Investigating relationships between *Pratylenchus penetrans* and *Fusarium verticillioides* on corn seedlings

By

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The root lesion nematode *Pratylenchus penetrans* and the ascomycetous *Fusarium verticillioides* both commonly infect corn seedlings shortly after planting and can be found ubiquitously in temperate regions around the globe. Understanding of the interaction between these two pathogens on corn is not well understood, although there is some evidence that co-infection may yield a synergistic disease response. The purpose of this investigation was to better understand the effects of the fungus upon the nematode in the corn host, and to determine if a synergistic disease effect on the development of the corn plant could be observed during the seedling stage. There was no evidence that the co-infestation of corn seedlings with the fungus and the nematode yielded a disease synergy, but across multiple different experiments, the presence of the fungus significantly reduced the ability of *P. penetrans* to colonize the corn seminal root system, as compared to the nematode alone. My study suggests that *F. verticillioides* influences parasitism by *P. penetrans*, which may in turn affect corn development in critical vegetative stages.

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CHAPTER ONE

Review of the Literature

Thesis organization:

This thesis is organized into three sections. The first chapter includes a review of the literature and statement of research objectives. Chapter two includes experiments in the growth chamber to elucidate whether *P. penetrans* and *F. verticillioides* interact to cause a disease synergy on corn seedlings, or if the co-infestation results in alterations in behavior on one pathogen by the other. Chapter three discusses in vitro and cellular investigations of the two pathogens to determine proximal relationships and a fungal preference assay.

Review of the Literature:

Pratylenchus penetrans

Pratylenchus penetrans is a migratory endoparasite that is capable of feeding and causing damage on a wide host range both from the outside and within a root (Zunke, 1990). Lesion nematodes can reduce the yields of a variety of agricultural crops, and can cause root discoloration, stunting, and chlorosis (Bernard and Laughlin, 1976; Dickerson et al., 1964; Smolik and Evenson, 1987; Todd and Oakley, 1996; Townshend, 1963a, 1963b). The eponymous lesions created at nematode feeding sites are visibly black or brown. Symptomology of plants infected by lesion nematodes is often confused or mistaken for other pathogens or abiotic factors, as stunting, chlorosis and wilting under heat can be attributed to other causes. Further, it is possible for lesion nematode species to cause root damage without creating aboveground symptoms, making nematode disease very difficult to spot.

Nematode feeding causes discolored, necrotic lesions to appear, which often expand longitudinally along the root, resulting in deterioration and collapse of cortical root tissue in the affected area along with root pruning and decrease of the root system in general. The lesion may expand and lead to girdling of the root, causing the death of distal sections of root beyond the lesioned area. Adult *P. penetrans* can vary in size, but have been reported to center at around 450µ in length, with J2 juveniles roughly half that size (Townshend, 1991). *Pratylenchus* species can vary in length between at least 330µ to ~800µ (Bhatti, 1984). In tobacco and strawberry, nematodes are capable of infecting well behind the root cap, sometimes focusing around or above the zone of root elongation, and have been shown to also feed upon root hairs in strawberry (Kurppa et al., 1985; Mountain, 1954; Zunke, 1990).

Root lesion nematode feeds upon cortical cells through the use of its needle-like stylet, which can rupture plant cell walls. As the nematode moves through the cortical parenchyma, it may leave a trail of necrotic tissue in its wake. Female nematodes commonly lay eggs in root tissue, although eggs may also be laid in the soil as well. Root lesion nematodes hatch from their eggs in the J2 stage; any vermiform nematodes in life stages that have emerged from the egg are infective and will begin to feed when in contact with root tissue. Like other phytopathogenic nematodes, *P. penetrans* is capable of producing an array of cell wall degrading enzymes including cellulases, which improve the ability of the nematode to colonize and feed upon plant tissue (Uehara et al., 2001)

Pratylenchus penetrans, unlike most other species in the *Pratylenchus* genus, is capable of sexual reproduction as well as parthenogenic reproduction, and completes its life cycle within roughly 30 days. Juveniles in the J2 stage emerge from eggs as they hatch, and will molt through two additional juvenile stages and finally to the adult stage. All vermiform life stages from J2-adult are infective, and can damage root tissues on any part of the root system, not just root tips or hairs. *Pratylenchus penetrans* primarily reproduces sexually, although parthenogenetic reproduction is also possible from an individual female (Thistlethwayte, 1970).

All vermiform stages of root lesion nematode (*Pratylenchus* species) are capable of feeding both ectoparastically and endoparasitically, utilizing their stylet to penetrate host cells, secrete saliva, and feed upon cytoplasm. When feeding on corn, *P. penetrans* exhibits some social behavior – nematodes encounter each other when browsing, probing, and thrusting epidermal cells and root hairs with their stylet, sometimes being dislodged by other feeding and browsing nematodes, and can often be found feeding on roots in concentrated areas (Rebois and Huettel, 1986). Although penetration of epidermal cells with the stylet is not always successful, *P. penetrans* may enter epidermal or cortical cells after puncturing, or may stay localized at a solitary feeding side ranging from one minute to multiple hours (Rebois and Huettel, 1986). Rebois and Huettel also observed *P. penetrans* entering and exiting damaged corn roots freely, sometimes leaving damaged root channels only to be replaced by another nematode. Male *P. penetrans* have been reported to exit roots of pea more frequently than females, perhaps resulting from

an attraction to females, and egress behaviors may not be dependent on the conditions of nearby roots (Wixted and MacGuidwin, 1990)

Nematode feeding does not pass the endodermis or enter vascular tissues. However, the endodermis is one of the first areas of the root to turn brown, likely due to higher concentrations of phenolic compounds at that location (Zunke, 1990). Despite the fact that nematode feeding is limited to the cortex of the root, *P. penetrans* vermiform stages possess the ability of moving within and outside the root in order to select the best available feeding site (Kurppa et al., 1985). When colonizing the root, the nematode may move either inter- or intracellularly, but after penetrating the root, they move intercellularly throughout the root (Zunke, 1990). Interestingly, although root lesion nematode is confined to cortical tissues, lesions may include damaged cells outside of the cortex, as lesions are caused by both physical and biochemical influences. The secretion of chemicals including β-glucosidase by *P. penetrans* resulted in the release and subsequent oxidation of phenolic compounds by the plant, which was concluded to be the primary causal agent of the browning and necrosis in lesioned areas (Townshend et al., 1989).

Pratylenchus species tend to exhibit the highest rates of population growth in the temperature range of 25-30 degrees Celsius. *Pratylenchus penetrans* reproduced most aggressively at 25 degrees on soybean, completing its life cycle in approximately 30 days. There is considerable variability in reproductive rates and environmental preferences across species within the *Pratylenchus* genus (Acosta and Malek, 1979). Nematode populations tend to increase as crops develop more suitable habitat for their colonization (Lamindia, 2002; Todd and Oakley, 1996). In

the spring, there is a large percentage of the nematode population in root fragments. The proportion of nematodes in roots will change over time as nematodes migrate from within roots to the soil and back again.

Plants infected by lesion nematodes often appear in clusters in the field; the patchy nature of lesion nematode infection is characteristic of many nematode species due to limited motility in the soil. It has been shown that nematode populations can be larger in areas of the field where farm tillage equipment enters first (Morgan et al., 2002), perhaps suggesting that the nematode may be transported from field to field by machinery that comes into contact with nematode-infested soil. Root lesion nematodes may overwinter in either soil or infested plant debris – once a field is infested, it is difficult to eradicate the pathogen due to its ability to persist in plant material or living freely in the soil.

In addition to being a significant problem in perennial agricultural systems, lesion nematodes are also known to multiply well on corn and soybean and other commonly rotated crops (Bélair et al., 2002). According to publicly available extension outreach materials, using damage thresholds to predict crop losses is still difficult across the *Pratlyenchus* host range, due to dependence on edaphic and environmental conditions, including soil nutrients, organic matter, moisture and temperature (U Illinois Extension, 1999).

Importance

Although reliable economic data on the global importance of nematode pests is lacking, there is enough information available to clearly illustrate that nematode

pests pose a significant threat to agriculture especially in tropical regions; agricultural losses in tropical and subtropical agroecosystems is as high as 14.6%, with losses of 8.8% in developed temperate regions (Nicol et al., 2011). In Nigeria, a 50% increase in root lesion nematode density was correlated with a 28.5% yield reduction of maize at harvest, illustrating potentially significant harms particularly in the developing world (Egunjobi et al., 1975).

Pratylenchus spp. is the most common nematode pest of corn in Midwestern states including Wisconsin, Iowa and Michigan (Warner, 2008; Morgan et al., 2002). *P. penetrans* has been correlated with reductions in maize root size, and height and diameter of plant stalks in Wisconsin fields, as well as being associated with reductions in potato yields in the state (Dickerson et al., 1964). Further, root lesion pressures on corn in Wisconsin are on the increase, due to changing insecticide use, more corn-on-corn rotations and the adoption of transgenics reducing soil insecticide use.

Economic losses due to all pathogenic nematode species on corn has been estimated at nearly $23bn per annum in the United States, with *Pratylenchus* species in the top three most important nematode pests along with the genera *Meloidogyne* and *Hoplolaimus* (Koenning et al., 1999). There is evidence that root lesion nematode may serve as a limiting factor for the growth of the corn plant; in South Africa, the use of nematicides led to a yield increase of between 33-128%, while in the US, yields increased from 10-54% (McDonald et al., 2005). As with other nematode pests on other hosts, a major challenge with lesion nematode on corn is

the fact that it can cause yield reductions without visible aboveground symptomology, making management difficult.

Root lesion nematode can be a pest of importance in organic vegetable and/or cereal regimes, with reported incidence of *Pratylenchus* species over 90% in Germany, with pressure building up over time as populations increase in the soil (Hallmann et al., 2007). These problems are exacerbated by sandy soils that facilitate nematode movement, and hence infection.

Corn

Corn is the highest value crop grown in Wisconsin (USDA NASS), and is essential to animal production, the fuel ethanol industry, and global food security. Some experts believe that nematodes are often underestimated as a pathogen to corn, as they pose a "silent threat" – the symptoms they cause on the host plant resemble the effects of other pathogens, and yield losses are often not attributed to the nematode pest (Holgado and Magnusson, 2012). The majority of farmers in Wisconsin have the potential to improve their yields, as *Pratylenchus* spp. have been found in over 90% of surveyed fields in the state in the months of June and July (MacGuidwin and Bender, 2012). *P. penetrans* also parasitizes and can increase populations on soybean, which is the most commonly rotated crop with corn in the American corn belt.

During the early vegetative growth stages of the corn plant, pathogens may be able to disrupt the transition between reliance on stores of kernel energy and the embryogenic seminal root system to dependence upon the adventitious root system.

Soil-born pathogens contact specific root parts to cause infection during the seedling stage of corn development: the mesocotyl, which attaches the radicle and seminal root system to the nodal roots and the shoots, and the seminal roots themselves. Despite the fact that the seminal roots can persist for the duration of a corn plant's development, even supporting a mutant variety to maturity that completely lacks adventitious roots (Hetz et al., 1996), the seminal roots diminish in importance after serving as the primary portion of the seedling rootstock for the first two weeks (Hochholdinger et al., 2004).

The ability of the seminal roots to provide water to the corn seedling through this transition is important as the plant develops past this sensitive period. Seminal root development in early seedling stages (14 days post-planting) has been shown to support plant resilience against root pruning (Andrew and Solanki, 1966), and diameter of the primary seminal root has been shown to be negatively correlated with lodging as the corn plant reaches maturity (Stamp et al., 1992). However, at later seedling stages (21 days post-planting), the proportion of seminal roots to total root weight was negatively related to final height and ear weight (Andrew and Solanki, 1966). Further, the proportion of seminal roots at later stages of development has been suggested to be negatively correlated with plant growth characteristics. In the greenhouse, adventitious roots at 28 and 25 days post-planting were positively correlated to resistance to root pulling and root clump weight, while seminal root weights were negatively correlated (Nass and Zuber, 1971). These results may indicate that the proportion of seminal roots to total roots is initially an indication of successful a seminal root growth, but later a measure of

successful adventitious root system development. Seminal roots decrease in importance as the adventitious roots vigorously grow to replace them, reducing the proportion of total root mass occupied by the seminal roots, but those seminal roots provide important developmental assistance to the plant as the adventitious roots are emerging and growing.

Corn is also sensitive to early developmental stages because it is not as effective as other crops at compensatory growth; if early plant growth is compromised, corn has difficulty recovering. Some evidence for this is present in negative correlations found between row spacing variability and yield at harvest (Martin et al., 2005; Nielsen, 2001), along with yield losses correlated with poor emergence and stand establishment (Liu et al., 2004). However, there have been conflicting findings on the relationship between spacing variability and yield, calling the relationship into question (Lauer and Rankin, 2004).

Nematodes are a ubiquitous parasite of corn; it is likely that most if not all corn plants grown around the world are parasitized by nematodes to some degree. Maize can often be enormously tolerant of nematode endoparasitism. Populations as high as 40,000 nematodes per gram of dry root can be found, albeit with concomitantly poor root performance, with as many as 20,000 individuals per gram of root commonly found in the field (Norton, 1983).

Lesion nematodes attack corn seminal roots when exposed to them immediately following germination and the emergence of the radicle - in corn, the seminal root system is the first to develop, which supports the seedling through important development and establishment phases. *Pratylenchus* species are first

exposed to mesocotyl and seminal root parts, as they are the first belowground tissues to develop in the corn plant, and the concentrations of lesion nematodes in seminal roots will grow throughout seedling stages but have been shown to decline after 30 days of plant development (Todd and Oakley, 1996). Corn plants transition from dependence upon kernel-based energy reserves to the dependence upon nodal roots as a seedling grows; adventitious roots appear around V2 and become the dominant player in the corn root system around V6 (Abendroth, 2011), and continue to grow during all vegetative stages. The adventitious roots grow very quickly after their appearance and will also be targeted by the nematodes, but due to their high rate of development, may "outgrow" any symptomology caused by the nematodes. Following the first 30 days of development, all of the population increase of *Pratylenchus neglectus* and *Pratylenchus scribneri* was shown to occur in the adventitious roots, not the seminal roots (Todd and Oakley, 1996). The seminal root system stops its growth in the late seedling stage and diminishes in importance to the plant as the nodal/adventitious root system develops around it. There is some evidence that *Pratylenchus penetrans* hatches from eggs at a higher rate when in the presence of young corn plants as compared to older ones (Pudasaini et al., 2008). The number of lesion nematodes present in the root system of a corn plant has been shown to be significantly correlated with final yield levels as well as seed test weight – particularly in the seminal root system (Todd and Oakley, 1996). Therefore, the initial growth stages of the plant and initial population densities of *Pratylenchus* species in the soil are important factors in determining the level of disease caused by lesion nematodes.

The relationship between lesion nematode species and maize is an old one, because *Pratylenchus hexincisus* is pathogenic on teosinte, the ancestor of modern cultivated corn (Norton, 1983). *Pratylenchus* species have different pathogenicities on different corn hybrids, but the factors that mediate the relationship between nematode and plant are not well known. There has been work suggesting that different cultivars of corn may be more tolerant to soils infested with *Pratylenchus* species, as different inbreds were shown to result in differential levels of nematode population development when exposed to *P. hexincisus* and *P. scribneri* (Smolik and Wicks, 1987). Little work has been done to develop crop cultivars resistant to root lesion nematode; there are some commercially available varieties of alfalfa that are resistant to *Pratylenchus penetrans* (Samac and Kinkel, 2001), but no work has been done in corn.

