AN ASSESSMENT OF NEMATODES AFFECTING WHEAT IN MONTANA

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Plant Pathology

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 2018

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DEDICATION

To all scientists who are always searching to get truth of this universe. To my martyred father, Talib, *in memoriam*, and all freedom martyrs whose blood lit the path to liberty. To my mother Bedriah, my wife Ghusoon, my children Samaa, Ahmed, Talib, Tiba, Noor, and all my friends who have always given me unconditional advice and support.

ACKNOWLEDGEMENTS

I would like to thank and show my deep appreciation to my adviser, Dr. Alan T. Dyer, for guiding me patiently through my PhD program. I would like also express my gratitude to my committee members: Dr. Michael Giroux, Dr. William Grey, Dr. John Martin, and Dr. Kris Lambert, for their contributions to my success and knowledge growing through these years. I would also like to thank my lab members Erin Gunnink Troth, Jeff Johnston, Nathaniel Ellis, and David May for their tireless help and support. Thank you also to the members of the Department of Plant Science & Plant Pathology who have helped me throughout these studies. Thank you to Dr. Richard Smiley (Oregon State University) and Eileen Carpenter (Montana State University). Thank you to the Montana Extension agents who helped me do my survey across Montana.

I am deeply grateful to the Republic of Iraq, especially the Establishment of Martyrs, for the scholarship and all the necessary support during my study. Thank you to the Ministry of Higher Education and Scientific Research, and the Iraqi Cultural office in Washington DC, for their help and support concerning formalities. Also, I would to thank the Montana Wheat and Barley Committee for funding these projects.

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ABSTRACT

Nematodes represent a major biological constraint on wheat and barley production worldwide, and yet no comprehensive assessment of plant parasitic nematodes associated with cereals has been conducted for Montana. To address this shortcoming, a survey was conducted to determine the species of plant parasitic nematodes associated with small grains, and an assessment of variation in virulence across 4 crops and 8 resistant wheat lines was conducted for *Pratylenchus neglectus* populations collected from across Montana. The survey, conducted across 11 counties, and found widespread distribution of *P. neglectus, Tylenchorhynchus* spp., and *Tylenchus* spp. within the state. Populations of *P. neglectus* were generally low (268 to 363 nematodes/kg of dry soil for 2015 and 2016, respectively). However, destructive populations were detected in 9 fields in both 2015 and 2016. In addition, populations of the cereal cyst nematode *Heterodera avenae* were detected. Cereal cyst nematode *Heterodera filipjevi*, a regulated pest, was detected in only one field. Additional species of parasitic nematode were detected, but rare. To assess the applicability of resistant crops and wheat lines for management of *P. neglectus*, greenhouse trials were conducted using nematode populations from within the state. In two trials conducted with resistant crops, significant interaction was detected between crops and populations of nematodes (ANOVA $P < 0.001$ and $P = 0.01$). In the first trial, populations from 3 counties were virulent on barley (mean reproductive factor = 10.9). Populations from other counties were either non-virulent on barley, or their inoculations were ineffective. In the second trial, 2 of the 3 populations were again virulent on barley (mean Rf = 4.4 and Rf = 10.7). Trials examining virulence across resistant wheat lines found no interactions between populations and wheat lines (ANOVA $P = 0.60$ and $P = 0.93$). While significant variation in reactions to the resistant lines were detected, none of the lines appeared particularly resistant to Montana populations, with mean Rf values of 13.1 and 15.4 for trials 1 and trials 2, respectively. Results suggest plant parasitic nematodes are localized problems, with *P. neglectus* and *Heterodera* species of particular concern, and that "resistant" wheat lines and barley may ineffective in managing *P. neglectus* in some regions of the state.

CHAPTER ONE

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized in three chapters. The first chapter contains the introduction, literature review, and research justification. Chapter two describes a survey of plant parasitic nematodes associated with wheat and barley fields in Montana. Chapter three includes experiments assessing variability in root lesion nematode populations from across Montana and in particular their virulence on common rotational crops and resistant wheat lines.

Cereal Crops

The Food and Agriculture Organization of the United Nations' (FAO) latest forecast for world cereal production in 2014 stands at 2.8 billion tons of cereals per year globally. Small grains including wheat, barley, oats, rye, triticale, rice and other species comprise the world's most significant source of food. Worldwide, wheat (*Triticum. aestivum* and *T. durum*) ranks as the third largest cereal staple in 2014 after corn and rice with global yield of 729 million tons, and barley (*Hordeum vulgare*) ranks a fifth largest cereal staple with global yield of 144 MT (USDA, 2016; FAO, 2016).Wheat and barley production in the United States primarily used for domestic consumption as well as for the export market.

Montana Wheat and Barley

Montana ranks third in production of both wheat and barley in the US. Wheat production of Montana in 2014 and 2015 were 5.7 and 5.0 million metric tons respectively, and 0.97 and 0.96 million metric tons of barley, respectively. Montana is also first in the nation in the production of certified organic wheat. Roughly 80% of Montana's wheat crop is exported out of the United States to purchasers worldwide. Montana produces three different classes of wheat: hard red winter, hard red spring and durum (WBC, 2016).

Available water is typically the biggest restriction to wheat production in the northern Great Plains of the United States, and this is especially true in Montana (Farahani et al., 1998; Lenssen, et al, 2007). The most common rotation with wheat in Montana is summer fallow, which is used to accumulate additional soil moisture for the next successive crop. Tillage during fallow periods may be done to control weeds; however, chemical fallowing in zero tillage systems improves soil water conservation, decreases soil erosion and allows for increased cropping intensity (Lenssen, et al, 2007). Common crop rotations for small grains in Montana include field pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietanum*), mustard (*Brassica juncea* and *Sinapis alba*), sunflower (*Helianthus annuus*), and safflower (*Carthamus tinctorius*) (Thomson et al, 1997; Miller et al.2002a, b, 2003a, b; Johnston et al. 2002; Lenssen, et al, 2007).

The Phylum Nematoda and Plant Parasitic Nematodes

Soil represents the most diverse ecosystem on earth, hosting more than 25% of all living species on our planet (Wolters, 2001; Decaens et.al 2006). A primary group of

metazoans within this ecosystem are the nematodes which are the most numerous multicellular organisms in the soil (Bongers and Ferris, 1999, Knox et al., 2004). The phylum Nematoda contains both free-living and parasitic nematode species. Plant parasitic nematodes (PPNs) comprise about 15% of the total number of named nematode species, of which there are more than 4,000 species (Wyss, 1997; Decraemer and Hunt, 2006). In 1998, Whitehead estimated that 10% of the world crop production was lost as a result of plant nematode damage (Whitehead, 1998). In particular, root-infesting nematodes not only directly reduce a plant's ability to withdraw water and nutrients from soil, but they may also interact synergistically in disease complexes with plant-pathogenic fungi that cause root diseases and wilts; these complexes result in more damage than would occur from either pathogen alone (Nicol et al., 2008; Dababat et.al 2015a,b).

In most cases, management of root infested nematodes is extremely challenging, particularly as the use of chemical nematicides has decreased (Wesemael et al, 2011). Genetic resistance to nematodes is perhaps the most desirable method of control of the pests, and resistance to some nematode species in some crops have been identified and exploited, but incorporating effective genes into commercial cultivars is a slow, arduous process. Disease management strategies other than genetic resistance, such as seed treatments, biological control agents, crop rotation, and fallow, are effective for some nematode species, but these strategies are not always environmentally or economically feasible (Dababat et.al 2015a,b).

