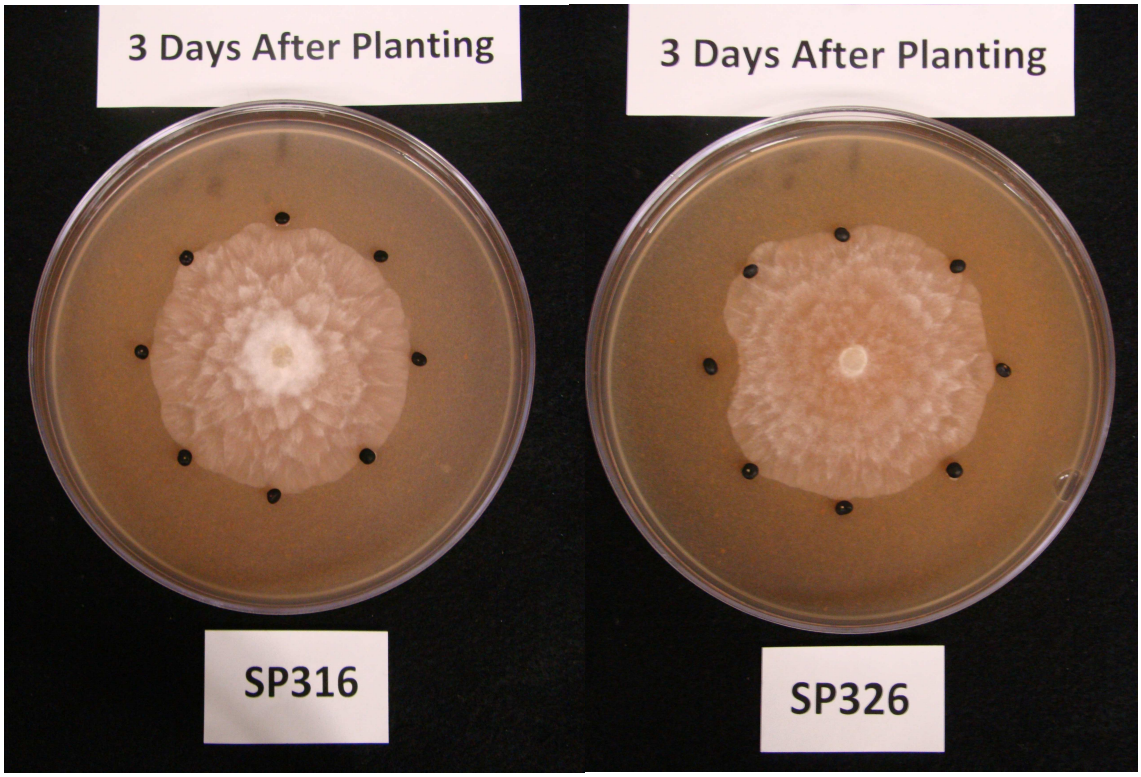

Figure 1.2. Asparagus seeds treated with fungicides and biocontrol agents on V8 agar plates inoculated with *Phytophthora asparagi* isolates SP316 (**A**) and SP326 (**B**), 3 days after planting.

Table 1.3. Seeds were soaked in each treatment for approximately 30 minutes, then air-dried in a laminar flow hood for approximately 1 hour. Seeds from each treatment were equally spaced in a randomized order around the plate, approximately 3 cm from the edge (Figure 1.2). Seeds from each treatment were also plated on sterile V8 agar plates as the uninoculated controls.

At 10 days after plating the seeds, inhibition of mycelial growth was measured as the distance between the seed and margin of mycelial growth along the radius of the agar plate. Seeds were removed from culture and germinated seeds were rated for disease severity. The number of seeds in each treatment that germinated on infested plates was compared with the number that germinated on the uninoculated plates. Seeds were considered successfully germinated if the hypocotyl had emerged or the radicle was > 5 mm long. Treatments were

scored according to a 0 to 3 rating scale (0 = no disease symptoms; 3 > 50% tissue water-soaked, mycelial growth present) (Table 1.4, Figure 1.3). Each treatment was replicated five times for each isolate and the uninoculated control, and the experiment was conducted three times.

Pathogen isolation. At the end of each study, plant tissue was surface disinfested in 0.525% sodium hypochlorite for approximately 60 seconds, rinsed in sterile distilled water, and air dried in a laminar flow hood for 1 to 3 minutes. Tissue sections were placed into AR-V8 agar. Plates were incubated for 3 to 7 days at room temperature (23-25°C) under continuous fluorescent lighting to confirm the presence of the pathogen. For the spear rot experiment, approximately 70% of the inoculated spears were sampled. Control spears with water-soaked areas were also sampled to confirm the absence of pathogens. The epidermal layer was peeled away and four small sections (approximately 5 x 5 mm) from the lesion margins were excised with a flame-sterilized scalpel and disinfested and plated in the method described above. For the seedling root rot experiments, approximately 44% and 56% of the inoculated plants were sampled in the isolate virulence and variety screening trials. Control plants with discolored and wilted roots were also sampled. Four small sections from lesion margins of root and crown tissue were removed, disinfested, and plated. For the seed treatment experiment, whole seeds were disinfested in the manner described above. After air-drying, radicle tissue was wounded using a flame-sterilized scalpel and plated. All inoculated seeds were sampled, and control seeds were also sampled if radicles had water-soaked or discolored tissue.

Statistical analysis. For all experiments, data was analyzed with a two-way analysis of variance (ANOVA) using the PROC MIXED procedure with the REPEATED statement of SAS version 9.3 (SAS Institute Inc., Cary, NC). Residuals were tested for normality and homogeneity of variance using PROC UNIVARIATE. Trial repetition was treated as a random affect

Table 1.4. Scale of disease ratings on successfully germinated asparagus seedlings inoculated with *Phytophthora asparagi*.

Rating	Symptoms
0	No disease
1	<50% radicle tissue water-soaked
2	>50% radicle tissue water-soaked
3	>50% radicle tissue water-soaked + mycelial growth present

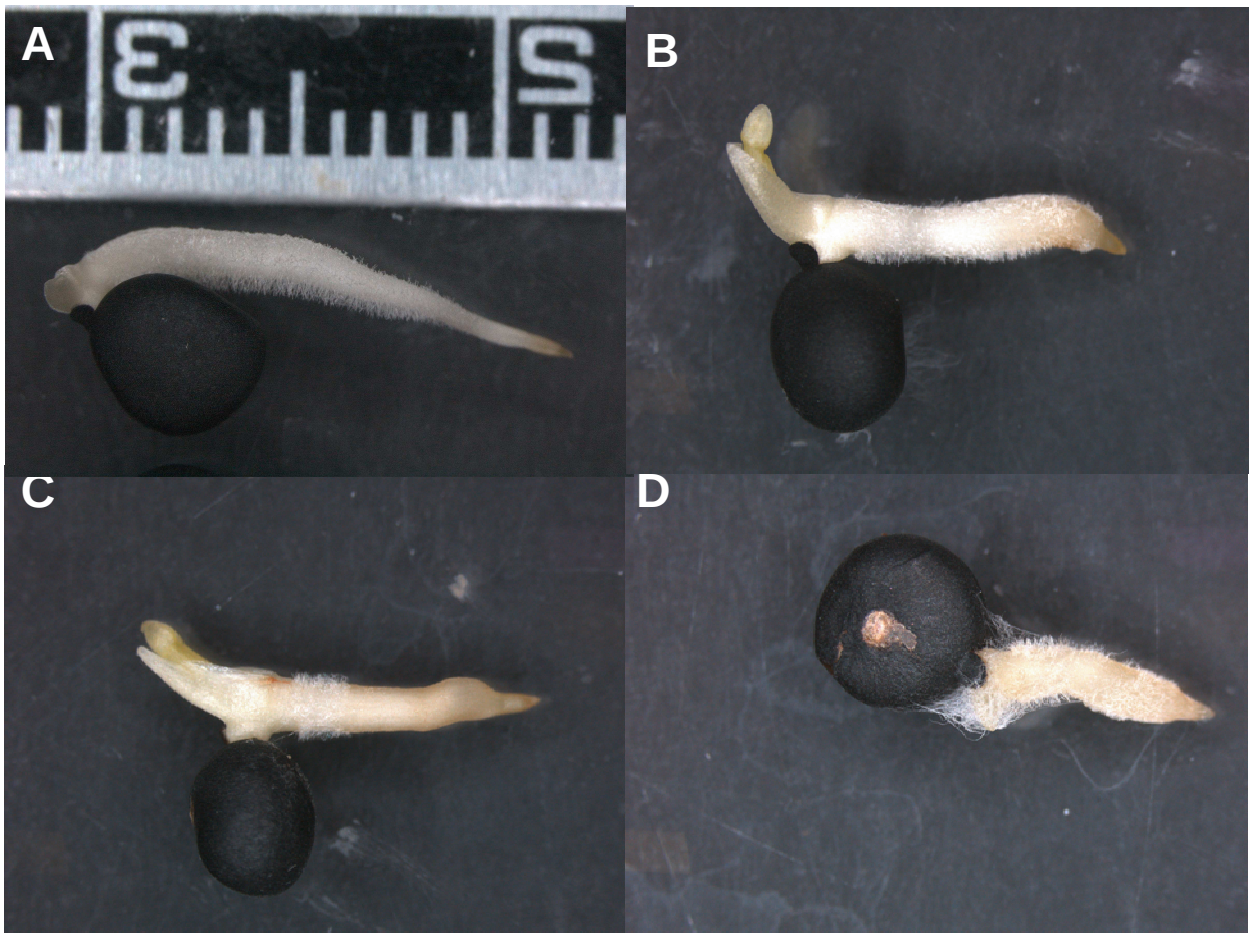

Figure 1.3. Symptoms of *Phytophthora asparagi* observed on germinated asparagus seedlings. (**A**) 0 = no disease symptoms, (**B**) 1 < 50% radicle tissue water-soaked, (**C**) 2 > 50% radicle tissue water-soaked, and (**D**) 3 > 50% radicle tissue water-soaked with mycelia growth.

in all experiments, but was excluded from the model analyzing the incidence data in the seed treatment trial. For the spear rot experiment, isolate was used as the group effect in the REPEATED statement. Multiple comparisons among the cultivar and isolate means were conducted using ANOVA and Tukey's adjustments were used for separation of means when effects were statistically significant at $P = 0.05$. For the seedling root rot experiments, the REPEATED statement was used with cultigen as the group effect for the AUDPC data in the virulence trial and the AUDPC and DSI data in the variety trial. Isolate was used as the group effect for the DSI data in the virulence trial. Data from the control plants were removed prior to analyses to avoid violating the assumption of equal variances. Multiple comparisons among the cultigen and isolate means were conducted using ANOVA and Tukey's adjustments were used in the virulence screen and CONTRAST statements in the cultigen trial for separation of means when effects were statistically significant at $P = 0.05$. For the seed treatment experiment, the REPEATED statement was used with treatment as the group effect. Data for both variables was square root transformed before analysis. Multiple comparisons among the isolate and treatment means were conducted using ANOVA and Tukey's adjustments were used when effects were statistically significant at $P = 0.05$.

