PLANT-PARASITIC NEMATODES IN FIELD PEA AND POTATO AND THEIR EFFECT ON PLANT GROWTH AND YIELD

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ABSTRACT

In this study, surveys were conducted in pea and potato fields in North Dakota and Central Minnesota to investigate the incidence and abundance of plant-parasitic nematodes in these fields. Moreover, the effect of the pin nematode, *Paratylenchus nanus*, on plant growth and yield of six field pea cultivars was determined under greenhouse conditions. Similarly, the influence of lesion nematode, *Pratylenchus penetrans*, and wilt fungi, *Fusarium oxysporum* alone and together on growth and yield of potato cultivar 'Red Norland', was evaluated in microplots under field conditions. The results indicate *Paratylenchus* spp. and *Pratylenchus* spp. are the most frequent nematodes, respectively, in pea and potato fields. Pin nematodes reproduced on field pea cultivars and caused up to 37% reduction in plant height and 40% reduction in yield. Additionally, both *P. penetrans* and *F. oxysporum* alone, and together had significant negative effect on growth and yield of potato.

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CHAPTER 1. INTRODUCTION

Plant-parasitic nematodes of economic importance can have detrimental effects on plant growth and yield potential of food crops. Plant-parasitic nematodes are reported to cause an estimated annual crop loss of $10 billion in the United States (US) (Chitwood 2003) and up to $157 billion globally (Singh et al. 2013). The damage caused by plant-parasitic nematodes under field conditions is often difficult to diagnose, above ground symptoms like yellowing, necrosis, stunting and patchy growth can be confused with symptoms of nutrient deficiencies or other soil problems and pathogens. Field peas and potatoes are among the important crops in temperate climate and North Dakota is a major producer of field peas in the US (United States Department of Agriculture National Agriculture Statistics Service [USDA-NASS 2017]). Similarly, North Dakota and Minnesota together contribute greatly to potato production in the USA (USDA-NASS 2017). Soil borne pathogens are among the major production constraints of both field peas and potatoes. There is limited information on incidence, densities and potential impact of soil borne, plant-parasitic nematodes on field peas and potatoes in the region.

The type and abundance of plant-parasitic nematodes are influenced by several factors such as crop type, soil physical and chemical properties, management practices and sampling time of the year (Yeates et al. 1999). Soil factors including soil texture, soil pH, soil structure, organic matter, aeration, and soil moisture affect the survival and pathogenicity of plant-parasitic nematodes (Norton 1989). To design effective nematode management strategies it is very crucial to have adequate information on soil-environmental factors influencing nematode reproduction and development.

Nematode surveys in pea fields in Canada indicated *Paratylenchus* spp., *Tylenchorhynchus* spp., and *Helicotylenchus* spp. as frequently occurring parasitic nematodes

(Sanwal 1971). In North Dakota-Minnesota potato fields, *Pratylenchus* spp. were identified as common parasitic nematodes (Yan et al. 2016; Baidoo et al. 2017). Plant-parasitic nematodes were reported to cause yield losses in potatoes (Castillo and Vovlas 2007) and field peas (Riga et al. 2008) in temperate regions. Root lesion nematode, *Pratylenchus penetrans* was reported to cause yield losses of 25 to 73% on potato cultivars (Olthof 1986). Similarly, *P. neglectus* and *P. thornei* along with *Paratylenchus hamatus* caused 75 to 90% yield losses in a pea field in Idaho (Riga et al. 2008).

Plant-parasitic nematodes can also interact with other organisms like fungui, bacteria, and viruses to increase damage to crops (Singh et al. 2013). Most reported nematode-fungal interactions are in crops such as cotton, potato, tobacco, banana, and tomato (Ravichandra 2013). In potato, the interaction of *P. penetrans* and *Verticillium dahliae* resulted in severe yield losses and increased potato early dying disease severity (Rowe and Powelson 2002).

In spite of the key role North Dakota and Minnesota play in potato and North Dakota plays in field pea production in the USA, limited knowledge exists on incidence and population densities of plant-parasitic nematodes and their impact on plant growth and yield of these crops. In addition, there is no information on the potential interaction of these nematodes with soil borne pathogens on crop growth and yield. Hence, the objectives of this study were:

1) To determine the occurrence and distribution of vermiform plant-parasitic nematodes and the relationship with soil factors in field pea (*Pisum sativum* L.) in North Dakota.
2) To determine the reproduction ability and impact of pin nematode, *Paratylenchus nanus* on field pea cultivars.

3) To determine the incidence of the plant-parasitic nematodes in potato (*Solanum tuberosum* L.) fields in central Minnesota.

4) To determine the effects of inoculation with *Pratylenchus penetrans* and *Fusarium oxysporum* alone or together on potato growth and yield.

CHAPTER 2. LITERATURE REVIEW

Field pea (*Pisum sativum* L.)

Background of field pea

Field pea (*Pisum sativum* L.) is a cool season legume crop cultivated in temperate regions, worldwide. Pea is one of the oldest food crop originated in the Near East and Mediterranean regions. It was domesticated over 9000 years ago since Neolithic period (Zohary and Hopf 1973: McPhee 2003). Field pea is used for both human consumption and livestock feed. Field pea contains 18-30 % protein, 35-50% starch, and 4-7 % fiber (McPhee 2003). It also has high amount of amino acids, lysine and tryptophan compared to cereal crops (McKay et al. 2003). It is frequently used as a rotational crop in temperate regions with cereal grain like wheat and has been reported to increase the yield of subsequent cereal crop by breaking the cereal pest cycles and improving the soil nitrogen (Carr et al. 2006; USDA-AMS 2009; Pavek 2012). Field pea was reported to increase the protein concentration of mixed forage by two to four percentage in forage crop mixture with small grains (McKay et al. 2003). Use of field pea as green manure improved the physical, chemical and biological properties of soil and productivity of successive crop (Fageria 2007).

Status of field pea production in the world, United States and North Dakota

Worldwide, field pea is the fourth highest produced food legumes after soybeans, peanuts, and dry beans (USDA Agriculture Marketing Service [USDA-AMS 2009]). In 2016 global production of field pea was 14.36 million tons (FAOSTAT 2018). Canada is the leading producer of field pea followed by Russia, China, and the United States of America (FAOSTAT 2018). The United States produced 1.37 million tons during 2016. In the United States, Montana, North Dakota, Washington, Idaho and Oregon are the important field pea producing states.

Among these States, North Dakota is one of the major producer of field pea with 560, 000 acres and production of 0.68 million tons with a monetary value of $ 131 million USD (United States Department of Agriculture National Agriculture Statistics Service [USDA-NASS 2017]). In 2016, 44% of total dry peas in the USA were produced in North Dakota (USDA-NASS 2017). In North Dakota, more than 70% of the field pea acreage is concentrated in the west-central counties (USDA-NASS 2017). Generally, two classes of field peas, green cotyledon type and yellow cotyledon type are grown in North Dakota (Table 2.1).

Table 2.1. Field pea cultivars commonly grown in North Dakota.

Cultivar	Market class	Days to maturity
Arcadia	Green	Early/Medium
Cruiser	Green	Medium
CDC Striker	Green	Medium
Majoret	Green	Medium
Aragorn	Green	Early
Bridger	Yellow	Early
Salamanca	Yellow	Medium
Agassiz	Yellow	Medium
CDC Meadow	Yellow	Medium
DS Admiral	Yellow	Early/Medium
Spider	Yellow	Medium

Biotic constraints of field pea production

Field pea is susceptible to more than 32 diseases including those caused by bacteria, fungi, and viruses (Hagedom and Kraft 2000). In the Northern great plains, major diseases and pests of field peas include blights (Aschochyta blight and bacterial blight), root rots (Ascochyta foot rot, Aphanomyces root rot, Fusarium root rot), stem rot (Sclerotinia stem rot), powdery mildew, Fusarium wilt, seed and seedling rot (Pythium and Rhizoctonia rot), pea seedborne mosaic virus, pea aphids, lygus bug, grasshoppers and nematodes. Among these diseases, root

rots are the most damaging in North Dakota field peas (Chittem et al. 2015). There have been limited studies on the plant-parasitic nematodes in North Dakota and their potential damage to field peas alone, or in association with other pathogens.

Nematode pests of field pea

Plant-parasitic nematodes are reported to cause an estimated annual crop loss of 10 billion USD in the USA (Chitwood 2003) and up to 157 billion USD globally (Singh et al. 2013). In Washington State, yield losses caused by *Heterodera goettingiana* were estimated to be 5 to 10% in green pea, whereas 1 to 5% losses were attributed to *Meloidogyne chitwoodi*, *M. hapla*, and *Pratylenchus* spp. during 1994 (Koenning et al. 1999). In Idaho, two species of lesion nematodes, *P. neglectus* and *P. thornei*, and one species of pin nematode, *Paratylenchus hamatus*, caused 75 to 90% yield losses to field peas under field conditions. Similarly, in a greenhouse study in Idaho, *P. neglectus*, *P. thornei*, and *P. hamatus* reduced plant height by 50 to 70% in field pea cultivars Columbian and Small Sieve (Riga et al. 2008). Stem and bulb nematode, *Ditylenchus dipsaci* reduced the biomass of yellow pea cultivars in greenhouse studies in Canada (Hajihassani et al. 2016). *Helicotylenchus vulgaris* together with *Heterodera goettingiana* and *P. thornei* affected pea crop growth in Worcestershire, England (Green and Dennis 1981). Similarly, *Helicotylenchus dihystera* was reported to reduce the yield of peas in Federal district, Brazil (Sharma et al. 1993). In India, field pea cultivars were observed to have susceptible to resistant reactions to *M. incognita* (Sharma et al. 2006). Charchar et al. (2008) reported *M. pisi* to be parasitic to field peas in Brazil. In Australia, field pea cultivars were reported to be susceptible to *P. penetrans* while resistant to *P. neglectus* (Vanstone 2007). Similarly, Smiley et al. (2014) observed that some cultivars of field pea were good hosts of *P. thornei*. During a survey in Idaho pea fields, *Pratylenchus* spp. and *Paratylenchus* spp. were

considered damaging and of economic importance to pea crop (Riga et al. 2008). Similarly, in pea fields of Eastern Ontario, Canada, *Helicotylenchus* spp., *Tylenchorhynchus* spp, *Pratylenchus* spp., and *Paratylenchus* spp. were found to be the predominant plant-parasitic nematodes while *Xiphinema* spp., *Criconemoides* spp., *Meloidogyne* spp and *Heterodera* spp. were less dominant (Sanwal 1971). Moreover, during a survey in Alberta, Canada, *Paratylenchus* spp., *Tylenchorhynchus* spp. and *Ditylenchus* spp. were determined as prominent nematodes in field peas (Hawn 1973). However, *Tylenchorhynchus* spp., *Longidorus* spp., *Rotylenchus* spp., and *Helicotylenchus* spp. occurred in lower densities in pea and bean fields in Scotland, hence, were considered of no economic importance (Boag 1980). Cyst nematode infection was reported to have negative impact on nodulation on peas (Taha and Raski 1969; Green 1985).

Nematode interactions with other pathogens of field pea

Nematodes interact with other organisms like fungi, bacteria and viruses to form a disease complex (Singh et al. 2013). Celetti et al. (1990) reported a significant positive relationship in incidence of *Fusarium solani* root infections and plant-parasitic nematodes: *Tylenchorhynhus* spp., *Helicotylenchus* spp., and *Paratylenchus* spp. in field pea soils on Prince Edward Island, Canada. Oyekan and Mitchell (1971) reported that *F. oxysporum* resistant field pea cultivars became susceptible with increased damage due to *P. penetrans* infection. Pea early-browning virus (B) was reported to be transmitted to pea seedlings by Stubby root nematode, *Trichodorus primitivus* (de Man) in Britain (Harrison 1966).

Nematode association with soil factors

The type and abundance of plant-parasitic nematodes were determined to be influenced by crop type, soil physical and chemical properties, management practices and sampling time of

the year (Yeates at al. 1999). Many soil factors including soil texture, soil pH, soil structure, organic matter, aeration, and soil moisture affected the survival and pathogenicity of plant-parasitic nematodes (Norton 1989). Plant-parasitic nematode genera or species were also reported to be correlated with different soil properties. *H. pseudorobustus* was positively correlated with % clay and pH while negatively with % silt and organic matter. Similarly, *Hoplolaimus galeatus*, *Tylenchorhynchus nudus* and *Xiphinema americanum* were negatively associated with soil pH in soybean fields in Iowa (Norton et al. 1971). However, *Tylenchorhynchus* spp. had a positive correlation with soil pH within a range of 5.0 to 6.5 in a native Iowa prairie (Schmitt 1969). Workneh et al. (1999) reported a significant negative correlation between *H. glycines* and % clay in no-tilled soil. *P. projectus* were reported to have negative correlation with soil pH (Thomas 1980). In organically farmed soil, only *Pratylenchus* spp. and *Xiphinema* spp. were correlated with soil factors (Chen et al. 2012).

Management of plant-parasitic nematodes

The best way to manage plant-parasitic nematodes is to prevent the infestation of non-infested fields. Nematode dissemination occurs during the movement of soil, plant tissue, farm machinery and equipment, water, animal, and wind from infested to non-infested field. Cultural practices to prevent nematode movement from one place to another include sanitation, use of certified clean plant material, quarantine and nematode free soil or planting media (Bird 1981). In a nematode infested field, an integrated nematode management approach can help to reduce nematode populations below damage threshold levels. Such an approach relies on combination of control tactics rather than using a single control measure. Integration of cultural practices such as crop rotation, cover crops, planting date, trap crops, rogueing or weed management may help to reduce the population densities of nematodes (Bird 1981; Brown 1978). Other nematode

management measures include use of organic soil amendments (Akhtar and Malik 2000), biological control agents (Siddiqui and Mahmood 1999) or host resistance (Williamson and Hussey 1996). Organic soil amendments such as crop residues and green manure may release compounds with nematicidal properties upon the decomposition of organic matter in soil and help in nematode reduction (Akhtar and Malik 2000). Similarly, biological control agents such as bacteria and fungi provide hostile environments for nematode reproduction or development through parasitism or trapping mechanism. Although biological control agents are environmentally safe and have been effective in controlling nematodes under laboratory conditions, their use in fields is limited due to lack of large scale production (Siddiqui and Mahmood 1999). Alternatively, Chemical nematicides can help to manage plant-parasitic nematodes, however, they have environmental issues (Fairbairn et al. 2007). In such scenario, use of host resistance can be an effective tool to manage parasitic nematodes. Recently, nematode resistance genes have been characterized which confer resistance to different plant-parasitic nematodes. Resistance gene mediated resistance provides hostile environments to nematode reproduction or development after infection. For instance, *Mi*-mediated resistance prevents the formation of giant cell in host plants, required for nematode infection and development, upon the invasion by *Meloidogyne incognita* (Williamson and Hussey 1996).

Potato (*Solanum tuberosum* L.)

Background of potato crop

Potato (*Solanum tuberosum* L.) originated about 8,000 years ago in South America was introduced into Europe around sixteenth century and spread to other parts of the world from Europe (Hawkes 1992; FAO 2009). Potatoes were introduced into the United States around 1621 A.D (National Potato Council 2018). It is the fourth important food crop worldwide after maize,

wheat, and rice (FAO 2018). Potatoes are grown in more than 100 countries worldwide under temperate, subtropical and tropical conditions, however, they are considered as cool temperature crop. Potatoes are good source of carbohydrate, vitamin C, potassium, phosphorus, and magnesium along with dietary antioxidants (FAO 2009). The United States ranked fifth in potato production worldwide with total production of 19.9 million tons in 2016 (FAO 2018). In the United States, Idaho, Washington, Wisconsin, North Dakota, Colorado, Oregon, Michigan, Minnesota, Maine, and California are the top ten potato producing states. North Dakota and Minnesota together produced 2.15 million tons in 2016 (USDA-NASS 2017).

Biotic constraints of potato production

Potato crops are severely affected by approximately forty soil borne diseases worldwide including those caused by soil inhabiting fungi, bacteria and nematodes (Fiers et al. 2012). Soil borne diseases of potato are important in the United States since many of them cause damage to tuber and roots (Gudmestad et al. 2007). Plant-parasitic nematodes are among the important pests of potato production, they can cause serious yield losses but remain unnoticed, in most cases. Above ground symptoms of nematode damage are rarely observed since most nematodes cause damage on roots and tubers (Hooker 1981). Seventy species of plant-parasitic nematodes belonging to twenty-four genera have been associated with potato crops (Jensen et al. 1979). Important plant-parasitic nematodes of potato crop in temperate region include potato cyst nematode (*Globodera rostochiensis* and *G. pallida*), Root knot nematode (*Meloidogyne hapla* and *M. chitwoodi*), false root knot nematode (*Nacobbus aberrans*), root lesion nematode (*Pratylenchus penetrans*), tuber rot nematode (*Ditylenchus destructor*), stem nematode (*Ditylenchus dipsaci*), and stubby root nematodes (*Trichodorus* spp. and *Paratrichodorus* spp.).

Many other plant-parasitic nematode species are also associated with potato crop, however, they are of less economic importance (Evans et al. 1992).

Lesion nematode (*Pratylenchus* spp.) in potato

Pratylenchus spp. are distributed worldwide and are important pests of potato in temperate, tropical and subtropical regions (Castillo and Vovlas 2007). Brown et al. (1980) reported six species of root lesion nematodes from potato roots including *P. crenatus*, *P. penetrans*, *P. scribneri*, *P. alleni*, *P. thornei*, and *P. neglectus* in Ohio. Yan et al. (2016) reported *P. scribneri* infestation in potato fields of North Dakota. Similarly, Baidoo et al. (2017) reported *P. penetrans* from potato fields in Minnesota. Kimpinski (1979) reported *P. penetrans* and *P. crenatus* as the dominant nematodes of potato crop in Prince Edward Island, Canada. In light sandy soil of Wisconsin, *P. penetrans* were reported to be the most damaging nematodes of potato crop (Dickerson et al. 1964). *Pratylenchus* spp. are migratory in nature and can feed both as ectoparasite and endoparasite. However, Feeding as endoparasite inside root tissue cells caused more damage than feeding from outside as ectoparasite (Castillo and Vovlas 2007).

Pratylenchus penetrans

P. penetrans is one of the economically important nematode in temperate regions. *P. penetrans* has a wide host range of over 350 plant species distributed in temperate regions in Europe, North America, Central and South America, Africa, Asia and Australia (Corbett 1973). *P. penetrans* are reported to cause serious damage of cereals, vegetables, fruits, grasses, and ornamental crops (Castillo and Vovlas 2007). *P. penetrans* completed its life cycle in 34-35 days at 24^{o} C on carrot callus (Wu et al. 2002). In clover root, single generation of *P. penetrans* was accomplished in 22 to 46 days at different temperature regimes. Temperature was an important factor determining the generation time of *P. penetrans* (Mizukubo and Adachi 1997). *P.*

penetrans was reported to be the most damaging nematode species of potato in a temperate climate (Castillo and Vovlas 2007). *P. penetrans* can infect roots, underground stems, stolons, and tubers. Symptoms of *P. penetrans* on roots involve brown necrotic lesions while on tubers are cross-lesions like those of common scab. *P. penetrans* infection leads to stunting and yellowing of potato crop and patchy growth of plants in heavily infested fields. In a heavily infested field in Norway, *P. penetrans* caused up to 50% yield losses (Holgado et al. 2009). Similarly, Olthof (1986) reported yield losses of 25 to 73% in Canada depending, upon potato cultivars. On cv. Superior of potato 30% yield losses was reported in Michigan (Bernard and Laughlin 1976). Moreover, *P. penetrans* was reported to suppress top growth and root mass of potato crop (Martin et al. 1982). Damage threshold of *P. penetrans* on potato was reported to be 1-2 *P. penetrans*/ cm^3 of soil (Castillo and Vovlas 2007). Bernard and Laughlin (1976) observed yield losses at 0.38 *P. penetrans*/ cm^3 of soil on cv. Superior cultivars. Similarly, Martin et al. (1982) reported that 0.56 *P. penetrans*/ cm^3 of soil could cause damage on potato growth.

Nematode fungal interactions

Atkinson (1892) was the first person to report the interaction of *Meloidogyne* spp. and *Fusarium* spp. causing severe Fusarium wilt of cotton. Considerable number of nematode fungal interactions have been reported in crops such as banana, cotton, potato, tobacco, cowpea, brinjal, and tomato. In okra and tomato crop, infection with *M. incognita* resulted high susceptibility to *Rhizoctonia solani* and root decay. In winter wheat, combined infection of *P. minyus* and *R. solani* lead to root rot, yellowing, stunting, and yield reduction. A disease complex, vascular wilt of cotton was caused by concomitant infection of *F. oxysporum* and *M. incognita*, *Rotylenchulus reniformis* or *Belonolaimus longicaudatus*. Similarly, vascular wilt of banana was caused due to combined infection of *Radopholus similis* and *F. oxysporum f.* spp. *cubense*. Moreover,

Pratylenchus spp. and *F. oxysporum f.* spp. *pisi* together resulted in vascular wilts of pea (Ravichandra 2013).

Potato early dying disease was reported to be more severe when *P. penetrans* and *Verticillium dahliae* were present together than when *V. dahliae* was alone (Rowe and Powelson 2002; Marin et al. 1982; MacGuidwin and Rouse 1990). Low levels of *P. penetrans* or *V. dahliae*, which caused little to damage when alone significantly increased disease severity and significantly lower tuber yield when present together. Nematode densities of 15, 50, and 150 *P. penetrans*/ 100 cm^3 of soil together with *V. dahliae* resulted in 36, 60 and 75% reduction in tuber yield (Martin et al. 1982). Under field conditions, 44 *P. penetrans*/ 100 cm^3 of soil did not reduce yield when acting alone, but reduced yield by 36% in the presence of *V. dahliae* (MacGuidwin and Rouse 1990). Similarly, 0.8 *P. penetrans*/ cm^3 of soil did not reduce tuber yield, but same density in combination with *V. dahliae* reduced the tuber yield by 51%. However, Burpee and Bloom (1978) did not find significant interactive effect of *P. penetrans* and *V. alboatrum* on plant growth and disease severity of Katahdin, Kennebec and Abnaki cultivars of potato. Nematode fungal interaction was observed to be species specific. *P. penetrans* and *V. dahliae* had significant interaction and were responsible for up to 39% yield loss while *P. crenatus* was not found to interact with *V. dahliae* (Riedel et al. 1985).

Ravichandra (2013) described the potential role of the nematode in the nematode fungal interaction as a wounding agent, host modifier, rhizosphere modifier and resistance breaker. He explained that nematode feeding leads to mechanical injury to roots, increases the root exudate production or creates galls/ lesions in roots or breaks the normal resistance of plant to other pathogens. Rowe and Powelson (2002) explained that nematode feeding and entry into root tissues increased root exudate production and the size of rhizosphere zone, leading to a change in

host physiology. Increased rhizosphere allows more fungal spores to attach to roots. Bowers et al. (1996) disproved earlier research indicating that nematode feeding injury provides direct pathway for fungal spores. In his observations, *P. pentrans* feeding on root tissues was not spatially related to entry of *V. dahliae*. Although exact mechanism of nematode fungal interactions are not clear, nematode attack increases stress on plant as well as changes host physiology making plants prone to infection by fungal pathogens (Rowe and Powelson 2002).

Management of nematodes in potato fields

Exclusion is the best approach for nematode management. Once the field is infested with economically important plant-parasitic nematodes it is difficult to completely eradicate them. Hence, sustainable and safe measures should be applied to suppress nematode populations below damage thresholds. Moreover, before applying any control measures, accurate identification of nematode to species level is desirable, some species within a genus can be more damaging than others (Castillo and Vovlas 2007).

Cultural practices such as crop rotation can help to suppress the population of lesion nematode, *Pratylenchus penetrans*, in potato fields. Chen et al. (1995) reported the suppression of *P. penetrans* in potato fields after a two-year rotation with alfalfa or clover. Similarly, in Canada, one-year rotation of potato with forage and grain pearl millet (*Pennisetum glaucum* L.) reduced *P. penetrans* populations and also increased potato yield in subsequent years (Belair et al. 2005). Organic amendments also can be used to control economically important nematodes in potato fields. Organic amendments such as green manure, crop residues, and farmyard manure provide conducive environment to micro-organisms which can parasitize plant-parasitic nematodes. Moreover, decomposition of organic matter can also produce compounds or chemicals toxic to nematodes (Castillo and Vovlas 2007). Conn and Lazarovitis (1999) observed

the reduction of *P. penetrans* populations in potato fields upon the application of poultry manure at 1,573 kg total N per hectare. Soil solarisation can also suppress parasitic nematodes in potato fields by heating soils under transparent plastic tarps. In Australia and Japan, *P. penetrans* populations were reduced using soil solarisation (Forter and Merriman 1985; Minagawa et al. 2004). In major potato producing states of the USA, farmers also use various chemical products to control nematodes and other pests in the potato crop. Various types of soil fumigants and non-fumigant nematicides can suppress nematode populations depending upon the application method and field condition. Pre-plant nematicides applied in soil should target the future root zone for effective nematode control. In Florida, non-fumigant nematicides of organo-carbamate group (product name – Vydate) in combination with fumigants have been more effective in lowering the incidence of stubby root nematode populations which vector tobacco rattle viruses causing corky ringspot disease of potato (Noling 2016). Resistant potato cultivars also can suppress the populations of parasitic nematodes present in infested fields (Castillo and Vovlas 2007). In conclusion, different management tactics are available for the control of plant-parasitic nematodes in potato crop, hence, selection of a method or combination of methods should be done after assessing the damage potential of nematodes present in field.