As with any pest problem, the first step in managing PPNs is to correctly identify the nematode species, as host range and resistance are often species-specific, and is vital to the effective use of quarantine or regulatory strategies to limit their spread (Coomans et al, 2001). Correct identification of species is hampered by the highly conserved morphology among PPN species (Coomans ,2000; Siddiqi ,2000; Subbotin ,2006; Palomares-Rius ,2014; Oliveira et.al, 2006; Gutiérrez-Gutiérrez et.al, 2010; Cantalapiedra-Navarrete et.al, 2013; Archidona-Yuste et.al, 2016; Nickle, 1991). Currently, three effective methods are available for identifying PPNs: morphological, molecular barcoding, and proteomic techniques (Wilkins et al. 1995; Ahmad & Babalola 2014). Morphological taxonomy, or identification of nematode species based on their physical characteristics, has been efficiently used for the precise identification of a wide range of PPN species (Palomares-Rius et al, 2014; Gutiérrez-Gutiérrez et al, 2010; Cantalapiedra-Navarrete et al, 2013; Archidona-Yuste et al, 2016; Ye et al, 2004; Gutiérrez-Gutiérrez et al, 2012; Gutiérrez-Gutiérrez et al, 2013; Archidona-Yuste et al, 2016). However, morphological identification of individual PPN to species is not always possible due to lack of expert taxonomic knowledge. More recently, molecular barcoding has gained favor as a methodological answer to the relative lack of available nematode taxonomists. Unfortunately, barcoding is not a panacea but is relatively new, and still needs taxonomists to validate performance across many genera.

PPNs are a well-known threat to production of small grain cereals in fields that are highly infested by these parasites. It has been estimated that nematodes have decreased worldwide productivity of small grains by 7.0%, 6.3% and 4.2% for wheat,

barley and oats, respectively (Sasser and Freckman 1987). A more recent evaluation reports yield losses equal to about 10% of worldwide production (Dixon et.al 2009). At least seventeen economically important species in three major genera (*Heterodera*, *Pratylenchus* and *Meloidogyne*) affect small grains (Nicol et al., 2008; Smiley and Nicol 2009). The most economically important PPNs on cereal crops are cereal cyst nematode (CCN, *Heterodera*) and root lesion nematode (RLN *Pratylenchus*) (see Table 1)

Table 1. Plant parasitic nematodes in Pacific Northwest on small grain crops which cause economic damage (Smiley, 2015).

Nematode	Species of substantial importance
Cereal cyst	*Heterodera avenae* and *H. filipjevi*
Root-knot	*Meloidogyne naasi*
Root-lesion	*Pratylenchus neglectus* and *P. thornei*
Stunt	Merlinius brevidens (*Tylenchorhynchus* spp.)

(Dababat et.al 2015). Other genera such as root knot (*Meloidogyne*), stem (*Ditylenchus*) and seed gall (*Anguina*) nematodes are present, but are less economically important than CCN and RLN (see Table 2). The most economically important CCN species are *Heterodera avenae*, *H. filipjevi* and *H. latipons* (Rivoal & Cook, 1993; McDonald & Nicol, 2005). For root lesion nematodes, there are at least eight significant species infesting small grains (Rivoal & Cook, 1993); the most economically important are *P. thornei*, *P. neglectus*, *P. penetrans* and *P. crenatus*. They are all polyphagous and have a worldwide distribution (Nicol et al., 2008).

Table 2. Other plant parasitic nematodes of widespread, but less serious, economic importance in the Pacific Northwest on small grains (Smiley, 2015).

Nematode	Species of moderate importance
Foliar	*Aphelenchoides* species and *Megadorus megadorus*
Dagger	*Xiphinema* species
Lance	*Hoplolaimus* species
Pin	*Paratylenchus* species
Ring	*Criconemoides*, *Criconema*, and *Criconemella* species
Root gall	*Subanguina radicicola*
Root knot	*Meloidogyne chitwoodi*
Root lesion	*Pratylenchus penetrans* and other species not shown above
Spiral	*Helicotylenchus* species
Stem	*Ditylenchus* species
Stunt	*Tylenchorhynchus* species
Stubby root	*Trichodorus* and *Paratrichodorus* species

Cereal Cyst Nematodes

CCNs are important soil-borne cereal crop pathogens; they are sedentary plant parasitic nematodes that infect wheat, barley and oat (*Avena sativa*) (Peng et al, 2013; Wu et al, 2014; Kumar et al, 2014; Long et al, 2013). They are found worldwide and cause significant economic yield losses in many countries, especially in dryland cereal systems (Nicol et al, 2003; Subbotin et al, 2010; Dababat et al, 2015a, b). CCNs can have synergistic negative effects in combination with other biotic and abiotic factors, such as fungal pathogens and water stress (Nicol et al.2004, 2006). Nicol (2002) reported CCNs cause yield losses as high as 15–20 % in Pakistan, 40–90 % in Saudi Arabia, 23 -50 % in

Australia, and 24 % in the USA. Losses due to cereal cyst nematodes have been estimated at $78 billion worldwide (Barker et al, 1998). Based on their global distribution, dominance in areas where cereal is grown, and their pathogenicity, CCNs are ranked as the main nematode pest affecting the world's food supply (Subbotin et al, 2010; Dababat et al, 2015a, b).

The cyst nematode genus *Heterodera* contains 12 species that attack the roots of cereals and grasses. Three of these species (*H. avenae*, *H. filipjevi*, and *H. latipons*) cause significant economic losses in small grain crops globally (Smiley, 2016). *H. avenae* is most common in temperate wheat-producing regions globally (Nicol et al, 2003; Nicol, 2002; Rivoal and Cook 1993 and Smiley and Nicol 2009). *Heterodera filipjevi* has been detected in eastern and northern Europe, central and west Asia, the Middle East, the Indian subcontinent, and North America (Rivoal et al. 2003; Smiley and Nicol 2009; Smiley et al. 2008). *Heterodera latipons* occurs mainly in the Mediterranean region, but is also observed in Asia and Europe (Abidou et al.2005; Smiley and Nicol 2009). The species *H. bifenestra* and *H. hordecalis* are associated with wheat but are considered less problematic (Smiley and Nicol, 2009).

Heterodera avenae and *H. filipjevi* are the only two species of cereal cyst nematodes documented in the Pacific Northwest region of the United States. Both species are present in Montana, Oregon, and Washington (Dyer et al, 2015; Smiley, 2016). *Heterodera avenae* has been documented in Idaho (Smiley, 2016). The two species may occur in fields either individually or as a mixture of the two species. Each species may be composed of different pathotypes (i.e., strains or races) (Smiley, 2016). Most CNN

accomplish only one generation per crop season in temperature regions. During infection of roots, juveniles access epidermal and cortical cells of young root segments in the zone of elongation. They invade the stele, where they activate the formation of a specialized feeding cell called a syncytium. Females are fertilized by males and host 100-600 eggs within their body (Smiley and Nicol, 2009).

Cereal cyst nematode pathotypes and races are distinguished by testing unknown populations against a variety of cereals in 'The International Cereal Test Assortment for Defining Cereal Cyst Nematode Pathotypes,' suggested by Andersen and Andersen (1982a) and updated by Rivoal and Cook (1993). The test recognizes three primary virulence populations based on host resistance reactions of three barley (*Hordeum vulgare* L.) cultivars (Andersen, 1961; Cook & Williams, 1972; Andersen & Andersen, 1982a). More barley, oat (*Avena sativa* L. and *A. sterilis* L.) and wheat (*Triticum aestivum* L. and *T. durum* L.) differentials are used to specify pathotypes within each of the three populations (Andersen & Andersen, 1982a; Sanchez & Zancada, 1987; Rivoal & Cook, 1993; Cook & Rivoal, 1998; Cook & Noel, 2002).