RESULTS

Susceptibility of commercial cultivars to spear rot. When spears were inoculated with *P. asparagi*, dark green, water-soaked lesions developed regardless of isolate. Occasionally, white mycelial growth was evident on the inoculated spears. All isolates were readily isolated from the spear tissue (64 to 95%). By the end of the 7-day incubation period, some spears developed a secondary infection on the scales and tip. Control spears either showed no water-

soaked lesions or only very small water-soaked areas along the base. *P. asparagi* was not recovered from water-soaked tissue on the control spears.

The interaction between cultivar and isolate ($P = 0.05$) did not have a significant effect on lesion area, but lesion size differed significantly among cultivars ($P = 0.05$) and isolates ($P = 0.05$). 'Jersey Supreme' spears had significantly larger lesions compared to 'Jersey Giant' and 'Jersey Knight.' 'Tiessen' spears had a larger average lesion area compared to 'Jersey Knight.' Lesion size was similar among 'Jersey Knight,' 'Jersey Giant,' and 'Millennium' and among 'Millennium,' Tiessen,' and 'Jersey Supreme' (Figure 1.4A). When spears were inoculated with isolates SP324, SP325, and SP3236, the lesions were larger than those resulting from isolates SP318 and C013. Spears inoculated with isolates SP316, SP317, SP319, and SP326 produced lesions that were statistically similar to all other isolates (Figure 1.4B).

Virulence of isolates on seedlings roots. Phytophthora rot symptoms appeared as translucent water-soaked tissue that was sometimes accompanied by a red brown discoloration. Symptoms were first observed on seedling roots at 7 days post inoculation (dpi). All isolates were readily isolated from infected seedling roots (69 to 100%). There were no significant differences among *P. asparagi* isolates (Figure 1.5) or among cultivars (Figure 1.6), but slight trends were observed. Isolates SP316 and SP317 caused the highest DSI values and C013, SP316, and SP317 had the highest AUDPC (Figure 1.5). Of the three cultivars tested, Millennium had the smallest DSI and AUDPC (Figure 1.6).

Susceptibility of cultivars and breeding lines to seedling root rot. Root rot symptoms were first observed at 7 dpi, and all cultigens were symptomatic at 14 dpi. The symptoms appeared as described above. All isolates were readily recovered from root tissue (78 to 100%).

The interaction between cultigen and *P. asparagi* isolate ($P = 0.05$) had a significant effect on DSI, but not on AUDPC (Table 1.5). SP317 inoculation resulted in significantly higher DSI in cultigens Cumulus, Jersey Supreme, Mondeo, NJ1191, and Pacific Challenger 1. SP316 caused higher DSI in 'Jersey Giant.' 'Jersey Supreme,' 'Pacific 2000,' and UG009 had the lowest DSI values, while 'Cumulus,' NJ1191, and 'UG005' had the highest DSI values when inoculated with SP316. 'Pacific Challenger 2' and '3 x Phy99' also had low DSI means when inoculated with SP316, although unequal variances affected the DSI significance. 'Jersey Giant,' 'Pacific 2000,' and 'UG009' were significantly less susceptible than 'NJ1191,' 'Mondeo,' and 'Cumulus' when inoculated with SP317. 'Pacific Challenger 2,' 'UG010,' and '74 x 22' also had low DSI means when inoculated with SP317, although variance size affected the DSI significance.

Main effects isolate ($P = 0.05$) and cultigen ($P = 0.05$) significantly affected both DSI and AUDPC. SP317 was significantly more virulent than SP316. 'Jersey Giant' and 'UG009' were significantly less susceptible than 'Cumulus,' 'NJ1191,' and 'UG005.' 'Pacific 2000' and 'UG009' had the smallest AUDPC values.

Efficacy of fungicides and biocontrol agents as seed treatments. At 10 dpi, both pathogens had colonized most of the agar disc area, including the area immediately surrounding seeds. Seeds from all treatments, except for *Bacillus subtilis*, were colonized with mycelium growth. *B. subtilis* growth was observed on the treated seeds and the agar surrounded the seeds. Average inhibition of mycelial growth caused by *B. subtilis* was observed surrounded the immediate area outside of the bacterial growth for both *P. asparagi* isolates. SP316 were readily isolated from seed tissues from most seed treatments (73 to 100%), and less readily from the *B. subtilis* treatment (40%). SP326 was readily isolated from seed tissues from most seed

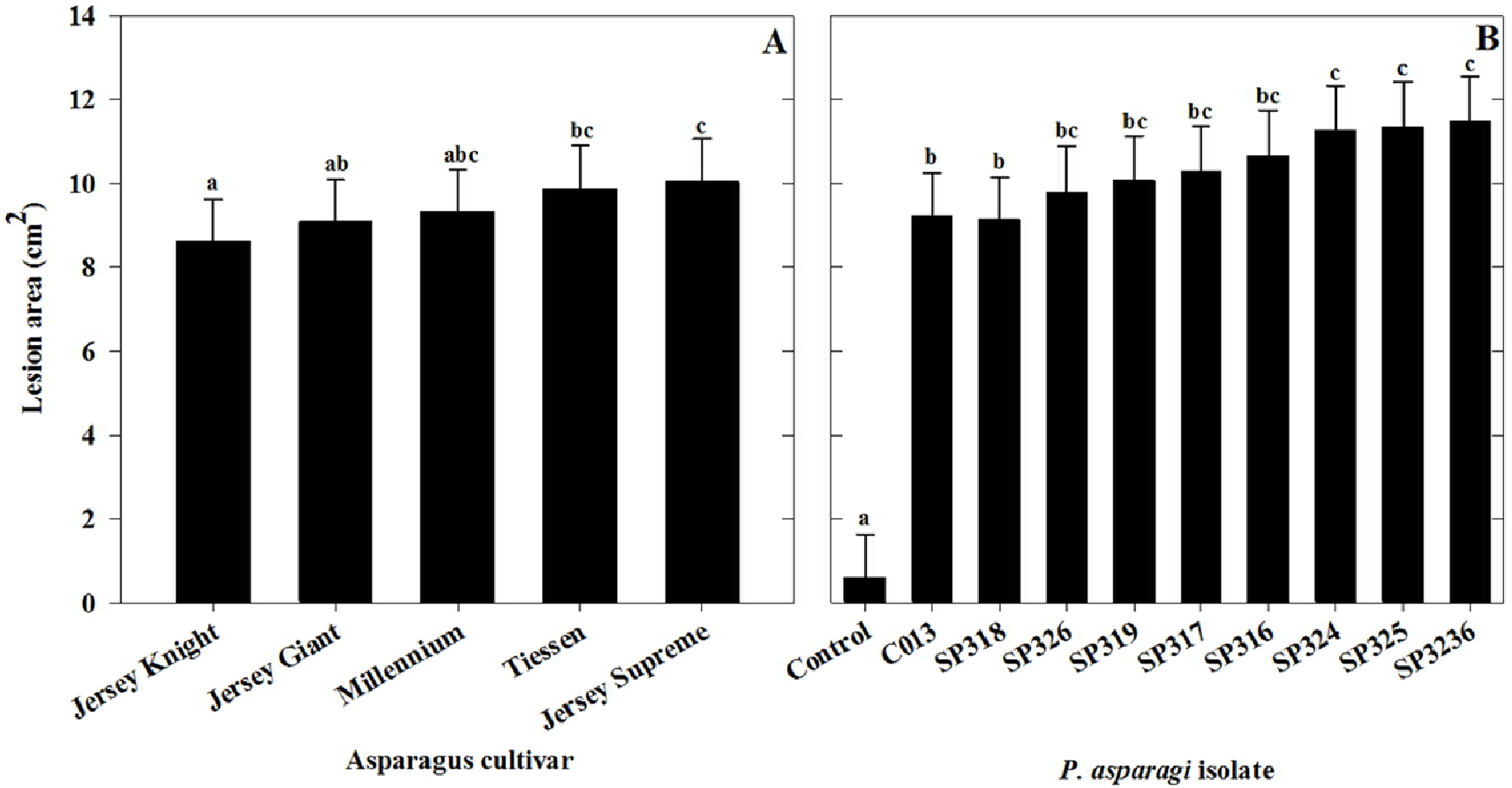

Figure 1.4. Effect of cultivar (**A**) and *Phytophthora asparagi* isolate (**B**) on mean lesion area on detached asparagus spears 7 days after inoculation. Bars with common letters are not significantly different, LSD *P*=0.05 (**A**) and Tukey's *P*=0.05 (**B**).

