PLANT-PARASITIC NEMATODES ON CORN (*ZEA MAYS* L.) AND SOYBEAN (*GLYCINE MAX* L.) IN NORTH DAKOTA

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Four studies were conducted to investigate plant-parasitic nematodes (PPN) of corn and soybean in ND. The first study investigated the incidence and abundance of vermiform PPN in ND corn fields in 2015 and 2016. Samples were collected from 300 corn fields across 20 counties. Seventy-two percent of the fields were positive for PPN. The major genera of PPN identified were *Helicotylenchus*, *Tylenchorhynchus*, *Paratylenchus*, *Pratylenchus*, *Heterodera*, *Xiphinema*, *Hoplolaimus*, and *Paratrichodorus*. The second study characterized SCN (SCN; *Heterodera glycines*) virulence phenotypes in ND. A total of 419 soybean fields across 22 counties were sampled during 2015, 2016, and 2017. Among these samples, 73 SCN field populations were successfully virulence phenotyped using the HG type tests. The HG types that were detected and confirmed in ND were HG type 0, 7, 2.5.7, 5.7, 1.2.5.7, and 2.7. The third study developed a new molecular method for detecting and identifying a new *Pratylenchus* sp. discovered in a soybean field in ND. A species-specific primer set, that can be used in both conventional and real-time polymerase chain reaction (PCR) assays, was designed from the internal transcribed spacer (ITS) region of ribosomal DNA. Laboratory experiments confirmed that the primers only amplified DNA of the target nematode species but not the non-target species used in the specificity tests. Practically, DNA from as little as a single nematode could be used to specifically identify the new *Pratylenchus* sp. using the molecular diagnostic methods developed in this study. The fourth study was conducted to ascertain resistance levels of 20 soybean cultivars to the new *Pratylenchus* sp. Combined results of four trials indicated that seven of the cultivars were moderately resistant, ten were moderately susceptible, four were susceptible, and none of the cultivars tested were resistant. Analysis of the habitat preference of the new *Pratylenchus* sp., revealed that above or close to 50% of the nematode population

resided in roots at nine weeks after planting for a majority of the cultivars evaluated. Results from these studies will be helpful in improving nematode detection and developing management strategies to control plant-parasitic nematodes in ND corn and soybean fields.

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DEDICATION

This work is dedicated to my father Ayub Khan, my mother Joushan Ara Hoque, and my wife Humayra Ferdous Shoshi. All three of whom never failed to believe in me and support me all the way throughout this endeavor. I would also like to dedicate this piece of work to the entire Chowdhury-Khan-Hoque family, as the first one to obtain a PhD degree, for the generation behind me and for all the hopes and dreams they will fulfill.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CCA	Canonical Correspondence Analysis
CRD	Completely Randomized Design
DNA	Deoxyribonucleic Acid
HSD	Honestly Significant Difference
ITS	Internal Transcribed Spacer
MR	Moderately Resistant Cultivar
MS	Moderately Susceptible Cultivar
ND	North Dakota
NDSU	North Dakota State University
PCR	Polymerase Chain Reaction
PPN	Plant-parasitic Nematodes
rDNA	Ribosomal Deoxyribonucleic Acid
RLN	Root-Lesion Nematodes
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
S	Susceptible Cultivar
SCN	Soybean Cyst Nematodes
US	United States
US$	United States Dollar
USA	United States of America
USDA-NASS	United States Department of Agriculture- National Agricultural Statitics service
UV	Ultra Violet

DISSERTATION ORGANIZATION

This dissertation elaborates on plant-parasitic nematode in North Dakota Corn fields. Furthermore, this research investigates soybean cyst nematodes (SCN; *Heterodera glycines*) and root-lesion nematodes (*Pratylenchus* spp.) collected from North Dakota soybean fields. There are five chapters in this dissertation. Chapter 1 is the literature review with introduction to corn, soybean and plant-parasitic nematodes. Chapter 2 describes the prevalence and distribution of plant-parasitic nematodes in North Dakota corn fields and their association with environmental variables. Chapter 3 described the virulence phenotypes of soybean cyst nematode in North Dakota soybean fields. Chapter 4 describes a new molecular identification method for identifying a new species of root-lesion nematodes. Chapter 5 describes the resistance levels of 21 soybean cultivars against the new species of root-lesion nematodes. Chapter 6 summarizes the findings of chapter 2, 3, 4, 5.

CHAPTER 1: LITERATURE REVIEW

Corn

Corn (*Zea mays* L.) is one of the oldest domesticated field crop that belongs to the Poaceae family (Hart and Lovis 2013). This cereal crop originated from Central America specifically in the Mexican region (Iltis and Doebly 1980) and was likely domesticated around 7,000 to 10,000 years ago from a Mexican wild grass known as Balsa Teosinte (Smith 1989). Domestication happened as ancient farmers native to the Balsa River Valley of Mexico started to store the seeds of the wild grass from the best yielding plants and sowing them the next year to achieve better yield. This selection process was probably the beginning of plant breeding (Pruitt 2016). Shortly after domestication, small scale maize production occured throughout North America and South America, likely through major trade routes. By the time Europeans arrived there were more than 300 distinct races of corn that were present in the Americas spanning from Chile to southern Canada (Hallauer 1987). Thus, Europeans learned about corn cultivation from the Natives of the region and helped spread maize production throughout the world. As a result, maize became a prominent part of many world cultures, allowing societies to grow and flourish (Pruitt 2016).

Modern corn hybrids are facultative short-day C4 plants. Each corn plant has a single relatively tall stalk with multiple large narrow leaves growing out of each node of the stalk. This monocot plant has a staminate (male) flower that is referred to as tassel, which terminates at the top end of the stem. The pistillate (female) flower, referred to as ears, grows out of the leaf nodes through a branch like structure called shank (Ritchie et al. 1993). After maturation, ears are harvested for kernel, cop, silk, and husk (Nelid and Newman 1990). Today, harvested corn products are used for livestock feed, human food, ethanol production, corn starch, and corn syrup

production (Pruitt 2016). Due to modern advances in plant breeding, corn is grown in most part of the world over a wide range of environmental conditions, from Chile to Canada and Russia (USDA-FAS), helping millions of people achieve food security and better livelihood.

Corn production

Corn has become one of the most produced staple crops in the world after wheat and rice. In 2019, more than 43.7 billion bushels of corn was produced in the world and a majority of this production occurred in the U.S. and China. The U.S. produced 13.7 billion bushels of corn in 2019; whereas, China produced 10.3 billion bushels (USDA-ERS 2020a). Thus, the U.S. was the leading producer of corn among the corn producing nations in 2019 with U.S. corn fields yielding an average of 168 bushels/acre. In the U.S. alone crop value of corn was more than $52.7 billion in 2019 (USDA-NASS 2020a). A majority of the U.S. corn production occurs in the U.S. Heartland region, which is often referred to as the U.S. corn belt (USDA-ERS 2020b). Leading corn producing states of the U.S. in 2019 were Iowa, Illinois, Nebraska, Minnesota, Indiana, Kansas, South Dakota, Missouri, North Dakota and Wisconsin.

In 2019, North Dakota was ranked as the ninth highest corn producing state of the nation. More than 3.5 million acres of corn were planted in North Dakota during 2015, accounting for more than US$1 billion. Generally, two types of corn are produced in North Dakota, which includes grain corn and silage corn. Thus, the top three grain-corn producing counties of North Dakota in 2019 were Sargent, Ransom and Richland counties; whereas, the top three silage-corn producing counties of North Dakota were Barnes, Griggs and Towner (USDA-NASS 2020). Thus, most of North Dakota's corn acreage is located in the east-central and south-eastern areas of the State (Callez-Torrez et al. 2018). Today, a majority of the corn produced in North Dakota is used as raw materials for human food, animal feed, and as a source of ethanol. However, this

was not always the case. Prior to the nineteen-nineties, corn production in North Dakota was limited. Over the past three decades, corn production in North Dakota nearly doubled due to the recent increase in demand for corn as a source of ethanol and livestock feed (Ransom 2010; NDCC 2020). Additionally, the introduction of early maturing cultivars and the recent change in weather pattern being more favorable to corn production have contributed to increase in corn acreage in the state. With the recent increase in North Dakota's corn acreage, various disease-related limiting factors associated with corn production are predicted to increase as well (Ransom 2010).

Corn diseases

Diseases of corn continue to reduce corn yield around the world (Farber et al. 2018). However, severity of diseases and yield loss due to diseases vary between locations and growing seasons. Moreover, crop production practices, environmental conditions, hybrid selection, previous disease history, and susceptibility of hybrids to diseases also influence corn yield losses due to diseases (Munkvold and White 2016). Although, catastrophic losses like the one that occurred in 1970 due to the introduction of race T resulting in the southern corn leaf blight epidemic (Ullstrup 1972) were rare, diseases of corn were reported to causes yield losses worth more than US$20.1 billion in the U.S. and Ontario, Canada during 2012 to 2015 (Mueller et al. 2016). Causal agents of these diseases include bacteria, fungi, plant-parasitic nematodes and other microbial organisms.

There are numerous diseases of corn that can reduce yield in the U.S. Among these diseases the top 10 diseases that caused the greatest yield loss during 2013 in Northern U.S. and Ontario Canada were seedling blights, Northern corn leaf blight, Goss's wilt, Gray leaf spot, root rots, *Fusarium* stalk rot, Southern rust, Common rust, plant-parasitic nematodes, and *Fusarium*

ear rot (Mueller et al. 2016). In North Dakota, the major known diseases of corn include corn rust, Northern corn leaf blight, and Goss's wilt. Although previous studies have investigated these three diseases of corn in North Dakota (Bauske 2018; Friskop et al. 2014; Ransom et al. 2016; Wise et al. 2019), there is little to no information about plant-parasitic nematodes associated with corn in North Dakota.

General introduction to plant-parasitic nematodes

Nematodes have existed on earth for more than a billion years (Wang et al. 1999). Thus, they are considered one of the most ancient and diverse animals on earth (Lambert and Bekal 2002). These soft bodied unsegmented roundworms are thought to have evolved from simple primitive microbial animals before the "Cambrian explosion" that occurred approximately 541 million years ago (Marshall 2006; Poiner 1983). Since, then these microscopic multicellular organisms have evolved to occupy all ecological niches from desert to the snowy mountains of the world (Hartman et al. 2015; Khan 2008; Ortiz 2019). Today, more than 7.5 billion nematodes can be found in a 2-acre field of any soil type in the top 20 cm of soil below the soil surface (Hartman et al. 2015; Ortiz 2019). These organisms can survive in these fields by feeding on a wide range of food sources that include plant residues, plant roots, fungal hyphae, algae, bacteria, protozoa, and other nematodes (Khan 2008). However, only a small portion of the nematode species described thus far in previous literature parasitize plants (Khan 2008; Ortiz 2019). These nematodes are generally referred to as plant-parasitic nematodes.

A distinguishing feature of plant-parasitic nematodes that separate them from other nematodes is the presence of a specialized hollow spear like feeding structure called a stylet that is visible when the nematode is viewed under a microscope (Mai et al. 1996). Although a majority of the plant-parasitic nematodes commonly found in crop fields can only be visualized

under a microscope, few species, such as the cyst nematodes in adult stage, can be seen without the aid of a microscope since they swell up as they reach the adult stage (Hartman et al. 2015; Lamber and Bekal 2002). Thus, some plant-parasitic nematodes' bodies swell up as they reach adult stage while others remain as vermiform throughout their life cycle (Lambert and Bekal 2002).

The life stages of a majority of plant-parasitic nematodes include an eggs stage, four juvenile stages, and finally the reproductive adult stage. The duration of the life cycle of plant-parasitic nematodes can range from a few days to almost one year depending on the genera and species (Bridge and Starr 2007). Plant-parasitic nematodes can reproduce sexually and/or asexually depending on the species, environmental condition and availability of food sources (Bridge and Starr 2007; Castillo and Volvas 2007). Plant-parasitic nematodes have different overwintering strategies depending on the species that includes dormancy (diapause and quiescence) and cyst formation through which adult dead females protect their eggs inside their bodies (Bridge and Starr 2007; Karssen and Moens 2006; Mai et al. 1996).

Moreover, some species of plant-parasitic nematodes lead a migratory ectoparasitic life style during which the nematode's body remain outside of the root tissue while feeding on cells of the plant roots using their stylet. This strategies allows these ectoparasites to migrate and graze between numerous plants. On the other hand, plant-parasitic nematodes that lead a migratory endoparasitic lifestyle can penetrate and invade the root-tissue to feed on plant cells (Lambert and Bekal 2002). They can complete their entire life cycle inside the roots or leave the root tissue and migrate to other roots (Castillo and Volvas 2007; Lambert and Bekal 2002). Similar to the migratory endoparasitic nematodes, sedentary endoparasitic nematode can also penetrate and invade the root tissue. However, sedentary endoparasites lose their ability to

migrate out of the root tissue as they swell up to house numerous eggs when they reach their adult stages (Bridge and Starr 2007; Lambert and Bekal 2002).

Environmental and soil abiotic factors that affect plant-parasitic nematodes

The plant-parasitic nematode prevalence, distribution, and species composition in soil communities were determined to be influenced by cropping history, environmental factors such as temperature and soil moisture, soil physical and chemical properties, farming practices, and management practices (Edwards et al. 1988; Neher 1999; Norton 1978; Simon 2015; Wallace 1971; Yeates et al. 1984). Free soil moisture is critical for nematode, survival, activity, and mobility (Bridge and Starr 2007; Wallace 1971). However, excessive soil moisture levels can limit nematode survival (Sultan and Ferris 1991). Additionally, soil moisture directly influences nematodes ability to parasitize host, Koenning and Barker (1995) reported that increase in soil moisture led to better penetration, hatching and mating of soybean cyst nematodes. Temperature, on the other hand, can stimulate nematode metabolic activity, feeding rate and root penetration. Furthermore, temperature may also influence nematodes' dormancy or diapause (Huang and Pereira 1994); thus, influencing the timing of events in nematodes' life cycle (Bridge and Starr 2007; Karssen and Moens 2006). The optimal soil temperature for nematode development can range between 15 to 32°C (Ortiz 2019); however, it can vary significantly depending on the species of plant-parasitic nematodes (Acosta et al. 1979).

Soil texture and other soil edaphic factors are important variables that can influence occurrence and distribution of plant-parasitic nematodes (Simon 2015; Chen et al. 2012; Norton 1978; Wallace 1971). The textural properties of soil can directly influence nematode directly influence nematode movement. According to Wallace (1971), larger nematodes with greater length and diameter required bigger soil particles that allow better pore size for nematodes to

optimally move through. Brodie (1976) reported that the larger *Belonolaimus longicaudatus* nematodes were more prevalent in soil with 5 to 7% clay; 87 to 88% sand, and 6 to 7% silt; whereas, the smaller *Pratylenchus brachyurus* nematodes were more prevalent in soil with 15 to 16% clay, 78 to 79% sand, and 6% silt. Furthermore, Norton and Hoffmann (1974) reported that pH could be used as a tool to predict where nematodes were more likely to occur, however, the effect of pH on nematode populations could vary between species.

Plant-parasitic nematodes associated with corn

Over 60 species of plant-parasitic nematodes are associated with corn in North America (Norton 1983). Among these, the species belonging to the genera of *Belonolaimus* (common name: sting), *Criconemella* (ring), *Helicotylenchus* (spiral), *Hoplolaimus* (lance), *Longidorus* (needle), *Meloidogyne* (root-knot), *Paratylenchus* (pin), *Pratylenchus* (root-lesion), *Paratrichodorus* (stubby root), *Tylenchorhynchus* (stunt) and *Xiphinema* (dagger) are commonly found in North American corn fields (Koenning et al. 1999; Norton 1983; Tylka et al. 2011). Although, *Belonolaimus*, *Criconemella*, *Helicotylenchus*, *Hoplolaimus*, *Longidorus*, *Paratylenchus*, *Paratrichodorus*, *Tylenchorhynchus* and *Xiphinema* are ectoparasites, *Meloidogyne* are a genus consisting of sedentary endoparasites, and *Pratylenchus* are migratory endoparasites (Bridge and Starr 2007; Lambert and Bekal 2002; Simon 2015). Although *Heterodera zeae* (corn cyst), a cyst forming nematode that parasitizes corn, has been detected in some states of the U.S (Koeenning et al. 1999), significant losses caused by this nematode is not projected to occur in North America (Koeenning et al. 1999) due to its apparent requirement for high soil temperature (Koenning et al. 1999; Windham 1998).

Although many species of plant-parasitic nematodes have been detected in North American corn fields, only three to seven genera were found to simultaneously exist in soil

communities of individual corn fields (Koenning et al. 1999; Norton 1983; Tylka et al. 2011). Moreover, some of these genera have a greater frequency and distribution than others (Simon 2015; Tylka et al. 2011). In the Midwestern state of Iowa, *Helicotylenchus*, *Pratylenchus*, and *Xiphinema* were reported to be the most prevalent genera in corn fields (Tylka et al. 2011). In Illinois, another Midwestern state, *Helicotylenchus* was also the most prevalent genus with *Pratylenchus* being the second most frequent in corn fields (Niblack 2009; Simon 2015). Similarly, in Ohio corn fields, *Paratylenchus*, *Helicotylenchus*, and *Pratylenchus* were the most prevalent genera.

Damage caused by plant-parasitic nematodes in corn fields

Plant-parasitic nematodes are one of the greatest threat to crop production worldwide (Sasser and Freckman 1987). They were reported to cause annual crop losses worth more than US$8 billion in the U.S. alone (Smiley 2005). However, yield losses caused by plant-parasitic nematodes often go unnoticed or are attributed to other causes, because symptoms caused by plant-parasitic nematodes on plants are similar to the symptoms caused by other biotic and abiotic diseases (Norton 1983; Tylka et al. 2011). In some southern states of the U.S. such as Florida, plant-parasitic nematodes have been reported to cause yield losses as high as 20% in corn fields in 1999. Moreover, spiral, root-knot, stubby root, and root-lesion nematodes were reported to causes 5 to 10% yield loss in corn fields of South Carolina (Koenning et al. 1999). In Georgia, annual grain corn yield losses as high as 7% were attributed to plant-parasitic nematodes.

In the Midwestern state of Iowa, state-wide yield losses were predicted to increase by 4.2% to 5.5%, if nematicides were not used to control these pathogens (SON crop loss assessment committee 1987). In the neighboring state of South Dakota, *Pratylenchus hexincisus*

was estimated to cause yield losses of 9.5 bushels per acre on dry land corn, while *P. scribneri* was estimated to cause yield losses of 4 bushels per acre in irrigated corn fields (Smolik and Evenson 1987). Corn yield loss survey conducted during 2012 to 2015, reported that plant-parasitic nematodes caused more than 294 million bushels of corn loss in the U.S. and Ontario, Canada (Mueller et al. 2016). Furthermore, Mueller et al. (2016) determined that plant-parasitic nematodes were among the top ten disease pathogen of corn in 2012-2013.

Management of plant-parasitic nematodes

One of the most important management tactic against plant-parasitic nematodes is the use of phytosanitary measures that prevent dispersal of plant-parasitic nematodes. Plant-parasitic nematodes dissemination occurs during movement of soil via farm machinery and equipment, animal, water, wind, and plant tissue from infested to non-infested field (Bird 1981). Thus, cultural practices such as quarantine measures, sanitation measures, use of certified clean plant material, and nematodes free soil are effective against plant-parasitic nematodes (Bird 1981; Nicol et al. 2011). However, an integrated pest management strategy that incorporates chemical, biological, cultural, and host plant resistance is optimal for practical management of plant-parasitic nematodes in crop fields (Hague and Gowen, 1987; Halbrendt and LaMondia 2004; Heald 1987; Kerry 1987).

Chemicals such as broad-spectrum fumigants have been effective in managing plant-parasitic nematodes populations. However, these chemical can be highly toxic for humans, animals, and the environment. Thus, chemicals such as methyl bromide, that were commonly used to control nematodes, weeds and other soil borne pathogen two decades ago, has been phased out and banned in countries like the U.S.in recent years (Tylka et al. 2012). However, recent studies such as Chellemi et al. (2013) have reported the efficacy of 1,3-dichloropropene

and chloropicrin in controlling plant-parasitic nematodes as alternative soil fumigants. Furthermore, protectant seed treatment nematicides such as Avicta® and Votivo® are commercially available products that are rated for controlling plant-parasitic nematodes (Simon 2015; Tylka et al. 2012). Avicta® is a contact nematicide, with the active ingredient abamectin, that is developed and sold by Syngenta, Whereas Votivo® is a biological control agent that uses a special strain of the bacteria *Basillus firmus*. The bacteria grow on the surface of root tissue, and reportedly create a thin film barrier around the root to prevent nematode penetration (Tylka et al. 2012). Although other biological control agents that are environmentally safe have been reported in previous literature, their large-scale production and field application is seemingly impractical due to intense labor requirements and high costs (Siddiqui and Mahmood 1999). Furthermore, long term stability and survival of the biological control agents in natural environments of cropping fields can be a limiting factor for such management strategies (Jatala 1986)

Other management strategies against plant-parasitic nematodes include soil amendments, cover crops, host resistance, and crop rotation (Akhter and Malik 2000; Williamson and Hussey 1996). Organic soil amendments such as green manure can reduce nematode population in soil due to the compounds released by these amendments upon decomposition that have nematicidal properties (Akhtar and Malik 2000). Similarly, cover crops can also reduce nematode population through nematicidal activity or by trapping nematodes (Kruger et al. 2013). However, dependence on soil amendment or cover crops alone to control nematodes seems not practical in large-scale crop production due to labor and cost requirements (Akhter and Malik 2000; Kruger et al. 2013).

Resistance of plants against plant-parasitic nematodes results in hostile environment for nematode reproduction and development during and after infection, which results in suppression of nematode populations (Niblack et al. 2005; Ortiz 2019; Oyekanmi and Fawole 2010). Non-host resistance can be utilized through crop rotation to control nematode populations. On the other hand, several nematode resistance genes have been characterized in host plants and integrated into commercial cultivars to control plant-parasitic nematodes (Williamson and Hussey 1996; Williamson et al. 2006). Thus, an integrated approach that includes, crop rotation, resistant cultivars, soil amendments, cover crops, and other cultural and chemical practices is optimal for controlling these soil borne pathogens (Niblack 2005; Orlando 2020; Siddiqui and Mahmood 1999).

Soybean

Soybean (*Glycine max* L. (Merr.)) is a leguminous crop that originated in northern and central China. It was first domesticated around the 11th century BC in China (Hymowitz 1970; Hymowitz and Newell 1981). However, until the late 17th century it was not introduced to the North America. In 1765, a seaman named Samuel Bowen first introduced soybean to the British colony of Georgia in North America (Hymowitz and Newell 1981). In 1904, George Washington Carver, an American chemist, discovered that soybeans are an extremely valuable source of protein and soil. Furthermore, he realized that this crop can benefit soil quality (Hyomowitz and Shurtleff 2005). Today, this annual herbaceous legume crop is a high value crop that provides for more than half of the world's oilseed and protein needs (Singh and Shivakumar 2010).

Soybean production

Processed soybeans are the world's largest source of animal feed protein and the second largest source of vegetable oil (USDA-NASS 2020). Soybean is also used to produce biodiesel

and other industrial products (Kumar et al. 2002). More than 86% of the world's soybean production occurs in North and South America (Chang et al. 2015; ASA 2019). Among the soybean producing nations of the world, the U.S. was the leading producer of soybeans during 2018 (USDA-NASS 2020). However, Brazil overtook the U.S. during 2019 as the leading producer of soybeans. In 2019, Brazil contributed 37% of the world's soybean production, followed by the U.S. with 28%, Argentina with 16%, China with 5%, and Paraguay with 3% (ASA 2019). In the U.S., North Dakota is the ninth largest soybean producer. In North Dakota, soybean is one of most widely planted crops with a value of production over US$2 billion. A majority of this production occurs in the eastern and southeastern counties of the State. In fact, during 2018, Cass and Stutsman county were the second and third highest soybean producing counties in the country (USDA-NASS 2020).

Similar to corn production history in North Dakota, soybean production has increased almost dramatically over the past few years in North Dakota (USDA-NASS 2020). With the increased production, yield limiting factors prevalent in North Dakota were predicted to increase as well (Bradley 2008; Ransom 2010). There are several biotic and abiotic yield limiting factors that can adversely affect soybean yield and seed quality. Such abiotic factors include, water stress, low temperature salt toxicity, and nutrient deficiency whereas the biotic factors include, insect pests, weeds, soil-borne pathogens and foliar pathogens of soybeans (Hartman et al. 2011).

Soybean diseases

According to Allen et al. (2017), the top 10 most destructive diseases of soybeans in northern U.S. and Ontario, Canada, in 2014 were soybean cyst nematodes (SCN), seedling diseases, sudden death syndrome, *Sclerotinia* stem rot, *Phytophthora* root and stem rot, *Septoria* brown spot, charcoal rot, brown stem rot, pod and stem blight. Among these diseases, soybean

cyst nematodes alone caused the greatest soybean yield reduction in northern U.S. and Ontario, Canada, during 2014; causing almost 50% more yield reduction than the second most destructive soybean disease which were seedling diseases. Moreover, SCN caused greatest yield loss in soybean production during 2010, 2011, 2012, and 2013 growing seasons as well (Allen et al. 2017). Furthermore, Allen et al. (2017), reported that other plant-parasitic nematodes such as root-lesion nematodes and root-knot nematodes caused the second most destructive nematode diseases of soybean in the southern U.S.

Soybean cyst nematodes

Soybean cyst nematodes, *Heterodera glycines* (Ichinohe 1955), was first discovered in northeast China (Li et al. 2011). In the Unites States, this sedentary endoparasitic nematodes (Niblack et al. 2006) was first discovered in 1954 in North Carolina (Riggs 1977; Winstead et al. 1955). Since then it has spread to almost all the soybean producing states of the country (Tylka and Marett 2017). In North Dakota, SCN was first detected in Richland County during 2003 (Bradley et al. 2004), and by 2017, this nematode has spread to almost all the major soybean producing counties of the state (Tylka and Marett 2017). Thus, SCN has become one of the major threats to soybean production in North Dakota.

The life cycle of soybean cyst nematodes has three main stages, which include egg, juvenile, and adult females (cyst) or males. Inside the egg, the single cell to multicellular embryo develops and differentiates into a first stage juvenile (J1). The J1 then develops and molts into second stage juvenile (J2) as it hatches from the egg (Niblack 2005). The J2 is the infective stage of SCN, that migrates to root tissue following the chemical gradients created by root exudates. The J2 then penetrates root elongation zone and root tips by using their hollow stylet to release cell wall degrading enzymes such as cellulases (Papademetriou and Bone 1983; Smant et al.

1998). After penetration, they migrate towards the vascular tissue intracellularly, while causing destruction through necrosis, to find a suitable feeding site. (Johnson et al. 1993; Riggs and Wrather 1992). Once they find a suitable feeding site, the juvenile releases effector proteins that can mimic endogenous plant cell signals that causes surrounding cells to undergo physiological changes and become a nutrient sink (Kandoth et al. 2011). Thus, forming a permanent feeding site called a syncytium (Johnson et al. 1993).

During syncytium formation, syncytial cell walls start to degrade and they fuse together establishing cytoplasmic continuum (Mitchum et al. 2013). The nematode then becomes sedentary as they start to swell up and undergo three more molting stages that includes J2 to J3, J3 to J4, and J4 to Adult. However, as the J3 juvenile differentiates into a male, it starts to become vermiform again. At the adult stage the male SCN is vermiform and can leave the root tissue 10 to 15 days after infection by J2. Whereas the females, continue to swell up into the characteristic lemon shape as they reach adulthood (Lauritis et al. 1983). As the females swells up the posterior end of the nematode burst out of the root tissue as the nematode ruptures the root epidermis (Raski 1950). The females then secrete pheromones that attracts the males and results in fertilization of eggs. A single female can produce hundreds of eggs inside its body; while some are released outside in a gelatinous matrix, others remain inside the female body (Sipes et al. 1992). After fertilization, the SCN females dies and the body eventually senescence and tans to become a protective hard shell called the cyst, that provides protection against desiccation and other predators to the eggs (Niblack et al. 2006). The SCN-eggs can survive inside the cyst for almost a decade (Inagaki and Tsutsumi 1971). In general, SCN can complete its life cycle in three to four weeks, thus multiple generations can occur in a single growing season (Niblack et al. 2006)

Symptoms caused by SCN often goes unnoticed as they mimic symptoms caused by other bacterial diseases, fungal diseases, herbicidal injury, drought stress, and/or nutrient deficiency. Moreover, upto 30% yield loss can occur in soybean fields due to SCN without any above-ground visual symptoms (Niblack 2005). However, yellowing and stunting can be caused by SCN in fields with high population densities. Furthermore, when plant roots are taken out of the soil and examined, adult lemon shaped white females can be visible to naked eye (Davis and Tylka 2000).

Management of SCN includes several different tactics such as crop rotation, cover crops, host resistance, chemical control, biological control. However, host resistance and crop rotation are the most commonly used strategy against SCN due to their effectiveness in controlling SCN population and their minimal environmental impact (Niblack et al. 2005; Oyekanmi and Fawole 2010). Compared to other plant-parasitic nematodes such as root-lesion nematodes and root-knot nematodes, SCN have a narrow host range. Other than soybeans, only few other leguminous crops and some weeds are parasitized by SCN (Creech et al. 2007; Johnson et al. 2008; Poromarto et al. 2011; Poromarto et al. 2015). Thus, Crop rotation with non-host crops such as corn and wheat can be effective in reducing SCN population densities in infested fields (Chen et al. 2001).

Several resistance genes that confer resistance to SCN has been identified from different soybean accessions in previous literature. They include the recessive genes designates as *rhg1*, *rhg2*, and *rhg3* (Caldwell 1960) and the dominant genes designated as *Rhg4* and *Rhg5* (Matson and Williams 1965; Rao-Arelli 1994). Quantitative trait loci (QTL) mapping studies revealed that genetic regions of *rhg1* and *Rhg4* contributes most of the SCN resistance in soybean cultivars (Concibido et al. 2004; Meksem et al. 2001). Furthermore, different resistance sources

have different *rhg1* allele and about 90% of the resistance sources in the U.S. use the *rhg1-b* allele to confer resistance against SCN (Brucker et al. 2005; Cook et al. 2012; Tylka and Mullaney 2018). The *rhg1-b* allele is derived from the plant introduction line PI 88788 (Tylka and Mullaney 2018). However, *rhg1-b* mediated resistance is not only dependent on the presence or absence of the gene, but also by copy number variation (Cook et al. 2012). On the other hand, the dominant *Rhg4* gene is required for complete resistance in some soybean cultivars (Brucker et al. 2005).

At present, SCN resistance in soybean is grouped into two types, which includes PI 88788-type resistance and Peking-type resistance. The PI 88788-type resistance only requires the *rhg1-b* allele to be functional; whereas, the Peking-type resistance requires both *rhg1-a* and *Rhg4* alleles to be functional (Liu et al. 2017; Meksem et al. 2001; Mitchum 2016). Moreover, the Peking type resistance convers a rapid localized hypersensitive response to SCN infection; whereas, PI-88788 provides a more prolonged response that affects the SCN third and fourth stage juveniles (Cooper et al. 2008; Kim and Riggs 1992; Klink et al. 2011).

On the pathogen side, SCN field populations are known to be genetically diverse. Thus, new virulent forms can develop when same resistance source is used continuously (Colgrove and Niblack 2008). Currently, virulence phenotypes of SCN is characterized by a standardized classification scheme referred to as ‘HG type’ tests (Niblack et al. 2002). HG refers to *H. glycines* and the type refers to seven different plant introductions with various forms of resistance. The HG type tests evaluates the reproductive potential of SCN populations on the seven different indicator lines compared to a susceptible check, which is referred to as female index that is expressed as a percentage (Howland et al. 2018; Niblack et al. 2002).

The seven plant Introductions suggested by Niblack et al. (2002) were PI 548402 ('Peking', indicator #1), PI 88788 (#2), PI 90763 (#3), PI 437654 (#4), PI 209332 (#5), PI 89772 (#6), and PI 548316 ('Cloud', #7). Among the seven indicator lines, PI 88788 (#2) is the most commonly used source of SCN resistance in North America. Additionally, Peking-type resistance derived from PI 548402 (#1) is the second most commonly used source of resistance. On the other hand, very few of the commercially available cultivars derive resistance from other plant introduction lines (McCarville et al. 2017; Tylka and Mullaney 2018).

In North Dakota the SCN virulent type that has been reported thus far is HG type 0, which is considered the least virulent type of SCN and does not attack any of the seven indicator lines. However, there has been several recent research reports of commonly used resistance sources such as PI 88788 and Peking (PI 548402) being overcome by SCN populations from other major soybean producing states in the Midwest, which includes Iowa, Kansas, Minnesota, Missouri, Nebraska, South Dakota and Wisconsin (Acharya et al. 2016; Broderick 2016; Chen et al. 2010; Howland et al. 2018; MacGudwin 2012; McCarville et al. 2017; Rzodkiewics 2010).

Root-lesion nematodes

Root-lesion nematodes, *Pratylenchus* spp., are one of the more destructive groups of plant-parasitic nematodes worldwide. They are ranked as the third most important group of plant-parasitic nematodes after root-knot nematodes and cyst nematodes (Jones et al. 2013). Like SCN and root-knot nematodes, root-lesion nematode can penetrate host root tissue; thus, they are endoparasitic in nature. However, unlike SCN and root-knot nematodes, root-lesion nematodes remain vermiform after hatching throughout their remaining life stages. Thus, they retain the ability to move out of the host tissue and parasitize another healthier section of the host tissue,

when there is a lack of food source or high competition in one section of the root (Castillo and Volvas 2007). Root-lesion nematodes are therefore referred to as migratory endoparasites.

The life cycle of root-lesion nematodes includes an egg stage, 4 juvenile stages, and an adult stage. The first molt of root-lesion nematodes occurs within the egg as the first stage juvenile (J1) molts into the second stage juvenile (J2). The J2 then hatches from the egg usually one week after deposition and then molts into J3, then to J4, and then to the adult stage, becoming either female or male. Generally, *Pratylenchus* spp. takes around 30 to 40 days from hatching to reach adult stage. Males are common in some species of *Pratylenchus*; whereas, absent or rare in other species (Duncans and Moens 2013). When the males are present, reproduction occurs sexually; however, when they are absent reproduction can occur parthenogenically (Orlando et al. 2020). Moreover, members of *Pratylenchus* spp. can reproduce sexually and/or asexually depending on the species of root-lesion nematodes, environmental conditions, and availability of nutritional sources. Hence, they are able to adapt to diverse environmental conditions (Agrios 2005; Castillo and Volvas 2007). Their ability to complete their entire life cycle inside root tissue helps them thrive in adverse environmental conditions such as semiarid and temperate growing regions (Elhady et al. 2019; May et al. 2016; Vanstone 1998).

Root-lesion nematodes are polyphagous with a wide host range. Members of this genus can parasitize and reproduce on over 400 species of host plants (Davis and MaGudwin 2000), which includes almost all of the most economically important crops of the world, such as soybean, corn, potato, rice, wheat, vegetables, and fruits (Castillo and Volvas 2007; Davis and MacGudwin 2000; USDA-NASS 2020).

Above ground symptoms caused by root-lesion nematodes in these host plants can be obscure due to the symptoms being similar to other bacterial diseases, fungal diseases, herbicidal injury, drought stress, and/or nutrient deficiency. However, in fields with high population density yellow oval to round patches can be observed as a result of nematode infestation (Davis and MacGudwin 2000). Below ground, the hallmark lesion symptom of root-lesion nematodes can be seen in roots of host plants. As root-lesion nematodes penetrate and migrate inside the root tissue, it uses mechanical movement of its stylet and enzyme secretions to degrade cell walls and feed on cytoplasm. As a result, brown lesions form along the point of entry and migration zone (Zunke 1990). Such lesions typically lead to necrotic areas that results in reduction of root growth. Reduced root growth can cause poor water and nutrient uptake by the plant and result in stunting and leaf chlorosis of host plants (Castillo and Volvas 2007; Duncans and Moens 2013).