The pathotype approach was first established to differentiate northern European populations of *H. avenae,* but has been found to be incomplete for characterizing virulence phenotypes in other regions. The original test array underrepresented the global polymorphism of this species (Cook & Noel, 2002; Nicol & Rivoal, 2008) and additional pathotypes have been reported. Thirty virulence phenotypes have been identified from 69 *H. avenae* populations tested worldwide (Cook & Rivoal, 1998), and further virulence phenotypes have yet to be assessed in many other countries (Mathur et al., 1974; Ibrahim

et al., 1999; Al-Hazmi et al., 2001; Peng et al., 2009). Mixtures of pathotypes have been observed within individual fields and geographic regions (Cook & Williams, 1972; Swarup & Sosa-Moss, 1990; Abidou et al., 2005; Handoo, 2002, Holgado et al., 2009; Yan & Smiley, 2009), and some have been reported to crossbreed (Andersen & Andersen, 1982a). Because of these phenotypic mixtures, the development of resistance to local populations of CCN has shifted to the use of differentials in resistance screening (Cook & Noel, 2002; Smiley & Nicol, 2009).

Root Lesion Nematodes

Root lesion nematodes (*Pratylenchus thornei* Sher & Allen and *P. neglectus* (Rensch) Filipjev Schuurmans & Stekhoven) pose a critical threat to dryland wheat production, globally. (Smiley et al. 2004a; Thompson et al. 2008). There are eight species of *Pratylenchus* that are pathogenic on wheat (De Waele and Elsen 2002; Nicol, 2002; Nicol et al., 2003; McDonald and Nicol, 2005; Castillo and Vovlas 2007), four of which (*P. crenatus, P. neglectus, P. penetrans, and P. thornei*) exist worldwide in temperate cereal-producing regions (Smiley and Nicole, 2009). Of these, *P. thornei* is considered most devastating, causing estimated yield losses of up to 85% in Australia, 70% in Israel, 37% in Mexico, and 50% in the United States (May et al.2016; Armstrong et al. 1993; Nicol and Ortiz-Monasterio, 2004; Smiley et al. 2005b). Estimated yield losses due to *P. neglectus* of up to 30% have been notified in Australia (Vanstone et al. 2008) and up to 37% in the Pacific Northwest region of the United States (Smiley et al. 2005a, b).

Pratylenchus species are migratory root endoparasites capable of feeding on a wide range of monocot and dicot host species (Loof 1978; Vanstone and Russ 2001a, b;

Vanstone et al., 2008). They live freely in soil, and while they may become entirely embedded in the root tissue, they always can migrate back into soil (Smiley and Nicole, 2009). RLN invade roots and move through root epidermal and cortical cells, which deteriorate and may predispose tissues to root-rotting fungi. As these lesions increase, they curtail the development of secondary roots, reducing absorption of water and nutrients. Synergism of root lesion nematodes, fungal pathogens, insect pests, and other plant parasitic nematodes have been observed (Lasserre et al., 1994; Taheri et al., 1994; Smiley et al., 2004a, b).

There are no distinctive foliar symptoms when plants are infected with RLN (Van Gundy et al., 1974; Dagan, 1984; Doyle et al., 1987; Thompson et al., 1995; Smiley et al., 2005a, b). Infected plants may show yellowing, wilting, stunting, reduced vitality and tillering, death of younger leaves, and reduced yield and grain quality. Yield losses are particularly substantial in plants under drought stress (Nicol and Ortiz-Monasterio 2004).

Root lesion nematodes that infect wheat are damaging even in the very driest rainfed wheat-producing zones. *Pratylenchus* species can stay alive in an inactive, dehydrated state (anhydrobiosis) in roots and soil during dry conditions (Glazer and Orion 1983; Talavera and Van stone, 2001). Populations of *Pratylenchus* often drop during long fallow periods between crops, but the nematodes have been observed at high rates of survival after long fallow periods (Orion et al., 1984; Talavera and Vanstone 2001, May et al., 2016). Both *P. neglectus* and *P. thornei* are parthenogenic: males are rare or absent. In contrast to this, species such as *P. penetrans* (Cobb) Filipjev and Schuurmans Stekhoven are amphimictic, with populations having both males and

females. *Pratylenchus* species have a vermiform body shape with average 0.5 mm long and 0.02 mm in diameter. Life cycles range from 45 to 65 days depending on species and environmental conditions. Females lay about one egg per day in root tissue or in soil. Juveniles of the J1 stage molt to become J2 while still inside the egg. J2 emerge from eggs after about one week after the egg is laid. Adults emerge after two more molts (J3 and J4), all stages of which are pathogenic.

Identification of *Pratylenchus* to the species level is an essential for most control strategies, particularly the development of resistant cultivars as resistance genes appear species specific (Smiley and Nicol, 2009). Nonetheless, identification is challenging because *Pratylenchus* species have substantial intraspecific variation and few morphological differences of taxonomic value. Accordingly, procedures to differentiate species based on comparative morphology (Loof 1978; Café Filho and Huang 1989; Handoo and Golden 1989) are often uncertain. New techniques have been established on proteins or DNA (Ibrahim et al., 1995; Ouri and Mizukubo 1999; Uehara et al., 1999; Andrés et al., 2000; Waeyenberge et al., 2000; Carta et al., 2001; Al-Banna et al., 2004; Carrasco-Ballesteros et al., 2007; Castillo and Vovlas 2007). In particular, PCR or RFLP procedures are useful for species identification (Yan et al, 2008).

The Root Lesion Nematode *Pratylenchus* spp. in Montana

A statewide soil survey in 2006-2007 found damaging populations of *Pratylenchus neglectus*, mainly in continuous winter wheat production areas of Montana, while *P. thornei* was not found (May et al., 2016). Statewide yield losses due to root lesion nematodes were 12% and 15% for winter wheat in 2006 and 2007, respectively. A

subsequent study conducted in 2008 to 2009 showed significant differences in reproductive success of *P. neglectus* among seven rotational crops. When nematode populations were assessed from spring to fall, populations did not change or decrease under fallow, barley, pea, lentil, and camelina rotations; populations increased under winter wheat and canola (May et al., 2016). Populations monitored from spring to spring declined for fallow, barley, pea, lentil, and camelina. Significant variation in RLN resistance was identified among Montana's most common barley cultivars (May et al., 2016).

Other Economically Significant Nematodes Identified in Montana

Root Knot Nematodes

Root knot nematodes (RKNs; *Meloidogyne* spp.), are economically significant worldwide (Nickle, 1991), and infect over 2,000 different plant species, including crops and wild plants. Root knot nematodes live part of their lives in soil, either as eggs or as second-stage larvae (J2). They later invade roots of susceptible hosts, and induce root swellings (Kyndt et al., 2014). A single gall can have several feeding sites and nematodes. Root galling acutely limits water and nutrient uptake, causing symptoms that include malnutrition, chlorosis, and stunting, causing large-scale losses in several crop plants. Four species, *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*, generate approximately 90% of the agronomic damage caused by root-knot nematodes in tropical and subtropical environments (James, 1991; Taylor and Sasser, 1978). Few field crops have been observed as infested with root-knot (*Meloidogyne naasi* and *M. chitwoodi*) nematodes in the semiarid Pacific Northwest (Smiley et al, 2004b).

Root knot nematode management in agriculture consists of soil fumigation, soil pasteurization, rotation with non-host crops, and the use of resistant varieties. Nonetheless, due to the environmental concerns associated with nematicidal soil fumigants, the high cost of thermal treatments, and few available resistant varieties, alternative methods of control, such as biological control and natural products, have attracted some attention. Nematicides of plant origin, including thiocyanates, glucosides, alkaloids, phenolics, fatty acids, and others, have been explored (Chitwood, 2002). Plant essential oils extracted from several plant species have been reported to have nematicidal activity and may provide potential alternative to currently used nematicides (Albuquerque et.al, 2007; Kong et. al 2006; Oka et. al, 2000; Onifade, 2007; Onifade 2008; Pandey et al, 2000; Park et al, 2007; Pérez et al 2003., Walker and Melin, 1996).