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CHAPTER 3. OCCURRENCE AND DISTRIBUTION OF VERMIFORM PLANT-PARASITIC NEMATODES AND THE RELATIONSHIP WITH SOIL FACTORS IN FIELD PEA (*PISUM SATIVUM* L.) IN NORTH DAKOTA

Abstract

Plant-parasitic nematodes restrict crop growth and cause yield losses of field peas. Nematode surveys were conducted in pea fields of North Dakota, a leading producer in the United States, from 2014 to 2017 to investigate their distribution, prevalence, abundance, and association with soil properties. A total of 243 soil samples were collected from 16 counties, and soil properties of 115 samples were analyzed to determine the association of nematode populations with soil factors (texture, organic matter, nutrients). Plant-parasitic nematodes, *Paratylenchus* (Absolute Frequency = 58 to 100%; Average densities = 470 to 1,558/ 200 g of soil; Highest Density = 7,114/ 200 g of soil) and *Tylenchorhynchus* (30 to 80%; 61 to 261; 1,980), were the most frequent and widely distributed. *Pratylenchus* and *Helicotylenchus* were identified in one-third counties and the average densities ranged from 43 to 224/ 200g of soil and 36 to 206/200 g of soil, respectively. Among the remaining nematodes identified, *Xiphinema* was found relatively frequently, but at low densities. *Hoplolaimus* and *Paratrichodorus* were rarely detected at lower densities in one or two counties. Canonical correspondence analysis revealed that soil factors explained 19% of the total variance of nematode genera abundance. The relationship between nematode abundance and soil factors such as sand and pH were significant ($P < 0.05$) while clay, silt, organic matter, and nutrients were not significant. This multi-year study conducted for the first time on nematodes associated with field peas and their relationship with soil factors in a major field pea production region of the U.S. serves as a guideline in designing effective nematode management strategies for this region.

Introduction

Field pea (*Pisum sativum* L.) is an important pulse crop with economic, nutritional, and agronomic benefits (McPhee 2003). It is commonly grown in rotation with cereal grains in temperate region of the United States (McPhee 2003; Carr et al. 2006). The U.S. is the fourth largest producer of field pea after Canada, Russia, and China (FAOSTAT 2018). The major field pea producing states in the U.S. include North Dakota, Montana, Washington, and Idaho. Two north central states, North Dakota and Montana together contributed 79 to 87% to the total national field pea production during 2014 to 2017 (USDA-NASS 2017).

Plant-parasitic nematodes (PPNs) have caused an estimated annual crop loss of 10 billion USD in the U.S. (Chitwood 2003) and 100 to 157 billion USD globally (Koenning et al. 1999; Chitwood 2003; Singh et al. 2013). Several morphological groups of PPNs are known to be present in pea fields in different parts of the world. Goodey et al. (1965) listed 32 species of PPNs associated with pea crop. Plant parasitic nematodes such as *Belonolaimus* spp., *Ditylenchus* spp., *Longidorus* spp., *Meloidogyne* spp., *Tylenchorhynchus* spp. (Goodey et al. 1965), *Heterodera goettingiana* (Handoo et al. 1994), *Hoplolaimus* spp. (Bridge and Starr 2007), *Helicotylenchus* spp., *Trichodorus* spp. (Green 1985), *Paratylenchus* spp. (Celetti et al. 1990; Riga et al. 2008), *Pratylenchus* spp. (Goodey et al. 1965; Celetti et al. 1990; Riga et al. 2008), and *Xiphinema* spp. (Lamberti et al. 1982) have been reported to be associated with field peas. In Eastern Ontario, Canada, *Helicotylenchus, Tylenchorhynchus*, *Pratylenchus*, and *Paratylenchus* were identified as frequently occurring nematode genera in pea fields (Sanwal 1971) while in Southern Alberta, *Ditylenchus*, *Tylenchorhynchus*, *Aphelenchus*, and *Paratylenchus* were predominant (Hawn 1973). The information on incidence and abundance of important PPNs in field pea is very limited in the field pea growing regions of the U.S. Nevertheless, *Pratylenchus*

and *Paratylenchus* were the common genera of PPNs in dryland pea in Latah and Nez Perce counties, Idaho, U.S (Riga et al. 2008) while *Heterodera*, *Meloidogyne*, and *Pratylenchus* were considered important to field pea production in Washington State, U.S (Koenning et al. 1999). Many plant-parasitic nematodes are known to be associated with field pea but only a few reports of nematode damage have been recorded around the world. In Washington, yield losses caused by *Heterodera goettingiana* were predicted to be 5 to 10% in green pea, whereas 1 to 5% losses were attributed to *Meloidogyne chitwoodi*, *M. hapla*, and *Pratylenchus* spp. in field pea during 1994 (Koenning et al. 1999). Similarly, *Pratylenchus neglectus*, *Pratylenchus thornei*, and *Paratylenchus hamatus* were responsible for stunting, chlorosis, and yield losses of 75 to 90% in two dryland pea fields in Latah County, Idaho. In addition, in greenhouse experiments, same three species reduced plant height of field pea cultivars by 50 to 70% (Riga et al. 2008). Besides, direct damage to a crop by feeding, nematodes can also cause indirect damage by interacting with other organisms like fungi and viruses (Green 1985; Singh et al. 2013).

The composition of nematode community is greatly influenced by host plant (Norton 1989). However, soil environmental variables such as soil texture, soil pH, organic matter, aeration, moisture, cation exchange capacity (Norton et al. 1971), soil nutrients, and soil temperature (Karuri et al. 2017) can also affect the type and population densities of PPNs. Among these soil factors, soil texture affects prevalence (Norton 1989; Yeates 1999; Workneh et al. 1999), population densities (Norton et al. 1971; Koenning et al. 1996; Workneh et al. 1999), reproduction (Griffin 1996; Koenning et al. 1996), migration, penetration capacity (Prot and Van Gundy 1981), and nematode pathogenicity (Griffin 1996). Yeates (1984) and Neher (1999) explained that soil properties including texture had more effect on nematodes than farming practice, either conventional or organic. However, it has also been suggested that cultivation

practice like tillage can change the structure of same textured soil and can influence the nematode population dynamics (Koenning et al. 1996; Workneh et al. 1999). Hence, in agricultural system, in addition to soil factors, some other factors such as cultivation practices and cropping history can also affect nematode community (Workneh et al. 1999).

In the U.S., farmers concern about PPNs and their potential impact in crop production is increasing because of huge investment in production activities. However, very less work has been done to identify the important group of PPNs and the threat posed by them in field pea. Currently, there is paucity of information on PPNs associated with field pea in the U.S. The existing knowledge on parasitic nematodes of this crop is solely based on small survey works covering few fields. Among the field pea producing states in the U.S., North Dakota is a principal producer in the nation; however, it is largely unknown about plant-parasitic nematodes associated with this crop in the state. Therefore, intensive nematode surveys are indispensable and the information obtained from such surveys on the type, distribution and abundance of nematode genera and species will be the guidelines essential to assess the damage potential on field pea and develop effective management strategies. Hence, the objectives of this study were-to 1) identify plant-parasitic nematodes associated with pea fields of North Dakota, 2) determine the incidence (occurrence frequency), abundance (population densities), and distribution of plant-parasitic nematodes in field pea growing regions of North Dakota, and 3) determine the relationship between nematode population densities and soil factors in pea fields. To our knowledge this is the first study describing the plant-parasitic nematodes in field peas in a region of the Northern Great Plains, and ascertaining their association with soil properties.

Materials and Methods

Soil sample collection

Nematode soil surveys were conducted in North Dakota pea fields encompassing North West, North Central, Central, and West Central regions where more than 70% of North Dakota pea production occurs (Fig. 3.1). Some fields were also surveyed in north eastern region of the state. A total of 243 soil samples were collected from 151 fields in 16 counties during 2014, 2015, 2016, and 2017 (Table 3.1). In general, one to three samples were collected from a field depending upon its size. Sampling was completed during the cropping season and immediately after harvest in the fall. Soil sampling was done arbitrarily for 58 samples during 2014 and for 91 samples during 2015 (Table 3.1). In 2016, 22 of 44 samples were taken from the previously sampled fields of the preceding years whereas in 2017, 40 of 50 samples were collected from fields not previously sampled.

Table 3.1. Total number of samples collected and counties covered during four years of sampling in pea fields in North Dakota

Year[a]	Number of samples	Number of fields	Counties[b]	Number of counties
2014	58	27	Burke, Divide, Williams, McHenry, Foster, Mountrail, Ward, Sheridan, McLean	9
2015	91	42	Mountrail, McLean, Renville, Ward, Burke, Divide, Williams, Foster, Sheridan, McHenry, Wells	11
2016	44	32	McLean, Mountrail, Wells, McHenry, Sheridan, Ward, Foster, McKenzie	8
2017	50	50	Divide, Williams, McKenzie, Mountrail, Ward, Renville, Bottineau, McLean, McHenry, Wells, Ramsey, Cavalier, Pembina	13
Total	243	151		16 (unique)

[a]Years in which samples were collected

[b]Name of counties from which samples were collected

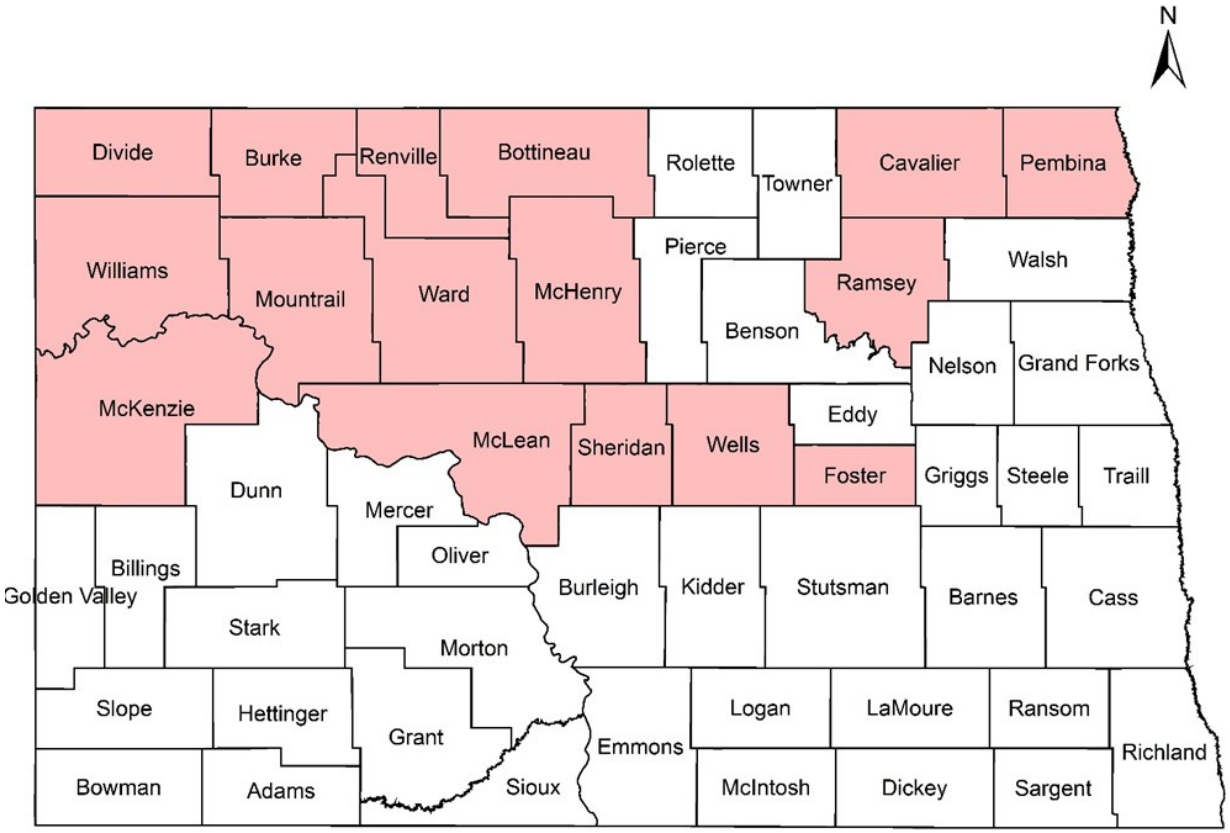

Fig.3.1. Map of North Dakota state showing counties sampled for vermiform plant-parasitic nematodes in field pea during 2014 to 2017. Sampled counties are represented by highlighted rose color.

Global Positioning System (GPS) co-ordinates were recorded for each sampling field using GPS navigator system (Garmin Drive 51 USA LM GPS Navigator System, OR, USA). Standard soil probes (2.5 cm diameter and 30 cm depth) were used to collect the representative soil sample from each sampling area in each field. Soil samples were collected in a zig-zag pattern with a distance of 5 m between two successive sample cores. For some of the larger fields more than one sample was collected from different directions of the field. In each sampling spot, the top soil of about 1-2 cm was removed and soil was collected up to a depth of 30 cm. Each soil sample consisted of 20 to 25 soil cores mixed together to obtain a composite sample. Soil

samples were sealed in a plastic bag, placed in a cooler during sampling and shipping and kept in a cold room at 4ºC. Nematode extractions were performed within two weeks of sampling to prevent changes in nematode populations.

Nematode extraction

Soil samples collected in 2014 were analyzed by Western Laboratories (Parma, Idaho) using standard procedures. Soil samples collected in other years were processed and analyzed in the Nematology Laboratory at North Dakota State University. Each of the soil samples was spread in a tray (36 cm x 27 cm) by breaking up large clumps of soil and removing crop residue and rocks, and mixed thoroughly. A sub-sample of 200 g was taken from each composite sample from which nematodes were extracted using sieving and decanting and sugar centrifugal-floatation technique (Jenkins 1964). Nematodes were collected in 20 to 25 ml tap water in a 50 ml suspension tube.

Plant-parasitic nematode identification and quantification

PPNs were identified to genus level based on the morphological characteristics: body shape and size, stylet type, stylet length, mouth type, lip region, esophageal overlap, vulva position, and tail type (Mai et al. 1996). Nematodes were categorized by genera and counted under an inverted transmitted light microscope at 100x magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY, USA). Finally, the number of PPN was expressed as the total number of individual nematodes of a genus in 200 g of soil.

Six genera of PPN either frequently occurring or rarely detected were further identified to species through molecular methods using nematode samples from 1 to 5 fields for each genus. For molecular identification, adult nematodes belonging to different genera were separately picked, and placed on concave glass slide with water, based on morphological features (Thorne

and Smolik 1971; Decramer 1980; Handoo and Golden 1992; Handoo 2000; Castillo and Vovlas 2007; Subbotin et al. 2015). Then, nematode DNA extraction was carried out according to the procedure described by Huang and Yan (2017). A single nematode was chopped in a concave glass slide, and nematode suspension (10 µl) was pipetted into a 0.5-ml sterile Eppendorf tube containing 10 µl of worm lysis buffer solution [2 µl of 10x PCR buffer, 2 µl of Proteinase K (600 µg/ml), and 6 µl of double-distilled water]. Eppendorf tubes holding chopped nematodes and lysis solution were incubated at -20ºC for 30 mins followed by 65ºC for 1 hour and then 95ºC for 10 mins. For each nematode genus, DNA was extracted individually from chopped pieces of single nematodes (n = 4 per field). The resulting DNA was then used immediately for PCR amplification using polymerase chain reaction (PCR) assays.

Molecular identification of nematodes to species level was achieved either by using species-specific PCR or by direct sequencing method (Table 3.2). Species-specific PCR was used to identify the species of lesion, stubby root, and lance nematode while direct sequencing was used to identify pin, spiral, dagger, and stunt nematode species. Species specific PCR was performed using *Pratylenchus neglectus* (primer set, Pn-ITS-F2/Pn-ITS-R2) (Yan et al. 2013), *P. scribneri* (PsF7/PsR7) (Huang and Yan 2017), *Paratrichodorus allius* (PaF11/PaR12) (Huang et al. 2017), and *Hoplolaimus stephanus* (Hs-1f/Hs-1r) (Ma et al. 2011) specific primer sets targeting the ITS region of rDNA. For direct sequencing technique, nematode DNA from two genomic regions D2-D3 region of 28S rRNA and ITS region of rDNA were amplified. The D2-D3 expansion region of 28S rRNA was amplified using primers D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Courtright et al. 2000) while the ITS region of rDNA amplified by primer set, rDNA1 (5′-ACGAGCCGAGTGATCCACCG-3′) and rDNA2 (5′-TTGATTACGTTCCCTGCCCTTT-3′)

(Cherry et al. 1997). For PCR reaction, template DNA (2 µl) was transferred into PCR tubes containing 18 µl of the PCR mixture consisting of 0.8 µl of each primer (10 µM), 0.4 µl dNTP, 1.2 µl $MgCl_2$, 4.0 µl 5x PCR buffer, and 0.15 U of Taq DNA Polymerase (Promega Corp., Madison, WI). PCR amplification was conducted with the following protocol: initial denaturation (94ºC for 3 min), followed by 40 cycles of denaturation at 94ºC for 45 s, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min, and a final extension for 10 min at 72ºC. After amplification, 2 µl of PCR product was mixed with 3 µl of 2x loading dye and a total of 5µl of the mixture was loaded in 2 % agarose gel for gel electrophoresis at 100 V for 25 min. The gel was visualized under UV light and images were taken using an AlphaImager Gel Documentation System (Proteinsimple Inc., Santa Clara, CA). After confirmation of the PCR amplification, amplified DNA was purified from the remaining PCR product using E.Z.N.A. Cycle Pure Kit (Omega BIO-TEK, Norcross, Georgia). Purified DNA was sent for DNA sequencing by GenScript (GenScript, Piscataway, NJ). DNA sequences were aligned using the sequence alignment tool, ClustalX, and the BLAST tool in NCBI (www.ncbi.nlm.nih.gov) was used to compare and identify similarity with the known nematode species sequences previously deposited in the GenBank database.

Soil property analysis

Soil property analysis was done for 115 soil samples, including 35 samples from 2014, 48 samples from 2015 and 2016, and 32 samples from 2017. A sub-sample of 500 g was prepared from each composite sample and were sent to Agvise Laboratory (Northwood, ND, USA) for soil property analysis. The soil pH, % organic matter, % sand, % silt, % clay particles, soil nitrogen, soil phosphorus and soil potassium of each sample were determined and reported from the laboratory.

Data analysis

Ecological parameters including Frequency, mean population density, relative density, and highest density were calculated to determine the incidence and abundance of plant-parasitic nematode genera in pea fields (Chen et al. 2012). Frequency of a nematode genus was expressed as number of samples containing a genus, divided by total number of samples collected, multiplied by 100. Mean population density of a nematode genus was calculated as average population density of a nematode genus in 200 g of soil. Relative density was expressed as average number of individuals of a genus divided by average number of individuals of all nematode genera, multiplied by 100 (Chen et al. 2012). Highest density was defined as the highest value from the range of population density of a nematode genus. Also, the Bray-Curtis dissimilarity indices/coefficients (Bray and Curtis 1957) were calculated to determine the dissimilarity/similarity of total plant-parasitic nematode genus abundance between counties in a sampling year. The formula for calculation is as follows:

$$\text{Similarity } (C_2) = 2W/A + B,$$

$$\text{Dissimilarity} = 1 - \text{Similarity},$$

Where C_2 = similarity index with a range of 0 to 1. A value of zero means no similarity between two sites and one means complete similarity between two sites for nematode abundance. Similarly, dissimilarity values also range from 0 to 1. However, a value of zero means no dissimilarity between two sites and one means complete dissimilarity between two sites.

A is the sum of nematode population densities of all the genera at site A,

B is the sum of nematode population densities of all the genera at site B, and

W is the sum of the lower nematode measures of each genus for the two compared sites.

The raw data for abundance of nematode genera were transformed using log (1+X) to maintain the homogeneity of variance between sites. Bray-Curtis dissimilarity matrix was generated based on dissimilarity indices. Then, hierarchical agglomerative cluster analysis using average linkage clustering algorithm (UPGMA) was done based on Bray-Curtis dissimilarity matrix for creating dendrogram to visualize the proximity of different sampled counties for nematode population structure using 'hclust' function of stats package of R (R Development Core Team 2017). Heat maps were also produced separately to visualize the abundance of each nematode genera in sampled counties for each sampling year using 'heatmap.2' function of gplots package of R (R Development Core Team 2017).

The Canonical Correspondence Analysis (CCA) was performed to determine the relationship between edaphic variables and abundance of nematode genera by using vegan package in R software (R Development Core Team 2017). Edaphic variables used for CCA included soil pH, % organic matter, % sand, % silt, % clay, soil nitrogen (ppm), soil phosphorus (ppm) and soil potassium (ppm) whereas nematode variables consisted of different nematode genera. Nematode genus rarely occurring in less than 5 soil samples were discarded prior to analysis. During analysis, Monte Carlo Permutation tests with 999 unrestricted permutations were performed to assess the statistical significance of CCA model and axes. Both model and axes were considered significant if the permutation test value was below 0.05. Moreover, in order to evaluate which edaphic variables significantly contributed to composition of nematode community a Monte Carlo test was performed. A CCA bi plot was produced where edaphic variables were represented by arrows with names and nematode variables by first three letters of nematode genus name (Fig. 3.4).

Results

Vermiform plant-parasitic nematodes in pea fields in North Dakota

Genus and species

Seven genera of plant-parasitic nematodes were detected in pea fields during the survey period. These genera include *Paratylenchus* (pin nematode), *Tylenchorhunchus* (stunt nematode), *Pratylenchus* (lesion nematode), *Xiphinema* (dagger nematode), *Helicotylenchus* (spiral nematode), *Hoplolaimus* (lance nematode), and *Paratrichodorus* (stubby root nematode). Based on the characteristics of DNA sequences, the species of pin, stunt, and spiral nematodes were identified as *Paratylenchus nanus*, *Tylenchorhynchus annulatus*, and *Helicotylenchus digonicus*, respectively (Table 3.2). The ITS region of pin nematodes showed 100% similarity to *P. nanus* upon sequence BLAST in NCBI (Table 3.2). Similarly, ITS region of stunt nematodes showed 99% affinity to *T. annulatus* (Table 3.2). The D2-D3 expansion region of spiral nematodes showed 99% identity to *H. digonicus* (Table 3.2). The species of lesion nematodes were identified as *Pratylenchus neglectus* and *P. scribneri* upon successful amplification of ITS region of rDNA with species specific primers Pn-ITS-F2/Pn-ITS-R2 (Yan *et al.*, 2013) and PsF7/PsR7 (Huang and Yan 2017), respectively (Table 3.2). Lance nematodes and stubby root nematodes were identified as *Hololaimus stephanus* and *Paratrichodorus allius*, respectively upon positive amplification of ITS region of rDNA with species specific primers Hs-1f/Hs-1r (Ma et al. 2011) and PaF11/PaR12 (Huang et al. 2017), respectively (Table 3.2).

Table 3.2. Molecular identification methods, accession numbers (GenBank deposited and compared), E value, and percent homogeneity for plant-parasitic nematode species identified from pea fields in North Dakota[a]

Nematode group	Identification method	Nematode species	Deposited accession no.	Compared accession no.	E value	Homogeneity
Pin	Direct sequencing	*Paratylenchus nanus*	MH236098	KF242264	0.0	100
Stunt	Direct sequencing	*Tylenchorhynchus annulatus*	MH379768	KJ461572	0.0	99
Spiral	Direct sequencing	*Helicotylenchus digonicus*	MH444651	KM347963	0.0	99
Lesion	Species-specific PCR	*Pratylenchus neglectus*	-	-	-	-
	Species-specific PCR	*P. scribneri*	-	-	-	-
Lance	Species-specific PCR	*Hoplolaimus stephanus*	-	-	-	-
Stubby root	Species-specific PCR	*Paratrichodorus allius*	-	-	-	-

[a]Deposited accession numbers and compared accession numbers denote to the distinct identification number of query sequence and the comparison sequence, respectively; E-value is the expect value and homogeneity refers to the percent similarity between query sequence and comparison sequence upon sequence BLAST in the NCBI database.