Damage Thresholds

In order to specify management options that are effective and economical for farmers, it is important to identify thresholds for nematode populations at which disease on crops begins to occur. Extrapolating from nematode threshold values in order to predict nematode damage can be difficult, as it depends on nematode species, corn cultivar, environmental conditions and ecological niches (Norton, 1983). Additionally, as with other plant pathogens, biochemical stressors can weaken plant defenses and resilience, and can affect the amount of disease caused by a particular nematode inoculation density; the health of the crop has an influential effect on root lesion damage thresholds. Initial population densities of

nematode species are the primary determinant of nematode disease on agricultural crops, followed by environmental factors. This is because of the low mobility of nematodes in field soil, their relatively low reproductive rates and the fact that there are not many roots on seedlings; as plants mature, compensatory root growth can reduce the impact of nematode damage (Barker and Olthof, 1976). In addition to damage variability caused by environmental and abiotic factors, there can be significant differences between nematode densities that cause disease in the field compared to disease-causing thresholds under greenhouse and growth chamber conditions (Barker and Olthof, 1976).

In the case of *Pratylenchus* species, there is an array of differing thresholds available to farmers. For example, *Pratylenchus brachyurus* reduced plant growth of maize at a level of 500 individuals per 5L pot, with penetration and nematode reproduction increasing as higher levels of inoculum (Chindo et al., 2006). For tobacco, a threshold of 500 individuals per kg of soil has been used (Reynolds et al., 2000). *Pratylenchus scribneri* was found to have approximately a 1% yield reduction in corn for every 1000 nematodes present per gram of root weight at harvest (Smolik and Evenson, 1987). Data from field experiments in Iowa made available through extension publications suggest that 1000 root lesion nematodes per 100 cc of soil is an appropriate benchmark damage threshold, but may vary significantly across different environmental conditions, cultivar used, and cultural practices employed by the farmer (Nyvall and Norton, 2011). In the warmer state of Delaware, university extension uses a threshold of 800 root lesion nematodes per 250 cc of soil during fall sampling, while the spring sampling threshold is anything

over 500 nematodes per 250 cc (Mulrooney, 2012). Quantitative recovery of nematodes from soil and roots is managed using a number of different techniques and with highly variable rates of success (Viglierchio and Schmitt, 1983; Whitehead and Hemming, 1965). In loamy sand soils, recovery rates of *Pratylenchus vulnus* from soil in controlled experiments was reported to center roughly around 30%, with changes across nematode species, soil type, and recovery method resulting in variation in recovery between ~2% and ~50% (Viglierchio and Schmitt, 1983).

Interactions With Other Pathogens

A synergistic interaction can be defined as an instance in which simultaneous infection with the two parasites results in damage that is greater than the sum of each pathogen in isolation (Back et al., 2002). This may manifest as significant disease symptoms caused by concurrent inoculation of both pathogens at levels of pathogen pressure where individual inoculation does not harm the plant (Rotenberg et al., 2004). Potato early dying, a synergy of *Pratylenchus penetrans* and *Verticillium dahlie,* is a well-studied example of this. *Pratylenchus* species are the nematodes most commonly found to be in association with fungal pathogens in disease complexes, along with the genera *Globodera, Heterodera, Meloidogyne* and *Rotylenchus*. The most common fungal genera in nematode-fungal disease complexes include *Fusarium, Verticillium, Phytophthora, Pythium* and *Rhizoctonia* (Back et al., 2002).

While plant parasitic nematodes are important pests of crop plants on an individual basis, a few genera, such as *Meloidogyne* and *Pratylenchus*, have been

shown to serve as essential components of disease complexes involving other types of pathogens. Some studies suggest that nematode infection increases the susceptibility of the plant to bacterial and fungal pathogens, while nematodes also can serve as vectors for viral, bacterial and fungal diseases (Bergeson et al., 1970; Dropkin, 1969; Griffin et al., 1993; Harrison, 1964; Hillocks, 1985; Jonathan et al., 1997; Kerry, 2000; Marley and Hillocks, 1994; Mayo and Bergeson, 1970; Riedel, 1988; Da Silva, 2010; Storey and Evans, 1987). Interestingly, in the "cauliflower disease" complex between an *Aphelenchoides* nematode species and a bacterium on strawberry, the combination of the two pathogens produce symptoms that do not present under infection of either pathogen individually (Crosse and Pitcher, 1952).

Synergistic disease resulting from simultaneous nematode and fungal infection has been observed for over a century. Root knot nematode and *Fusarium* wilt of cotton forms a disease complex that was first documented in 1892 – a disease complex that is still relevant today (Atkinson, 1892). The *Fusarium* – *Meloidogyne* complex has since been identified as extant within many other crops other than cotton, including beans (France and Abawi, 1994), chickpeas (Maheshwari et al., 1995), tomatoes (Abawi and Barker, 1984), and coffee (Bertrand et al., 2000). Experiments done on coffee, tomato and bean utilized a staggered inoculation method, however, where the fungus was introduced to the plant weeks after the nematode. There is evidence that in the case of *Meloidogyne-Phytophthora* interactions on alfalfa, nematode reproduction rates are not altered if the fungus is post-inoculated, but reproduction is reduced if the fungus is introduced prior to the

nematode; this disease complex also showed increased levels of plant damage when the nematode was inoculated first (Griffin et al., 1993).

There are multiple ways by which nematode infection can facilitate further disease development caused by secondary pathogens. All plant pathogenic nematodes feed by the use of a needle-like stylet, sometimes causing mechanical damage to host cells (Rebois and Huettel, 1986), which is thought to sometimes serve as an infection court for bacterial or fungal entry; however, mechanical wounding does not always result in increased fungal infection (Hart and Endo, 1981). Beyond simple mechanical development of infection courts, nematodes can also secrete effectors and otherwise induce biochemical changes in the plant that may predispose the crop to secondary infection; further, nematodes can cause changes to the types of root exudates produced by plants, that may stimulate fungal germination or alter the community composition of the rhizosphere in ways that can promote successful infection by other pathogens (Bergeson, 1972; Bowers et al., 1996).

Potato early dying (PED) is one of the most well known nematode-fungal synergies in agricultural systems. PED can result in the disruption of a variety of plant functions, including transpiration, carbon assimilation, photosynthesis and stomatal conductance, and can also lead to a reduction in tuber weights of up to 75% and the reduction of specific gravity values, which is an important processing characteristic for potatoes (MacGuidwin and Rouse, 1990; Rotenberg et al., 2004; Saeed et al., 1997). *Verticillium* passes through cortical root tissue on its way to the vasculature of the plant, which suggests that root lesion nematodes may provide an

avenue by which the fungus can enter the xylem (Mountain and McKeen, 1965) aside from the common fungal entry method of targeting undifferentiated tissue at the root tip in order to avoid the endodermal barrier. Some evidence is present that *Verticilllium* may interact with *P. scribneri* at high inoculum levels, as well as *P. neglectus*, so the ability of the fungus to interact with numerous species in the *Pratylenchus* genus other than *P. penetrans* is not entirely clear (Hafez et al., 1999; Riedel et al., 1985). *Pratylenchus* species and *V. dahlie* can also combine to exacerbate disease symptoms on both strawberry and mint (Faulkner, 1970; Mckinley and Talboys, 1979).

In peppermint, it was shown that *Pratylenchus minus* exacerbated the occurrence and severity of peppermint wilt when co-inoculated with *Verticillium dahlie*, even when the pathogens were exposed to separate, isolated sections of root. This suggests that the effect of the two pathogens on one another is likely other than a simple mechanical creation of infection courts by the nematode; rather, nematode infection may be creating systemic biochemical changes in the plant that improve the ability of the fungus to cause disease in remote locations of the root (Faulkner, 1970; Rotenberg et al., 2004).

The timing of pathogen inoculation matters for the course of the disease complex as well. There is evidence that when *V. dahliae* is introduced following the nematode, disease progress may not differ from when the fungus is inoculated on its own. As nematodes enter a potato root and progress through the cortex, the plant seals over the entry wound with lignified tissue that may prohibit the entry and proliferation of the fungus (Storey and Evans, 1987).

Pratylenchus penetrans is a pathogen of corn of significant importance, not only for its ubiquity in Wisconsin and ability to cause disease and yield reductions, but also because of its association with fungal pathogens in disease complexes that further exacerbate crop harm. It is important to better understand how *P. penetrans* relates to other common fungal pathogens on corn in Wisconsin, in order to better manage corn fields for the prevention of yield loss.

Fusarium verticillioides

Fusarium verticillioides, previously known as *Fusarium moniliforme*, has been known to be pathogenic on corn since at least 1904, as reported by JL Sheldon (Sheldon, 1904). It is the most commonly found fungal pathogen of maize, and can be recovered from most fields in plant debris following harvest (Leslie, 1996). *Fusarium verticillioides*, along with other anamorphs of *G. fujikuroi*, are the most commonly associated fungi with corn grown in temperate regions, including North America (Munkvold and Desjardins, 1997). *F. verticillioides* is capable of causing multiple different diseases on different parts of the corn plant, including ear rot, stalk rot and root rot and seedling disease (Limber, 1927; Munkvold and Desjardins, 1997). The species name of the fungus was effectively changed from *moniliforme* back to the original *verticillioides* in 2003, at the behest of the International Society for Plant Pathology and the International Committee on the Taxonomy of Fungi, which has lead to uncertainty about the applicability of prior studies of *moniliforme* to the current species (Venturini et al., 2011). This is the generally accepted nomenclature for fungal isolates retrieved from corn.

Seedling disease caused by *Fusarium verticillioides* can result from either exposure to soilborne fungal structures surviving on plant debris, or from systemic infection of diseased kernels (Munkvold et al., 1997a). The disease manifests as reduced root and shoot development or emergence (Desjardins et al., 1995; Ocamb and Kommedahl, 1994). Stalk rot occurs when the fungus grows parasitically within the growing stalk, leaving a discolored and weakened physical structure that can increase risk for lodging and crop loss, becoming more severe as the plant further develops (Limber, 1927; Osunlaja, 1990).

Ear rot is also of significant importance, as increased infection by *Fusarium verticillioides* can lead to the accumulation of fumonisins, a potent and harmful class of mycotoxin, as well as decreases in grain quality and yield (Munkvold et al., 1997b). *Fusarium verticillioides* produces mycotoxins called fumonisins, which are harmful to mammals including humans. Fumonisins are known to be the causal agent of equine leukoencephalomalacia and porcine pulmonary edema, and have been linked to esophageal cancer in humans in areas with high levels of fumonisin contamination of corn (Norred, 1993).

After the initial association of *F. verticillioides* with seedling blight of corn in 1904, controversy ensued regarding the causal nature of the fungus to the disease, which was ultimately settled when *F. verticillioides* was determined to be a causal agent using new techniques (Kommedahl and Windels, 1981; Yates et al., 2003). This is due to the high level of variation in disease depending on corn cultivar, fungal isolate and environmental conditions, which create significant variability in seedling disease outcomes (Bacon et al., 1994).

Different corn genotypes possess highly variable levels of susceptibility to the fungus, which results in a wide range of disease across different infected cultivars. This variation in susceptibility is thought to be under genetic control (King and Scott, 1981). Resistance is understood to function in two main phases: the ability to prevent or slow fungal entry into the plant, and the resistance to the proliferation and expansion of fungal biomass throughout plant tissue. However, there are no known fully-resistant cultivars of corn to *Fusarium* ear rot that are commercially available (Afolabi et al., 2007; Alessandra et al., 2010).

F. verticillioides can infect the corn plant through multiple pathways. The fungus can cause infection through the silks; after airborne or water-splashed conidia come into contact the silks, the fungus travels down the silks to infect individual corn kernels, usually only causing damage on a subset of the infected kernels (Headrick and Pataky, 1991; Munkvold et al., 1997a). In parts of the world where seed is saved, it is possible for these infected kernels to be re-planted the following year. Because these systemically infected seeds harbor the fungus internally, eradication via seed treatment is very difficult (Limber, 1927).

Another important inoculation pathway involves insect damage: in temperate areas, the incidence of both asymptomatic infection with *Fusarium verticillioides* and ear rot disease was correlated with the level of insect injury (Munkvold and Desjardins, 1997; Munkvold et al., 1999). The fungus is also capable of entering maize ears and causing ear rot through husk damage caused by birds and hail, in addition to insects (Bakan et al., 2002).

In addition to other pathways, *F. verticillioides* systemically and locally infects corn plants through the soil. The fungus can overwinter in contaminated crop residue and infect corn kernels upon planting, or can systemically infect the plant through seed that has been infected (Bacon and Hinton, 1996). Seed transmission of *F. verticillioides,* either through internal or external kernel infection, is primarily associated with seedling disease, while it may also lead to systemic infection of the plant, potentially causing either stalk or kernel rot (Munkvold and Desjardins, 1997). The relative importance of seedborne or soilborne inoculum sources, as the cause of systemic infection by *F. verticillioides*, is not well understood (Leslie et al., 1990; Oren et al., 2003; Rheeder and Marasas, 1998).

Early in the process of seedling infection by soil-borne fungal propagules, *F. verticillioides* colonizes and is limited to basal organs of the plant, such as mesocotyl, roots and crown, before eventually moving up into aboveground plant parts. Through the use of fluorescence microscopy, Oren et al found that at 25-35 days post planting, after an initial phase of infection confined to the lateral roots and mesocotyl, *F. verticillioides* will rapidly expand within the mesocotyl and the main seminal root and initiate rotting (Oren et al., 2003). Passing the crown into the stalk requires evading host defenses and is likely contingent upon favorable environmental conditions, senescence functions being initiated in the host, and additional sources of inoculum entering through the husks via airborne conidia (Lawrence et al., 1981; Venturini et al., 2011; Wilke et al., 2007). Although there is some evidence that corn plants appear to be able to outgrow systemic infection by the fungus during early stages of growth and avoid the development of symptoms,

the infection may turn symptomatic under abiotic stress conditions (Venturini et al., 2011).

It is also important to mention that *Fusarium verticillioides* is an effective facultative saprophyte, not an obligate parasite (Bacon et al., 2001). The fungus can survive effectively by feeding off of maize stover or other plant debris, while producing conidia that serve as inoculum for infection of future crops. While it does not produce chlamydospores, structures of hardened sections of mycelia appear to improve survival capabilities (Kommedahl and Windels, 1981). The fungus has the ability to infect within three days of planting, and often targets lateral roots and the mesocotyl, which connects the seed and developing seminal roots with the crown and shoots of the plant (Murillo et al., 1999; Oren et al., 2003).

Endophytic Behavior

Fusarium verticillioides is known to be pathogenic on corn, causing significant disease and yield reductions, but it is also increasingly understood to operate asymptomatically within the corn plant as an endophyte (Bacon and Hinton, 1996; Bacon et al., 1992; Munkvold and Carlton, 1997; Munkvold et al., 1997b; Yates et al., 1997). Importantly, it is not well understood why *F. verticillioides* commonly causes asymptomatic infection, how it causes rotting when infection does lead to disease, and the determining factors for whether fungal colonization leads to symptom development (Oren et al., 2003). Because of its ubiquity in corn plants around the world and the commonly asymptomatic nature of systemic infection, *Fusarium verticillioides* can be considered an endophyte of corn under many circumstances.

Despite the fact the *Fusarium verticillioides* does not confer detrimental effects upon plant growth when operating as an endophtye, and has been at times shown to boost plant development under certain circumstances (Yates et al., 1997), there are important negative considerations to endophytic growth. Fumonisins may accumulate in kernels of corn, even if the plant has been infected asymptomatically (Munkvold and Desjardins, 1997; Munkvold et al., 1997b; Nelson et al., 1993), and it is also possible for what starts as asymptomatic infection to progress into disease if abiotic stresses are encountered, or other biotic variables change (Abbas et al., 2006; Bacon and Nelson, 1994; Venturini et al., 2011). Indeed, it has been posited that the endophytic colonization of maize by *F. verticillioides* offers a significant challenge to the eradication of fumonisin contamination of grain because the fungus does not produce visible plant or kernel symptoms that can be utilized to cull contaminated grain (Fandohan et al., 2004).