Stunt Nematodes. Stunt nematodes (*Tylenchorhynchus* Cobb, 1913) are a large group of plant parasitic nematodes. They are obligate migratory ectoparasites, and under some conditions they are endoparasites (Steiner, 1937; Siddiqi, 2000, Khan, 2008). *Tylenchorhynchus* species are surface feeders because they have short stylets and feed on epidermal cells (Khan, 2008). Symptoms include stunting of roots, yellowing of leaves, stunted foliage, defoliation, and wilt (Mai et al, 1996). Hosts include many agricultural crops and native plants (Siddiqi, 2000). Species number has been listed between 129 (Fortuner & Luc, 1987) and 145 (Ebsary, 1991). Esbary also reported that the genera *Bitylenchus*, *Telotylenchus*, *Quinisulcius*, *Dolichorhynchus*, *Trilineellus*, *Divittus*, *Morasinema*, *Tessellus*, *Neodolichorhynchus*, *Prodolichorhynchus*, and *Mulkorhynchus* are synonymous with *Tylenchorhynchus.* Stunt nematodes (*Tylenchorhynchus clarus* and

Geocenamus brevidens) have been discovered in 35% of fields of the Pacific Northwest, sometimes in large and damaging populations (Smiley et al, 2004a).

Spiral Nematodes. The *Helicotylenchus* genus was first identified by Steiner (1945) and more than 200 species now share this genus (Marais, 2001), called spiral nematodes due to the spiral shape they take when heat-shocked. The genus is found worldwide (Reis et al., 2010). Because of the large number of species and substantial intraspecific variation, it is hard to identify species of *Helicotylenchus* (Fortuner et al., 1981; Fortuner, 1984a, b; Marais, 2001). Many species of this genus are pathogenic nematodes (Subbotin et al, 2011). They may be ectoparasites, semi-endoparasites and even migratory endoparasites (Decraemer and Hunt, 2006). Semi-endoparasites insert their stylets and part of the head inside the root while feeding (Thorn, 1961). Symptoms of juveniles and adults feeding are small discolored lesions in the root cortex and other underground parts. Lesions in the cortex cause death of the cells, and high populations causes damage on roots (Mai & Mullin, 1996).). In general, plants infected with *Helicotylenchus* display subtle symptoms, but infestations may lead to secondary infections from other pathogens (Yeates and Wouts, 1992).

Dagger Nematodes. The *Xiphinema* genus (Cobb, 1913) contains a varied group of migratory ectoparasitic nematode species (Coomans, 1996; Lamberti et al, 2000). Injury by *Xiphinema* spp. to host plants is generated via direct feeding on root cells, and through the transmission of nepoviruses (genus *Nepovirus*, family Comoviridae). A number of *Xiphinema* species vector some of the most important plant viruses, such as

Arabic mosaic virus (*ArMV*), *Grapevine fanleaf virus* (*GFLV*), *Strawberry latent ringspot virus* (*SLRV*), *Cherry leaf roll virus* (*CLRV*), or *Peach rosette mosaic virus* (*PMV*) (Taylor and Brown, 1997). Because *Xiphinema* spp have substantial morphological diversity, the genus was divided into two groups (Loof and Luc, 1990; Luc et al, 1998; Lamberti et al, 2000; Coomans et al, 2001): (1) the *Xiphinema americanum*-group, which comprises a complex of approximately 60 species, and (2) the *X*. non-*americanum*-group, which comprises a complex of approximately 215 species, (Loof and Luc, 1990; Loof et al, 1996).

Eggs of *Xiphinema* spp. are placed individually in the soil near the roots of host plants, and hatch as first stage juveniles (J1) (Abawi and Mai, 1990). Some dagger nematode species have three juvenile stages, while others have four (Halbrendt and Brown, 1992). Generally, symptoms of *Xiphinema* feeding belowground are swellings on roots, delayed root growth, and decay of the apical meristem on roots. The primary aboveground symptom is stunting (Meyer and Hugo, 1994). Dagger nematodes have been a known pest for some time in the semiarid Pacific Northwest (Smiley et al, 2004a).

Pin Nematodes. *Paratylenchus* spp. Micoletzky, 1922 are obligate ectoparasites on roots of plants, spread globally and associated with numerous plant hosts (Raski, 1991; Siddiqi, 2000; Van den Berg et al, 2014). They feed on epidermal cells of roots or cortical tissues of roots and can reduce plant vigor, decrease yields, and reduce yield quality (Siddiqi, 2000; Andrássy, 1985; Braun and Lownsbery, 1975; Brzesk, 1976, 1995; Loof, 1975; MacDonald, 1976). More than 120 species of *Paratylenchus* have been studied and most of them are very small in body size (0.2- 0.6 mm) (Siddiqi, 2000;

Brzeski & Háněl, 2000; Van Den Berg et al, 2014). The morphological identification of this genus is, like other genera, still arduous because of overlapping diagnostic characterstics (Geraert, 1965; Brzeski & Háněl, 2000), many of which are subject to variation under environmental stress, such as temperature and population size (Fisher, 1965). To date, very few characteristics can be used for species identification, such as stylet length, body length, vulva position, lateral lines, head shape and vulval lateral flaps, etc. Molecular identification is in early stages of development (Van den Berg et al, 2014). Few field crops (winter wheat, cereal and broadleaf fields) were reported infested with pin nematodes, *Paratylenchus* spp., in the semiarid Pacific Northwest (Smiley et al, 2004a).

Lance Nematodes. Lance nematodes (*Hoplolaimus* spp. Von Daday, 1905) are migratory ecto, endo and semi-endo plant parasites. They are visually noteworthy as their stylet knobs are tulip-shaped, they have a large body length (0.9 - 2 mm), and a very widespread distribution within the United States, feeding on the roots of several field crop, grass, and tree species (Lewis et al., 1976; Koenning et al, 1999; Lewis and Fassuliotis, 1982; Robbins, 1982). Today, *Hoplolaimus* has more than 30 recognized species (Sher, 1963; Handoo and Golden, 1992; Perry and Moens, 2013).

Thesis Justification

Plant parasitic nematodes are widespread and can generate serious crop losses. Various species of plant parasitic nematodes have been reported to infect wheat and barley roots in many regions around the world. Root lesion nematode *Pratylenchus*

neglectus and stunt nematodes *Tylenchorhynchus* spp are the only migratory species to have been reported in wheat roots in Montana. However, the status of other plant parasitic nematodes species on wheat and barley are unknown in Montana's fields, a deficiency that this research seeks to address. In addition, while the presence of *P. neglectus* in Montana was known, the relative aggressiveness of Montana's root lesion nematodes populations across the state was unclear. The central goal of this research was to survey the plant parasitic nematodes species associated with Montana's wheat and barley fields and, if applicable, quantify any pathotypes in the most important plant parasitic nematodes species, such as root lesion nematodes and cyst. The results of this study provide important baseline information on the identification, pervasiveness, and distribution of plant parasitic nematodes in Montana's wheat and barley fields, the findings from which may guide management decisions in the future research. The threat of PPN in Montana cereals was largely unknown, outside of a 2006-2007 survey conducted by Wendy A. Johnson, which prioritized evaluation of root lesion nematodes. Because of the lack of comprehensive research of all PPN in Montana, it was necessary to conduct a nematode survey of Montana cereal fields. The objectives of this research were to:

1. Identify plant parasitic nematodes associated with wheat and barley in Montana's fields.
2. Determine average incidence and distribution of plant parasitic nematodes species in Montana's counties.

3. Determine variation in virulence among populations of *Pratylenchus neglectus* in Montana on several sources of resistance in wheat and on several Montana crops.