Frequencies and densities of PPN genera

Paratylenchus were the most frequent nematodes with highest abundance in all years of the survey. They were identified in 100% of the samples in 2016 followed by 92% in 2017, 79% in 2014, and 58% in 2015 (Table 3.3). The abundance or mean population density of *Paratylenchus* for positive samples in survey years was between 470 to 1,557 individuals/ 200 g of soil (Table 3.3). However, across the survey period, population density of *Paratylenchus* ranged from 17 to 7,114 individuals/ 200 g of soil in positive samples (Table 3.3). In 2016 and 2017, thirty four percent of the positive samples for *Paratylenchus* had higher nematode numbers than the mean population density of *Paratylenchus* in those years. Similarly, 32% and 30% of the positive samples were identified with higher nematode numbers than the average population density of *Paratylenchus* in 2014 and 2015, respectively. The contribution of *Paratylenchus* to plant-parasitic nematode communities in pea fields based on relative density was higher (59% to 76%) than any other plant-parasitic nematodes detected (Table 3.3).

Tylenchorhynchus were the second most frequent nematodes identified in all survey years. They were most frequent (78 to 80%) during 2016 and 2017 compared to other years: 40% and 30% for 2014 and 2015, respectively (Table 3.3). The abundance or mean population density of *Tylenchorhynchus* was highest during 2014 (261/ 200 g of soil) and lowest for 2015 (61/ 200 g of soil) (Table 3.3). Nevertheless, the population density of this nematode ranged from 17 to 1,980/ 200 g of soil across the survey. In general, 25% to 43% of the positive samples for *Tylenchorhynchus* had higher nematode numbers than the average population density of this nematode. Based on the relative density, *Tylenchorhynchus* contribution to PPN communities in field pea was second to *Paratylenchus* in most survey years (Table 3.3).

Helicotylenchus, *Pratylenchus*, and *Xiphinema* were the commonly detected genera of PPNs after *Paratylenchus* and *Tylenchorhynchus* during the survey. *Helicotylenchus* were common in 34% of the samples in 2017, followed by 23% in 2016, 22% in 2015, and 9% in 2014 (Table 3.3). Density of *Helicotylenchus* ranged from 16 to 1,100/ 200 g of soil in positive samples. *Pratylenchus* were identified in 28% of the samples in 2017, followed by 23% in 2016, 10% in 2014, and 2% in 2015 (Table 3.3). Density of this nematode was in range of 17 to 1,980/ 200 g of soil. *Xiphinema* were detected in 23% to 24% of the samples in 2016 and 2017 while 6% to 8% of the samples in 2014 and 2015 (Table 3.3). Density of *Xiphinema* reached as high as 130/ 200 g of soil (Table 3.3).

Rest of the plant parasitic nematodes: *Hoplolaimus* and *Paratrichodorus* nematodes were detected least frequently and not observed in all the survey years, contributing least to nematode faunae in field peas. *Hoplolaimus* was observed only during 2016 in 11% of the samples with a density reaching up to 106/ 200 g of soil (Table 3.3). Likewise, *Paratrichodorus* was observed only in 1 of 44 samples during 2016 at a density up to 22/ 200 g soil (Table 3.3).

Table 3.3. Frequencies and densities of nematode genera during sampling years, 2014 to 2017 in pea fields of North Dakota[a]

Nematode genus	No. of positive samples	Frequency (%)	Mean population density or abundance (#/200 g)	Relative density (%)	Highest population density (#/200 g)
			2014 (N = 58)		
Paratylenchus	46	79.31	908.69	68.69	6,840
Tylenchorhynchus	23	39.65	260.86	19.71	1,980
Pratylenchus	6	10.34	43.33	3.27	100
Xiphinema	5	8.60	26.00	1.96	60
Helicotylenchus	5	8.62	64.00	4.83	120
			2015 (N = 91)		
Paratylenchus	53	58.24	470.45	73.16	4,294
Tylenchorhynchus	27	29.67	60.59	9.42	207
Helicotylenchus	20	21.97	35.80	5.56	173
Xiphinema	9	9.89	22.66	3.52	52
Pratylenchus	2	2.19	53.50	8.32	88
			2016 (N = 44)		
Paratylenchus	44	100.00	1557.52	76.22	7,114
Tylenchorhynchus	35	79.50	149.22	7.30	685
Helicotylenchus	10	22.70	144.10	7.05	739
Pratylenchus	10	22.70	104.50	5.11	559
Xiphinema	10	22.70	24.70	1.20	70
Hoplolaimus	5	11.40	41.20	2.01	106
Paratrichodorus	1	2.30	22.00	1.07	22
			2017 (N = 50)		
Paratylenchus	46	92	835.02	58.80	3,666
Tylenchorhynchus	39	78	114.25	8.02	780
Pratylenchus	14	28	223.64	15.77	1,980
Xiphinema	12	24	40.50	2.88	130
Helicotylenchus	27	34	205.52	14.50	1,100
			All years (N = 243)		
Paratylenchus	189	77.77	918.91	64.58	7,114
Tylenchorhynchus	124	51.02	139.63	9.81	1,980
Helicotylenchus	62	25.51	129.45	9.09	1,110
Xiphinema	36	14.81	29.63	2.08	130
Pratylenchus	32	13.11	141.9	9.97	1,980
Hoplolaimus	5	2.05	41.20	2.89	106
Paratrichodorus	1	0.41	22	1.54	22

[a]Frequency = (number of samples positive for a genus) / (total number of samples collected during that period) x 100; Mean population density / abundance = average population density of a genus in 200 g of soil; Relative density = (Mean population density of a genus) / (sum of measures of mean population densities of all genera) x 100; Highest density = highest value from the range of population density of a nematode genus (Chen *et al.*, 2012).

Nematode distribution and abundance in counties over the four years survey

Paratylenchus were widely distributed in all sampled counties (N=16) followed by *Tylenchorhynchus* (15), *Helicotylenchus* (13), *Pratylenchus* (12), *Xiphinema* (11), *Hoplolaimus* (2), and *Paratrichodorus* (1) (Fig. 2.2). The heatmap shows the high abundance of *Paratylenchus* and *Tylenchorhynchus* nematodes in most of the sampled counties (Fig. 2.2). *Helicotylenchus* were detected at higher densities in Mountrail, Sheridan, Bottineau, and Ward County (Fig. 2.2). The abundance of *Pratylenchus* were higher in counties such as Bottineau, Wells, and McHenry (Fig. 2.2). Among the commonly identified genera, *Xiphinema* were present at lower densities in all the detected counties. Similarly, rarely detected nematodes, *Hoplolaimus* and *Paratrichodorus* were also present at lower densities in the detected counties. *Hoplolaimus* were found in Ward and McHenry County whereas *Paratrichodorus* in only Ward County (Fig. 2.2). The most nematode genera (7) were identified in Ward County (Fig. 2.2). There was an average detection of five nematode genera in all surveyed counties. Counties of Bottineau, Divide, McHenry, McKenzie, McLean, Mountrail, Sheridan, Wells and Ward had five or more nematode genera detected.

Sampled counties were grouped into clusters in the dendrogram based on similarity for type and abundance of PPN genera. A cut-off was arbitrarily determined so that counties with higher affinity for PPN genera could be considered as a cluster (cut line not physically drawn in dendrograms of Fig. 3.3) (Jackson et al. 2010). Three major clusters were generated in dendrogram at a dissimilarity coefficient of 0.5 (Fig. 3.3). The first cluster in the dendrogram had three counties which shared approximately 55% similarity for PPNs genera (Fig. 3.3). The second cluster was the largest with nine counties and they had approximately 70% affinity for genera of PPNs (Fig. 3.3). Moreover, there were three sub-groups in this largest cluster and the

counties in sub-group of third cluster, Bottineau, Wells, Sheridan, McLean, and Mountrail, had highest level of similarity (approximately 93 to 95%) for type and abundance of PPNs genera (Fig. 3.3). The third and last cluster had four counties with approximately 70% identity for PPNs genera (Fig. 3.3).

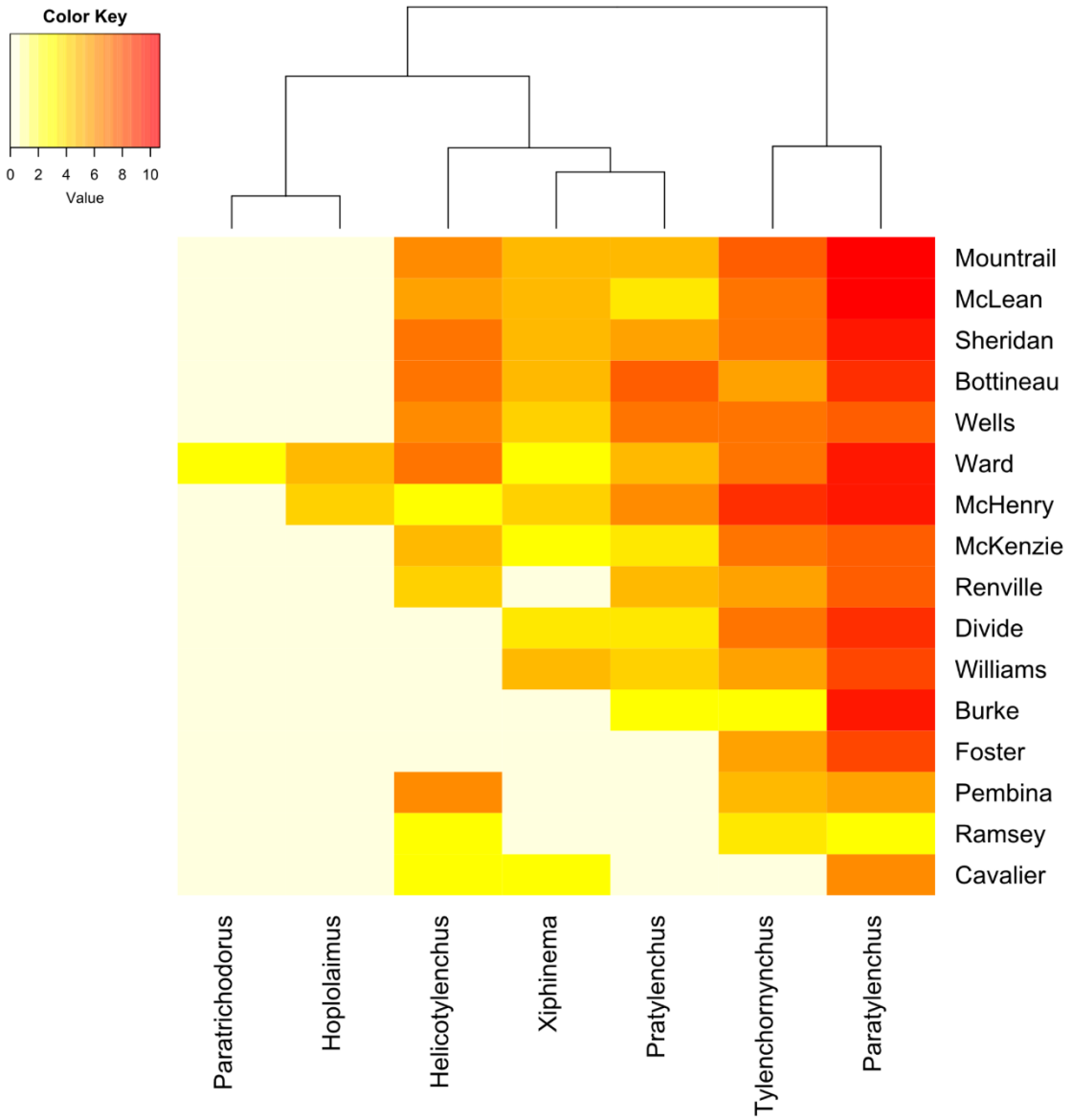

Fig. 3.2. Heatmap showing the abundance of individual plant-parasitic nematode genera in different sampled counties during entire survey period. Dendrogram on the top of heat map represents the clustering of nematode genera based on their abundance in sampled counties. On the right hand side and in the bottom of the heat maps are the names of the counties sampled and the genera of nematodes found in the sampling period. Color key indicates the normalized nematode abundances in 200 g of soil. Dark red color represents the highest abundance of a genera in the corresponding county and with decreasing nematode number the color intensity decreases to light whittish yellow color indicating absence of a nematode genus.

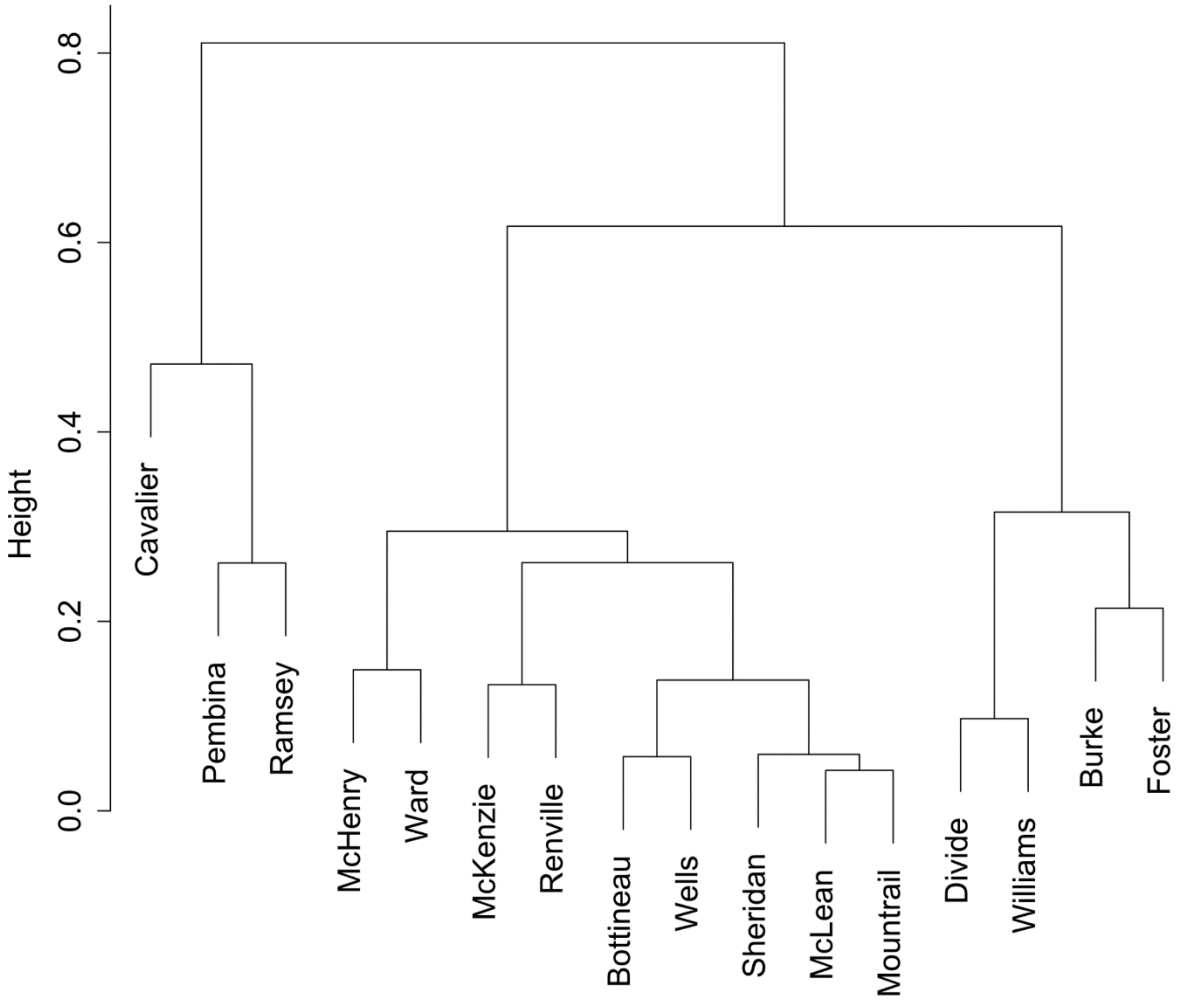

Fig. 3.3. Dendrogram showing clustering of counties based on Bray-Curtis dissimilarity values for type and abundance of nematode genera in each of the sampled counties during the entire survey period. Height bar on the left hand side of each dendrogram represents dissimilarity values from 0 to 1. A value of 0 means no dissimilarity or complete similarity between two compared sites (counties or group of counties) and 1 means complete dissimilarity or no similarity between two compared sites.

Relationship of nematode genera abundance with edaphic variables

Soil texture analysis identified five classes of soils in assayed pea field samples: clay, clay loam, loam, sandy loam and sandy clay loam. The predominant type of soil were sandy loam, sandy clay loam and loam. Soil pH ranged from 4.5 to 8.4 where 14.0% of the assayed soil samples had pH less than 5.5, 41.7% had between 5.5 to 7.0 and 44.3% had more than 7.0. Organic matter contents in the tested soil samples had a range of 1.1 to 6.3. The percentage of sand, silt, and clay ranged from 13 to 84%, 10 to 59%, and 6 to 42%, respectively.

Canonical correspondence analysis was used to measure the relative importance of edaphic factors in explaining the variation in nematode genera abundance. Edaphic variables considered in this study explained 19% of total variation in nematode genus abundance whereas remaining 81% of the variation remained unexplainable. The first two canonical axes were statistically significant (CCA1, $P = 0.001$; CCA2, $P = 0.02$) and the cumulative percentage of variance explained by these axes in the nematode genera-soil data was 83.8%. Among two axis, the first axis accounted for 56.1 % of the variance and the second axis accounted for 27.7% of the variance (Fig. 3.4). Two edaphic variables, % sand ($P = 0.001$), and pH ($P = 0.001$) were only significantly related to the abundance of PPNs genera in field pea soil which is also indicated by the length of arrows (longer) pointing these variables in the CCA bi-plot (Fig. 3.4). In the CCA bi-plot, % sand was related to positive side of axis 2 while pH was related to positive side of axis 1 (Fig. 3.4). There was a positive correlation for the abundance of nematode genera such as *Pratylenchus*, *Tylenchorhynchus* and *Xiphinema* with sand. Similarly, *Helicotylenchus* and *Tylenchorhynchus* were positively correlated with pH. However, densities of *Paratylenchus* were negatively associated with sand and pH (Fig. 3.4).

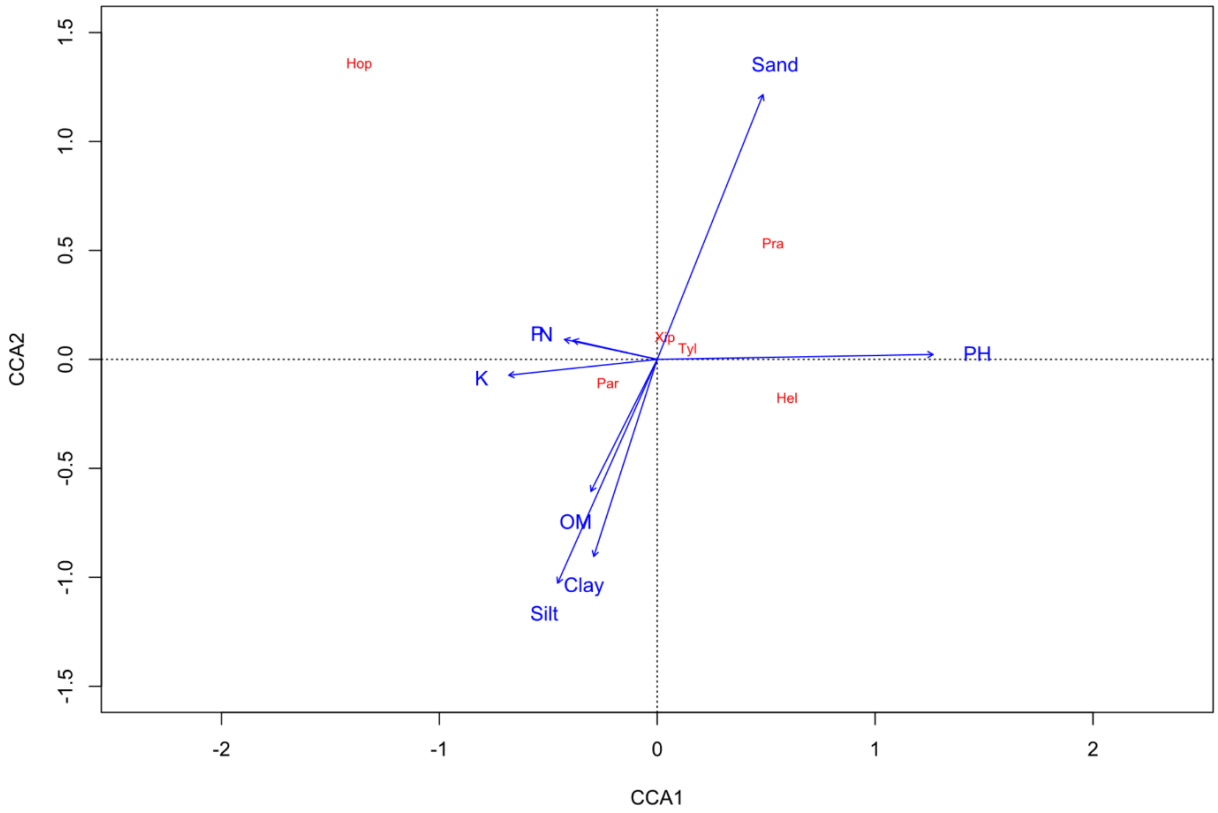

Fig. 3.4. Canonical correspondence analysis (CCA) bi-plot showing the relationship between nematode genera abundance and soil variables. Soil variables are represented by blue colored arrows. Nematode genera are represented by first three letters of genera name with red color: *Pratylenchus* (Pra), *Tylenchorhynchus* (Tyl), *Xiphinema* (Xip), *Helicotylenchus* (Hel), *Paratylenchus* (Par), and *Hoplolaimus* (Hop). The first axis (CCA1) explains 56.1% of the variance while the second axis (CCA2) explains 27.6% of the variance.

Discussion

This is the first comprehensive work done to study the distribution, occurrence, and abundance of PPNs in pea fields in North Dakota, principal producer of field pea in the U.S. A total of seven genera of PPNs were identified in this study, encompassing the endoparasite (*Pratylenchus*), ectoparasites (*Paratylenchus*, *Tylenchorhynchus*, *Helicotylenchus*, *Xiphinema*, *Paratrichodorus*) and semi-endoparasite (*Hoplolaimus*). Nematode genera identified in this study are also known to be pathogenic to peas elsewhere in the world (Goodey et al. 1965; Green

1985; Riga et al. 2008). In the current work, seven species were characterized for six genera, including *Pratylenchus neglectus*, *P. scribneri*, *Paratylenchus nanus*, *Tylenchorhynchus annulatus*, *Helicotylenchus digonicus*, *Hoplolaimus stephanus*, and *Paratrichodorus allius*. The pathogenicity of most of the identified species is unknown to field pea, however, *P. neglectus* has been previously reported to be economically important to field pea in the Pacific Northwest region of the U.S. (Riga et al. 2008). In North Dakota, some of the species identified during this survey have been reported as important pathogens in crops such as wheat, soybean, and potato. (Yan et al. 2016a; 2016b; 2016c). However, to our knowledge, *P. nanus*, *H. stephanus*, *and P. allius* identified during this survey are the first detection in field pea in the U.S. while all the identified species are the first record in pea fields in North Dakota. Moreover, stunt nematodes identified in this study, *Tylenchorhynchus annulatus* (accession number MH379668) is the first report in the state of North Dakota.

Pin nematodes, *Paratylenchus* were identified as the dominant plant-parasitic nematodes in terms of frequency and density in pea fields. The mean population density and incidence of these nematodes reached as high as 1,558/ 200 g of soil and 100%, respectively. In previously conducted surveys in Canadian pea fields, *Paratylenchus* were reported as predominant nematodes, however, the incidence was comparatively lesser than in present survey, 57% incidence in Alberta (Hawn 1973) and 55% in Eastern Ontario (Sanwal 1971). In general, *Paratylenchus* nematodes are considered as minor pathogens (Berry and Coop 2000), however, at higher densities and in the presence of susceptible host they can be damaging (Coursen and Jenkins 1958; Braun and Lownsbery 1975). For instance, in Missouri, soybean yields were negatively correlated with pre-plant *P. projectus* populations of approximately 400/ 200 g of soil (Niblack 1992). Moreover, in a previous greenhouse assay, *P. hamatus* was reported to impact

growth of field pea cultivars, Columbian and Small Sieve at the initial density of 400/ 200 g of soil (Riga et al. 2008). In the absence of ND region specific economic damage threshold for *Paratylenchus* nematodes, it is difficult to determine what population levels are damaging in field pea, however based on previous studies *Paratylenchus* population at higher densities should be considered seriously. In a preliminary greenhouse study, *P. nanus* identified in this survey increased by 10-fold and 5-fold in Columbian and Cooper cultivars, respectively (Upadhaya et al. unpublished). Hence, further research will be needed to determine the pathogenicity of this species to field pea cultivars.