Annual economic losses caused by *Pratylenchus* spp. was estimated to be US$51 million on wheat in the Pacific Northwest region of the United States alone (Smiley 2010). In Australia, yield losses due to *Pratylenchus* spp. was reported to be as high as 85% in some wheat fields (Nicol et al. 2003). In Norway, *P. penetrans* were reported to reduce potato yield by 50% in infested fields (Holgado et al. 2009). In Ghana, *P coffeae* was reported to cause 60% yield loss in plantain production (Green et al. 2004). In India, *P. indicus* is reported to cause yield losses of up to 28% in rice fields (Prasad et al. 1987). In soybean fields of Brazil, *P. brachyurus* was reported to cause 21% yield reduction (Lima et al. 2015). In Germany, *P. crenatus*, *P. neglectus*, and *P. penetrans* were reported to be the dominant taxa of plant-parasitic nematodes in soybean fields (Elhady et al. 2019). Thus, this nematode genus is present in almost all the major crop producing regions of the world, causing significant economic losses to growers.

Management of root-lesion nematodes is difficult since tools available for management of these nematodes are limited (May et al. 2016). Management strategies such as crop rotation can be challenging due to the broad host range of these plant-parasitic nematodes. Additionally, host range of each species within the genus can vary with the species; thus, choice of crops available for rotation can vary significantly depending on the species (Castillo and Volvas 2007; Schmitt and Barker 1981). Host resistance has been reported to effectively control root-lesion nematode populations (Castillo and Volvas 2007; Schmitt and Barker 1981; Thompson et al. 2020). However, cultivars and varieties vary in their ability to suppress root-lesion nematode populations. Moreover, cultivars and varieties that are resistant to one species of root-lesion nematodes are not necessarily resistant to all species of root-lesion nematodes (Schmitt and Barker 1981; Sheedy et al. 2008; Smiley et al. 2014; Thompson et al. 2008). Chemical nematicides such as Aldicarb (Temik 15) have shown efficacy against root-lesion nematodes in research studies (Smiley et al. 2014). However, many of these fumigants and nematicides are removed or being phased out of the market due to their negative environmental impact and human health risk (Barker and Koenning 1998). Moreover, cost of using such nematicides to manage nematode populations in large-scale crop production can be greater than the economic benefits (Lima et al. 2015).

The North Dakota State University Nematology Group performed soil surveys of different soybean fields during 2015 and 2016 to determine the prevalence and distribution of plant-parasitic nematodes in North Dakota soybean fields (Yan et al. 2017). Six soil samples were collected from a soybean field in Walcott, Richland County, ND during the surveys. After extracting nematodes from these samples, it became evident that root-lesion nematodes were present in all of these samples with a population density ranging from 125 to 2,000 per kg of soil.

Morphological measurement of adult males and females as well as DNA sequencing of two genomic regions revealed that this nematode differs from other known species of root-lesion nematodes in both morphology and DNA sequences, allowing us to conclude that this is a new species of root-lesion nematode that has never been reported in previous literature (Yan et al. 2017). A preliminary greenhouse study was conducted to show that soybean is a host of this new species, (Yan et al. 2017). In this study, naturally infested soil samples were planted with the local soybean cultivar Barnes [*Glycine max* (L.) Merr. cv. Barnes], which is highly susceptible to soybean cyst nematode (Poromarto et al. 2015). The postharvest population density of the new species of root-lesion nematodes increased more than five-fold compared to the preplant population density (Yan et al. 2017), indicating that the new species of root-lesion nematodes can parasitize and reproduces well on the soybean cultivar Barnes.

Justification of study

Corn and soybean are two important crops that help millions of people achieve food security and better livelihood (Hartman et al. 2011). In North Dakota, value of corn production was worth more than US$1 billion in 2018, whereas, value of soybean production was closer to US$2 billion in 2018 (USDA-NASS 2020). Soil-borne pathogens such as plant-parasitic nematode (PPN) have been reported to cause significant yield losses in both soybean and corn production in the U.S. (Allen et al. 2017; Mueller et al. 2016). Moreover, yield losses caused by PPN significantly varied with the species of PPN present and their population densities (Koenning et al. 1999). On the other hand, environmental variables such as soil edaphic factors, rainfall, and temperature can influence the community composition of PPN and affect their survival and pathogenicity (Chen et al. 2012; Edwards et al. 1988). However, prior to this study

there was little to no information available on PPN prevalence and abundance in North Dakota corn fields.

Soybean cyst nematodes (SCN; *Heterodera glycines*) continues to be the greatest threat to soybean production in the U.S. (Allen et al. 2017). Management of SCN can be difficult due to its persistence in infested fields for a long period of time. Nonetheless, crop rotation and host resistance are effective management strategies against SCN (Niblack 2005). However, continuous use of the same resistance sources can lead to SCN populations overcoming that resistance source (Brucker et al. 2005; Mitchum 2016). Hence, monitoring virulence phenotypes of SCN is of paramount importance for better management of this soil-borne pathogen.

Root-lesion nematodes (RLN; *Pratylenchus* spp.) are considered the third most destructive group of plant-parasitic nematodes after SCN and root-knot nematodes (Jones et al. 2013). A new species of root-lesion nematodes was discovered recently from a soybean field in Walcott, Richland County, North Dakota (Yan et al. 2017). Prior to this study, the only way to identify this new RLN species was through morphological measurements and/or DNA sequencing. Morphological measurements of a large number of specimens can be tedious and time consuming even for an experienced nematologists. Similarly, DNA sequencing can be expensive, time consuming, and may require samples to be sent to sequencing laboratories or companies. On the other hand, molecular techniques such as species-specific PCR and real-time PCR can be effective and efficient identification methods for accurate detection of PPN.Yan et al. (2017) reported that the new species of root-lesion nematodes reproduced well on the soybean cultivar Barnes. However, there is no information available on the resistance levels of other soybean cultivars to the new species of root-lesion nematodes.

Hence, the objectives of this study were to:

1. Determine incidence, abundance and distribution of vermiform plant-parasitic nematodes in corn fields and investigate whether soil edaphic factors and climatic factors influence nematode populations in ND corn fields.
2. Investigate the virulence diversity of SCN in North Dakota to determine if SCN populations from North Dakota overcome the resistance provided by common resistance sources such as PI 878788 and Peking.
3. Develop conventional and real-time PCR assays with species-specific primers for rapid and sensitive detection of the new species of *Pratylenchus*, and to evaluate the capability of the assays to distinguish this new species from other commonly found *Pratylenchus* spp. in ND soybean fields.
4. Evaluate resistance levels of twenty soybean cultivars to the new species of root-lesion nematodes detected in Walcott, Richland County, ND and to determine the habitat preference of the new root-lesion nematode species by comparing the numbers of nematodes present in roots and in soil for each cultivar.

Results of these studies will provide critical information on plant-parasitic nematodes in corn and soybean fields of North Dakota. Such information will help in developing management strategies against these soil borne pathogens of corn and soybean.

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CHAPTER 2: OCCURRENCE OF VERMIFORM PLANT-PARASITIC NEMATODES IN NORTH DAKOTA CORN FIELDS AND IMPACT OF ENVIRONMENTAL AND SOIL FACTORS[1]

Abstract

Plant-parasitic nematodes (PPN) are an important group of pathogens that can negatively affect corn production. In this study, nematode surveys were conducted to assess the incidence and abundance of PPN in corn fields of North Dakota. Samples were collected from 300 corn fields across 20 counties during 2015 and 2016. Seventy-two percent of the fields were positive for PPN. The major genera of PPN identified were *Helicotylenchus* (incidence: 52%; mean density: 1,513 nematodes per kg soil; greatest density: 16,910 nematode per kg of soil), *Tylenchorhynchus* (37%; 687; 9,500), *Paratylenchus* (31%; 1,484; 7,800), *Pratylenchus* (20%; 399; 2,125), *Heterodera* (9%; 555; 4,500), *Xiphinema* (8%; 330; 900), *Hoplolaimus* (3%; 294; 500), and *Paratrichodorus* (1%; 124; 200). Neighboring counties had greater similarity in PPN diversity than counties that are further apart, with western, northeastern and southeastern counties forming clusters of similar nematode occurrence and diversity. Canonical correspondence analysis was conducted to determine the association between incidence and abundance of these PPN populations and various soil edaphic and climatic factors. The analysis revealed that *Hoplolaimus*, *Paratrichodorus*, *Paratylenchus* and *Pratylenchus* were positively correlated with soil temperature, rainfall, and percent sand while *Helicotylenchus*,

[1] The material in this chapter was co-authored by Intiaz Amin Chowdhury (Department of Plant Pathology, North Dakota State University), Dr. Guiping Yan (Department of Plant Pathology, North Dakota State University) and Dr. Andrew Friskop (Department of Plant Pathology, North Dakota State University). Intiaz Amin Chowdhury had primary responsibility for collecting soil samples from the field, sample processing, and data analysis. Intiaz Amin Chowdhury was the primary developer of the conclusions that are advanced here. Intiaz Amin Chowdhury also drafted and revised all versions of this chapter. Dr. Andrew Friskop helped by checking data analysis, data interpretation and served as a proofreader. Dr. Guiping Yan served as principle investigator and corresponding author and evaluated the statistical analysis conducted by Intiaz Amin Chowdhury. This paper has been published in Canadian Journal of Plant Pathology.

Tylenchorhynchus and *Xiphinema* were positively correlated with pH, percent clay, and percent organic matter. This is the first report of an extensive investigation of PPN communities in North Dakota corn fields. According to our results, *Helicotylenchus*, *Paratylenchus*, *Pratylenchus* and *Tylenchorhynchus* nematodes have a potential to impact corn yield. Research findings will be useful in future field experiments to determine the impact of PPN on corn production in the Northern Great Plains.

Keywords: Corn, plant-parasitic nematode community, *Helicotylenchus*, *Paratylenchus*, *Hoplolaimus*, *Paratrichodorus*, *Pratylenchus*, *Tylenchorhynchus*, *Xiphinema*, canonical correspondence analysis, soil factor, climatic factor.

Introduction

Corn (*Zea mays* L.) has become one of the most produced staple crops in the world, and the United States (US) is the world's leading producer of this crop. In the US alone, the crop value for corn is 51.45 billion US dollars (USDA-NASS 2018). In the state of North Dakota (ND), corn acreage has nearly doubled over the last three decades, due to the recent increase in demand for corn as a source of ethanol and livestock feed (NDCC 2018; Ransom 2010). The introduction of early maturing corn hybrids and the recent weather pattern being more favorable are also contributing to increased corn acreage in the state (Ransom 2010). During 2017, more than 3 million acres were planted with corn in ND, accounting for more than 1 billion dollars in crop value (USDA-NASS 2018). Most of the acreage of corn is located in the east central and southeastern areas of the state (Calles-Torrez et al. 2018). With the recent increase in North Dakota's corn production, the various disease related limiting factors of corn production are predicted to increase as well (Ransom 2010), and plant-parasitic nematodes are a group of pathogens that can limit corn production (Tylka et al. 2011; Windham and Edwards 1999).

Plant-parasitic nematodes are one of the greatest threats to crop production worldwide (Sasser and Freckman 1987). In the US alone, they were reported to cause annual crop losses of more than 8 billion dollars (Smiley 2005). These soil borne parasites can also cause significant economic losses for corn growers, but losses often go unnoticed or are attributed to other causes (Norton 1983; Tylka et al. 2011). Plant-parasitic nematodes were reported to cause yield losses as high as 20% in corn fields in the southern US (Koenning et al. 1999). In the mid-western state of Iowa during 1987, statewide corn yield losses were predicted to increase from 4.2 to 5.5%, if nematicides are not used to control these pathogens (SON Crop Loss Assessment Committee 1987). In the neighboring state of South Dakota, *Pratylenchus hexincisus* was estimated to cause yield losses of 9.5 bushels per acre on dry land corn, while *P. scribneri* was estimated to cause yield losses of 4 bushels per acre in irrigated corn fields (Smolik and Evenson 1987). A more recent study reported that plant-parasitic nematodes caused more than 294 million bushels of corn loss in the US and Ontario, Canada during the years of 2013 to 2015 (Mueller et al. 2016).

Plant-parasitic nematodes' potential to cause economic loss can vary according to the species present and their population density (Koenning et al. 1999). Over 60 species of plant-parasitic nematodes are associated with corn in North America (Norton 1983). Among these, species of *Belonolaimus, Criconemella, Helicotylenchus, Hoplolaimus, Longidorus, Meloidogyne, Paratylenchus, Pratylenchus, Paratrichodorus Tylenchorhynchus,* and *Xiphinema* are most common in corn fields. However, only three to seven of these genera were found simultaneously in soil communities of individual corn fields (Koenning et al. 1999; Norton 1983; Tylka et al. 2011). Some of these genera have greater frequency and abundance than others. In corn fields of Iowa, *Helicotylenchus, Pratylenchus* and *Xiphinema* were reported the most prevalent (Tylka et al. 2011). Similarly, in Illinois corn fields, *Helicotylenchus* was also the most

prevalent genus with *Pratylenchus* being the second most frequent in these fields (Niblack 2009; Simon 2015). In corn fields of Ohio, *Paratylenchus, Helicotylenchus* and *Pratylenchus* were the most prevalent genera and populations were considered to be of moderate risk (Simon 2015). Tylka et al. (2011) also demonstrated that a few of these genera such as *Xiphinema* and *Longidorous,* exceeded damage thresholds previously established by the Plant Nematologist Don C. Norton at Iowa State University.

The pathogenicity, occurrence, frequency, distribution, and species composition of plant-parasitic nematodes can vary considerably across Midwestern states. This is often attributed to variations in environmental conditions, soil properties, pathogen virulence, and host genotypes (Alby et al. 1983; Chen et al. 2012; Edwards et al. 1988; Kandel et al. 2013; Norton 1983; Simon 2015). Among the variables that influence soil nematode communities, soil properties (including texture) had a greater influence on soil nematode communities than farming practices (Neher 1999; Yeates 1984). Soil texture directly affects nematode reproduction, migration and penetration capacity (Griffin 1996; Koenning et al. 1996). In addition to these variables, climatic factors such as temperature and rainfall had a direct or indirect effect on plant-parasitic nematode community composition and their ability to cause diseases (Fleming et al. 2016; Kandel et al. 2013; Karuri et al. 2017). Temperature can stimulate nematode metabolic activity, feeding rate and root penetration, whereas soil moisture can influence nematode infection rate (Fleming et al. 2016; Karuri et al. 2017). With North Dakota being the second coldest state in the US, it is important to investigate the population composition and impact of plant-parasitic nematodes in this environment.

There is also little to no information available on plant-parasitic nematodes in North Dakota corn fields. Proper assessment of plant-parasitic nematodes distribution throughout the

state as well as determining their population densities are prerequisites of efficient management planning. Hence, the objectives of this study were to 1) determine incidence, abundance and distribution of vermiform plant-parasitic nematodes in corn fields and 2) investigate whether soil edaphic factors such as pH, organic matter, sand, silt, and clay as well as climatic factors such as temperature and rainfall influence nematode populations in ND corn fields. To our knowledge, this is the first comprehensive study that describes the plant-parasitic nematodes in soil communities of corn fields in North Dakota.

Materials and methods

Soil sample collection and processing

Field surveys were conducted in 2015 and 2016 by collecting soil samples from North Dakota (ND) corn fields during the cropping season (May to August). Sampling was conducted in a zig-zag pattern starting 76 meters (100 paces) away from the field border/ field entrance closest to the motorway and walking 38 meters (50 paces) between each sampling point changing direction each time. One soil core was collected from each sampling point using 2.5 cm diameter soil probes (Gempler's model L Sampler, Madison, WI, USA) from depths of 1 to 30 cm below soil surface around the plant root system. Soil cores were taken from approximately 20 to 25 sampling points in each field, covering an area of 1 to 2 ha. The soil samples from each field were combined in a plastic bag to represent a field composite sample (approximately 1-2 kg of soil). The samples were stored in a cooler during transport and then kept in a cold storage room at 4 °C until processing.

A total of 300 randomly selected corn fields (200 and 100 in 2015 and 2016, respectively) were surveyed across 20 major corn producing counties (Barnes, Cass, Dickey, Foster, Grand Forks, Griggs, LaMaoure, Logan, McIntosh, Mountrail, McHenry, Nelson,

Ramsey, Richland, Sargent, Stutsman, Traill, Walsh, Ward and Wells) from all seven North Dakota Corn Council (NDCC) production districts (Fig. 2.1A). The North Dakota Corn Utilization Council (NDCC 2018) divides the state (includes all the counties) into seven districts based on annual production of the crop. The least annual corn production district encompasses the most counties, while the largest district includes a single county (USDA-NASS 2018). The number of samples collected from each district of North Dakota was determined based on the districts' annual corn production. Fifty-five to 65 samples were selected from each of the districts (1, 2, 3, and 4) that had the highest amount of corn acreage. Twenty to 25 samples were collected from each of the remaining three districts with lower corn acreage (Fig. 2.1A).

Geospatial referencing survey sites

Latitude and longitude coordinates were recorded for each field in degrees, minutes and seconds and converted to decimal degrees. Elevation and the nearest city were also recorded for each location. The sampling sites were then plotted into North Dakota county map (Fig. 2.1B) using geographic information system (GIS) techniques with ArcMap Software version 10.5 (Environmental Systems Research Institute, Redlands, CA, USA).

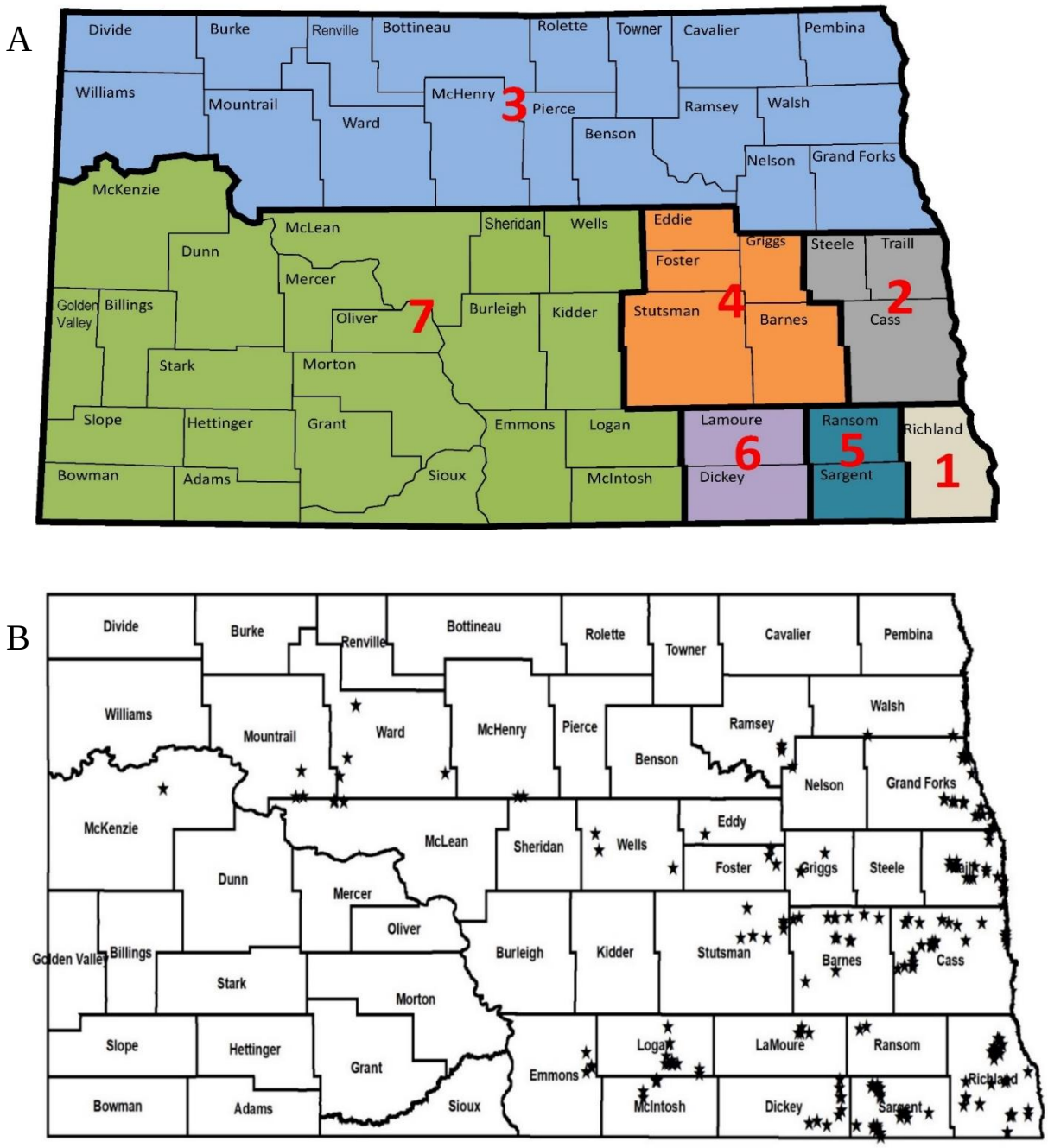


Fig. 2.1. Map of North Dakota counties showing (A) North Dakota corn council districts (B) location of the corn fields surveyed for plant-parasitic nematodes during 2015 and 2016. The numbers with red color in Fig. 2.1A represent North Dakota corn council (NDCC) district numbers 1-7; for example, 1 represents NDCC district 1.

Soil property and weather data collection

The composite soil sample from fields testing positive for plant-parasitic nematodes was thoroughly mixed and a 500 gram subsample was sent to Agvise laboratories (Benson, MN, USA) for soil analysis. Standard laboratory protocols were used to determine soil pH, percentage organic matter, percentage sand, percentage silt, and percentage clay particles of each soil sample. Samples were then classified into textural classes based on the percentage of sand, silt and clay of each sample. Annual rainfall and soil temperature (10 cm below soil surface) data were collected as 10-year averages from nearest North Dakota Agricultural Weather Network (NDAWN, www.ndawn.ndsu.nodak.edu) stations, each of which was within a 20 mile radius of their corresponding field.

Nematode extraction, identification and enumeration

A 200 g subsample from each composite field sample was processed for extracting vermiform nematodes using the standard hand sieving, decanting and sucrose centrifugal-floatation method (Jenkins 1964). The extracted nematodes were collected as a 20-25 ml suspension in a 50 ml nematode suspension vial. Using an inverted compound light microscope (at x 40 magnification) (Focus Precision Instruments, Cavaletti Court Victoria, MN, USA), the nematodes were subsequently identified to genus level based on genus descriptions provided by Fortuner (1988) and Mai et al. (1996). The nematode density of each genus was then reported (nematodes in 200g of soil x 5) as nematode number per kg of soil. The nematode population densities of *Helicotylenchus*, *Paratylenchus*, *Pratylenchus*, and *Xiphinema* for the positive samples were plotted as a histogram containing a reference of their respective economic damage threshold reported from Iowa, Illinois and Ohio specific corn fields (Niblack 2009; Simon 2015; Tylka et al. 2011).

Species identification

Five of the most prevalent and economically important plant-parasitic nematode genera detected in North Dakota corn fields were further identified to species level. To achieve this, a single specimen was chopped in a concave glass containing 10 µl of double-distilled water. Immediately, the suspension containing nematode pieces was pipetted into a 0.5 µl centrifuge tube containing 10 µl of lysis buffer (2 µl of 10 X PCR buffer, 2 µl of proteinase K (600 µg/ ml) and 6 µl of double-distilled water). The centrifuge tubes were then incubated at -20ºC for at least 30 minutes followed by incubation at 65ºC for 1 hour and termination at 95 °C for 10 min. The resulting DNA samples were then stored at -20 ºC (Huang and Yan 2017). DNA were extracted in this manner from up to 15 field samples per genus previously classified based on morphology. After DNA extraction, 4 to 5 biological replicates per sample were used for species-specific polymerase chain reaction (PCR) (Table 2.1). *Hoplolaimus*, *Paratrichodorus* and *Pratylenchus* genera were identified to species level using species-specific PCR. The DNA of the nematode specimens that could not be amplified with the available primers were sent for direct sequencing (Table 2.1).

Table 2.1. Plant-parasitic nematode species, identification method, GenBank accession numbers, E values and percentage homogeneity for plant-parasitic nematodes identified in North Dakota corn fields using molecular methods[a].

Nematode genus	Common name	Identification method	Nematode Species	Deposited accession	Compared accession	E value	Homogeneity
Helicotylenchus	Spiral	Direct sequencing	*Helicotylenchus microlobus*	MH672688	KM506867	0.0	99
Hoplolaimus	Lance	Species-specific PCR	*Hoplolaimus stephanus*	-	-	-	-
Paratylenchus	Pin	Direct sequencing	*Paratylenchus nanus*	MH672687	KY468901	0.0	100
Pratylenchus	Root-lesion	Species-specific PCR	*Pratylenchus scribneri*	-	-	-	-
		Species-specific PCR	*P. neglectus*	-	-	-	-
Paratrichodorus	Stubby root	Species-specific PCR	*Paratrichodorus allius*	-	-	-	-

[a] Deposited accession number is the distinct identifier assigned to the query (DNA) sequence submitted to GenBank and compared accession number refers to the sequence used for comparison. E value is the expected value. Homogeneity refers to percentage of similarity between the query sequence and the comparison sequence, indicating species identity. Homogeneity was determined using sequence BLAST in the NCBI database.

The primers that amplified the *Hoplolaimus* DNA were Hs-1f/Hs-1r (forward/reverse), specific for *Hoplolaimus stephanus* (Ma et al. 2011). *Paratrichodorus* DNA were amplified using PaF11/PaR12 primer set specific for *Paratrichodorus allius* (Huang et al. 2017). Some *Pratylenchus* DNA samples were amplified with Pn-ITS-F2/Pn-ITS-R2 (Yan et al. 2013) specific for *Pratylenchus neglectus*, whereas, others were amplified with PsF7/PsR7 (Huang and Yan 2017) for *P. scribenri*. All of these primers were targeting the ITS region of rDNA. On the other hand, *Helicotylenchus* and *Paratylenchus* genera were identified to species level using the direct sequencing method. To achieve this, ITS region of the rDNA was amplified using forward primer, 5´-ACGAGCCGAGTGATCCACCG-3´, called rDNA1 and reverse primer, 5´-TTGATTACGTTCCCTGCCCTTT-3´, called rDNA2 (Cherry et al. 1997). The D2-D3 expansion region of 28S rRNA was also amplified using forward primer 5´-ACAAGTACCGTGAGGGAAAGTTG-3´, called D2A and reverse primer 5´-TCGGAAGGAACCAGCTACTA-3´, called D3B (Courtright et al. 2000).

The PCR reaction mixture consisted of 2 µl DNA template, 0.8 µl of each of forward and reverse primers (each at 10 µM), 1.2 µl $MgCl_2$, 0.4 µl dNTP, 4.0 µl 5× PCR buffer, and 0.15 U of Taq DNA Polymerase (Promega Corp., Madison, WI, USA). The PCR reaction conditions were: 94ºC for 3 min (initial denaturation), followed by 40 cycles of 94ºC for 45 s (denaturation), 55ºC for 1 min (annealing) and 72ºC for 1 min (extension), and a final extension of 10 min at 72ºC. To confirm amplification, 2 µl of PCR product was mixed with 3 µl of 2X loading dye; the 5 µl mixture was then loaded onto 2% agarose gel for gel electrophoresis at 100 V for 20-25 min. After PCR products were separated, the gel was visualized and photographed under UV light using AlphaImager Gel Documentation System (ProteinSimple Inc., Santa Clara, CA, USA). Once successful PCR amplification was confirmed, the amplified DNA were purified from the

remaining PCR products using E.Z.N.A Cycle Pure Kit (Omega BIO-TEK, Norcross, GA, USA). The purified DNA was then sent for DNA sequencing by GenScript (GenScript, Piscataway, NJ, USA). The DNA sequences were then deposited to the GenBank database. Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology and Information (NCBI) was used to compare the sequences with previously deposited sequences and to identify sequence similarity with other known species (www.ncbi.nlm.nih.gov).

Population data analysis

The incidence (number of samples containing a nematode genus/number of samples collected x 100), mean population density (mean number of nematodes of a genus/kg of soil in positive samples), prominence value [mean density x square root (incidence)] and relative prominence value (prominence value of a nematode genus/sum of prominence values for all nematode genera x 100) of each nematode genus were calculated for the seven corn production (NDCC) districts (Chen et al. 2012; Karuri et al. 2017; Norton 1978), and presented in Table 2.2 and Table 2.3. The population density for each county was also calculated and tested for normality using Shapiro-Wilk test, then log transformed (log X+1) to reduce heterogeneity of variance. To visualize this log transformed density of each genus in sampled counties, a heatmap was generated using heatmp.2 function of gplots package in R statistical software (Development Core Team R 2013).

Table 2.2. Incidence and population density of major plant-parasitic nematode genera in corn fields in corn production districts of North Dakota[a].

PPN Genera	*Helicotylenchus*	*Hoplolaimus*	*Paratylenchus*	*Pratylenchus*	*Paratrichodorus*	*Tylenchorhynchus*	*Xiphinema*
			ND Corn Production District 1				
Incidence[b]	72.3	3.1	16.9	16.9	0.0	30.7	1.5
Density[c]	1,818.4	275.0	1,607.3	569.1	0.0	654.5	300.0
			ND Corn Production District 2				
Incidence[b]	40.4	0.0	31.6	26.3	1.8	33.3	7.0
Density[c]	1,968.0	0.0	1,567.5	306.7	120.0	392.9	171.3
			ND Corn Production District 3				
Incidence[b]	39.7	0.0	32.8	10.3	3.4	31.0	3.4
Density[c]	1,145.9	0.0	2,818.4	370.0	237.5	449.4	145.0
			ND Corn Production District 4				
Incidence[b]	53.7	3.7	38.9	16.7	3.7	42.6	13.0
Density[c]	844.3	150.0	788.6	224.4	137.5	1,389.8	430.0
			ND Corn Production District 5				
Incidence[b]	65.0	25.0	10.0	30.0	10.0	50.0	10.0
Density[c]	1,641.5	360.0	925.0	578.3	125.0	617.5	310.0
			ND Corn Production District 6				
Incidence[b]	42.9	0.0	42.9	39.3	0.0	25.0	7.1
Density[c]	2,322.5	0.0	434.6	367.3	0.0	450.0	205.0
			ND Corn Production District 7				
Incidence[b]	44.4	0.0	55.6	11.1	0.0	61.1	33.3
Density[c]	460.6	0.0	1,495.0	650.0	0.0	466.4	433.3
			All North Dakota Corn Production Districts				
Incidence[b]	51.7	3.0	31.0	20.0	1.3	36.7	8.0
Density[c]	1,512.6	294.4	1,484.1	398.5	123.8	687.2	329.7

[a] *Heterodera glycines* being an obligate parasite of soybeans, has been excluded from this table.
[b] Incidence = (number of samples containing a genus)/(number of samples collected) x 100.
[c] Density (mean population density) = (mean number of nematodes of a genus/kg of soil in positive samples)

Similarity in nematode diversity between geospatial communities

The plant-parasitic nematode communities of each of the NDCC districts and all the counties surveyed were further compared using the Bray and Curtis similarity index [S = (2W/(A+B)], where S can range from 0 (no similarity) to 1 (complete similarity), A is the sum of population densities of all genera in one community, B is the sum of population densities of all

genera in another community, and W is the sum of lower population densities of common genera in both communities (Bray and Curtis 1957; Chen et al. 2012; Norton 1978). These similarity index calculations were performed using the Vegan Package in R statistical software (Development Core Team R 2013; Karuri et al. 2017; Oksanen 2015). The similarity between corn production districts were presented in Table 2.4 and the extreme values of similarity between counties were presented in Table 2.5. To better visualize the similarity index results of the counties surveyed and to determine if regional nematode occurrence patterns exist, hierarchical cluster analysis was also conducted on Bray-Curtis dissimilarity index (1-similarity index) and then plotted as a dendrogram using the 'hclust' function of R (Bray and Curtis 1957; Development Core Team R 2013).

Influence of environmental variables on nematode community composition

After ensuring population data had unimodal distribution using Detrended Correspondence Analysis (Greenacre 2010; Ter Braak 1986), Canonical Correspondence Analysis (CCA) was performed to define and visualize the relationship between plant-parasitic nematode population densities of individual genera and seven environmental variables in R statistical software (Angers et al. 1999; Development Core Team R 2013; Karuri et al. 2017; Oksanen 2015; Ter Braak 1986). This eigenvalue ordination technique integrates ordination and multiple regression techniques, allowing simultaneous analysis of community attributes, such as plant-parasitic nematode genera, to environmental variables (Angers et al. 1999). Variables included soil parameters of pH, percent sand, percent silt, percent clay, and percent organic matter (OM), and environmental variables of rainfall (cm) and soil temperature (°C). As suggested by Ter Braak and Verdonschot (1995), log transformed nematode population data were used to perform CCA analysis. To assess the statistical significance ($P < 0.05$) of the effect

of environmental variables on nematode communities, Monte Carlo permutation tests were performed with 999 unrestricted permutations in R (Development Core Team R 2013; Ter Braak and Verdonschot 1995). This test was also used to determine which environmental variables had the strongest influence on plant-parasitic nematode communities of ND corn fields. Finally, a CCA bi-plot was generated, in which the blue arrows represented the environmental variables, the genera were represented as first 5 letters of their genus name in red, and the black dots represented the field sites.

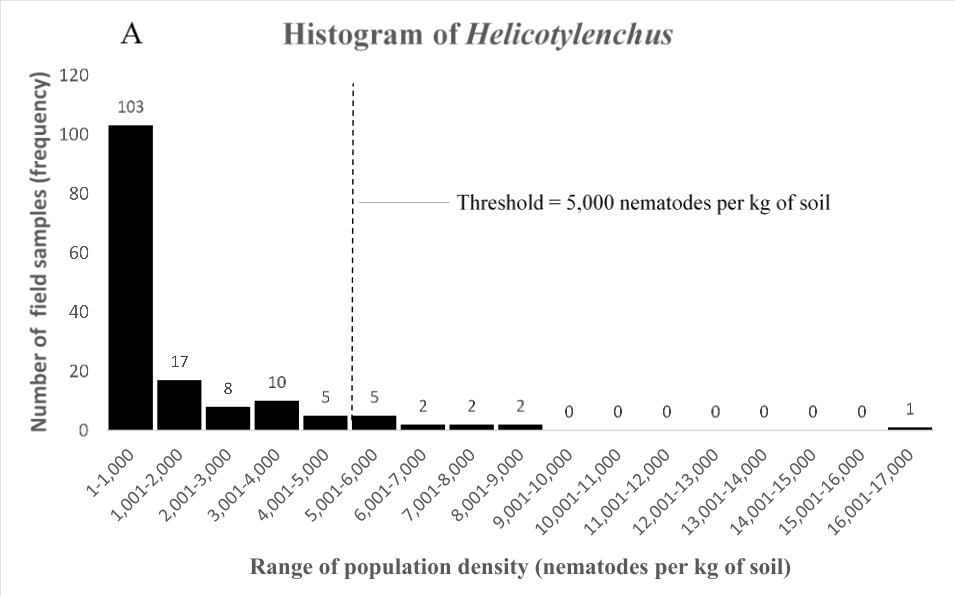
Results

Plant-parasitic nematodes in North Dakota corn fields

During the survey period of 2015 and 2016, eight major genera of plant-parasitic nematodes were detected in soil samples collected from 300 corn fields. These genera include *Helicotylenchus, Hoplolaimus, Paratrichodorus, Paratylenchus*, *Pratylenchus*, *Tylenchorhynchus* and *Xiphinema*. Juveniles of *Heterodera glycines* were also detected in these fields. Due to successful amplification of the ITS region of rDNA, *Pratylenchus* field populations were identified as *P. scribneri* and *P. neglectus* using the species-specific primers PsF7/PsR7 (Huang and Yan 2017) and Pn-ITS-F2/Pn-ITS-R2 (Yan et al. 2013), respectively. Similarly, the species-specific primers Hs-1f/Hs-1r (Ma et al. 2011) and PaF11/PaR12 (Huang et al. 2017) confirmed the species of *Hoplolaimus* and *Paratrichodorus* as *H. stephanus* and *P. allius* (Table 2.1). *Helicotylenchus* and *Paratylenchus* DNA sequence characteristically identified them to their respective species as *H. microlobus* (accession number: MH672688) and *P. nanus* (accession number: MH672687) (Table 2.1).