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CHAPTER TWO

IDENTIFICATION OF PLANT PARASITIC NEMATODES ASSOCIATED WITH WHEAT AND BARLEY IN MONTANA'S FIELDS

Abstract

Nematodes represent a major biological constraint on wheat and barley production worldwide and yet no comprehensive survey of plant parasitic nematode associated with wheat and barley production has been conducted for Montana. To address this shortcoming, a survey was conducted across eleven wheat- and barley-producing counties, involving 55 and 53 fields in 2015 and 2016, respectively. The survey found widespread distribution of *Pratylenchus neglectus, Tylenchorhynchus* spp., and *Tylenchus* spp. across the state. Populations of *P. neglectus* were generally low (268 to 363 nematodes/kg of dry soil for 2015 and 2016, respectively). However, destructive populations were detected in 9 fields in both 2015 and 2016. The impacts of *Tylenchorhynchus* spp. and *Tylenchus* spp. on crops are unclear. In addition, populations of the cereal cyst nematode *Heterodera avenae* were detected in 14 fields in 2015, and 5 fields in 2016; *H. avenae* was detected in Big Horn, Carter, Dawson, Judith Basin and Yellowstone counties. The cereal cyst nematode *Heterodera filipjevi* was detected in only one field, in Chouteau County. Nine other genera of plant parasitic nematodes were detected by the survey: *Aphelenchus, Haplolaimus, Helicotylenchus, Heterodera, Paratylenchus, Pratylenchus, Tylenchorhynchus, Tylenchus*, and *Xiphinema*. Their populations and distribution were limited, and therefore their impacts small grain

production are likely minimal. This work establishes that populations of *P. neglectus* are at lower levels than previously reported, and that populations of cereal cyst nematodes have restricted distributions at this time.

Introduction

Cereals represent the world's most significant source of food. Approximately 70% of all cultivated land is planted to cereal crops. By 2030, the world population is expected to growth to 8 billion. To meet the need of this growing population, it will require world wheat (*Triticum aestivum*) production to increase from 584 million metric tons (1995– 1999 average) to 860 million metric tons (Marathee and Gomez-MacPherson, 2001). The world nutritional deficit over this time span is expected to increase 2.5-fold, mostly impacting the developing world where 84% of the population increase is predicted to occur. Addressing these crop production demands will not be solved solely through improved yield potentials, but will require addressing the major biological sources of crop losses including plant pathogens, nematodes and insect pests (Nicol and Rivoal, 2008).

With a land area of 147,040 square miles, Montana is the fourth largest state in the United States after Alaska, Texas, and California (Infoplease, 2016), and has substantial variation in geography and climate. The state plays a significant role in wheat and barley production in the United States. Wheat production in Montana for 2014 and 2015 were 5.7 and 5.0 million metric tons, respectively. Montana ranked third in USA after North Dakota and Kansas. Barley production in Montana for 2014 and 2015 were

0.97 and 0.96 million metric tons respectively. Again, Montana ranked third in USA after North Dakota and Idaho (Statista, 2016; U.S. Grains Council, 2016).

Within the soil profile plant-parasitic nematodes (PPNs) are the most plentiful metazoans (Knox et al., 2004), and represent significant restrictions on cereal crop production with at least seventeen important nematode species. The most impactful of these come from three major genera: *Heterodera*, *Pratylenchus* and *Meloidogyne* (Nicol and Rivoal, 2008). Increased awareness of these nematodes and their impacts on small grains has developed in recent years. In 1998, Whitehead reported that 10% of the world crop production is lost as a result of plant parasitic nematodes (Whitehead, 1998). In addition, synergisms between these nematodes and other plant pathogens, especially soil borne fungi, often result increased damage relative to either pathogen alone (Nicol and Rivoal, 2008).

The most economically damaging nematodes for cereal crops are cereal cyst nematode (CCN, *Heterodera* spp.) and root lesion nematode (RLN, *Pratylenchus* spp). Other nematode species such as root knot (*Meloidogyne* spp.), stem (*Ditylenchus* spp.) and seed gall (*Anguina spp.*) are less economically important than CCN and RLN, either due to more restricted distributions or due to less damaging feeding habits. The most economically important CCN are *Heterodera avenae*, *H. filipjevi* and *H. latipons* (Rivoal & Cook, 1993; McDonald & Nicol, 2005). For RLN, there are at least eight species infesting small grains (Rivoal & Cook, 1993), but the most economically important are *P. thornei*, *P. neglectus*, *P. penetrans* and *P. crenatus*. These RLN species are polyphagous and widely distributed (Nicol and Rivoal, 2008).

In 2006 and 2007, a survey was conducted assessing distribution and importance of the root lesion nematode, *Pratylenchus neglectu*s (May et al, 2016). This work was invaluable as it showed for the first time that the RLN, *P. neglectus,* occurred in significant numbers throughout Montana, causing particularly heavy losses in winter wheat production areas (May et al, 2016). Since 2007, cropping systems in Montana have significantly changed, partly due to the economic pressures RLN placed on wheat monocultures, and partly due to changes in economics of legumes versus small grains. Today legumes, which are resistant to *P. neglectus,* amount to over 526,000 hectares, as opposed to approximately 121,000 acres in 2007 (https://www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=MONTANA). This change along with fact that the initial survey was not a comprehensive survey of plant parasitic nematode drives the current work to appraise the distribution and impacts of PPNs on Montana's small grains.

Materials and Methods

A survey of PPNs in Montana's small grain acreage was conducted in 2015 and 2016. The survey was conducted across eleven counties chosen based on to their large acreages of small grain production and geographic location in Montana; i.e., they well-represented the main small grain-growing regions of the state (Montana Agricultural Statistics, 2015). The surveyed counties represent approximately 40% of total wheat acreage in any given year (Montana Agricultural Statistics, 2015). Fifty-five fields were surveyed in 2015, and fifty-three were surveyed in 2016. The eleven counties surveyed

were: Bighorn, Carter, Chouteau, Dawson, Gallatin, Hill, Judith Basin, Liberty, Teton, Toole and Yellowstone (Figure 1). For each county, five wheat and/or barley fields were chosen. For each field, a minimum of twenty subsamples were gathered to generate a composite bulk sample, totaling a minimum volume of 2000 g. Subsamples were gathered by sampling at least 100 meters into the field, from a circle 100 meters in diameter. For each subsample, 2.5 cm-diameter soil cores were taken, to a depth of 30 cm. Sampling locations were chosen randomly, eschewing areas that displayed compaction by equipment (like tractors, combines, trucks, etc.) or were affected by a body of water, such as a pond or ditch. Information on previous cropping systems was collected from the grower for each field sampled. Soil type, moisture, and elevation were collected for the sites based on GPS data of field locations. Soil data was assessed based on GPS points taken for field samples and SoilWeb (UCDavis, NRCS-USDA, University of California).

In addition to soil samples, ten intact root systems were collected for each field from within the same field area. Soil cores were taken using a soil sampler with footstep (JMC Environmentalist's Subsoil Probe). Collected samples of both plants and soil were kept in plastic coolers during transport and kept refrigerated at 5 °C in the laboratory until nematodes were extracted.

Nematode Extraction from Soil Samples

Plant parasitic nematodes were extracted by Whitehead-Hemming tray method (Whitehead & Hemming, 1965; May et al, 2016), by soil sieving (Cobb, 1918), and by sucrose-centrifugation (Jenkins, 1964). The specific protocols are detailed below.

Whitehead-Hemming Method (Heretofore referred to as the Whitehead method). Twenty subsamples for one field were mixed together to make a bulk sample, and a coarse sieve (4.76 mm) was then used to remove large stones, gravel, and organic matter. A 200 g sample was taken from the sieved, bulked sample. This was spread on a Kimwipe lab tissue (Kimtech 30 x 30 cm.) over a plastic mesh screen (1 mm openings) on a metal frame (phosphor-bronze gauze with 25 mm openings), resting in a pool of shallow water in plastic trays (dimensions: 43w x 34l x 10d cm). Extractions occurred over 48 hours at 20° C. The nematodes were then concentrated by passing the nematode suspension through a 20 μm mesh sieve, and then rinsing the captured nematodes into 50 ml falcon tubes using 35 - 40 ml of sterile tap water.