Tylenchorhynchus nematodes, with huge potential to cause economic losses in crops, were the second most prevailing nematodes. *Tylenchornhynchus* nematodes were found in 30 to 78% of the samples with mean population density reaching up to 261/ 200 g of soil and a high density up to 1,980/200 g of soil. Previously, high incidence of the *Tylenchorhynchus* were also reported in pea fields in Eastern Ontario, Canada (80%) (Sanwal 1971) and Alberta, Canada (68%) (Hawn 1973). In addition, *Tylenchorhynchus* nematodes were also frequent (73%) in Syrian legume crops (Greco et al. 1984). Although, *Tylenchorhynchus* nematodes were present at higher densities in present survey the level of risk posed by them to field pea is unknown. Stunt nematode species, *T. annulatus*, identified in this survey has been previously reported in wheat and corn in Idaho State, U.S (Hafez et al. 2010) while in legumes like pigeon pea and cowpea in Trindad (Bala 1984). This suggests that this species is associated with both cereal crops and leguminous crops, and this justifies the presence of *T. annulatus* in field peas in this region where cereals and legumes are frequently grown in rotation.

Helicotylenchus and *Pratylenchus* nematodes which are considered to be economically important nematodes in many crops were relatively less frequent compared to *Paratylenchus* and

Tylenchorhynchus. Moreover, if compared to a similar survey in Eastern Ontario, Canada, with 90% incidence of *Helicotylenchus* and 70% occurrence of *Pratylenchus* (Sanwal 1971), detection frequency of these nematodes (<35 %) in the present survey can be considered low. However, during survey years, mean population density of *Helicotylenchus* and *Pratylenchus* reached as high as 206 and 224/ 200 g of soil, respectively. Moreover, some fields were also identified with very high density of *Helicotylenchus* nematodes reaching up to 1,100/200 g of soil and *Pratylenchus* nematodes up to 1,980/ 200 g of soil. Considering these facts, both of these nematodes can be considered important to field pea in North Dakota. Previously, species of *Helicotylenchus* such as *H. vulgaris* (Green and Dennis 1981) and *H. dihystera* (Sharma et al. 1993) were reported to be pathogens of pea crop in England and Brazil, respectively. However, in field pea in North Dakota different species of *Helicotylenchus*, *H. digonicus*, was identified. This particular species has been reported in pea (Sanwal 1971) and forage legumes like alfa alfalfa and clover (Townshed 1973). Species of *Pratylenchus*, *P. neglectus* identified in present study, has previously reported as pathogen of field pea based on greenhouse assays and field evaluations (Riga et al. 2008). In North Dakota, this species has been previously reported in wheat crop (Yan et al. 2016a). In a preliminary greenhouse assays, *P. neglectus* from pea fields, were also observed to successfully reproduce in field pea cultivars such as Columbian and Arcadia, multiplying up to six times at the end of a crop cycle (Upadhaya et al. unpublished).

Xiphinema nematodes with several species known to vector plant viruses, were detected at lower average densities compared to most detected nematodes during this survey. The densities of *Xiphinema* with more than 100 nematodes/100 cm^3 soil has been shown to cause considerable losses to some crops in organically farmed fields (Chen et al. 2012). In our survey, the highest density of *Xiphinema* was 130/ 200 g of soil in a field in Williams County. Several

species of *Xiphinema* are known to vector different plant viruses (Brown et al. 1995), however, there are no available reports of viral transmission by this nematode in field pea in the U.S. *Paratrichodorus*, which can also transmit plant virus, was detected only once in Ward County during 2016 at a low density of 22/ 200 g soil. *P. allius* identified in this study, is commonly known to transmit *Tobacco rattle virus* (TRV) causing corky ringspot disease of potato in North Dakota (Yan et al. 2016b). *Hoplolaimus* nematodes, responsible for crop losses in the U.S., were detected at lower incidence level and in only one of the survey years. The species of *Hoplolaimus*, *H. stephanus* identified in pea crop, was previously reported in soybean crop in North Dakota (Yan et al. 2016c).

Paratylenchus and *Tylenchorhynchus* nematodes were widespread in sampled counties. *Helicotylenchus*, *Pratylenchus*, and *Xiphinema* occurred approximately in one-third of the sampled counties while others were detected rarely in one or two counties. Counties such as Cavalier, Pembina, and Ramsey were separately clustered together because only three genera were identified in each of these counties as well as the densities of dominant nematodes such as *Paratylenchus* and *Tylenchorhynchus* were lower. Similarly, a separate cluster was generated for Counties like Divide, Williams, Burke, and Foster since they had higher densities of *Paratylenchus* but lower densities of other genera. Remaining nine counties were clustered together because they had almost comparable densities for five commonly identified nematode genera in this survey. In North Dakota, legumes such as field peas are frequently grown in rotation with cereal crops (McPhee 2003). However, due to absence of information on some other factors affecting nematode community in fields it is hard to explain about variability in nematode type and number among the counties. Previously, Chen et al. (2012) reported higher similarity for nematode abundance among the physically closer regions. However, in this survey

distantly located counties such as Bottineau, located in northern side of the state, and Wells, in central region, were observed to have more than 95% similarity for type and densities of nematode genera.

The role of soil environmental factors comes second to host plant in determining the nematode population dynamics (Norton 1989; Cadet et al. 2004). Previously, researchers identified edaphic factors such as soil texture, organic matter, pH (Norton et al. 1971; Kimpinski and Willis 1981), and nutrients (Cadet et al. 2004) as important variables influencing the densities of nematodes in soil. Although, soil characteristics influence the densities of PPNs, selected soil variables in this study were successful in explaining 19% of total variance in abundance of nematode genera whereas huge amount of variance remained unexplainable. This suggests that besides the selected explanatory variables, some other factors could be crucial for the nematode genera abundance. Hence, identification of such factors is indispensable to understand more about population dynamics of PPN genera in field peas. In Northern Great Plains, factors such as type of farming practice, cultivar type, and climatic variables could add to variation in nematode communities.

In general, there is some agreement that coarse textured soil favor the reproduction and movement of nematodes in soil (Koenning et al. 1996). In line with this fact, in the current work, % sand was statistically associated with the abundance of nematode genera. We observed the positive correlation of *Pratylenchus*, *Tylenchorhynchus*, and *Xiphinema* numbers with % sand. Similar to our observation, densities of *Pratylenchus* and *Xiphinema* were positively correlated with % sand in sugarcane fields in South Africa (Cadet et al. 2004). In current work, *Helicotylenchus* and *Tylenchorhynchus* were also positively correlated with pH. Likewise, earlier works also have reported similar correlation of *Helicotylenchus* (Cadet et al. 2004) and

Tylenchorhnychus with soil pH (Schmitt 1969). In the present study, smaller sized nematodes, *Paratylenchus* were only negatively correlated with % sand and pH. Similar to this observation, Thomas (1980) also reported the negative correlation between numbers of *P. projectus* and soil pH. Results indicating negative correlation of smaller sized nematodes while positive correlation of comparatively larger sized nematodes with % sand warrant future studies on finding the role of particle sizes to movement, feeding, and reproduction of different sized nematodes.

This study for the first time identified the genera and species of PPNs in pea fields of North Dakota, a major field pea production state, through a multi-year survey. Incidence and population density of nematodes varied among the genus, counties, and samples. The mean population density of some economically important genera were lower, however, some field samples were identified with higher densities of those nematodes. Higher incidence and abundance of some genera in survey years warrants further research to evaluate the damage potential and develop damage threshold level of these nematode populations in field pea. Description on nematodes associated with field pea, their incidence and population density in this region can also serve as important guideline to other field pea producing states in the northern region of the U.S. Furthermore, results of relationship between soil properties and genera abundance demonstrated that sand and pH are the important soil parameters describing variation in nematode population.

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CHAPTER 4. REPRODUCTION AND EFFECT OF PIN NEMATODE, *PARATYLENCHUS NANUS* ON SELECTED FIELD PEA CULTIVARS

Abstract

Greenhouse experiments were conducted to determine reproduction ability and effect of *Paratylenchus nanus* from North Dakota on field pea cultivars. Reproduction of *P. nanus* was determined on seven field pea cultivars using naturally infested field soils at low (1,500/kg of soil) and high (4,500/ kg soil) initial pin nematode densities. Nematode effect was evaluated at 4,500 *P. nanus*/kg of soil by artificially inoculating *P. nanus* on six field pea cultivars. Reproduction of *P. nanus* was observed higher at low density compared to high density. At low density, Reproductive Factor (RF) ranged from 1.10 to 11.20. However, at high density, RF ranged from 1.20 to 2.50. The results with high density in naturally infested soil were comparable to those from artificially inoculated experiment at the same level. Moreover, in first repetition of effect experiment, *P. nanus* caused reduction ($P < 0.05$) of plant height in Arcadia, Cruiser, and Bridger and seed yield in Arcadia and Cruiser. Plant height and yield were reduced most in Arcadia by 18 and 28%, respectively. In second repetition, plant height, shoot weight, and yield were significantly reduced for most tested cultivars. Plant height and shoot weight reductions were the highest in Arcadia by 37% and 53%, respectively, while yield was reduced most by 40% in Columbia. This research shows the damage potential of *P. nanus* on field peas, and is the important step towards developing effective management strategies to improve the productivity of this leguminous crop.

Introduction

Pin nematodes, *Paratylenchus* spp. are smaller plant parasitic nematodes (Raski 1975; Geraert 1965) associated with diverse plant species worldwide (Van Den Berg et al. 2014). They

are migratory in nature and feed ectoparasitically in root epidermis, root hairs (Rhoades and Linford 1961) and as deep as root cortex (Braun and Lownsbery 1975). *Paratylenchus* spp. can parasitize on plant roots and cause detrimental effect on plant growth: *P. hamatus* on wheat, pea (Riga et al. 2008), and celery (Lownsbery et al. 1952); *P. neoambycephalus* on myrobalan plum seedlings (Braun and Lownsberry 1975), apple and apricot seedlings (Fisher 1967); *P. projectus* on tobacco and tall fescue (Coursen and Jenkins 1958); and *P. nanus* on perennial rye grass (Bell 1999). Soil surveys in pea fields of North Dakota from 2014 to 2017 identified *Paratylenchus* spp. as the dominant plant parasitic nematodes based on the frequency of occurrence and population densities (Upadhaya et al. 2018, unpublished). The average population density of *Paratylenchus* spp. was 4,595/ kg of soil during 2014 to 2017 survey period. Pin nematode populations on some farms of North Dakota were identified as *P. nanus* based on the morphometric and molecular examinations (Upadhaya et al. 2016).

Paratylenchus nanus Cobb, 1923 was reported in Europe (Brzeski 1995), North America (Raski 1975), Australia (Fisher 1966), Africa (Van den Berg et al. 2014), Asia (Esmaeili and Heydari 2017), and even in Antartica (Ryss et al. 2005). *P. nanus* has a strikingly different Juvenile-4 stage, devoid of a prominent stylet, while other larval stages have a clear feeding stylet (Ghaderi et al. 2014). J-4 is a non-feeding resistant stage like in some other *Paratylenchus* spp. which can be explained as a form of self-regulation to unfavorable environmental conditions (Bell and Watson 2001a; Rhoades and Linford 1961). *P. nanus* is known to be associated with grasses, fruits, vegetables, and cereals (Fisher 1965; Raski 1975; Knight et al. 1997; Bell and Watson 2001a; Esmaeili and Heydari 2017). Grasses such as Italian rye grass, perennial rye grass, and cocksfoot are good hosts of *P. nanus* (Bell and Watson 2001b). Moreover, apple and apricot seedlings were known to be hosts of *P. nanus* population in Australia (Fisher 1965).

Previous studies have shown the negative effect of *P. nanus* on grasses and fruits but their impact on food legumes is largely unknown.

North Dakota is the major producer of field peas in the United States (USDA-NASS 2018). Many plant-parasitic nematodes are associated with field peas in ND, and pin nematodes are the common and dominant nematodes which were identified as *Paratylenchus nanus* (Upadhaya et al. 2016). However, the role of pin nematode populations to field peas in North Dakota is not known. Hence, the objectives of this study were to 1) evaluate the reproduction ability of *P. nanus* in field pea cultivars, using naturally infested pea field soil in greenhouse conditions and 2) evaluate the effect of *P. nanus* on plant growth and yield of field pea cultivars through artificial inoculation in greenhouse conditions.

Materials and Methods

Pin nematode species identification and confirmation

Morphological identification

In fall of 2015, twenty-seven soil samples were collected from eleven pea fields in North Dakota. Sixty-three percent of these soil samples had only pin nematodes as the plant-parasitic nematodes (Upadhaya et al. 2018, unpublished). Morphometric measurements of pin nematode adult females (total: n = 32) from four pea fields, in North Dakota were taken. Morphological measurements included total body length (µm), stylet length (µm), esophagus length (µm), body diameter (µm), tail length (µm), V% (vulva position from anterior end in % of body length), a [body length/greatest body width], b [body length/ distance from anterior end to junction of esophagus and intestine], and c [body length/ tail length] (Thorne and Smolik 1971; Raski 1975). Nematodes were kept in glass slides under a modular microscope (Zeiss Axio Scope.A1; Zeiss, Oberkochen, Germany) and images were taken at different magnifications (200X, 400X and

800X) depending upon the body parts. Measurements were made on those images using a software program, ZEN 2 lite (Carl Zeiss Microscopy, Germany). These measurements were then compared with those of pin nematode species in published papers (Thorne and Smolik 1971; Raski 1975).

Molecular confirmation

In order to confirm the species identity of the pin nematodes, molecular examination was also performed. Adult female pin nematodes were handpicked based on their morphological features (Raski 1975). A single nematode was chopped in a concave glass slide and nematode suspension (10 µl) was pipetted into a 0.5-ml sterile Eppendorf tube containing 10 µl of worm lysis buffer solution [2 µl of 10x PCR buffer, 2 µl of Proteinase K (600 µg/ml), and 6 µl of double-distilled water]. Tubes containing chopped nematodes and lysis solution were incubated at -20ºC for 30 mins followed by 65ºC for 1 hour and then 95ºC for 10 min (Huang and Yan 2017). DNA was extracted individually from chopped pieces of single nematodes (n = 4).
The ITS region of rDNA was amplified by a universal primer set, rDNA1 (5´-ACGAGCCGAGTGATCCACCG-3´) and rDNA2 (5´-TTGATTACGTTCCCTGCCCTTT-3´) (Cherry et al. 1997). Template DNA (2 µl) was transferred into 18 µl of the polymerase chain reaction (PCR) mixture consisting of 0.8 µl of each primer (10 µM), 0.4 µl dNTP, 1.2 µl $MgCl_2$, 4.0 µl 5x PCR buffer, 0.15 U of Taq DNA Polymerase (Promega Corp., Madison, WI). PCR cycle conditions consisted of initial denaturation (94ºC for 3 min), followed by 40 cycles of denaturation at 94ºC for 45 s, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min, and a final extension for at 72ºC for 10 min. After completion of PCR cycles, 2 µl of PCR product was mixed with 3 µl of 2x loading dye and a total of 5µl of the mixture was loaded in 2 % agarose gel. Gel electrophoresis was done at 100 V for 25 min. The gel was visualized under UV light

and images were taken using an AlphaImager Gel Documentation System (Proteinsimple Inc., Santa Clara, CA). After confirmation of the PCR amplification, amplified DNA was purified from the remaining PCR product using E.Z.N.A. Cycle Pure Kit (Omega BIO-TEK, Norcross, Georgia). Purified DNA was sent for DNA sequencing by GenScript (GenScript, Piscataway, NJ). DNA sequences were aligned using sequence alignment tool, ClustalX, and the BLAST tool in NCBI (www.ncbi.nlm.nih.gov) was used to compare and identify similarity with the known nematode species sequences previously deposited in the GenBank database.

Reproduction ability of *P. nanus* using infested field soil

Soil collection and processing

Naturally infested soil with pin nematode, *P. nanus* was collected from a pea field to determine the reproduction ability of this nematode using field pea cultivars in greenhouse conditions. Infested soil was mixed thoroughly for hours so that uniformity for initial nematode densities could be maintained in each experimental pot or unit. Three sub-samples of 0.2 kg were prepared from the mixed soil. Average of nematode densities in those sub-samples was determined after nematode extraction using Sugar Centrifugal-floatation technique (Jenkins 1964). The average nematode densities per kg of soil were calculated which were used as initial nematode densities for reproduction ability experiments.

Greenhouse experiments

Two greenhouse experiments were conducted using naturally infested soils at different starting densities of *P. nanus* with 16 hours day light and average temperature of 22 ºC. In the first experiment the initial density of *P. nanus* was low (1,500/ kg of soil) while in the second experiment it was high (4,500/ kg of soil). Both of these experiments were repeated (Table 4.1).

Table 4.1. Experimental details (experiment type, soil, initial population densities and experimental period) for pin nematode, *P. nanus* reproduction and effect in greenhouse experiments.

Experiment type[a]	Soil[b]	Level	Pi/ kg of soil[c]	Repetition (trial)	Planting date	Harvesting date
Reproduction ability	Naturally infested soil	Low	1,500	1	4/23/2016	7/19/2016
			1,500	2	12/29/2016	3/24/2017
		High	4,500	1	4/23/2016	7/19/2016
			4,500	2	12/29/2016	3/24/2017
Nematode effect	Sterilized soil	High	4,500	1	3/18/2016	5/31/2016
			4,500	2	12/5/2016	2/19/2017

[a]In reproduction ability experiments, the reproductive factor (final population/ initial population per kg of soil) of *P. nanus* was calculated. In effect experiments, plant growth parameters (plant height, dry root weights, and dry shoot weights), yield and final nematode populations were recorded.
[b]Naturally infested soil indicates the use of field soil positive for *P. nanus* as inoculum in reproduction experiments. On the other hand, nematodes were artificially inoculated to sterilized soil, which was then used as inoculum for effect experiments.
[c]Pi refers to initial population of *P. nanus* at the time of planting.

A total of seven field pea cultivars: Columbia, Cooper, Bridger, Arcadia, Cruiser, Aragorn, and Salamanca were used in these experiments. Seeds of these cultivars were pre-germinated for 4-5 days by placing them in petri-dishes with wet paper. Clay pots (15 cm x 15 cm) were filled with naturally infested soil with the initial nematode populations as explained earlier. Each pot with soil was fertilized with one tea spoon of slow release formulation 14-14-16 NPK and mixed thoroughly. After fertilizer application, a single pre-germinated seed of a cultivar was placed at a depth of 3-4 cm in each of the filled pots. Each cultivar was replicated four times during both experiments. Experiments were completely randomized in blocks and placed in greenhouse benches. Plants were allowed to grow in the greenhouse for 12 weeks and the experiments were terminated on 13th week. Plant tops were cut near to soil surface and the

soils with roots were placed in plastic bags which were stored at 4^{o}C until nematode extractions within a week.

Nematode extraction

Each soil plus root sample collected from a single pot with a plant was placed in a tray (36 cm x 27 cm), and mixed thoroughly keeping the roots separately. A sub-sample of 0.2 kg was taken from each sample from which nematodes were extracted using Sugar Centrifugal-floatation technique (Jenkins 1964). During extraction process roots were also rinsed with water to get all the nematodes from the soil around the roots. Nematodes were collected in 20 to 25 ml tap water in 50 ml suspension tube. All the *P. nanus* juvenile-4 without prominent stylet and other life stages with stylet were counted together under an inverted transmitted light microscope at 100X magnification. (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY, USA). Finally, number of *P. nanus* nematodes was expressed as total number of individuals in 1 kg of soil.

Reproductive factor and ratings

Nematode reproductive factor (RF) on each experimental unit, individual pot with a pea plant, was calculated by dividing the final population by initial population of *P. nanus* in kg of soil. Average RF of nematodes on a treatment, cultivar, is a mean RF of four replications of each cultivar. In order to determine the preference of vermiform nematode on pulse crops including peas, Hajihassani et al. (2016) have used four host groups: non-host (RF < 1), poor host (RF = 1 to 2), good host (RF = 2 to 4), and excellent host (RF > 4). In this study, four susceptible/resistant groups were designated to simplify the interpretation of reproductive factor on different cultivars: Resistance (RF = 0 to 0.9), moderately resistance (RF = 1.0 to 1.9), moderately susceptible (RF = 2.0 to 3.9) and susceptible (RF = 4).

Data analysis

Levene's test of homogeneity of variance was performed using PROC GLM of SAS to determine whether the data from repeated trials could be combined for further analysis (SAS Institute Inc., Cary, NC). Repeated trials for both low and high nematode densities were confirmed to be homogenous ($P > 0.05$) and therefore combined analysis on reproductive factors of *P. nanus* were performed using PROC GLM of SAS 9.4. Mean separations were performed using *F*-protected least significant difference (LSD) at $P < 0.05$ to determine the significant differences in reproductive factors of nematodes in tested pea cultivars. Furthermore, simple t-test was performed to compare reproductive factors of nematodes across cultivars in two experiments with different initial densities at 95% confidence interval using SAS 9.4.

Effect of pin nematodes on plant growth and yield

Inoculum preparation and experiment set up

P. nanus population collected from the same field as described earlier in reproduction ability experiment, were reared and increased on field pea cultivars Columbian and Cooper in greenhouse conditions. After 11-week incubation period, *P. nanus* was extracted from soil with plants using a Whitehead tray method (Whitehead and Hemming 1965). Whitehead tray method was preferred for extracting nematodes to be used as inoculum because extracted nematodes do not suffer stress like those obtained by Sugar Centrifugal-floatation technique, which involves use of sugar and multiple centrifugations steps. After nematode extraction, *P. nanus* population was concentrated to 300 nematodes/ ml with all vermiform life stages and stored at 4ºC as inoculum.

Two rounds of experiments were conducted to determine the effect of *P. nanus* on growth and yield of field pea cultivars using sterilized soil. Field pea cultivars used in these experiments are described in detail in Table 4.2.

Table 4.2. Field pea cultivars used in this study.

Genotypes	Market class[a]	Days to maturity	Originator[b]	Growing regions[c]
Columbia	Green	Medium	Campbell Soup Co.	PNW (ID, WA)
Cooper	Green	Late	Innoseeds B.V. Co.	ND
Arcadia	Green	Early/Medium	Lantmannen SW Seed Co.	MT, ND
Cruiser	Green	Medium	ProGene	PNW (WA), MT, ND
Aragorn	Green	Early	ProGene	PNW (ID, WA), MT, ND
Bridger	Yellow	Early	Legume Logic	MT, ND, SD
Salamanca	Yellow	Medium	Legume Logic	ND

[a]Green and yellow field pea cultivars are categorized based on seed cotyledon color.
[b]Originator refers to the developer of those field pea cultivars.
[c]PNW refers to the states of Pacific North West region (Washington, Idaho, and Oregon). ND, MT and SD indicates the states of North Dakota, Montana, and South Dakota, respectively. These data were obtained from field pea varietal trial extension bulletins from North Dakota State University (Kandel et al. 2016), South Dakota State University (Graham et al. 2017), Montana State University (Mohammed and Chen 2017), University of Idaho, Washington State University, and USDA-ARS Pullman, Washington (USA Dry Pea and Lentil Council 2012).

Clay pots were filled with 1 kg of sterilized soil and one tea spoon of slow release formulation 14-14-14 N-P-K was put in each pot and mixed well. A single pre-germinated seed of a field pea cultivar was placed in the center of a filled pot at 3-4 cm depth. Nematode suspension of 15 ml, approximately 4,500 *P. nanus*/ kg of soil, were pipetted in holes around the pre-germinated seeds to facilitate nematode invasion. Holes were covered with appropriate amounts of sterilized soil after inoculation. Staking was done in each pot after 3 weeks of planting to prevent the lodging of plants. Both nematode inoculated and non-inoculated control

of all cultivars were replicated four times and were completely randomized in blocks in a greenhouse bench. Plants were allowed to grow in greenhouse for 10 weeks and the experiments were terminated on 11th week.

Data collection

Data on plant parameters and final nematode populations were collected to determine the effect of *P. nanus* on field pea cultivars. Plant height was measured at the end of experimental period. Other plant parameters: shoots, roots and seed yield were determined after drying these parts at 80^{o}C for 48 hours in an incubator (VWR International, PA 19087 USA). Nematodes were extracted from a sub-sample of 0.2 kg from each pot using Sugar Centrifugal-floatation technique (Jenkins 1964). *P. nanus* was collected and counted using the same method described in the reproduction ability experiment. Final population of *P. nanus* was expressed as total number of individuals in 1 kg of soil.

Data analysis

Data obtained from the first and second round of nematode effect experiments were analyzed separately because they were found to be heterogeneous after Levene's test of homogeneity of variance ($P < 0.05$). Simple t-tests were performed to compare the average plant parameters: height, dry shoot weight, dry root weight and dry seed yield of nematode inoculated cultivars with non-inoculated control of corresponding cultivar at $P < 0.05$ using SAS 9.4 (SAS Institute Inc., Cary, NC). Plant parameters of each nematode treated cultivar treatment was only compared with respective non-inoculated control to ascertain the significant difference in those parameters.