Further, whether *F. verticillioides* infection confers beneficial properties to the host plant, likely through the stimulation of plant growth hormone production, is still in question; there is evidence that when functioning as an endophyte, *Fusarium verticillioides* not only does not induce plant disease, but actually possesses the ability to improve plant growth (Bacon et al., 2008; Mańka, 1979; Munkvold and Desjardins, 1997; Yates et al., 1995, 2005). It is importance to limit the colonization by *F. verticillioides* because fumonisins can accumulate even under asymptomatic infection, but if the fungus causes improved growth characteristics as an endophyte, complete eradication of the fungus may possibly have some negative effects under conditions that favor endophytic colonization. This effect has been

encountered before in other crops; much effort and expense was undertaken to remove endophytes from fescue, only to reduce the fitness of the crop to the point of being unusable as a forage (Rice et al., 1990).

In addition, there is some evidence that endophytic *F. verticillioides* can protect a corn plant against infection by more virulent and destructive pathogens, including *F. graminearum* and *Aspergillus flavus*, which in turn produce aflatoxins – another harmful class of mycotoxins (Van Wyk et al., 1988; Zummo and Scott, 1992). Lee et al found that co-inoculation of an endophytic strain of *F. verticillioides* with *Ustilago maydis* resulted in significant decreases in levels of corn smut, and increases in plant growth (Lee et al., 2009). However, this effect was not observed when *F. verticillioides* was pre- or post-inoculated with the pathogen, suggesting that the protective effect may not be a result of systemic acquired resistance in the plant.

When *F. verticillioides* is systemically present in infected corn seed, it has been shown to protect the kernel against colonization by other fungi including *F. graminearum* and *Dipolodia* species (Rheeder et al., 1990; Van Wyk et al., 1988). Van Wyk et al found that preinoculation with *F. verticillioides* before exposure to *F. graminearum* resulted in significantly greater seedling weights than when plants were exposed to *F. graminearum* without preinoculation, suggesting that *F. verticillioides* may protect seedlings from infection or at least slow disease development of other pathogens. Analysis of infected kernels from the field showed that *Fusarium verticillioides* kernels were strongly negatively correlated with coinfection by *Diplodia* species, as well as with *F. graminearum*, suggesting a

protective effect may be at play (Rheeder et al., 1990). Further, seed treatment with both live and dead particles of *F. verticillioides* has been found to offer a protective effect against future *F. verticillioides*-induced stalk rot, as caused by direct inoculation of the pathogen into plant stalks at later stages of development (Martins et al., 2014).

At a cellular level, *Fusarium verticillioides* alters its behavior in the host plant when operating as an endophyte compared to infecting as a pathogen; mycelia expand through the plant via intercellular movement in the endophyte mode, while symptomatic infections are correlated with intracellular and intercellular movement (Bacon and Hinton, 1996). The fungus is also known to induce plant biochemical changes under asymptomatic infection, causing increased deposition of lignin in shoots compared to non-infected plants, which may possibly alter the success rate of secondary infections (Yates et al., 1997). Asymptomatic infection of maize by *F. verticillioides* has been documented in many different cultivars (Bacon et al., 2001), although it is known that pathogenicity of the fungus on corn is dependent upon fungal isolate and cultivar. *F. verticillioides* is heterothallic and possesses a high level of genetic variability (Gohari et al., 2008), which may be a determinant of the variability in disease and endophytic behavior across different fungal isolates.

Fusarium verticillioides and Pratylenchus penetrans

Pratylenchus penetrans and *Fusarium verticillioides* colonize the roots of corn plants. The fungus and the nematode both commonly persist in soil; root lesion

nematode is able to overwinter in soil-borne root fragments as well as in the soil proper (Townshend, 1984), while *F. verticillioides* is capable of surviving in soil as a facultative saprophyte (Bacon et al., 2001). At the time of planting, both pathogens are present in soil and, in infested soils, encounter the corn plant immediately. The fungus and the nematode are both known to affect corn seedlings soon after encountering the plants, with the fungus capable of causing visible seed rot after three days of exposure (Yates et al., 2003) and some nematodes beginning to feed inside root tissues three days after infestation (Rebois and Huettel, 1986). *P. penetrans* is common across vast geographical areas, as is *F. moniliforme* (Munkvold et al., 1997a), particularly in temperate areas including the Midwest of North America. Overlap of the two pathogens in field systems is thus expected to be quite significant.

Both *Fusarium verticillioides* and *Pratylenchus penetrans* are virulent on corn in Wisconsin individually, and it is presupposed that there may be an interaction between the two, although evidence for a quantitative disease synergy is mixed (Jordaan et al., 1987; Palmer, 1974; Da Silva, 2010). Prior studies that have investigated the relationship between these two pathogens on corn have found conflicting evidence of effects, with only one study presenting evidence for a quantitative disease synergy where the combination of the two pathogens resulted in a level of disease greater than the sum of the two individual pathogen treatments (Da Silva, 2010).

Effects of the two pathogens upon each other in the corn plant have also been contradictory in the literature. Two studies illustrated a suppressive effect on the

nematode from the fungus (Palmer, 1974; Da Silva, 2010), one showing an augmentative effect on *Pratylenchus* spp. *zeae* and *brachyurus* from the fungus (Jordaan et al., 1987), and one research abstract showing no evidence of any combined effects whatsoever (Roth and Boothroyd, 1976). The relationship between these pathogens has not been adequately investigated and is not well understood.

There are various ways by which *Pratylenchus penetrans* and *Fusarium verticillioides* might interact within a plant to cause disease synergy – mechanisms that have been well explored in other disease complexes. In the process of the nematode feeding on cortical root tissues, it is thought that the weakening of the cortex and the accumulation of various compounds may render the increasingly susceptible to fungal attack (Riedel, 1988). Furthermore, the simple act of feeding by a migratory endoparasitic nematode such as *P. penetrans* creates microscopic wounds in root tissues that may serve as infection courts for fungal colonization and channels for movement. This is an observed phenomenon on sugar beet between *Rhizoctonia schachtii* and *Heterodera schachtii* (AG et al., 1969). Attempts have been made to mimic the mechanical wounding of feeding lesion nematodes on plant roots, which have indicated that this physical wounding phenomenon can be capable of inciting increased disease in combination with fungal inoculation (Inagaki and Powell, 1969). It is also possible that root wounding may result in changes in root exudation, which may have the ability to stimulate fungal germination and chemotaxis towards plant tissues (Riedel, 1988).

However, there are also known instances where nematode-fungal interactions can occur in a systemic manner – where physical separation between points of entry of the two pathogens still results in synergistic disease effects. This has been shown to be the case with Potato Early Dying, where *Verticillium dahlie* and *Pratylenchus penetrans* form a disease complex on potato (Rotenberg et al., 2004). In this case, it is unlikely that physical damage caused by nematode feeding is the primary causal mechanism of the disease synergy.

It is important to more accurately understand the relationship between these two pathogens upon each other in corn plants to be able to provide accurate and applicable management guidelines to growers who will encounter these ubiquitous pathogens in their fields. At the moment, farmers may manage for the nematode pest or the fungal pathogen at an individual level, but if the two create a disease synergy, it will be necessary for growers to assess the burden of both pathogens in order to manage for them at the same time. In addition, better understanding of any interaction between *P. penetrans* and *Fusarium* on corn may be of aid in moving to more biologically-based pest control strategies, avoiding the use of potentially harmful nematicides and fungicides.

Research Objectives:

The objectives of this research were to:

1. Elucidate the effect of *Fusarium verticillioides* upon the nematode *Pratylenchus penetrans* in the corn plant

2. Investigate whether the combination of these two pathogens results in a disease synergy in corn, as measured by plant growth metrics through the seedling stage.

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CHAPTER TWO

Investigating disease synergy between *P. penetrans* and *F. verticillioides* on corn in the growth chamber

Abstract

Pratylenchus penetrans and *Fusarium verticillioides* are both common soilborne pathogens of corn and infect corn seedlings shortly after planting. It is presupposed that there may be an interaction between the two pathogens, but the literature is sparse and conflicted on this topic. The purpose of this investigation was to better understand the effects of the fungus upon the nematode in the corn host, and to determine if a synergistic disease effect on the development of the corn plant could be observed during the seedling stage. There was no significant interaction for disease in a single experiment conducted four times, or in three additional experiments conducted with an alternate method of fungal infestation and three different nematode inoculum levels, but there were some indications that further study is warranted. In multiple different experiments, the presence of the fungus significantly reduced the ability of *P. penetrans* to colonize the corn seminal root system, as compared to the nematode alone. My study suggests that *F. verticillioides* influences parasitism by *P. penetrans*, which may in turn affect corn development in critical vegetative stages.

Introduction

Pratylenchus spp. (root lesion nematode) is an important nematode parasite of corn in a wide range of hosts all over the world. The most common and important pest nematode genus of corn in the Midwest corn belt (Koenning et al., 1999; Norton, 1983), *Pratylenchus* occurs throughout temperate regions worldwide. *Pratylenchus penetrans* damages epidermal and cortical cells by puncturing them with its stylet, releasing plant-degrading enzymes such as cellulases (Uehara et al., 2001), and entering the root to feed. The majority of nematodes enter roots as endoparasites (Zunke, 1990), causing necrotic lesions, which normally expand longitudinally along the root, but can girdle roots as well, leading to the collapse of cortical tissues, root pruning and general decrease in root vitality and root system size. The damage caused by root lesion nematode is confined to epidermal and cortical tissues but lesions can involve cells not directly encountered by the nematode (Townshend et al., 1989). *Pratylenchus penetrans* within seminal roots have the greatest influence on corn seedlings; nematode density within the seminal root 30 days after planting was a better predictor of yield loss than population densities in the root at mid-season (Todd and Oakley, 1996).

Fusarium verticillioides is also a common pathogen on corn in Wisconsin and throughout the world (Norred, 1993). The fungus damages similar organs as the root lesion nematode, colonizing mesocotyl and root tissues in the early stages of corn plant development. Unlike *P. penetrans*, the fungus can enter the vascular system (Nelson, 1992) and spread past the crown towards systemic establishment in stalks, cobs and kernel tissues (Munkvold et al., 1997; Oren et al., 2003).

The fungus grows intercellularly as an endophyte or pathogen, but the factors that determine whether the fungus operates endophytically or pathogenically are not well understood (Yates et al., 1997). It is also presupposed that fungal hyphae, while operating as an endophyte, are in fact biochemically active rather than dormant and can be responsible for the accumulation of mycotoxins within plant tissue (Bacon and Hinton, 1996). Regardless of growth form, F. verticilliodes can produce fumonisins, secondary metabolites harmful to livestock and humans (Abbas et al., 2006; Bacon et al., 2008). Fumonisins are known to play a role in fungal virulence, but are likely not sufficient to cause disease on their own (Desjardins et al., 1995).

Both *F. verticilliodies* and *P. penetrans* are active in the seminal roots and mesocotyl of corn seedlings as plants transition between reliance on stores of kernel energy and the embryogenic seminal root system to a functional adventitious root system. Adventitious roots appear around V2 and become the dominant player in the corn root system around V6 (Abendroth, 2011). The ability of the seminal roots to provide water to the corn seedling through this transition is important as the plant develops past this sensitive period; seminal root development in early seedling stages (14 days) has been shown to support plant resilience against root pruning (Andrew and Solanki, 1966), and diameter of the primary seminal root has been shown to be negatively correlated with lodging as the corn plant reaches maturity (Stamp et al., 1992).

Disease complexes between *P. penetrans* and other fungi have been demonstrated for potato (MacGuidwin and Rouse, 1990; Rotenberg et al., 2004;

Saeed et al., 1997), but whether this nematode interacts with *F verticillioides* on corn is not well understood. One study concluded these two pathogens interacted synergistically to cause disease (Da Silva, 2010) and another reported no interaction (Palmer, 1974). For other crop-fungus-nematode systems, endophytes repressed nematode reproduction (Hallman and Sikora, 1996), so it is possible that *F. verticillioides* may influence P. penetrans, but it is also possible that *P. penetrans* may affect whether the fungus colonizes endophytically, potentially with a protective effect, or causes disease. It is important to understand these relationships in order to provide growers with accurate and effective management strategies. In the case of synergistic disease interactions, growers need to pay attention to and manage for the control of both pathogens at the same time, instead of one or the other. The objectives of my work were to 1.) confirm that P. penetrans and F. verticilliodes interact synergistically to cause disease to corn seedlings and 2.) determine if F. verticilliodes influences endoparasitism of corn by P. penetrans.

Materials and Methods

Experiments were performed in the growth chambers of Russell Laboratories at the University of Wisconsin-Madison, maintained at 28 degrees C, with a photoperiod of 14 hours under GE Hi-Lumen XL fluorescent bulbs. Each pot was fertilized at the time of planting. Pots were bottom watered daily with each pot placed in a separate saucer to limit contamination from any leaching that may occur.

Each experiment was conducted as a randomized complete block design with six replications and four treatments: *F. verticilliodes* alone, *P. penetrans* alone, both

pathogens together, and a no-pathogen control. One experiment, repeated four times, used inoculum of *F. verticilliodes* applied as a condial dip and a run time of 24 days. A series of three experiments, without repetition, used a cornmeal-based inoculum and a run time of 25 days.

Fusarium verticillioides A-1099 MAT-1, originally isolated from field corn in Kansas, was utilized in fungal treatments of all trials. *Fusarium* cultures were maintained at 4°C on Synthetic Nutrient Agar (SNA) and transferred every three months, while cultures for inoculum collection were grown on potato dextrose agar (PDA). A Wisconsin isolate of *P. penetrans* was used for all experiments. Nematodes were reared monoxenically on sweet corn cultivar "IO Chief" root explants incubated on Gamborg's B5 medium with vitamins and without plant growth hormones. Three to four month old cultures of *P. penetrans* were utilized for the collection of experimental inoculum, which consisted of all nematode life stages.

Soil utilized in growth chamber experiments was a Plainfield loamy sand collected from the Hancock Agricultural Research Station. The soil was sieved to remove debris and root fragments and steam pasteurization in an autoclave for one hr reaching a soil temperature of 80° C. Soil was then dried for a minimum of one week before storage, and was rested for a minimum of one month before use in experiments.

Experiments using a conidial soak method of infestation for F. verticilliodes

A condial soak infestation method adapted from Munkvold and Carlton (1997) was used for an experiment repeated four times (hereafter referred to as

trials). *Fusarium verticillioides* was cultured on PDA at ambient temperature in the dark for one week, before plugs were aseptically placed in CMC broth for agitation and formation of conidia. After five days of shaking in CMC broth for the development of conidia, hemocytometer counts of conidial density were performed and conidial concentrations adjusted with sterile water and Tween 20 to reach a density of $1x10^7$ conidia per mL and concentration of 0.1% Tween 20. The same adjustments of Tween and sterile water were made to negative controls.

Seed of Pioneer field corn hybrid P9917XR without seed treatment was disinfested in 95% EtOH for five minutes, then in 1.0% sodium hypochlorite for 30 minutes, followed by rinsing in three changes of sterile distilled water. Disinfested seed was immersed in the conidial suspension or the negative control for 14 hours, then air dried (Munkvold and Carlton, 1997). After infestation, seeds were then pre-germinated for 48 hours in sterile petri dishes with sterilized filter paper and sterile distilled water. One seed with a radicle length of 1 cm were placed into a 2.5-cm deep depression in the center of each plastic container filled with 200 cm^3 pasteurized soil, *P. penetrans* was added directly upon the seed at planting and holes were backfilled with additional pasteurized soil. Nematode inoculum was collected by incubating roots for 24 hours in Baermann funnels. Within 24 hours of collection, the nematode suspension was counted and approximately 1000 nematodes of all life stages were added in 1 ml of water to each container. Control containers received 1 ml of water without nematodes.