Incidence and population density of plant-parasitic nematodes

Seventy-two percent of the corn fields surveyed in North Dakota tested positive for plant-parasitic nematodes. In these corn fields, *Helicotylenchus* was identified in 52% of the 300 fields surveyed with a mean population density of 1,513 nematodes/kg of soil in positive samples (Table 2.2). The population densities for this nematode varied from 100 to 16,910 nematodes/kg of soil, with the highest density detected in Dickey County of NDCC District 6 (see Fig. 2.1 to identify which counties belong to each district). However, majority of the samples had *Helicotylenchus* population densities below 9,000 nematodes per kg of soil (Fig. 2.2A). The relative prominence (RP) value, which indicated the contribution of a nematode genus to the plant-parasitic nematode community, was also high (38%) for this genus (Table 2.3).



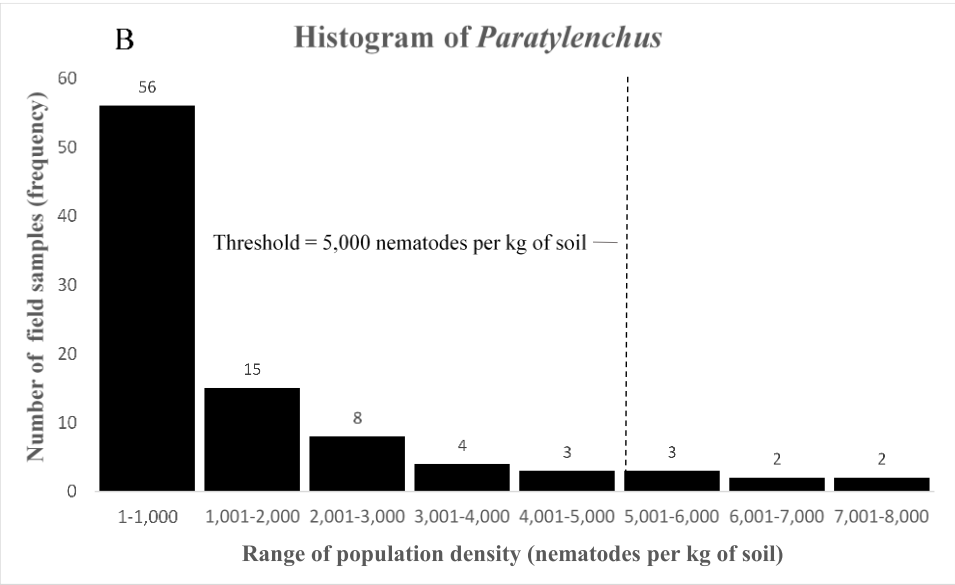


Fig. 2.2. Population density distribution of commonly detected plant-parasitic nematode genera, *Helicotylenchus* (A), *Paratylenchus* (B) and *Pratylenchus* (C), in North Dakota corn field samples presented as a histogram, with a reference of their corresponding economic damage threshold reported from Iowa, Illinois and Ohio corn fields (Niblack 2009; Simon 2015; Tylka et al. 2011). For each chart, the X-axis represents the range of population density for the respective nematode genus, and the Y-axis represents the number of field samples containing their corresponding nematode genus that had a population density within the range, with the exact number displayed above their corresponding bar. The potential damage threshold for each genus is represented by the dotted vertical line in their corresponding chart, and the field samples to the right of the dotted line have their corresponding nematode genus' population densities that are greater than the threshold.

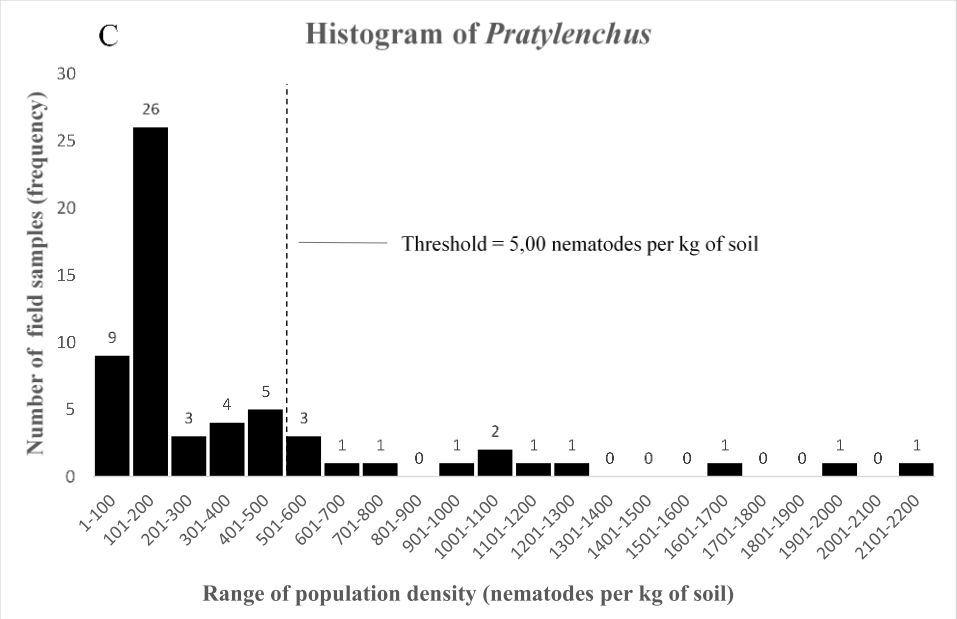


Fig. 2.2. Population density distribution of commonly detected plant-parasitic nematode genera, *Helicotylenchus* (A), *Paratylenchus* (B) and *Pratylenchus* (C), in North Dakota corn field samples presented as a histogram, with a reference of their corresponding economic damage threshold reported from Iowa, Illinois and Ohio corn fields (Niblack 2009; Simon 2015; Tylka et al. 2011) (continued). For each chart, the X-axis represents the range of population density for the respective nematode genus, and the Y-axis represents the number of field samples containing their corresponding nematode genus that had a population density within the range, with the exact number displayed above their corresponding bar. The potential damage threshold for each genus is represented by the dotted vertical line in their corresponding chart, and the field samples to the right of the dotted line have their corresponding nematode genus' population densities that are greater than the threshold.

Table 2.3. Prominence of major plant-parasitic nematode genera in corn fields in corn production districts of North Dakota.

PPN Genera	*Helicotylenchus*	*Hoplolaimus*	*Paratylenchus*	*Pratylenchus*	*Paratrichodorus*	*Tylenchorhynchus*	*Xiphinema*
ND Corn Production District 1							
PV [a]	1,546.3	48.2	661.2	234.1	0.0	363.1	37.2
RP [b]	48.8	1.5	20.9	7.4	0.0	11.5	1.2
ND Corn Production District 2							
PV [a]	1,250.1	0.0	880.9	157.3	15.9	226.8	45.4
RP [b]	45.3	0.0	31.9	5.7	0.6	8.2	1.6
ND Corn Production District 3							
PV [a]	721.6	0.0	1,613.1	119.0	44.1	250.4	26.9
RP [b]	25.8	0.0	57.6	4.2	1.6	8.9	1.0
ND Corn Production District 4							
PV [a]	618.7	28.9	491.8	91.6	26.5	907.0	154.8
RP [b]	26.7	1.2	21.2	4.0	1.1	39.1	6.7
ND Corn Production District 5							
PV [a]	1,323.5	180.0	292.5	316.8	39.5	436.6	98.0
RP [b]	47.9	6.5	10.6	11.5	1.4	15.8	3.5
ND Corn Production District 6							
PV [a]	1,520.4	0.0	284.5	230.2	0.0	225.0	54.8
RP [b]	65.7	0.0	12.3	9.9	0.0	9.7	2.4
ND Corn Production District 7							
PV [a]	307.1	0.0	1,114.3	216.7	0.0	364.6	250.2
RP [b]	13.6	0.0	49.5	9.6	0.0	16.2	11.1
All North Dakota Corn Production Districts							
PV [a]	1,087.3	51.0	826.3	178.2	14.3	416.1	93.3
RP [b]	38.4	1.8	29.2	6.3	0.5	14.7	3.3

[a] Prominence values (PV) = mean density x square root (incidence)
[b] Relative prominence values (RP) = (prominence value of a nematode genus)/(sum of prominence values for all nematode genera) x 100.

Tylenchorhynchus was detected in 37% of the fields surveyed, with a mean population density of 687 nematodes/ kg of soil (Table 2.2) and a relative prominence value of 15% (Table 3). *Tylenchorhynchus* nematode density in these fields was as high as 9,500 nematodes/ kg of soil, detected in Stutsman County of NDCC District 4 (Fig. 2.1). *Paratylenchus*, on the other hand, was identified in 31% of the samples collected (Table 2.2). Density of this nematode was as high as 7,800 nematodes/kg of soil, detected in Ward County of NDCC district 3, with a mean

population density of 1,484 nematodes/kg of soil (Table 2.2). Greater than 50% of the *Paratylenchus* positive samples had *Paratylenchus* population densities below 1000 nematodes per kg of soil (Fig. 2.2B). Thus, the nematode had a RP value of 29% in corn fields (Table 2.3). *Pratylenchus* was detected in 20% of the fields surveyed, with a mean population density of 399 nematodes/kg of soil. The highest density (2,125 nematodes/kg of soil) for this nematode (Fig. 2.2C) was detected in Sargent County of NDCC district 5 (Fig. 2.1).

Xiphinema, Hoplolaimus and *Paratrichodorus* were detected in less than 8% of the samples collected, at a mean population density of 330, 294 and 124 nematodes/kg of soil, respectively. During our survey, a corn field in Logan County of NDCC District 7 had the highest population density of *Xiphinema* (900 nematodes/kg of soil); whereas, in Sargent County, the highest densities of *Hoplolaimus* (500) and *Paratrichodorus* (200) were detected (Fig. 2.1).

Distribution of plant-parasitic nematodes

During the survey period of 2015 and 2016, all eight genera identified in this survey were detected in NDCC Districts 4 and 5. In Districts 2 and 3 all genera but *Hoplolaimus* were detected. *Paratrichodorus* was the only genus not detected in District 1. In Districts 6 and 7, six genera were detected with *Paratrichodorus* and *Hoplolaimus* being the only genera not detected. The relative prominence values also varied between the districts. The highest RP value for *Helicotylenchus* (65.7%), *Hoplolaimus* (6.5%), *Paratylenchus* (57.6%), *Pratylenchus* (11.5%), *Paratrichodorus* (1.6%), *Tylenchorhynchus* (39.1%), and *Xiphinema* (11.1%) was observed in NDCC Districts 6, 5, 3, 5, 3, 4 and 7, respectively.

When considering the counties, Sargent (number of genera detected: 8), Richland (7), Cass (7), Stutsman (6) and Foster (6) counties had the most diverse communities in the samples

collected (Fig. 2.3). Five out of the eight genera were detected in Dickey, Grand Forks, Logan, McIntosh and Traill counties, whereas four genera were detected in Barnes, Griggs, McHenry, Ward and Wells counties. Less than four genera were detected in Mountrail, Nelson, LaMoure, Ramsey and Walsh counties (Fig. 2.3). *Tylenchorhynchus* and *Paratylenchus* were the most widely distributed genera being detected in all but Nelson and Walsh County, respectively (Fig. 2.3). *Helicotylenchus* was also widely distributed, being detected in all except Griggs and LaMoure counties. However, greater number of counties had high *Helicotylenchus* mean population densities than the number of counties with high mean population densities of *Tylenchorhynchus* and *Paratylenchus* (Fig. 2.3). On the other hand, *Pratylenchus* populations were found in 14 counties, with Sargent, Richland, Stutsman, Traill, Dickey, Logan and McIntosh counties having high mean population densities. Populations of *Xiphinema* were detected in 12 counties, with Logan, Foster, Stutsman and Wells counties having high mean population densities. The least common genera were *Paratrichodorus*, which was found only in Cass, Foster, Griggs and Sargent counties, and *Hoplolaimus*, which was detected in Richland, Sargent and Stutsman counties only (Fig. 2.3).

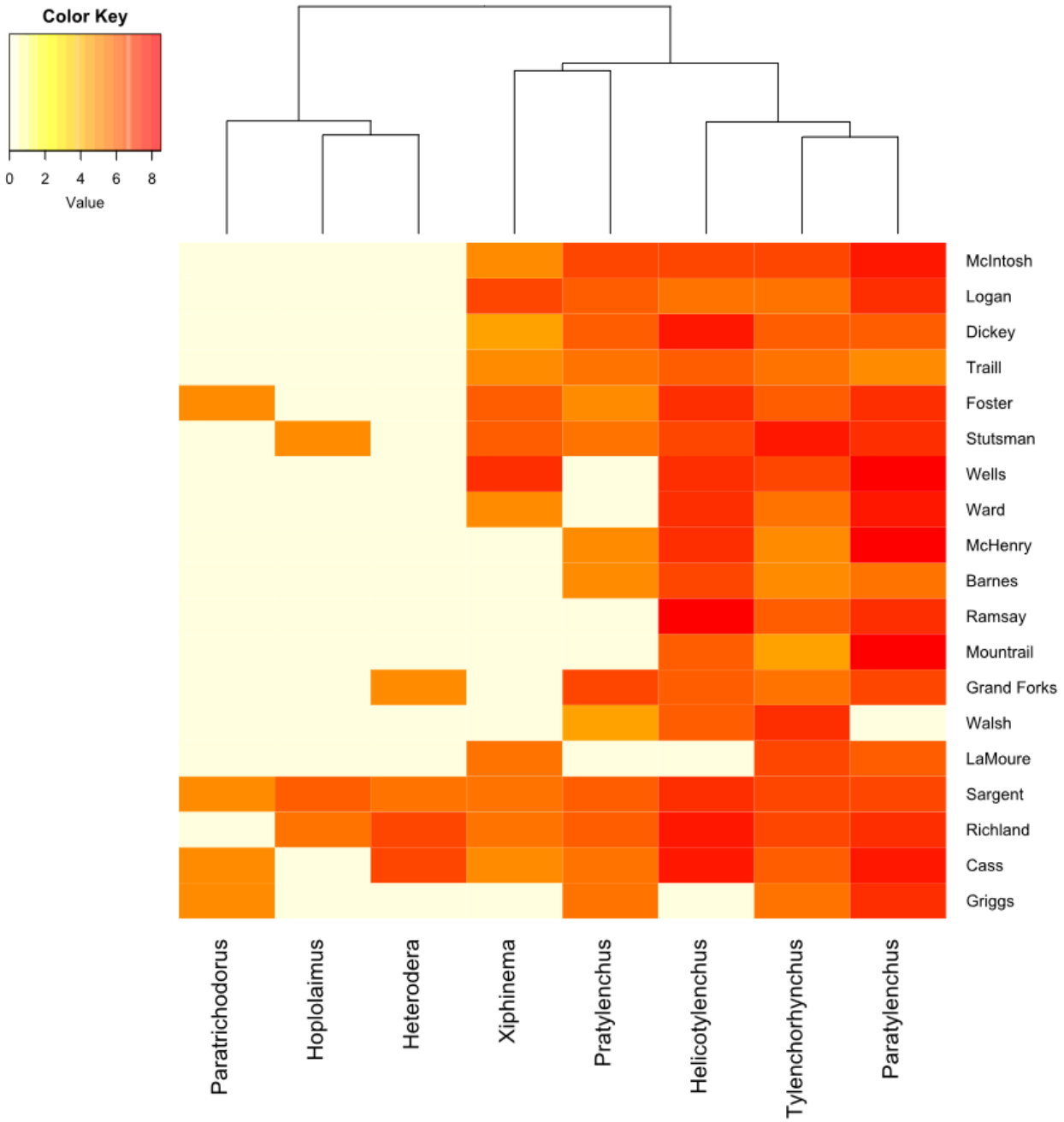


Fig. 2.3. Heatmap displaying normalized mean population density (nematodes per kg of soil) of individual plant parasitic nematode genus for each of the counties surveyed in this study. The greatest densities are indicated by deep red color, whereas the lowest densities are indicated by light red; thus, as the nematode density decreases the color intensity also decreases, with yellowish white representing absence of nematode genus (labeled at the bottom) in their corresponding county (labeled on the right). The nematode genera dendogram (labeled on the top) indicates clustering of nematode genera based on their similarity of mean population densities. Nelson County with less than two samples collected was excluded from the heatmap; less than two genera were detected in this county as well.

Similarity among nematode communities

Plant-parasitic nematode communities (incidence and population density of each genus) in most of the NDCC districts surveyed were similar to their neighboring districts, however, as distance increased between the districts, their Bray-Curtis similarity index also decreased (see Fig. 2.1 for locations of counties). The highest similarity in nematode communities was observed between the Districts of 1 and 2 (similarity index: 0.88) as well as between the Districts of 1 and 5 (0.83); all of which were neighboring districts (Table 2.4). Similar trends were also evident when analyzing the communities of each of the counties surveyed using Bray-Curtis similarity index. For instance, the neighboring counties of Richland and Sargent had the highest similarity index (0.86), likewise the neighboring counties of Mountrail and McHenry and the neighboring counties of Richland and Cass had similarity index values above 0.80, whereas, the western counties of McHenry and the southeastern county of LaMoure had the lowest similarity index (0.18) (Table 2.5).

Table 2.4. Bray and Curtis similarity index of the plant-parasitic nematodes among the seven North Dakota corn production districts[a].

NDCC	District 1	District 2	District 3	District 4	District 5	District 6
District 2	0.88					
District 3	0.69	0.74				
District 4	0.56	0.56	0.55			
District 5	0.83	0.76	0.66	0.70		
District 6	0.66	0.73	0.56	0.56	0.72	
District 7	0.66	0.65	0.66	0.63	0.66	0.53

[a] Bray and Curtis similarity index denoted as S = 2W/ (A+B), here S can range from 0 (no similarity) to 1 (complete similarity), A is the sum of population densities of all genera in one district, B is the sum of population densities of all genera in another district, and W is the sum of lower population densities of common genera in both communities (Bray and Curtis 1957; Norton 1978; Chen et al. 2012).

Table 2.5. The extreme values of Bray and Curtis similarity index of the plant-parasitic nematodes among the counties surveyed[a].

Counties	Similarity index value
Richland and Sargent	0.86
Richland and Cass	0.85
McHenry and Wells	0.83
McHenry and Mountrail	0.82
Mountrail and Walsh	0.20
McHenry and Walsh	0.19
McHenry and LaMoure	0.18

[a] Bray and Curtis similarity index denoted as S = 2W/ (A+B), here S can range from 0 (no similarity) to 1 (complete similarity), A is the sum of population densities of all genera in one county, B is the sum of population densities of all genera in another county, and W is the sum of lower population densities of common genera in both communities (Bray and Curtis 1957; Norton 1978; Chen et al. 2012). The pair counties with similarity index values greater than or equal to 0.8, as well as pair of counties with similarity index values less than or equal to 0.2 were presented in this table.

Clustering of these results reinforced these trends, as well as indicated that most of the southeastern and northeastern and western counties sampled, form their own clusters (Fig. 2.4). However, there were exceptions, for example, the southeastern county of Dickey and the northeastern county of Ramsey generated a sub-cluster, and similarly, the southcentral county of McIntosh formed a sub-cluster with the northcentral county of Ward. The first cluster with three counties in the dendogram had approximately 55% similarity in the genera detected. The second cluster also had 55% similarity, and was the largest cluster with two sub-clusters; one of which had Mountrail, McHenry, Wells, McIntosh and Ward counties, with 65% similarity in communities among each other. The third cluster with four counties only had 50 % similarity in the nematode communities detected (Fig. 2.4).

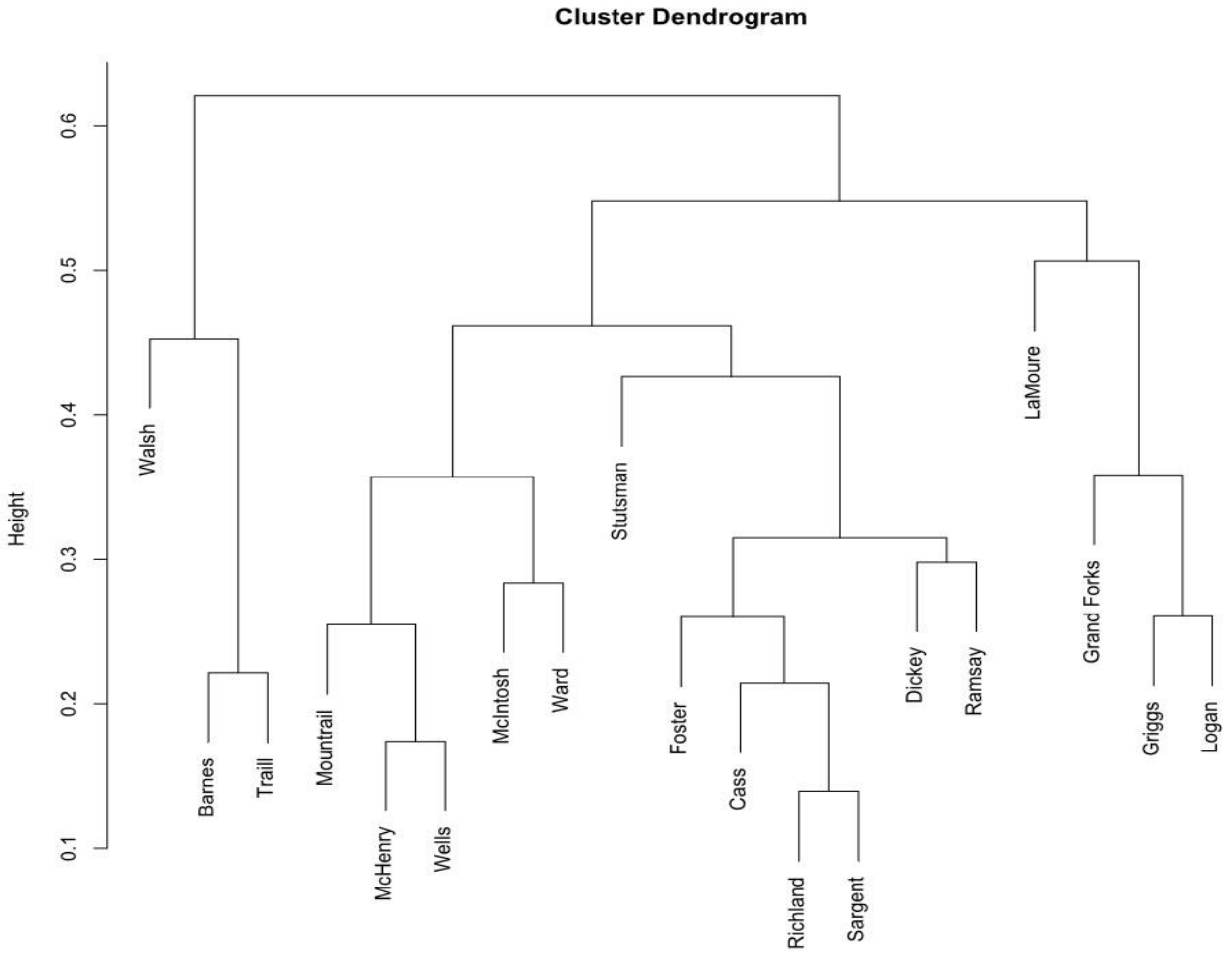


Fig. 2.4. Dendogram of North Dakota counties surveyed for plant-parasitic nematodes in corn fields, displaying similarity of nematode genera found in these counties. The height bar represents dissimilarity values with 0 indicating no dissimilarity or complete similarity whereas 1 indicates complete dissimilarity or no similarity. Hierarchical clustering method was applied to Bray and Curtis dissimilarity matrix of nematode abundance in corn fields. Nelson County with less than two samples collected have been excluded.

Association of environmental variables with plant-parasitic nematode incidence and population density

Nine different classes of soil textures were recorded, including clay, clay loam, loam, loamy sand, sandy clay loam, sandy loam, silty clay, silty clay loam and silt loam. *Helicotylenchus*, was present in most of these soil textural classes except silty clay soil and with a slightly higher population density in sandy loam soil. *Tylenchorhynchus* was also present in

most soil textural classes except clay, silty clay and silty loam soils. Comparable results also were observed with *Paratylenchus*, which was present in loamy sand, sandy loam, sandy clay loam, clay loam and loam soil textural classes. In contrast, *Pratylenchus* was only present in soil with silty loam, sandy loam and loam soil textural classes, with a slightly higher density in silty loam soil. The pH in these samples varied from 4.4 to 8.4, whereas percent organic matter, percent sand, percent silt and percent clay ranged from 1.1 to 7.1%, 12 to 84%, 10 to 59% and 5 to 61%, respectively. The 10-year average rainfall of the sample sites varied from 35.4 to 51.0 cm, and the average bare soil temperature among sites varied by only 2.7 °C.

Canonical correspondence analysis revealed that approximately 22 % of the total inter-site variation in distribution and population density of plant-parasitic nematode genera was explained by the seven edaphic and climatic variables. Fifty-six percent of the total variation in nematode-environment relationship was elucidated by the first canonical axis (CCA1), and 23% of the variance was explained by the second canonical axis (CCA2). Permutation test confirmed both axes were statistically significant at $P = 0.01$. Permutation test also revealed that pH, percent sand, percent clay, soil temperature and rainfall significantly ($P = 0.01$) influenced the composition of genera in the sampling sites. The tests also indicated that percent organic matter influenced nematode composition albeit at a lower significance level ($P = 0.05$); whereas, percent silt was aliased by the statistical software, since the variable was co-linear (redundant). According to the CCA bi-plot scores, the variables that were positively correlated with CCA1, were percent clay, percent sand and percent organic matter; whereas rainfall, soil temperature and percent sand were positively correlated with CCA2. Soil pH was positively correlated with CCA 3. Length of the arrows (environmental gradient) in the bi-plot indicated strength of the correlation between the environmental variables and genera composition (Fig.2.5). High positive

correlation was recorded between *Hoplolaimus* and percent sand, *Pratylenchus* and weather variables (rainfall and soil temperature), *Tylenchorhynchus* and pH, and *Xiphinema* and percent organic matter; as indicated by the proximity of the variables to the genera in the bi-plot (Fig. 2.5). The bi-plot also revealed clear distinction between the influence of soil property variables (pH, percent sand, percent clay and percent organic matter) and weather variables (rainfall and soil temperature), since the weather variables were grouped in the negative direction of CCA1 and positive direction of CCA2.

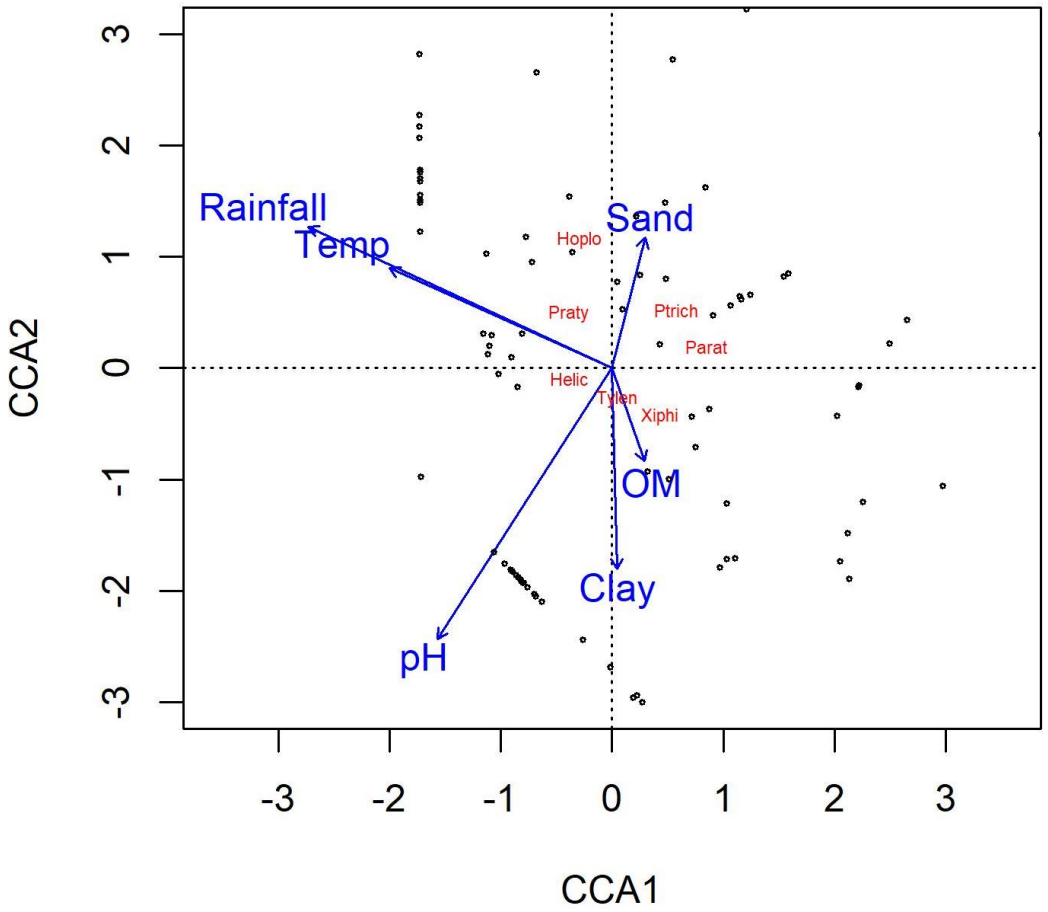


Fig. 2.5. Canonical correspondence analysis (CCA) bi-plot representing the relationship between plant-parasitic nematode genera composition in North Dakota corn fields and various soil edaphic and climatic factors. Sample sites are indicated by the black circles. Nematode genera are indicated by the red letters, which include *Helicotylenchus* (Helic), *Hoplolaimus* (Hoplo), *Paratrichodorus* (Ptrich), *Paratylenchus* (Parat), *Pratylenchus* (Parat), *Tylenchorhynchus* (Tylen) and *Xiphinema* (Xiphi). The blue arrows represent the environmental variables (rainfall, temperature, sand, organic matter, clay, and pH).

Discussion

Plant-parasitic nematodes are soil dwelling pests that are proven to parasitize and cause yield loss in corn (Koenning et al. 1999; Norton 1983). This study was the first extensive investigation, based on the number of soil samples and the area of sampling, of plant-parasitic nematode communities in ND corn field soils, providing an insight into population dynamic for a very cold climatic region in the US. The eight major genera identified in this study were similar to those previously reported in corn producing regions of other Midwestern states (Niblack 2009; Simon 2015; Tylka et al. 2011; Wysong and Kerr 1984). For instance, studies performed in Iowa and Ohio identified nine (*Criconemella, Helicotylenchus, Hoplolaimus, Longidorus, Paratylenchus, Pratylenchus, Paratrichodorus Tylenchorhynchus*, and *Xiphinema*) and ten (*Helicotylenchus, Heterodera glycines, Tylenchorhynchus*, *Paratylenchus, Pratylenchus, Xiphinema, Hoplolaimus, Criconemella, Trichodorus and Tylenchids*) genera, respectively. Therefore, other than the absence of *Longidorus*, *Criconemella* and *Trichodorus*, the genera found in ND corn fields were similar to those found in other surveyed states of the Midwest (Simon 2015; Tylka et al. 2011).

The six species identified in this study were all first reports of plant-parasitic nematode species in ND corn fields. Some of the species have been characterized in corn fields in other states, including *P. scribenri* common in major Midwestern corn producing states (MacGuidwin and Stanger 1991; Simon 2015; Todd and Oakley 1996; Waudo and Norton 1986;), *P. neglectus* in Kansas (Todd and Oakley 1996), whereas *P. allius* was in Ohio (Lopez-Nicora et al. 2014), and *H. microlobus* in Iowa (Castaner 1965) were reported to parasitize corn roots. Some of the species identified in this study are also known to parasitize other important crops in ND. *Pratylenchus scribenri* has been detected in a ND potato field (Yan et al. 2016a), *P. neglectus* in

a wheat field (Yan et al. 2016c), *Hoplolaimus stephanus* in a soybean field (Yan et al. 2016b), *Paratrichodorus allius* in a wheat field (Yan et al. 2016d), and *Helicotylenchus microlobus* detected in a soybean field (Yan et al. 2017).

Helicotylenchus, *Tylenchorhynchus* and *Paratylenchus* were the most prominent genera identified in corn fields in ND. These ecto-parasitic nematodes are one of the most prevalent nematodes in soil communities of field crops globally (Fleming et al. 2016; Simard et al. 2008; Simon 2015; Tylka et al. 2011). *Helicotylenchus* was the most common PPN being identified in over half of the surveyed fields in ND. A higher incidence of this nematode has also been reported in corn fields of Iowa (77.4%) and Ohio (94%) (Simon 2015; Tylka et al. 2011). *Tylenchorhynchus,* another globally common plant-parasitic nematode, was present in 36.7% of the surveyed fields in ND. The incidence of this nematode is comparable to incidence levels in Ohio (48%) and much higher than what has been reported in Iowa (4.8%) fields. A higher incidence of *Paratylenchus* in ND (31%) again aligns with the level observed in Ohio (57%), but not that of Iowa (0.6%) corn fields (Simon 2015; Tylka et al. 2011). In Ohio, Simon (2015) collected soil samples from arbitrarily selected corn fields; whereas in Iowa, Tylka et al. (2011) assayed soil samples submitted by corn growers, who were perhaps suspecting a nematode problem in their fields. Thus, the sampling strategy of ND corn survey has greater similarity with Ohio corn survey rather than Iowa corn survey, which could potentially explain why *Tylenchorhynchus* and *Paratylenchus* incidence levels in ND corn fields are closer to Ohio corn fields (Simon 2015; Tylka et al. 2011).

Pratylenchus is one of the more destructive endo-parasitic nematode prevalent in a variety of crop fields worldwide, and corn is one of the reported hosts of this pest (Grabau and Chen 2016; Schmitt and Barker 1981; Simon 2015). For example, *Pratylenchus scribneri* can

cause significant reduction in root health and size of corn (Simon 2015; Todd 1991; Waudo and Norton 1986). *Pratylenchus* was found in 51 and 80 % of Iowa and Ohio corn fields (Simon 2015; Tylka et al. 2011); whereas, the incidence of this nematode in ND corn fields was considerably less, with some reports of higher densities. Also, the *Pratylenchus* population density and prominence values may have been underestimated since root samples were not collected (due to limitation of performing a large extensive survey in commercial fields). However, this study does enable us to ascertain the relative prevalence and the species present in ND corn fields. In Iowa and Ohio corn fields the incidence of *Hoplolaimus, Paratrichodorus* and *Xiphinema* were 8 and 4 %, 0 and 13 %, and 37 and 42 %, respectively. However, in ND corn fields, the population density and frequency for these nematodes were very low. This suggests that these three genera are probably not a threat to ND corn growers.

Equally important to documenting PPN incidence in corn fields is determining population densities that can be used for action level thresholds. Nematode thresholds for corn have not been developed for North Dakota, however, other Midwestern states have developed threshold data for a few of the PPN presented here. With the understanding that North Dakota has a different climate, soil types, and corn maturity groups, population density data for a few PPN will be compared to threshold values reported in Iowa and Ohio. Iowa specific nematode threshold for *Helicotylenchus* is approximately 2,500 – 5,000 nematodes/kg of soil (Tylka et al. 2011). Approximately 6 % of the ND corn fields surveyed had *Helicotylenchus* population densities within the 2,500 to 5,000 threshold range and 4 % of the fields had population densities above 5,000 nematodes/kg of soil (Fig. 2.2A). The Ohio threshold for *Paratylenchus* indicates significant risk occurs when population densities are at approximately 2,505 to 5,000 nematodes/kg of soil (Simon 2015). In this survey, approximately 3 % of the fields had

Paratylenchus population densities within the 2,505 to 5,000 threshold range and another 3 % of the fields had population densities above 5,000 nematodes/kg of soil (Fig. 2.2B). In Illinois, soybean and corn growers are recommended to consider *Pratylenchus* to be of significant risk at population densities above 500 nematodes/kg of soil (Niblack 2009). In North Dakota, eight (Cass, Dickey, Grand Forks, Griggs, Logan, Richland, Sargent and Stutsman) out of the 20 counties surveyed had at least one field with a *Pratylenchus* population density above this threshold (Fig. 2.2C). The aforementioned thresholds for the three PPN described above indicate that North Dakota corn growers should monitor nematode populations to reduce economic damage. Also, more investigations into determining PPN action thresholds are needed, especially for important PPN genera that occur frequently in the state. On the other hand, specific population levels at which *Tylenchorhynchus* would be of risk were not established in Iowa, Ohio and Illinois (Niblack, 2009; Simon, 2015; Tylka et al., 2011) and therefore comparisons cannot be made with the ND survey. However, low numbers of *Tylenchorhynchus* are generally considered a mild pathogen risk.