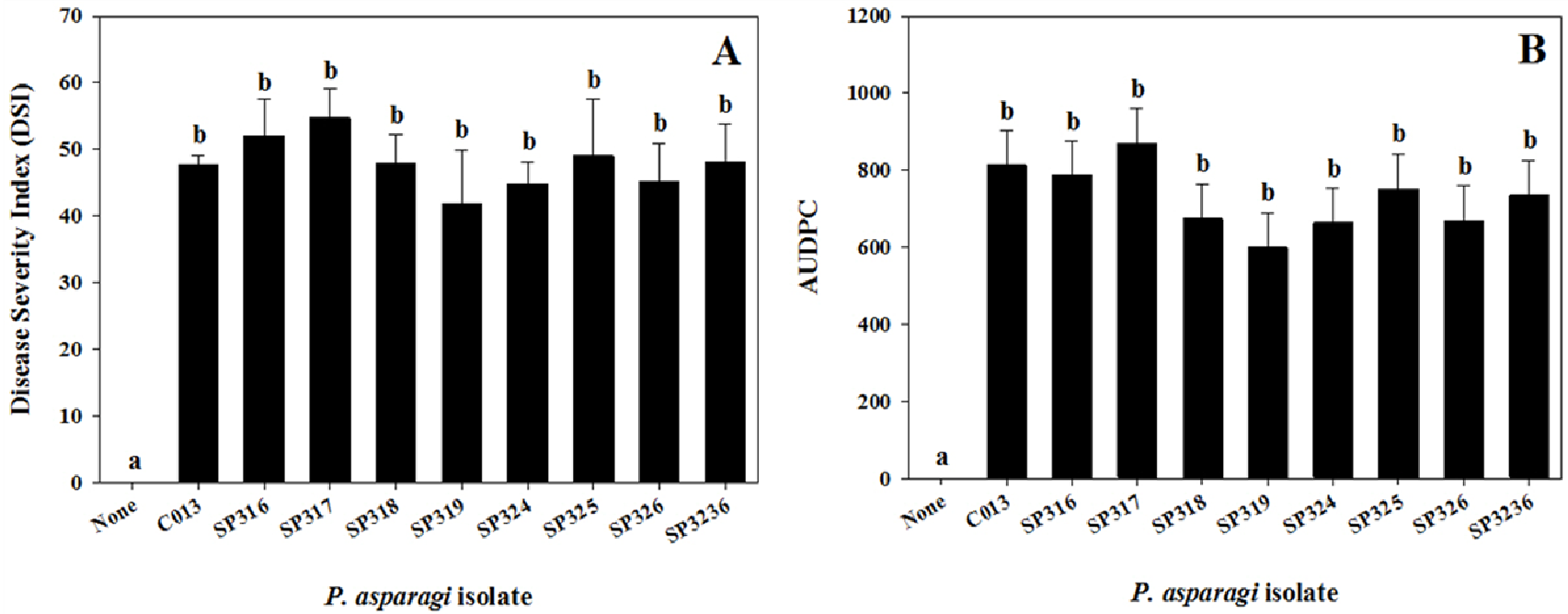

Figure 1.5. Effect of *Phytophthora asparagi* isolates on disease severity index (DSI) where 0 = no disease and 100 = all seedlings roots with > 50% of their roots water-soaked (**A**) and area under the disease progress curve (AUDPC) values (**B**). Bars with common letters are not significantly different, LSD *P*=0.05.

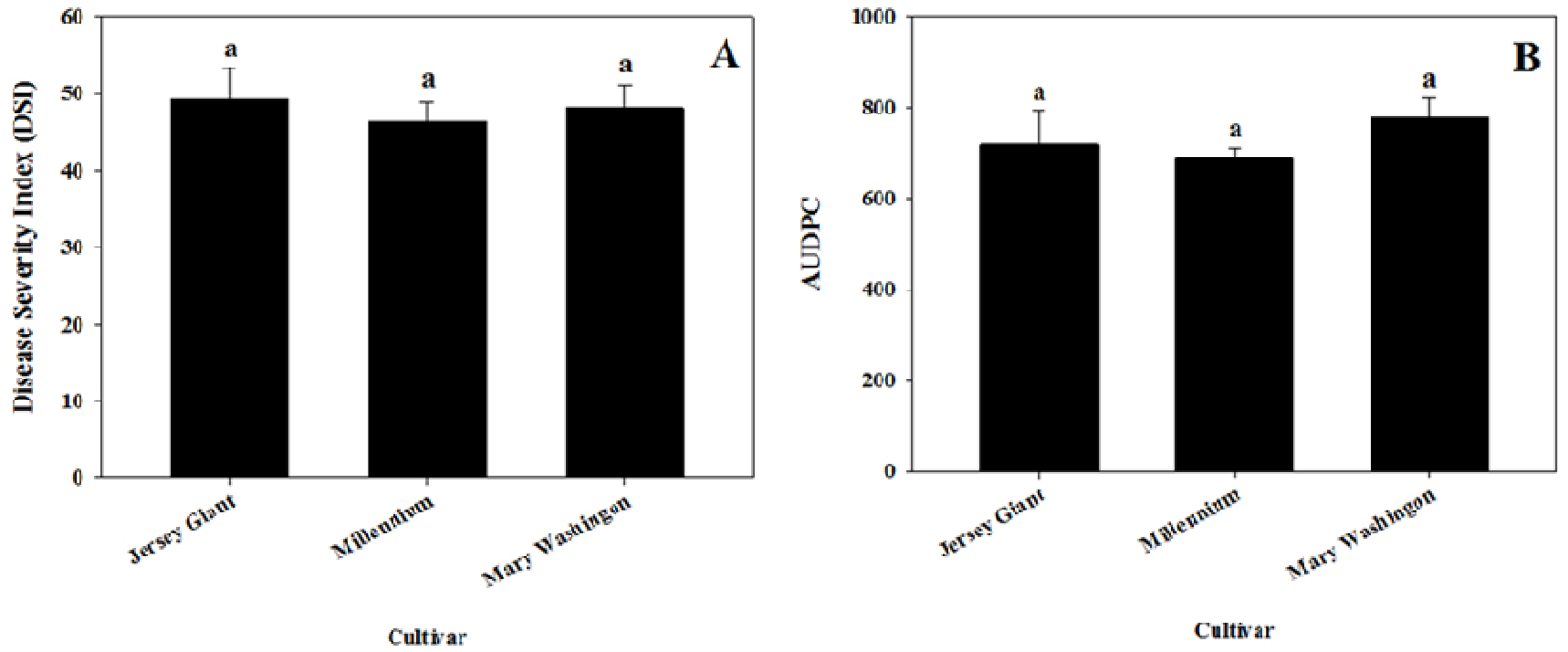

Figure 1.6. Effect of cultivars on disease severity index (DSI) where 0 = no disease and 100 = all seedlings with > 50% of their roots water-soaked (**A**) and area under the disease progress curve (AUDPC) values (**B**). Bars with common letters are not significantly different, LSD *P*=0.05.

treatments (73 to 93%), less readily from the mefenoxam and DPX-QGU42 treatments (57 and 60%), and infrequently from the *B. subtilis* treatment (7%).

The interaction between isolate and treatment ($P = 0.05$) significantly affected seed germination. *P. asparagi* isolates were statistically similar within treatments, although germination of seeds inoculated with SP316 was usually lower than those inoculated with SP326 (Table 1.6). Mefenoxam, DPX-QGU42, and *B. subtilis* treatments had the highest germination when seeds were inoculated with SP316 (Table 1.6). The remaining treatments were statistically similar to the untreated seeds. Mefenoxam and *B. subtilis* were the only treatments with higher germination compared to the untreated control when seeds were inoculated with SP326 (Table 1.6). Both main effects isolate and treatments ($P = 0.05$) were significant for seed germination. Seeds inoculated with SP316 had significantly lower germination (approximately 18%) compared to those inoculated with SP326 (42%). Mefenoxam (approximately 73%) and *B. subtilis* (approximately 97%) were most effective in increasing germination percentage compared to the untreated control (approximately 1%).

The interaction between isolate and treatment ($P = 0.05$) did not significantly affect disease incidence, but the main effects isolate ($P = 0.05$) and treatment ($P = 0.05$) were significant. Seeds inoculated with SP316 (approximately 79%) had higher root rot incidence than those inoculated with SP326 (approximately 63%). *B. subtilis*-treated seeds had the lowest disease incidence (Table 1.6), although seedling radicals in the treatment group appeared stunted and discolored. All remaining treatments were statistically similar to the untreated control.

Of the seedlings inoculated with SP316 and that germinated successfully, mandipropamid (N = 2) and *B. subtilis* (N = 13) had the highest percentage of 0 and 1 disease severity ratings (100% and 85%, respectively) (Figure 1.7). All seedlings treated with mefenoxam (N = 5) and

Table 1.5. Average disease severity index (DSI) and area under the disease progress curve (AUDPC) of asparagus seedlings infected with *Phytophthora asparagi* isolates at 28 days post inoculation.

Cultigen	Disease severity index[z]				AUDPC	
	P. asparagi SP316		*P. asparagi* SP317			
Millennium	50.0	ABC[y]	65.3	BCDE[y]	1130.2	BCD[x]
UG005	72.2	C	68.1	CDE	1227.4	D
UG009	36.1	A	40.3	ABC	585.8	AB
UG010	40.3	AB	44.4	ABCDE	663.5	ABC
UG020	47.2	ABC	48.6	BCDE	824.0	BCD
Jersey Giant	40.3	*AB	31.9	A	544.5	ABC
NJ938	44.4	AB	55.6	ABCDE	743.8	ABCD
NJ941	44.5	AB	54.2	ABCDE	853.2	BCD
NJ1191	55.6	BC	68.0	*DE	896.9	CD
Jersey Supreme	37.5	A	61.1	*CDE	717.0	C
Mondeo	44.4	AB	70.8	*DE	999.0	CD
Pacific Challenger 1	43.1	AB	65.3	*CDE	947.9	CD
Pacific Challenger 2	44.0	ABC	42.0	ABCDE	805.8	ABCD
Pacific 2000	31.9	A	44.4	ABC	449.7	A
3 x Phy99	37.8	AB	64.9	CDE	827.6	BCD
74 x 22	54.2	ABC	43.1	ABCD	753.5	BC
Bacchus	55.6	ABC	66.6	CDE	904.2	CD
Cumulus	57.0	BC	70.8	*E	909.0	CD

[z]Seedlings were given disease ratings (0 to 4) that were used to calculate a disease severity index (DSI) between 0 and 100, where 0 = no disease and 100 = all seedlings with >50% of their roots water-soaked. Each DSI represents the average of three repeated tests with six asparagus seedlings per cultigen/isolate combination per test.