Infection of seed by the fungus was confirmed after surface disinfestation with 0.5% sodium hypochlorite for 3 minutes (Munkvold and Carlton, 1997). Ten

each of infested and control seed were plated on plates of potato dextrose agar (PDA) for fungal isolation or detection of contaminants. Plates were incubated at room temperature in the dark for 7 days. All infested seeds were infected with fungus and all non-infested seeds showed no microbial activity after one week. This exercise was repeated once.

Data Collection

Plant height was measured every 4 days until the termination of experiments at 24 days post planting, using height of the tallest leaf from the soil line. Plants were removed from the containers at harvest, shaken free of soil, and thoroughly rinsed until all soil was cleared from the root. Cleaned roots were separated into adventitious and seminal root systems, and scanned separately using an Epson Perfection V700 scanner. The mesocotyl was kept with the seminal root system for all analyses. Roots were spread in the scanning bed with 500 ml water to minimize root overlap and were imaged with a monochrome blue background from a rectangle of blue polycarbonate plastic, as per the instructions of the WinRhizo package. Water in the scanning bed was changed between every plant. High resolution TIFF images were analyzed for root growth and color characteristics using WinRhizo software (Regent Instruments Inc., Canada). Root growth parameters analyzed include total root length, average diameter, surface area, root volume, tips, forks and crossings. Shoots were dried at ambient temperature for one week, then for 48 hours in an oven at 60° C and weighed.

The WinRhizo software's capability of performing color analysis were used to determine if there were differences in discoloration of plant mesocotyl across treatments (Fig. 1). The analysis of color characteristics by WinRhizo required the establishment of "color classes", which drive the software's ability to differentiate between image background, healthy root and diseased root. These classes were defined by selecting image sections that exemplified discolored or diseased root, healthy root, and background portions of the image. The same color classes were saved and used across all repetitions of this experiment.

After scanning, seminal and adventitious root portions were then assayed for nematodes, with the mesocotyl grouped with the seminal roots. Seminal and adventitious root systems were cut into 1-cm lengths and incubated on separate Baermann funnels for 48 hrs. After nematode collection, the root segments were dried at ambient temperature for 1 wk, then for 48 hrs at 60° C before weighing. Soil from each pot was assayed for nematodes using a sieving and centrifugation-flotation technique. Nested sieves were used so that soil-borne root fragments remained on a 250-µm sieve, and nematodes free in the soil collected on a 38-µm sieve. Contents of the 250-µm sieve were incubated on Baermann funnels as described for the intact root systems and contents of the 38-µm sieve were cleaned by centrifugation in a sucrose solution of 1.14 specific gravity. Nematodes collected from the supernatant were rinsed well in water and dispensed into test tubes for storage for 1 to 5 days. Nematodes recovered from incubated root systems, root pieces, and soil were viewed using a stereomicroscope and counted.

Experiments using sand/corn meal for incubation of Fungal inoculum

For three experiments, *F. verticilliodes* was delivered in a sterilized mixture of sand and cornmeal. Corn meal has been utilized as a vehicle for infesting both field and greenhouse soil with *Fusarium* species in a variety of agricultural systems (Bevivino et al., 2000; Keinath, 1995; McLean and Lawrence, 1993; Miles and Wilcoxson, 1984; Munkvold and O'Mara, 2002; Rollins et al., 1999; Da Silva, 2010). Following Munkvold and O'Mara's modification of the technique of Desjardins et al. (Desjardins et al., 1995), a mix of 1,900 cm^3 sand and 380 cm^3 corn meal in autoclave bags with 110 ml of water was sterilized for 1 hour on two consecutive days. Bags were then inoculated with 2 ml of conidial suspension in CMC broth ($2x10^7$ conidia/mL), and incubated in the dark with thorough mixing daily. After 7 days, the fungal-infested sand/cornmeal mixture or the non-inoculated sand/corn meal mixture was incorporated into pasteurized field soil at a rate of 10% vol:vol. Fungal-infested or control soils were then placed in 500 cm^3 pots with filter paper in the bottom to reduce drainage and soil loss from the pots. Pioneer P9917XR seed was disinfested as described previously and placed in "rag dolls" made of paper towels moistened with water for 2 days to germinate. Seeds with radicle lengths of 1 cm were planted at a depth of 2.5 cm into containers filled with the soil-sand/cornmeal inoculum mixture and the holes backfilled with additional mixture.

Inoculum of P. *penetrans* was collected as described previously except that the inoculum also contained nematodes collected by rinsing the surface of culture plates with water. Control treatments utilized rinsate from non-inoculated plates of

Gamborg's media. The inoculum was adjusted to different concentrations for each experiment. The experiments used an inoculum of ca. 1000, 2000, and 3000 nematodes in 1 ml of water, respectively. For all experiments, the inoculum was placed on top of the seed before seeds were covered with soil. Seeds in treatments not including nematodes were wet with 1 ml of Gamborg's rinsate from nematode-free root explants cultures.

Plant Metrics

Plant height was measured over time until the termination of experiments at 25 days post planting. Height, measured as the distance from the soil line to the tip of the tallest leaf, was collected every 5 days. After 25 days, plants were removed from pots and scanned as described previously except that no data on root discoloration was collected. After scanning, the seminal root system only was assayed for nematodes as described previously. Seminal and adventitious root systems were then dried at ambient temperature for one week, then for 48 hours in a 140 C oven before weighing. Soil and root fragments from each container were assayed as described previously. Samples were counted within 7 days of collection.

Statistical Analysis

Data were analyzed using the SAS 9.4 statistical software package (SAS Institute, Inc., Cary, NC). Over the seven studies reported here, nine plants died: three in trial 3 of the conidial soak experiment, and 3 in each of the 2000-nematode and 3000-nematode experiments in the cornmeal-sand series. Plant metric data for

the dead plants were analyzed as zeros. The means of all plant metrics across each of the four treatments were compared utilizing PROC MIXED in SAS, with blocks (within experiment) considered to be a random factor and experiment, treatment, and the interaction considered to be fixed.

For the four repeated trials utilizing the conidial soak fungal infestation method, the variances were assessed for heterogeneity using a log-likelihood heterogeneity test. The four trials were combined when possible. Where variances were heterogeneous or when experiment-treatment interaction terms were significant, each trial was assessed individually. Means were compared utilizing Tukey-Kramer Least Significant Differences, at $P < 0.05$ and also at $P < 0.10$. Diagnostic plots were utilized utilizing 'plot' and 'univariate' procs, in order to determine if the normality, constant variance and outlier assumptions had been violated. Transformations of the data were used when required to satisfy assumptions; the natural log transform was most commonly used, with occasional use of 'squared' and 'e^x' data transformations.

The three experiments using sand/corn meal fungal infestation and three nematode inoculum levels were analyzed separately. Data were transformed as necessary.. Data from these three experiments were also analyzed using the 'meta' package in R statistical software (R Foundation for Statistical Computing, Vienna, Austria). This meta-analysis was performed to assess whether the number of nematodes recovered from the seminal roots of the corn plant were different across the nematode-only and nematode-plus-fungus infestation treatments. The meta-analysis pools mean and standard deviation data of control and experimental

treatments across similar trials, utilizing a heterogeneity test to assess whether the trials are comparable enough to warrant pooling under a meta-analysis. The analysis weights the results of the trials, and combines them into a random effects model to compare treatments.

For all studies, nematode recovery data from root parts, soil, and soil-borne root fragments were assessed across the nematode-only treatment and the fungus-plus-nematode treatment, without consideration for the two treatments without nematode infestation. These data were analyzed as described above

Results

Objective 1: Effects on Plant Metrics

Shoot metrics showed differences across treatments in all except the fourth trial in the conidial soak experiment, but treatment effects were not consistent. Dry shoot weight was reduced in the nematode-only treatment in trial 2, and in the fungus-only treatment in trial 3 (Fig. 2). The co-infested treatment was not different from the control in trial 2. In trial 3, shoot weight reductions, as compared to the control, were the same for the fungus-only and co-infested treatment. There was a two-fold difference in mean shoot weights among the trials. Plant heights were influenced by treatment in trials 1 and 3 with mildly significant trends in trial 2 (Table 1). Height reductions in the co-infested treatments were different from the control, but similar to one of the pathogen-only treatments. In trial 1, the co-infested treatment was not different from the nematode-only treatment, and in trial 3 the combination treatment was not different from the fungus-only treatment. The

integrated value of plant height over the experiment showed results similar to the daily values.

There were no differences in shoot weight across treatments in any of the three experiments utilizing the sand/cornmeal fungal infestation method (Fig. 3). Plant heights where higher but shoot weights were lower in the sand/cornmeal experiments than the conidial soak experiments (Table 2). With a level of 1000 nematodes per plant, there were no differences among treatments for plant height. With a level of 2000 nematodes per plant, the nematode-only treatment reduced plant height around V-2 to V-3 (10-15 days post planting) relative to the fungus-only treatment but not the control or the co-infested treatment (Table 2). For the experiment using 3000 nematodes, height at the V-5 stage (25 days post planting) was greater for the pathogen-only treatments than the control or co-infested treatments, but the results were influenced by the early (3 days after planting) death of two control plants and one co-infested plant.

Root system metrics varied among trials of the conidial soak experiment with few statistically significant differences among treatments. Seminal root lengths were smaller ($P = 0.07$) in the control as compared to the other treatments in trial 2. No other differences in total root or adventitious root lengths were detected. Total dry root weight was greater for the fungus-only treatment in trial 1 (Fig. 4) compared to the co-infested treatment. The fungus-only and co-infested treatments had the lowest ($P = 0.01$) total root weight in trial 2. Seminal root weights showed the same differences, as did total root surface area, average root diameter, and total root volume (data not shown). There were no differences among treatments in any

trial for total root length, number of root tips, number of root forks, or number of root crossings (Table 3).

Root metrics varied greatly across the sand/cornmeal experiments and few differences among treatments were noted (Table 4). At 2000 nematodes per pot, total root length and root volume were less (P = 0.08) for the nematode-only than the fungus-only treatment. Total root system length was smaller in the control and co-infested treatments compared to plants inoculated with *F. verticillioides* alone; average root diameter was smaller in the fungus-only treatment compared to the nematode treatment and control (Table 5). No other differences in plant growth metrics were detected, including seminal root and total root system weights.

There was only one weakly significant difference in root disease levels among treatments for one trial (Table 6). Mesocotyls in trial four were more diseased in the fungus-only treatment compared to the negative control (P = 0.0761), In general, the mesocotyl was more symptomatic than roots, sometimes with diseased percentages greater than seminal or adventitious roots by over 20-fold.

Objective 2: Effects of Fungus on Nematode

In general, plants infected with both the fungus and nematode supported fewer nematodes than plants infected by the fungus alone for the conidial soak experiment. The number of nematodes recovered from the entire root system was lower (P = 0.09) for the combined treatment in trials 1 and 3. Additionally, the number of nematodes recovered from seminal roots in trial 3 (P = 0.02) and from

adventitious roots in trial 2 (P = 0.06) was lower for co-infected plants (Table 7). For all trials and treatments, nematodes recovered from roots and soil represented all life stages.

Across the three experiments utilizing the sand/cornmeal fungal infestation method only one difference was detected among treatments. With 2000 nematodes, fewer (P = 0.09) nematodes were recovered from root systems of plants co-infested with the fungus (Table 8). Nematodes collected from these experiments were also varied across life stages, with a mix of life stages and genders. The meta-analysis of all three sand/cornmeal fungal infestation experiments showed fewer nematodes for the co-infested treatment (P= 0.01). The heterogeneity test justified pooling experiments for the meta-analysis (P = 0.97).

Discussion

Despite instances of fungus-induced and nematode-induced reductions in plant metrics, we did not see enhanced disease or evidence for a synergistic disease interaction between *P. penetrans* and *F. verticillioides*. There was one instance where adding the fungus negated the effect of the nematode on shoot growth (Fig. 2), and one instance where adding the nematode eliminated the beneficial effect of the fungus (Table 5). A consistent pathogen-only effect was only observed in one trial, where both the individual fungal infestation and co-infested treatments reduced plant vigor (Table 1). On the whole, my experiments exhibited either a failure of the two pathogens to increase plant disease, or a "wipe out" elimination of disease effects when they were combined. My results are similar to work by Palmer

and MacDonald (1974) who found that combined infestation resulted in no differences from the control at 25 and 27 C. At 22 C, these authors showed F. verticilliodes reduced root and shoot weight alone, but not in the presence of the nematode. Relatedly, Jordaan et al. investigated a similar complex but with *F. verticillioides* and *P. brachyurus* and *P. zeae* instead of *P. penetrans,* and found no evidence of an interaction for disease and some evidence that the presence of the fungus suppressed increase of the nematode (Jordaan et al., 1987).

My results are in conflict with Da Silva (2013) who found a synergistic interaction of F. verticilliodes and P. penetrans, but his study took a very different approach. Da Silva's study infested all pots with both nematodes and the fungus, utilizing the mixed sand/cornmeal fungal infestation method, and "removed" nematodes and fungi from his control pots through the use of various nematicidal and fungicidal seed treatments. He used three different combinations of fungicide and nematicide for his control treatments; often only one of those controls exhibited any differences compared to his co-infested treatment, while the co-infested treatment was only different from both of the single infestation treatments in the case of root tips and root forks. Further, Da Silva used a higher concentration of nematode inoculum (4000 individuals) in a smaller pot volume.

My experiments confirmed reports that one genotype of *F. verticillioides* can be an endophyte and pathogen to corn (Bacon et al., 2008; Mańka, 1979; Yates et al., 2005). The fungus reduced plant root and shoot development in trial three, but had no effect in other trials, and significantly improved root length in one of the corn meal infestation experiments (Table 5). There is evidence that *Fusarium*

verticillioides can increase above- and below-ground growth, possibly due to hormone production (Bacon et al., 2008; Mańka, 1979; Yates et al., 1997, 2005). The mechanisms and triggers that determine whether this fungus functions endophytically or pathogenically are not well understood, and I cannot discern a pattern of variation across my experiments that lends explanation to the phenomenon. My studies did not suggest P. penetrans plays a role in determining the behavior of the fungus in planta.

While the nematode and the fungus did not combine to reduce plant development, the presence of the fungus had an inhibitory effect on the number of nematodes residing in seminal roots. In alignment with these results, Da Silva found a reduction in nematode recovery from plants co-inoculated with *F. verticillioides* (Da Silva, 2010). This effect was consistent even though it was not statistically significant in all of my experiments. Seminal roots are important to the early development of the corn plant and nematode population density in seminal roots was shown to be predictive of yield (Todd and Oakley, 1996), so it is important to elucidate the inhibitory effect of the fungus on the nematode and to determine if the effect is modified by environmental conditions and differences in pathogen pressure.

While nematode recovery was relatively low within my experiments, the number of nematodes recovered per gram of dry root were between two to 20 times higher than the maximum recovery of Da Silva (18.7 individuals/g), who utilized the same combination of pathogens, but at a rate of 4000 nematodes per pot (Da Silva, 2010). Rates of nematode recovery from soil in my experiments, while low, were

comparable to recovery from treatments not utilizing nematicides in Da Silva's work. This being said, there are areas of my experimental process that may have resulted in nematode loss. It is possible that nematodes may not have come optimally into contact with the radicle and seminal root system, as the nematode inoculum was pipetted directly on the kernel at the time of planting and not in the soil below the kernel, where the roots were to develop. Nematodes may have been lost during rinsing of corn roots to prepare for WinRhizo analysis, although the period of time between rinsing, imaging and nematode collection on Baermann funnels was minimized as much as possible.