As the distance between NDCC districts and counties increases, the geographic location changes significantly, leading to differing environmental conditions such as soil properties, rainfall and temperature between these counties, which could influence the distribution and makeup of nematode communities (Edwards et al. 1988; Kandel et al. 2013; Norton 1983; Simon 2015). Therefore, in this study, as the spatial distance between North Dakota counties and districts increased, similarity in nematode community decreased, with western, northeastern and southeastern counties forming clusters of similar nematode occurrence and diversity. Such clusters could be explained by the fact that the samples collected from the western counties such as Mountrail, Ward and McHenry were from the Missouri River Plateau area, which is

characterized by a topological elevation and dry land conditions (Bailey 1926). Nematodes of comparatively smaller size, such as *Paratylenchus*, are more frequent and abundant in corn fields of this region. Higher *Paratylenchus* population were also found in pea fields of western ND (Upadhaya et al. 2018). Soil samples obtained from the southeastern region of ND tend to be sandy and sandy loam, thus corn fields of this region are characterized by higher diversity of PPN found. Almost all the genera identified in this study can also be found in southeastern counties such as Sargent, Richland and Cass counties, with *Hoplolaimus*, *Paratrichodorus* and *Heterodera* being only found in southeastern counties. On the other hand, soil samples taken from northeastern ND along the Red River have a higher amount of clay particles (Bailey 1926). These counties are characterized by comparatively lower diversity of PPN in corn fields. Therefore, soil texture from these three regions could help explain similar plant-parasitic nematode diversity in each cluster. Counties within such regions can have similar nematode management plan implemented due to higher similarity in PPN diversity.

In this study, the environmental variables, pH, percent sand, percent silt, percent organic matter, percent clay, rainfall and soil temperature, explained 22 % of the variation in plant-parasitic nematode genera composition. Other variables such as tillage, cropping sequence, host susceptibility, nematode virulence may have been responsible for the remaining variation in nematode composition between field sites (Edwards et al. 1988; Neher 1999; Norton and Hoffmann 1974; Yeates 1984). It is crucial to identify these variables and understand their influence on nematode communities for better management of these pests. During this survey, canonical correspondence analysis revealed that pH, rainfall and soil temperature had the strongest influence on incidence and population density of North Dakota corn fields' nematode genera.

Canonical correspondence analysis results also indicated that *Hoplolaimus* incidence and abundance were greatly influenced by percent sand. This could be attributed to the fact that as the nematode's length and diameter increase, the optimum pore and particle size also need to increase for optimal nematode movement to occur (Wallace 1971). Since *Hoplolaimus* has comparatively larger size, it may be more prevalent in sandy soils of corn fields in ND.

Significant positive correlations of pH with *Helicotylenchus, Tylenchorhynchus* and *Xiphinema* were observed in this study. In the pH range of 8.7 to 4.4, the population densities of these three nematodes were greater in soil with high pH than in soil with low pH. Only 6% of the fields infested with these nematodes had pH lower than 6. According to Norton and Hoffmann (1974), pH could be used as a tool to predict where nematodes were more likely to occur, however, effect of pH on nematode populations could vary with species. For instance, *X. americanum* and *H. platyurus* populations increased with increasing pH from 4.5 to 7.4 whereas *X. chambersi* population decreased with increasing pH from 4.5 to 6.4; on the contrary, *H. pseudorobustus* were in its greatest population at a pH range of 7.5 to 7.9 (Norton and Hoffmann 1974). Additionally, Schmitt and Norton (1969) recorded increasing abundance of *T. maximus* as pH increased from 5.0 to 6.5, which is consistent with this study.

Canonical correspondence analysis results also indicated that rainfall and soil temperature were positively associated with *Pratylenchus*, *Hoplolaimus*, *Paratrichodorus* and *Paratylenchus*. However, *Pratylenchus* had the strongest correlation with these weather variables. In agreement with this observation, *P. scribneri* population density was reported to increase with increasing temperature from 20 to 37.5 °C on soybean roots (Dickerson 1979). Similar effects of temperature were also recorded for other species of *Pratylenchus* (Florini et al. 1987; Kandel et al. 2013; Kimpinski and Willis 1981). Another study significantly correlated distribution and

abundance *P. neglectus* populations with soil moisture (influenced by rainfall) in wheat fields (Kimpinski et al. 1976).

In conclusion, results from this study indicated that eight different genera of plant-parasitic nematodes were present in 20 major corn producing counties of North Dakota. The species identified for the first time in these corn fields were *Helicotylenchus microlobus, Hoplolaimus stephanus, Paratrichodorus allius, Paratylenchus nanus, Pratylenchus scribneri* and *P. neglectus*. Neighboring counties had similar PPN incidence and population density; however, as the distance between these counties increased similarity in PPN diversity decreased. Significant correlations between nematode genera and various environmental factors were also observed. Higher incidence and population density of *Helicotylenchus, Paratylenchus, Pratylenchus* and *Tylenchorhynchus* nematodes have a potential to impact corn yield. Therefore, further studies in ND are required to assess the species-specific yield losses and determine species-specific damage thresholds.

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CHAPTER 3: CHARACTERIZATION OF VIRULENCE PHENOTYPES OF SOYBEAN CYST NEMATODE (*HETERODERA GLYCINES*) POPULATIONS IN NORTH DAKOTA

Abstract

Soybean cyst nematode (SCN; *Heterodera glycines*) continues to be the greatest threat to soybean production in the United States. Since host resistance is the primary strategy used to control SCN, knowledge of the SCN virulence phenotypes (HG types) is necessary for choosing sources of resistance for SCN management. To characterize SCN virulence phenotypes in North Dakota, a total of 419 soybean fields across 22 counties were sampled during 2015, 2016, and 2017. SCN was detected in 42% of the fields sampled and the population densities in these samples ranged from 30 to 92,800 eggs and juveniles per 100 cm^3 of soil. The SCN populations from some of the infested fields were then virulence phenotyped with seven soybean indicator lines and a susceptible check (Barnes) using the HG type tests. Overall, 73 SCN field populations were successfully virulence phenotyped. The HG types detected in North Dakota were HG type 0 (frequency rate: 36%), 7 (27%), 2.5.7 (19%), 5.7 (11%), 1.2.5.7 (4%), and 2.7 (2%). However, prior to this study only HG type 0 was detected in North Dakota. The designation of each of the HG types detected was then validated in this study by repeating the HG type tests for thirty-three arbitrarily selected samples. Additionally, repetition of the tests proved that there were several SCN populations in North Dakota that successfully reproduced on indicator lines #2 (PI 88788) and #1 (PI 548402), the most widely used sources of resistance. This research for the first time reports several new HG types detected in North Dakota and confirms that the virulence of SCN populations is shifting and overcoming resistance,

highlighting the necessity of utilization of different resistance sources, rotation of resistance sources, and identification of novel resistance sources for SCN management in North Dakota.

Keywords: *Glycine max*, *Heterodera glycines*, HG types, North Dakota, resistance, soybean, soybean cyst nematode, indicator lines, survey, virulence phenotypes.

Introduction

Soybean (*Glycine max* L. (Merr.)) is a leguminous crop that plays a key role in achieving global food security (Hartman et al. 2011). It is an important source of protein and oil for humans and animals. Processed soybeans are the world's largest source of animal feed protein and the second largest source of vegetable oil (USDA-ERS 2019), and the United Sates is the leading producer of soybeans in the world (FAOSTAT 2019). In North Dakota, it is one of most widely planted crops with a value of production over $2 billion (USDA-NASS 2019).

Among the various biotic factors that threaten soybean production, soybean cyst nematode, *Heterodera glycines* (Ichinohe 1955) continues to be the most economically devastating pathogen for soybean producers worldwide. This sedentary endoparasitic nematode is responsible for more than 1.2 billion dollars in yield losses in the U.S. alone (Koenning and Wrather 2010). A more recent study has revealed that soybean cyst nematode (SCN) caused yield losses of up to 617.4 billion bushels in 28 U.S. states and Ontario, Canada during 2010 to 2014 (Allen et al. 2017). However, losses often go unnoticed since SCN can cause more than 30% yield loss without any noticeable above-ground symptoms (Koenning and Wrather 2010; Wang et al. 2003).

In addition to causing serious yield loss, SCN has the ability to spread at an alarming rate. Since this nematode's first discovery in 1899 in north-east China (Li et al. 2011) and characterization in Japan in 1915 (Hori 1916), it has spread to all the major soybean producing

regions of the world (Yan and Baidoo 2018). SCN was first discovered in the U.S. during 1954 in North Carolina (Winstead et al. 1955) and by 2014, this nematode has spread to every soybean producing state in the U.S. except New York and West Virginia (Tylka and Marett 2014). Since then, SCN was detected in Cayuga County, New York in 2016 (Wang et al. 2017). SCN was first found in Richland County, North Dakota in 2003 (Bradley et al. 2004). Since then, infestation of SCN has spread to at least 19 soybean producing counties by 2015 (Yan et al. 2015) and the SCN population densities in these fields were as high as 21,540 eggs and juveniles per 100 cm^3 of soil (Chowdhury et al. 2016). SCN continues to spread quickly as soybean production expands to other counties of North Dakota (Tylka and Marett 2017). Thus, SCN has the potential to become North Dakota growers' biggest disease problem if it is not detected early and managed proactively.

Resistant cultivars combined with crop rotation are the two primary methods for controlling SCN (Mueller et al. 2016; Niblack et al. 2003). However, SCN is known to be genetically diverse nematode populations and can develop new virulent forms due to continuous use of the same resistance source (Colgrove and Niblack 2008; Yan and Baidoo 2018). These new virulent forms can have higher levels of resistance and can overcome the prevailing resistance genes that are commonly used for control (Colgrove and Niblack 2008). Thus, characterizing the occurrence and distribution of SCN virulence phenotypes is essential for developing management strategies based on the use of resistant cultivars derived from durable resistance sources.

To characterize the virulence phenotypes of SCN, a standardized classification scheme was proposed by Niblack et al. (2002) referred to as 'HG type' tests. HG refers to *H. glycines* and the type refers to seven different 'Plant Introductions' with various forms of resistance.

These seven ‘Plant Introductions’ suggested by Niblack et al. (2002) are resistance sources which include PI 548402 (‘Peking’, indicator #1), PI 88788 (#2), PI 90763 (#3), PI 437654 (#4), PI 209332 (#5), PI 89772 (#6), and PI 548316 (‘Cloud’, #7). Thus, the HG type tests classify SCN populations’ virulence phenotypes by assessing the reproductive potential of SCN populations on the seven different indicator lines compared to a susceptible check, which is referred to as female index (FI) that is expressed as a percentage (Howland et al. 2018; Niblack et al. 2002).

Among the seven indicator lines, PI 88788 (#2) is the most commonly used source of SCN resistance in North America. Additionally, Peking-type resistance derived from PI 548402 (#1) is the second most commonly used source of resistance, however, very few of the commercially available cultivars derive resistance from other plant introduction lines (McCarville et al. 2017; Yan and Baidoo 2018). In North Dakota the virulent type that has been reported thus far is HG type 0, which is considered the least virulent type of SCN and does not attack any of the seven indicator lines. However, there has been several recent research reports of commonly used resistance sources such as PI 88788 and Peking (PI 548402) being overcome by SCN populations from other major soybean producing states in the Midwest, which includes Kansas (Rzodkiewics 2010), Missouri (Howland et al. 2018), Iowa (McCarville et al. 2017), Nebraska (Broderick 2016) and Wisconsin (MacGudwin 2012). Additionally, the neighboring states of Minnesota and South Dakota have also reported that the virulence of SCN populations are shifting and the number of SCN populations that are virulent on PI 88788 is increasing (Acharya et al. 2016, Chen et al. 2010, Zheng et al. 2006). Hence, the objective of this study was to investigate the virulence diversity of SCN by characterizing the HG types occurring in North

Dakota to determine if SCN populations from North Dakota can overcome the resistance provided by common resistance sources such as PI 878788 and Peking.

Materials and methods

Soil sample collection

During 2015, 2016, and 2017 soil samples were collected from North Dakota soybean fields or fields with a history of soybean production. The sampled fields were selected arbitrarily from each of the major soybean producing counties of North Dakota. Sampling frequency from each county was weighted based on their soybean acreage (Niblack et al. 2003). Thus, greater number of samples were collected from counties with higher soybean production. Counties of western and south-western North Dakota were not sampled due to very low soybean acreage in these counties. Across the three years, a total of 419 soybean fields from 22 counties of North Dakota were selected and sampled. The majority of these samples were collected during April-May and August-September each year.

In each field, soil sampling was focused towards field entrances, along field borders and the edge of spots with yellow and stunted plants to increase the likelihood of detecting SCN in the representative soil sample. Soil sampling was conducted using a 2.5 cm diameter soil probe with a probe length of 30 cm (Gempler's model L sampler, Madison, WI), but when the field's soil was too compacted, a shovel was used. Approximately 100 to 200 cm^3 of soil were collected from each sampling point and each soil sample was a composite of the soil collected from 20 to 25 sampling points in each field, covering an area of 2 to 2.5 ha. For each field, soil samples from all the sampling points were then composited in a plastic bag and labeled with the field number and collection date. The samples were stored in a cooler during transport and then stored in a cold room at 4°C until processing. Latitude and longitude coordinates were recorded for

each field in decimal degrees. Additionally, elevation and the nearest city were recorded for each location.

Soil sample processing, nematode extraction, and enumeration

Each soil sample was first thoroughly mixed by hand, and a 100 cm^3 subsample was collected from each sample for nematode extraction. Adult females and/or cysts of *H. glycines* were isolated from these subsamples using the sieving and decanting method described by Krusberg et al. (1994). According to this method, the 100 cm^3 subsample was stirred in a beaker with tap water (1000 ml) and after 30s the mixture was poured through a stack of sieves (710 µm and 250 µm apertures; VWR, Radnor, PA) to separate larger organic and inorganic soil particles from smaller soil particles and SCN adult females and/or cysts. Next, the smaller soil particles and adult females and/or cysts were transferred into a customized cylindrical PVC pipe that has an opening at the top but the bottom is lined 250 µm aperture sieve. The adult females and/or cysts at the bottom of the PVC pipe were then crushed using a rubber stopper attached to a drill press (MasterForce Drill Press, Menards, Fargo, ND) following the procedures described by Faghihi and Ferris (2000). Liberated eggs and juveniles (J2) were then collected from the crushed adult female and/or cysts in a 20 µm aperture sieve and enumerated using an inverted compound light microscope (at 100× magnification; Focus Precision Instruments, Cavaletti Court Victoria, MN).

Inoculum preparation and soil infestation

Eggs and juveniles collected from the samples with a low population density (less than 400 eggs and juveniles/100 cm^3 of soil) were arbitrarily selected for SCN population increase by inoculating the susceptible cultivar Barnes in controlled greenhouse conditions. Barnes [*Glycine max* (L.) Merr. cv. Barnes] is a local soybean cultivar that is equally as susceptible as Lee 74

(Poromarto et al. 2015), a standard cultivar that is widely used as a susceptible check for HG type testing (Niblack et al. 2002). After the population increase, the SCN white females from the susceptible check were collected and crushed. The liberated eggs and juveniles were then used as inoculum for HG typing tests that were conducted by artificially inoculating autoclaved sandy soil.

Samples with population densities higher than 400 eggs and juveniles per 100 cm^3 of soil were first manually mixed for 30 to 60 mins to break up soil clumps and then further mixed for 20 to 30 mins using a motorized cement mixer (Kushlan Products LLC, Houston TX) to ensure uniform distribution of nematodes throughout the soil sample. These thoroughly mixed soil samples were then used for HG typing tests that were conducted with naturally infested soil. However, some of the field samples with very high population density were HG typed by artificially inoculating autoclaved sandy soil after eggs were extracted and collected from the samples. Overall, seventy-three SCN field populations were virulence phenotyped including assays conducted with artificially infested and naturally infested soil.

HG type determination

Modified HG typing tests were conducted according to standardized protocols suggested by Niblack et al. 2002. Each HG type test included four replications of each of the seven indicator lines, PI 548402 (‘Peking’, indicator #1), PI 88788 (#2), PI 90763 (#3), PI 437654 (#4), PI 209332 (#5), PI 89772 (#6), and PI 548316 (‘Cloud’, #7), as test lines and a susceptible check (Barnes). Seeds of each indicator line and the susceptible check were pre-germinated for four to five days by placing them in Petri dishes (Falcon, Corning, NY) with wet filter paper (Whatman, Darmstadt, Germany). A single germinated seed for each experimental unit was then planted into a cone type container (3.8-cm in diameter and 21-cm in height; Stuewe and Sons, Inc. Tangent,

OR) containing about 100 cm^3 of thoroughly mixed naturally infested soil. For artificial infestation, the cone type containers were filled up with autoclaved sandy soil and artificially inoculated with SCN eggs and juveniles obtained from crushed SCN adult females and/or cysts, following procedures suggested by Niblack et al. (2002). Inoculation was performed during planting by making 2 to 3 holes at a 5 cm depth around the seedling in the container and transferring a total of 5 ml nematode suspension that contained 2,000 SCN eggs and juveniles using a 5 ml pipette. The holes containing the inoculum as well as the seedling were then covered up with a thin layer of moist sterilized sandy soil. The same procedure was also followed during the previously mentioned second round of HG type tests for confirmation purposes. After planting, the containers were put into plastic racks with 14 X 7 wells and arranged in a completely randomized design. They were then maintained in a greenhouse growth chamber (GR64, Conviron, Winnipeg, Manitoba, Canada) for 30 to 35 days at a constant temperature of 27 °C and a daylight period of 16 hours.

Out of the seventy-three field populations evaluated, fifty-one of the SCN field populations were HG typed by planting the HG type indicator lines and the susceptible cultivar into naturally infested soil (Table 3.1). Additionally, twenty-two SCN field populations with low density in naturally infested soil were reared on the susceptible soybean cultivar to increase the population density, which were then used for HG typing tests that were conducted by artificially infesting autoclaved sandy soil (Table 3.2). Furthermore, thirty-three SCN populations were arbitrarily selected and HG type tested for a second time to confirm the HG types and validate the accuracy of the first iteration (Table 3.3). These repeated HG types tests were all conducted by artificially infesting autoclaved sandy soil.

During harvest, the above soil portion of the plants were removed and adult females and/or cysts from both roots and soil were collected using the sieving and decanting method described by Krusberg et al. (1994). The roots and soil from each container were emptied into a 4-liter bucket, which was then filled to ¾ of its capacity with water. This allowed the soil to easily dislodge from the root surface. The roots were then gently placed on top of a 710-µm-pore sieve that was stacked on top of a 250-µm-pore sieve, and subjected to high-pressure water spray to dislodge the SCN females attached to the roots; which were collected in the 250-µm-pore sieve. The remaining soil and water were then stirred and poured immediately through both the sieves, stacked in the same order, to collect the newly formed females dislodged from the roots and/or old cysts from the infested soil. The extracted nematodes were then examined, and the newly formed white (majority) or light-yellow females (few) were enumerated under a dissecting microscope (SM 100 Series, Swift Optical Instrument, INC. TX, US) in a petri dish with grids containing the nematode suspension. For each indicator line, a female index (FI) was calculated as follows: FI = (average no. of white females found on the indicator line/average no. of females found on the susceptible check) x 100. A FI cutoff of 10 was used because a FI value of less than 10 on a test line would not be able to maintain themselves, at least within a single growing season and may result in false positive, as suggested by Golden et al. (1970) and Niblack et al. (2002). Therefore, the indicator lines with FI $\geq$ 10 were considered to have positive host compatibility, whereas the indicator lines that had FI < 10 were considered as negative host incompatibility.

Table 3.1. Heterodera glycines virulence phenotypes detected in North Dakota determined by HG type tests conducted with seven indicator lines and the susceptible check planted in naturally infested field soil[a].

Sample ID	County	Egg count[b] (100 cm^3)	Female index (FI)[c]							No. females on susceptible[d]	HG type[e]
			PI 548402 (#1)	PI 88788 (#2)	PI 90763 (#3)	PI 437654 (#4)	PI 209332 (#5)	PI 89772 (#6)	PI 548316 (#7)		
Ken	Richland	2,800	10.0	25.5	2.2	0.1	46.3	0.3	40.3	583	1.2.5.7
N 32	Richland	12,200	1.6	9.7	0.3	0.2	2.0	0.2	14.0	668	7
N 48	Cass	12,200	5.8	14.1	0.4	0.0	19.4	0.6	25.1	509	2.5.7
SCN 2EF	Cass	4,200	0.6	2.9	0.2	0.1	1.5	1.3	2.7	981	0
SCN 3	Cass	9,600	1.1	2.7	1.3	0.3	5.4	1.5	10.4	968	7
SCN 4	Cass	13,600	2.4	8.5	3.7	0.5	5.6	1.4	10.3	1,568	7
SCN 7	Cass	700	1.0	11.9	0.3	0.7	22.0	0.7	38.0	296	2.5.7
SCN 14	Cass	750	0.0	0.4	0.0	0.0	3.5	0.0	7.3	391	0
SCN 22	Cass	1,800	0.0	0.7	0.0	0.0	2.2	0.3	11.2	381	7
SCN 24	Cass	840	0.0	0.0	0.0	0.0	0.0	0.0	0.1	617	0
SCN 49	Richland	450	1.1	3.8	0.0	0.0	2.5	0.0	26.7	275	7
SCN 52	Richland	630	5.7	16.4	0.2	0.0	2.8	0.2	26.7	307	2.7
SCN 52*	Richland	3,800	0.1	6.5	0.0	0.0	14.0	0.0	60.7	541	5.7
SCN 55	Richland	460	1.4	11.5	0.0	0.0	26.2	1.0	34.7	234	2.5.7
SCN 55*	Richland	12,300	2.0	9.9	0.0	0.0	15.5	0.0	61.1	461	5.7
SCN 59	Richland	780	1.1	6.0	3.9	0.1	5.1	1.3	7.6	340	0
SCN 61	Richland	1,190	0.1	2.4	0.0	0.0	1.4	0.0	0.7	549	0
SCN 62	Richland	2,700	0.0	1.7	0.0	0.0	10.3	0.0	10.5	332	5.7
SCN 63	Richland	1,050	1.6	23.5	0.0	0.0	18.7	0.7	25.3	352	2.5.7
SCN 78	Richland	1,368	0.0	22.3	0.0	0.0	13.8	0.0	21.1	220	2.5.7
SCN 79	Richland	3,630	0.1	9.7	0.1	0.1	10.2	0.0	15.2	534	5.7
SCN 81	Richland	1,260	0.0	0.1	0.0	0.0	0.2	0.0	0.1	518	0
SCN 82	Richland	610	0.0	1.2	0.0	0.0	1.6	0.0	0.2	620	0
SCN 95	Richland	720	0.0	8.8	0.0	0.0	5.9	0.0	16.0	190	7
SCN 102	Richland	1,020	0.0	0.0	0.0	0.0	0.0	0.0	0.3	223	0
SCN 103	Richland	1,680	0.0	0.9	0.0	0.0	3.5	0.0	4.9	143	0
SCN 131	Cass	400	0.0	2.4	0.0	0.1	2.2	0.0	1.9	247	0
SCN 154	Cass	500	0.0	2.4	0.0	0.0	0.6	0.0	7.4	116	0
SCN 156	Cass	8,700	1.9	7.0	0.0	0.0	27.0	0.7	60.0	541	5.7
SCN 180	Cass	664	2.0	39.1	0.0	0.0	38.9	0.0	56.9	172	2.5.7
SCN 246	Cass	400	3.8	10.7	0.0	0.0	3.6	0.0	16.1	93	2.7*
SCN 280	Barnes	680	0.0	0.0	0.0	0.0	0.0	0.0	0.1	236	0
SCN 285	Steele	2,583	0.5	3.5	0.0	0.0	10.8	0.0	31.6	1,430	5.7
SCN 325	GF[f]	300	0.0	0.0	0.0	0.0	0.0	0.0	49.8	54	7
SCN 329	Traill	5,200	0.8	8.2	0.0	0.0	4.4	0.1	6.8	1,603	0
SCN 331	Traill	1,200	0.0	5.3	0.1	0.0	0.2	0.0	5.8	384	0
SCN 333	Traill	2,800	0.0	3.1	0.0	0.0	1.1	0.0	11.3	331	7
SCN 334	Traill	7,600	1.7	4.4	0.1	0.0	1.0	0.1	17.3	1,954	7

Table 3.1. Heterodera glycines virulence phenotypes detected in North Dakota determined by HG type tests conducted with seven indicator lines and the susceptible check planted in naturally infested field soil[a] (continued).

Sample ID	County	Egg count[b] (100 cm^3)	Female index (FI)[c] PI 548402 (#1)	PI 88788 (#2)	PI 90763 (#3)	PI 437654 (#4)	PI 209332 (#5)	PI 89772 (#6)	PI 548316 (#7)	No. females on susceptible[d]	HG type[e]
SCN 339	Traill	1,200	0.5	7.3	2.5	0.1	2.8	0.4	6.3	371	0
SCN 342	Traill	42,500	25.5	39.7	0.0	0.2	10.5	0.0	35.5	325	1.2.5.7
SCN 344	Traill	4,600	25.4	30.5	3.0	2.6	10.0	2.7	31.4	742	1.2.5.7
SCN 350	Traill	8,600	0.0	17.7	0.0	0.0	14.6	0.0	28.1	1,037	2.5.7
SCN 351	Traill	2,400	0.0	2.4	0.0	0.0	2.1	0.0	12.6	731	7
SCN 352	Traill	4,100	0.1	2.2	0.0	0.1	0.0	0.0	23.4	491	7
SCN 355	Steele	15,500	0.1	0.5	0.0	0.0	0.5	0.0	0.0	425	0
SCN 357	Steele	19,200	0.0	8.5	0.0	0.1	12.8	0.0	55.5	1,252	5.7
SCN 370	GF[f]	1,100	0.0	4.0	0.0	0.0	0.0	0.0	20.2	100	7
SCN 390	Steele	700	9.5	27.7	4.1	0.0	31.2	6.7	19.1	227	2.5.7
SCN 391	Steele	2,300	0.0	0.2	0.0	0.0	0.0	0.0	0.0	712	0
SCN 392	Steele	5,600	0.1	18.1	0.0	0.0	26.4	0.0	46.0	346	2.5.7
SCN 394	Steele	2,200	0.2	1.3	0.1	0.0	8.1	0.0	27.7	907	7

[a] Samples with population densities higher than 400 eggs and juveniles per 100 cm3 of soil were HG typed by planting the seven indicator lines and the susceptible check in naturally infested soil. Average no. of white females for each of the seven indicator lines and the susceptible check were calculated from four replicates.

[b] Density of SCN eggs and juveniles in 100 cm3 of naturally infested original samples.

[c] FI = (average no. of white females found on the indicator line/average no. of white females found on the susceptible check) x 100.

[d] Average number of white females in the susceptible check Barnes.

[e] HG types from assays in which the average number of white females on the susceptible check was greater than 50 but less than 100 are indicated with the asterisks (*) symbol.

[f] GF refers to Grand Forks County of North Dakota

Table 3.2. *Heterodera glycines* virulence phenotypes detected from additional field soil samples in North Dakota determined by HG type tests conducted with seven indicator lines and the susceptible check planted in artificially infested sandy soil[a]

		Female index (FI)[b]								
Sample ID	County	PI 548402 (#1)	PI 88788 (#2)	PI 90763 (#3)	PI 437654 (#4)	PI 209332 (#5)	PI 89772 (#6)	PI 548316 (#7)	No. females on susceptible[c]	HG type[d]
Arne	Richland	1.3	4.8	0.2	0.0	2.6	0.0	8.8	898	0
Castleton	Cass	0.0	1.1	0.0	0.0	0.2	0.1	1.2	568	0
GregShort	Richland	0.8	10.8	0.0	0.0	11.6	0.1	14.8	955	2.5.7
GregTall	Richland	0.0	7.0	0.0	0.0	9.5	0.0	23.3	831	7
Lee	Richland	0.0	1.1	0.0	0.0	2.0	0.0	4.7	261	0
Miller	Richland	0.0	5.9	0.0	0.0	6.1	0.0	8.9	110	0
Prosper	Cass	0.0	0.5	0.0	0.0	0.7	0.0	4.9	919	0
SCN 2WF	Cass	5.6	3.9	0.0	0.0	2.2	0.0	11.7	205	7
SCN 16	Cass	0.0	27.4	0.0	0.0	37.0	0.0	55.0	173	2.5.7
SCN 38	Cass	0.0	17.7	0.0	0.0	33.8	0.0	44.0	233	2.5.7
SCN 46	Richland	0.0	5.0	0.1	0.0	3.5	0.0	18.6	762	7
SCN 48	Richland	0.0	1.7	0.0	0.0	0.0	0.0	10.7	121	7
SCN 50	Richland	0.0	0.6	0.2	0.0	0.0	0.0	15.9	131	7
SCN 53	Richland	0.0	8.0	0.0	0.0	0.0	0.0	0.0	477	0
SCN 101	Richland	0.0	4.5	0.0	0.0	2.0	0.0	4.0	100	0
SCN 157	Richland	0.0	0.0	0.0	0.0	5.8	0.0	6.8	51	0*
SCN 182	Cass	0.1	5.9	0.1	0.0	3.5	0.1	2.9	963	0
SCN 220	Cass	0.0	2.6	0.0	0.0	1.6	0.0	12.1	64	7*
SCN 326	Grand Forks	0.0	3.0	0.0	0.0	13.4	0.0	25.4	209	5.7
SCN 330	Traill	0.6	11.6	0.2	0.0	15.3	0.0	19.1	238	2.5.7
SCN 332	Traill	0.3	1.9	0.1	0.0	1.3	0.5	29.0	405	7
SCN 353	Traill	0.6	32.6	0.2	0.0	42.0	0.0	13.6	216	2.5.7

[a] Additional samples with population densities lower than 400 eggs and juveniles per 100 cm^3 of soil were HG typed by planting the seven indicator lines and the susceptible check in artificially inoculated soil. Average no. of white females for each of the seven indicator lines and the susceptible check was calculated from four replicates.

[b] FI = (average no. of white females found on the indicator line/average no. of white females found on the susceptible check) x 100.

[c] Average number of white females in the susceptible check Barnes.

[d] HG types from assays in which the average number of white females on the susceptible check was greater than 50 but less than 100 are indicated with the asterisks (*) symbol.

Table 3.3. Repeated HG type tests conducted to confirm the virulence phenotypes detected in North Dakota by planting soybean lines and the susceptible check in artificially infested sandy soil[a]

		Females index (FI)[b]								
Sample ID	County	PI 548402 (#1)	PI 88788 (#2)	PI 90763 (#3)	PI 437654 (#4)	PI 209332 (#5)	PI 89772 (#6)	PI 548316 (#7)	No. females on susceptible[c]	HG type[d]
Arne's	Richland	1.6	3.7	0.0	0.0	6.2	0.0	7.3	109	0
Castleton	Cass	0.0	5.2	0.0	0.0	1.8	0.0	3.6	217	0
Greg Short	Richland	6.2	15.5	0.0	0.0	24.0	0.0	24.0	60	2.5.7*
Greg Tall	Richland	0.0	8.9	0.0	0.0	9.8	0.0	27.4	156	7
Ken's	Richland	12.2	17.9	1.6	0.0	20.1	0.4	19.8	92	1.2.5.7*
Lee's	Richland	0.1	4.7	0.0	0.0	6.4	0.1	6.8	409	0
Prosper	Cass	0.0	0.0	0.0	0.0	0.5	0.0	1.4	105	0
SCN 2WF	Cass	0.0	2.5	0.0	0.0	1.5	0.0	25.7	99	7*
SCN HG 7	Cass	0.7	10.0	0.0	0.0	16.3	0.0	26.6	335	2.5.7
SCN 16	Cass	0.3	13.7	0.0	0.0	24.6	0.0	50.6	181	2.5.7
SCN 38	Cass	0.0	27.0	0.0	0.0	35.0	0.0	24.0	53	2.5.7*
SCN 46	Richland	0.0	7.1	0.0	0.0	5.9	0.0	28.0	511	7
SCN 48	Richland	0.0	0.0	0.0	0.0	0.1	0.0	13.3	218	7
SCN 50	Richland	0.2	1.6	0.0	0.0	2.3	0.1	17.8	490	7
SCN 52*	Richland	0.0	4.4	0.0	0.0	10.3	0.0	27.7	120	5.7
SCN 101	Richland	0.0	1.9	0.0	0.0	3.8	0.0	3.7	431	0
SCN 102	Richland	0.0	5.9	0.0	0.0	0.3	0.0	44.3	81	0*
SCN 103	Richland	0.0	6.6	0.0	0.0	7.0	0.0	8.6	198	0
SCN 180	Cass	0.4	10.0	0.2	0.0	15.7	0.0	24.7	352	2.5.7
SCN 246	Cass	0.2	10.1	0.5	0.0	4.3	0.1	11.1	222	2.7
SCN 280	Barnes	0.0	0.0	0.0	0.0	0.0	0.0	0.3	106	0
SCN 285	Steele	0.0	0.0	0.0	0.0	10.3	0.0	15.0	104	5.7
SCN 325	Grand Forks	0.0	0.0	0.0	0.0	0.0	0.0	49.8	54	7*
SCN 330	Traill	0.0	11.7	0.0	0.0	10.0	0.0	13.9	81	2.5.7*
SCN 333	Traill	0.0	0.0	0.0	0.0	4.3	0.0	15.5	55	7*
SCN 339	Traill	0.0	9.3	0.1	0.0	7.4	0.0	3.8	210	0
SCN 342	Traill	27.2	48.8	1.2	0.0	40.7	3.4	34.2	81	1.2.5.7*
SCN 344	Traill	39.0	30.1	0.1	0.3	39.4	3.1	29.8	281	1.2.5.7
SCN 350	Traill	0.0	10.1	0.0	0.0	10.1	0.0	14.4	232	2.5.7
SCN 351	Traill	0.0	1.2	0.0	0.0	8.6	0.0	9.7	185	0
SCN 355	Steele	0.0	0.0	0.0	0.0	1.9	0.0	3.9	52	0*
SCN 357	Steele	0.0	7.2	0.0	0.3	9.6	0.0	12.5	234	7
SCN 392	Steele	0.3	13.5	0.1	0.0	19.0	0.0	30.9	173	2.5.7

[a] Within the study period we were not able to repeat the HG type tests for all 73 of the SCN populations tested, however the occurrence of each of the HG types detected was validated, by repeating the HG type tests for thirty-three arbitrarily selected samples. Average no. of white females for each of the seven indicator lines and the susceptible check was calculated from four replicates.

[b] FI = (average no. of white females found on the indicator line/average no. of white females found on the susceptible check) x 100.

[c] Average number of white females in the susceptible check Barnes.

[d] HG types from assays in which the average number of white females on the susceptible check was greater than 50 but less than 100 are indicated with the asterisks (*) symbol.