Results

P. nanus identification and confirmation

Morphometric measurements of individuals (n = 32) included body length (range = 300 to 395 µm, average = 350 µm), stylet (24.80 to 31.30, 27.50), esophagus length (63.90 to 82.10, 74.06), body diameter (14.30 to 23.40, 17.90), tail length (20.10 to 28.20, 24.70), a (16.80 to 23.50, 19.75), b (3.13 to 4.52, 3.64), c (13.17 to 15.43, 14.16), and V% (83.30 to 86, 84.80). The head region was round and tail terminus was subacute and blunt but not digitate. These characters are important to separate them from morphologically closely related species, *P. projectus* which has truncated head and often digitate tail (Ghaderi et al. 2014). Males were not observed in our populations, however, Raski (1975) reported males for this species and claimed to be rarely observed. These morphometrics are within the range of those described for *Paratylenchus nanus* by Thorne and Smolik (1971). Molecular analysis of Internal Transcribed Spacer (ITS) region of rDNA showed that sequence (MH236098, 828 bp) was 100% identical with one population of *P. nanus* from South Africa (KF242264), 99% identical with two populations of *P. nanus* from South Africa (KF242263 and KF242266) and two from South Korea (KY468906 and KY468910). Moreover, this sequence shared 87% sequence identity with other pin nematode species, *P. hamatus*. The molecular examination and morphological measurements confirmed the species of pin nematode from the ND pea fields as *P. nanus*.

Reproduction ability of *P. nanus* on field pea cultivars using naturally infested soil

P. nanus reproduction was the highest (RF = 11.20) on Columbia cultivar with significantly high ($P < 0.05$) reproductive factor value compared to other tested field pea cultivars at initial density of 1,500 *P. nanus*/kg of soil. (Fig. 4.1). Similarly, *P. nanus* reproduction on Arcadia (RF = 4.80) and Cooper (4.30) was greater than 4, indicating that these

two cultivars were susceptible. Other cultivars including Cruiser (3.80), Bridger (3.40), and Aragorn (2.50) had RF values between 2 to 3.90 suggesting a moderately susceptible reaction (Fig. 4.1). Among all the tested cultivars, *P. nanus* reproduced least in Salamanca (RF = 1.10) (Fig. 4.1). Statistical analysis showed that the RF of *P. nanus* was not significantly ($P > 0.05$) different among Arcadia, Cooper, Cruiser and Bridger. (Fig. 4.1). Similarly, RF of *P. nanus* was not significantly ($P > 0.05$) different between Aragorn and Salamanca (Fig. 4.1).

Reproductive factor of *P. nanus* averaged across cultivars was statistically lower ($P = 0.0001$) at high initial inoculum density compared to low inoculum density. *P. nanus* reproduction was reduced by 5-fold in Columbia cultivar at high initial inoculum density of 4,500 *P. nanus*/ kg of soil compared to low initial density of 1,500 *P. nanus*/ kg of soil. Similarly, approximately 2-fold reduction was observed in Arcadia, Cooper, Bridger, and cruiser. However, Salamanca, which favored least reproduction at lower density, behaved in almost similar way at higher initial density. *P. nanus* reproductive factor was between 2 to 3.90 on Arcadia (RF = 2.50), Cruiser (2.50), and Columbia (2) with a moderately susceptible reaction while other cultivars, Cooper (1.90), Bridger (1.90), Aragorn (1.60), and Salamanca (1.20), were moderately resistance with RF between 1 to 1.90 (Fig. 4.2). Reproductive factor values of Arcadia, Cruiser, Columbia, Cooper, and Bridger were significantly higher ($P < 0.05$) than Salamanca while significant difference was not observed in RF values of Aragorn and Salamanca (Fig. 4.2).

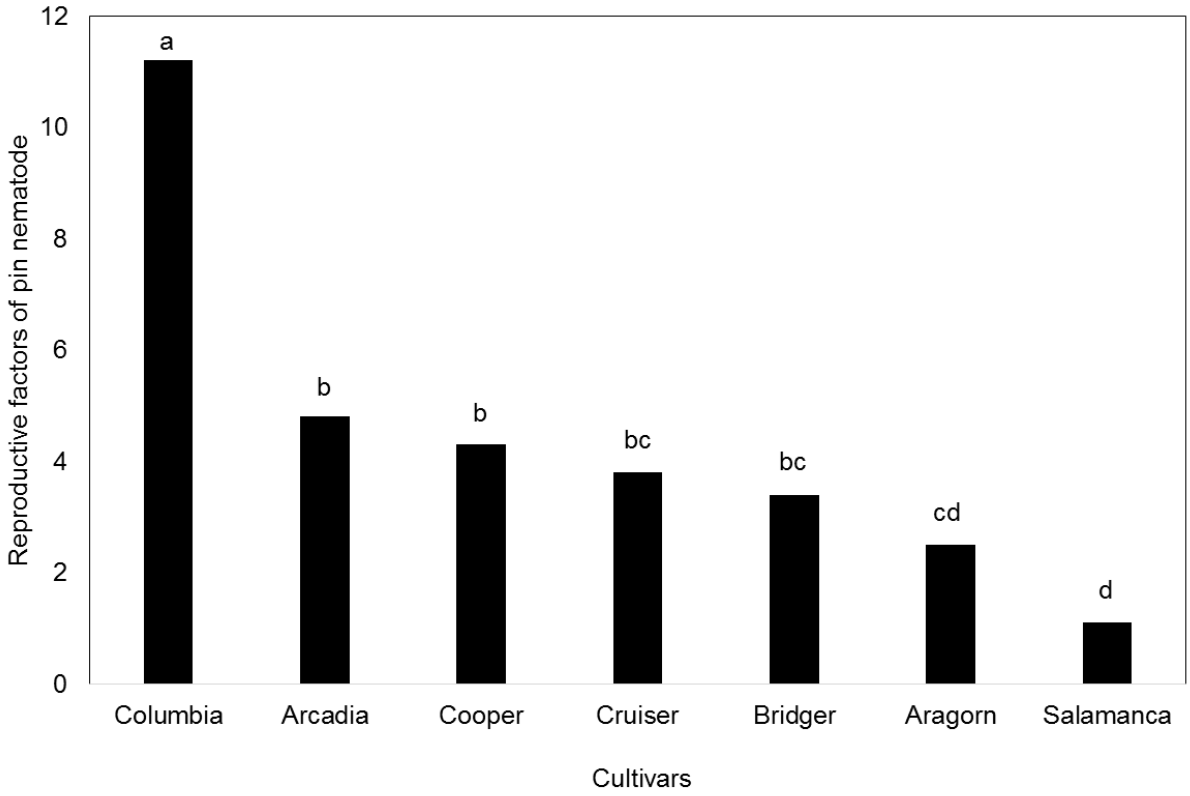

Fig. 4.1. Average reproductive factor (RF) values (final nematode density divided by initial density) of *P. nanus* on field pea cultivars grown in naturally infested field soil with 1,500 *P. nanus*/ kg of soil at the time of planting in greenhouse conditions. RF values are the mean of two trials for each cultivar (n = 8). Mean RF values with same letter are not significantly different according to F-protected least significant different test ($P = 0.05$). Final nematode density in each pot with a single plant was determined after harvesting the trial on 13th week after planting.

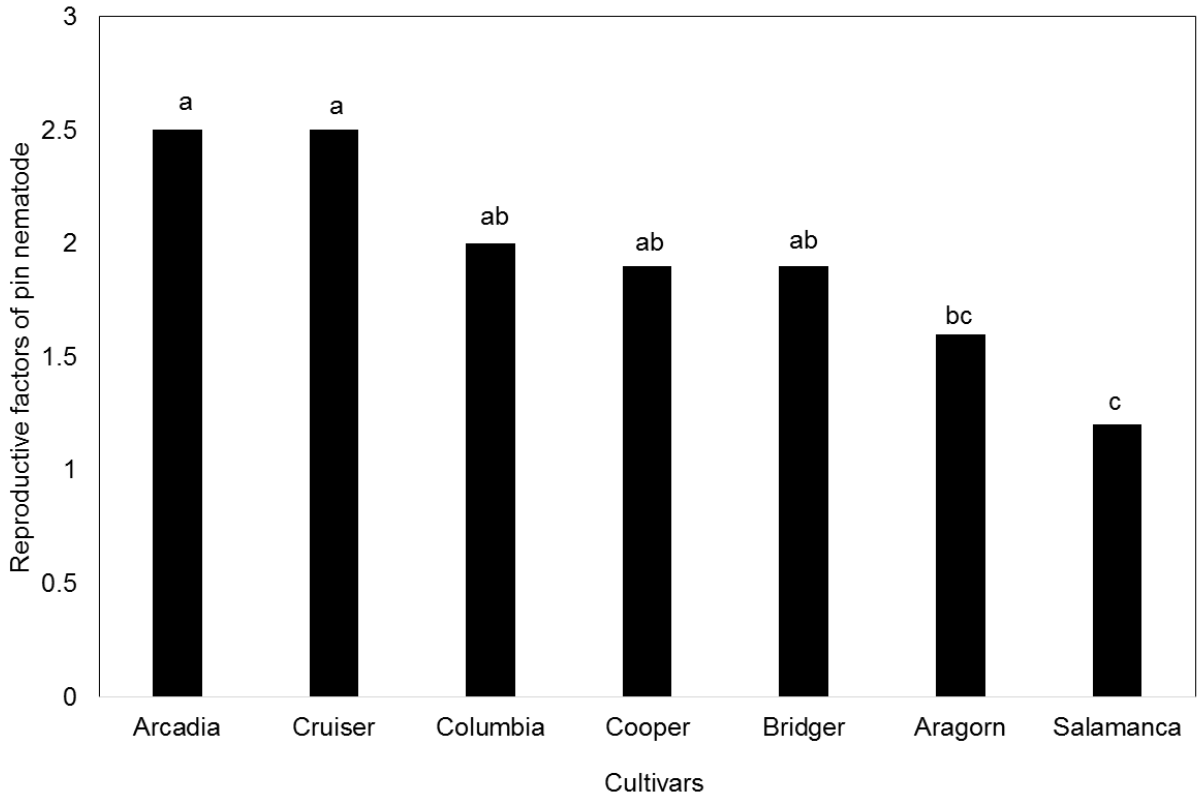

Fig. 4.2. Average reproductive factor (RF) values (final nematode density divided by initial density) of *P. nanus* on field pea cultivars grown in naturally infested field soil with 4,500 *P. nanus*/ kg of soil at the time of planting in greenhouse conditions. RF values are the mean of two trials for each cultivar (n = 8). Mean RF values with same letter are not significantly different according to F-protected least significant different test (P = 0.05). Final nematode density in each pot with a single plant was determined after harvesting the trial on 13th week after planting.

Effect of *P. nanus* on plant growth and yield using artificially inoculated soil

In first repetition of experiment, plant parameters, plant height and dry seed yield, were reduced for some field pea cultivars at inoculation density of 4,500 *P. nanus/* kg of soil. Both plant height and dry seed yield were significantly lower ($P < 0.05$) in Arcadia and Cruiser while only plant height in Bridger compared to corresponding non-inoculated cultivar (Table 4.3). The highest reduction of plant height and dry seed yield was by 18 and 28%, respectively in Arcadia (Table 4.5). The average final population of *P. nanus* reached as high as 9,656/ kg of soil for Cruiser and least for Aragorn (2,938/ kg of soil) (Table 4.6). The highest reproductive factor was

slightly above two for Cruiser (RF = 2.14) and the lowest was below one for Aragorn (Table 4.6).

In second repetition of experiment, nematode inoculation resulted in statistically lower ($P < 0.05$) plant height, dry shoot weight, and dry seed yield for Columbia, Arcadia, and Aragorn compared to their respective non-inoculated controls (Table 4.4). Similarly, for Cruiser statistically lower ($P < 0.05$) plant height and dry seed yield was observed (Table 4.4). Moreover, all the plant parameters were statistically reduced ($P < 0.05$) for Bridger compared to its control while only the plant height was reduced for Salamanca (Table 4.4). The highest reduction of plant height, stem weight, root weight, and yield was by 37, 53, 50, and 40%, respectively, across all the tested cultivars (Table 4.5). Average final population of *P. nanus* was the highest for Columbian (9,428/ kg of soil) and least for Aragorn (3,892) (Table 4.6). Similar to first round of experiment, the highest reproductive factor was slightly more than two for Columbian and the lowest was less than one for Aragorn (Table 4.6).

Table 4.3. Average plant growth (plant height, dry shoot weight, dry root weight) and average dry seed yield of five field pea cultivars, inoculated with *P. nanus* (4,500/ kg of soil) and corresponding non-inoculated control in the first repetition of nematode effect experiment[a].

Cultivars	Height (cm)		Dry shoot wt. (g)		Dry root wt. (g)		Dry seed yield (g)	
	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
Arcadia	39.81**	48.57	2.16	2.54	0.23	0.23	2.82**	3.90
Cruiser	38.54*	45.33	2.43	2.38	0.21	0.23	2.74*	3.53
Bridger	40.95*	46.79	1.62	2.02	0.18	0.20	3.32	3.03
Aragorn	30.16	32.82	1.51	2.02	0.14	0.13	1.87	2.56
Salamanca	42.22	45.40	1.55	1.86	0.20	0.21	2.41	3.02

[a]T-test was done to compare the nematode inoculated treatment of each cultivar (n = 4) with corresponding non-inoculated treatment of the same cultivar (n = 4). Symbols,* and **, represent the significant difference for plant parameters between nematode inoculated and non- inoculated treatments of each cultivar at $P = 0.05$ and 0.01, respectively.

Table 4.4. Average plant growth (plant height, dry shoot weight, dry root weight) and average dry seed yield of six field pea cultivars, inoculated with *P. nanus* (4,500/ kg of soil) and corresponding non-inoculated control in the second repetition of nematode effect experiment[a].

Cultivars	Height (cm)		Dry shoot wt. (g)		Dry root wt. (g)		Dry seed yield (g)	
	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control
Columbia	57.78*	90.17	1.06*	1.83	0.18	0.26	2.34*	3.91
Arcadia	26.67**	42.54	0.99**	2.12	0.07	0.10	2.06*	3.01
Cruiser	32.38*	41.27	1.34**	1.83	0.08	0.11	2.98*	4.14
Bridger	31.11*	43.81	1.17*	1.91	0.10*	0.20	2.24*	3.39
Aragorn	30.48*	37.46	1.28***	1.86	0.08	0.14	2.17*	3.29
Salamanca	42.54*	53.34	2.08	2.68	0.11	0.17	3.24	3.48

[a]T-test was done to compare the nematode inoculated treatment of each cultivar (n = 4) with corresponding non-inoculated treatment (n = 4) of the same cultivar. Symbols,*, ** and ***, represent the significant difference for plant parameters between nematode inoculated and non-inoculated treatments of each cultivar at $P = 0.05$, 0.01, and 0.001, respectively.

Table 4.5. Percentage reduction of measured plant parameters of field pea cultivars inoculated with 4,500 *P. nanus*/ kg of soil in the two nematode effect trials.

Cultivar	Reduction in plant parameters (in percentage)a							
	Height (%)		Dry stem wt. (%)		Dry root wt. (%)		Dry seed yield (%)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Columbia	-	36	-	42	-	31	-	40
Arcadia	18	37	15	53	0	30	28	32
Cruiser	15	22	0	27	9	27	22	28
Bridger	12	29	20	39	8	50	0	34
Aragorn	8	19	25	31	0	43	27	34
Salamanca	7	20	17	22	1	35	20	7

[a]Plant growth of each cultivar inoculated with 4,500 *P. nanus*/ kg of soils was compared with corresponding non-inoculated control of each cultivar in two respective trials.

Table 4.6. Average final populations and average reproductive factors of *P. nanus* at the time of harvest in the two nematode effect trials, artificially inoculated with 4,500 *P. nanus*/kg of soil at the time of planting[y].

Cultivars	Trial 1		Trial 2	
	Final population/ kg of soil	Reproductive factor (RF)	Final population/ kg of soil	Reproductive factor (RF)
Columbia	-	-	9,428a	2.09a
Cruiser	9,656a	2.14a	6,742b	1.49b
Arcadia	9,073a	2.01a	4,325bc	0.96bc
Bridger	6,779ab	1.50ab	6,403bc	1.42 bc
Salamanca	5,163ab	1.14ab	4,138c	0.91c
Aragorn	2,938b	0.65b	3,892c	0.86c

[y]Final nematode populations are the mean of *P. nanus* population per kg of soil in each cultivar within a trial (n = 4 reps) at the time of harvest. Similarly, reproductive factor values are the mean of four replicates of each nematode inoculated cultivar within a trial. Final *P. nanus* populations and reproductive factor values with same letter within a column are not significantly different according to F-protected least significant different test ($P = 0.05$). Nematodes were inoculated at the root zone of a single pea seedling in a soil filled clay pot during planting. Both greenhouse trials were harvested on 11th week after planting and inoculation. Nematode extraction was done using Sugar Centrifugal-floatation technique (Jenkins 1964).

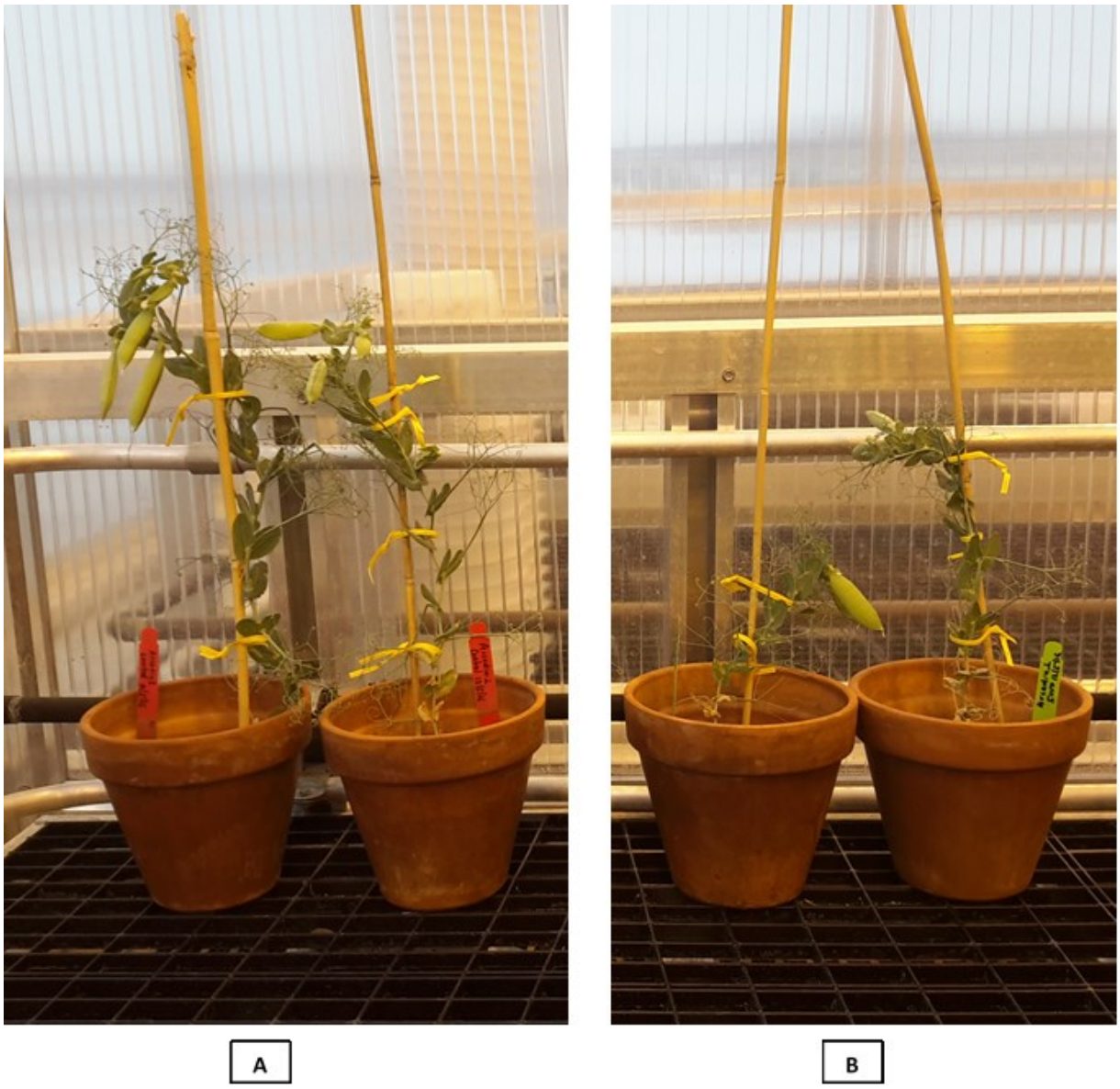

Fig 4.3. Plant growth comparison of field pea cultivar, Arcadia at 40 days after planting and pin nematode, *P. nanus* inoculation. A) Pea plants without *P. nanus* inoculation, while (B) pea plants were artificially inoculated with 4,500 *P. nanus*/kg of soil.

Discussion

This study describes the preference and effect of pin nematode, *Paratylenchus nanus* on field pea cultivars commonly grown in major field pea producing states of the USA. *P. nanus*

was confirmed to be able to reproduce on some field pea cultivars and have negative impact on plant growth and seed yield in controlled greenhouse conditions.

Reproduction ability experiments using naturally infested soils showed that *P. nanus* could survive and propagate in field pea cultivars. This comes in agreement with the association of *P. nanus* with cereals, grasses, and fruits (Raski 1975). Bell and Watson (2001b) also indicated the reproduction of *P. nanus* on grasses such as *Dactylis glomerata*, *Lolium perenne*, and *Lolium multiflorum* in pot experiments. Moreover, other species of pin nematodes such as *P. hamatus* and *P. projectus* reproduced in cereals (Riga et al. 2008; Niblack 1992) while *P. neoambycephalus* in *Prunus cerasifera* and *Malus domestica* (Braun and Lownsbery 1975; Fisher 1967). Based on the results of reproduction ability experiments, *P. nanus* reproduction potential varied with cultivar type. Variable response of field pea cultivars to *P. nanus* infection could be due to differences in genotypes. In line with these results, differential hosting abilities of field pea cultivars to *Ditylenchus dipsaci* was observed in greenhouse studies in Canada (Hajihassani et al. 2016).

Reproductive factor (RF) of *P. nanus* was observed to be influenced by the initial nematode density at the time of planting. In this study, RF values at the low starting density (1,500 *P. nanus*/ kg of naturally infested soil) were generally higher compared to those at high densities (4,500 *P. nanus*/ kg of same soil). Such variation in reproduction rate could be due to more intraspecific competition for food at the high starting density than at the low density. Nematode competition for feeding sites/ food can limit their reproduction (Duncan and Ferris 1983). Brinkman et al. (2005) observed the intraspecific competition of *Pratylenchus penetrans* leading to lower females than males at high inoculation densities. Our results agree with different reproduction rate and rapid multiplication rate of *P. projectus* at lower initial density

(1,000/plant) compared to higher (5,000 or 10,000/plant) on tobacco (Coursen and Jenkins 1958). Moreover, Braun and Lownsbery (1975) detected statistically higher RF of *P. neoambycephalus* on Myrobalan plum seedlings at lower starting densities compared to higher ones (Braun and Lownsbery 1975). Results from our study including others in different parts of the world indicate that *Paratylenchus* spp. have potential to multiply quickly at lower starting populations compared to higher starting populations.

In the present study, *P. nanus* caused detrimental effect on plant growth and seed yield of pea cultivars during artificial inoculation. Pea plant stunting was observed from early growth till harvest as indicated in Fig. 3 and Fig. 4 in the *P. nanus* inoculated pots. Similar stunting was caused by other species of pin nematode, *Paratylenchus projectus* on tobacco and tall fescue plants (Coursen and Jenkins 1958). *P. nanus* caused reduction of dry shoot weight of some pea cultivars. In agreement with such reduction, in a greenhouse study in Australia, *P. nanus* caused decrease in shoot dry matter of perennial rye grass at an initial inoculation density of 950 *P. nanus*/ tube of 20 x 2.5 cm with two plants (Bell 1999). Moreover, seed yield of field pea cultivars were also reduced in this study. Niblack (1992) also reported yield reduction of soybean cultivars due to pin nematode, *P. projectus* at an initial nematode density of 2,000/kg of soil in Missouri. In Idaho, pin nematode, *P. hamatus* was able to negatively impact plant growth and yield of Columbian and Small Sieve cultivars of field pea in greenhouse conditions at the initial density of 400/ 200 g of soil (Riga et al. 2008).

In this study, plant growth and yield were observed to be reduced during artificial inoculation but the nematode reproductive factor (RF) in both trials could reach only up to two or slightly more. Similar observations were made in Canada, where the RF of *D. dispsaci* in field pea cultivars (Admiral and Bronco) and bean cultivars (Windbreaker and Envoy) reached up to

2.5 and less than 2, respectively. However, aboveground biomass of both crops were significantly reduced by *D. dipsaci* (Hajihassani et al. 2016). No any reason was given for such observations (Hajihassani et al. 2016). These lower RF values of *P. nanus* during harvest time could be due to decline of nematode population because of intraspecific competition for food. This explanation was actually supported by lower RF values of *P. nanus* in both naturally infested soil and artificial inoculation at the same high nematode density (4,500 *P. nanus*/ kg of soil). Nematode competition could play a negative role in their development and propagation (Duncan and Ferris 1983; Brinkman et al. 2005). In nematode effect experiments, high number of *P. nanus* (4,500/ kg of soil) were inoculated directly around the root zone of seedlings. Based on the images of pea plants in Fig. 3, nematode damage started from early growth stage. Pin nematodes feed on roots and started to multiply but by the time population started to increase a single pea plant was not able to support the increased nematode numbers. Eventually, with poor plant growth and availability of less food at the latter half of growing period, nematode population could not increase to the maximum as expected and then decreased by the end of harvest. This assumption was supported by increase of *P. projectus* during mid growing season and again reduction to more than half at the end of harvest (Niblack 1992).