Further investigations would be greatly improved by quantifying fungal biomass within the plant, particularly seminal and adventitious roots, to track colonization as the corn root develops. This would be best performed with real-time PCR techniques or by quantifying fungal-specific sterols. It would also be worthwhile to investigate fungal colonization of aboveground tissues; it is surmised that the crown of the plant represents a barrier that slows the ability of the fungus to colonize systemically (Kedera et al., 1994; Munkvold et al., 1997), and it would be helpful to utilize accurate tools that could pinpoint under what conditions and timings the fungus is able to progress from roots to shoots. Molecular methods would also provide a highly sensitive methodology to quantify fungal colonization across treatments, and thus verify that controls are free of *F. verticillioides* or other opportunistic, ubiquitous *Fusarium* species.

Experimental temperature is an additional consideration in any interaction study, and would offer an interesting avenue for further research. The growth

chamber was kept in a favorable temperature range for *Pratylenchus penetrans*, but lower temperatures tend to exacerbate fungal damage via the retardation of seed germination rates (Palmer, 1974; Yates et al., 2003). Repetition of these experiments at lower temperatures may offer further illustrations of how these pathogens interact in an arena where fungal damage is generally at a higher level. The observed effect of fungal reduction of nematode recovery may stay the same or may become altered under conditions that are more favorable to fungal infection and disease development.

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FIGURE 1. WinRhizo image of a typical seminal root system including mesocotyl, overlaid with the WinRhizo software's algorithmic identification of roots split apart into different diameter classes (delineated by different colors).

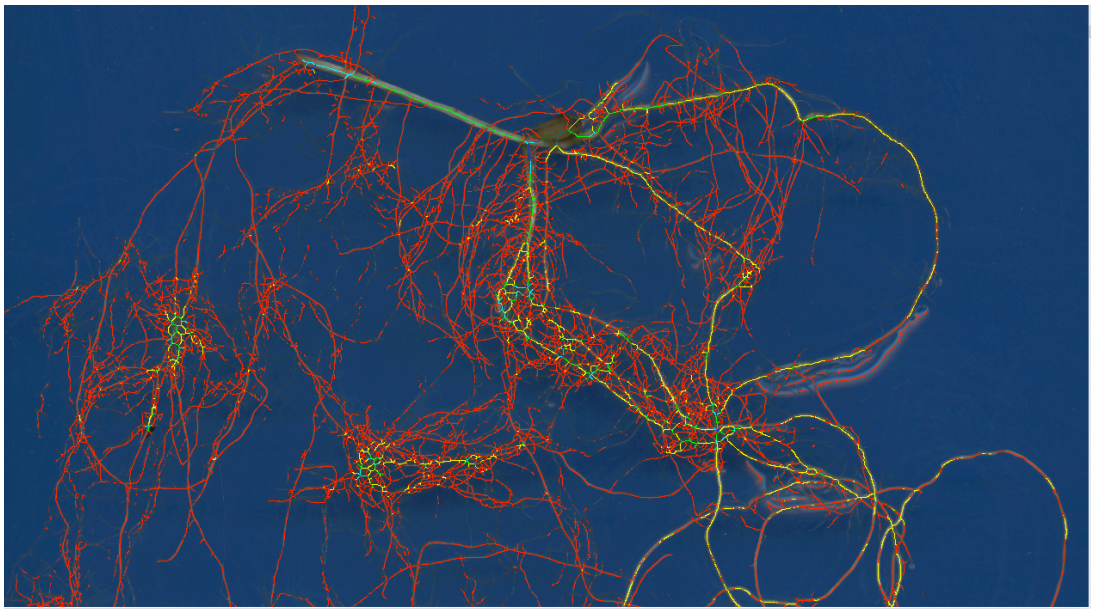

FIGURE 2. Shoot weights (g) by treatment for four repeated trials utilizing a conidial soak fungal infestation method. Treatments include control, *F. verticillioides* alone (FV), *Pratylenchus penetrans* along (PP), and the co-infested treatment (FV+PP). Bars indicate one standard error from the mean.

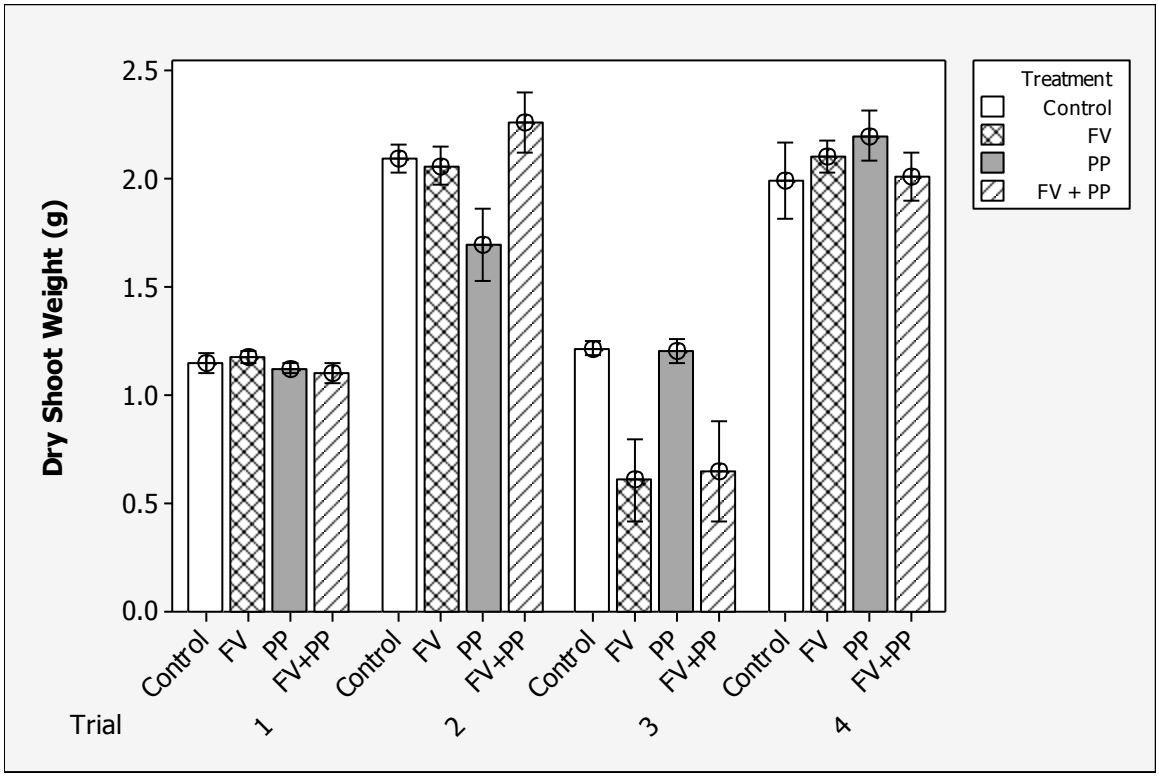

TABLE 1. Height of tallest leaf at six time points post-infection (DPI), across four inoculation treatments, and the integrated area under the plant growth curve (AUC) for the trial. For Trial 3, data were transformed (squared) to satisfy normality and constant variance assumptions. For trial two, data at days 12 and 16 were transformed to satisfy assumptions. Tukey-Kramer least significant differences were used to compare treatment at either $P < 0.05$ or $P < 0.10$, depending on the significance level of the treatment main effect.

Trial:	4 DPI	8 DPI	12 DPI	16 DPI	20 DPI	24 DPI	AUC
Trial 1	***P* = 0.018**	***P* = 0.12**	***P* = 0.24**	***P* = 0.97**	***P* = 0.97**	***P* = 0.52**	***P* = 0.29**
control	3.8 a**	13.6	17.0	21.8	22.6	23.8	362.7
FV	3.8 a**	13.8	16.9	21.7	22.5	23.6	362.2
PP	3.5 ab**	13.3	16.5	21.5	22.5	24.2	357.2
FV+PP	2.9 b**	12.5	16.0	21.7	22.7	23.5	350.3
Trial 2	***P* = 0.47**	***P* = 0.22**	***P* = 0.064**	***P* = 0.071**	***P* = 0.072**	***P* = 0.60**	***P* = 0.056**
control	5.1	9.2	20.3 ab*	23.0 ab*	30.6 ab*	32.6	418.0 ab*
FV	5.3	9.7	20.2 ab*	22.8 ab*	29.8 ab*	32.2	415.1 ab*
PP	4.4	8.0	16.8 b*	19.9 b*	28.4 b*	31.9	373.8 b*
FV+PP	5.2	9.4	20.7 a*	23.3 a*	30.9 a*	33.6	425.0 a*
Trial 3	***P* = 0.007**	***P* = 0.0021**	***P* = 0.0027**	***P* = 0.0062**	***P* = 0.018**	***P* = 0.042**	***P* = 0.0056**
control	3.9 a**	15.0 a**	21.5 a**	23.5 a**	25.9 a**	27.1 a**	413.5 a**
FV	1.9 b**	6.3 b**	10.5 b**	13.8 b**	16.6 ab**	18.9 b**	233.8 c**
PP	3.4 ab**	14.5 a**	21.1 a**	22.8 ab**	24.4 ab**	27.1 a**	398.8 ab**
FV+PP	2.0 b**	7.2 b**	11.2 b**	13.2 b**	14.9 b**	17.3 ab**	228.6 bc**
Trial 4	***P* = 0.88**	***P* = 0.70**	***P* = 0.78**	***P* = 0.52**	***P* = 0.63**	***P* = 0.82**	***P* = 0.65**
control	2.8	10.2	16.4	25.0	28.0	31.4	392.6
FV	2.8	11.0	17.1	26.0	28.8	31.9	406.2
PP	2.9	10.8	16.9	25.9	28.4	31.9	403.7
FV+PP	2.7	10.9	17.2	26.3	28.8	32.0	407.3

FIGURE 3. Shoot weights (g) by treatment for three experiments utilizing a mixed sand/cornmeal fungal infestation method. Nematode inoculum levels differed across the experiments, at levels of 1000, 2000 and 3000 nematodes per pot. Treatments include control, *F. verticillioides* alone (FV), *Pratylenchus penetrans* along (PP), and the co-infested treatment (FV+PP). Bars indicate one standard error from the mean.

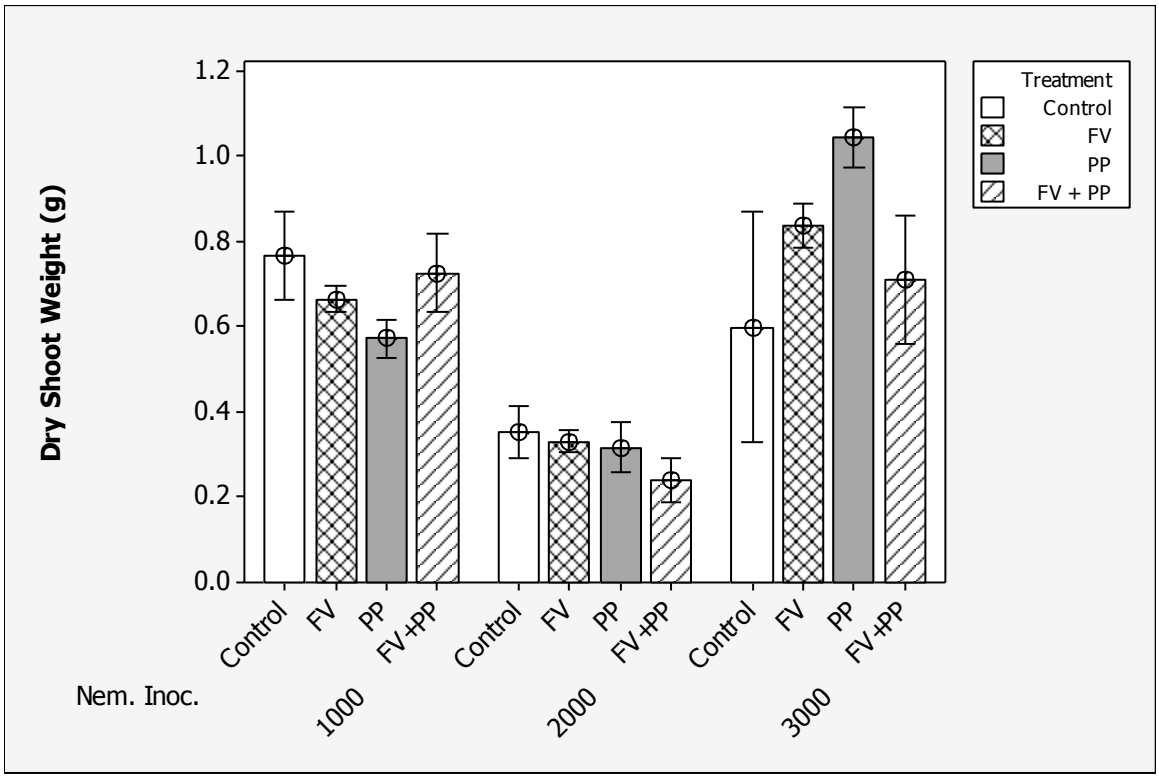

TABLE 2. Height of tallest leaf (cm) at five time points post-infection (DPI), for an experiment utilizing a mixed sand/cornmeal fungal infestation and 2000 nematode individuals per pot. Treatments include control, *F. verticillioides* alone (FV), *P. penetrans* alone (PP), and the co-infested treatment (FV+PP). Comparisons of means were conducted utilizing Tukey-Kramer least significant differences at $P < 0.05$ or $P < 0.10$, depending on the significance level of the treatment main effect.

	5 DPI	**10 DPI**	**15 DPI**	**20 DPI**	**25 DPI**
2000 nem	P = 0.550	P = 0.094	P = 0.035	P = 0.221	P = 0.253
Control	2.3	12.1 ab*	18.7 ab**	26.1	37.0
FV	3.2	14.9 a*	23.7 a**	29.5	37.7
PP	2.6	10.3 b*	15.5 b**	23.5	34.4
FV+PP	3.1	11.5 ab*	17.4 ab**	21.4	28.2

FIGURE 4. Dry seminal root and total root weights (g) by treatment for four repeated trials utilizing a conidial soak fungal infestation method. Treatments include control, *F. verticillioides* alone (FV), *P penetrans* alone (PP), and the co-infested treatment (FV+PP).