Sieving and Sucrose Centrifugation. For each field, a 100 g sample was taken from the mixed bulk soil. This was mixed with 500 ml sterile tap water by pouring the suspension between two beakers ten times. A beaker with the soil sample was then whisked using a stainless laboratory spatula and heavy particles were allowed to settle for 15 seconds. The resulting suspension was then poured through stacked USA standard testing sieves, size No. 20 /No. 500 (841 μm /25 μm). Gentle tapping on the side of No. 500 sieve was done to aid drainage. A water bottle was used to gently wash nematodes

and to transfer them into a 50 ml centrifuge tube. Water was added to the tubes to equalize volumes at 40 ml. The resulting tubes were spun at 1700 RPM for 5 minutes. Supernatant was removed, leaving 5ml of water and pellet. Tubes were then filled with sucrose solution at a concentration of 454 g sugar/liter of deionized water at room temperature. A spatula was used to break up pellet of soil, mixing it into suspension, and tubes were centrifuged again at 1000 RPM for 60 seconds. Finally, the supernatant containing the nematodes was poured through the No. 500 sieve to separate the nematodes from the sucrose, gently rinsed with tap water, and decanted into vials.

Sieving Method for Cyst Nematodes. A 200 g subsample of the mixed bulk soil was mixed with 500 ml tap water using a spatula. The large particles in the suspension were allowed to settle for 10 seconds, and the remaining supernatant was poured through No. 35/No. 60 (500/250 μm) stacked sieves. The collected material on the fine sieve was rinsed gently with tap water and transfer to labeled vials to await examinations for cysts.

Statistical Analysis of Soil and Cropping Systems

Analyses of field environmental conditions were performed via single-factor ANOVA for: field soil type, elevation, mean annual temperature, mean annual relative humidity, and previous crop in 2015 and 2016. These analyses were conducted for populations of the 3 most common nematode genera: *Tylenchus* spp., *Tylenchorhynchus* spp., and *P. neglectus*. Other nematode species were excluded as they did not appear in sufficient fields to achieve statistical strength. Populations were natural-log transformed

to normalize the dataset. Analysis was conducted for all trials using RStudio version 3.2.3 (Crawley, 2012).

Morphological Nematode Identification and Quantification

Classical morphological characters were used to identify nematodes to genus by using 10x and 40x magnifications on a Nikon Eclipse 50i microscope (Melville, N.Y.) (Nickle, 1991; Handoo et al, 2014; Loof and Luc, 1990; Siddiqi, 1963; Van den Berg et al, 2014 and Wouts and Knight, 1993). A Leica 10445929 0.5x dissecting microscope was used to check samples for cereal cysts. Nematodes were removed for examination by using a dental pick (K-Files, size #08, 25mm, [07-0871558] Patterson Brand) and dissecting microscope.

A McMaster Counting Slide (Chalex Corporation, Wallowa, OR) was used to count nematodes. The counting chamber was filled with nematode suspension (2ml) and examined using a Nikon Eclipse 50i microscope. Resulting counts were converted to nematodes per kilogram of soil, and were adjust to account for soil moisture. Percent soil moisture was determined by drying 100 g of fresh soil at 70 °C for 48 h. Processing of samples occurred within 1 day to 2 weeks of each samples collection (May et al, 2016).

Molecular Nematode Identification

Single Nematode DNA extraction. The following protocol was adapted from Kelley Thomas' Lab (Department of Molecular Cellular and Biomedical Sciences and Program in Genetics University of New Hampshire): For each genus detected within a field, five representative nematodes were isolated using a dental pick and Leica

dissecting microscope. Each nematode was then processed individually. For this, glass slides were sterilized by ETOH 70% and DNA Away solution. Slides were used to clean the surface of the nematode by dipping nematode into 5 µl deionized water drops dispensed on the slides surface. Air flow pipe was used to dry the cleansed nematode. The dried nematode was then cut into two halves using a pipette tip which also delivered 5 µl Worm Lysis Buffer: 1M Tris (10 µl), 1M $MgCl_2$ (2.5 µl), 1M KCl (50 µl), Tween 20 (4.5 µl), 1% (w/v) gelatin (50 µl), 20mg/ml Proteinase K (3.3 µl) and ddH2O (879.7 µl). By pipette tip, all nematode tissues were moved from the slide by sucking the lysis buffer along with the nematode tissues and placing them in an eppendorf tube containing an addition 15 µl of lysis buffer. The tube was then placed in a -80 °C) freezer for 10 minutes, incubated at 60C for 1 hour, heated to 95 °C for 15 minutes, and finally cooled to 4 °C. Two µl of the resulting solution was used as template for PCR reactions.

SSU rDNA Amplification. The protocol from Holterman et al., 2006 was followed to amplify small subunit rDNA from nematodes:

SSU rDNA was developed as 2 half overlapping fragments using 3 universal and 1 nematode-specific primer (1912R), the latter contained to avoid amplification of non-target eukaryotic SSU rDNA. For the first fragment, either the primer 988F (5’ -ctcaaagattaagccatgc- 3’) or the primer 1096F (5’-ggtaattctggagctaatac-3’) was used in mix with the primer 1912R (5’ -tttacggtcagaactaggg- 3’). The second fragment was developed with primers 1813F (5’-ctgcgtgagaggtgaaat-3’) and 2646R (5’-gctaccttgttacgactttt-3’). The PCR profile was used: 94 °C for 5 min; 5 x (94 °C, 30 s; 45 °C, 30 s; 72 °C, 70 s) and by 35x (94 °C, 30 s; 54 °C, 30 s; 72 °C, 70 s) and 72 °C, 5 min.

The amplifications used a master mix (25 µl) made by mixing together each H_2O (13.38 µl), flexi 5x buffer (5 µl), MgCl2 (2 µl), 10mM dntp (0.5 µl), 10µM F (1 µl), 10µM R (1 µl), and go taq (0.125 µl). 2 µl DNA/tube will be taken. Gel-purified amplification outputs were sent to the MCLAB (San Francisco) for sequencing. Resulting sequences were then compared to published SSU rDNA sequences to identify genera and species using Basic Local Alignment Search Tool (BLAST) in https://blast.ncbi.nlm.nih.gov/Blast.cgi and the GenBank database (Holterman et.al 2006). https://blast.ncbi.nlm.nih.gov/Blast.cgi https://blast.ncbi.nlm.nih.gov/Blast.cgi

Results

In 2015, plant parasitic nematodes were detected in 80.0% and 98.2% of soil samples by using sugar-centrifugation and Whitehead methods, respectively. Similarly, in 2016, plant parasitic nematodes were detected in 86.8% and 98.1% of soil samples by using sugar-centrifugation and Whitehead methods, respectively. Seven species of plant parasitic nematodes were identified using both sugar-centrifugation and Whitehead methods in 2015, and eight species were identified by both sugar-centrifugation and Whitehead methods in 2016. An additional five plant parasitic species were detected in very low numbers using both methods in 2015 and 2016. Also, two cereal cyst nematode species were detected via the sieving method.

For 2015, identified nematode species included *Pratylenchus neglectus*, *Heterodera filipjevi, Heterodera avenae, Tylenchorhynchus* spp., *Tylenchus* spp., *Helicotylenchus* spp., *Haplolaimus* spp., *Paratylenchus* spp., and *Xiphinema* spp. These

same species were detected in 2016 along with Pin nematodes *Gracilacus* spp (Figure 2). The five rare unknown species belonged to the genera *Aphelenchoides* spp., *Aphelenchus* spp., *Ditylenchus* spp., *Scutellonema* spp. and *Meloidogyne* spp. In most cases, species and genera identified based on morphology were confirmed by molecular barcoding methods (Table 1).