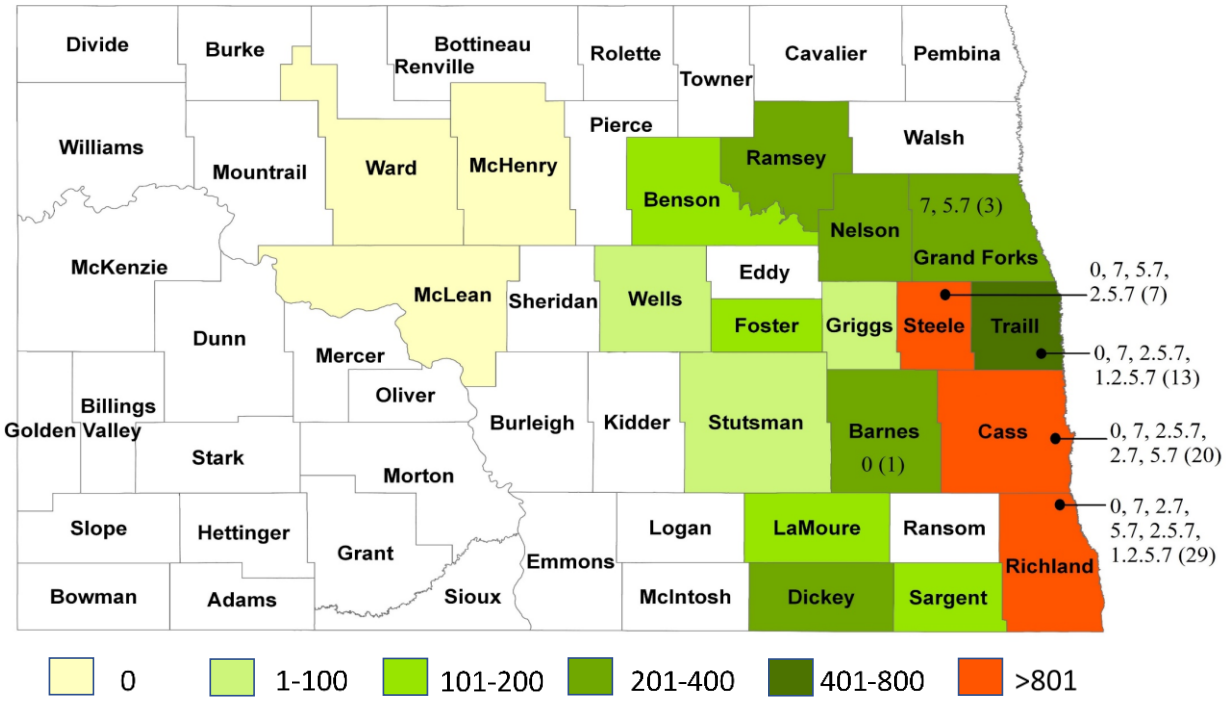

Fig. 3.1. Distribution of *Heterodera glycines* and their virulence phenotypes (HG types) in North Dakota (ND). Each of the 22 major soybean producing counties, that were surveyed, were color coded based on their mean SCN population density in SCN positive fields. The color codes and their corresponding mean SCN population density range in eggs and juveniles/100 cm^3 of soil are shown on the bottom of the map. The population density of SCN eggs and juveniles in SCN positive individual fields of ND ranged from 90 to 92,800 eggs and juveniles per 100 cm^3 of soil. The numbers within each county represents the HG types found in those counties, and the number inside the parenthesis represents the number of samples tested from those counties.

Data analysis

The average number of white females from the four replicates of each indicator line and the susceptible check were used to calculate the FI value. The statistical software SAS 9.4 (SAS Institute, Cary, NC) was used to analyze the data and determine the descriptive statistics. Additionally, SAS 9.4 was used to determine the Pearson's product moment correlation between the FI values of different indicator lines (Acharya et al. 2016; Niblack et al. 2003). The samples in which SCN could not be detected were excluded from analysis. The mean population density in SCN positive fields and the HG types detected in each county were plotted into a North

Dakota county map (Fig. 3.1) using geographic information system techniques with ArcGIS Pro software (Environmental Systems Research Institute, Redlands, CA).

Results

Out of the 419 soybean fields sampled, 176 (42%) fields were tested positive for the SCN. The SCN positive samples came from 14 of the 22 counties sampled. The SCN population densities of these samples ranged from 30 to 92,800 eggs and juveniles per 100 cm^3 of soil. The majority of the SCN positive counties were in the south-eastern part of North Dakota (Fig. 3.1), and the sample with the greatest population density was collected from a field in the south-eastern county, Richland. The average population density from the positive samples was 3,873 eggs and juveniles per 100 cm^3 of soil. Out of the 176 SCN positive fields detected, 79 (45%) had a population density greater than 500 eggs and juveniles per 100 cm^3 of soil. A total of 73 SCN field populations were successfully virulence phenotyped using HG typing tests. Although the remaining samples did have detectable levels of SCN eggs and juveniles, they did not produce enough eggs and juveniles for HG typing tests within the period of this study.

Out of the 73 SCN populations tested, 3 (4.1%) were able to parasitize PI 548402 (HG type indicator line #1) having a FI $\geq$ 10 (Table 3.1 and 3.4). Two of these populations originated from Traill County and the other one was from Richland County (Table 3.1). The FI for all three of these populations on PI 548402 were from 10 to 26% (Table 3.1 and 3.4). These three populations were also HG typed for a second time to confirm their HG type designations. In the repeated tests, the FI of the two samples from Traill County on PI 548402 were 27 and 39%, whereas the sample from Richland County had a FI of 12% (Table 3.3).

Twenty-six percent of the SCN populations tested were able to produce FI $\geq$ 10% on PI 88788 (#2). The FI among the SCN populations that were able to parasitize PI 88788 ranged

from 11 to 40% (Table 3.1, 3.2, and 3.4). The highest FI on PI 88788 was produced by a SCN population from Traill County (Table 3.1). Moreover, this population produced the highest FI in HG type tests conducted for repetition purposes as well (Table 3.3). The remaining SCN populations that attacked PI 88788 were collected from Cass, Richland, Steele, and Traill counties, and the percentage of SCN populations that attacked PI 88788 in each of these counties were 30, 21, 29, and 39%, respectively. Furthermore, three of the SCN populations from Traill County and one of the populations from Cass County had FI values ≥ 30% on PI88788 (Table 3.5).

The soybean line PI 209332 (#5) had the second-highest number of SCN populations from North Dakota. Thirty-four percent of the SCN populations tested had FI ≥ 10% on PI 209332. The highest FI among the SCN populations reared on PI 209332 was 46% (Table 3.4), and this population was collected from a field in Richland County (Table 3.1). This SCN population had the third-highest FI (20%) among the repeated HG type tests conducted (Table 3.3). The remaining SCN populations that attacked PI 209332 had FI values between 10 and 42% (Tables 3.1 and 3.2). These field populations were collected from soybean fields in Cass, Grand Forks, Richland, Steele, and Traill counties. The percentage of SCN populations in each of these counties with FI ≥ 10% on PI 209332 were 30, 33, 35, 57, and 39%, respectively. Furthermore, three SCN populations from Cass County and one of the populations from each of Richland, Steele, and Traill counties produced aggressive response on PI 209332, having FI ≥ 30% (Table 3.5).

Table 3.4. Univariate descriptive statistics of female index (FI) of *Heterodera glycines* populations from North Dakota that produced FI ≥ 10 on soybean indicator lines with resistance to *H. glycines*[a]

	Female index				
Soybean indicator line[b]	FI ≥ 10%[c]	Minimum observed	Maximum observed	Mean	Standard deviation
PI 548402 (#1)	4.1	10.0	25.5	20.3	7.3
PI 88788 (#2)	26.0	10.8	39.7	21.5	9.1
PI 209332 (#5)	34.2	10.0	46.3	21.7	11.0
PI 548316 (#7)	64.4	10.3	61.1	27.3	15.4

[a] Female indices from the repeated HG type tests were not included in the analysis as they are repeat experiments conducted for the confirmation purpose.
[b] None of the *H. glycines* populations were able to produce FI ≥ 10 on soybean indicator lines PI 90763 (#3), PI 437654 (#4) and PI 89772 (#6). Therefore, they were not included in the table.
[c] Percentage of population having a female index greater than or equal to 10.

The HG type indicator line #7 (PI 548316) was the most commonly infected soybean indicator line in North Dakota. More than 64% of the SCN populations tested had FI values ≥ 10% on PI 548316. The highest FI among the SCN populations virulent on PI 548316 was 61% (Table 3.4), and two samples from Richland County had this FI value (Table 3.1). The HG type test for one of these SCN populations (sample ID: SCN 52*) was repeated. In the repeated experiment the SCN population with the sample ID 52* also had high FI value on PI 548316 (Table 3.3). The remaining SCN samples that attacked PI 548316 had FI values between 10 and 60% (Tables 3.1 and 3.2). These SCN samples originated from Cass, Grand Forks, Richland, Steel, and Traill counties (Table 3.5). The percentage of SCN populations in each of these counties with FI ≥ 10% on PI 548316 were 60, 100, 59, 71, and 77%, respectively. Furthermore, 25, 33, 3, 43, and 15% of the SCN samples collected from Cass, Grand Forks, Richland, Steel, and Traill counties, respectively were found to have FI ≥ 30% on PI 548316 (Table 3.5). None of the SCN populations tested had a FI ≥ 10% on PI 90763 (#3), PI 437654 (#4), or PI 89772 (#6). The greatest FI produced on each of these indicator lines were 4, 3, and 7%, respectively (Table 3.1).

Table 3.5. Percentage of *Heterodera glycines* populations from North Dakota with female index (FI) ≥ 10% or ≥ 30% on resistant soybean lines in different counties of North Dakota[a]

	Counties											
	Barnes		Cass		Grand Forks		Richland		Steele		Traill	
Soybean indicator lines	FI ≥ 10	FI ≥ 30	FI ≥ 10	FI ≥ 30	FI ≥ 10	FI ≥ 30	FI ≥ 10	FI ≥ 30	FI ≥ 10	FI ≥ 30	FI ≥ 10	FI ≥ 30
PI 548402 (#1)	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0	15.4	0.0
PI 88788 (#2)	0.0	0.0	30.0	5.0	0.0	0.0	20.7	0.0	28.6	0.0	38.5	23.1
PI 209332 (#5)	0.0	0.0	30.0	15.0	33.3	0.0	34.5	3.4	57.1	14.3	38.5	7.7
PI 548316 (#7)	0.0	0.0	60.0	25.0	100.0	33.3	58.6	3.4	71.4	42.9	76.9	15.4
Total[b] (HG tested)[c]	3 (1)		58 (20)		8 (3)		66 (29)		7 (7)		16 (13)	

[a] Female indices from the repeated HG type tests were excluded from this table as they are repeat experiments conducted for the confirmation purpose.

[b] Total number of *Heterodera glycines* positive samples are outside the parenthesis.

[c] Total number of samples used in HG typing assays are in parenthesis.

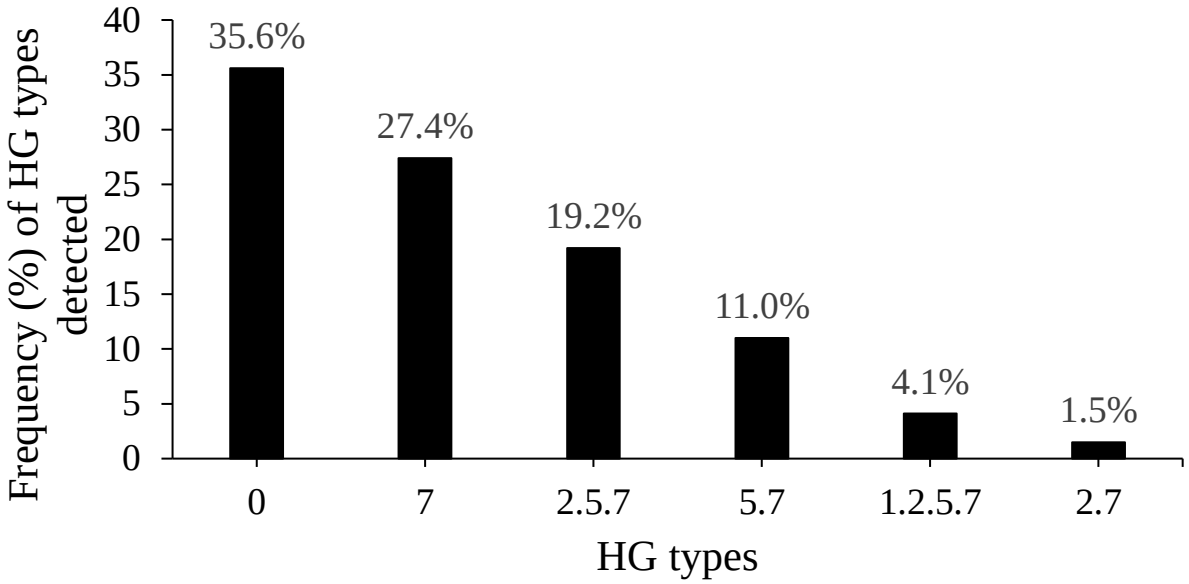

Fig. 3.2. Frequency of *Heterodera glycines* types detected in North Dakota from the 73 field populations tested. The x-axis represents *Heterodera glycines* types detected in North Dakota, and the y-axis represents the frequency of those HG types.

A total of six different HG types were detected in North Dakota and the most commonly detected SCN virulence phenotype was HG type 0. Out of the 73 SCN field populations tested, 36% were HG type 0, 27% were HG type 7, 19% were HG type 2.5.7, 11% were HG type 5.7, 4% were HG type 1.2.5.7, and 2% were HG type 2.7 (Fig. 3.2). Thirty-one of the 33 SCN populations that were HG type tested had the same HG type in both first and second tests (Table 3.3). One of the samples from Traill County was HG type 7 in the first test; however, in the second test, this sample was HG type 0 with a FI of 9.7% on PI 548316 (#7), barely short of the cutoff of FI ≥ 10% (Tables 3.1 and 3.3). Similarly, a sample from Steele County was HG type 5.7 in the first test, whereas, in the second test this sample's HG type was determined to be 7 with a FI of 9.6% on PI 209332 (#5; Tables 3.1 and 3.3)

Overall, 10, 8, 7, 3, 2, and 1 samples with HG type 0, 2.5.7, 7, 1.2.5.7, 5.7 and 2.7 in the first round of HG type tests, respectively were repeated and confirmed through the second round of the HG type tests (Table 3). The diversity in virulence phenotypes of SCN populations from

North Dakota varied between and within counties. Richland County had the greatest diversity in virulence phenotypes detected in North Dakota. In fact, each of the six different HG types detected in North Dakota was detected in Richland County. On the other hand, only one HG type was detected in Barnes County. Cass, Traill, and Steele counties had four different HG types detected, whereas, SCN populations of Grand Forks County only had two HG types (Fig. 3.1).

The FI values produced on the indicator lines PI 88788 (#2), PI 209332 (#5), and PI 548316 (#7) were positively correlated ($P < 0.0001$) with each other (Table 3.6). Additionally, there was a positive correlation between the FI values of PI 88788 (#2) and PI 548402 (#1) ($P < 0.0001$; Table 3.6). Although positive correlations ($P < 0.05$) were observed between the FI values of PI 548402 (#1), PI 90763 (#3) and PI 89772 (#6) (Table 3.6), stronger correlations were observed between the FI values of indicator lines #3 and #6 as well as #1 and #4 ($P < 0.0001$)

Table 3.6. Correlation coefficient among soybean lines with resistance to soybean cyst nematode based on female index (FI)[a] from 73 SCN field populations in North Dakota

Soybean lines	PI 548402 (#1)	PI 88788 (#2)	PI 90763 (#3)	PI 437654 (#4)	PI 209332 (#5)	PI 89772 (#6)
PI 88788 (#2)	0.61**[b]					
PI 90763 (#3)	0.41*	0.27*				
PI 437654 (#4)	0.64**	0.30*	0.44**			
PI 209332 (#5)	0.21ns	0.80**	0.18ns	0.03ns		
PI 89772 (#6)	0.41*	0.31*	0.74**	0.37*	0.25*	
PI 548316 (#7)	0.22ns[c]	0.50**	-0.03ns	0.11ns	0.64**	0.04ns

[a] Female index = (average no. of white females found on the indicator line/ average no. of white females on the susceptible check) x 100.
[b] * = $P < 0.05$, ** = $P < 0.0001$.
[c] ns = not significant, $P > 0.05$.

Discussion

This study investigated and characterized SCN virulence phenotypes prevalent in North Dakota State, one of the major soybean producing states in the U.S. SCN populations from North

Dakota were demonstrated for the first time to have positive host compatibility with soybean indicator lines that have resistance to SCN. The indicator line PI 88788 (#2) is the most commonly used source of resistance in the U.S. (Joos et al. 2013; Mitchum 2016; Tylka and Mullaney 2016; Yan and Baidoo 2018) and our results indicated that 26% of the SCN populations tested were virulent against this line. Repetition of the HG type tests conducted confirmed that there were some SCN populations in North Dakota that adapted to the most commonly used SCN resistance source PI 88788 (#2). Additionally, 4% of the SCN populations tested in this study were virulent on PI 548402 (#1), which is another common source of resistance (Joos et al. 2013; Mitchum 2016; Tylka and Mullaney 2016). Increased virulence on these commonly used resistance sources directly correlate with yield reduction in cultivars deriving resistance from them (Howland et al. 2018; McCarville et al. 2017). On the other hand, very few SCN populations in the upper Midwest (Acharya et al. 2016; MacCarville et al. 2017; MacGuidwin 2012; Niblack et al. 2003; Zheng et al. 2006) and none of the populations from North Dakota were virulent against resistance sources such as PI 90763 (#3) or PI 437654 (#4). This highlights the importance of rotating between resistance sources in commercially available SCN-resistant cultivars for North Dakota growers because SCN populations can become virulent on commonly used resistance sources such as PI 88788 (#2).

Monitoring the diversity and distribution of SCN virulence phenotypes in the state is crucial for directing breeding programs and establishing management recommendations for growers (Howland et al. 2018; Niblack et al. 2002). Our results indicate there has been a shift in SCN virulence phenotypes in North Dakota, since its first detection in Richland County during 2003 (Bradley et al. 2004). Prior to this study, only HG type 0 was identified in infested fields of North Dakota (Poromarto and Nelson 2009). Since then, we detected a total of six different HG

types, including 0, 7, 2.7, 5.7, 2.5.7, and 1.2.5.7, in the soil samples collected during 2015, 2016, and 2017. Although our results showed HG type 0 to be the most prevalent virulence phenotype in North Dakota, HG types 7 and 2.5.7 were the second and third most prevalent HG types.

When looking at the HG types detected on a regional basis, similar results were observed in the HG type surveys of neighboring states, South Dakota and Minnesota, where HG types 0, 7, and 2.5.7 were the three most prevalent HG types (Acharya et al. 2016; Zheng et al. 2006). Additionally, in these neighboring states, PI 548316 (#7) was the most commonly parasitized indicator line followed by PI 88788 (#2) and PI 209332 (#5) (Acharya et al. 2016; Zheng et al. 2006), which is similar to this study. Thus, a regional pattern in HG type diversity is observed and may be attributed to similarity in cropping practices, available cultivars, and environmental conditions.

Although, 4% of the SCN populations tested in South Dakota had FI ≥ 10% on both PI 90763 (#3) and PI 89772 (#6) (Acharya et al. 2016), none of the SCN populations from North Dakota had a FI ≥ 10% on either of these two indicator lines. A possible explanation for this difference is that SCN was detected in South Dakota much earlier than North Dakota, consequently South Dakota has a longer history of planting SCN-resistant cultivars (Acharya et al. 2016; Smolik et al. 1996). In fact, in other midwestern states such as Kansas, Missouri, and Nebraska that have a much longer history of SCN infestation and planting resistant cultivars, several super virulent SCN populations have been detected (Broderick 2016; Mitchum et al. 2007; Rzodkiewicz 2010). These super virulent populations are virulent against all six of the seven indicator lines used in HG type testing, except for PI 437654 (#4). Furthermore, a super virulent population of SCN that reproduced on all seven indicator lines has been reported in China (Lian et al. 2017) that has the greatest history of SCN infestation (Li et al. 2011).

Nevertheless, North Dakota has a shorter history of SCN infestation and subsequent resistant cultivar use, which may partially explain why none of the SCN populations from North Dakota were able to adapt to PI 90763 (#3) and PI 89772 (#6).

However, history of planting resistant cultivars may not be the only factor that drives shifts in SCN virulence, as demonstrated by China's SCN virulence phenotype diversities in Northern and central regions. Despite having little to no pattern or history of using SCN-resistant cultivars prior to 1997, there was greater diversity in SCN virulence phenotypes in the central regions than in Northern China (Liu et al. 1997; Zheng et al. 2006). Therefore, it is possible that other factors such as climatic conditions in different geographical regions influence the fitness of SCN virulence phenotypes in each region, resulting in different SCN diversities in different regions (Zheng et al. 2006). Thus, lower SCN virulence phenotype diversity in North Dakota compared to neighboring states, may be partially due to North Dakota's climate, which is the second coldest state in the U.S.

One distinction of this study compared to a majority of the previously published SCN virulence phenotype surveys is the use of naturally infested soil as well as artificially inoculated sterilized soil in which HG type indicator lines and the susceptible check were planted during the HG type tests (Acharya et al. 2016; Howland et al. 2018; Niblack et al. 2002). Although artificially inoculating sterilized soil ensures only SCN is infesting the soil in which the soybean seedlings are planted, it takes considerable time to increase the SCN population to a sufficient level for inoculation purposes. Our results have shown if the field population density is higher than 400 eggs/100 cm^3 of soil, HG type tests can be conducted with naturally infested soil. Moreover, using naturally infested soil may better emulate field soil conditions and also make testing results available to growers quicker without increasing population first. An advantage of

using artificial inoculum is that an equal amount of inoculum can be added to each of the indicator lines as well as the susceptible cultivar. Although, it is difficult to achieve even distribution of inoculum in naturally infested soil, it can be obtained by thoroughly mixing the naturally infested soil prior to planting. For this study, we diligently ensured even distribution of inoculum by thoroughly mixing the soil by hand first to break up soil clumps, and then using a motorized cement mixer to mix the soil for 30 to 60 minutes depending on the soil texture.

Furthermore, to validate the designation of the HG types determined in this study, we repeated the HG type tests for 33 arbitrarily selected SCN populations. These repeated HG type tests were conducted by artificially inoculating sterilized sandy soil during planting Although within the study period we were not able to repeat the HG type tests for all the SCN populations, the HG types detected in the second iteration of the tests were consistent with the first iteration. Thus, suggesting that naturally infested soil can be used to conduct HG type tests, since similar results were observed when the same population was HG typed by using naturally infested soil and artificially inoculated soil.

The occurrence and frequency of SCN virulence phenotypes varied among the counties in North Dakota. The greatest diversity in SCN virulence phenotypes was detected in the south-eastern county, Richland. This may be attributed to Richland County having one of the highest soybean acreage and the longest history of SCN infestation in North Dakota (Bradley et al. 2004; USDA-NASS 2019). Richland County also shares its border with South Dakota and Minnesota, both of which has a longer history of SCN infestation than North Dakota (Macdonald et al. 1980; Smolik et al. 1996). This may explain why Richland county is the first North Dakota county to have detectable levels of SCN infestation (Bradley et al. 2004). Another such south-eastern North Dakota county that shares a border with Richland is Cass County. This county has the

highest soybean acreage in North Dakota (USDA-NASS 2019) and the second highest diversity in SCN virulence phenotypes according to our results. Furthermore, these eastern and south-eastern counties of North Dakota are characterized by higher precipitation and warmer climatic conditions than western counties of North Dakota (Chowdhury et al. 2019), which may influence the fitness of the virulence phenotypes under these climatic conditions in these counties.

A statistically significant positive correlation at significance level of was observed between the FI of the HG type indicator lines #2, #5 and #7. Similar results were reported in previous studies where significant positive correlations were found between the FI values on the indicator lines #2, #5, and #7 (Acharya et al. 2016; Niblack et al. 2003; Zheng et al. 2006). The reason may be that these three indicator lines belong to a group that share similar resistance mechanism and confer similar type of resistance response to SCN. The HG type indicator lines #5 and #7 were reported to belong to the PI 88788-type resistance group (Colgrove and Niblack 2008).

On the other hand, previous studies also reported positive correlations between the FI values on the indicator lines #1, #3, and #6 (Acharya et al. 2016; Niblack et al. 2003; Zheng et al. 2006). Moreover, the indicator lines #3 and #6 belonged to the Peking-type (#1) resistance group because these three indicator lines (#1, #3, and #6) shared similar resistance mechanism and conferred similar type of resistance response to SCN (Colgrove and Niblack 2008). In this study, positive correlations exist between the FI values of the indicator lines #1, #3, and #6. However, positive correlations between the FI values of the indicator #1 and #2 as well as #1 and #4 were also observed at a high significance level. These results maybe be explained by the fact that only three populations from North Dakota attacked the indicator line #1 having a FI $\geq$ 10% and all three of these populations also attacked indicator line #2. It is possible that the fields from which

these three populations were collected may have a history of using soybean cultivars that derived resistance from the indicator lines #1 as well as #2. Thus, the three SCN populations were able to overcome the resistance derived from both these indicator lines, as a result, they had higher FI on both the indicator lines in our HG type tests, leading to a stronger correlation between them in our correlation analysis. However, a majority of the SCN populations that attacked indicator line # 5 and #7 did not attack indicator lines #1. Thus, significant correlation between the FI values of indicator lines #1, # 5, and #7 were not observed.

Nonetheless, correlation analysis findings suggest, for example, the SCN populations that are able to overcome the resistance conferred by PI 88788 (#2) will most likely overcome the resistance conferred by PI 209332 (#5) and PI 548316 (#7). Thus, breeding efforts should consider resistance sources other than PI 209332 and PI 548316 to effectively manage SCN populations that are virulent against PI 88788. Furthermore, rotating between sources of resistance may delay the shift in SCN virulence phenotypes as the chances of SCN adapting to any particular resistance group get reduced (Acharya et al. 2016).

In conclusion, this study reports a shift in SCN virulence phenotypes in North Dakota has occurred since its first detection in 2003 (Bradley et al. 2004). This is a first report of five new HG types, namely 7, 2.5.7, 1.2.5.7, 5.7, and 2.7, that were detected in SCN field populations from North Dakota, in addition to HG type 0. The repeated HG type tests validated the occurrence of each of these HG types in North Dakota. Repetition of the HG type tests proved that there are several SCN populations in North Dakota that can successfully reproduce on indicator line #2 (PI 88788), the most widely used source of resistance (Yan and Baidoo 2018). This underscores the importance of further utilizing resistance sources other than PI 88788 in future breeding efforts. However, correlation analysis suggests that SCN populations that are

adapted to PI 88788 are more likely to adapt to resistance from PI 209332 and PI 548316, since they belong to the same resistance group. Thus, highlighting the importance of rotating between resistance groups to delay the adaptation of SCN (Acharya et al. 2016; Colgrove and Niblack 2008). Since only three population from North Dakota had FI ≥ 10% on PI 548402 (Peking), the indicator lines in the Peking-type resistance group can be an effective source of resistance to rotate with cultivars deriving resistance from PI 88788-type resistance group. Growers can also use SCN-resistant cultivar such as Hartwig (Anand 1992), which derives resistance from PI 437654 since none of the populations tested in this study had a FI ≥ 10% on PI 437654. However, previous greenhouse studies have shown that SCN populations can adapt to resistant cultivars that derives resistance from PI 437654 within a few successive generations (Colgrove and Niblack 2008). Thus, an integrated approach that includes crop rotation, utilization of SCN-resistant cultivar, and rotation between sources of resistance in SCN-resistant cultivars is highly recommended.

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CHAPTER 4: DEVELOPMENT OF REAL-TIME AND CONVENTIONAL PCR ASSAYS FOR IDENTIFYING A NEW SPECIES OF ROOT-LESION NEMATODE

Abstract

Root-lesion nematodes, *Pratylenchus* spp., are an important group of plant-parasitic nematodes that have a wide host range and worldwide distribution., An accurate and efficient identification method is required for their risk assessment and management. Distinction between root-lesion nematode species is difficult and time-consuming based on morphology using traditional microscopic methods. Hence, a rapid and accurate molecular method was developed in this study for detecting and identifying a new species of root-lesion nematode recently discovered in a soybean field in North Dakota. A species-specific primer set, that can be used in both conventional and quantitative real-time polymerase chain reaction (PCR) assays for identification of the new *Pratylenchus* sp., was designed from the internal transcribed spacer (ITS) region of ribosomal DNA. The specificity of the primer set was evaluated by both in silico analysis and laboratory PCR experiments. Results showed that only the target species DNA was exclusively amplified in both conventional and real-time PCR assays but none of the DNA from other *Pratylenchus* spp. and non-*Pratylenchus* control species, used in the specificity tests, were amplified. Detection sensitivity analysis revealed that the conventional PCR was able to detect an equivalent to 1/8 of the DNA of a single nematode, whereas, real-time PCR with higher sensitivity detected an equivalent to 1/32 of the DNA of a single nematode. According to the generated standard curve the amplification efficiency of the primer set in real-time PCR was 94% with a R^2 value of 0.95. To determine the ability of the assays to distinguish the new *Pratylenchus* sp. from other *Pratylenchus* spp. commonly detected in North Dakota (ND) soybean fields, a total 21 soil samples were tested from seven major soybean producing counties

of ND. Both conventional and real-time PCR assays successfully amplified the DNA of the target nematode and discriminated it from *P. scribneri*, *P. neglectus*, and another new *Pratylenchus* sp. from ND soybean fields. This is the first report of a species-speccific detection method for identification of the new *Pratylenchus* sp. recently discovered in ND. The new PCR assays developed in this study are rapid and accurate, thus, suitable for use in diagnostic laboratories for the detection of field infestations with the new *Pratylenchus* sp.

Keywords: Detection, DNA, endomigratory, identification, in silico analysis, ITS rDNA, conventional PCR, *Pratylenchus*, real-time PCR, and root-lesion nematode.

Introduction

Root-lesion nematodes, *Pratylenchus* spp. are one of the most economically important nematode pests of crops worldwide (Sasser and freckman 19870). These plant-parasitic nematodes have a migratory endoparasites nature, ability to reproduce sexually and/or asexually, excellent adaptability to diverse environmental conditions, and broad host range (Castillo and Volvas 2007). Thus, they are ranked as the third most important group of plant-parasitic nematodes after root-knot nematodes and cyst nematodes (Jones et al. 2013). Furthermore, there are over 70 described species of *Pratylenchus* affecting crops of major economic importance (Davis and MacGudwin 2000), including cereals, coffee, fruits, ornamental crops, peanuts, soybeans, and vegetables, resulting in serious economic losses for growers throughout the world (Castillo and Volvas 2007; Davis and MacGudwin 2000).

Numerous studies have reported high yield losses caused by root-lesion nematodes in infested crop fields. In Australia, *P. thornei* was reported to cause yield losses in wheat as high as 85% (Taylor et al. 1999). In Oregon USA, *P. neglectus* reduced the yield of intolerant wheat cultivars by 36% (Smiley et al. 2005). In Norway, *P. penetrans* caused tuber lesions and reduced

potato yield by 50% in affected fields (Holgado et al. 2009). In Brazil, *P. brachyurus* was reported to cause 21% yield reduction in soybean fields (Lima et al. 2015). Hence, appropriate and effective management of these nematode diseases is critical for improving crop production worldwide. The most commonly used management strategies against *Pratylenchus* spp. include crop rotation and host resistance (Thompson et al. 2008). Choice of crops available for rotation depends on the identity of the species of root-lesion nematode present in the field because the host range of each *Pratylenchus* sp. can vary and only a few plant species and cultivars are known to be immune or possess some level of resistance against these plant-parasitic nematodes (Castillo and Volvas 2007).

The identity of root-lesion nematodes is often determined using traditional microscopic methods, which utilizes subtle morphological differences such as vulval position, stylet length, body length, postuterine sac length, and lip annule number (Castillo and Volvas 2007; Handoo et al. 1989). However, such methods are highly time-consuming because it requires experienced nematologists to collect detailed microscopic measurements from multiple nematode specimens from each field to generate mean measurement values that could be used for accurate species identification (Castillo and Volvas 2007; Handoo et al. 1989). Furthermore, multiple species of *Pratylenchus* can coexist in a single field, making it more challenging to identify *Pratylenchus* spp. (Kimpinski 1979; Luc 1987; Wheeler et al. 1992). On the other hand, molecular technologies provide several rapid, reliable, and efficient DNA-based diagnostic methods for the species identification of root-lesion nematodes.

One of the first such molecular diagnostic studies on root-lesion nematodes was described by Pinochet et al. (1994) that identified and differentiated isolates of *P. vulnus* using random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR). Since then,

numerous studies have identified and differentiated *Pratylenchus* spp. to their species level using DNA-based molecular diagnostic methods such as PCR restriction fragment length polymorphism (RFLP), species-specific PCR, and DNA sequencing (Al-Banna et al. 2004; Baidoo et al. 2017; Huang and Yan 2017; Oliveira et al. 2017; Uehara et al. 1998; Yan et al. 2008, 2012, 2013). The RFLP potion of the RFLP-PCR is an additional time-consuming step that is not amenable to commercial high-throughput applications (Yan et al. 2008). Although recent advances in sequencing technology simplified the sequencing process, it can still be relatively expensive when a large number of samples need to be diagnosed. Species-specific PCR being a simple, rapid, and relatively inexpensive method, is commonly used in nematology research for diagnostic purposes (Huang and Yan 2017; Jones et al. 2013).

Conventional species-specific PCR method uses PCR amplification with species-specific primers followed by gel electrophoresis to successfully identify the species of the target organism based on bands visible in agarose gel. These species-specific primers are often designed based on sequence information of ribosomal DNA (rDNA) such as D2-D3 expansion region of 28S rDNA and internal transcribed spacer (ITS) of rDNA (Castillo and Volvas 2007; Huang and Yan 2017). Additionally, specific genes such as cytochrome c oxidase gene, β-1,4-endoglucanase gene, and major sperm protein gene are commonly used to design species-specific primers in plant-nematology (Castillo and Volvas 2007).

Al-Banna et al. (2004) developed conventional PCR assays with five forward species-specific primers, PNEG, PSCR, PPEN, PTHO, and PVUL, to identify and differentiate *P. neglectus*, *P. scribneri*, *P. penetrans*, *P. thornei*, and *P. vulnus*, respectively. Each of the five forward primers and a single common reverse primer were designed from the D3 expansion region of 28S rDNA. However, the difference in amplicon size between some of the target

species) was as little as two base-pairs. Thus, Yan et al. (2008) modified and designed the PNEG-F1/D3B5 primer set, that could specifically identify *P. neglectus* by conventional PCR. Additionally, Yan et al. (2008) optimized the amplification conditions for the PTHO/D3B primer set to successfully identify *P. thornei*. Mekete et al. (2011) designed the PP5 primer set from ITS rDNA region to identify *P. penetrans* using conventional PCR and multiplex PCR assays. Huang and Yan (2017) designed the PSF7/PSR7 primer set from the ITS-rDNA region to identify *P. scribneri*. Moreover, Huang and Yan (2017) reported that the PSF7/PSR7 primer set was equally effective in identifying *P. scribneri* using conventional as well as real-time PCR. Compared to conventional PCR, real-time PCR is a molecular diagnostic technique that has higher detection sensitivity, provides quantitative data, and allows real-time data monitoring (Whittwer et al. 1997). Many other studies have also developed species-specific primers for detection of root-lesion nematode species such as *P. brachyurus, P. coffeae*, *P. crenatus, P. loosi, P. neglectus*, *P. penetrans*, *P. scribneri, P. thornei,* and *P. zeae* using species-specific PCR or real-time PCR (Arora et al. 2020; Baidoo et al. 2017; Berry et al. 2008; Machado et al. 2007; Uehara et al. 1998; Yan et al. 2012, 2013).

In North Dakota, during 2015 and 2016, soil surveys of soybean fields were conducted to determine the prevalence and distribution of plant-parasitic nematodes in these fields (Yan et al. 2017a). During the surveys, six soil samples were collected from a soybean field in Walcott, Richland County, ND. After extracting nematodes from these samples, it was evident that root-lesion nematodes were present in all of these samples with population densities ranging from 125 to 2,000 nematodes per kg of soil. Morphological measurement of adult male and female, as well as DNA sequencing of 28S D2-D3 (GenBank accession: KX889989) and ITS rDNA (KX889990), revealed that this nematode differs from other known species of root-lesion

nematodes in both morphology and DNA sequences (Yan et al. 2017a). Greenhouse experiments proved that this isolate of *Pratylenchus* reproduced well on soybean with a reproduction factor (final population/initial population) as high as 5.02. Thus, allowing us to conclude that, this is a new species of *Pratylenchus* that had never been reported in the literature prior to 2017, consequently, no molecular diagnostic method exists to identify this nematode species prior to this study. In order to detect and distinguish the new species of *Pratylenchus* from other *Pratylenchus* spp., new species-specific primers need to be exploited for the development of an efficient and sensitive detection system.