[y]DSI values with the same letter are not significantly different among cultigens for each isolate (within columns). $P = 0.05$.

*Isolates within a cultigen are significantly different (within rows). $P = 0.05$

[x]AUDPC values with the same letter are not significantly different between cultigens. $P = 0.05$.

Streptomyces lydicus (N = 1) received a severity rating of 3 when inoculated with SP316 (Figure 1.7). All seedlings in the fluopicolide (N = 2) and V-10208 (N = 1) treatments received a rating of 2 when inoculated with SP316 (Figure 1.7). More than half of the seedlings treated with DPX-QGU42 (N = 10) and inoculated with SP316 were given a rating of 3, and the remaining seedlings were given either a rating of 1 or 2 (Figure 1.7). No seedlings germinated successfully in the untreated control when inoculated with SP316, and therefore not given a disease severity

Table 1.6. The effect of *Phytophthora asparagi* isolate and seed treatment on germination and disease incidence.

Treatment	Germination (%)[z]				Disease incidence (%)	
	P. asparagi SP316		*P. asparagi* SP326			
Untreated	0.0	A[y]	8.3	A	71.6	B[x]
Mefenoxam	50.0	BCD	100.0	B	75.7	B
Fluopicolide	20.0	ABCD	23.3	AB	93.1	B
Mandipropamid	13.3	ABCD	40.0	AB	82.3	B
V-10208	11.1	AB	55.6	AB	93.1	B
DPX-QGU42	76.7	CD	53.3	AB	78.1	B
Streptomyces lydicus	11.1	ABC	52.8	AB	88.1	B
Bacillus subtilis	100.0	D	93.3	B	13.0	A

[z]Germination percentages were calculated for each treatment as $(G_I/G_0)*100$, where G_I = number of inoculated seeds that germinated and G_0 = number of uninoculated seeds that germinated

[y] Germination percentages with the same letter are not significantly different among *P. asparagi* isolates for each treatment (lower case) or between treatments for each isolate (upper case). Tukey's, *P*=0.05.

[x] Disease incidence values with the same letter are not significantly different between treatments. Tukey's, *P*=0.05.

rating. All seedlings treated with *B. subtilis* (N = 12) and inoculated with SP326 received a rating of 0, except for one seedling that was given a rating of 1 (Figure 1.8). Approximately 27% and 20% of seedlings treated with mefenoxam (N = 11) and *S. lydicus* (N = 5) were given a rating of 0 when inoculated with SP326 (Figure 1.8). Most of the seedlings (approximately 83%) inoculated with SP326 and treated with mandipropamid (N = 6) were given a rating of 1 (Figure 1.8). Varying numbers of seedlings in the remaining seed treatment groups were given ratings between 1 and 3. Only one seedling in the untreated control successfully germinated and was given a rating of 1 (Figure 1.8).

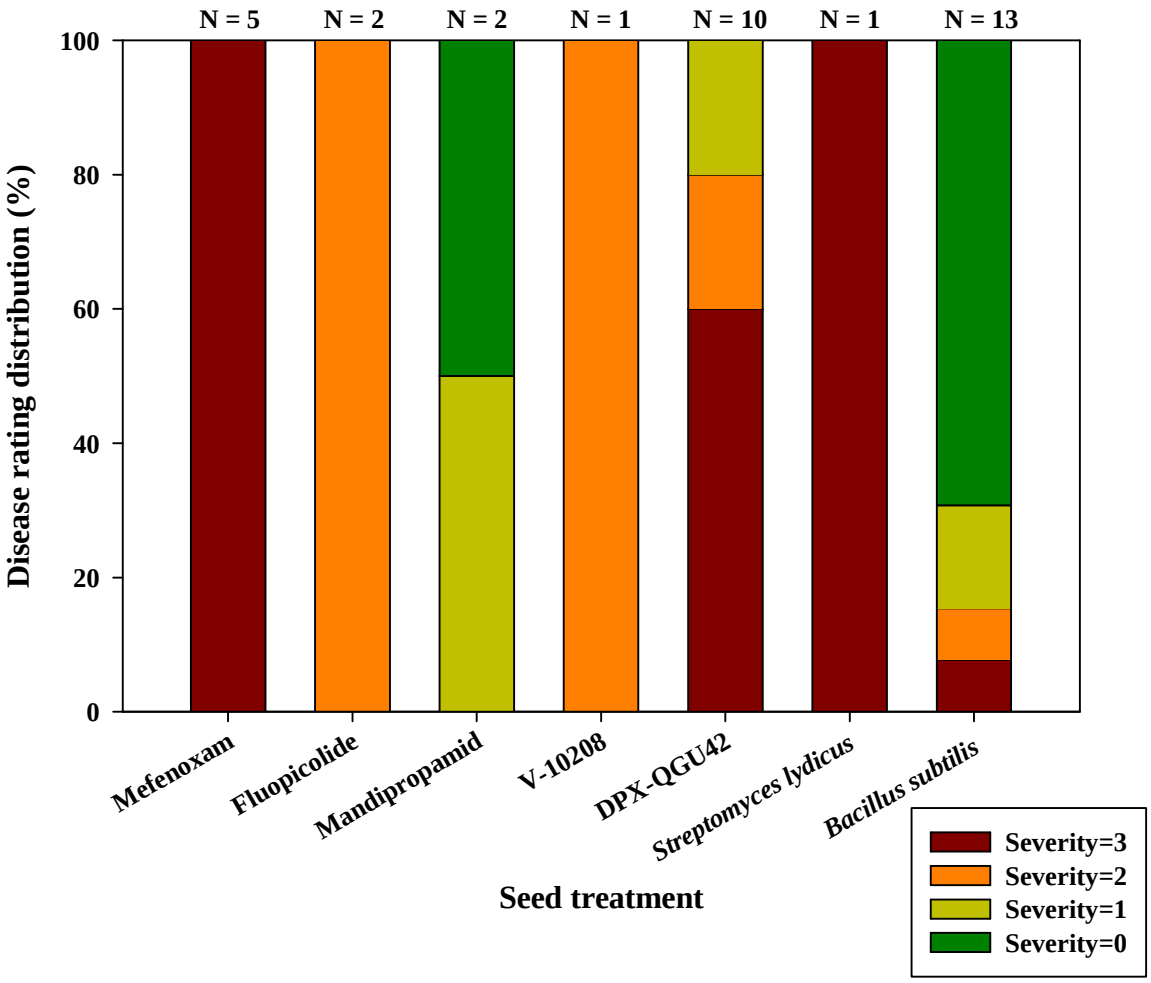

Figure 1.7. The distribution of disease rating scores of germinated seedlings inoculated with *Phytophthora asparagi* SP316 and treated with fungicides and biocontrol agents.

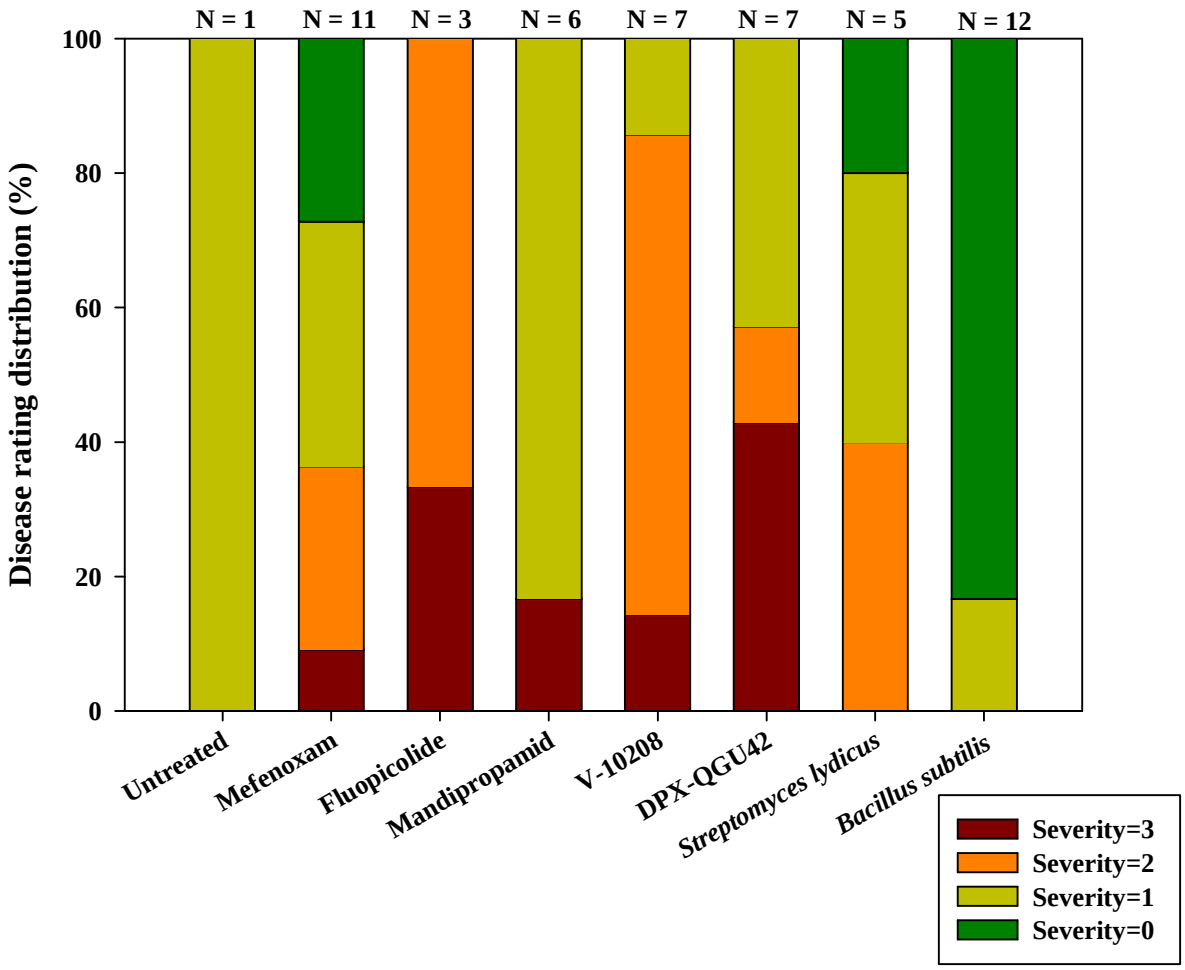

Figure 1.8. The distribution of disease rating scores of germinated seedlings inoculated with *Phytophthora asparagi* SP326 and treated with fungicides and biocontrol agents.