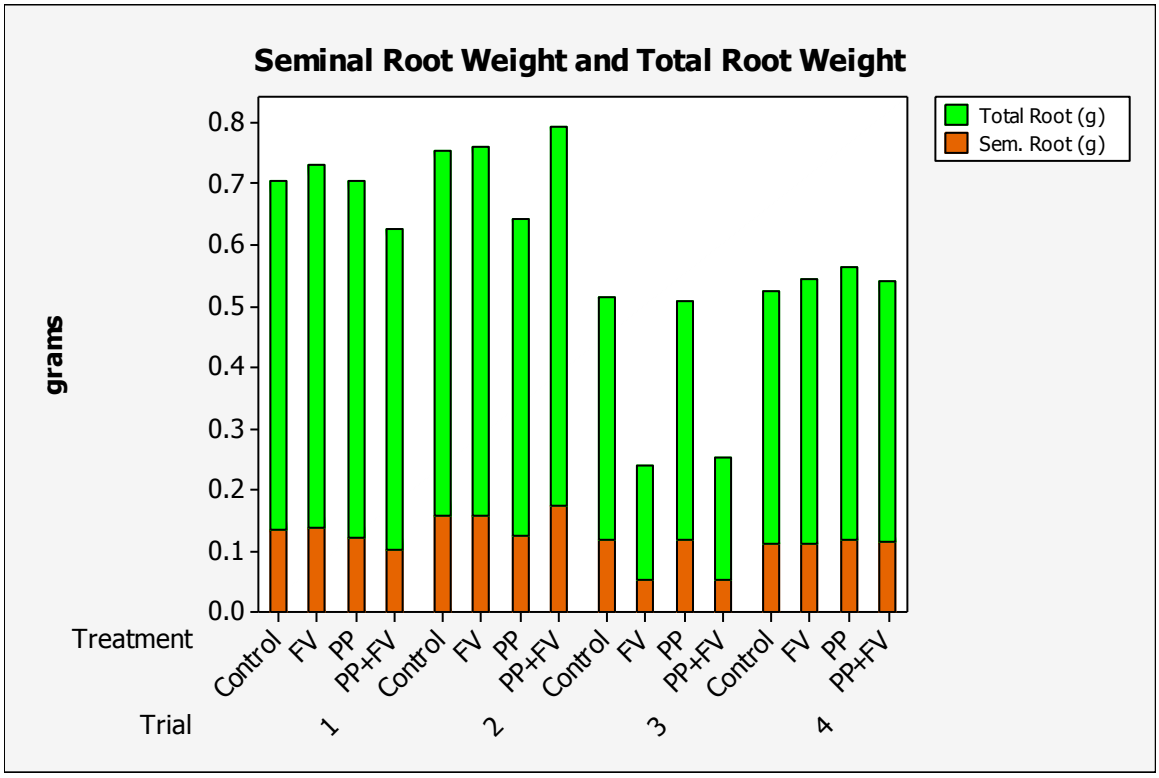

TABLE 3. Grand mean of the length of the total root system, adventitious roots (Adv.) and seminal root system including mesocotyl (Sem.), as well as tips, forks and crossings from the total root system for four trials utilizing a conidial soak method of *Fusarium verticilliodes* infestation.

Trial:	Length	Adv. Length	Sem. Length	Tips	Forks	Crossings
1	1493.2	770.7	722.5	14728	31732	6657
2	2213.8	1072	1141.9	17288	45478	10677
3	2050	1086	964	22130	36467	9895
4	2460.8	1124	1336.7	12117	33354	6866

TABLE 4. Grand mean of the length of the total root system, and tips, forks and crossings from the total root system for three experiments utilizing a mixed sand/cornmeal method of fungal infestation, at three nematode inoculum levels (1000, 2000, and 3000 individuals per pot).

Nem Inoc:	Length (cm)	Total Tips	Total Forks	Total Crossings
3000	3346	13926	31132	7033
2000	924.8	5179	10344	1598
1000	1447	9135	25741	4811

TABLE 5. Root metrics by treatment for an experiment using a sand/cornmeal infestation method for *Fusarium verticilliodes* and 2000 *Pratylenchus penetrans* per pot. FV = *F. verticillioides*, PP = *P. penetrans*, and FV + PP = co-infested treatment. Means were compared using Tukey-Kramer least significant differences at $P < 0.10$.

	Length (cm)	Diameter (cm)	Volume (cm^3)	Tips	Forks	Crossings
Control	811 b	1.44 a	4.71	5666	11220	1755
FV	1320 a	1.09 ab	3.33	6063	12353	1939
PP	840 ab	1.28 ab	3.49	5276	9400	1361
FV + PP	726 b	0.93 b	3.03	3712	8402	1336

FIGURE 5. Dry seminal root and total root weights (g) by treatment for three experiments utilizing a mixed sand/cornmeal method of Fusarium verticilliodes infestation, and three inoculum levels of *Pratylenchus penetrans*. Treatments include control, *F. verticillioides* alone (FV), *P penetrans* alone (PP), and the co-infested treatment (FV+PP).

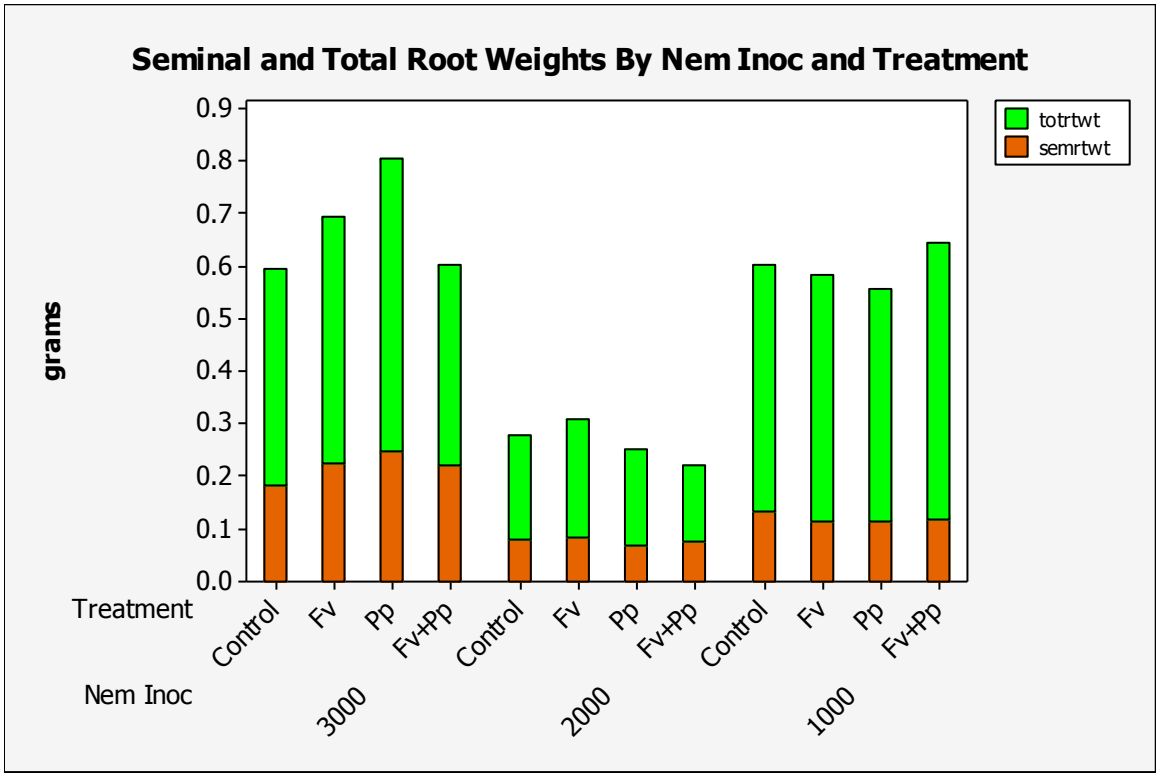

TABLE 6. Percentage of adventitious (Adv.), seminal (Sem.), and mesocotyl (Meso.) root portions that were discolored as analyzed by the WinRhizo software package, for four repeated trials utilizing a conidial kernel soak infestation method for *Fusarium verticiliodes*. Comparisons of means were conducted utilizing Tukey-Kramer least significant differences at $P < 0.10$.

	Percent Diseased		
	Adventitious	Seminal	Mesocotyl
Trial 1			
Control	2.37	0.85	9.29
FV	2.28	0.85	7.87
PP	2.27	0.96	7.32
FV + PP	2.49	1.03	14.47
Trial 2			
Control	1.62	1.23	26.94
FV	1.62	1.23	22.64
PP	1.89	1.43	24.46
FV + PP	1.6	1.28	20.54
Trial 3			
Control	2.29	0.83	8.18
FV	3.97	0.88	26.24
PP	2.52	0.87	8.67
FV + PP	1.64	0.83	15.31
Trial 4			
Control	3.12	1.02	1.615 b*
FV	2.96	1.16	10.72 a*
PP	2.83	1.24	5.65 ab*
FV + PP	2.95	1.32	9.52 ab*

TABLE 7. Mean number of nematodes recovered from *Pratylenchus. penetrans* individual infested treatment (PP) and *Fusarium verticillioides* and *P. penetrans* co-infested treatment (FV+PP) for four repeated trials. All trials utilized a conidial soak infestation method for *F. verticilliodes*. Comparisons of means were conducted utilizing Tukey-Kramer least significant differences at P < 0.05, (**) or P < 0.10 (*).

	Mean Root Count	Seminal Root Count
Trial 1		
PP	98*	92*
FV+PP	76	70
Trial 2		
PP	48	41
FV+PP	37	30
Trial 3		
PP	19**	14*
FV+PP	7	6
Trial 4		
PP	91	83
FV+PP	80	73

TABLE 8. Count of nematodes recovered from *P. penetrans* individual infested treatment (PP) and *F. verticillioides* and *P. penetrans* co-infested treatment (FV+PP) across three experiments utilizing a mixed sand/cornmeal inoculation method. Experiments infested plants with *P. penetrans* at three different levels: 1000, 2000 and 3000 nematodes per pot. A Tukey-Kramer test was utilized as for above.

	Mean Root Count	Seminal Root Count
3000 nem		
PP	17.67	17.67
FV+PP	8.83	8.83
2000 nem		
PP	34.58*	31.33
FV+PP	18.58*	17.25
1000 nem		
PP	50.70	50.70
FV+PP	41.00	41.00

CHAPTER THREE

Infection of Corn Seedlings by *Fusarium verticilliodes* and *Pratylenchus penetrans*

Abstract

Corn is a common host to the fungus *Fusarium verticillioides* and the nematode *Pratylenchus penetrans*, which are both known to operate within cortical root tissues of seedlings at early infection stages. The ability of one pathogen to affect the behavior of the other is not well understood, and cellular-level investigation of the relationship between the two pathogens in corn has never been reported. Fluorescence microscopy and an in-vitro fungal biopredisposition assay were used to visualize the two pathogens on 7-day-old seminal corn root explants. The fungus and nematode were observed to invade corn cortical tissues close to each other, and no differences in behavior between co-inoculated and singly inoculated treatments were seen. There was weak evidence that the fungus grew towards nematode-infested corn roots over control roots on nutrient rich media, but no differences were detected on nutrient poor water agar. These results indicate that the two pathogens can be found in proximity to each other during important early stages of corn seedling growth, but further study is needed to solidify their effects upon each other.

Introduction

Pratylenchus penetrans and *Fusarium verticillioides* colonize the roots of corn plants. The fungus and the nematode persist in soil; root lesion nematode is able to overwinter in soil-borne root fragments as well as in the soil proper (Townshend, 1984), while *F. verticillioides* is capable of surviving as a facultative saprophyte (Bacon et al., 2001). Both pathogens infect corn plants within days of planting, with the fungus capable of causing visible seed rot after three days of exposure and mycelia have been observed on roots 6 days after exposure (Yates and Jaworski, 2000; Yates et al., 2003). *P. penetrans* is common across vast geographical areas, as is *F. moniliforme* (Nelson, 1992; Nelson et al., 1993), particularly in temperate areas including the Midwest of North America. Overlap of the two pathogens in field systems is thus expected to be quite significant.

P. penetrans is a migratory endoparasite, known to feed from both within and outside host roots, and can cause cellular damage when moving intracellularly within cortical root tissues (Oyekan et al., 1972; Vovlas et al., 1990). The nematode is not known to penetrate the root stele. All vermiform stages of root lesion nematode (*Pratylenchus* species) are capable of feeding both ectoparastically and endoparasitically, utilizing their stylet to penetrate host cells, secrete saliva, and feed upon cytoplasm. Male *P. penetrans* have been reported to exit roots of pea more frequently than females, perhaps resulting from an attraction to females, and egress behaviors may not be dependent on the conditions of nearby roots (Wixted and MacGuidwin, 1990).

Like *P. penetrans*, F*. verticillioides* interacts with corn roots in multiple ways: growing through tissues intracellularly as a pathogen or between cells as an endophyte (Bacon and Hinton, 1996). Intercellular growth is less destructive as the hyphae progress through apoplastic extracellular spaces, perhaps minimizing plant response, while intracellular growth results in cellular damage. Given that *F. verticillioides* has been reported to function both inter- and intracellularly, its effects upon nematodes in these two different phases of development are of interest. Analysis of the path and structures of fungal growth, as well as the location and behavior of *P. pentrans* in cortical tissues, would offer insight into whether the fungus and the nematode are directly interacting. Our objectives for this research were to (1) determine if *F. verticilliodes* and *P. penetrans* operate in close physical proximity in the cortex of corn roots, and (2) determine if the presence of *P. penetrans* affects the growth of *F. verticilliodes* pre- and post-infection of corn roots.

Materials and Methods

Two in vitro studies of living roots inoculated with *Fusarium verticillioides* and *Pratylenchus penetrans* were undertaken, utilizing Pioneer P9917XR corn seed without seed treatment. Seeds were disinfested in 95% EtOH for five minutes, then in 1.0% sodium hypochlorite for 30 minutes, followed by rinsing in three changes of sterile distilled water. Disinfested seeds were then placed on individual Petri dishes of nutrient agar and germinated until the radicle was 1-2 cm long, in order to assess for fungal or bacterial contaminants

Spatial relationship of F. verticilliodes and P. penetrans inside roots

A green fluorescent protein-expressing mutant strain of *F. verticillioides* (TXI-76), was provided by Dr. Gary Munkvold, Iowa State University. This strain, transformed with a gene for hygromycin-B resistance and for the expression of GFP (Wilke et al., 2007), was brought to the University of Wisconsin-Madison on silica pellet and reinvigorated for GFP expression by culturing and selection on potato dextrose agar (PDA). The fungus was maintained at 4° C on Synthetic Nutrient Agar medium (SNA), and cultured on PDA in the dark for one wk for inoculation purposes.

A Wisconsin isolate of *P. penetrans* was used for this study. Nematodes were reared monoxenically on sweet corn cultivar "IO Chief" root explants grown on Gamborg's B5 medium with vitamins and without plant growth hormones for three to four months, before being collected to be used as inoculum. All vermiform life stages of *P. penetrans* were collected aseptically from cultured roots incubated in sterile water for 24 hours.

Axenic germinated seeds were transferred to Petri dishes with water agar when the radicle was 1-2 cm long and grown as intact plants. The fungus and/or the nematode were inoculated at the time of transfer. Each inoculation treatment was replicated in three Petri dishes. A $1cm^2$ plug of TXI-76 culture on PDA was placed aseptically on the radicle of each corn plant. Nematodes were inoculated concurrently. All vermiform life stages of *P. penetrans* were collected aseptically from cultured roots incubated in sterile water for 24 hours. Nematodes were counted and the water volume adjusted so as to deliver 200 nematodes in 0.5ml of

water to the radicle of the plant; control plants received 0.5ml of sterile water. The Petri dishes with plants were incubated in ambient temperature and light.

After 7 d, plant roots were washed in water to remove surface mycelia and 1 cm-long sections of root were excised and sectioned longitudinally using a double-edged razor blade and mounted in water on microscope slides. Two sections per plant, one at the root tip of the radicle and another ca. two inches behind the root tip, were examined for ca. thirty minutes each using an Olympus BX60 trinocular brightfield / fluorescence microscope, with a 100 watt epifluorescence lamp and GFP filter cube with an excitation wavelength of 395 nm. Images were taken with an Olympus DP73 camera and the Olympus cellSans Standard 1.9 software package. All nematodes observed within plant tissue were moving and/or actively feeding.

Root choice assay for F. verticilliodes

A Petri dish study was conducted to determine if infection of root explants by *P. penetrans* influenced the growth of and infection of roots by the fungus. Following disinfestation, 1 cm-long root segments were excised from seeds and transferred to Gamborg's B5 media with vitamins and without plant growth hormones. After the roots were ca. 5 cm-long, they were exposed to plugs from a 3 month-old nematode culture. After one week, 1-5 cm-long root segments were excised and placed on either side of six replicate PDA or water-agar Petri dishes, equidistant from the center of the plate, with one nematode-infested root segment on one side of the plate and one non-infected control root on the opposing side, each 3-cm away from the center.