The objective of this research was to develop conventional and real-time PCR assays with species-specific primers for rapid, reliable, and sensitive detection of the new species of *Pratylenchus*, and to evaluate the capability of the assays to distinguish and discriminate between this new species and other commonly found *Pratylenchus* spp. in ND soybean fields.

Materials and methods

Soil sampling and nematode extraction from soil

Soil samples were collected from a soybean field in Walcott, Richland County, ND, that was previously reported to have been infested with the new species of *Pratylenchus* investigated in this study (Yan et al. 2017a). Nematodes were then extracted from the soil samples using the standard hand sieving, decanting, and sugar centrifugal-flotation method (Jenkins 1964). Briefly, this method relies principally on agitating the soil in tap water (200 ml) through stirring and pouring the mixture through a stack of sieves (250 µm and 20 µm apertures) to separate larger organic and inorganic soil particles from nematodes and smaller soil particles. Next, the smaller soil particles and nematodes that are collected in the 20-µm-aperture sieve were separated from each other by suspending them in a 1.3 M sugar solution (American Crystal Sugar Company,

Moorhead, MN). Once suspended, the solution was centrifuged at 4,000 RPM for 30 s. The supernatant containing the vermiform nematodes was poured through 20-µm-aperture sieve to remove the sugar solution and the nematodes were then rinsed with tap water and collected in 15 ml tap water for quantification, and the pellet containing the soil particles was discarded. Subsequently, root-lesion nematodes were identified and enumerated based on their key morphological features (Castillo and Volvas 2007; Mai et al. 1996) under a dissecting microscope (Zeiss Stemi 305 lab microscope; Zeiss, Thornwood, NY) at 80 X magnification.

Root-lesion nematode isolation and DNA extraction

Individuals of the *Pratylenchus* spp. identified to genus level were then isolated from the nematode suspension, and the proteinase K method described by Kumari and Subbotin (2012) was then used to extract DNA from the nematodes. According to this method, individual nematodes were hand-picked using a dental pick, placed into a concave slide containing 10 µl of sterilized double-distilled water, and cut into two pieces using the dental pick under the dissecting microscope. The 10 µl suspension containing the nematode pieces was then pipetted into a 0.5 ml sterile centrifuge tube containing 10 µl of extraction buffer (2 µl of 10 X PCR buffer, 2 µl of proteinase K [µg/ml], and 6 µl double-distilled water). The tubes containing the extraction buffer and the nematode suspension were then vortexed and frozen at -20 °C for at least 30 min. Subsequently, the tubes were incubated at 65 °C in a water bath for 1 h followed by incubation at 95 °C for 10 min to inactivate the proteinase K (Kumari and Subbotin 2012).

Species-specific PCR primer design and development

The internal transcribed spacer (ITS) rDNA sequence of the new species of *Pratylenchus*, with the accession number KX889989 (Yan et al. 2017a), was acquired from GenBank. Additionally, 16 ITS rDNA sequences from 11 other *Pratylenchus* spp. were collected from

GenBank. These ITS sequences with the corresponding accession numbers contained two isolates each of *P. agilis* (FJ712891.1 and KC952982.1), *P. neglectus* (LC030328.1 and LC030323.1), *P. pseudocoffeae* (LC030337.1 and LC030339.1), *P. scribneri* (KT873860.1 and KX842626.1), and *P. thornei* (FR692305.1 and FR692304.1) and one isolate each of *P. alleni* (JX081545.2), *P. gutierrezi* (KT971367.1), *P. hippeastri* (KR029085.1), *P. jaehni* (FJ712937.1), *P. loosi* (KY424222.1), and *P. penetrans* (LC030336.1). The ITS sequence of the new species of *Pratylenchus* together with the ITS sequences of the 11 other *Pratylenchus* spp. were aligned using the Clustal W tool of the BioEdit software version 7.0.9.0 (Hall 1999). The multiple sequence alignment was used to identify and design a pair of primers, IC-ITS1F (forward, 5'-TGTGTGCGAATGTTCCTG-3') and IC-ITS1R (reverse, 5'-CGTATGTTTTATATGGGGACTC-3'), within the diverse region of ITS rDNA among the different species of *Pratylenchus*. The primer set was then assessed on the basis of optimal annealing temperature, GC content, and potential for duplex formation through hairpin formation, self-dimerization, and heterodimer formation using OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc).

The primers were initially screened for specificity using the BLAST search function of National Center for Biotechnology Information (NCBI) to determine if the primers matched with other non-target nucleotide sequences in the nucleotide collection (nr/nt) database. Moreover, the specificity of the primer set was predicted in silico against the 16 ITS rDNA sequences used to design the primer in addition to the ITS sequences of 16 other important *Pratylenchus* spp. isolates collected from GenBank (Table 4.1). Primer specificity was determined in silico by evaluating the primer-template duplex stability values (ΔG) calculated using the OligoAnalyzer 3.1, as described by Schroeder et al. (2006) and Yan et al. (2012). The primers were synthesized

by Eurofin MWG Operon LLC (Huntsville, AL). The performance of the designed primer set in PCR amplification was then evaluated at different annealing temperatures (56, 58 and 60 °C) using DNA from the new *Pratylenchus* sp. along with DNA from *P. neglectus*, *P. penetrans*, *P. scribneri*, and *P. thornei*, and 58 °C was determined to be the optimal annealing temperature at which the primer pair performed the best.

Detection specificity of the primers in PCR

The designed primer set was further evaluated for detection specificity through conventional and real-time PCR using DNA from multiple isolates of the target species as well as multiple isolates of five other confirmed species of *Pratylenchus* (Table 4.2). They include two isolates of *P. scribneri* and *P. neglectus*, one isolate of *P. thornei* and *P. penetrans*, and one isolate of another newly identified *Pratylenchus* sp. (Table 4.2, sample ID: Hg 51). Furthermore, DNA from eight other genera of plant-parasitic nematodes including *Paratylenchus* sp., *Paratrichodorus* sp., *Tylenchorhynchus* sp., *Helicotylenchus* sp., *Heterodera glycines*, *H. schatii*, *Hoplolaimus* sp., and *Xiphinema* sp. as well as two genera of non-plant-parasitic nematodes were used in the specificity test (Table 4.2, sample ID: NPN1 and NPN2). For each nematode population used in the specificity testing, three independent DNA extractions were conducted and used as biological replicates.

Table 4.1. Isolates of *Pratylecnhus* spp. and their corresponding GenBank accession numbers of internal transcribed spacer (ITS) sequences used for analysis of primer-template duplex stability (ΔG) for predicting the specificity of primers, IC-ITS1F and IC-ITS1R

Species	GenBank accession	Isolate or clone[a]	Origin	Sequence length (bp)	ΔG (kcal/mole)[b]	
					IC-ITS1F	IC-ITS1R
Pratylenchus sp.[c]	KX889990.1	Hg50	ND, USA	1,126	-33.5	-38.4
Pratylenchus sp.[d]	KY200666.1	Hg51	ND, USA	981	ins	ins
P. agilis	KC952982.1	SX/Clone7	P.R. China	882	ins	ins
P. agilis	FJ712891.1	PagKL5/Clone5	Belgium	880	ins	ins
P. alleni	JX081545.2	N/A	Canada	1,080	ins	ins
P. bolivianus	HM469446.1	TW2	P.R. China	1,163	ins	ins
P. brachyurus	MH020807.1	AD70	Spain	627	ins	ins
P. coffeae	JX046940.1	YT/Clone4	P.R. China	1,102	ins	ins
P. convallariae	HM469448.1	N/A	P.R. China	722	ins	ins
P. crenatus	LC030310.1	He1/Clone1	Japan	928	ins	ins
P. crenatus	LC030308.1	Pcr-H01/Clone1	Japan	958	ins	ins
P. fallax	KY828273.1	V4C/Clone180	Belgium	755	ins	ins
P. goodeyi	KF700243.1	CICR-Cot.Warud	India	782	ins	ins
P. gutierrezi	KT971367.1	O22_1	Spain	906	ins	ins
P. hippeastri	KR029085.1	QIXIA	China	1,185	ins	ins
P. jaehni	FJ712937.1	PjaKL1/Clone	Belgium	997	ins	ins
P. lentis	AM933154.1	Individual Nematode 10	Italy	703	ins	ins
P. loosi	KY424222.1	EX11	P.R. China	1,212	ins	ins
P. mediterraneus	FR692306.1	N/A	Italy	946	ins	ins
P. neglectus	LC030328.1	NM1/Clone8	Japan	852	-14.4	ins
P. neglectus	LC030323.1	HT2KU1/Clone1	Japan	871	-14.4	ins
P. neglectus	LC030325.1	HKf1/Clone6	Japan	855	-14.4	ins
P. penetrans	LC030336.1	HM1/Clone3	Japan	874	ins	ins
P. pinguicaudatus	KY828261.1	T572/Clone168	Belgium	762	ins	ins
P. pratensis	KY828311.1	T616/Clone 232	Belgium	807	ins	ins
P. pseudocoffeae	LC030337.1	Pps-KM1/Clone1	Japan	1,090	ins	ins
P. pseudocoffeae	LC030339.1	Pps-KM1/Clone7	Japan	1,090	ins	ins
P. scribneri	KT873860.1	ND	ND, USA	1,103	ins	ins
P. scribneri	KX842626.1	F21	ND, USA	1,103	ins	ins
P. scribneri	JX046934.1	XC/Clone2	P.R. China	957	ins	ins
P. thornei	FR692305.1	Pt_Je_SP_cl13	Italy	1,001	ins	ins
P. thornei	FR692304.1	PT_SI_IT_cl19	Italy	974	ins	ins
P. vulnus	KY424232.1	JSR	China	925	ins	ins
P. zeae	FJ643590.1	N/A	Republic of China	967	ins	ins

[a] Names of isolates or clones were obtained from GenBank. N/A indicates that the information is not available in GenBank.

[b] Values of ΔG < -31 kcal/mol indicate stable primer-template duplex formation for PCR amplification, conversely, values > -31 kcal/mol indicate poor primer-template duplex stability for PCR amplification; 'ins' indicate very poor or insignificant primer-template duplex stability values.

[c] The isolate Hg50 is the target species of *Pratylenchus* investigated in this study.

[d] The isolate Hg51 is another species of *Pratylenchus* newly identified in North Dakota (ND), USA, which is different from the target species.

Conventional PCR assay

The presence and quality of the template in DNA extracts used in this study were verified using conventional PCR amplification of the ITS1 rDNA with the universal primer set

rDNA2/rDNA1.58s (Cherry et al. 1997). The PCR amplification conditions were initial denaturing at 94 °C for 3 min followed by 40 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; with a final extension at 72 °C for 10 min, as suggested by Cherry et al. (1997). The Bio-Rad T100 thermal cycler (Hercules, CA) was used to conduct all PCR amplification reactions for this study. For the newly designed primer set, IC-ITS1F/IC-ITS1R, the species-specific conventional PCR reaction mixture (20 µl) consisted of 1.5 µl of DNA template, 0.5 µM forward and reverse primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1X Green GoTaq Flexi buffer, 1 unit of GoTaq Flexi DNA polymerase (Promega Corp., Madison. WI), and 11.2 µl of double-distilled water. The amplification conditions for the designed primer set were initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturing at 94 °C for 40 s, annealing at 58 °C for 50 s, extension at 72 °C for 1 min, and a final extension for 10 min at 72 °C. After DNA amplification, 8 µl of the PCR products were added to the wells of a 2% agarose gel and stained with ethidium bromide. The PCR products in the gel were then separated by gel electrophoresis for 30 min at 90 V. The banding pattern of the PCR products was visualized under UV light and photographed using AlphaImager Gel Documentation system (Proteinsimple Inc., Santa Clara, CA). For each sample, conventional PCR reactions were conducted with three biological replicates.

Real-time PCR assay

To determine whether the IC-ITS1F/IC-ITS1R primer set can be used in real-time PCR assay for identification of the new species of *Pratylenchus*, a SYBR-based real-time PCR assay was conducted. In the real-time PCR assay, a 12 µl qPCR reaction mixture containing 1.5 µl of DNA template, 6 µl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.5 µl of each forward and reverse primer (10 µM), and 3.5 µl of nuclease-free PCR grade water was used. In

each experiment, non-template controls containing nuclease-free PCR grade water in place of DNA were included. The real-time PCR amplification was performed using the Bio-Rad CFX96 Touch Real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). The cycling conditions for the real-time PCR amplification were incubation at 95 °C for 4 min followed by 35 cycles of 95 °C for 10 s and 58 °C for 30 s. A melting curve temperature profile was generated by increasing the temperature of the reaction from 65 to 95 °C with a ramp rate of 0.5 °C s^{-1} to evaluate the amplification specificity. Each reaction was run in triplicate as technical replicates and three independent biological replicates were assessed for each sample, thus generating nine observations per sample in real-time PCR. The Bio-Rad CFX Manager Software (V3.1) was used to analyze the real-time PCR data, and the quantification cycle value (Cq) for each reaction was determined at default settings. The real-time PCR product identity was also checked by electrophoresis on a 2% agarose gel as described above.

Detection sensitivity of PCR assays

The sensitivity of the assays was investigated to establish a detection limit for the conventional and real-time PCR assays. The sensitivity, which refers to the minimum number of target nematode individuals that could produce visible amplicons in the gel electrophoresis portion of the conventional PCR assay or generate a Cq value lower than 34 in the real-time PCR assay (Huang et al. 2017b; Huang and Yan 2017), was evaluated using a sequential two-fold serial dilution of DNA extracted from four individuals of the target nematode. Thus, DNA extracted from four nematodes were diluted nine times down to 1/64 equivalent of a single nematode (4, 2, 1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64). In a real-time PCR assay, each sequential dilution from three independent DNA extracts was then used as biological replicates and the PCR assay was conducted in triplicates for each dilution of each biological replicate as technical

replicates. This resulted in nine observations for each dilution level in real-time PCR. The Cq values from each dilution level were then plotted against the corresponding $\log_{10}$ of equivalent target nematode individuals to generate a standard curve, from which coefficient of determination (R^2) and amplification efficiency were calculated using the equation $E= 10^{(1/-m)} – 1$, where m is the slope of the equation. The same DNA templates from the sequential serial dilution of the first biological replicate were used to determine the detection limit for the assay designed for conventional PCR.

Isolation, identification and verification of root-lesion nematode species collected from soybean fields

During 2019, twenty soil samples were collected from ND soybean fields, or fields with a history of soybean production, that were suspected to be infested with other *Pratylenchus* spp. based on our previous research (Akhter 2020, Chowdhury et al. 2019, and unpublished data). One of these 20 soil samples was collected from a soybean field neighboring the field in which the new *Pratylenchus* sp. was first discovered. Additionally, in 2019, a soil sample was also collected from the field in which the new *Pratylenchus* sp. was first discovered. Nematodes from these samples were used to test whether the PCR assays could accurately detect and differentiate this new species from other species of *Pratylenchus* (Table 4.3). In each field, sampling was conducted in a zig-zag pattern starting 76 m (100 paces) away from the field entrance, by collecting soil from 20-25 sampling points using a 2.5 cm diameter soil probe from depths of 1-30 cm below the soil surface (Chowdhury et al. 2019). After compositing and thoroughly mixing the soil samples collected from each field, nematodes were extracted from a 100 cm^3 subsample using the sugar centrifugal-flotation method (Jenkins 1964) described previously. Nematodes belonging to the genus *Pratylenchus* were identified and enumerated based on morphological

features using a compound microscope (100 X magnification) and the density of *Pratylenchus* sp. in the 100 cm^3 soil subsample was calculated. Individuals of *Pratylenchus* sp. were then isolated from each sample using a dental pick, and DNA was extracted using the proteinase K method (Kumari and Subbotin 2012), as described previously. For each sample, single nematode DNA extractions were conducted independently from three different individuals of *Pratylenchus* as three biological replicates. Presence of DNA was confirmed using PCR with the universal primer set rDNA2/rDNA1.58s (Cherry et al. 1997). Conventional species-specific PCR was then conducted to identify the *Pratylenchus* sp. in each sample. The primers used in species-specific PCR included PsF7/PsR7 (Huang and Yan 2017), PNEG-F1/D3B5 (Yan et al. 2008), PTHO/D3B (Yan et al. 2008), and PP5F/PP5R (Mekete et al. 2011), which are specific to *P. scribneri*, *P. neglectus*, *P. thornei*, and *P. penetrans*, respectively. The amplification conditions for the species-specific PCR followed the corresponding authors' recommendations. Moreover, each DNA sample was tested in the conventional and real-time PCR assays with the newly designed primer set IC-ITS1F/ITS1R to confirm the specificity of the new PCR assays. The banding pattern of the PCR products in the conventional PCR assay and the Cq values from the real-time PCR assay were then recorded.

Results

Species-specific PCR primer design

The multiple sequence alignment of the ITS rDNA from *Pratylenchus* spp. revealed unique regions of polymorphism in the target nematode genome. The species-specific primer set, IC-ITS1F/IC-ITS1R was designed based on these unique regions of polymorphism for the detection of the new species of *Pratylenchus* investigated in this study. The primer set was expected to amplify a specific 194-bp fragment of the ITS rDNA of the new species of

Pratylenchus. The percent GC content for the 18-bp forward primer (IC-ITS1F) was 50%, whereas the percent GC content for the 22-bp reverse primer (IC-ITS1R) was 40.9%. The predicted annealing temperatures for IC-ITS1F and IC-ITS1R were estimated as 57.6 and 58.9 °C, respectively. Additionally, the primers had a low potential for duplex formation through hairpin formation, self-dimerization, and heterodimer formation. Using the BLAST search function of the nr/nt database of NCBI for the initial specificity screening revealed that the primer sequences have no perfect match with nontarget plant-parasitic or non-plant-parasitic nematodes and no strong similarity to other soil microorganisms.

The IC-ITS1F and the IC-ITS1R primers were predicted to have strong and specific annealing with the ITS rDNA sequence of the target *Pratylenchus* sp. based on primer-template duplex stability (ΔG) values of the in silico analysis (Table 4.1). Since ΔG values less than -31 kcal/mol predict stable primer-template hybrid formation, the IC-ITS1F and IC-ITS1R primers, having a ΔG value of -33.5 and -38.4, respectively, were predicted to hybridize with the ITS rDNA sequence of the target species (Huang et al. 2017b; Yan et al. 2012). Values of ΔG higher than 31 kcal/mol are indicative of unstable primer-template binding resulting in unidirectional or non-logarithmic amplification which are not competitive with specific amplification. Thus, IC-ITS1F forward primer was predicted to hybridize poorly with ITS rDNA sequences of the three isolates of *P. neglectus* used in silico analysis, having a ΔG value of -14.4. On the other hand, the IC-ITS1R reverse primer was predicted to form insignificant (ΔG = ins) hybrid with ITS rDNA sequences of *P. neglectus* (Huang et al. 2017b; Yan et al. 2012). In addition to that, both the primers were not predicted to result in primer-template duplexes with the ITS rDNA sequences of any other *Pratylenchus* spp. used in silico analysis. Hence, the primer set IC-ITS1F/IC-ITS1R

was predicted to specifically amplify the DNA of the new species of *Pratylenchus* detected in ND and therefore was selected for further laboratory experiments.

Primer specificity

Laboratory conventional and real-time PCR assays confirmed the prediction of in silico analysis about the specificity of the newly designed primers. As expected, the IC-ITS1F/IC-ITS1R primer set amplified a specific 194-bp DNA fragment of the target species of *Pratylenchus*. A single specific amplicon was observed in the conventional PCR assay with all four populations of the target *Pratylenchus* sp. used in the specificity tests (Table 4.2, sample ID: Hg50-1, Hg50-2, Hg50-3, and Hg50-4). Additionally, all DNA extracted from different numbers of the target individuals (4, 2, 1, and 0.5) from the same population produced the specific amplicon in conventional PCR (Table 4.2, Hg50-4). No amplification was observed in the conventional PCR assay with non-target control species consisting of multiple isolates of five other confirmed *Pratylenchus* spp., eight other genera of plant-parasitic nematodes, and two genera of non-plant-parasitic nematodes. Conventional PCR also amplified DNA from each of the target and non-target species using the universal primers rDNA2/rDNA1.58s, confirming presence of template DNA in each of the samples tested. As expected, real-time PCR amplifications with the non-target control species yielded no fluorescence signal, thus Cq values were not detectable. However, Cq values ranging from 26.43±0.05 to 30.64±0.50 were recorded from real-time PCR with the various populations of the target species of *Pratylenchus* at varying number of target individuals in DNA extracts (Table 4.2). Melting curve analysis revealed a single melting peak at 81.5 °C and no secondary curves confirming a single amplicon specific for each of the four populations of the target *Pratylenchus* sp. (Fig. 4.1). For both conventional and

real-time PCR assays, no amplicons were generated in non-template controls containing water instead of DNA.

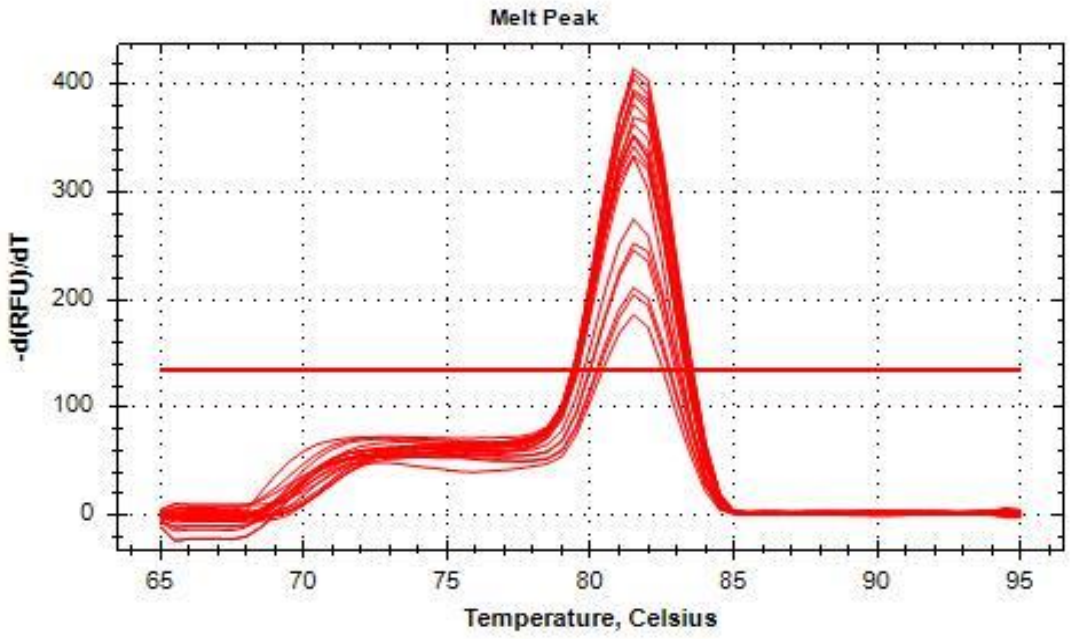


Fig. 4.1. Melting curve profiles of the new *Pratylenchus* sp. recently discovered in North Dakota. Amplicons with a single peak at a melting temperature of 81.5 °C were observed for different populations of the target species.

Table 4.2. Taxon, geographic origin, current crop, and polymerase chain reaction (PCR) assays of the control nematode species used to test the specificity of IC-ITSF/ITS1R primer set designed to identify and detect the new species of *Pratylenchus* detected in a soybean field of North Dakota

Sample ID[a]	Species	Origin	Crop[b]	# of Nema[c]	PCR Assay	
					Conventional[d]	RT (Cq)[e]
Ps1	*Pratylenchus scribneri*	Sargent, ND, USA	Corn	2	-	N/A
Ps2	*P. scribneri*	Sargent, ND, USA	Potato	2	-	N/A
Pn1	*P. neglectus*	Richland, ND, USA	Corn	2	-	N/A
Pn2	*P. neglectus*	Bottineau, ND, USA	Field Pea	2	-	N/A
Pt	*P. thornei*	OR, USA	Wheat	2	-	N/A
Pp	*P. penetrans*	Sherburne, MN, USA	Potato	2	-	N/A
Tyl	*Tylenchorhychus* sp.	Richland, ND, USA	Corn	2	-	N/A
Spi	*Helicotylenchus* sp.	Richland, ND, USA	Soybean	2	-	N/A
Xph	*Xiphinema* sp.	Sargent, ND, USA	Potato	2	-	N/A
Prt	*Paratylenchus* sp.	McIntosh, ND, USA	Corn	2	-	N/A
Ptr	*Paratrichodorus* sp.	Sargent, ND, USA	Potato	2	-	N/A
Hop	*Hoplolaimus* sp.	Sargent, ND, USA	Soybean	2	-	N/A
SCN	*Heterodera glycines*	Richland, ND, USA	Soybean	2	-	N/A
SBCN	*H. schachtii*	Richland, MT, USA	Sugarbeet	2	-	N/A
NPN1	Non-plant parasitic nematode 1	Richland, ND, USA	Corn	2	-	N/A
NPN2	Non-plant parasitic nematode 2	Richland, ND, USA	Soybean	2	-	N/A
Hg51[f]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	2	-	N/A
Hg50-1[g]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	2	+	28.91±0.40
Hg50-2[g]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	2	+	27.70 ± 0.10
Hg50-3[g]	*Pratylenchus* sp.	Richland, ND, USA	Corn	2	+	28.02±0.59
Hg50-4[h]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	4	+	26.43±0.05
Hg50-4[h]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	2	+	27.60±0.20
Hg50-4[h]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	1	+	29.87±0.50
Hg50-4[h]	*Pratylenchus* sp.	Richland, ND, USA	Soybean	0.5	+	30.64±0.50

[a] Each of the nematode samples used in the primer specificity tests were collected by the North Dakota State University Nematology Laboratory from different field locations in North Dakota, Minnesota, and Montana except for the *P. thornei* isolate (sample ID: Pt), which was acquired from Oregon State University. Samples with the sample ID Hg50-1-4 are target species and the remaining samples in the table are non-target control species.
[b] The crop that was planted during the growing season, in which the samples were collected.
[c] For each of the control species used in the specificity tests, DNA was extracted from 2 individuals. For the target nematode species used in the specificity tests, DNA was extracted from 0.5, 1, 2, and 4 individuals.
[d] The positive (+) and negative (-) symbols indicate that the target amplicon was detected or not detected, respectively, in conventional PCR assays with the designed primer specific to the new species of *Pratylenchus* investigated in this study.
[e] The quantification cycle (Cq) value has been presented as the mean ± standard deviation of three technical replicates.
[f] Hg51 isolate is another newly identified *Pratylenchus* species from North Dakota that is different from the target species (Yan et al. 2017b).
[g] Hg50-1 and Hg50-3 are populations of the target *Pratylenchus* species that have been reared on soybean and corn, respectively, under greenhouse conditions for population maintenance and nematode culture. Hg50-2 is a population of the target *Pratylenchus* species that has been extracted from soil collected from the infested soybean field (Yan et al. 2017a).
[h] Hg50-4 is another population of the target *Pratylenchus* species that has been reared on soybean under greenhouse conditions. This population is included in the specificity test to initially determine whether DNA extracted from varying number of individuals affected the performance of the primers.

Detection sensitivity of the PCR assays

The sensitivity was determined using DNA from each dilution level of a sequential two-fold serial dilution of DNA extracted from an equivalent of four target nematode individuals in both conventional and real-time PCR assays. As expected, with each dilution of template DNA the intensity of PCR bands gradually decreased in the conventional PCR assay, and the Cq

values were continuously increased in the real-time PCR assay (Fig. 4.2). According to gel electrophoresis of conventional PCR products on 2% agarose gel, the assay was able to amplify the DNA of four individuals diluted down five times (4, 2, 1, 1/2, 1/4, and 1/8) to an equivalent of 1/8 of a single nematode. However, DNA from 6^{th} (1/16), 7^{th} (1/32), and 8^{th} (1/64) dilutions were not amplified by the conventional PCR indicated by the absence of bands on 2% agarose gel, suggesting that the IC-ITS1F/IC-ITS1R primer set could detect an equivalent to 1/8 of the DNA of a single nematode. In the real-time PCR assay, DNA from each dilution level was used to generate a standard curve. The equation for the standard curve was determined as $y = -3.474x + 29.002$, with an R^2 value of 0.9467 and an efficiency (E) of 94.02%. Thus, the standard curve generated showed a strong negative correlation between the Cq values from real-time PCR and the log values of numbers of the target nematode (Fig. 4.3). Since the amplification efficiency and the R^2 value were high and within the acceptable range, the IC-ITS1F/ IC-ITS1R primer set was considered suitable for real-time PCR assay. The sensitivity of the real-time PCR assay was also determined. The assay was able to amplify the DNA of four individuals diluted seven times (4, 2, 1, 1/2, 1/4, 1/8, 1/16, and 1/32), with the Cq values ranging from 26.57±0.30 to 33.74±0.32 (Fig. 4.2) and the DNA from the 8^{th} dilution (1/64) was not amplified, suggesting this primer set could detect an equivalent to 1/32 of the DNA of a single nematode in the real-time PCR assay.

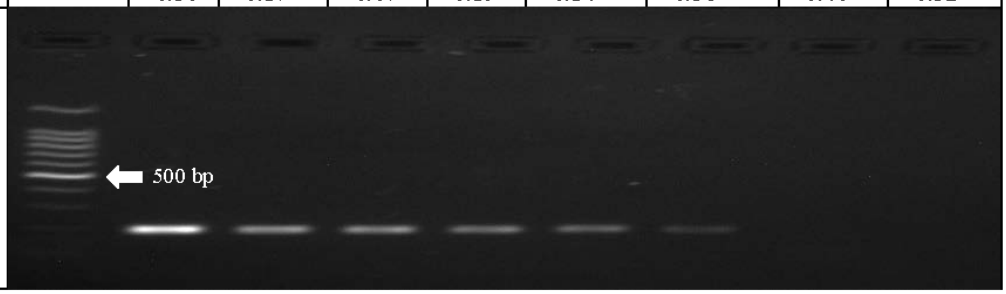
DNA serial dilution	Ladder (100 bp)	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
# of nematode		4	2	1	1/2	1/4	1/8	1/16	1/32
Real-time PCR		26.57 ±0.30	27.54 ±0.17	28.64 ±0.07	30.16 ±0.19	31.02 ±0.14	32.07 ±0.56	33.00 ±0.40	33.74 ±0.32
Conventional PCR									

Fig. 4.2. Amplification sensitivity of the IC-ITS1F/IC-ITS1R primer set in conventional polymerase chain reaction (PCR) compared to real-time PCR. The quantification cycle (Cq) value is presented as the mean ± standard deviation of three technical replicates of one of the biological replicates used for standard curve development. DNA was extracted from 4 nematodes and sequential two-fold serial dilutions (2, 1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64) were conducted, but the last dilution level 1:256 (1/64) was not included in this figure.

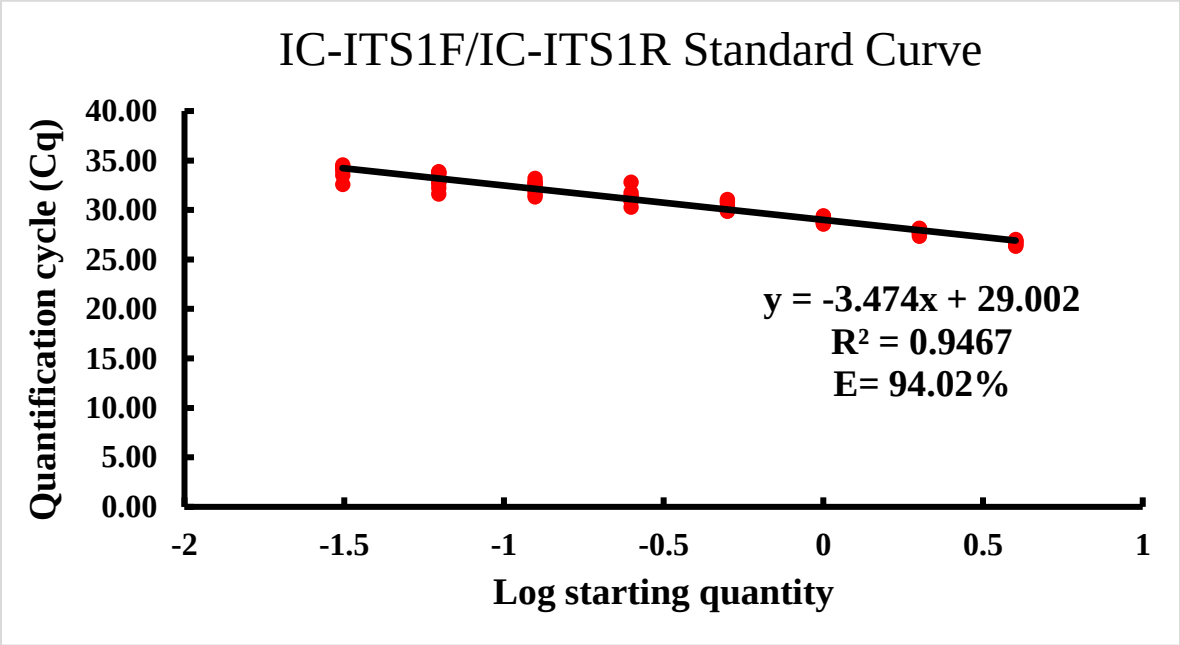


Fig. 4.3. Standard curve of the real-time PCR assay for the new species of *Pratylenchus* detected in a soybean field of North Dakota: quantification cycle number (Cq) plotted against the log number of individuals of target *Pratylenchus* sp. by sequential two-fold dilutions (4, 2, 1, 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64). Each dot represents an independent reaction run in triplicate for three biological replicates at each dilution level. The efficiency (E) of amplification was calculated as $E = 1^{1/-m} - 1$, where *m* indicates the slope.

Identification of *Pratylenchus* spp. collected from soybean fields

During 2019, the 21 soil samples collected from soybean fields across seven soybean producing counties of ND (Cass, Dickey, Grand Forks, Nelson, Richland, Sargent, and Wells), were all positive with *Pratylenchus* spp. The population densities of *Pratylenchus* spp. in these field soil samples ranged from 360 to 15 nematodes per 100 cm^3 of soil (Table 4.3). Out of the 20 samples, 12 were determined to be *P. neglectus* whereas seven were determined to be *P. scribneri* through published species-specific PCR assays. The IC-ITS1F/IC-ITS1R primer set designed in this study did not amplify any of those 19 samples, however it was able to amplify the DNA of root-lesion nematodes obtained from a field sample that was collected from a field that is neighboring the field where the new species of *Pratylenchus* was first detected.

Table 4.3. Identification of *Pratylenchus* spp. collected from 21 soybean fields in North Dakota by species-specific polymerase chain reaction (PCR) assays[a]

Field ID	Crop	County Origin	Density in 100 cm^3 of Soil[b]	Conventional[c]			RT[d]	Species Identity
				PNEG-F1/ D3B5	PsF7/ PsR7	IC-ITS1F/ IC-ITS1R	IC-ITS1F/ IC-ITS1R (Cq)[e]	
SCN 372	Soybean	Grand Forks	100	+	-	-	N/A	*P. neglectus*
SCN 366	Dry bean	Grand Forks	60	+	-	-	N/A	*P. neglectus*
SCN 311	Soybean	Nelson	30	+	-	-	N/A	*P. neglectus*
SCN 388	Soybean	Grand Forks	25	+	-	-	N/A	*P. neglectus*
SCN 207	Soybean	Cass	242	+	-	-	N/A	*P. neglectus*
Russ Field	Soybean	Wells	203	+	-	-	N/A	*P. neglectus*
SCN 188 W	Corn	Cass	45	+	-	-	N/A	*P. neglectus*
SCN 188 E	Corn	Cass	45	+	-	-	N/A	*P. neglectus*
SCN 310	Soybean	Nelson	31	+	-	-	N/A	*P. neglectus*
SCN 215	Soybean	Grand Forks	120	+	-	-	N/A	*P. neglectus*
SCN 222	Soybean	Cass	100	+	-	-	N/A	*P. neglectus*
SCN 7	Soybean	Cass	60	+	-	-	N/A	*P. neglectus*
SCN 55	Corn	Richland	75	-	+	-	N/A	*P. scribneri*
50 RL 1	Soybean	Richland	21	-	+	-	N/A	*P. scribneri*
50 RL 2	Soybean	Richland	28	-	+	-	N/A	*P. scribneri*
50 RL 4	Soybean	Richland	360	-	+	-	N/A	*P. scribneri*
C3L	Soybean	Sargent	75	-	+	-	N/A	*P. scribneri*
C14L	Soybean	Dickey	140	-	+	-	N/A	*P. scribneri*
SCN 48	Soybean	Richland	15	-	+	-	N/A	*P. scribneri*
HG50-0[f]	Soybean	Richland	360	-	-	+	27.7 ± 0.1	*Praytylenchus* sp.
50 RL 3[g]	Soybean	Richland	342	-	-	+	27.4 ± 0.1	*Praytylenchus* sp.