DISCUSSION

Phytophthora asparagi is a homothallic species, and molecular tests suggest that Michigan populations have risen from one clonal lineage (Saude et al., 2008). Other *Phytophthora* spp., such as *P. capsici*, have substantial genetic variation within populations and virulence varies between isolates. Pathogen virulence, however, is not solely based on pathogen genotype, but is also influenced by host genetic variability. A previous study concluded that *P. asparagi* virulence levels were similar across Michigan isolates. The asparagus spears used were from one all-male hybrid, and did not represent genetic differences between cultigens or within open-pollinated cultivars. In the current study, the results suggest that cultigens affected the virulence levels among *P. asparagi* isolates.

The differences in isolate virulence on five cultivars of asparagus spears were significant, although the interaction between cultivar and isolate was not. When all nine isolates were screened for virulence on seedling roots of three cultivars, there were no statistical variations between cultivars or isolates. However, in the cultigen screen, there were significant differences in virulence between SP316 and SP317, depending on the cultigen. The increased diversity of asparagus genotypes likely made the interaction between isolate and host detectable.

Host resistance against *Phytophthora* spp. is a valuable management tool in crops such as soybean (Lin et al., 2013) and pepper (Mallard et al., 2013). True host resistance against *P. asparagi* has not been developed in asparagus, but a breeding program in New Zealand has identified several breeding lines with tolerance to *P. megasperma* var. *sojae* and *P. cryptogea*. Although all cultigens used in this study were susceptible to *P. asparagi*, several varieties were relatively less susceptible, including the New Zealand cultivar Pacific 2000. 'Jersey Giant' and UG009 were least susceptible. Other cultigens from the Pacific, Jersey, and Guelph lines were

highly susceptible to *P. asparagi*, indicating that while each line contains better hybrids, an entire line of hybrids that is consistently superior to the other breeding programs has not yet been developed.

When asparagus seeds were inoculated with *P. asparagi* isolates in vitro, damping-off incidence was high for all seed treatments except for *Bacillus subtilis*. *B. subtilis* seeds also germinated at higher percentages than most of the treatments. Seeds not affected by Phytophthora symptoms were surrounded by *B. subtilis* growth, which antagonized mycelial growth on the agar. This is consistent with prior studies, where *B. subtilis* effectively inhibited *Phytophthora* spp. in vitro (Chung et al., 2008). *B. subtilis* produces several antibiotics with antifungal and antibacterial properties, which has resulted in effective control of a wide range of plant pathogens in laboratory and greenhouse conditions (Berger et al., 1996; Chung et al., 2008). A number of seedling radicles within the *B. subtilis* treatment group were stunted and discolored, with an absence of root hairs. Because the in vitro conditions were highly favorable for the bacterium, it is likely that bacterial growth overwhelmed the young seedling tissues. *B. subtilis* should be studied further in soil-based assays to determine if the seed treatment is safe for young asparagus roots before it can be recommended for use on seeds or young seedlings growing in nursery fields.

Mefenoxam increased germination in plates inoculated with either isolate. Both isolates were previously reported to be mefanoxam-sensitive (Saude et al., 2008). Mefanoxam has been used to successfully control Phytophthora root rot as a crown soak and as a soil drench (Falloon and Fraser, 1991; Green et al., 2008) and is registered for use in production fields. Our results suggest that mefanoxam has the potential to successfully protect young seedlings from Phytophthora root rot as well.

In this study, the unequal number of germinated seedlings across treatments made germination and disease incidence a more reliable measure of treatment effectiveness than disease severity. The same active ingredients used in this study could also be applied to seedling roots following germination to measure the effect of fungicides and biocontrol agents on Phytophthora root rot severity. In vitro conditions, with an abundant nutrient supply, lack of competition and antagonism from microbial communities, and favorable environmental conditions for growth and infection may have increased the pathogens ability to rapidly colonize seed tissues from what would normally occur in soil. Future research should examine the efficacy of these and other seed treatments in soil-based assays to better represent field conditions.

In order to effectively manage Phytophthora spear and root rot, growers are advised to combine control strategies. Careful selection of asparagus cultivars and chemical and biological treatments is useful in an integrated approach to disease management. Our results suggest that certain hybrids from the Guelph, Jersey, and New Zealand breeding programs and mefanoxam may be helpful in management programs.

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LITERATURE CITED

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CHAPTER II

THE EFFECT OF CULTIGENS AND FUNGICIDES AND BIOLOGICAL AGENTS ON FUSARIUM ROOT ROT OF ASPARAGUS.

ABSTRACT

Fusarium crown and root rot (caused by *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum)* is considered to be the most economically limiting disease to asparagus crops worldwide. Pathogenic isolates of *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* are nearly ubiquitous in soils, and methods to protect asparagus from disease are limited. *F. oxysporum* f. sp. *asparagi* (isolate FOA-50) and *F. proliferatum* (isolate P-67) were used to inoculate seedlings of 11 cultigens under controlled conditions. Significant differences were detected in isolate virulence and cultigen susceptibility. The *F. oxysporum* f. sp. *asparagi* isolate was more virulent than the *F. proliferatum* isolate. Reduced susceptibility to Fusarium crown and root rot was identified in commercial cultivars Jersey Supreme, Mondeo, and Mary Washington, and breeding line UG005. The interaction between pathogen and cultigen had a significant effect on disease severity. Commercial cultivars Jersey Supreme and Mondeo had reduced susceptibility to both pathogens. Five seed treatments, including fungicides and biocontrol agents, were evaluated against FOA-50 and P-67 on 'Jersey Giant'. No significant difference in germination was detected between treatments, but the biocontrol, *Bacillus subtilis*, reduced disease incidence compared to the remaining treatments. This is the first reported study on the efficacy of seed treatments against *Fusarium spp.* on asparagus. Future studies should evaluate these and additional seed treatments to determine their efficacy in managing disease in greenhouse and field conditions.

INTRODUCTION

F. oxysporum Schlectend.:Fr. f. sp. *asparagi* Cohen & Heald and *F. proliferatum* (Matushima) Niremburg are the primary causal agents of Fusarium crown and root rot, which is considered to be the most economically limiting disease to asparagus crops worldwide (Elmer, 2001a; Elmer, 2001b; Schreuder et al., 1995; Van Bakel and Kerstens, 1970; Wong and Jeffries, 2006). *F. proliferatum* can colonize below- and above-ground tissues of asparagus plants and is commonly found on mature crowns, while *F. oxysporum* f. sp. *asparagi* usually infects young plants (Elmer et al., 1996; Burgess, 1981; Elmer, 2001a; Keulder, 1999). Fusarium crown and root rot symptoms begin as reddish brown discoloration in the rhizome, shoot, or storage root vascular tissues (Zandstra et al., 1992). As the disease progresses, the feeder roots completely rot, storage roots become hollow, and the fern stand turns yellow and senesces (Elmer et al., 1996; Zandstra et al., 1992).

F. oxysporum abundantly produces chlamydospores, but can also survive as hyphae on plant residues in and on the soil. There is no known teleomorph of *F. oxysporum* (Burgess, 1981). Forty-three vegetative compatibility groups (VCGs) have been reported in *F. oxysporum* f. sp. *asparagi* (Elmer et al., 1996) and it is believed that each VCG is a clonal lineage (Correll, 1991). The pathogenicity of *F. oxysporum* on asparagus does not appear to correspond with vegetative compatibility, indicating that pathogenicity on asparagus may be a common trait in *F. oxysporum* (Elmer, 2001a; Elmer et al., 1996).

F. proliferatum (Teleomorph *G. fujikuroi* var. *intermedia* Kuhlman) is mating population "D" of the *Gibberella fujikuroi* complex (Elmer, 1995; Samuels et al., 2001). Perithecia have not been found in nature (Elmer, 2001a). The anamorph has not been found to produce chlamydospores and it overwinters as mycelia on plant debris.

Pathogenic isolates of *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* are nearly ubiquitous within the major asparagus producing regions, so choosing fields with pathogen-free soils may not be an option to prevent Fusarium crown and root rot (Hartung et al., 1990; Elmer, 2001a). Planting disease-free crowns from fumigated nurseries, applying protective crown soaks, and following good cultural practices to maintain stand vigor are the best management strategies available (Drost, 1997; Elmer, 2001b; Morrison et al., 2011; Zandstra et al., 1992). Maintaining crop health by managing foliar pathogens, viruses, insects, and weeds and selecting safe herbicides is also important to reduce susceptibility to Fusarium crown and root rot (Elmer, 2001b; Hausbeck et al., 1999; Johnson and Lunden, 1992; Morrison et al., 2011; Zandstra et al., 1992).

Host resistance against *Fusarium* spp. is a valuable management tool in crops such as wheat (Burgess et al., 2001) and snap beans (Snapp et al., 2003). Currently, all *A. officinalis* cultivars are susceptible to *F. oxysporum* f. sp. *asparagi* and *F. proliferatum.* All-male hybrids from Rutgers University have higher plant vigor than open-pollinated cultivars, which might offer some protection against disease (Ellison and Kinelski, 1985). In vitro screens have identified tolerance to *Fusarium* spp. in the ornamentals *A. densiflorus* 'Sprengeri,' and 'Myersii', however sexual incompatibility between the two species has prevented hybrids from being deveoped (Dan and Stephens, 1995; Stephens and Elmer, 1988; Stephens et al., 1989). As resistant cultivars are identified and developed, cultivar selection could become a useful management tool for growers.