Pathogen Incidence

Mean nematode density for the rest of the document will refer to the number of juvenile and adult nematodes per kg dry soil (nematodes/kg). Among the plant parasitic nematodes, *Pratylenchus neglectus* had a very high incidence, occurring in 30.9% (sugar-centrifugation) and 74.5% (Whitehead) of the sampled fields in 2015, and 62.2% and 75.4% of the fields in 2016. *Pratylenchus neglectus* mean density across fields was 172 (sugar-centrifugation) and 268 (Whitehead) nematodes/kg in 2015; 196 and 377 nematodes/kg, respectively, in 2016. Populations of RLNs were highest in Teton County in 2015 and 2016 (506 and 969 nematodes/kg, respectively; Figure 3) and were lowest in Judith Basin County (65 nematodes/kg) in 2015 and Toole county (17 nematodes/kg) in 2016. Among fields sampled in 2015 and 2016, damaging populations as determined by sugar-centrifugation (populations above 1000 nematodes/kg) were found in 5 fields (9% of fields), all of which occurred in either Gallatin or Teton counties. As assessed by the Whitehead tray method in both 2015 and 2016, damaging populations were detected in 9 fields (17% of fields), which occurred in Big Horn, Gallatin, Chouteau, Dawson, Hill and Teton counties.

Cereal cyst nematode species *Heterodera filipjevi* was detected in one field in both 2015 and 2016 (incidence of 1.8% and 1.9%, respectively) (Figure 4). The species *Heterodera avenae* was detected in 10 fields in 2015 (18.1% incidence) and four fields (7.5% incidence) in 2016. For *Heterodera filipjevi* and *Heterodera avenae,* the average number of cysts were 27 and 28 cysts, respectively per kg dry soil in 2015. In 2016 mean number of cysts were much lower at 0.3 (*H. filipjevi*) and 14 cysts (*H. avenae*) per kg dry soil. Juveniles of *Heterodera filipjevi* averaged 15 and 0 per kg dry soil, by sugar-centrifugation and Whitehead methods, respectively, in 2015. The averages number of juveniles of *Heterodera avenae* were 2 per kg dry soil, for both methods in 2016.

Tylenchorhynchus spp. had a very high incidence of isolation (Figure 5), being found in 54.5% and 89.0% of the sites by using sugar-centrifugation and Whitehead methods, respectively, in 2015, and in 52.8% and 69.8% of the fields in 2016. The mean of density of *Tylenchorhynchus* spp. was 317 (sugar-centrifugation) and 423 (Whitehead) nematodes per kg dry soil in 2015; in 2016, 196 (sugar-centrifugation) and 309 (Whitehead) nematodes per kg dry soil were observed. Similarly, *Tylenchus* spp. had a very high incidence, being found in 43.6% and 72.7% of the locations by using sugar-centrifugation and Whitehead methods, respectively, in 2015, and in 30.1% and 22.6% of the fields in 2016. *Tylenchus* spp. density averaged 825 (sugar-centrifugation) and 552 (Whitehead) nematodes per kg dry soil in 2015. In 2016 these numbers were much lower, at 79 (sugar-centrifugation) to 59 (Whitehead) juveniles and adults per kg dry soil in 2016.

Incidence of the other important species, *Helicotylenchus* spp., *Xiphinema* spp., *Paratylenchus* spp., and *Haplolaimus* spp, were 14.5%, 3.6%, 1.8%, and 1.8%, respectively, as observed by sugar-centrifugation in 2015; the Whitehead method generated 14.5%, 0%, 10.9%, and 7.2% incidence, respectively. In 2016, incidence of *Helicotylenchus* spp., *Xiphinema* spp., *Paratylenchus* spp., and *Haplolaimus* spp., and *Gracilacus* spp. were 13.2%, 5.6%, 5.6%, 3.7%, and 0%, respectively, by sugar-centrifugation. Incidence using the Whitehead method in 2016 was 15.0%, 1.8%, 1.8%, 3.7%, and 1.8%, respectively. Density of the other cereal parasitic species, *Helicotylenchus* spp., *Xiphinema* spp., *Paratylenchus* spp. and *Haplolaimus* spp. in 2015 were 89, 18, 20, and 2 juveniles and adults per kg dry soil, respectively, by using sugar-centrifugation, and 114, 0, 10, and 4 juveniles and adults per kg dry soil, respectively, by the Whitehead method. Density of *Helicotylenchus* spp., *Xiphinema* spp., *Paratylenchus* spp. and *Haplolaimus* spp. in 2016 were 64, 9, 7, and 5 juveniles and adults per kg dry soil, respectively, by using sugar-centrifugation, and 112, 2, 4, and 8 juveniles and adults per kg dry soil, respectively, via the Whitehead method.

Other important plant parasitic nematodes that were detected at rare incidence and density include: *Aphelenchoides* spp., *Aphelenchus* spp., *Ditylenchus* spp., *Scutellonema* spp., and *Meloidogyne* spp (data not presented).

Effect of Soil and Cropping Systems.

An analysis of environmental factors (temperature, moisture, and elevation) found no associations between environmental factors and densities and distributions of plant parasitic species (data not shown) with the exception of a negative correlation between

altitude of fields and density of *Tylenchorhynchus* spp. (r = -0.341, *P*= 0.012) and (r = -0.478, *P*= 0.002) as determined by sugar-centrifugation and Whitehead extraction methods in 2016, respectively.

In 2015, previous crops did not significantly predict the density of *P. neglectus* as determined by sugar-centrifugation and Whitehead methods (*P*= 0.635 and *P*= 0.233 respectively, ANOVA). Previous crops did not significantly affect the density of *Tylenchorhynchus* spp. (*P*= 0.441, ANOVA), as determined by sugar-centrifugation, but previous crops significantly affected the density of *Tylenchorhynchus* spp. (*P*= 0.007, ANOVA) as determined by Whitehead method (Figure 5). A follow-up Tukey's test of multiple comparisons of means identified differences between sugar beet and barley (mean nematodes/kg = 0 and 119, respectively, *P*=0.03), spring wheat and fallow (mean nematodes/kg = 146 and 296, respectively, *P*= 0.04), sugar beet and fallow (mean nematodes/kg = 0 and 296, respectively, *P*= 0.01) and winter wheat and sugar beet (mean nematodes/kg = 772.5 and 0, respectively, *P*= 0.028). Previous crops did not significantly affect the density of *Tylenchus* spp. (*P*= 0.094 and *P*= 0.622) as determined by sugar-centrifugation and Whitehead methods, respectively.

In 2016, previous crops did not significantly affect the density of *P. neglectus* (*P*= 0.251, ANOVA) as extracted by sugar-centrifugation, but they significantly affected the density of *P. neglectus* as extracted by the Whitehead method (*P*= 0.012, ANOVA). Tukey's multiple comparisons of means identified a significant difference between winter wheat and alfalfa (mean nematodes/kg of =478 and 0, respectively, *P*= 0.05). Previous crops did not significantly affect the density of *Tylenchorhynchus* spp. as extracted by

sugar-centrifugation and the Whitehead method (*P*= 0.35 and *P*= 0.61, respectively, ANOVA). In addition, previous crops did not significantly affect the density of *Tylenchus spp.* that extracted by sugar-centrifugation and Whitehead methods (*P*= 0.32 and *P*= 0.62, respectively, ANOVA).

The effect of soil type was assessed by ANOVA. In 2015, soil type did not significantly affect the density of *P. neglectus* (*P*= 0.25 (sugar-centrifugation) and *P*= 0.70 (Whitehead)), or of *Tylenchorhynchus* spp. (*P*= 0.48, ANOVA) as extracted by sugar-centrifugation; however, a significant effect on the density of *Tylenchorhynchus* spp. was observed (*P*= 0.007, ANOVA) as extracted by the Whitehead method. Tukey's multiple comparisons of means identified significant differences between silt loam - clay loam (mean nematodes/kg =44 and 501, respectively, *P*= 0.007), silt loam – loam (mean nematodes/kg =44 and 271, respectively, *P*=0.01), and silt clay loam –silt loam (mean nematodes/kg = 631 and 44, respectively, *P*=0.04). Soil types did not significantly affect the density of *Tylenchus* spp. as extracted by sugar-centrifugation and the Whitehead method (*P*= 0.102 and *P*= 0.401, respectively, ANOVA).