A 1-cm^2 plug of *F verticillioides* A-1099 MAT-1 (Kansas), from the periphery of one-week-old cultures, were placed in the center of each dish. Dishes were kept in the dark at ambient temperature. Fungal growth from the center of the plate towards the nematode-infected and control roots was measured at 4 and 6 days post inoculation. Growth was measured from the edge of the inoculated agar plug to the widest point of fungal growth directly in line with the nematode-infested or non-infested roots.

Data were analyzed using PROC MIXED in the SAS statistical software package (SAS, Cary, NC). A paired t-test was utilized to determine differences in growth towards the two nematode treatments, paired by petri plate. Data satisfied assumptions of constant variance, normal distribution and lack of outliers so were not transformed before analysis. Results from the two assays on the two different media types were analyzed separately.

Results

The GFP-expressing mutant *F. verticillioides* was clearly seen in all roots with fungal treatment. Conidia were observed on the surface of one plant and within roots of the same plant, in both sections. When singly inoculated, the fungus grew intercellularly within undifferentiated tissue in the root tip and in the zone of elongation (Figure 1). The fungus colonized root hairs and a single hypha grew throughout the entire length of a single root hair (Fig. 2), and then expanded within cortical and vascular tissues of the plant (not shown). Disorganized, intracellular hyphal growth and conidia were observed within the root cortex and vasculature as

well in the zone of elongation, but were not observed in the root tip. Single conidia were present but microconidial chains on monophialides were not seen; conidiation patterns of *F. verticillioides* can vary by isolate, but conidia have been reported on root tissues of corn in prior studies (Oren et al., 2003). No macroconidia were observed in any of the roots exposed to *F. verticillioides*; this fungus produces aerial mycelia in PDA culture, suggesting it is a "mycelial type" and not a "pionnotal type" that produces many macroconidia and less aerial mycelium (Nelson, 1992). A single branching hypha could be observed exiting the root away from the hyphal mass within root tissue, exhibiting fungal entry/exit from mid-root (Figure 3), and entry/exit from the root tip was also observed (Figure 1). No runner hyphae were observed.

Pratylenchus penetrans and *F. verticillioides* were observed in close proximity to each other in co-inoculated roots. Nematodes were actively feeding and moving close to inter- and intra-cellular fungal growth, and were near to conidia present in root tissue. Autofluorescent lipids in the nematode were orange in color, in contrast to the green GFP-expressing fungal mutant (Figure 4). Nematodes were found throughout the cortex even in regions with extreme intercellular and intracellular fungal growth and conidiogenesis. Non-motile nematodes or specimens with patterns of autofluorescence associated with death (Forge and MacGuidwin, 1989) were not observed. In the absence of *Fusarium verticillioides*, behavior of *P. penetrans* was similar to that observed in co-infected roots (Figures 5, 6). A nematode was observed leaving the root after feeding in cortical tissues (Figure 6).

The fungal preference assay provided weak evidence that *F. verticillioides* may differentially grow towards roots that have been exposed to the nematode *P. penetrans*. After six days, the inoculated plug of *Fusarium verticillioides* had grown beyond the point of contacting both nematode-infested and control root segments. On Gamborg's media, the fungus overtook the root segments on day seven. Branching of root segments into some lateral root growth could be observed on Gamborg's plates (Figure 7). There was weak evidence (P = 0.10) that at six days on Gamborg's media, the fungus grew more towards root segments with *Pratylenchus penetrans*, as compared to non-infected roots. No differences in fungal growth were detected at 4 days on Gamborg's media or on either day for roots on water agar (Table 1).

Discussion

Neither *Fusarium verticillioides* nor *Pratylenchus penetrans* appear to alter their behavior when in close proximity in vivo, suggesting the pathogens may not affect each other within the root. An abstract presented by Roth and Boothroyd, who investigated disease at the plant scale in greenhouse work, found no evidence of altered nematode colonization of corn roots under co-inoculations (Roth and Boothroyd, 1976), which supports the notion that the pathogens may not influence each other. Some studies found fewer nematodes in fungus-infected roots after weeks of growth and speculated an antagonism (Jordaan et al., 1987; Da Silva, 2010), but did not examine the spatial dynamics of infection. It is important to connect fungal-nematode behavior at the cellular level with population dynamics of

F. verticillioides and *P. penetrans* during the first month of corn development, as the understanding afforded by microscopic and macroscopic work could combine to create a clearer picture of how the pathogens interact.

Fusarium. verticillioides is capable of differentially colonizing corn in two separate modes (Bacon and Hinton, 1996; Oren et al., 2003): intercellularly as an endophyte, and intracellularly as a pathogen, and I was able to observe both modes at seven days post-inoculation. In the greenhouse, *F. verticillioides* altered corn root cellular structure at seven days post-infestation (Yates et al., 1997), and sustained intracellular growth at 14 days (Bacon and Hinton, 1996). The fact that I observed relatively high levels of intracellular hyphal growth after seven days may be in part due to the fact that petri plates were incubated under ambient, indirect light, or that the fungus was infested directly on the root and grown in vitro. Low-light conditions have been reported to significantly increase the rate of colonization and aggressiveness of *F. verticillioides* on corn in greenhouse work (Oren et al., 2003), suggesting that plant stress may be an important determining factor as to whether the fungus operates as an endophyte or as a pathogen. No runner hyphae were observed, but they may have been washed off in the brief rinse before the root sections were viewed under the microscope. Although no direct repressive or facilitative effects were observed between the two pathogens, the observed close physical relationship between them suggests that further study with more samples over a longer time period may be warranted to establish a better understanding of how the pathogens relate to each other. It is possible that the fungus switches from endophyte to pathogen under conditions of plant stress (Bacon et al., 2008; Oren et

al., 2003), but my observations showed no indications of altered fungal behavior due to nematode presence. However, we saw no evidence that the nematode was a stressor of plant function at this stage of plant development.

Through analysis of a root preference assay, there was weak evidence that the fungus may prefer to grow towards roots that have been exposed to the nematode *P. penetrans*; further study over longer time periods, including observation and quantification of nematodes within roots, are needed to verify this. It is possible that the nematodes themselves secrete effectors during infection that serve to engage fungal chemotaxis or encourage germination of spores; it is also possible that infestation with *P. penetrans* indirectly affects biochemistry by altering root exudates of the corn plant. Differentially analyzing root exudate composition in nematode infested and control plants, and in turn fungal responses to these exudates, would help to illustrate the relationship of the fungus to nematode-infested corn roots. Study of fungal responses to nematode effectors and root exudate changes would further elucidate whether this relationship is an important factor for fungal colonization of corn. It is important to note that the fungal attraction towards nematode-infested roots only occurred on nutrient-rich Gamborg's growth medium, and not on water agar, perhaps indicating that the fungus may not be responding to compounds secreted by the nematode.

These visualization studies were conducted under a short time frame that falls within important developmental stages of corn seedlings; in corn, the seminal root system ceases growing relatively early, and serves as important initial point of entry for both *P. penetrans* and *F. verticillioides*. Understanding infection events in

this timescale can offer insights into later damage or repression of plant development, but are not directly representative of field conditions. In vitro systems offer significantly improved control against contamination by ubiquitous *Fusarium* species that may cause problems in greenhouse and field experiments, and provide an opportunity to study root and disease development over time without destructive sampling. However, roots grown in vitro are presented with a different conditions compared to the field setting, which are important considerations when considering how in vitro findings may translate to a field-level disease complex.

Studying GFP-expressing mutant *F. verticillioides* with *P. penetrans* in vitro is powerful for investigating a presently clouded understanding of the two pathogens, because the pathogens remain alive while under observation. Time-lapse microscopy would provide an avenue to understand the spatial and temporal dynamics of fungal growth and nematode movement in roots, and determine if the nematode influences pathogenesis by the fungus or the fungus alters feeding by the nematode. Other methods of visualizing co-infected roots have significant drawbacks; staining roots concurrently for fungi and nematodes is difficult, and also requires clearing of root cells, precluding any opportunity to observe growth and behavior of the pathogens over time. In addition to time-lapse methods, measuring nematode root colonization at multiple time steps, as well as taking snapshots over time of fungal and nematode associations at a cellular level, would improve understanding of how these two important pathogens interact during sensitive stages of root development.

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FIGURE 1. In undifferentiated root tip, *Fusarium verticilliodes* grew intercellularly (A) and entered/exited from root cap (B). Fungal growth continued intercellularly through zone of elongation (C).

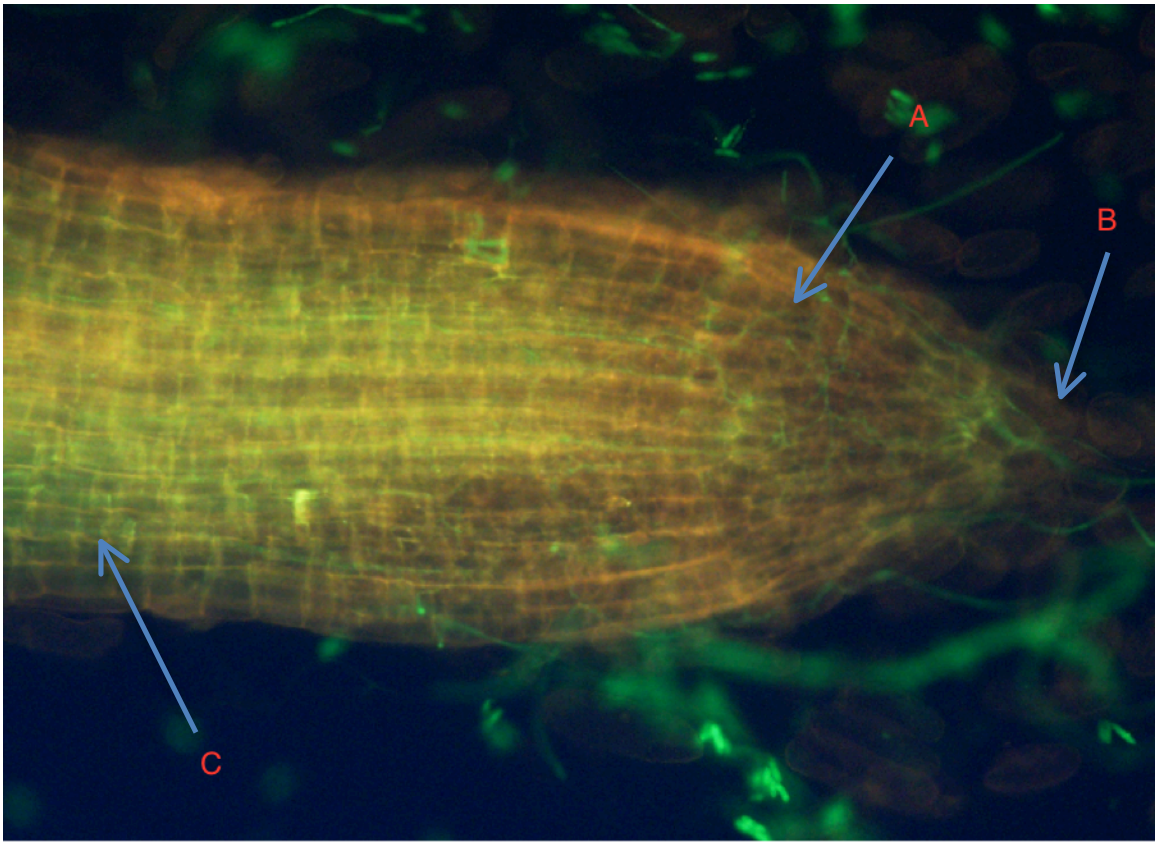

FIGURE 2. Colonization by *Fusarium verticilliodes* of a root hair by a single hypha. This hypha continued through root cortex intercellularly (not shown).

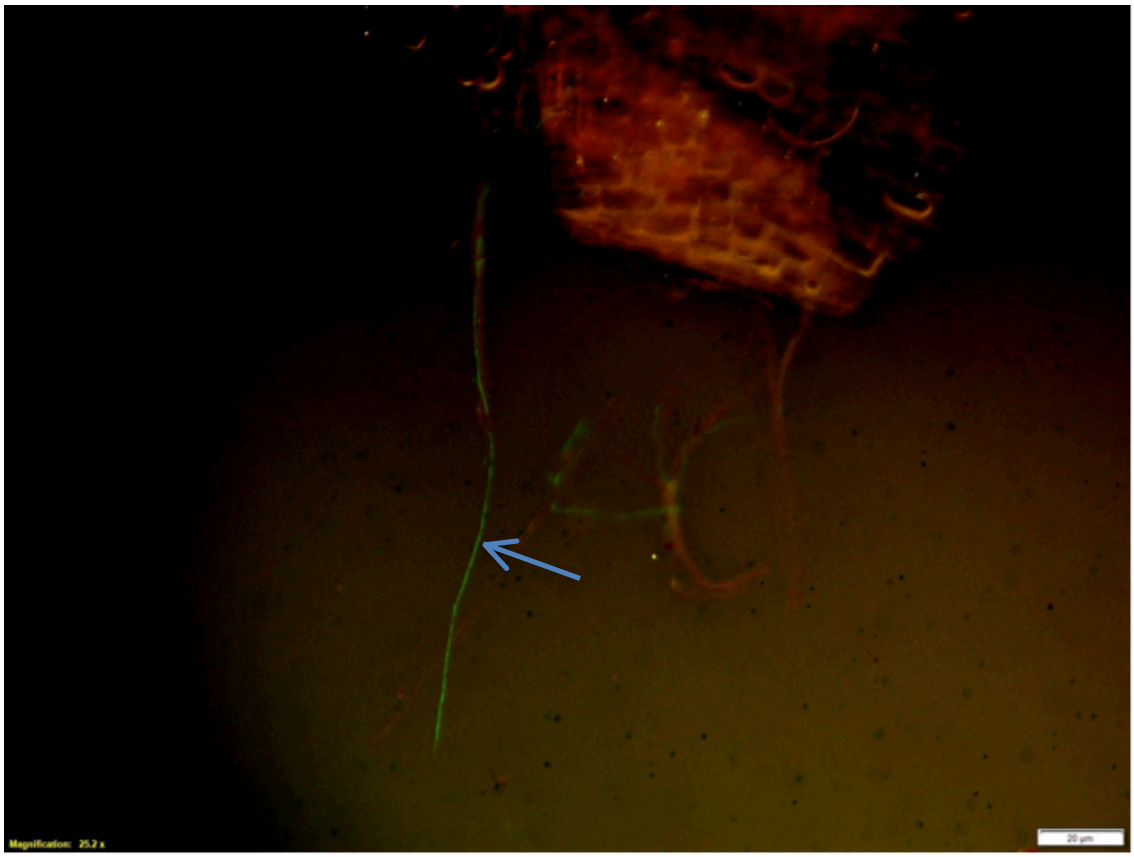

FIGURE 3. Emergence of *Fusarium verticilliodes* hypha from root, through cortex and epidermis (A). Intracellular growth of *F. verticilliodes* (B). Stele of transected corn root can be observed in the center of the root tissue, with the highest concentration of fluorescing fungal material.