In conclusion, this study provides the evidence that *P. nanus* populations from pea fields in North Dakota can reproduce well in some field pea cultivars such as Columbia, Arcadia, Cooper, and Cruiser. However, the reproduction rate of *P. nanus* was dependent upon initial density at the time of planting. Hence, further research may be required to confirm this differential rate of reproduction in field pea cultivars. Moreover, *P. nanus* populations caused harmful impact on plant growth and seed yield of field pea cultivars. In future, screening of more

diverse cultivars is required considering the effect of *P. nanus* on plant growth even with slow reproduction of *P. nanus* at the high inoculation density used in this study.

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CHAPTER 5. PLANT-PARASITIC NEMATODES IN POTATO FIELDS AND THE EFFECTS OF CO-INOCULATION WITH *PRATYLENCHUS PENETRANS* AND *FUSARIUM OXYSPORUM* ON POTATO GROWTH AND YIELD

Abstract

Plant-parasitic nematode surveys were conducted on potato fields of Central Minnesota where 43 soil and root samples were collected from 11 fields during 2015 and 2016 growing seasons. Root lesion nematode, *Pratylenchus* spp. were the most frequent (incidence = 57% in soil) and their density reached up to 204/ 200 g of soil and 1100/ g of fresh roots. During the survey, *Pratylenchus penetrans* (Pp) and *Fusarium oxysporum* (Fo) were present in some fields showing stunted and patchy growth. Therefore, a micro-plot study was carried out during 2016 and 2017 growing season at the Sand Plain Research Farm in Becker, Minnesota to evaluate the effects of these pathogens individually or co-inoculation on growth and yield of potato. *P. penetrans* at 200 (low), 800 (medium), 2,000 (high) or 3,000 (very high) nematodes per 5 kg of soil and *F. oxysporum* at 5 (low), 10 (medium), 20 (high) or 30 (very high) colonized barley seeds per 5 kg soil were either inoculated individually or together at same level. Nematode or fungus alone or co-inoculation at 'high' and 'very high' densities significantly (P<0.05) reduced all parameters of plant growth and yield in both years. However, co-inoculation at 'medium' density significantly (P<0.05) reduced yield, plant height, and dry stem weight in both years. Nevertheless, in most cases, the effect (P<0.05) of co-inoculation on plant growth, yield was not significantly higher than individual pathogens. Similarly, plant health as assessed by % wilting, necrosis, chlorosis; and nematode reproduction were not significantly (P<0.05) different between co-inoculation and lone pathogens in most cases. In conclusion, both pathogens have significant negative effects on potato growth and yield; however, in most cases, the presence of both

pathogens together did not significantly (P<0.05) increase damage than lone pathogens. This study demonstrates that both pathogens can significantly reduce growth and yield of potato in this region. Hence, management of these pathogens would help to improve potato production in this region.

Introduction

Potato crop is the fourth important food crop worldwide after maize, wheat, and rice (FAO 2018). Potato crops are severely affected by approximately forty soil borne diseases worldwide including soil inhabiting fungi, bacteria, and nematodes (Fiers et al. 2012). Seventy species of plant-parasitic nematodes belonging to twenty four genera are reported to be associated with potato crops (Jensen et al. 1979). Depending upon the environmental conditions, the economically important nematode species in potato include *Globodera* spp., *Pratylenchus* spp., *Paratrichodorus* spp., *Trichodorus* spp., *Ditylenchus* spp., *Meloidogyne* spp., and *Belonolaimus* spp. (Holgado and Magnusson 2012; Brodie et al. 1993; Crow et al. 2000). In temperate regions, *Pratylenchus* spp. are the most common nematode pests of potato (Florini and Loria 1990; Brown et al. 1980; Castillo and Vovlas 2007). Similarly, among the soil borne fungal pathogens, *Fusarium* spp. are one of the important pathogens of potato crop (Secor and Salas 2001; Gachango et al. 2012; Fiers et al. 2012).

Many species of *Pratylenchus* are reported to be associated with potato crop worldwide (Castillo and Vovlas 2007). In the state of Ohio,USA, up to six species of *Pratylenchus* including *P. crenatus*, *P. penetrans*, *P. scribneri*, *P. alleni*, *P. thornei*, and *P. neglectus* were isolated from field potato root samples (Brown et al. 1980). Similarly, in North Dakota, *P. scribneri* was reported to occur in potato crop (Yan et al. 2016). Likewise, in Minnesota (Baidoo et al. 2017), Wisconsin (MacGuidwin and Rouse 1990), Washington (Ingham et al. 2005), and Idaho (Hafez

et al. 2010), *P. penetrans* is an important plant-parasitic nematode of potato crop. Several species of *Pratylenchus* can cause negative impact to potato (Mahran et al. 2010; Castillo and Vovlas 2007). Among the *Pratylenchus* spp., *Pratylenchus penetrans* is the most damaging in potato crop (Waeyenberge et al. 2009; Castillo and Vovlas 2007). This nematode has a wide host range and is distributed throughout the temperate regions (Castillo and Vovlas 2007; Corbett 1973; Loof 1991). *P. penetrans* aused significant damage on growth and yield of potato crops (Castillo and Vovlas 2007; Bernard and Laughlin 1976), and was responsible for yield losses up to 50% in an affected potato field in Norway (Holgado et al. 2009). *P. penetrans* penetrates on root tissue and migrates in root cells feeding cell contents, ultimately reducing the ability of infected roots to absorb water and nutrients normally (Castillo and Vovlas 2007).

In North Eastern United States, the most prevalent and pathogenic *Fusarium* species infecting potato crop include *F. sambucinum*, *F. solani*, and *F. oxysporum* (Hanson et al. 1996). In North Dakota, *F. graminearum* was reported to be frequently occurring and responsible for dry rot of potato tubers (Ali et al. 2005). However, in Michigan, *F. oxysporum* was the most dominant in seed potato tubers and was pathogenic to potato cultivar, Dark Red Norland (Gachango et al. 2012). Similarly, *F. oxysporum* was the most serious pathogen of potato causing *Fusarium* wilt disease and significant yield losses in Tunisia (Trabelsi et al. 2016; Ammar et al. 2017). Wilt causing pathogens invade the growing roots at early crop growth and move through the water conducting tissues of roots and stem. Finally, infection leads to symptoms such as wilting, yellowing, necrosis, vascular discoloration and ultimate death of vines and plant (Rowe and Powelson 2002; Hwang and Evans 1985). *F. oxysporum* can result in wilting of potato plant, loss of tuber yield (McKay 1926; Bibsy 1919) and tuber dry rot in field or storage (Secor and Salas 2001; Gachango et al. 2012). Moreover, it also caused collapse of plants near the end of

growing season (Bisby 1919). *Fusarium* spp. can also lead to reduction in plant height of potato crop (Hwang and Evans 1985). *Fusarium* spp. were reported to cause up to 25% yield losses in potato fields and occasionally up to 60% tuber losses in storage condition (Desjardins 2006; Secor and Salas 2001).

Nematodes and fungi interactions have been reported in many crops including cotton, banana, tobacco, cowpea, brinjal, tomato, and potato (Ravichandra 2013). The mechanism of nematode and fungus interaction is complex and largely unknown. However, nematode and fungus interactions were demonstrated to be species-specific (Bowers et al. 1996; Rowe and Powelson 2002). Many researchers have described the interaction between plant-parasitic nematodes and soil borne fungal pathogens resulting in synergistic or additive or antagonistic reactions on growth, yield and disease development (Martin et al. 1982; Jorgenson 1970; Rowe et al. 1987). Most frequently reported one was lesion nematode, *P. penetrans* and wilt fungi, *Verticillium dahliae* interaction on causing potato early dying symptoms along with reduction of tuber quality and yield (Rowe et. al 1987; Martin et al. 1982).

In some potato fields of Central Minnesota, growers observed poor growth or no growth of potatoes in patchy patterns. Those fields were detected to have higher occurrence frequencies and densities of plant-parasitic nematode, *P. penetrans* and fungal pathogen, *F. oxysporum*. Hence, the objectives of this study were to 1) determine the incidence or occurrence frequency of plant-parasitic nematodes in some potato fields of Central Minnesota and 2) determine the effect of two soil borne pathogens, *P. penetrans* and *F. oxysporum* individually and together on growth and yield of the potato cultivar Red Norland through micro-plot experiments in field conditions. This research will help to identifying important parasitic nematodes of potato crop in Central

Minnesota. Moreover, the micro-plot study will evaluate the damage potential of *P. penetrans* and *F. oxysporum* on potato crop in infested fields in this region.

Materials and Methods

Plant-parasitic nematodes in potato fields

Field sampling

Nematode surveys were conducted in potato fields of Central Minnesota upon the request of growers. A total of 43 soil plus root samples were collected from 11 potato fields during 2015 and 2016 growing seasons. In general, four samples were collected from each field because the fields were large in size. Standard soil probes (2.5 cm diameter and 30 cm depth) were used to collect the representative soil sample from each sampling area in each field. Soil samples were collected in a zig-zig pattern with 5 m distance between two successive sample cores. In each sampling spot, a sample core was probed along the root zone up to a depth of 30 cm discarding the top dry soil of about 1-2 cm. A single composite sample from each sampling area consisted of 20 to 25 soil cores mixed together. Root samples were collected by uprooting the potato plant along with tops. Both soil and root samples were sealed in a plastic bag, placed in a cooler during sampling and shipping, and kept in a cold room at 4ºC before nematode extraction.

Nematode assays

Before extracting nematodes from soil, each composite sample was mixed thoroughly discarding dried crop residues and rocks. After soil mixing, nematodes were extracted from a sub-sample of 200 g from each composite sample using sieving and decanting and sugar centrifugal-floatation technique (Jenkins 1964). Nematodes from root samples were extracted separately using Whitehead tray method (Whitehead and Hemming 1965). Roots were rinsed gently to remove soil particles around the root zone. Then, roots were chopped into 1- cm pieces

and spread in paper towels placed over mesh on coated metallic frame. Metallic frame (coated) holding mesh, paper towels and chopped roots were placed in a plastic tray (45 cm x 35 cm). Approximately 1 liter water was poured into the plastic tray just enough to submerge the chopped roots. After 48 hours, nematodes were collected from the water. Nematodes from soil and roots were separately collected in 20 to 25 ml tap water in a 50 ml suspension tube. Plant-parasitic nematodes were identified to genus level based on the morphological characteristics (Mai et al. 1996) and counted under an inverted transmitted light microscope at 100x magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY, USA). Finally, nematode numbers were expressed as the total number of nematodes of a genus in 200 g of soil or 1 g of fresh roots.

Effects of co-inoculation with *P. penetrans* and *F. oxysporum* on potato growth and yield

Micro-plot establishment

Two micro-plot trials were conducted during Mid-May to Mid-August of 2016 and 2017 in field conditions at the Sandplain Research Farm, Becker, Minnesota. The Sandplain Research Farm is the research station of University of Minnesota. Monthly mean precipitation at Becker was 4.0 and 2.8 mm during May to August of 2016 and 2017, respectively. Mean monthly high temperature exceeded 27ºC during the peak growing month of July in both years. Experiments were performed using terracotta clay pots holding approximately 5 kg of steam sterilized light sandy loam soil. Clay pots were installed below soil surface leaving 5 cm above the ground after planting and pathogen inoculation in the micro-plot site. Distance between column to column in the micro plot was 1.5 m and pot to pot in a column was kept 0.75 m apart. Mico-plot was fenced after experiment set up to prevent the damage due to rabbits and deer.

Pathogen identification and inoculum preparation

Lesion nematodes, *Pratylenchus* spp. were identified to species level using species-specific polymerase chain reaction (PCR). Nematodes isolated from soil and root samples were handpicked based on morphological characters (Mai et al. 1996). Nematode DNA was extracted independently from chopped pieces of single nematodes (n = 12) using Proteinase K method as described by Huang and Yan (2017). The D2-D3 expansion region of 28S rRNA was amplified using a *P. penetrans* specific primer set, PP5 forward primer (5´-ACATGGTCGACACGGTGATA-3´) and PP5 reverse primer (5´-TGTTGCGCAAATCCTGTTTA) which produces an amplified fragment of approximately 520 bp (Mekete et al. 2011). Template DNA (1.50 µl) was transferred into 14.50 µl of the PCR mixture [0.64 µl of each primer (10 µM), 0.32 µl dNTP, 0.96 µl $MgCl_2$, 3.2 µl 5 x Green GoTaq Flexi buffer, 0.12 U of GoTaq Flexi DNA polymerase (Promega Corp., Madison, WI)]. Amplification conditions were initial denaturation (94ºC for 3 min), followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 57ºC for 1.5 min, and extension at 72ºC for 1.5 min, and a final extension at 72ºC for 10 min. After amplification, PCR products were run in 2% agarose gel at 100V for 25 min. Finally, the gel was visualized under UV light and images were captured using an AlphaImager Gel Documentation System (Proteinsimple Inc., Santa Clara, CA).

Identification of fungal pathogen to species level was done using molecular techniques and colony morphology characters. For molecular identification, fungal DNA was extracted from soil and fungal mycelia isolated from infected tissue grown on potato-dextrose agar. DNA from soil was extracted using the MoBio PowerSoil DNA Isolation Kit (MOBIO Laboratories Inc, Carlsbad, CA) based on the manufacturer's protocol. Total fungal DNA from mycelium was

extracted using the MP Biomedical FASTDNA Kit (MP Biomedical, California, USA) according to the manufacturer's protocol. *F. oxysporum* specific primer set, FOF1 forward primer (5′-ACATACCACTTGTTGCCTCG-3′) and FOF2 reverse primer (5′-CGCCAATCAATTTGAGGAACG-3′) were used to amplify the ITS region of rDNA (Mishra et al. 2003). Each PCR tube consisted of 1.0 µl template DNA, 12.8 µl double distilled water, 0.6 µl of each primer (10 µM), 0.4 µl dNTP, 1.2 µl $Mgcl_2$, 3.2 µl 5x Green GoTaq Flexi buffer, 0.2 U of GoTaq Flexi DNA polymerase (Promega Corp., Madison, WI). PCR cycles consisted of initial denaturation at 94ºC for 3 min, 40 cycles of denaturation at 94ºC for 1 min, annealing at 57ºC for 50 s and extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min. Finally, PCR products were run in agarose gel and visualized under UV light as described earlier.

In order to identify the fungal pathogen using morphological characters fungal pathogens were isolated from infected stem tissues and cultures were established. For pathogen isolation, first stem tissues were thoroughly washed in sterilized water. Then, infected tissues were cut into small pieces (5 mm squares) and surface sterilized using 10% Clorox (Narayanasamy 2011). Surface sterilized tissues (1-2) were then aseptically transferred into half-PDA medium [Potato Dextrose (9.75 g), Agar (3.75 g), and Deionized water (500 ml)]. Culture plates with infected tissues were then incubated at room temperature (22-27ºC) to facilitate pathogen growth. After mycelium growth, in order to obtain the pure culture, fungal hyphae at the edge of colony were transferred into half-PDA media using hyphal tipping technique (Narayanasamy 2011). Then, the fungal pathogen was characterized based on both macroscopic and microscopic characters. Macroscopic characters included colony color and colony growth whereas microscopic characters included micro conidia shape and septa number (Leslie and Summerell 2008). For microscopic characterization, a small section of agar with fungal parts was picked up and

crushed in slides. Fungal parts including micro and macro conidia developed were then visualized under the compound microscope microscope (Zeiss Axio Scope A1; Zeiss, Oberkochen, Germany).

Nematode inoculum was prepared by rearing *P. penetrans* in sterilized carrot disks in laboratory conditions and also in susceptible potato cultivars in greenhouse conditions which were originally obtained from a potato field in Becker, Minnesota. Sterilized carrot disks were inoculated with 3 to 6 *P. penetrans* individuals per disk including both males and females. After inoculation, these carrot disks were kept in incubator at 22ºC for about 5 to 6 months to allow enough time to increase in number. After 5-6 months all the carrot disks were harvested and 0.001% final concentration of streptomycin was used to inhibit bacterial growth in nematode suspension. Collected nematodes were stored at 4ºC until using within 2-3 days. *P. penetrans* were also reared in susceptible potato cultivar, Red Norland in the greenhouse with 16 hrs day light at an average temperature of 22ºC. Potato plants were harvested after a crop cycle at 90 days after planting. *P. penetrans* was recovered from root tissues using the Whitehead tray method. Nematodes recovered from roots were also stored in similar way as described earlier for nematodes from carrot disks. Nematodes from carrot cultures and root tissues were mixed together and *P. penetrans* densities were determined from 1 ml of aliquots. Dilutions were made appropriately to get 200, 800, 2,000 and 3,000 *P. penetrans*/ pot/ plant in 20 ml of nematode suspension. *F. oxysporum* inoculum was provided by Dr. Gary Secor (Professor and Plant pathologist, NDSU) as fully infected barley seeds.

Preparation of tubers for planting

Red potato cultivar, Red Norland was selected for the entire experiment based on their susceptibility to *P. penetrans* and *F. oxysporum* (personal communication with Dr. Gary Secor,

Plant Pathology, NDSU). Seed potato tubers were provided by potato research facilities at the North Dakota State University, obtained from seed potato farms. In order to facilitate the sprouting, tubers were spread in plastic trays with moist paper towels in the bottom for 15-20 days at room temperature of 22ºC. This practice of pre-sprouting allows quick plant growth with roots available for nematodes to feed after inoculation. Sprouted tubers were cut into 2 to 3 halves each with at least a single sprout. Cutting of tubers was done 3-4 days before planting in order to provide adequate time for healing of cut sections.

Treatments and experimental design

In first year, ten treatments including a non-inoculated control were selected while in second year three more treatment with very high pathogen levels were added. Each treatment was replicated ten times and experiments were randomized in complete block design in both years. In order to interpret the results easily, treatments were also categorized as follows: Control [no pathogen inoculated], low pathogens [200 *P. penetrans* (Pp) or 5 *F. oxysporum* infected barley seeds (FOBS) or 200 Pp + 5 FOBS], medium pathogens [800 Pp or 10 FOBS or 800 Pp + 10 FOBS], high pathogens [2,000 Pp or 20 FOBS or 2,000 Pp + 20 FOBS], and very high pathogens [3,000 Pp or 30 FOBS or 3,000 Pp + 30 FOBS] per pot per plant as indicated in Table 5.1. In order to simplify the interpretations, pre-plant densities of *P. penetrans* can also be expressed as follows: 8 Pp (low), 32 Pp (medium), 80 Pp (high), and 120 Pp (very high) per 200 g of soil considering a total of 5,000 g of soil in each pot. In addition, 200 g of the light sandy loam soil used in this experiment was approximately equal to 100 cubic centimeter of soil.

Table 5.1. Pathogen levels and treatments used in micro-plot studies conducted in Becker, Minnesota during 2016 and 2017 growing seasons.

Soil type	Pathogen level[a]	Treatments	*Pratylenchus penetrans* or *Fusarium oxysporum* per pot per plant[b]
Steam autoclaved field soil	Low level	Low *P. penetrans* (Pp)	200 individuals of Pp per pot or 8 Pp per 200 g of soil
		Low *F. Oxysporum* (Fo)	5 *F. oxysporum* infected barley seed per pot (FOBS)
		Low Pp + Low Fo	200 Pp (8 Pp/ 200 g) + 5 FOBS
	Medium level	Medium Pp	800 Pp or 32 Pp/ 200 g
		Medium Fo	10 FOBS
		Medium Pp + Medium Fo	800 Pp (32 Pp/ 200 g) + 10 FOBS
	High level	High Pp	2,000 Pp or 80 Pp/ 200 g
		High Po	20 FOBS
		High Pp + High Fo	2,000 Pp (80 Pp/ 200 g) + 20 FOBS
	Very high level	Very high Pp	3,000 Pp or 120 Pp/ 200 g
		Very high Fo	30 FOBS
		Very high Pp + Very High Fo	3,000 Pp (120 Pp/ 200 g) + 30 FOBS
	Control	Non-inoculated control	None

[a]Three pathogen levels including low, medium and high were used in first year of mico-plot trials while very high level was added in second year along with those used in the first year.
[b]*P. penetrans* and *F. oxysporum* alone or together were inoculated in terracotta clay pots holding 5 kg of soil. The soil type used during entire experiment was light sandy loam soil. Each pot had a single potato plant. Pp represents for *P. penetrans* and FOBS represents *F. oxysporum* infected barley seeds.

Pathogen inoculation

Before pathogen inoculation, Clay pots were filled two third (3 kg of soil), with autoclaved light sandy loam soil. For treatments involving both fungal and nematode infestation, first, *F. oxysporum* infected barley seeds were spread in center over which sprouted cut tuber pieces were placed with a thin layer of soil in between. Then, soil holes were created around the tuber piece where nematode inoculum was added and finally tuber piece was covered with an appropriate amount of sterilized soil with sprouts barely visible from soil layer. In order to infest

soil with only one pathogen the same process described above was followed discarding the step for infestation with other pathogen.

Irrigation, fertilization, weeding and harvesting

Sprinkle irrigation was done twice a week. Recommended dose of fertilizers per plant was put in split dose at the time of planting and 45 days after planting. At the time of planting fertilizer was put in soil filled pot and mixed thoroughly with soil. While during second fertilization, fertilizer was placed on the top soil in the pot. Weeding of micro-plot was done manually for three times at different time periods depending upon weed growth. Experiments were terminated on 14th week after planting.

Soil and plant sample processing after harvest

Soils in individual pots along with plant parts were placed in separate plastic bags and brought to the Nematology Laboratory at the North Dakota State University. Soil and plant parts were processed for further plant parameter measurements and nematode extractions. Soil from each experimental unit, single pot, was mixed thoroughly and 200 g of sub-sample was prepared for nematode assays. Similarly, roots were also collected for nematode extraction. Other plant parts, tuber and stem were collected separately to measure fresh tuber yield and dry stem weight.

Nematode extraction from soil and roots after harvest

Nematodes were extracted from a sub-sample of 200 g from each individual pot sample using sieving and decanting and sugar centrifugal-floatation technique (Jenkins 1964). Nematodes from root samples were extracted separately. Roots were rinsed gently to remove soil particles around the root zone. Then, roots were chopped into 1-cm pieces and nematodes from roots were extracted using Whitehead tray method (Whitehead and Hemming 1965) after 48 hours. Nematodes from soil and roots were separately collected in 20 to 25 ml tap water in a 50

ml suspension tube. *P. penetrans* was counted under an inverted transmitted light microscope at 100x magnification (Zeiss Axiovert 25, Carl Zeiss Microscopy, NY, USA). Nematode numbers from soil were expressed as the total number of *P. penetrans* in 5 kg of total soil. Finally, nematode numbers from total soil were added with those from roots to calculate the final population of *P. penetrans* reproduced in individual plant per pot.

Data collection

Plant height (cm) and plant health were measured after 12 weeks of planting. Plant health based on percentage of wilting, chlorosis and necrosis of a plant was determined using 0 to 5 rating scale based on percentage of wilting, chlorosis and necrosis symptom on plant (Kotcon et al. 1985). A value of 0 = no symptoms, 1 = < 25% of foliage with wilting, chlorosis and necrosis, 2 = 25 to 49%, 3 = 50 to 74%, 4 = 75 to 100% of foliage with the above symptoms, and 5 = complete death of plant. Dry root weight was measured for all the treatments by collecting the roots after nematode extraction from roots. Roots and stems were dried at 80ºC for 48 hours before measuring dry weight while tubers were measured as fresh tuber weights. Nematode reproduction expressed as reproductive factor (Rf) was calculated by dividing the final nematode population (total in 5 kg soil plus all roots) by the initial nematode population during inoculation.

Data analysis

Analysis on different parameters (plant height, dry root weight, dry stem weight, yield, plant health, nematode reproductive factor) of treatments (average of replications, N = 10) were performed using PROC GLM of SAS 9.4 (SAS Institute Inc., Cary, NC). Mean separation was performed using *F*-protected least significant difference (LSD) at $P<0.05$ to determine the significant differences in plant parameters among treatments.

Results

Pathogen identification

Based on the species specific primers (PP5F/PP5R), root lesion nematodes were identified as *P. penetrans* with the amplified fragment size of approximately 520 bp from the D2-D3 region of the 28S rRNA. This band size matched with that of *P. penetrans*, produced using the same primer set (PP5F/PP5R) as described originally by Mekete et al. (2011). Similarly, fungal pathogen was identified as *F. oxysporum* using species specific primers (FOF1/FOR1) producing the fragment size of approximately 340 bp. The amplified bands from ITS region of rDNA of the fungal pathogen had the same fragment sizes as described by Mishra et al. (2003). Moreover, fungal colonies in pure culture plates had dark violet pigmentation. Microconidia were abundant compared to macroconidia. Macroconidia were usually two to three septate, straight to slightly curved while microconidia were usually zero to one septate, oval or elliptical. These morphological features of fungal pathogen were similar to those described for *F. oxysporum* by Leslie and Summerell (2008).