Research of chemical and biological strategies has shown varying levels of disease control. Historically, sodium chloride was believed to control the Fusarium crown and root rot (Elmer, 2001a), but growth chamber, greenhouse, and field studies have not resulted in

consistent disease control. NaCl does not appear to benefit healthy stands, the mode of action and environmental impact is unknown, and it exacerbates Phytophthora crown and root rot (Elmer, 2004; Morrison et al., 2011; Reid et al., 2001). Therefore it is not currently recommended for use. Seed disinfestation with benomyl in acetone is useful to remove seed-borne *Fusarium* spp. (Damicone, 1981), but does not provide protection from other soil-borne pathogens. Fungicides such as Cannonball and Topsin, applied as a crown soak, have resulted in improved spear and fern production (Hausbeck and Cortright, 2009). Biological agents including nonpathogenic *Fusarium* spp., arbuscular mycorrhizae, and *Trichoderma harzianum* have reduced disease and increased root weight in some studies, but in other trials these treatments did not affect crown health (Arriola et al., 2000; Counts and Hausbeck, 2008; Elmer, 2004; Elmer, 2008).

Young asparagus seedlings are especially susceptible to *F. oxysporum* f. sp. *asparagi* (Elmer et al., 1996 and Keulder, 1999). Nursery beds are sometimes fumigated to remove pathogen populations, and growers are advised to surface disinfest their seeds before planting (Zandstra et al., 1992). Currently, Michigan growers do not use fungicides or biocontrols to protect seedlings grown in nursery fields (Hausbeck, personal communication, 2014). Identifying and developing effective seed treatments against Fusarium crown and root rot would be useful for producing disease-free crowns.

The objectives of this study were to: *i*) screen seedling varieties for relative susceptibility and *ii)* test chemical and biological products for efficacy as seed treatments against *Fusarium* spp.

MATERIALS AND METHODS

***Fusarium* spp. and inoculum preparation.** Michigan isolates of *F. oxysporum* f. sp. *asparagi* (FOA-50) and *F. proliferatum* (P-67) from the collection of M. K. Hausbeck were taken from long-term storage by aseptically transferring infested silica crystals to PDA (39 g potato dextrose agar and 1000 ml distilled water) plates. Cultures were maintained at room temperature (23 ± 2°C) under continuous fluorescent lighting and transferred every 7 to 10 days.

Spore suspensions were prepared by flooding cultures after fungal growth covered the entire disc with sterile distilled water and scraping with a flame-sterilized glass rod. The resulting spore suspensions were collected and diluted to 10^6 conidia/ml for FOA-50 and 10^5 conidia/ml for P-67 using a hemocytometer (Stephens and Elmer, 1988). Suspensions were used within 30 minutes of preparation.

Seedling inoculation and incubation. Asparagus seeds were surface disinfested using the method of Damicone et al. (1981) by agitating in solutions of 25,000 ppm benomyl in acetone on a rotary shaker (1000 rpm) for 24 hours, then rinsing 3 times in acetone and 3 times in distilled water. Seeds were then agitated in 1.23% sodium hypochlorite at 1000 rpm for 1 hour and rinsed 3 times in distilled water. After being allowed to air dry for 1 to 2 hours in a laminar flow hood, the seeds were placed on water agar (16 g agar and 1000 ml distilled water) and germinated in the dark at room temperature for 7 to 14 days. Once hypocotyls emerged, the seeds were planted in 13 ml of Hoagland's agar (Stephens and Elmer, 1988) in test tubes (25 x 150 mm) and grown under continuous fluorescent light at room temperature.

When seedlings were approximately 11 cm in height and secondary branches with cladophylls had formed (approximately 2 weeks), a 0.5 ml aliquot of FOA-50 (10^6 conidia/ml) or P-67 (10^5 conidia/ml) was placed directly on the agar of each test tube. Following inoculation,

seedlings were allowed to grow and infection to develop for 4 weeks under laboratory conditions described above.

Susceptibility of cultivars and breeding lines to seedling root rot. Eleven cultivars and breeding lines (Table 2.1) were selected to compare relative susceptibility against *F. oxysporum* f. sp. *asparagi* and *F. proliferatum*. Seeds were disinfested and germinated using the methods described above. When the seedlings were approximately 11 cm in height and secondary branches with cladophylls had formed (approximately 2 weeks), conidial suspensions (0.5 ml) were added to test tubes. Sterile distilled water (0.5 ml) was used for uninoculated controls. Four seedlings were used for each cultigen. Seedling test tubes were arranged in a randomized complete block design in test tube racks.

Table 2.1. Asparagus cultivars and breeding lines tested for susceptibility to *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum*.

Cultigen	Source
Millennium	University of Guelph
UG005	University of Guelph
UG009	University of Guelph
UG010	University of Guelph
UG020	University of Guelph
Mary Washington	Eden Brothers
NJ 938	Rutgers University
NJ 941	Rutgers University
NJ 1191	Rutgers University
Jersey Supreme*	Vilmorin
Mondeo*	Vilmorin

*Packaged seeds were pretreated with thiram.

Seedlings were rated for disease incidence and severity of root rot every 7 days beginning 7 days post inoculation (dpi). Disease incidence was recorded, and severity was measured as a visual estimate of the area (%) of each root system with *Fusarium* lesions. For the final rating (week 4), seedlings were removed from the test tubes and the roots were mechanically separated

from the agar. The area under the disease progress curve (AUDPC) was calculated (Madden et al., 2007) to obtain the cumulative severity (%) throughout the experiments. The experiment was conducted 4 times.

Efficacy of fungicides and biocontrol agents as seed treatments. The methods used by Broders et al. (2007) were adapted to screen three fungicides and two biocontrol agents for efficacy as seed treatments in vitro. A mycelial plug (7-mm in diameter) of FOA-50 or P-67 was placed in the center of 15-cm-diameter petri plate containing 50 ml of PDA. Seeds of 'Millennium' were surface disinfested according to the methods of Damicone et al. (1981) and allowed to air dry in a laminar flow hood (approximately 1 hour). Treatments were applied to seed lots (0.33 g) at the rates listed in Table 2.2. Seeds soaked in the treatments for approximately 30 minutes, then air-dried in a laminar flow hood for approximately 1 hour. Seeds from each treatment were equally spaced in a randomized order around the plate, approximately 3 cm from the edge (Figure 2.1).

At 11 days after plating the seeds, inhibition of mycelial growth was measured as the distance between the seed and margin of mycelial growth along the radius of the agar plate. The seeds were removed from culture and successfully germinated seeds were rated for disease severity. The number of seeds that successfully germinated on inoculated plates was compared with the number that germinated on the uninoculated plates. Seeds were considered successfully germinated if the hypocotyl had emerged or the radicle was > 5 mm long. Treatments were scored according to a 0 to 3 rating scale, where 0 = 100% germination with no disease symptoms and 3 = all seed tissues colonized (Table 2.3, Figure 2.2). Each treatment was replicated 5 times for each isolate and the uninoculated control, and the experiment was conducted a total of 4 times: twice with 'Jersey Giant.'

Table 2.2. Fungicides and biocontrol agents tested for efficacy against *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum*.

Treatment	Active ingredient	Rate/100 lb seed	Rate/20 seeds (0.48 g)
Untreated	Sterile deionized water		600.0 µl
Dynasty	azoxystrobin	44.4 ml	0.47 µl
Fontelis	penthiopyrad	29.6 ml	0.31 µl
Maxim	fludionoxil	4.5 g	0.05 mg
Actinovate AG	*Streptomyces lydicus*[*]	340.2 g	3.60 mg
Serenade Soil	*Bacillus subtilis*[*]	813.2 ml	8.61 µl

[*] Biological control agent

Table 2.3. Scale of disease ratings on successfully germinated asparagus seedlings inoculated with *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum.*

Rating	Symptoms
0	no disease
1	<50% radicle tissue covered in lesions
2	>50% radicle covered in lesions
3	All viable seed tissues covered in lesions

Pathogen isolation. At the end of the observation period for the seedling root rot experiment, small sections of root tissue from lesion margins were plated on Komada's agar (1000 ml distilled water, 20 g $_{D}$-galactose, 15.0 g agar, 2.0 g $_{L}$-asparagine, 1.0 g K_2HPO_4, 1.0 g pentachloronitrobenzene, 0.5 g KCl, 0.5 g $MgSO_4 \bullet 7H_2O$, 0.1 g Fe-Na-EDTA, 10 ml oxgall solution, and 6 ml streptomycin), a media selective for *Fusarium* spp. growth. All seedlings were sampled.

For the seed treatment experiment, whole seeds were disinfested in 0.525% sodium hypochlorite for approximately 60 seconds, rinsed in sterile distilled water, and air dried in a laminar flow hood for 1 to 3 minutes. Radicle tissue was wounded using a flame-sterilized scalpel and plated in the method described above. All inoculated seeds were sampled, and control seeds were also sampled if radicles had water-soaked or discolored tissue.