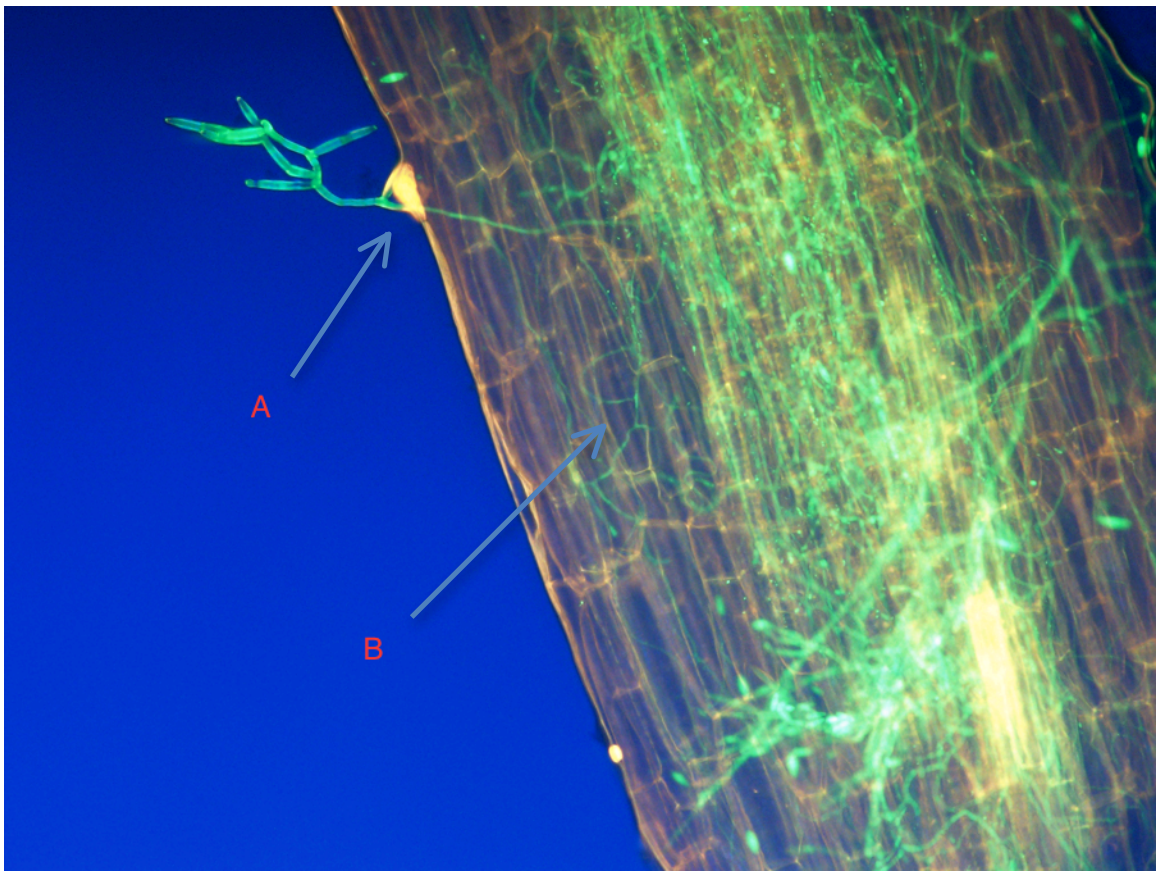

FIGURE 4: *Pratylenchus penetrans* individual in association with *Fusarium verticillioides* microconidia (A, arrows). *P. penetrans* elongated along root cortex parallel to stele amidst heavy intracellular fungal colonization (B). *P. penetrans* individual observed in corn root cortex near inter- and intracellular infection by *F. verticillioides* (C).

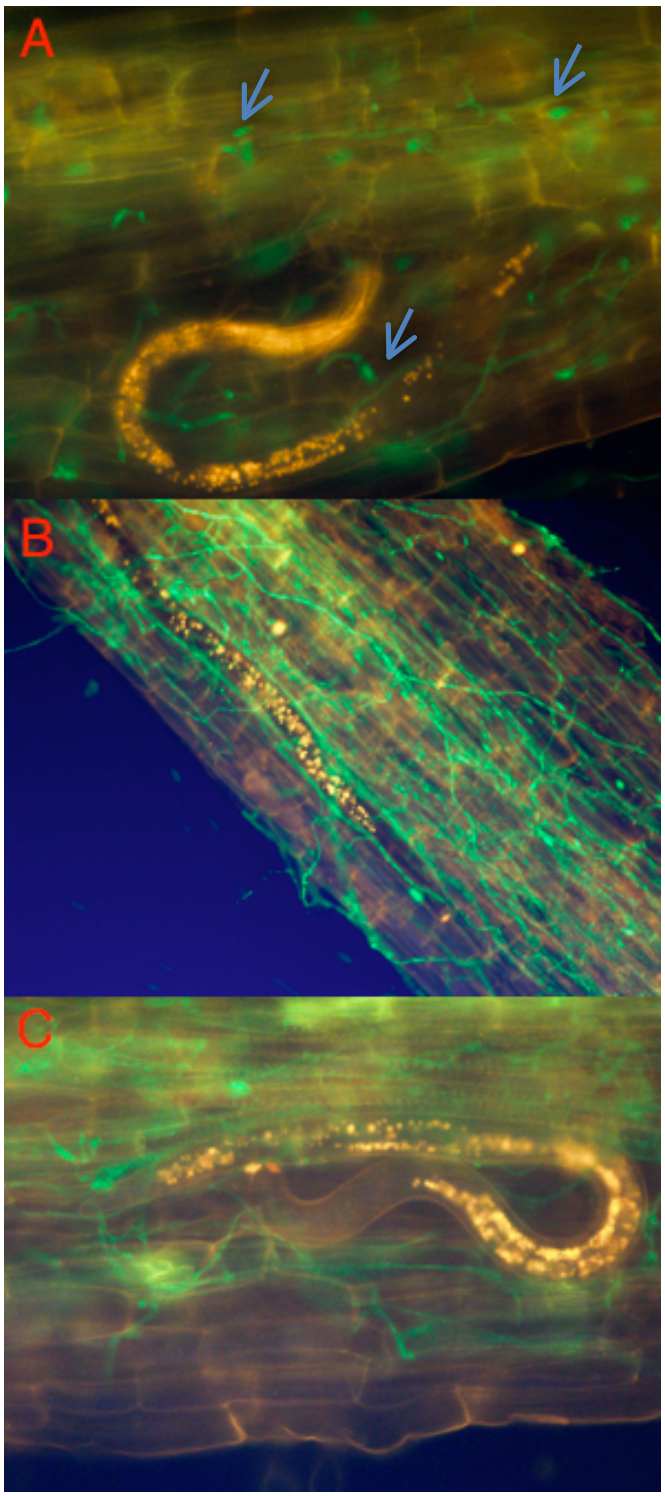

FIGURE 5. *Pratylenchus penetrans* emerging from corn cortex after having fed endoparasitically (A). The same nematode under fluorescence microscopy, taken at the same time as image A (B).

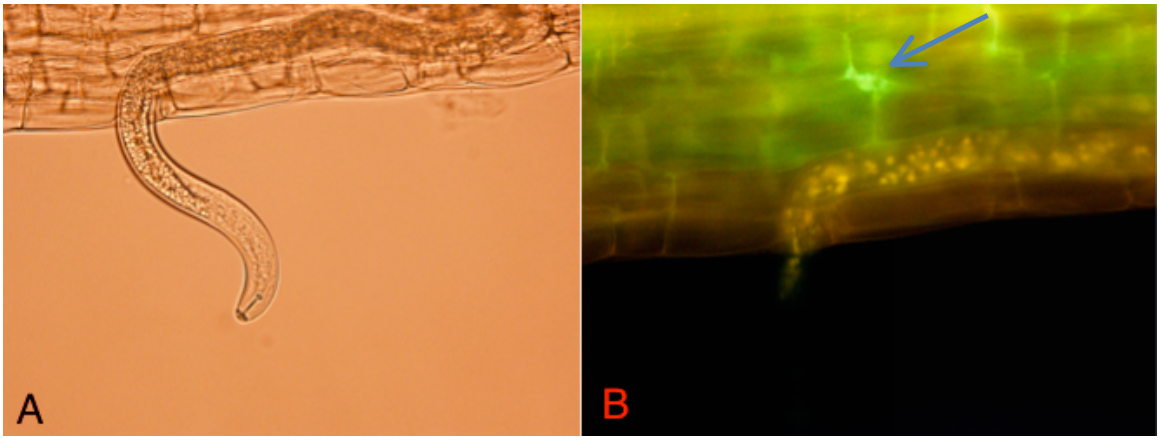

FIGURE 6. *Pratylenchus penetrans* individual feeding as an endoparasite on cortical root tissues in zone of elongation.

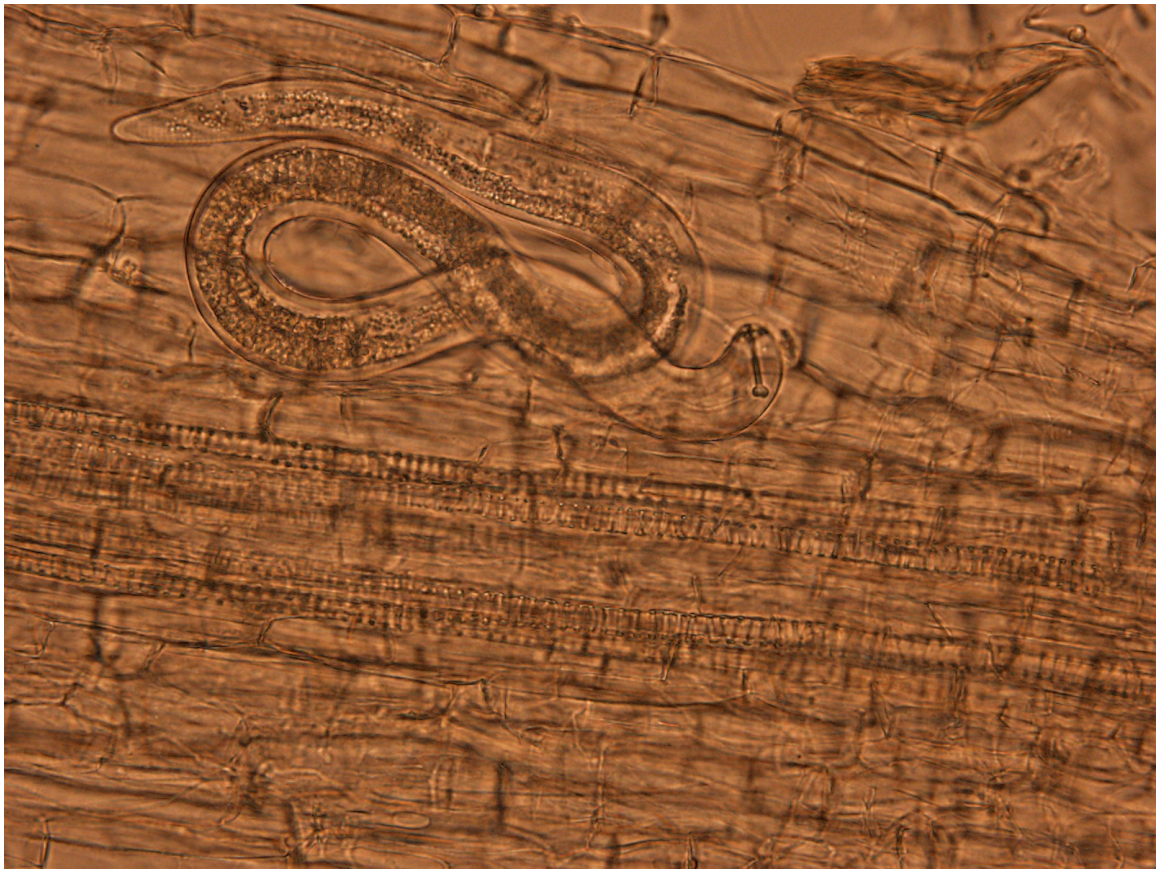

FIGURE 7. Fungal root preference assay on Gamborg's media at day three. Root segments can be seen on either side of the plate. Lateral root branching of control treatment can be seen on the right side of the plate (A).

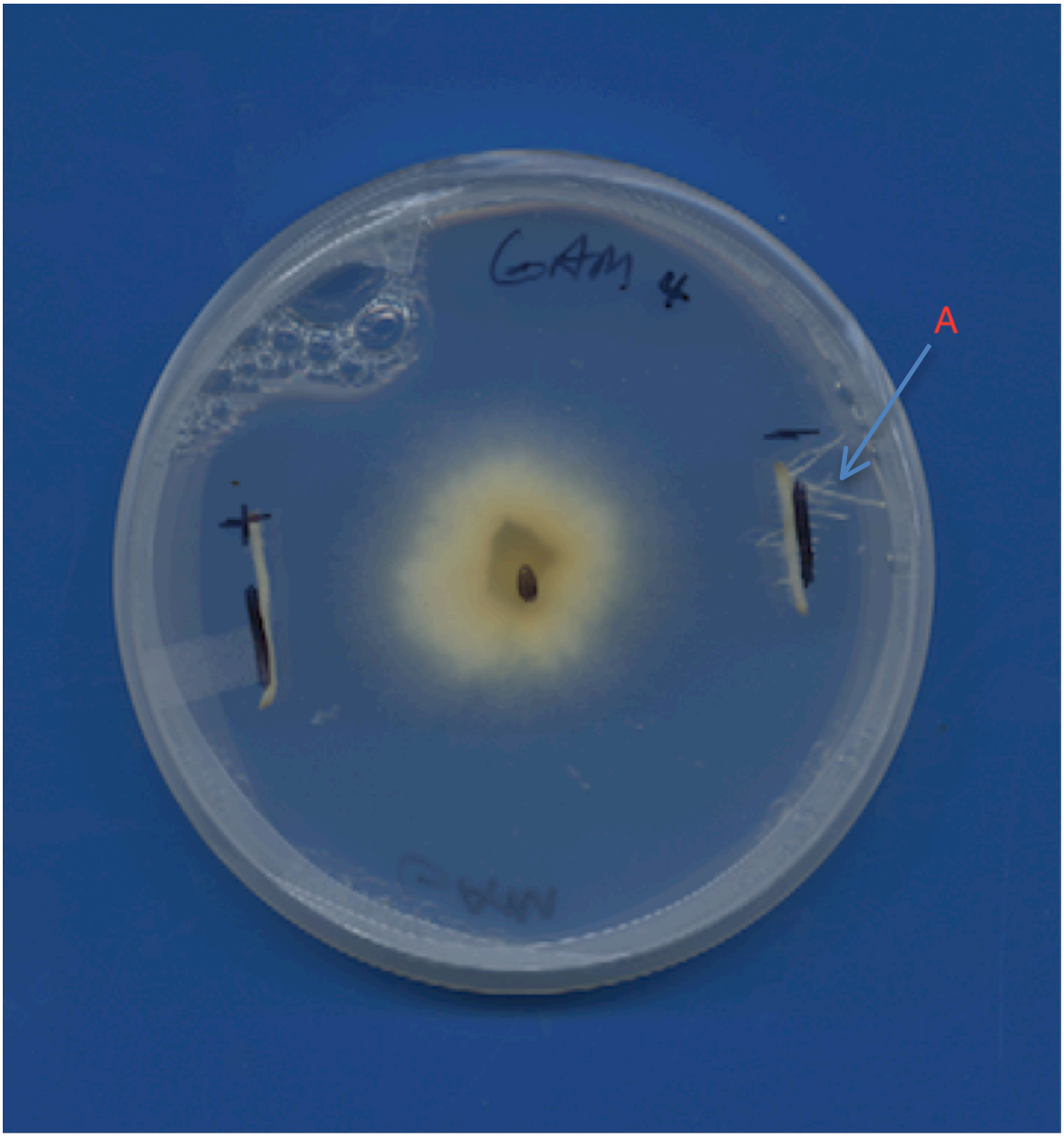

Table 1: Radial growth of *Fusarium verticillioides* towards either nematode-infected or control roots at 4 and 6 days post infestation.

Media Type	Colony Radius (cm)	
GAMBORG'S	DAY 4	DAY 6
P value:	*P* = 0.74	*P* = 0.10
Nematode-infected root	2.03	2.87
Control root	2.00	2.68
WATER AGAR		
P value:	*P* = 0.52	*P* = 0.50
Nematode-infected root	2.08	3.02
Control root	2.13	3.08

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