Occurrence and population density of vermiform plant-parasitic nematode genera in potato fields of Becker, Minnesota

A total of eight genera of plant-parasitic nematodes were detected in soil and root samples in potato fields. These genera include *Pratylenchus* (lesion nematode), *Helicotylenchus* (spiral nematode), *Heterodera* (cyst nematode), *Tylenchorhynchus* (stunt nematode), *Paratrichodorus* (stubby root nematode), *Hoplolaimus* (lance nematode), *Mesocriconema* (ring nematode), and *Paratylenchus* (pin nematode) (Table 5.2).

Pratylenchus nematodes were the most frequent among all the plant-parasitic nematodes. They occurred in 57% of the soil samples and 100% of the root samples (Table 5.2). They were

the only plant-parasitic nematode species found in root samples. The density of *Pratylenchus* reached up to 204/ 200 g of soil and 1,100/ g of fresh roots (Table 5.2). *Helicotylenchus* was detected in 30% of the soil samples with a density as high as 218/ 200 g of soil. The vermiform stage of soybean cyst nematode was identified in 23% of the soil samples where the density reached up to 64/ 200 g of soil. The remaining five nematode genera, *Tylenchorhynchus*, *Paratrichodorus*, *Hoplolaimus*, *Mesocriconema*, and *Paratylenchus*, were rarely detected at lower densities, except *Mesocriconema* (Table 5.2). The density of *Mesocriconema* reached 267/ 200 g of soil in a soil sample (Table 5.2).

Table 5.2. Plant-parasitic nematode genera in soil and root samples collected from potato fields during 2015 and 2016 growing seasons in Becker, Minnesota[a].

Sample #	Soil or root	Number of plant-parasitic nematodes[b]							
		Pratylenchus	*Heterodera*	*Helicotylenchus*	*Tylenchorhynchus*	*Paratrichodorus*	*Hoplolaimus*	*Mesocriconema*	*Paratylenchus*
1	Soil	47	0	0	0	0	0	0	0
2	Soil	0	0	0	0	0	0	0	0
3	Soil	76	0	0	0	0	0	0	0
4	Soil	0	0	16	0	0	0	0	0
5	Soil	122	0	0	0	0	0	0	0
6	Soil	121	15	15	0	0	0	0	15
7	Soil	62	0	0	16	0	0	0	0
8	Soil	48	0	0	0	0	0	0	0
9	Soil	0	0	0	0	0	0	0	0
10	Soil	0	0	0	0	0	0	0	0
11	Soil	16	0	0	0	0	0	0	0
12	Soil	0	0	0	31	0	0	0	0
13	Soil	0	0	0	0	0	0	0	0
14	Soil	49	0	0	0	0	0	0	0
15	Soil	204	0	0	0	0	15	0	0
16	Soil	0	0	50	0	0	0	267	0
17	Soil	0	0	50	0	0	0	133	0
18	Soil	0	0	0	0	17	0	0	0
19	Soil	16	0	0	0	0	33	0	0
20	Soil	0	0	15	0	0	0	0	0
21	Soil	0	0	204	16	0	0	0	0
22	Soil	0	0	218	0	0	0	0	0

Table 5.2. Plant-parasitic nematode genera in soil and root samples collected from potato fields during 2015 and 2016 growing seasons in Becker, Minnesota[a] (continued)

Sample #	Soil or root	Number of plant-parasitic nematodes[b]							
		Pratylenchus	*Heterodera*	*Helicotylenchus*	*Tylenchorhynchus*	*Paratrichodorus*	*Hoplolaimus*	*Mesocriconema*	*Paratylenchus*
23	Soil	15	0	0	0	0	0	0	0
24	Soil	176	64	0	0	0	0	0	0
25	Soil	39	52	0	0	0	0	0	0
26	Soil	13	53	13	0	0	0	0	0
27	Soil	12	25	12	0	37	0	0	0
28	Soil	25	0	0	0	0	0	0	0
29	Soil	0	57	0	0	0	0	0	0
30	Soil	11	56	0	0	0	0	0	0
31	Root	60	0	0	0	0	0	0	0
32	Root	84	0	0	0	0	0	0	0
33	Root	173	0	0	0	0	0	0	0
34	Root	32	0	0	0	0	0	0	0
35	Root	25	0	0	0	0	0	0	0
36	Root	18	0	0	0	0	0	0	0
37	Root	904	0	0	0	0	0	0	0
38	Root	213	0	0	0	0	0	0	0
39	Root	179	0	0	0	0	0	0	0
40	Root	58	0	0	0	0	0	0	0
41	Root	1,100	0	0	0	0	0	0	0
42	Root	1,018	0	0	0	0	0	0	0
43	Root	484	0	0	0	0	0	0	0

[a]A total of 25 soil samples were collected in 2015 whereas 18 soil and root samples in 2016.
[b]Density of different plant-parasitic nematode genera per 200 g of soil or 1 g of fresh roots.

Effect of pathogens on plant growth and yield - first year experiment

Inoculation with either *P. penetrans* or *F. oxysporum* alone, or both, at 'low' densities [(8Pp per 200 g of soil) / 200 *P. penetrans* (Pp) or 5 *F. oxysporum* infected barley seeds (FOBS) per pot per plant] did not significantly reduce any of the plant parameters compared to non-inoculated control (Table 5.3). However, at 'medium' pathogen density [(32 Pp per 200 g of soil) / 800 Pp or 10 FOBS], *P. penetrans* significantly ($P < 0.05$) decreased plant height (19%), whereas *F. oxysporum* significantly reduced root (35%) and stem (30%) weights (Table 5.3 and Table 5.4). But, co-inoculation of both pathogens reduced ($P < 0.05$) plant height (12%), stem weight (28%), and tuber yield (35%) when compared to non-inoculated control (Table 5.3, Table 5.4, and Fig. 5.1). Moreover, at 'high' nematode density [(80 Pp per 200 g of soil) / 2,000 Pp] plant parameters: height, root weight, stem weight and yield were reduced ($P < 0.05$) by 27, 42, 28, and 29%, respectively, while the reduction ($P < 0.05$) of the same parameters were by 21, 38, 36, 35%, respectively, due to 'high' fungal density (20 FOBS). Similarly, co-inoculation resulted in 26, 43, 44 and 43 % reduction ($P < 0.05$) in height, root weight, stem weight and yield, respectively (Table 5.3, Table 5.4, and Fig. 5.1). Increasing the initial inoculum level of *P. penetrans* from 'low' to 'high' level result in a statistically significant reduction of plant height and root weight (Table 5.3). Similarly, the effect of *F. oxysporum* on plant height and stem weight significantly increased ($P < 0.05$) with increasing fungal density (Table 5.3).

Effect of pathogens on plant health and nematode reproduction - first year experiment

Pathogen effects on the plant health was assessed based on chlorosis, necrosis, and wilting of plants using 0-5 rating scale. Disease severity on plant was only significantly greater ($P < 0.05$) on plants co-inoculated with both pathogens at 'high' level compared to non-inoculated plants (Table 5.5). Nonetheless, there was no significant difference between the

effects of co-inoculation of pathogens and the individual pathogens in terms of disease severity. Nematode reproduction rates ranged from 1.5 to 3.6 (Table 5.5). High density (20 FOBS) of *F. oxysporum* significantly increased ($P < 0.05$) *P. penetrans* final population in co-inoculated treatment compared to the treatment with *P. penetrans* alone (Table 5.5). However, *P. penetrans* final populations were not significantly influenced by *F. oxysporum* at low and medium pathogen co-inoculation densities.

Effect of pathogens on plant growth and yield - second year experiment

Likewise to year one, treatments at 'low' pathogen inoculations either alone or together did not significantly reduce the plant parameters except the co-inoculation level on root weight (18%) (Table 5.3 and Table 5.4). But, at 'medium' pathogen level, *P. penetrans* reduced ($P < 0.05$) the plant height and root weight by 15% and 17%, respectively, whereas *F. oxysporum* did not reduce any plant parameter (Table 5.3 and Table 5.4). Nevertheless, co-inoculation caused decline ($P < 0.05$) of plant height, root weight, stem weight, and tuber yield by 21%, 31%, 27%, and 25%, respectively, compared to control (Table 5.3, Table 5.4 and Fig. 5.2). Similarly, plant parameters: plant height, root weight, stem weight, and tuber yield were reduced by 33, 35, 33 and 34%, respectively by 'high' nematode while 13, 23, 28, and 22%, respectively by 'high' fungus. Moreover, co-inoculation caused significant reduction of the same parameters by 30, 37, 38, and 44%, respectively (Table 5.3, Table 5.4, and Fig. 5.2). However, there were no significant differences in the plant growth parameters and yield between 'high' and 'very high' levels (Table 5.3). At 'very high' pathogen densities, nematode caused 23, 35, 30 and 33% reduction of plant height, root weight, stem weight, and tuber yield, respectively while the same parameters were reduced by 15, 24, 29 and 23%, respectively by fungus. Likewise, co-inoculation reduced those parameters by 32, 37, 42 and 42%, respectively (Table 5.3, Table 5.4,

and Fig. 5.2). Increase in initial inoculum level of *P. penetrans* alone from 'low' level to 'very high' progressively increased the effect on plant growth and yield (Table 5.3). Similarly, increasing fungal inoculum density to 'very high' level led to a significant reduction ($P < 0.05$) in plant height and stem weight whereas co-inoculation caused significant reduction ($P < 0.05$) of plant height, root weight, and tuber yield (Table 5.3).

Effect of pathogens on plant health and nematode reproduction – second year experiment

In year two, compared to non-inoculated control, there was significantly ($P < 0.05$) higher disease effect at all co-inoculation levels except the 'low' level. Similarly, individual pathogens at 'high' and 'very high' densities significantly ($P < 0.05$) increased disease symptoms. However, no significant differences were observed among the treatments at a particular level just as recorded in the previous year. In second year, nematode multiplication rates ranged from 3.1 to 4.8 (Table 5.5). There were no statistically significant differences between *P. penetrans* alone or co-inoculation of both pathogens in the multiplication of *P. penetrans* within a particular level. Moreover, nematode multiplication rates were not significantly different among the different *P. penetrans* inoculation levels.

Table 5.3. Average plant growth (plant height, dry root weight, and dry stem weight) of Red Norland potato for thirteen treatments with non-inoculated control, low, medium, high and very high pathogen densities of *P. penetrans*, *F. oxysporum* or both during 2016 and 2017 micro-plot trials[y].

Treatments[z]	Plant growth parameters					
	Plant height (cm)		Dry root weight (g)		Dry stem weight (g)	
	2016	2017	2016	2017	2016	2017
Non-inoculated control	52.06 a	46.86 a	1.85 a	1.73 a	9.23 a	6.89 a
Low Pp	50.50 ab	44.19 abc	1.86 a	1.51 ab	8.31 ab	6.25 ab
Low Fo	48.90 ab	45.84 ab	1.67 ab	1.52 ab	8.05 abc	6.92 a
Low Pp + Low Fo	47.74 ab	43.30 abc	1.56 abc	1.42 bc	7.85 abc	5.97 abc
Med. Pp	42.05 cd	39.87 cd	1.49 abc	1.43 bc	7.95 abc	6.27 ab
Med. Fo	48.26 ab	43.05 abc	1.21 bc	1.50 ab	6.46 cd	6.01 ab
Med. Pp + Med. Fo	45.70 bc	36.83 de	1.33 abc	1.20 cd	6.06 d	5.01 bc
High Pp	38.23 d	31.38 e	1.07 c	1.12 d	6.69 bcd	4.61 bc
High Fo	41.02 cd	40.66 bcd	1.14 bc	1.33 bcd	5.87 d	4.98 bc
High Pp + High Fo	38.34 d	32.68 e	1.05 c	1.09 d	5.13 d	4.29 bc
V. High Pp	-	35.93 de	-	1.12 d	-	4.81 bc
V. High Fo	-	39.96 cd	-	1.31 bcd	-	4.87 bc
V. High Pp + V. High Fo	-	31.97 e	-	1.09 d	-	4.00 c

[y] Data on plant height of potato plants were recorded during the crop growing period at 12 weeks after planting while dry root weight and dry stem weight were collected after plant/ soil processing and nematode extractions. All the parameters are the average of ten replication for each treatments. Means of different parameters followed by same letter within a column are not significantly different according to F- protected least significant different test ($P < 0.05$).

[z] Pathogen densities for each treatments are provided in table 5.1. Pp represents *P. penetrans* and Fo represents *F. oxysporum*. Three treatments with very high pathogen densities were added in second year experiment.

Table 5.4. Percent reduction of plant growth parameters and tuber yield of Red Norland potato in pathogen inoculated treatments compared to non-inoculated control during 2016 and 2017 micro plot trials[a].

Treatments[b]	Reduction of plant growth parameters						Reduction of yield	
	Plant height (%)		Dry root weight (%)		Dry stem weight (%)		Tuber weight (%)	
	2016	2017	2016	2017	2016	2017	2016	2017
Non-inoculated control	0	0	0	0	0	0	0	0
Low Pp	3	6	0	13	10	9	13	6
Low Fo	6	2	10	12	13	0	12	3
Low Pp + Low Fo	8	8	16	18	15	13	20	10
Med. Pp	19	15	19	17	14	9	27	13
Med. Fo	7	8	35	13	30	13	23	10
Med Pp + Med. Fo	12	21	28	31	34	27	35	25
High Pp	27	33	42	35	28	33	29	34
High Fo	21	13	38	23	36	28	35	22
High Pp + High Fo	26	30	43	37	44	38	43	44
V. High Pp	-	23	-	35	-	30	-	33
V. High Fo	-	15	-	24	-	29	-	23
V. High Pp + V. High Fo	-	32	-	37	-	42	-	42

[a] Plant height data were collected during harvesting while others after harvest of trial.
[b] Pathogen densities in ten different treatments are provided in table 5.1. Pp represents *P. penetrans* and Fo represents *F. oxysporum*.

Table 5.5. Disease severity, nematode final population and nematode reproductive factor for different treatments during 2016 and 2017 micro plot trials[x].

Treatment	Disease severity[y]		Final nematode population per pot per plant		Nematode Reproductive factor (RF)[z]	
	2016	2017	2016	2017	2016	2017
Non-inoculated control	1.50 bc	0.90 d	-		-	-
Low Pp	1.00 c	1.30 bcd	308 d	750 d	1.50 b	3.75 a
Low Fo	1.60 bc	1.40 bcd	-	-	-	-
Low Pp + Low Fo	1.70 abc	1.20 cd	729 d	626 d	3.60 a	3.13 a
Med. Pp	1.30 bc	1.30 bcd	2,708 c	3,870 c	3.30 a	4.83 a
Med. Fo	1.90 ab	1.60 abcd	-	-	-	-
Med. Pp + Med. Fo	1.80 ab	1.80 abc	2,373 c	3,836 c	2.90 ab	4.79 a
High Pp	1.80 ab	2.08 abc	4,569 b	6,668 b	2.20 ab	3.28 a
High Fo	1.90 ab	2.14 ab	-	-	-	-
High Pp + High Fo	2.40 a	2.13 ab	5,464 a	8,268 ab	2.70 ab	3.98 a
V. High Pp	-	2.10 abc	-	8,771 ab	-	3.11 a
V. High Fo	-	2.48 a	-	-	-	-
V. High Pp + V. High Fo	-	2.42 a	-	9,690 a	-	3.31 a

[x]Disease severity, final nematode population and nematode reproductive factor values for each treatment are the mean of ten replications of each treatment.

[y] Disease severity is based on 0 to 5 rating scale (Kotcon et al. 1985). A value of 0 = no symptoms, 1 = < 25% of foliage with wilting, chlorosis, necrosis, 2 = 25 to 49%, 3 = 50 to 74%, 4 = 75 to 100%, and 5 = complete death of plant.

[z] Nematode Reproductive factor (RF) is calculated by dividing the final nematode population per pot per plant by initial nematode population inoculated during planting. Initial nematode populations for different treatments are mentioned in detail in Table 5.1.

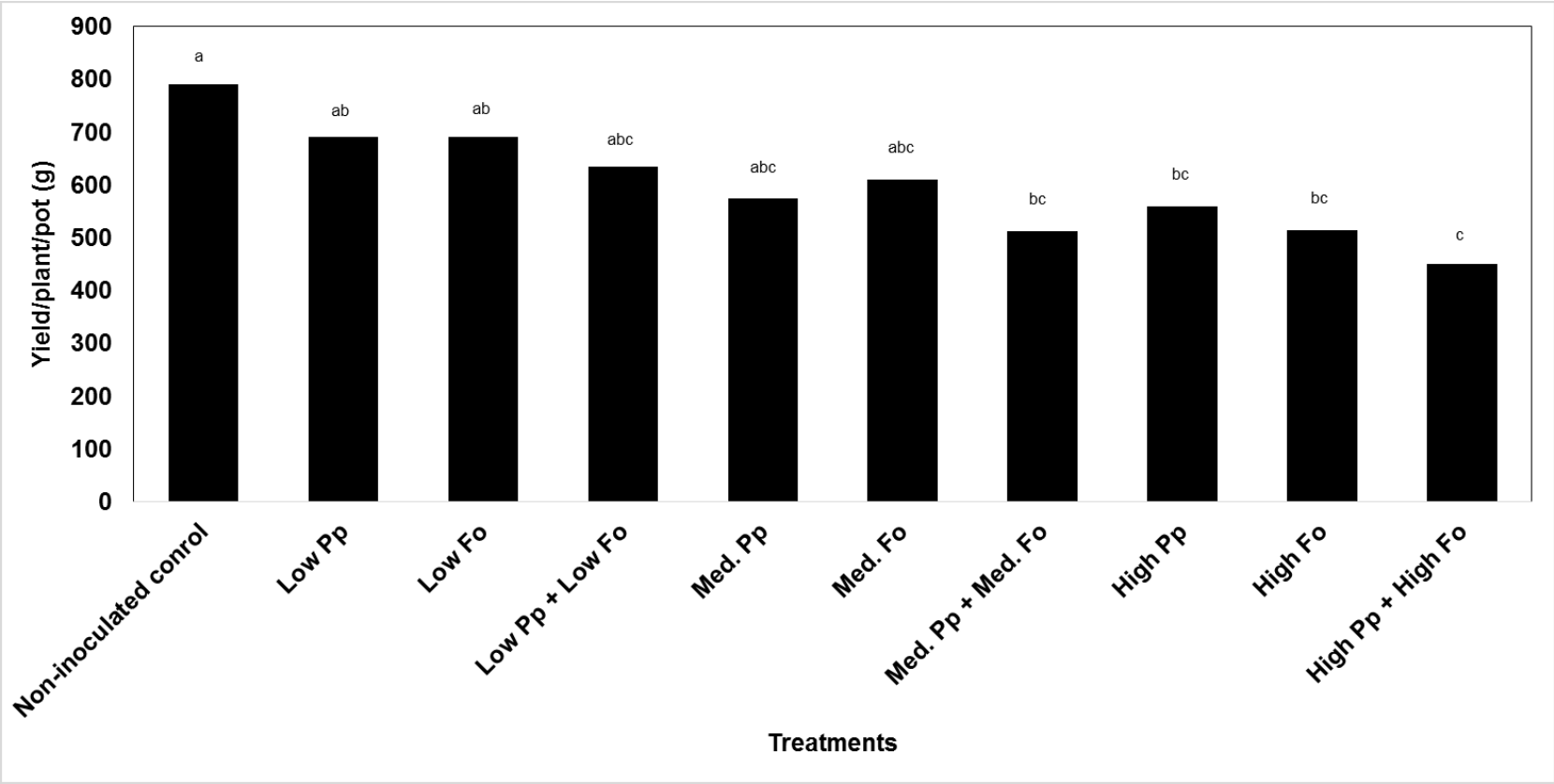

Fig.5.1. Tuber yield (in gram) per pot per plant of Red Norland potato in ten treatments of micro-plot trial during 2016 growing season. Yield is the average of ten replications for each treatment. Yields with same letters are not significantly different according to F-protected least significant different test ($P < 0.05$). Pp represents the root lesion nematode, *P. penetrans* and Fo represents the fungal pathogen, *F. oxysporum*. The density of pathogen for each treatment was provided in Table 5.1.

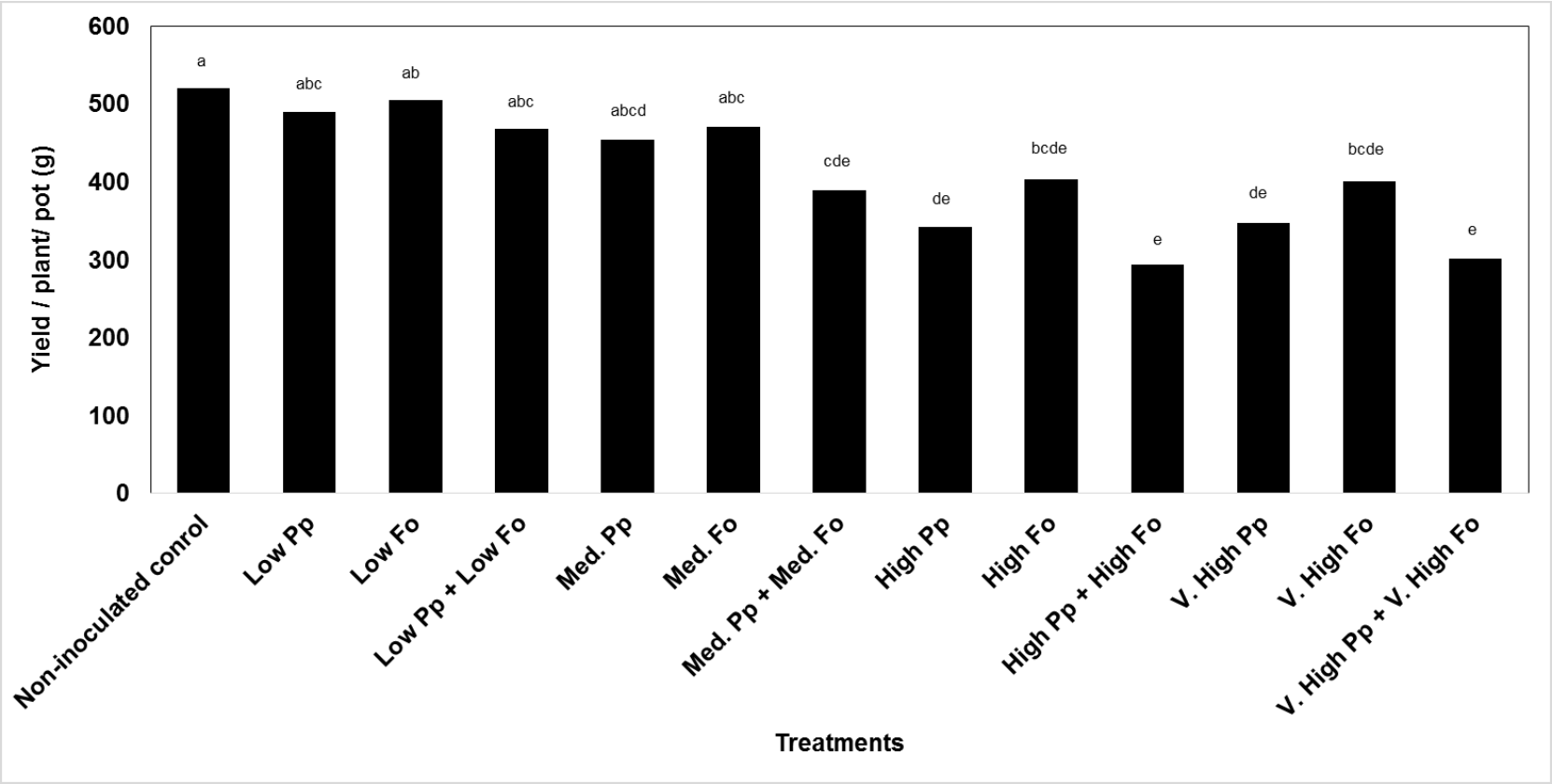

Fig.5.2. Tuber yield (in gram) per pot per plant of Red Norland potato in thirteen treatments of micro-plot trial during 2017 growing season. Yield is the average of ten replications for each treatment. Yields with same letters are not significantly different according to F-protected least significant different test ($P < 0.05$). Pp represents the root lesion nematode, *P. penetrans* and Fo represents the fungal pathogen, *F. oxysporum*. The density of pathogen for each treatment was provided in Table 5.1.