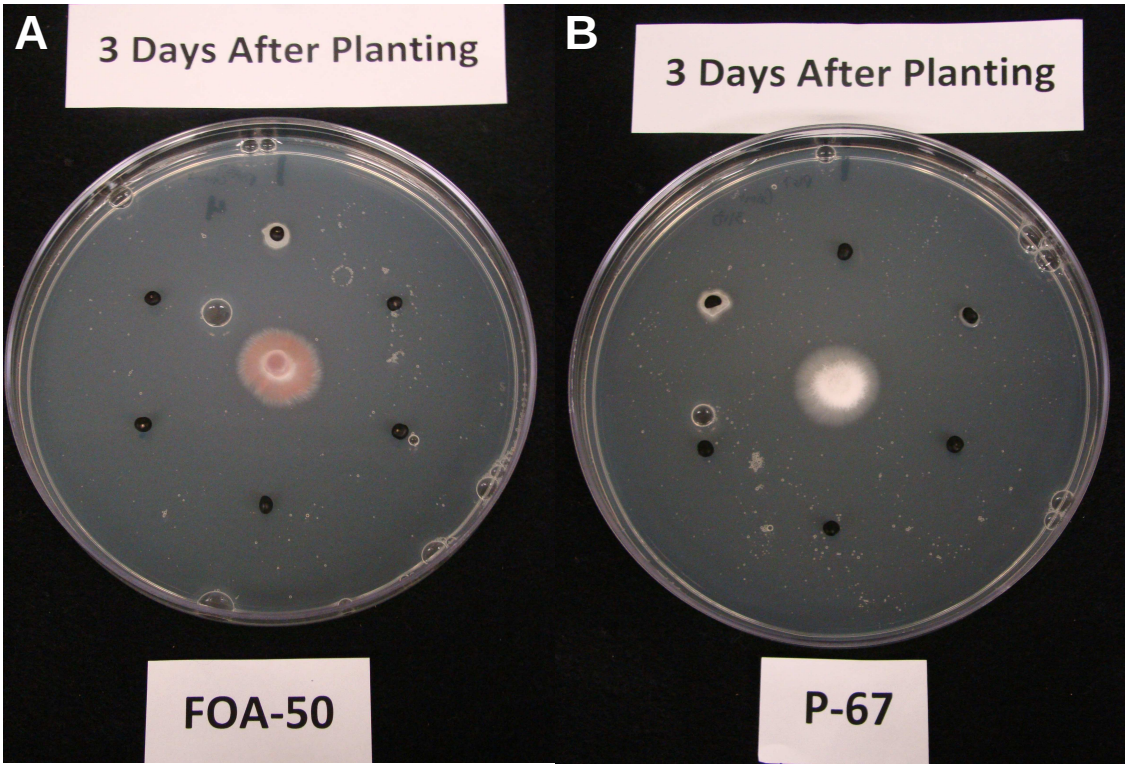

Figure 2.1. Asparagus seeds treated with fungicides and biocontrol agents on V8 agar plates inoculated with *Fusarium oxysporum* f. sp. *asparagi* (**A**) and *F. proliferatum* (**B**), 3 days after planting.

Statistical analysis. In the cultigen experiment, disease severity data was tested by analysis of variance (ANOVA) using the PROC GLIMMIX procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). The PROC MIXED procedure was used to analyze AUDPC data. Trial repetition was treated as a random affect for both data sets. Cultigen was treated as the group effect in the REPEATED statement for AUDPC data. Residuals were tested for normality and homogeneity of variance using PROC UNIVARIATE. Data from the control plants were removed prior to analyses to avoid violating the assumption of equal variances. Multiple comparisons among the cultigen means were conducted using ANOVA and Tukey's adjustments were used for separation of means when effects were statistically significant at $P = 0.05$.

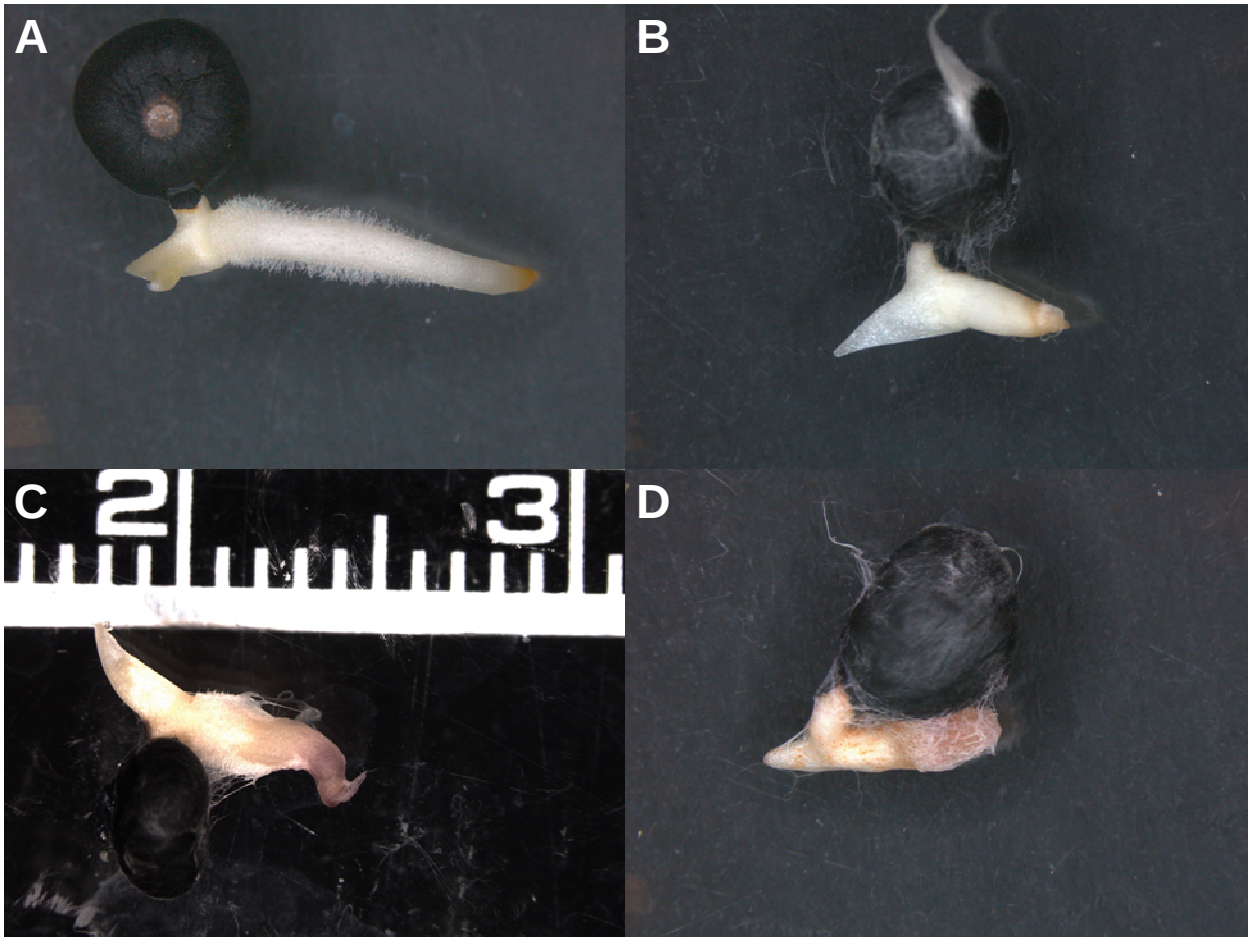

Figure 2.2. *Fusarium* symptoms observed on germinated asparagus seedlings. (**A**) 0 = no disease symptoms, (**B**) 1 = lesions present on <50% radicle tissue, (**C**) 2 = lesions present on >50% radicle tissue, and (**D**) 3 = all seed tissues covered in lesions.

For the seed treatment experiment, germination data was tested by analysis of variance (ANOVA) using the PROC MIXED procedure. Treatment was treated as the group effect in the REPEATED statement. Residuals were tested for normality and homogeneity of variance using PROC UNIVARIATE. Data was square root transformed prior to analysis to avoid violating the assumption of equal variances. Disease incidence data were analyzed using analysis of variance with the AOV command of R version 3.01 (The R Foundation for Statistical Computing, Vienna, Austria). Multiple comparisons among the cultigen means were conducted using a Fischer's Least Significant Differences (LSD) test (Agricolae package) at $P = 0.05$.

RESULTS

Susceptibility of cultivars and breeding lines to seedling root rot. At four weeks post inoculation, all inoculated plants were diseased. Seedlings infected with both *Fusarium* spp. developed dark red-brown lesions with distinct margins. Root tissue of uninoculated controls remained vigorous without discoloration. The respective pathogen was successfully isolated from seedlings infected with *F. oxysporum* f. sp. *asparagi* and *F. proliferatum*. An unidentified *Fusarium* spp. was also isolated from approximately 3% of the asymptomatic uninoculated controls (NJ938 and NJ1191).

The interaction between pathogen and asparagus cultigen was significant ($P > 0.05$) for root rot severity. The main effects, cultigen ($P = 0.05$) and isolate ($P = 0.05$) were significant. *F. oxysporum* f. sp. *asparagi* caused significantly higher severity in cultivars Mary Washington and Mondeo and breeding lines NJ941, UG009, and UG020 compared to *F. proliferatum.* *F. proliferatum* caused higher disease severity in UG010. NJ941 was most susceptible to both pathogens. 'Jersey Supreme' was significantly healthier than 'Millennium,' UG009, NJ1191, and NJ941 when inoculated with *F. oxysporum* f. sp. *asparagi.* UG005, UG009, UG020, 'Mondeo,' and NJ938 were less susceptible than NJ941 and NJ1191 when inoculated with *F. oxysporum* f. sp. *asparagi*. Cultivars Mary Washington, Mondeo, Supreme, and breeding line UG020 were significantly less susceptible than NJ938, NJ941, NJ1191, and UG010 when inoculated with *F. proliferatum* (Table 2.4).

The interaction between pathogen and cultigen significantly affected AUDPC. Infection by *F. oxysporum* f. sp. *asparagi* resulted in a higher AUDPC than *F. proliferatum* in most cultigens, but was only significantly higher in NJ941. There were no significant differences between cultigens when inoculated with *F. proliferatum* (Table 2.5).

Efficacy of fungicides and biocontrol agents as seed treatments. At 11 days post inoculation, both pathogens had colonized most of the agar disc area, including the area immediately surrounding seeds. Seeds from all treatments, except for *Bacillus subtilis*, were colonized with mycelial growth. *B. subtilis* growth was observed on the treated seeds and the agar surrounded the seeds. Average inhibition of mycelial growth caused by *B. subtilis* was observed at an average length of 8.5 mm from the bacterial growth in plates inoculated with *F. oxysporum* f. sp. *asparagi* and immediately surrounding the bacterial growth in *F. proliferatum* cultures. *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* were readily isolated from seed tissues in all treatments (100%), except for the *B. subtilis* treatment (0 and 10%).

The interaction between pathogen and treatment ($P = 0.05$) was did not significantly affect germination or incidence. The main effect pathogen ($P = 0.05$) significantly affected germination, but main effect treatment ($P = 0.05$) was not significant. Both pathogens reduced seed germination compared to the uninoculated seeds, and *F. proliferatum* infection resulted in significantly lower germination (2.5%) than seeds inoculated with *F. oxysporum* f. sp. *asparagi* (40%). Pathogen ($P = 0.05$) did not have a significant effect on disease incidence. Seeds treated with *B. subtilis* had significantly lower disease incidence than the remaining treatments (Table 2.6). All germinated seedlings inoculated with *F. proliferatum* were given the highest severity rating. Of the seedlings inoculated with *F. oxysporum* f. sp. *asparagi*, only one that was treated with *B. subtilis* (N = 1) had a severity rating of 0 (Figure 2.2). However, the seedling was stunted with discoloration at the radicle tip. All germinated seedlings treated with azoxystrobin (N = 3), penthiopyrad (N = 3), and *Streptomyces lydicus* (N = 2) were given a rating of 3 (Figure 2.2).