[a] For each samples, DNA was extracted from a single individual of *Pratylenchus* using the proteinase K method (Kumari and Subbotin 2012).
[b] Nematodes were extracted from 100 cm^3 soil sub-samples using centrifugal sugar floatation method, and the population density of *Pratylenchus* was determined under a microscope based on their morphological features (Castillo and Volvas 2007; Mai and Mullin 1996).
[c] Each DNA sample was used in conventional PCR assays with 5 different species-specific primer sets to identify the species of *Pratylenchus* in each sample. The primer sets include PNEG-F1/ D3B5 (Yan et al. 2008), PsF7/PsR7 (Huang and Yan 2017), PTHO/D3B (Yan et al. 2008), PP5F/PP5R (Mekete et al. 2011), and IC-ITS1F/IC-ITS1R (this study) that are specific to *P. neglectus*, *P. scribneri*, *P. thornei*, *P. penetrans*, and the new species of *Pratylenchus* (Hg50), respectively. However, none of the samples were positive for *P. thornei* and *P. penetrans*, thus results of those PCR assays were not included in this table.
[d] Each sample was tested using real-time PCR assays with the IC-ITS1F/IC-ITS1R primer set, which is specific to the new species of *Pratylenchus*.
[e] Quantification cycle (Cq) value is presented as the mean ± standard deviation of three replicates.
[f] Hg50-0 is the soybean field where the new species of *Pratylenchus* was originally detected.
[g] 50 RL 3 is a neighboring field of Hg50-0.

Discussion

Pratylenchus spp. are one of the most economically important pests of field crops (Jones et al. 2013; Scmitt and Barker 1981). However, the potential of root-lesion nematodes to cause economic loss can vary according to the species present and their population density (Koenning et al. 1999). Therefore, diagnostic assays that can effectively identify the species of *Pratylenchus* are of utmost importance to facilitate their management. Conventional and real-time PCR

continues to be amongst the most important diagnostic tools in plant nematology research (Jones et al. 2013), since they are relatively inexpensive, less time consuming, accurate, and easy to use.

Both conventional and real-time PCR have advantages of their own, compared to each other. While conventional PCR is more inexpensive, easy to use, and less affected by PCR inhibitors (Schrader et al. 2012), real-time PCR is less time consuming, more sensitive, allows continuous monitoring of data, and provides quantitative data (Yan et al. 2012). Thus, we designed and developed a conventional PCR assay, as well as a SYBR Green-I based real-time PCR assay, for identification and quantification of a new species of *Pratylenchus* recently detected in a soybean field in Walcott, Richland County, ND (Yan et al. 2017a). The IC-ITS1F/IC-ITS1R primer set designed in this study was able to specifically and sensitively amplify the DNA of the new *Pratylenchus* species in both conventional and real-time PCR assays. Moreover, the designed primer set was able to successfully detect and differentiate the new species *Pratylenchus* from other confirmed *Pratylenchus* spp. collected from 20 other soybean fields of North Dakota. Hence, we report for the first time, two efficient molecular methods for accurate detection and identification of a new species of *Pratylenchus* detected in ND (Yan et al. 2017a).

The IC-ITS1F/IC-ITS1R species-specific primer set was designed from the ITS rDNA region of the target nematode's genome. Numerous ITS-region based PCR assays have been developed to identify and differentiate plant-parasitic nematodes to their species level (Arora et al. 2020; De. Weerdt et al. 2011; Huang et al. 2017a, b, 2018a, 2019; Huang and Yan 2017; Li et al. 2015; Subbotin et al. 2005;). Compared to 28S rDNA and 18S rDNA that are conserved through evolution, the ITS rDNA region is considered to be more variable among genera and species of plant-parasitic nematodes and other soil-borne organisms (Huang and Yan 2017; De

Luca et al. 2011; Subbotin and Moeens 2006). Although, several studies have developed species-specific primers that are designed from the 18S rDNA, as well as 28S rDNA (Al Banna et al. 2004; Baidoo et al. 2017; Yan et al. 2008), the ITS rDNA sequences are known to have greater variability between different *Pratylenchus* spp. (De Luca et al. 2011; Huang and Yan 2017; Uehara et al. 1998). Several other nematode genes such as mitochondrial cytochrome oxidase 1, mitochondrial cytochrome oxidase 2, 16D10 effector gene, and chorismate mutase effector gene have been used, in recent studies, to design species-specific primers for identification of nematode species (Blouin 2002; Gorny et al. 2019; Mattiuci et al. 2003; Seesao et al. 2017; Valentini et al. 2006; Yu et al. 2011). However, in our literature review we found that more ITS rDNA sequences of plant-parasitic nematodes and *Pratylenchus* spp. were available in GenBank and previous literature compared to these genes, which facilitated our sequence analysis and comparison when designed the primers.

The performance of species-specific primers in PCR is substantially influenced by primer length, melting temperature (T_m), GC content, and potential for self-dimer or primer dimer formation (Dieffenbach et al. 1993). The specificity of a primer pair is generally controlled by their sequence length and the annealing temperature used in PCR reaction. Primers that are 18-24 bp in length are considered to be the best in being sequence specific if the annealing temperature of the PCR reaction is set within 5 °C of the primer T_m (Dieffenbach et al. 1993; Singh and Kumar 2012). On the other hand, primers with 50-60% GC content ensure optimal binding strength of primer-template duplex because the G and C bases have stronger hydrogen bonding, and the binding strength of the primer-template duplex determines the efficiency of annealing (Rychlik et al. 1990). Moreover, the presence of G or C bases at the 5’ and 3’ end ensures a GC clamp and promotes specific binding. Another important factor to consider when designing a

primer is the potential of the primers to form secondary structures. Presence of complementary sequence within the primer length can result in hairpin or self-dimer formation, whereas homology between the forward and reverse primer can lead to primer dimer formation (Erlich et al. 1991). These secondary structures can directly compete with the template in PCR, negatively affecting species-specific amplification (Singh and Kumar 2012). Thus, the IC-ITS1F and the IC-ITS1R primers were designed to have optimal primer length, melting temperature (T_m), and GC content, as well as low potential for secondary structure formation.

Numerous previous molecular diagnostic studies have used in silico analysis to predict the performance of primers regarding specificity against multiple isolates of their respective target species as well as multiple isolates of non-target species (Arora et al. 2020; Delcourt et al. 2016; Huang et al. 2017b; Okubara et al. 2008; Schroerder et al. 2006; Yan et al. 2008, 2012, 2013). The in silico analysis provided us an opportunity to predict the specificity of the primers against the DNA sequences of multiple isolates of 26 other important species of *Pratylenchus* originating from different regions of the world. Results of in silico analysis predicted that the primers were highly specific to the target *Pratylenchus* sp. The specificity of the primers was further confirmed through laboratory PCR assays with multiple populations of the target nematodes as well as multiple isolates of five other *Pratylenchus* spp. along with eight other genera of non-target control species. Since DNA from each of the target nematodes population were amplified by the designed primers and none of the non-target species' DNA were amplified, the results of the PCR specificity tests were consistent with the predictions obtained from the in silico analysis. Thus, this allows us to conclude that IC-ITSF/IC-ITS1R primer set can be successfully used in both conventional and real-time PCR assays to distinguish and

identify the new species of *Pratylenchus* (Yan et al. 2017a) from other plant-parasitic nematode species.

Primers that are specific for other *Pratylenchus* spp. in conventional PCR have been described before (Al-Banna et al. 2004; Castillo and Volvas 2007; Yan et al. 2008). However, not all of those primers could directly be used in real-time PCR due to non-specific amplifications being detected by the greater amplification sensitivity of real-time PCR (Huang and Yan 2017; Yan et al. 2013). However, the species-specific primers designed in this study can be used in both conventional PCR and real-time PCR for species identification. Applicability of the primers in real-time PCR was demonstrated by the high inverse linear relationship observed between the Cq values and the log values of nematode numbers ($R^2 = 0.95$) revealed by the standard curve generated. Moreover, the standard curve also revealed that the primers had good amplification efficiency (E = 94%), reflecting on the robustness of real-time PCR assay developed.

Practically, DNA from as little as a single nematode could be used to specifically identify the new *Pratylenchus* sp. using the molecular diagnostic methods developed in this study. As expected, the real-time PCR assay with greater sensitivity could detect an equivalent of 1/32 of a single nematode; whereas, the conventional PCR could detect an equivalent of 1/8 of a single nematode. These results are consistent with previous studies where the sensitivity of primers was found to be greater in real-time PCR assay than the conventional PCR assay (Huang et al. 2017b; Haung and Yan 2017). Moreover, the sensitivity of the IC-ITS1F/IC-ITS1R primer set in conventional and real-time PCR assays was comparable to other nematode related PCR assays reported in previous literature, where DNA from less than a single nematode individual could be detected (Berry et al. 2008; Jeszke et al. 2015; Mokrinin et al. 2013; DeWeerdt et al. 2011;

Huang et al. 2017b; Haung and Yan 2017; Yan et al. 2012). For examples, the real-time PCR assay developed by Berry et al. (2008) could detect as little as 2.5% (1/40) of the DNA of a *P. zeae* individual. On the other hand, the conventional PCR assay developed by Huang and Yan et al. (2017) could detect an equivalent of 1/4 of the DNA of a single *P. scribneri* individual. The slight difference in Cq values observed between the biological replicates used in our real-time PCR assay might be explained by the variation in copy number of ITS rDNA between different life stages of the target nematode species (Lopes et al. 2019; Oliviera et al. 2017).

The assays developed in this study were able to distinguish between the new *Pratylenchus* sp. and other *Pratylenchus* spp. commonly present in ND soybean fields. Among the 20 soybean fields of ND, from which soil samples were collected, a majority were infested with *P. scribneri* or *P. neglectus*. Although, several studies reported that multiple species of *Pratylenchus* could coexist in an individual cropping field (Luc 1987; Kimpinski 1979; Wheeler et al. 1992), none of the fields surveyed in our study were infested with more than one species of *Pratylenchus*. The IC-ITS1F/IC-ITS1R primer set did not amplify the DNA of *P. scribneri* or *P. neglectus* obtained from these field samples. However, it was able to amplify the DNA of a *Pratylenchus* sp. obtained from a soil sample (Table 4.3, sample ID: 50 RL 3) collected from a neighboring field of the soybean field that was originally infested with the new *Pratylenchus* sp. (Yan et al. 2017a). Therefore, this study reports for the first time that another soybean field (50 RL 3) of North Dakota is infested with the new *Pratylenchus* sp.

In conclusion, the PCR assays developed in this study provides a rapid, specific, and sensitive detection method for the new species *Pratylenchus* detected in ND. Both the specificity and sensitivity of the primers designed were confirmed through extensive testing. Moreover, the standard curve analysis revealed that the primers had a good amplification efficiency. Thus, IC-

ITS1F/IC-ITS1R primer set is suitable for use in both diagnostic laboratories as well as research laboratories for the detection of field infestation with this nematode species. Such information would be valuable for improving nematode detection to develop nematode management strategies. Moreover, this study will help in future development of PCR assays that can identify and quantify this nematode species from DNA extracts of soil samples. This will simplify the diagnosis process by avoiding the nematodes extraction from soil prior to DNA extraction.

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CHAPTER 5: EVALUATION OF SOYBEAN CULTIVARS FOR RESISTANCE TO A NEW *PRATYLENCHUS* SP. DETECTED IN NORTH DAKOTA

Abstract

Root-lesion nematodes, *Pratylenchus* spp., are one of important groups of plant-parasitic nematodes that has a worldwide distribution and a broad host range including soybeans. One of the key management strategies against root-lesion nematodes is host resistance. Hence, two greenhouse experiments were conducted in this study to ascertain resistance levels of soybean cultivars to the new species of root-lesion nematodes detected in ND (Experiment 1) and the habitat preference of the new root-lesion nematode species (Experiment 2). A total of twenty soybean cultivars and the positive control Barnes accompanying a non-planted control, each with five replicates, were evaluated in both experiments with naturally infested soil. Experiment 1 was harvested at fifteen weeks after planting while Experiment 2 was harvested earlier at 9 weeks to aid in separation of root and soil, and both experiments were repeated once. The ratio of the postharvest population density of a test cultivar relative to the postharvest population density of a susceptible check was used to scale resistance rating. The cultivar NS 1911NR2 consistently produced the greatest postharvest population densities in both trials of Experiment 1 and Experiment 2 and therefore it was selected as the susceptible check. The cultivar NS 60083NXR2 also showed susceptible reaction in all the trials. Whereas, cultivars such as 3408RR2YN and NS 0651NR2 were the least susceptible in all the trials with resistant or moderately resistant rating. Combined results of all trials indicated that seven of the cultivars, including 3408RR2YN, H09X7, NS 051NR2, NS 1742NLL, NS 61493NXR2, S03-G9, and S06-Q9, were moderately resistant. Ten of the cultivars, including 0616R2X, 20915N, 41-10, Barnes, INT 51449NRZX, LS-1335NRR2X, NS 0081NR2, NS 1291NLL, NS 2013NLL, and S12-R3,

were moderately susceptible. Four of the cultivars, including NS 1911NR2, NS 60083NXR2, NS 62002NXR2, and S07-Q4X, were susceptible. However, none of the cultivars evaluated were classified as resistant. Analysis of the habitat preference of the new species in roots versus soil revealed that above or close to 50% of the root-lesion nematodes resided in the root habitat and the remaining in soil at nine weeks after planting for a majority of the cultivars evaluated. These results provide us an insight into the virulence of the new root-lesion nematodes species identified in ND, against commercial soybean cultivars. Such information has practical implication for soybean growers and researchers in finding best performing, least susceptible cultivars to minimize yield loss.

Keywords: Cultivar, disease management, field crops, legume, nematode reproduction, North Dakota, *Pratylenchus*, host resistance, root-lesion nematodes, and soybean.

Introduction

Soybean (*Glycine max* L. (Merr.)) is a globally important crop that plays an essential role in achieving global food security (Hartman et al. 2011). This leguminous crop is an important source of vegetable oil and high-quality vegetable proteins for humans in the world today. Moreover, it is the largest source of animal feed protein in the world (USDA-ERS 2020). However, production of this valuable crop is limited by several biotic and abiotic factors. Such abiotic factors include frost damage, nutritional deficiency, salt toxicity, and water stress, while biotic factors include insect pests, fungal pathogens, plant-parasitic nematodes, weeds and other diseases (Hartman et al. 2011). Among the abiotic factors, plant-parasitic nematodes are one of the most important limiting factors of soybean production (Koenning and Wrather 2010).

Root-lesion nematodes, *Pratylenchus* spp., are one of the destructive groups of plant-parasitic nematodes worldwide. They are ranked as the third most important group of plant-

parasitic nematodes after root-knot nematodes and cyst nematodes (Jones et al. 2013). These vermiform soil-borne pathogens are multicellular unsegmented roundworms that feed on plant roots (Lambert and Bekal 2002). They are endoparasitic in nature as they can penetrate the root system of host crop, feed, and reproduce inside root tissue. Unlike other endoparasitic nematodes such as root-knot nematodes and cyst nematodes, root-lesion nematodes are migratory endoparasites. Thus, they can move between cells and feed on cell cytoplasm resulting in root tissue necrosis and extensive lesion. However, once the nutritional sources in one area of root cortex are depleted, root-lesion nematodes can migrate into a new area with better nutritional sources and less competition (Castillo and Volvas 2007). Hence, they can cause loss of root function and resulting in nutrient and moisture deficiency symptoms in susceptible hosts (Thompson et al. 2020).

Members of *Pratylenchus* spp. can reproduce sexually and/or asexually depending on the specific species, environmental conditions, and availability of nutritional sources (Agrios 2005; Castillo and Volvas 2007). Thus, they are able to adapt to a diverse set of climatic regions of the world. Furthermore, their ability to undergo all life stages of their life cycle inside the root-tissue of the host plants enables them to thrive in adverse environmental conditions such as semiarid growing regions and temperate growing regions (Elhady et al. 2019; May et al. 2016; Vanstone 1998). Nevertheless, in such growing regions extreme temperatures and lack of moisture can limit other ectoparasitic and free-living nematodes (May et al. 2016; Vanstone et al. 1998).

Root-lesion nematodes have a wide host range that includes over 400 host plant species (Davis and MacGudwin 2000). They can parasitize and reproduce on almost all of the major crops of the world including soybean, corn, potato, rice, wheat, vegetables, and fruits (Castillo and Volvas 2007; Davis and MacGudwin 2000; USDA-ERS 2020). Thus, root-lesion

nematodes' ability to survive in diverse environmental conditions, worldwide distribution, and broad host range enables them to cause serious economic losses for growers throughout the world (Castillo and Volvas 2007; Jones et al. 2013; Vanstone et al. 1998).

Numerous studies, in previous literature, have reported high yield losses caused by root-lesion nematodes in infested crop fields. In Australia, Mexico, and Israel, root-lesion nematodes such as *P. thornei* were reported to cause yield losses in wheat production as high as 85%, 37%, and 70%, respectively (Nicol et al. 2003; Orion et al 1984; Taylor et al. 1999). In Norway, *P. penetrans* was reported to reduce potato yield by 50 % in affected fields (Holgado et al. 2009). In Ghana, *P coffeae* was reported to cause 60% yield loss in plantain production (Green et al. 2004). In India, *P. indicus* was reported to cause yield losses of up to 28% in rice fields (Prasad et al. 1987). When it comes to soybean fields, root-lesion nematodes such as *P. brachyurus* was reported to cause 21% yield reduction in Brazilian soybean production (Lime et al. 2015). In Germany, *P. crenatus*, *P. neglectus*, and *P. penetrans* were reported to be the dominant taxa of plant-parasitic nematodes in soybean fields (Elhady et al. 2019). In North Carolina of the United States, *P. brachyurus* was reported to reduce soybean yields by 31% (Ross et al. 1967). Thus, immediate, appropriate and effective management of these nematode diseases is critical for improving crop production worldwide.

Management of root-lesion nematodes is challenging because tools available for practical management of these plant-parasitic nematodes are limited (May et al. 2016). Crop rotation is a management tactic commonly used to limit pathogen population increase in infested fields. However, effective use of crop rotation to control root-lesion nematodes is difficult due to the broad host range of *Pratylenchus* spp. Moreover, choice of crops available for rotation can vary significantly depending on the species of root-lesion nematodes (Castillo and Volvas 2007;

Schmitt and Barker 1981). Historically, broad spectrum soil fumigants such as chloropicrin, metam-sodium and methyl bromide have shown efficacy in controlling nematode populations (Castillo and Volvas 2007; Rich et al. 2004). However, many of these fumigants and nematicides are removed or being phased out of the market due to their negative environmental impact and human health risk (Barker and Koenning 1998). Furthermore, cost of using chemical treatments to control root-lesion nematodes in large scale crop production can be greater than the economic benefits. Thus, chemical nematicides are not commonly recommended in sustainable management strategies of root-lesion nematodes, especially for large scale production of low value crops (Lima et al. 2017). On the other hand, host resistance is a promising eco-friendly option for managing root-lesion nematode populations.

Host resistance has been reported to effectively reduce population development of root-lesion nematodes in previous studies (Castillo and Volvas 2007; Schmitt and Barker 1981; Thompson et al. 2020). Moreover, cultivars of host crops can vary in their ability to suppress root-lesion nematode populations and they can be ranked from resistant to susceptible based on nematode reproduction in each cultivar (Acosta et al. 1979; Santos et al. 2015; Sheedy and Thompson 2009; Smiley et al. 2014a, b). However, virulence of root-lesion nematodes can also vary depending on different species of *Pratylenchus* (Acosta et al. 1979; Castillo and Volvas 2007). For example, cultivars of wheat that were resistant to *P. neglectus* were not necessarily resistant to *P. thornei* (Sheedy et al. 2008; Thompson et al. 2008). Similarly, Acosta et al. (1979) reported a soybean cultivar that was resistant to *P. neglectus* but susceptible to *P. scribneri*. Thus, cultivars that suppress the reproduction of one species of root-lesion nematodes do not necessarily suppress the development of all other root-lesion nematode species (Schmitt and Barker 1981; Sheedy et al. 2008; Smiley et al. 2014a; Thompson et al. 2008).

In North Dakota, during 2015 and 2016, soil surveys of different soybean fields were conducted by the North Dakota State University nematology group to determine the prevalence and distribution of plant-parasitic nematode in these fields (Yan et al. 2017). During the surveys, six soil samples were collected from a soybean field in Walcott, Richland County, ND. After extracting nematodes from these samples, it was evident that root-lesion nematodes were present in all of these samples with a population density ranging from 125 to 2,000 per kg of soil. Morphological measurement of adult males and females as well as DNA sequencing of two genomic regions revealed that this nematode differs from other known species of root lesion nematodes in both morphology and DNA sequences, allowing us to conclude that this is a new species of root-lesion nematode that has never been reported in previous literature (Yan et al. 2017).

To demonstrate that soybean is a host of this new species, a preliminary greenhouse study was conducted (Yan et al. 2017). In this study, naturally infested soil samples were planted with the local soybean cultivar Barnes, which is susceptible to soybean cyst nematode (Pormarto et al. 2015). After 15 weeks of growth at 22°C in the greenhouse, the postharvest population density of the new species of root-lesion nematodes increased more than five-fold compared to the preplant population density (Yan et al. 2017). Thus, this indicated that the new species of root-lesion nematodes parasitized and reproduced well on the soybean cultivar Barnes. However, the impact of the new root-lesion nematodes species on other soybean cultivars, except Barnes is still unknown. Consequently, the resistance reactions of these cultivars to the new root-lesion nematode species are still undetermined. Therefore, the objective of this research was to evaluate resistance levels of twenty soybean cultivars to the new species of root-lesion nematodes detected in Walcott, Richland County, ND and to determine the habitat preference of the new

root-lesion nematode species by comparing the numbers of nematodes present in roots and in soil for each cultivar.

Materials and methods

Soil sample collection and processing

During the 2018 and 2019 soybean growing seasons, composite soil samples were collected from a soybean field in Walcott, Richland County, ND, that was previously reported to be infested with the new species of root-lesion nematode (Yan et al. 2017). Sampling was conducted using a standard soil probe (2.5 cm diameter and 30 cm depth), but when the soil was too compacted, a shovel was used to collect the soil samples from the same depth in the rhizosphere. Samples were collected from 20-25 sampling points in the field, walking up to 10 m between successive sampling points and following a zig-zag pattern. For each sampling point, 1-2 cm of dry surface soil was discarded, and a soil core was probed up to a depth of 30 cm along the rhizosphere. The soil cores were then composited in a Rubbermaid storage box and immediately transported to the North Dakota State University Nematology Laboratory. The composite soil sample was first mixed by hand for one hour to break down soil clumps. Subsequently, a motorized cement mixer (Kushlan Products LLC, Houston TX) was used for one hour to ensure even distribution of the nematode population throughout the soil sample. Afterwards, three 0.2 kg sub-samples were collected from the mixed soil sample to determine the preplant nematode density.

Nematode extraction, identification, and preplant population density determination

Nematodes were extracted from the three sub-samples using the Whitehead tray nematode extraction technique. This method principally relies on active movement of migratory nematodes from the moist soil sample into the surrounding water (Whitehead and Hemming

1965). For each sub-sample, the extracted nematodes were accumulated in a 20-µm-aperture sieve and collected as a 20-25 ml nematode suspension, in a 50 ml plastic vial (Capitol vial Inc, Nashville, AL). Subsequently, the root-lesion nematodes in the nematode suspension were identified to genus level based on genus description provided by Mai et al. (1996), under an inverted light microscope (Focus Precision Instruments, Cavaletti Court Victoria, MN, USA) at 100 X magnification. The density of root-lesion nematodes in each sub-sample were then recorded as the total number of individuals per kg of soil. The average population density of root-lesion nematode from the three sub-samples were then used as the preplant population density (Pi) for reproduction ability experiments.

Root-lesion nematode species confirmation

In order to confirm the species identity of root-lesion nematodes, the species-specific PCR based molecular diagnostic method, described in the previous chapter, was used. According to this method, DNA was extracted from a single specimen of root-lesion nematode using the Proteinase K method (Kumari and Subbotin 2012), which involves chopping the nematode in a 10 µl of double distilled (dd)H_2O then adding it to a centrifuge tube containing 2 µl of 10X PCR buffer, 2 µl proteinase K (600 µg/ml), and 6 µl of ddH2O. The tubes were then stored at -20°C for 30 minutes and then incubated at 65°C for 1 hour followed by 95°C for 10 minutes. DNA was extracted in this manner from three individuals of root-lesion nematodes obtained from each sub-sample.

To confirm the presence of template in each DNA extract, a set of universal primers (rDNA2/rDNA1.58s) was used to amplify the internal transcribed spacer (ITS) region of rDNA, and the polymerase chain reaction (PCR) amplification was conducted as described by Cherry et al. (1997). After confirming presence of DNA template, species-specific PCR was conducted

with the forward IC-ITS1F (5'-TGTGTGCGAATGTTCCTG-3') and the reverse IC-ITS1R (5'-CGTATGTTTTATATGGGGACTC-3') primers. The IC-ITS1F/IC-ITS1R primer set, developed in the study described in chapter 4 of this dissertation, is specific to the new species of root-lesion nematode. The reaction mixture for this species-specific PCR was comprised of 1.5 µl of DNA template, 0.5 µM forward and reverse primers, 0.2 mM dNTP, 1.5 mM MgCl2, 1X Green GoTaq Flexi buffer, 1 unit of GoTaq Flexi DNA polymerase (Promega Corp., Madison. WI), and 11.2 µl of double-distilled water. The amplification was carried out under the conditions of initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturing at 94 °C for 40 s, annealing at 58 °C for 50 s, extension at 72 °C for 1 min, and a final extension for 10 min at 72 °C. The PCR amplifications were performed on the Bio-Rad T100 thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). The banding pattern of PCR products in 2% agarose gel were visualized after separation using the AlphaImager gel documentation system (Proteinsimple Inc., Santa Clara, CA).

Furthermore, each DNA sample was subjected to species-specific PCR with primers specific to other species of root-lesion nematodes that have been detected in North Dakota and Minnesota (Baidoo et al. 2017; Yan et al. 2016a, b) to exclude the possibility of being other species. The primers used in species-specific PCR included PsF7/PsR7 (Huang and Yan 2017), PNEG-F1/D3B5 (Yan et al. 2008), PTHO/D3B (Al-Banna et al. 2004), and PP5F/PP5R (Mekete et al. 2011), which are specific to *P. scribneri*, *P. neglectus*, *P. thornei*, and *P. penetrans*, respectively. The amplification conditions for the species-specific PCR followed the corresponding authors' recommendations.

Plant materials

A total of twenty different soybean cultivars used in the region were selected for cultivar resistance screening experiments conducted in a greenhouse. Additionally, two controls were selected, which included the cultivar Barnes as a positive control and unplanted control as a negative control. Details of the cultivars used in the greenhouse experiments are listed in Table 5.1. Seeds from each of these cultivars and the positive control were pre-germinated for 4 to 5 days in a petri dish containing a wet filter paper so that adequate roots were available for nematodes to feed on immediately after planting (KC 2019; Upadhaya et al. 2019).

Table 5.1. Soybean cultivars used in this study[w]

Name of cultivars	Originator[x]	Maturity group[y]
0616R2X	Channel	0.6
20915N	Integra	0.9
3408RR2YN	Thunder seed	0.8
41-10	Proseed	1.1
Barnes	North Dakota State University	0.3
H09X7	Hefty	0.9
INT 51449NRZX	Northstar Genetics	N/A[z]
LS-1335NRR2X	Legacy	1.3
NS 0081NR2	Northstar Genetics	0.8
NS 0651NR2	Northstar Genetics	0.6
NS 1291NLL	Northstar Genetics	1.2
NS 1742NLL	Northstar Genetics	1.7
NS 1911NR2	Northstar Genetics	1.9
NS 2013NLL	Northstar Genetics	2.0
NS 60083NXR2	Northstar Genetics	0.8
NS 61493NXR2	Northstar Genetics	1.4
NS 62002NXR2	Northstar Genetics	2.0
S03-G9	Syngenta	0.3
S06-Q9	Syngenta	0.6
S07-Q4X	Syngenta	0.7
S12-R3	Syngenta	1.2

[w] A total of 20 cultivars and the positive control "Barnes" were evaluated to ascertain their resistance levels against the new species of root-lesion nematode detected in Walcott, Richland County, ND. Furthermore, the habitat preference of this new nematode species in roots versus soil at nine weeks after planting was ascertained using the same 20 soybean cultivars and the positive control.

[x] Originator refers to the developer of the soybean cultivars.

[y] Maturity of a soybean cultivar refers to the length of time from planting to physiological maturity. Maturity groups are assigned to soybean cultivars by their developers to indicate the geographical zones that the cultivars are best adapted for (Motzinis and Conley et al. 2017).

[z] N/A = not available.

Greenhouse experiments

Immediately before planting, the composite soil sample, naturally infested with the new root-lesion nematode species, was transported from the cold storage to the greenhouse station. Subsequently, a slow release fertilizer "Multicote 4" (Haifa chemicals, Israel) with the formulation 14-14-16 N-P-K was added to the composite soil sample according to the ratio recommended by manufacturer, which is one tea spoon (~ 5g) of fertilizer per kg of soil. The naturally infested soil and the fertilizer were then mixed thoroughly by hand for an hour to ensure even distribution of nematode population and fertilizer throughout the soil sample. Afterwards, for the first experiment large plastic cone-type containers (6.6-cm diameter X 26.1-cm height, Stuewe and Sons, Inc. Tangent, OR) were filled with approximately 0.5 kg of the naturally infested soil. Each cone-type container (except non-planted container) was then planted with a single pre-germinated seed. Five replicates of each cultivar and the positive and negative controls were planted in this manner. The cone-type containers were then placed in the greenhouse benches in a completely randomized block experimental design. The plants were then grown in the greenhouse for 15 weeks at an average temperature of 22 °C and a photoperiod of 16-hour daylight.

To ascertain the habitat preference of this new root-lesion nematode species, a second independent experiment was conducted with the same twenty cultivars and controls in five replicates. During this second experiment composite soil samples were again collected from the field infested with the new root-lesion nematode species, preplant population density was determined from three sub-samples (Table 5.2), and the species of root-lesion nematode infesting the soil sample was confirmed. Thus, a majority of the experimental procedure for the second experiment was similar to the first experiment. However, the second experiment was conducted

by planting a single pre-germinated seed in a small cone-type container (2.8-cm in diameter and 20.3-cm in height; Stuewe and Sons, Inc. Tangent, OR) containing approximately 0.17 kg of naturally infested soil and the plants were grown in the greenhouse for nine weeks at an average temperature of 22 °C and a photoperiod of 16-hour daylight. The completely randomized block experimental design was also used in the second experiments. Both first and second experiments were repeated once to confirm the results (Table 5.2).

Table 5.2. Experimental details of greenhouse bioassays for the new species of root-lesion nematode detected in North Dakota.

Experiment[v]	Repetition (trial)[w]	Pi/kg of soil[x]	Experiment duration[y]	Nematode extraction type[z]
Experiment 1	1	1,575	15 weeks	Whitehead tray
	2	2,650	15 weeks	Whitehead tray
Experiment 2	1	1,950	9 weeks	Whitehead tray & Centrifugal floatation
	2	1,310	9 weeks	Whitehead tray & Centrifugal floatation

[v] Experiment 1 refers to the greenhouse bioassays conducted to ascertain resistance levels of soybean cultivars to the new species of root-lesion nematode detected in Walcott, Richland County ND; whereas, Experiment 2 refers to the greenhouse bioassays conducted to ascertain the habitat preference of the new root-lesion nematode species.
[w] Each experiment was repeated, thus two trials were conducted for each experiment.
[x] Pi refers to the initial population density of the new root-lesion nematode in naturally infested soil, used to conduct each experiment.
[y] In both trials of the Experiment 1, it became evident that it was difficult to separate and obtain intact root system from soil at 15 weeks after planting because the cultivars reached maturity and the root system became weak and easily fragmented. Thus, both trials of Experiment 2 were harvested at nine weeks after planting.
[z] In Experiment 1 after harvest, the roots were cut into 1 to 2 cm pieces and mixed thoroughly with the soil; nematodes were then extracted from the root + soil mixture using the Whitehead tray extraction method (Whitehead and Hemming 1965). In Experiment 2 the root system was separated from soil and nematodes were then extracted from the root system and the soil separately using the Whitehead tray extraction method and standard hand-sieving, decanting, and sucrose centrifugal-floatation method (Jenkins 1964), respectively.

Nematode extraction, identification, and final population density determination

During harvest of each experiment, the above-soil portion of each plant was cut off and discarded. Then the soil and the roots were collected into plastic bags and kept in a cold storage room at 4°C until nematode extraction. During nematode extraction from the harvested materials of the first experiment and its repeat experiment, the roots were cut into 1 to 2 cm pieces using a sharp scissor and mixed thoroughly with the soil. Nematodes were then extracted from 0.2 kg of the root-soil mixture, over a 48-hour period, using the Whitehead tray nematode extraction

method (Whitehead and Hemming 1965). The extracted nematodes were then accumulated in a 20-µm-aperture sieve and collected in a nematode suspension vial. The root-lesion nematodes were then identified and quantified based on their morphological features (Mai et al. 1996) under an inverted light microscope (Focus Precision Instruments, Cavaletti Court Victoria, MN, USA) at 100 X magnification. The postharvest densities of root-lesion nematodes were then averaged across the five replicates of each cultivar and the non-planted control. The mean postharvest population density for each cultivar was then tallied as the total number of individuals per kg of soil and roots and recorded in Table 5.3.

During nematode extraction from the harvested materials of the second experiment and its repeat experiment, each individual cone-type container was gently emptied into a 3-litre jug containing water; as a result, the soil particles were easily dislodged from roots without much fragmentation of roots. The root system of each plant was then washed into the jug to further remove any remaining soil particles attached to the root system. Subsequently, the roots were cut into 1-cm pieces, from which nematodes were extracted using the Whitehead tray nematode extraction method (Whitehead and Hemming 1965). From the remaining soil in the water jug, nematodes were then extracted using the standard hand-sieving, decanting, and sucrose centrifugal-floatation method (Jenkins 1964), described in the previous chapter. The extracted nematodes from the roots and the soil were then kept in separate nematode suspension vials. The root-lesion nematodes in each of the suspension were then identified and quantified based on their morphological features under an inverted light microscope. The final population density of root-lesion nematodes in roots and in soil were then recorded separately. The total number of individuals in roots and soil of each cultivar were combined and averaged across the five

replicates. The combined mean postharvest population density for each cultivar was then tallied as the total number of individuals per kg of soil and roots and recorded in Table 5.3.

Resistance rating of soybean cultivars

The cultivar with the highest postharvest mean population density in all trials of the two experiments was considered as the susceptible check. Resistance ratings were then scaled on the basis of postharvest population density of the new root-lesion nematode species in a test line relative to the postharvest mean population density in the susceptible check (Smiley et al. 2014a). The ratio of the postharvest population density in the test line relative to the susceptible check was then expressed as a percentage and recorded. The ratio of the postharvest population density for each cultivar and the non-planted control was then averaged across the five replicates and reported in Table 5.4. The host ranking for each cultivar was then categorized into four classes based on the ratio as described by Smiley et al. (2014a). The four resistance rating classes were resistant = R (postharvest mean population density of root-lesion nematode ≤ 25% of the susceptible check), moderately resistant = MR (26-50%), moderately susceptible = MS (51-75%), and susceptible = S (≥ 76%).