Discussion

Eight genera of plant-parasitic nematodes including *Pratylenchus* spp., *Helicotylenchus* spp., *Heterodera* spp., *Tylenchorhynchus* spp., *Paratrichodorus* spp., *Hoplolaimus* spp., *Mesocriconema* spp., and *Paratylenchus* spp. were detected in potato fields of Becker, Minnesota. Previous study in Ohio potato fields also reported eight genera of plant-parasitic nematodes (Brown et al. 1980). However, we detected *Heterodera* spp., *Hoplolaimus* spp., and *Mesocriconema* spp. which were not reported during their survey. Both surveys suggest *Pratylenchus* spp. are the most frequent and dominant nematodes in potato fields. In our survey, *Pratylenchus* spp. were present in 57% of soil samples while in Ohio survey they were observed in 65% of the soil and 85% of the root samples. We also identified *Pratylenchus* spp. in 100% of the root samples but these included those obtained from the problematic areas of the fields with stunted plant growth. Other plant-parasitic nematode genera were detected infrequently in both surveys. Moreover, *P. penetrans* was considered to be the most damaging nematode in light sandy soil of Wisconsin potato fields (Dickerson et al. 1964). Additionally, *P. penetrans* and *P. crenatus* appeared to be the dominant nematodes of potato crop in Prince Edward Island, Canada (Kimpinski 1979). Our results, including those from other researchers from Northeastern United States and Canada, suggest that *Pratylenchus* spp. are an important pest of potato crop in these regions. In Scandinavian regions, *Pratylenchus* spp. including a few other nematodes such as *Globodera* spp., *Trichodorus* spp., *Paratrichodorus* spp., and *Ditylenchus* spp. were considered important nematodes of potato crop (Holgado and Magnusson 2012).

The effect of root lesion nematode, *P. penetrans* or wilt fungus, *F. oxysporum* individually or combination was evaluated on growth and yield of red skinned potato cultivar, Red Norland in micro-plots in field conditions. The effect of these pathogens individually on

potato crop and together on other crop hosts has been studied. However, to our knowledge the effect of both pathogens together on potato cultivar, Red Norland has not been reported in the United States. In this study, either *P. penetrans* or *F. oxysporum* or together reduced growth and yield of potato crop, in light sandy loam soil at most pathogen densities. The negative effect on plant growth and yield increased with increasing density of pathogens individually or together. In most cases, co-inoculation of both pathogens caused comparatively more harmful effect on growth parameters and yield than individual pathogen alone. However, there was no significant interactive effect of co-inoculation on plant growth, tuber yield, and plant health.

In this study, significant effect of nematode on plant growth started to appear at pre-plant density of 32 *P. penetrans*/ 200 g of soil (approx. 32/100 cc soil or 800 Pp per pot). We only observed the effect on plant height at 32 Pp/ 200 g of soil. Similar, to our findings Martin et al. (1982) observed the reduction on top growth at 56 Pp/ 100 cc soil. However, Bernard and Laughlin (1976) found the suppression of other growth attributes such as root weights and tuber yield at pre plant density of 38 Pp/ 100 cc soil. In present study, negative effect on all growth parameter (plant height, stem weight, root weight) and yield was observed at pre-plant densities equal to or higher than 80 Pp / 200 g of soil (approx. 80 Pp/ 100 cc soil) or 2000 Pp per pot. We report 27 to 33% and 35 to 42% reduction of plant height and root weight respectively in Red Norland cv. at 80 Pp/ 200 g of soil. In contrast, at similar inoculum density (81 Pp/ 100 cc soil) Bernard and Laughlin (1976) observed no reduction of top growth but 54% and 45% reduction of root weight in Superior and Kennebec, cultivars respectively in Michigan. However, in Ohio on same Superior cv., Martin et al. (1982) observed both top growth and root reduction at densities both lower and higher than 80 Pp/ 200 g of soil in this study. In their study, *P. penetrans* suppressed top growth and root by 37% and 38% at 56 Pp / 100 cc of soil and 48%

and 35% at 151 Pp/ 100 cc of soil. These differences observed on effect of *P. penetrans* on plant growth might be due to differences in virulence of *P. penetrans* or growing conditions in Minnesota, Michigan and Ohio considering all being micro-plot studies in fields.

In this study, yield losses ranged from 29 to 34% at 'high' density of *P. penetrans* (80 Pp/ 200 g of soil or approx. 80 Pp/ 100 cc of soil). However, in micro-plot studies in Canada, yield losses were even higher and ranged from 25 to 73% depending upon potato cultivars (Olthof 1986). Similar to our study, 30% yield loss was reported on Superior cv. while no losses on Russet Burbank at similar inoculation density (81 Pp/ 100 cc of soil) (Bernard and Laughlin 1976). Moreover, in infected fields of Norway, up to 50% yield losses were observed (Holgado et al. 2009). All these previous findings provided more evidence to yield reduction of certain of certain potato cultivars due to *P. penetrans*. Our results also suggest that in a suitable condition, effect of *P. penetrans* on yield of potato could vary depending upon the pre-plant densities. Moreover, pre-plant densities of *P. penetrans* (80 or 120 Pp/ 200 g of soil) which caused yield losses in micro-plot studies were within the range of those densities (60 to 160 Pp/ 200 g of soil) found in some problematic fields with stunted plant growth. This suggests that *P. penetrans* in some potato fields in Becker, Minnesota might be causing serious yield damage. Decline in growth and yield due to *P. penetrans* infection may be due to loss of normal ability of infected roots to uptake water and nutrients as required by crop. It was suggested that feeding by *Pratylenchus* spp. inside root tissues could collapse infected cells and tissues increasing stress on crops (Rowe and Powelson 2002; Smiley and Machado 2009).

F. oxysporum at 'high' and 'very high' inoculation densities caused significant reduction of plant growth and yield losses. However, at 'low' and 'medium' level significant reductions were not observed in most cases. This could simply be explained that lower inoculum levels

were not enough to cause damage to potato crop. In field conditions, up to 25% yield losses were reported to be caused by *Fusarium* spp. while in storage damage to tubers can reach up to 60% (Desjardins 2006; Secor and Salas 2001). While in our micro-plot studies we found 22 to 35% yield losses, similar to those reported for field conditions. Dry conditions of Becker, Minnesota with relatively low precipitations during the growing period might have helped to increase the damage caused *by P. penetrans* or *F. oxysporum* individually on potato crop.

In present study, we did not observe significant interactive effect of co-inoculation at any level compared to individual pathogen alone at the same level of inoculation. This contrasts with the suggestion that nematode and fungus interaction effects were observed at the pathogen level where individual pathogens caused no effect or very little effect when acting alone (Rowe and Powelson 2002; Martin et al. 1982). Nevertheless, in most cases of this study pathogens during co-inoculation were producing more negative effect than individual pathogens. Like our observation, Burpee and Bloom (1978), did not found significant interactive effect of *V. alboatrum* and *P. penetrans* on potato crop. But, he also observed slightly higher effect on tuber yield during co-inoculation of *V. alboatrum* and *P. penetrans* than individual pathogens. In contrast, Jorgenson (1970) observed negative role of *F. oxysporum* during co-inoculation with *H. schachtii* leading to lesser damage on sugarbeet crop than *H. schachtii* alone.

In this study, plant health assessed as wilting, chlorosis and necrosis were not correlated with reduction of plant parameters for some treatments. Similar observations were made during nematode-fungus interaction studies in potato by Martin et al. (1982) and Burpee and Bloom (1978). It is questionable for such observation in our study. In addition, in this study, plant health assessment was done only one time at the later end of growing season. Hence, we suggest that in

future plant health assessment should be done at least one more time from early growth stage rather than only one time at the later end of growing season.

There was no significant interactive effect of co-inoculation on plant health or disease development and nematode multiplication in the present study. Consistent with our result, Burpee and Bloom (1978) did not observe significant interaction of *P. penetrans* and *V. albo-atrum* on potato early dying disease development in Katahdin, Kennebec and Abnaki cultivars. However, significant interaction of *P. penetrans* and *V. dahliae* was observed in development of potato early dying symptoms on Russet Burbank and Superior cultivar of potato (Riedel et al. 1985; Rowe and Powelson 2002; MacGuidwin and Rouse 1990; Martin et al. 1982). Therefore, it could be inferred that interactive effect on disease development may be influenced by type of cultivar grown since many researchers observed interaction on specific potato cultivars. In this study, in most of the cases the reproduction of *P. penetrans* was not significantly affected due to presence of *F. oxysporum* during co-inoculation compared to *P. penetrans* alone. Conversely, reproduction rate of *P. penetrans* was reduced in nematode species mixture of *P. scribneri* and *P. penetrans* in the presence of *V. dahliae* on Superior cv. of potato in Ohio (Wheeler and Riedel 1994). Similarly, *F. oxysporum* inhibited the reproduction of *H. schachtii* in sugarbeet (Jorgenson 1970). However, we neither observed significant reduction nor increment but only slight numeric increment in reproduction rate of *P. penetrans* in the presence of *F. oxysporum*. However, Mountain and McKeen (1962) noticed significantly positive role *V. dahliae* in reproduction rate of *P. penetrans* in eggplant and tomato. Although the mechanism of interaction between nematode and fungus is not clearly understood till now, researchers have agreed that either pathogen infection increases stress on plant changing host physiology and making more prone to infection by another pathogen. Moreover, it was also claimed that root

lesion nematode feeding led to higher root exudate production and increased rhizosphere zone, facilitating germination of more fungal spores (Rowe and Powelson 2002).

We conclude that *P. penetrans* and *F. oxysporum* both individually and together can cause effect on plant growth, yield and plant health. However, in most cases, presence of both pathogens were not observed to have significant increment in damage compared to lone pathogen on Red Norland cultivar of potato. Considering slightly higher negative effect of co-inoculation than individual pathogen in micro-plot studies using autoclaved field soil, future research should be carried out in field conditions using more crop cultivars where both pathogens are present. Based on the findings of this research management of these pathogens would be considered worthy in order to improve potato production in this region.

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CHAPTER 6. SUMMARY

In North Dakota pea field soils, eight genera of plant-parasitic nematodes including *Paratylenchus*, *Tylenchorhynchus*, *Pratylenchus*, *Helicotylenchus*, *Xiphinema*, *Hoplolaimus*, *Meliodogyne*, and *Paratrichodorus* were identified during the surveys from 2014 to 2017. Among eight group of parasitic nematodes, *Paratylenchus* and *Tylenchorhynchus* were the top two dominant nematodes based on incidence, distribution and population densities. Moreover, greenhouse experiment indicated that the dominant parasitic nematode, pin nematode, *Paratylenchus nanus* could reproduce in selected field pea cultivars with a differential rate of reproduction at two initial population densities. *P. nanus* was also demonstrated to cause reduction on growth and yield of some field pea cultivars.

Nematode soil surveys conducted in potato fields of Central Minnesota during 2015 to 2016 indicated *Pratylenchus* as the dominant nematode based on incidence and population densities. Other genera of plant-parasitic nematodes identified in potato fields included *Heterodera*, *Helicotylenchus*, *Tylenchorhynchus*, *Paratrichodorus*, *Hoplolaimus*, *Mesocriconema*, and *Paratylenchus*. Micro-plot trials conducted to examine the effect of lesion nematode, *Pratylenchus penetrans* and wilt fungi, *Fusarium oxysporum* on potato growth and yield showed that these pathogens have ability to cause detrimental effect on potato crop when acting alone or together. However, these pathogens were not observed to have significant interactive effect on potato growth and yield when present together compared to present alone.

APPENDIX. ACTIVITIES DURING AND AFTER HARVEST OF MICRO-PLOT TRIALS

Fig A1. Drilling holes using an automated driller at the micro-plot trial site.

Fig. A2. Potato plants in micro-plot trial at the Sandplain Research Farm, Becker, Minnesota.

A **B** **C**

Fig. A3. A) A plant with no obvious visual symptom under the non-inoculated treatment (A) and wilting, necrosis, and chlorosis of potato plants inoculated with both *P. penetrans* and *F. oxysporum* at the 'high' pathogen density (B and C).

A **B**

Fig. A4. Images (A and B) showing browning and necrosis of roots inoculated with both *P. penetrans* and *F. oxysporum* at the 'very high' pathogen density.

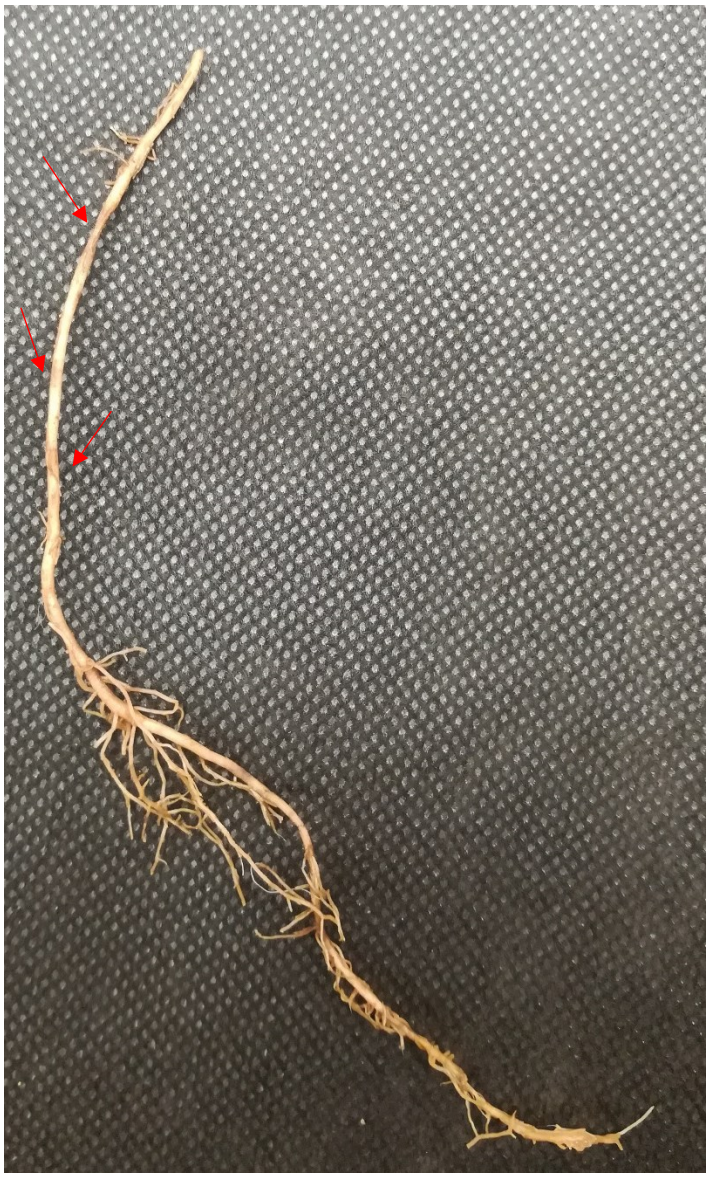


Fig. A5. An image showing necrotic areas, with red arrows, in a single root fiber due to *P. penetrans* infection in root tissues.

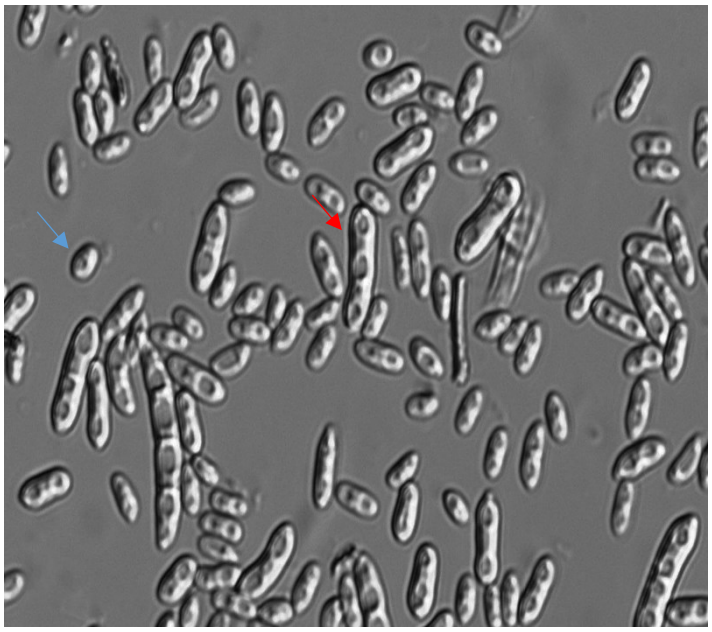
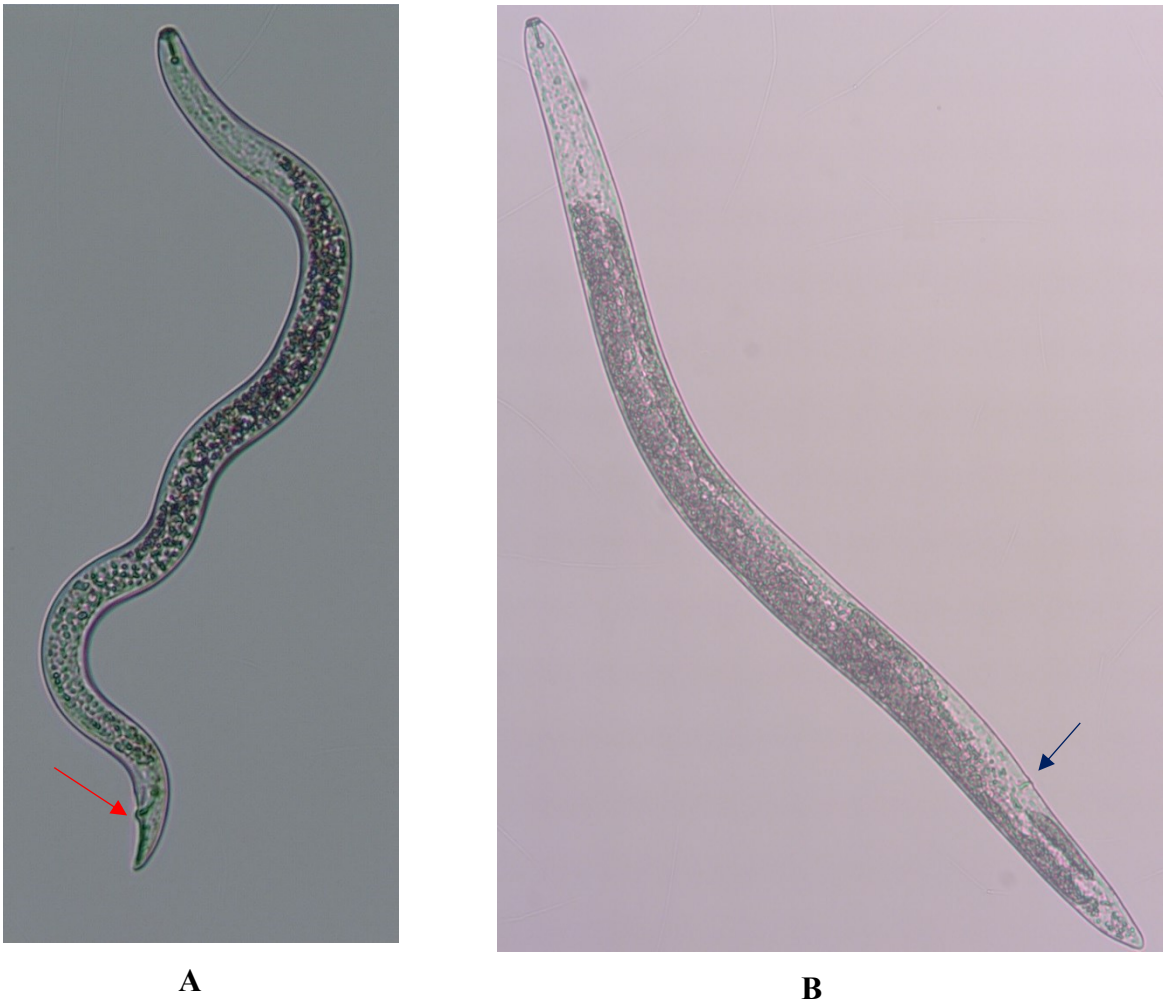


Fig. A6. An image showing macro and microconidia of *F. oxysporum*. Blue arrow shows microconidia while red arrow indicates macroconidia.



A B

Fig. A7. A) Male of *P. penetrans*. Red arrow shows the spicules which is the diagnostic character of male. B) Female of *P. penetrans*. Dark Blue arrow indicates the vulva slit, diagnostic character of female.

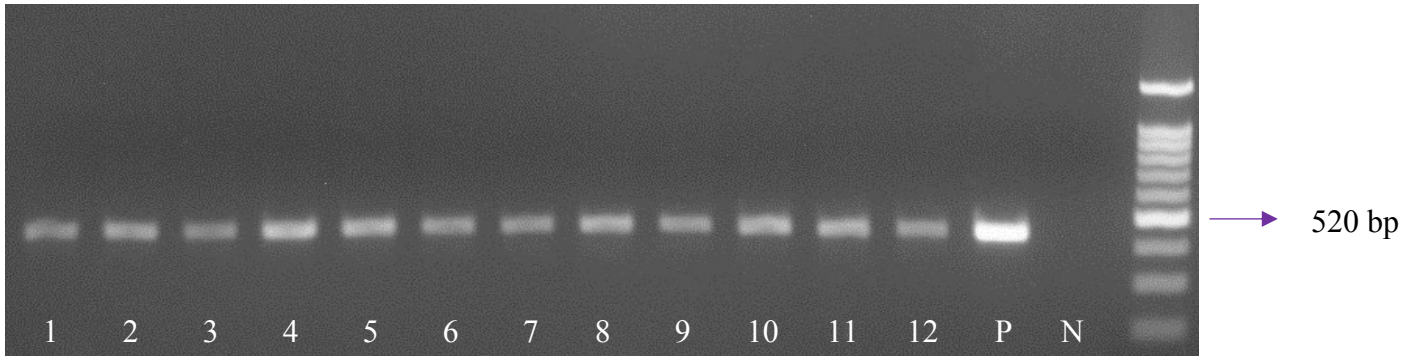


Fig. A8. Identification of *P. penetrans* from DNA extracted from single individuals by conventional polymerase chain reaction using species-specific primers. DNAs were amplified with the *P. penetrans*-specific primer set PP5F/PP5R (approx. 520 bp). M indicates 100-bp DNA ladder (Promega Corp.). Lanes 1 to 12 indicates amplified DNA from single lesion nematode individuals, Pp represents *P. penetrans* DNA, and NC represents non-template control with sterilized double-distilled water instead of DNA in PCR mixture.

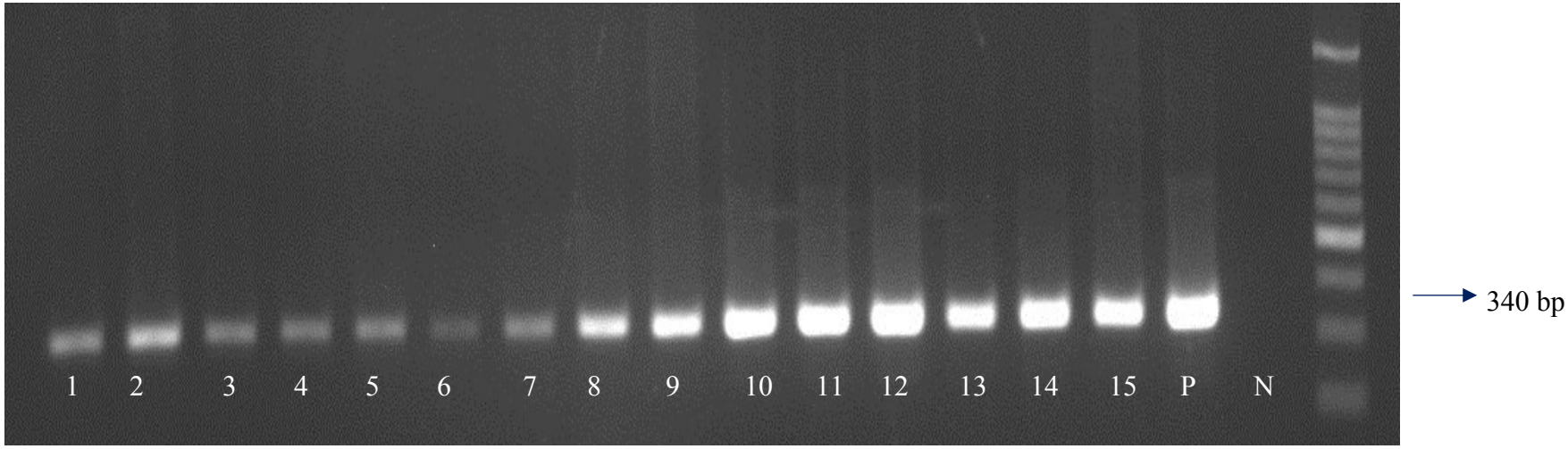


Fig. A9. Identification of *F. oxysporum* from DNA extracted from soil and mycelium grown on potato dextrose agar medium by conventional polymerase chain reaction using species-specific primers. DNAs were amplified with the *F. oxysporum* specific primer set FOF1/FOR1 (340 bp). M indicates 100-bp DNA ladder (Promega Corp.). Lanes 1 to 9 indicates amplified DNA from soil, lane 10 to 15 represents amplified DNA from mycelium, PC represents positive control for *F. oxysporum* DNA, and NC represents non-template control with sterilized double-distilled water instead of DNA in PCR mixture.