Table 2.4. Average disease severity (%) of asparagus seedlings infected with *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum* at 28 days post inoculation.

Cultigen	Disease severity (%)[x]			
	F. oxysporum f. sp. *asparagi*		*F. proliferatum*	
Millennium	14.8	BC[y]	12.7	BCD
UG005	11.5	AB	10.6	ABC
UG009	15.0	*BC	12.4	ABCD
UG010	11.5	AB	16.2	*D
UG020	12.7	*AB	10.0	AB
Mary Washington	14.2	*ABC	7.1	A
NJ938	13.3	AB	14.5	CD
NJ941	34.2	*D	28.0	E
NJ1191	18.6	C	15.9	D
Jersey Supreme	10.3	A	8.9	AB
Mondeo	12.1	*AB	8.0	A

[x]Seedlings were rated by a visual estimation of the percentage of root system infected. Each disease severity value represents the average of 4 repeated tests with 4 replicate asparagus seedlings per pathogen/cultigen combination per test.

[y]Disease severity values with the same letter are not significantly different among cultigens for each pathogen (within columns). Tukey's, P=0.05.

*Pathogens within a cultigen are significantly different (within rows). LSD, P = 0.05

Table 2.5. Area under the disease progress curve (AUDPC) of asparagus seedlings infected with *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum*.

Cultigen	AUDPC[x]			
	F. oxysporum f. sp. *asparagi*		*F. proliferatum*	
Millennium	318.3	A[y]	233.0	A
UG005	365.3	A	242.8	A
UG009	271.3	A	262.5	A
UG010	248.3	A	220.9	A
UG020	309.5	A	225.3	A
Mary Washington	340.2	A	249.4	A
NJ938	352.6	A	356.6	A
NJ941	1097.2	*B	448.0	A
NJ1191	378.9	A	296.6	A
Jersey Supreme	362.0	A	263.6	A
Mondeo	254.8	A	251.6	A

[x]AUDPC values were calculated from weekly severity ratings over the period of 4 weeks.

[y]AUDPC values with the same letter are not significantly different among pathogens for each cultigen (lower case) or between cultigens for each pathogen (upper case). Tukey's, P=0.05.

*Pathogens within a cultigen are significantly different (within rows). LSD, P = 0.05

Table 2.6. The effect of *Fusarium* spp. and seed treatment on germination and disease incidence.

Treatment	Germination (%)[z]		Disease incidence (%)
	F. oxysporum f. sp. *asparagi*	*F. proliferatum*	
Untreated	33.3	10.0	100.0 B[y]
Azoxystrobin	75.0	50.0	100.0 B
Penthiopyrad	62.5	0.0	93.8 B
Fludionoxil	58.3	0.0	100.0 B
Streptomyces lydicus	50.0	10.0	100.0 B
Bacillus subtilis	50.0	0.0	6.3 A

[z]Germination percentages were calculated for each treatment as $(G_I/G_0)*100$, where G_I = number of inoculated seeds that germinated and G_0 = number of uninoculated seeds that germinated

[y]Disease incidence values with the same letter are not significantly different among seed treatments. LSD, P=0.05.

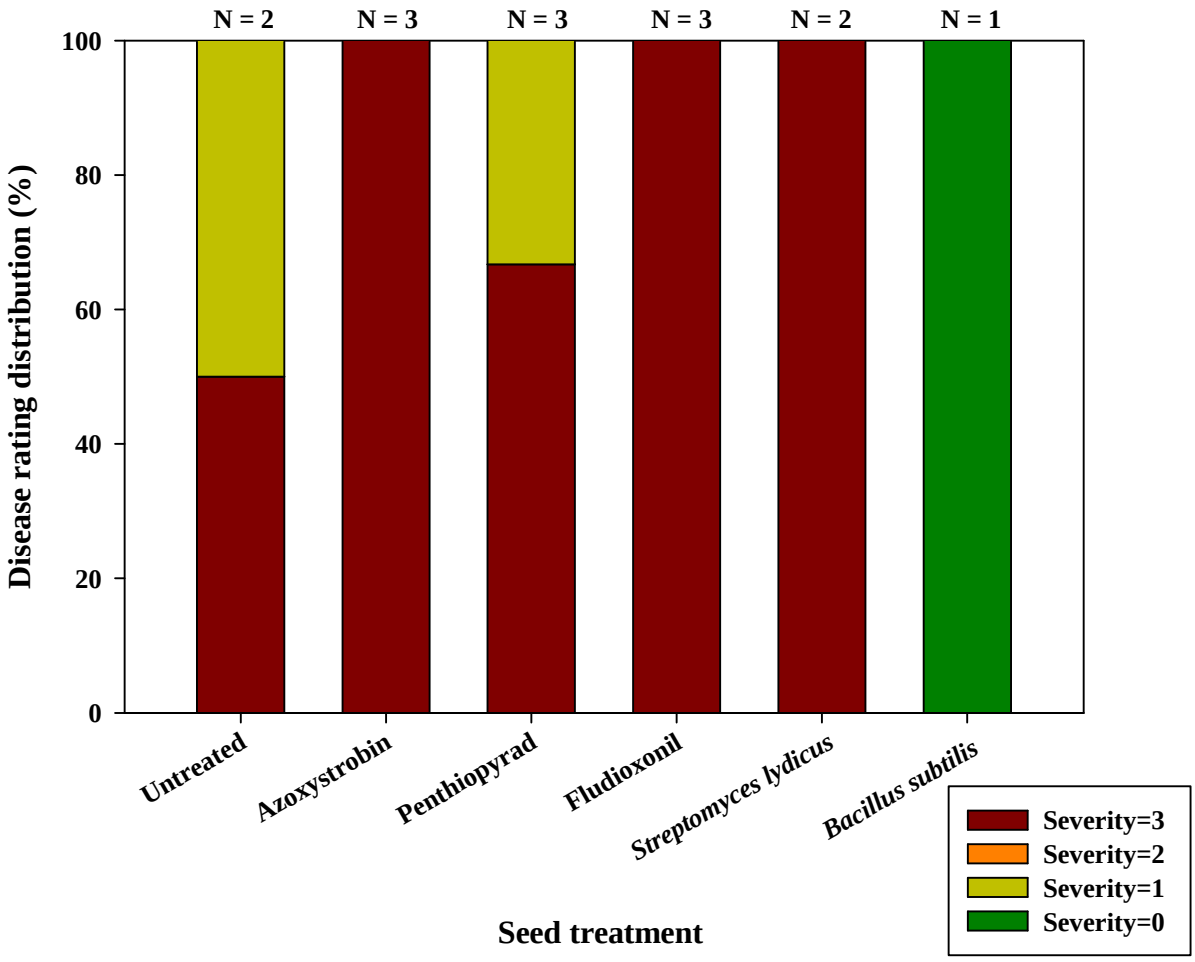

Figure 2.3. The distribution of disease rating scores of germinated seedlings inoculated with *Fusarium oxysporum* f. sp. *asparagi* and treated with fungicides and biocontrol agents.

DISCUSSION

Effective management strategies against Fusarium crown and root rot are limited, and currently the best control methods are good cultural practices (Drost, 1997; Elmer, 2001b; Zandstra et al., 1992). Resistant cultivars, a useful tool to manage disease, are not currently available in *Asparagus officinalis*. In this study, all cultigens were susceptible to both *Fusarium* species. However, cultigens Jersey Supreme, Mondeo, and UG020 were less susceptible to both pathogens; NJ938, UG005, and UG010 were less susceptible to *F oxysporum* f. sp. *asparagi*; and Mary Washington was less susceptible to *F. proliferatum*. NJ941 was consistently the most susceptible to Fusarium crown and root rot. This information may be valuable to growers as well as to breeding programs for cultivar selection.

When asparagus seeds were inoculated with *Fusarium* spp. in vitro, damping-off incidence was high for all seed treatments except for *Bacillus subtilis*. Seeds not affected by Fusarium symptoms were surrounded by *B. subtilis* growth, which antagonized mycelial growth on the agar. This is consistent with prior studies, where *B. subtilis* effectively inhibited *Fusarium* spp. in vitro (Chung et al., 2008). *B. subtilis* produces several antibiotics with antifungal and antibacterial properties, which has resulted in effective control of a wide range of plant pathogens in laboratory and greenhouse conditions (Berger et al., 1996; Chung et al., 2008). A number of seedling radicles within the *B. subtilis* treatment group were stunted and discolored, with an absence of root hairs. Because the in vitro conditions were highly favorable for the bacterium, it is likely that bacterial growth overwhelmed the young seedling tissues. *B. subtilis* should be studied further in soil-based assays to determine if the seed treatment is safe for young asparagus roots before it can be recommended for use on seeds or young seedlings growing in nursery fields.

In this study, the unequal number of germinated seedlings across treatments made disease incidence a more reliable measure of treatment effectiveness than disease severity. The same active ingredients used in this study could also be applied to seedling roots following germination to measure the effect of fungicides and biocontrol agents on Fusarium root rot severity. In vitro conditions, with an abundant nutrient supply, lack of competition and antagonism from microbial communities, and favorable environmental conditions for growth and infection may have increased the pathogens ability to rapidly colonize seed tissues from what would normally occur in soil. Future research should examine the efficacy of these and other seed treatments in soil-based assays to better represent field conditions.

In order to effectively manage Fusarium crown and root rot, growers are advised to combine control strategies. Careful selection of asparagus cultivars and integrating chemical and biological treatments is useful in a coordinated approach to disease management. The results of this study suggest that specific hybrids from the Guelph, Jersey, and German asparagus lines may be helpful in asparagus cultivation.

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LITERATURE CITED

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