Data analysis

The statistical analysis was performed using the statistical software SAS 9.4 (SAS Institute Inc., Cary, NC). Leven's test was performed on the postharvest population density and the ratio of the postharvest population density to test the homogeneity of variances between the four trials conducted for Experiment 1 and Experiment 2. Although, the postharvest population densities did not have homogeneous variance, the ratio of the postharvest population density were homogeneous in their variances. Thus, postharvest mean population densities were analyzed separately for each trial, whereas, the ratio of the postharvest population density were

analyzed separately as well as by combining the results of the four trials of Experiment 1 and Experiment 2 for resistance rating. Tukey's honestly significant difference (HSD) test was conducted on the PROC GLM platform to perform mean separation of the ratios of the postharvest population densities for each trial at $\alpha = 0.05$ significance level. During the analysis of habitat preference, two sample t-test was conducted to determine if mean population density in roots was significantly different than mean population density in soil for each cultivar.

Results

Root-lesion nematode identification and quantification

The root-lesion nematodes extracted from naturally infested soil were identified and quantified to genus level based on morphological characters that separate them from other plant-parasitic nematodes. These morphological characters include flat sclerotized lip region, relatively short dark stomato stylet with basal knobs, dorsal overlap of esophagus with intestine, vulva position at 70-85% of body length from the anterior in adult stage, and tail region not pointed (Fortuner 1988; Mai et al. 1996). All vermiform life stages of root-lesion nematodes including juveniles, females and males were quantified to determine population densities. The new root-lesion nematode specific PCR primer set IC-ITS1F/IC-ITS1R, developed in the study described in chapter 4 of this dissertation, was able to amplify a single 194 bp amplicon that was visualized after gel electrophoresis under UV light. However, species-specific PCR with PsF7/PsR7 (Huang and Yan 2017), PNEG-F1/D3B5 (Yan et al. 2008), PTHO/D3B (Al-Banna et al. 2004), and PP5F/PP5R (Mekete et al. 2011) primer sets, that are specific to *P. scribneri*, *P. neglectus*, *P. thornei*, and *P. penetrans*, respectively, did not produce any amplification. Thus, this confirmed the identity of the root-lesion nematodes in the naturally infested soil samples used in this study to set up greenhouse bioassays as the new species of root-lesion nematode (Yan et al. 2017).

Postharvest population densities of the new root-lesion nematode species in soybean cultivars

In both trials of the two greenhouse experiments, the postharvest mean population densities of the new root-lesion nematode species were greater than their respective preplant population densities for each of the 21 cultivars tested (Table 5.3). Furthermore, in each of the trials of Experiment 1 and Experiment 2, the postharvest mean population densities of root-lesion nematodes determined from each of the soybean cultivars were greater than the postharvest mean population densities of root-lesion nematodes determined from the non-planted soil, that were used as the negative control (Table 5.3).

In both trials of Experiment 1 the postharvest mean population densities of root-lesion nematodes determined from the cultivar NS 1911NR2 were the highest, whereas the postharvest mean population densities of root-lesion nematodes determined from the cultivar 3408RR2YN were the lowest (Table 5.3). In trial 1 of Experiment 1, these postharvest mean population densities ranged from 5,345 (cultivar: NS 1911NR2) to 1,529 (3408RR2YN) root-lesion nematodes per kg of soil and roots. In trial 2 of Experiment 1, the postharvest mean population densities ranged from 13,612 (NS 1911NR2) to 1,785 (3408RR2YN) root-lesion nematodes per kg of soil and roots (Table 5.3).

Table 5.3. Postharvest population densities of the new root-lesion nematode species from soybean cultivars assayed in greenhouse experiments[v].

	Experiment 1		Experiment 2	
Cultivars[w]	Trial 1[x]	Trial 2[x]	Trial 1[y]	Trial 2[y]
0616R2X	3,064	6,871	5,859	3,647
20915N	1,894	9,620	6,998	3,000
3408RR2YN	1,529	1,785	3,206	1,882
41-10	2,844	6,390	4,985	3,088
Barnes	3,076	6,813	5,195	3,647
H09X7	2,800	2,180	4,475	2,376
INT 51449NRZX	3,855	6,481	5,407	3,941
LS-1335NRR2X	3,000	7,535	5,619	3,265
NS 0081NR2	3,313	7,041	4,869	3,118
NS 0651NR2	1,594	2,500	1,857	1,794
NS 1291NLL	3,030	7,682	3,718	2,794
NS 1742NLL	2,068	4,120	4,129	2,294
NS 1911NR2	5,345	13,612	6,947	4,265
NS 2013NLL	2,920	3,657	3,997	3,111
NS 60083NXR2	4,948	11,724	6,398	4,265
NS 61493NXR2	2,498	4,053	2,338	2,012
NS 62002NXR2	3,863	7,770	6,941	3,647
S03-G9	2,502	4,696	4,302	2,441
S06-Q9	2,593	5,170	3,178	1,985
S07-Q4X	4,545	7,726	6,966	3,235
S12-R3	3,245	6,995	4,235	2,676
Non-planted soil	1,114[z]	871[z]	682[z]	218[z]

[v] Experiment 1 refers to the greenhouse bioassays conducted to ascertain resistance levels of soybean cultivars to the new species of root-lesion nematodes detected in Walcott, Richland County ND; whereas Experiment 2 refers to the greenhouse bioassays conducted to ascertain the habitat preference of the new root-lesion nematode species. Each experiment was repeated, which is indicated in this table by "trial 1" and "trial 2".

[w] A total of 20 soybean cultivars and the positive control "Barnes" were evaluated. Additionally, a non-planted control was included in each greenhouse bioassay as a negative control.

[x] The postharvest population densities of root-lesion nematodes were determined in Experiment 1 by extracting nematodes from the root + soil mixture as described in Table 5.2. The final population densities of the root-lesion nematodes from five replicates of each cultivar were then averaged and reported as total number of individuals per kg of soil and roots.

[y] The postharvest population densities of root-lesion nematodes were determined in Experiment 2 by adding the populations of root-lesion nematodes in roots and in soil. The final population densities of root-lesion nematodes from five replicates of each cultivar were then averaged and reported in this table as total number of individuals per kg of soil and roots.

[z] From the five replicates of non-planted soil used as the negative control in each bioassay, postharvest mean population density in soil is reported in this table.

Similarly, in trial 2 of Experiment 2, the cultivar NS 1911NR2 had the greatest postharvest mean population density (4,265 root-lesion nematodes per kg of soil). However, in trial 1 of Experiment 2, the cultivar 20915N had slightly higher postharvest mean population density (6,998) than the cultivar NS 1911NR2 (6,947). It is also noted that the cultivar 20915N did produce a high postharvest mean population density of root-lesion nematodes in trial 2 of Experiment 1 and trial 2 of Experiment 2 (Table 5.3). In Experiment 2, the cultivar NS 0651NR2 had the lowest postharvest mean population density of root-lesion nematodes in trial 1 (1,857) and in trial 2 (1,794). Additionally, this cultivar had the second and third lowest postharvest mean population density in Experiment 1 trial 1 and trial 2, respectively. According to the Levene's test, variances in postharvest population densities were not homogeneous between the trials of Experiment 1 and 2 and therefore the postharvest population densities determined from each of the trials were not combined and analyzed.

Resistance reactions of soybean cultivars to the new species of root-lesion nematode

Among the 21 cultivars evaluated, the cultivar NS 1911NR2 consistently produced the greatest postharvest mean population density of root-lesion nematodes in both trials of Experiment 1 and Experiment 2 (Table 5.3); therefore, it was selected as the susceptible check for scaling resistance ratings. Tukey's honestly significant difference (HSD) mean separation test at $\alpha = 0.05$ significance level, revealed that significant differences were evident in the ratios of postharvest population densities relative to the susceptible check across the 21 soybean cultivars in both trials of Experiment 1 and Experiment 2 (Table 5.4).

Table 5.4. Host ranking of twenty soybean cultivars and the positive control "Barnes" to the new species of root-lesion nematode detected in North Dakota[v].

	Experiment 1						Experiment 2					
	Trial 1			Trial 2			Trial 1			Trial 2		
Cultivars	Ratio (%)[y]		Rating[z]	Ratio (%)[y]		Rating[z]	Ratio (%)[y]		Rating[z]	Ratio (%)[y]		Rating[z]
0616R2X	57	abcde	MS	50	bcde	MR	84	ab	S	86	abc	S
20915N	35	de	MR	71	abc	MS	100	a	S	70	abcd	MS
3408RR2YN	29	e	MR	13	ef	R	46	abc	MR	44	d	MR
41-10	53	bcde	MS	47	cde	MR	72	ab	MS	72	abcd	MS
Barnes	58	abcde	MS	50	bcde	MR	75	ab	MS	86	abc	S
H09X7	52	bcde	MS	16	ef	R	64	abc	MS	56	bcd	MS
INT 51449NRZX	72	abcd	MS	48	bcde	MR	78	ab	S	92	ab	S
LS-1335NRR2X	56	bcde	MS	55	bcd	MS	81	ab	S	77	abcd	S
NS 0081NR2	62	abcde	MS	52	bcde	MS	70	ab	MS	73	abcd	MS
NS 0651NR2	30	de	MR	18	def	R	27	bc	MR	42	de	MR
NS 1291NLL	57	bcde	MS	56	bcd	MS	54	abc	MS	66	abcd	MS
NS 1742NLL	39	de	MR	30	def	MR	59	abc	MS	54	cd	MS
NS 1911NR2[w]	100	a	S	100	a	S	100	a	S	100	a	S
NS 2013NLL	55	bcde	MS	27	def	MR	58	abc	MS	73	abcd	MS
NS 60083NXR2	93	ab	S	86	ab	S	92	a	S	100	a	S
NS 61493NXR2	47	cde	MR	30	def	MR	34	bc	MR	47	d	MR
NS 62002NXR2	72	abcd	MS	57	bcd	MS	100	a	S	86	abc	S
S03-G9	47	cde	MR	34	cdef	MR	62	abc	MS	57	bcd	MS
S06-Q9	49	cde	MR	38	cdef	MR	46	abc	MR	47	d	MR
S07-Q4X	85	abc	S	57	bcd	MS	100	a	S	76	abcd	S
S12-R3	61	abcde	MS	51	bcde	MS	61	abc	MS	63	abcd	MS
Non-planted soil	21	e	…	6	f	…	9	c	…	5	de	…
$HSD_{0.05}$[x]	0.4271		…	0.3907		…	0.5832		…	0.3809		…
P>F	<0.0001		…	<0.0001		…	<0.0001		…	<0.0001		…

[v] Experiment 1 refers to the greenhouse bioassays conducted to ascertain resistance levels of soybean cultivars to the new species of root-lesion nematodes detected in Walcott, Richland County ND; whereas, Experiment 2 refers to the greenhouse bioassays conducted to mainly ascertain the habitat preference of the new root-lesion nematode species together with resistance responses. Each experiment was repeated, which is indicated in this table by "trial 1" and "trial 2".

[w] The cultivar NS 1911NR2 consistently produced the greatest postharvest population density of root-lesion nematodes in both trials of Experiment 1 and of Experiment 2 and therefore it was selected as the susceptible check in this study.

[x] Minimum significant difference ($P < 0.05$), determined from Tukey's honestly significant difference (HSD) test.

[y] Ratio of population densities (%) = (postharvest population density in a test line/ postharvest population density of the susceptible check) X 100. The ratio of postharvest population densities for each cultivar and the non-planted control was then averaged across the five replicates and reported in this table. Numbers followed by the same letter within a column are not significantly different at $\alpha = 0.05$ as determined by Tukey's honestly significant difference test.

[z] The host ranking for each cultivar was categorized into four classes based on the ratio of postharvest population densities as described by Smiley et al. (2014): Resistant = R (postharvest population density of root-lesion nematode < 25 % of the susceptible check NS 1911NR2), Moderately resistant = MR (26-50 %), Moderately susceptible = MS (51-75 %), and Susceptible = S (≥ 76 %).

In addition to the cultivar NS 1911NR2, the cultivar NS 60083NXR2 had susceptible reaction to the new species in both trials of Experiment 1 and Experiment 2 having ratios of postharvest population densities ≥ 76% of the susceptible check. Moreover, the mean ratios of postharvest population densities of the cultivars NS 1911NR2 and NS 60083NXR2 were not significantly ($P < 0.05$) different from each other in all the trials. On the other hand, the cultivars

3408RR2YN and NS 0651NR2 had resistant reaction in trial 2 of Experiment 1 having the ratios of postharvest population densities < 25% of the susceptible check (Table 5.4). However, in trial 1 of Experiment 1 and in both trials of Experiment 2, these two cultivars had moderately resistant (ratio: 26% to 50%) reaction to this new species. Additionally, the cultivars NS 61493NXR2 and S06-Q9 had moderately resistant reaction in both trials of Experiment 1 and 2.

Among the soybean cultivars tested, a majority of the cultivars had a moderately susceptible (51% to 75%) reaction to the new species of root-lesion nematodes in each of the trials of Experiment 1 and Experiment 2. Three of the cultivars, NS 0081NR2, NS 1291NLL, and S12-R3, consistently had moderately susceptible reaction in both trials of Experiment 1 and Experiment 2. Similarly, the cultivars Barnes, LS-1335NRR2X, and NS 2013NLL had moderately susceptible reaction in three out of the four trials conducted for Experiment 1 and Experiment 2. In the remaining trial Barnes and LS-1335NRR2X were susceptible, whereas, NS 2013 NLL was moderately resistant (Table 5.4). The cultivars 0616R2X and NS 62002NXR2 had moderately susceptible reaction in both trials of Experiment 1. However, these two cultivars had susceptible reaction in both trials of Experiment 2 (Table 5.4).

The Levene's test revealed that the variances in ratios of postharvest population densities relative to the susceptible check were homogeneous between the trials of Experiment 1 and Experiment 2 and therefore the ratios of postharvest population densities determined from each of the trials of both experiments were combined, averaged, and analyzed for each cultivar. According to the results of combined analysis, none of the soybean cultivars were resistant to the new species of root lesion nematodes (Fig. 5.1). Seven of the cultivars, including 3408RR2YN, H09X7, NS 051NR2, NS 1742NLL, NS 61493NXR2, S03-G9, and S06-Q9, were moderately resistant. Ten of the cultivars, including 0616R2X, 20915N, 41-10, Barnes, INT 51449NRZX,

LS-1335NRR2X, NS 0081NR2, NS 1291NLL, NS 2013NLL, and S12-R3, were moderately susceptible. Four of the cultivars, including NS 1911NR2, NS 60083NXR2, NS 62002NXR2, and S07-Q4X, were susceptible.

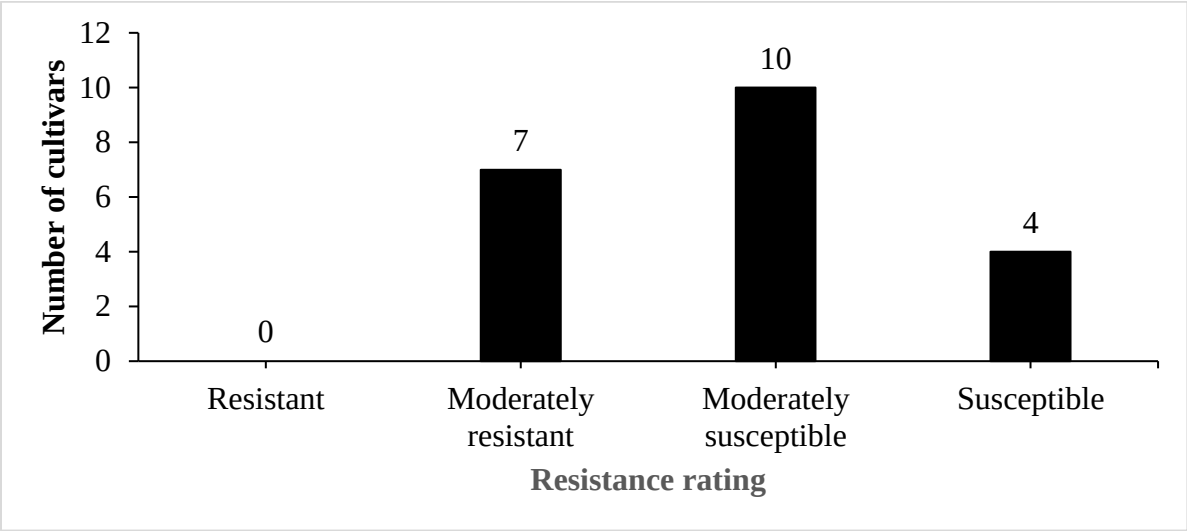

Fig. 5.1. Classification of the resistance responses of soybean cultivars to the new species of root-lesion nematode detected in Walcott, Richland County, ND. Ratio of postharvest population densities obtained from each cultivar compared to susceptible check NS 1911NR2 was used for scaling resistance Average ratio of postharvest population densities from 4 trials of two experiments were used to evaluate each of the 20 soybean cultivars and the positive control Barnes. The host ranking for each cultivar was then categorized into four classes based on the ratios as described by Smiley et al. (2014): Resistant = R (postharvest population density of root-lesion nematode < 25 % of the susceptible check NS 1911NR2), Moderately resistant = MR (26-50 %), Moderately susceptible = MS (51-75 %), and Susceptible = S (≥ 76 %).

Soil versus root habitat preference of the new root-lesion nematode species

Postharvest mean populations of the new root-lesion nematode species in root habitat were not significantly different ($P < 0.05$) from the mean population in soil habitat for a majority of the soybean cultivars tested in both trials of Experiment 2 (Table 5.5). However, the cultivars NS 60083NXR2 and S07-Q4X did have significantly different ($P < 0.05$) mean population densities in roots compared to soil in both trials. In trial 1, the cultivar 60083NXR2 had significantly lower proportion (27%) of root-lesion nematode population in root habitat compared to the soil habitat, whereas in trial 2, this cultivar had significantly higher proportion

(65%) of the population in roots (Table 5.5). On the contrary, in trial 1, the cultivar S06-Q4X had 73% of the population in roots, whereas in trial 2, this cultivar had 43% of the population in roots. Additionally, the cultivars NS 1742NLL and NS 1911NR2 in trial 1 and the cultivar NS 0081NR2 in trial 2 had significantly ($P < 0.05$) higher population of the root-lesion nematodes in roots. On the other hand, the cultivar 41-10 had significantly ($P < 0.05$) lower population of root-lesion nematodes in roots compared to soil in trial 1 of Experiment 2. However, it is evident that a majority of the cultivars tested in trial 1 and trial 2 of Experiment 2 had above or close to 50% of the total postharvest root-lesion nematode population in root habitat and the remaining proportion in soil habitat.

Table 5.5. Soil versus root habitat preference of the new root-lesion nematode (RLN) species detected in North Dakota[x].

	Trial 1			Trial 2		
	Mean RLN individuals in		Proportion in	Mean RLN individuals in		Proportion in
Cultivars	Soil[y]	Roots[y]	roots (%)[z]	Soil[y]	Roots[y]	roots (%)[z]
0616R2X	349	647	65.0	265	355	57.3
20915N	683	506	42.6	250	260	51.0
3408RR2YN	287	258	47.3	170	150	46.9
41-10	535	312	36.9*	305	220	41.9
Barnes	396	487	55.2	310	310	50.0
H09X7	467	294	38.6	231	173	42.8
INT 51449NRZX	492	427	46.5	350	320	47.8
LS-1335NRR2X	387	569	59.5	310	245	44.1
NS 0081NR2	368	460	55.6	170	360	67.9*
NS 0651NR2	154	162	51.3	150	155	50.8
NS 1291NLL	290	342	54.1	205	270	56.8
NS 1742NLL	248	454	64.7*	180	210	53.8
NS 1911NR2	439	742	62.8*	340	385	53.1
NS 2013NLL	296	383	56.4	215	314	59.3
NS 60083NXR2	792	295	27.2*	255	470	64.8*
NS 61493NXR2	232	165	41.5	160	182	53.2
NS 62002NXR2	541	639	54.1	330	290	46.8
S03-G9	381	350	47.9	210	205	49.4
S06-Q9	261	279	51.7	100	238	70.4*
S07-Q4X	320	864	73.0*	315	235	42.7*
S12-R3	258	462	64.2	175	280	61.5

[x] In both trials of Experiment 2, nematodes were extracted from soil and roots separately using sucrose centrifugal-floatation method (Jenkins 1964) and Whitehead tray method (Whitehead and Hemming 1965), respectively.

[y] From each soil cone-type container containing a single plant, total number of root-lesion nematode individuals in roots and in soil were quantified and averaged from five replicates of each cultivar.

[z] Proportion in roots = (number of RLN in roots/sum of RLN individuals in roots and in soil) X 100. Proportions of RLN in roots from cultivars that had significantly different ($\alpha < 0.05$) values were denoted by asterisk (*).

Discussion

This study is the first report on resistance levels of soybean cultivars to the new species of root-lesion nematodes detected in a soybean field in Walcott, Richland County ND. The new species of root-lesion nematode was demonstrated for the first time to invade and parasitize the root systems of 20 different soybean cultivars, in addition to the cultivar Barnes, that was previously reported to be a host of these endoparasitic nematodes (Yan et al. 2017). Furthermore, the habitat preference of this new root-lesion nematode species at nine weeks after planting was evaluated in the present study.

Previously, several root-lesion nematode species, including *P. brachyurus*, *P.* crenatus, *P. hexincisus*, *P. neglectus*, P. *penetrans*, and *P. scribneri*, were reported to invade and parasitize soybean (Acosta and Malek 1979; Castillo and Volvas 2007; Nicol et al. 2011; Schmitt and Barker 1981). Moreover, previous studies on these *Pratylenchus* spp. revealed a wide range of susceptibility among soybean cultivars (Acosta et al. 1979; de Almeida et al. 2014; Elhady et al. 2019; Lindsey et al. 1971; Santos et al. 2015; Schmitt and Barker 1981; Zirakparvar 1982). For instance, Acosta et al. (1979) evaluated and confirmed resistance levels of 12 soybean cultivars to *P. scribneri* under greenhouse conditions. Among these 12 cultivars, three were found to be resistant, three were highly susceptible, one was intermediate in susceptibility, and the remaining four were reported to support limited reproduction. Thus, these four cultivars could be considered as moderately resistant (Acosta et al. 1979). Another such study, Schmitt and Barker (1976), screened 25 southern soybean cultivars against *P. brachyurus* under greenhouse conditions. Among these 25 cultivars, nine were relatively resistant to *P. brachyurus*, seven were highly susceptible but tolerant, and nine were highly susceptible but intolerant (Schmitt and Barker 1976).

Similarly, Elhady et al. (2019) evaluated ten soybean cultivars against a mixed population of *P. crenatus* and *P. neglectus* and found different degrees of susceptibility among soybean cultivars. Of the ten cultivars evaluated by Elhady et al. (2019), three were reported to have the greatest postharvest population density and considered to be the most susceptible, two were reported as least susceptible, and the rest were intermediate in susceptibility (Elhady et al. 2019). In line with these results, our results indicate that the resistance levels of soybean cultivars to the new species of root-lesion nematodes varied between the cultivars. Variable responses of soybean cultivars to the new species of root-lesion nematode infection could be due to differences in cultivar genotypes. Several studies have explored soybean genotypes and differential lines to investigate soybean resistance against plant-parasitic nematodes such as soybean cyst nematodes and root-knot nematodes (Anand et al. 1985; Hua et al. 2018; Niblack et al. 2002; Ravelombola et al. 2020). However, few such studies were conducted to investigate *Pratylenchus*-soybean pathosystem (Rios et al. 2016). Thus, such investigations are an avenue that can be further explored for the new species of root-lesion nematodes in future studies.

In the present study, soybean cultivars were planted in soil naturally infested with the new species of root-lesion nematodes. Several studies in previous literature used sterilized soil that was artificially inoculated with root-lesion nematodes during planting (Acosta and Malek 1979; de Almeida et al. 2014; Lindsey and Carins 1971; Zirakparvar 1982). Artificially inoculating sterilized soil can ensure only a single species of root-lesion nematodes are infesting the soil in which seedlings are planted. Previous studies have indicated that there is a possibility of multiple species of root-lesion nematodes coexisting in the same field (Luc 1987; Wheeler et al. 1992). However, molecular identification of the root-lesion nematode specimens collected from the naturally infested soil used in this study indicated that only the new species of root-

lesion nematodes are infesting the soil. In greenhouse experiments with naturally infested soil equal amount of inoculum in each experimental unit can be achieved through homogenization of the inoculum throughout the soil sample prior to potting (Biela et al. 2016). Although, it is difficult to achieve even distribution of inoculum in naturally infested soil, it can be obtained through thorough mixing. In our study, we meticulously ensured even distribution of inoculum through thorough mixing.

Similar to our study, several studies in previous literature used naturally infested soil in cultivar screening bioassays that evaluated resistance levels of host crops against root-lesion nematodes (Akhter 2019; Biela et al. 2016; Forge et al. 2000; Kok and Coenen 1996; Motta et al. 2007). For example, Biela et al. 2016 evaluated the host status of 26 rice genotypes to *P. brachyurus* using naturally infested soil under greenhouse conditions. Similarly, Kok and Coenen (1996) evaluated the susceptibility of oil seed and fiber crops to *P. penetrans*. They conducted two greenhouse experiments, one of which was conducted with naturally infested soil and the other one was conducted with artificial inoculum.

To achieve the two objectives of this study, two experiments were conducted under greenhouse conditions. Since the objective of Experiment 1 was to evaluate the resistance levels of soybean cultivars to the new species of root-lesion nematodes, both trials of the experiment were harvested at fifteen weeks after planting as the plants reached maturity. Previous studies conducted by the North Dakota State University Nematology group have indicated that early maturing soybean cultivars such as Barnes reach maturity at approximately fifteen weeks after planting under greenhouse conditions (Unpublished data). Furthermore, Yan et al. (2017) found that the population density of the new species of root-lesion nematodes increased up to 5 times, compared to preplant density, when incubated with the cultivar Barnes for fifteen weeks under

greenhouse conditions. Several other studies in previous literature used the 15-16 weeks duration between planting and harvesting for greenhouse bioassays conducted to ascertain resistance levels of cultivars against root-lesion nematodes (Hafez et al. 2000; Smiley et al. 2014b; Thompson et al. 2020; Zirakparvar 1982).

Elhady et al. (2019) evaluated soybean cultivars for their susceptibility to root-lesion nematodes, solely based on postharvest population density in roots. Similar to the Experiment 2 of our study, Elhady et al. (2019) also harvested and evaluated the soybean cultivars at nine weeks after planting. Moreover, Santos et al. (2015) indicated that the greatest population density of root-lesion nematodes in soybean roots were observed nine to eleven weeks after planting. However, the cultivar evaluated by Santos et al. (2015) was a late maturing cultivar, primarily used in South American countries such as Brazil. Since the cultivars used in our study are generally early maturing cultivars that are rated for shorter number of days to maturity, both trials of Experiment 2 were harvested at nine weeks after planting.

Compared to Experiment 1, smaller cone-type containers were used in Experiment 2 because it became evident from both trials of Experiment 1 that it was extremely difficult to separate and obtain intact root system from soil, as the root system started to deteriorate when the plant reaches maturity. Since the objective of Experiment 2 was to evaluate the habitat preference of the new root-lesion nematode species in roots compared to soil, each replicate was planted in a small cone-type container containing 0.17 kg of soil. After harvest, we were able to gently empty the entire container into a 3-liter jug containing water. As a result, the soil particles were easily dislodged from roots without much fragmentation of roots. Thus, with greater vigor of the root system and the easy separation of soil particles from roots, we were able to accurately

quantify the new species of root-lesion nematodes in roots and in soil separately, formed in the entire container.

In previous literature, several studies have used reproductive factors to evaluate and rank resistance levels of host crops against plant-parasitic nematodes (Dababat et al. 2019; Sheedy and Thompson 2009; Upadhaya et al. 2019). Reproductive factor (RF) refers to the ratio of the postharvest population density relative to the preplant population density. However, previous research indicated that reproductive factors of the same cultivar could vary significantly with differing preplant population densities of nematodes (Santos et al. 2015; Schmitt and barker 1981; Upadhaya et al. 2019). For example, Upadhaya et al. (2019) found that the RF of *Paratylenchus nanus* on the cultivar Columbia was 2.0 when the preplant population density was 4,500 *P. nanus* per kg of soil. However, when the preplant population density of *P. nanus* was decreased to 1,500 nematodes per kg of soil, the RF value increased to 11.2 for the cultivar Columbia. Consequently, the host ranking of the cultivar Columba varied with the preplant population densities. Similarly, Schmitt and Barker (1981) found that the RF of *P. brachyurus* was significantly lower for the soybean cultivars Forrest and Essex, when the preplant population density was approximately 6,000 nematodes per kg of soil, compared to the preplant population density of 2,000 nematodes per kg of soil. Thus, RF was not used in our study to evaluate resistance levels of soybean cultivars against the new species of root-lesion nematodes.

In the present study, the ratio of the postharvest population density of a test cultivar relative to the postharvest population density of a susceptible check was used to scale resistance rating. Thus, differences in preplant population densities did not play a role in the resistance rating method used in this study. This method has been used to screen wheat cultivars for resistance against root-lesion nematodes in previous studies (Singh 2020; Smiley et al. 2014a).

Moreover, a similar method that uses the ratio of postharvest white females in a test cultivar relative to a susceptible check is commonly used to evaluate resistance levels of soybean cultivars and breeding lines against *Heterodera glycines* (Acharya et al. 2017; Hua et al. 2018; Koenning 2004; Schmitt and Shannon 1992; St-Amour et al. 2020). Furthermore, the resistance rating method used in this study may be easily adopted in future studies that evaluate resistance levels of soybean cultivars against the new species of root-lesion nematodes under field conditions. The pre-sowing population densities of plant-parasitic nematodes can have high spatial variation in cropping fields (Nicol et al. 1999; Reeves et al. 2020). Thus, using RF based ranking of host cultivars may be challenging under field conditions because all experimental sites may not have the same preplant population densities (Reeves et al. 2020).

According to our results, seven of the cultivars consistently produced the same resistance reaction in all four trials of the two experiments. However, the remaining cultivars did not have the same resistance reactions in all four trials. Nonetheless, it is important to note that the resistance reactions for most of the remaining cultivars did not vary significantly between the trials. For example, in the first and second iterations of the experiment the cultivars 3408RR2YN and NS 0651NR2 were moderately resistant in trial 1 of Experiment 1 and both trials of Experiment 2, while being resistant in trial 2 of Experiment 2. Similarly, the cultivars 0616R2X and NS 62002NXR2 were moderately susceptible in both trials of Experiment 1, while being susceptible in Experiment 2. The variation in resistance ratings between trials of the experiments could be attributed to slight inconsistencies of the environmental conditions in the greenhouse, such as minor variations in air temperature, air currents, soil moisture, and time of the year the trials were conducted on.

When analyzing the root versus soil habitat preference of the new root-lesion nematode species, it became evident that considerable variations occurred between the cultivars, even the replicates of the same cultivar. Moreover, for some cultivars significant variations occurred between the two trials of Experiment 2. For example, in trial 1 the cultivar S06-Q4X had significantly higher proportion of the population in roots whereas in trial 2 significantly lower proportion of the population were in roots compared to soil. On the other hand, the cultivar NS 60083NXR2 had significantly lower proportion of the population in roots in trial 1 whereas in trial 2 this cultivar had significantly higher proportion of the population in roots compared to soil. Such variations could be attributed to individual plants vigor and the proliferation of the root system. During postharvest processing, we realized through visual observation that plants with greater vigor of root system during harvest had higher proportion of the postharvest population density in roots. However, root mass measurements were not taken.

Thus, habitat preference analysis results indicate that a constant scaling factor cannot be used to account for the ratio of soil vs root habitat preference of the new root-lesion nematode species. However, our results did indicate a significant proportion of the new root-lesion nematode population resided in roots. In fact, in majority of the cultivars, above or close to 50% of the new species of root-lesion nematodes were recovered and quantified from the root habitat. Thus, it is of paramount importance to assay both roots and soil when determining the total population of the new root-lesion nematode in a sample Further studies will be conducted to ascertain the relationship between the new species of root-lesion nematodes and plant yield and plant growth parameters.

Based on the results presented in this study, it can be concluded that the susceptibility of soybean cultivars to the new species of root-lesion nematodes is greater for some cultivars than

others. Similar resistance reactions observed in all four trials of the experiments conducted in this study, confirmed that cultivars such as NS 1911NR2 and NS 60083NXR2 were the most susceptible, while cultivars such as 3408RR2YN and NS 0651NR2 were the least susceptible. Analysis of the habitat preference of the new species of root-lesion nematodes, revealed that above or close to 50% of the root-lesion nematodes resided in the root habitat at nine weeks after planting for a majority of the cultivars evaluated in this study. However, further research is needed to evaluate the performance of these cultivars under field conditions. These results provide us an insight into the virulence of the new species of root-lesion nematodes discovered in ND, in commercial soybean cultivars. Such research can have significant practical implication for soybean growers and researchers in finding best resistant cultivars to minimize yield loss.

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CHAPTER 6: SUMMARY

In North Dakota (ND), eight different genera of plant-parasitic nematodes (PPN) were present in corn fields of 20 major corn producing counties. Among these PPN genera, *Helicotylenchus*, *Paratylenchus*, *Pratylenchus* and *Tylenchorhynchu* were the top four most dominant nematodes based on incidence, distribution and abundance. The species identified for the first time in ND corn fields were *Helicotylenchus microlobus*, *Hoplolaimus stephanus*, *Paratrichodorus allius*, *Paratylenchus nanus*, *Pratylenchus scribneri* and *P. neglectus*. Significant correlations between nematode genera and various environmental factors were also observed. Canonical correspondence analysis revealed that approximately 22 % of the total inter-site variation in distribution and population densities of PPN genera was explained by the seven edaphic and climatic variables evaluated in this study.

In North Dakota soybean fields, a shift in soybean cyst nematode (SCN; *Hterodera glycines*) virulence phenotypes has occured since its first detection in 2003. Thus, we report for the first time, five new HG types, namely 7, 2.5.7, 1.2.5.7, 5.7, and 2.7, that were detected in SCN field populations from North Dakota, in addition to HG type 0 reported previously. Repitition of the HG type tests validated the occurrence of each of these HG types in North Dakota. Moreover, the repeated HG type tests proved that there were several SCN populations in North Dakota that successfully reproduced on indicator line #2 (PI 88788), the most widely used source of resistance.

The PCR assays developed in this study provides a rapid, specific, and sensitive detection method for the new species of root-lesion nematode (*Pratylenchus* sp.) detected in ND. The IC-ITS1F/IC-ITS1R primer set developed in this study can be used in both conventional and real-time PCR assays to specifically and sensitively identify the new *Pratylenchus* sp. Detection

sensitivity analysis revealed that the conventional PCR assay was able to detect an equivalent to 1/8 of the DNA of a single nematode, whereas, the real-time PCR assay with higher sensitivity could detect an equivalent to 1/32 of the DNA of a single nematode. Moreover, the species-specific primer set could distinguish and discriminate between the new *Pratylenchus* sp. and other *Pratylenchus* spp. commonly detected in ND soybean fields.

Susceptibility of soybean cultivars to the new species of root-lesion nematodes varied between the cultivars evaluated in this study. Similar resistance reactions observed in all four trials of the experiments conducted in this study, confirmed that cultivars such as NS 1911NR2 and NS 60083NXR2 were the most susceptible, while cultivars such as 3408RR2YN and NS 0651NR2 were the least susceptible. Combined results of all the trials indicated that seven of the cultivars were moderately resistant, ten of the cultivars were moderately susceptible, four of the cultivars were susceptible, but none of them were resistant. Analysis of the habitat preference in roots versus soil revealed that above or close to 50% of the new *Pratylenchus* sp. resided in the root habitat at nine weeks after planting for a majority of these cultivars.