In 2016, soil type did not significantly affect the density of *P. neglectus* (*P*= 0.12, ANOVA) as extracted by sugar-centrifugation, but a significant effect on the density of *P. neglectus* extracted by the Whitehead method was observed (*P*= 0.02, ANOVA). Tukey multiple comparisons of means identified significant differences between loam-clay loam (mean nematodes/kg = 632 and 216, respectively, *P*= 0.02). Soil type did not significantly affect the density of *Tylenchorhynchus* spp. as extracted by sugar-centrifugation and the Whitehead method (*P*= 0.14 and *P*= 0.43, respectively, ANOVA).

Similarly, soil type did not significantly affect the density of *Tylenchus* spp. as extracted by sugar-centrifugation and the Whitehead method (P= 0.092 and P= 0.79, respectively, ANOVA).

Extraction Methods Correlate Significantly

The results of the sugar-centrifugation extraction method significantly correlated with the Whitehead method for densities of *Pratylenchus neglectus* in both 2015 (R^2 =0.33, $P < 0.001$) and 2016 (R^2 =0.496, $P < 0.001$). Populations detected by the Whitehead method were significantly greater (mean = 268 and 376 nematodes in 2015 and 2016, respectively) than those detected with sugar extractions (mean = 171 and 196 nematodes in 2015 and 2016, respectively). Similarly, both methods correlated significantly for density of *Tylenchorhynchus* spp. in 2015 (R^2 =0.173, $P < 0.01$) and 2016 (R^2 =0.435, $P < 0.001$). Populations detected by the Whitehead tray method were significantly greater (mean = 422 and 309 nematodes in 2015 and 2016, respectively) than those detected with sugar extractions (mean = 317 and 196 nematodes in 2015 and 2016, respectively). Results were similar for densities of *Tylenchus* sp.; the two methods significantly correlated in 2015 (R^2 =0.733, $P < 0.001$) and 2016 (R^2 =0.17, $P < 0.01$). Mean populations detected by the Whitehead tray method were 552 and 58 nematodes in 2015 and 2016, respectively, and by sugar-centrifugation were 825 and 78 nematodes in 2015 and 2016, respectively.

Discussion

This study is the first comprehensive survey for plant parasitic nematodes associated with Montana's wheat and barley fields. It provides the first documentation for the existence, incidence, density, and geographic distribution of many plant-parasitic nematodes associated with wheat and barley for the state. Root lesion nematodes *Pratylenchus neglectus*, stunt nematodes *Tylenchorhynchus* spp., and *Tylenchus* spp. were found broadly distributed across Montana's wheat and barley growing regions (Figures 2 and 3). Both *Pratylenchus neglectus* and *Tylenchorhynchus* spp. have been previously reported as being widespread (May et al, 2016). Our results document *Tylenchus* spp. as new species of concern for the state. The survey also confirmed the presence of cereal cysts nematodes, as previously reported (Smiley, 2010; Dyer et al, 2015). Previously undocumented species for Montana's small grains reported here include *Merlinius joctus*, *Helicotylenchus vulgaris*, *Helicotylenchus varicaudatus, Xiphinema elongatum*, *Xiphinema brevicolle*, *Paratylenchus nanus*, *Haplolaimus galeatus*, *Gracilacus epacris*, *Aphelenchoides fragariae*, *Aphelenchus* spp., *Ditylenchus* spp., and *Scutellonema bradys.*

This study provides critical new insights into *Pratylenchus neglectus* dynamics since the first survey was conducted in 2006 and 2007 (May et al, 2016). Density of *P. neglectus* are significantly less than those previously reported; *P. neglectus* populations in this survey were approximately tenfold lower than densities recorded in 2006 and 2007 (May et al. 2016). At the time of the first survey, wheat production was intense, and crop rotation was limited to non-existent in the state. Through economics and active

promotion, rotational crops including peas and barley have gained tremendous favor in the state. Our previous research has shown these crops provide considerable benefits in the management of root lesion nematodes (May et al, 2016). Crop rotations have not eliminated this pest, however, and root lesion nematodes will continue to be a threat to wheat production in the state, necessitating the use of resistant rotations and the incorporation of resistance into locally adapted wheat cultivars. The current survey confirms previous findings that *P. neglectus* prefers winter wheat, and also documents a preference for loamy soils over heavier clay loam soils.

This study provides the first information on the distribution of cereal cyst nematodes *Heterodera filipjevi* and *Heterodera avenae* in Montana's wheat and barley fields (Figure 4). The presence of cereal cyst nematode *Heterodera avenae* has been previous reported in Montana by Smiley and Yan in 2010, and *Heterodera filipjevi* was first reported in Montana by us in 2015 (Dyer et al, 2015). The isolation of *H. fillipjevi* to a single field suggests a recent arrival in the state, which indicates that an effort made to limit its spread will be important in the future. This species is considered more destructive that *H. avenae,* as it affects winter wheat much more severely (Smiley and Guiping, 2015; Smiley, 2016). Good management tools are available for both of these pests as determined by plant pathologists from neighboring states (Smiley et al, 2011; Smiley et al, 2013; Smiley, 2016). These include information on relative susceptibility of wheat cultivars and yield response curves for nematode population sizes. These need to be locally validated.

Survey results show that *Tylenchorhynchus* spp. is present in all eleven counties surveyed. This nematode is an ectoparasite, limiting the time of activity for the species in the field. This likely limits its ability to damage crops including wheat (Nickle, 1991). Similar statements could be made about *Tylenchus* spp., which are also widespread in Montana's wheat and barley fields. Little information is available on their impacts on crop species and wheat in particular. Weak preferences were documented for *Tylenchorhynchus* spp. to fields located at lower altitudes, and winter wheat crops. Populations of *Tylenchorhynchus* spp. tended to be higher in non-silty soils as well. No preferences were discerned for *Tylenchus* spp.

Other damaging plant parasitic nematodes, including *Helicotylenchus* spp., *Paratylenchus* spp., *Haplolaimus* spp., *Xiphinema* spp., are less widespread and are at low enough numbers in Montana's wheat and barley fields that they are unlikely to be a significant threat at this time. Their presence may pose future risks as cropping methods evolve that may favor their population increase. This is especially true for *Xiphinema* spp., which is a noted virus vector. Other plant parasitic nematodes identified include *Meloidogyne* spp, *Aphelenchoides* spp., *Aphelenchus* spp, *Gracilacus* spp., *Ditylenchus* spp, and *Scutellonema* spp. These were all documented, but were so rare in Montana's wheat and barley fields that they are of little concern.

For this survey, two methods were used for the extraction of migratory nematodes: sugar-centrifugation and the Whitehead tray method. The sugar-centrifugation method is a very rapid method for the extraction of many samples in a short time; thus, it is one of the most common techniques used in the United States for the

extraction of soil nematodes. It also improves extractions for some larger nematode species (Viglierchio and Schmitt 1983; Barker, 1985). The results from the two methods regularly correlated with each other for this survey, but those extracted by Whitehead tray method were significantly higher than those by sugar-centrifugation. In other words, the Whitehead method was a more effective and sensitive extraction method than sugar-centrifugation for the species found in this survey. Previous studies have documented similar results (Freckman and Virginia, 1993). These extraction methods are dynamically different. The sugar centrifugation method is a passive extraction technique due to the centrifugation, whereas the Whitehead method is an active extraction technique that is reliant on nematode behavior. As such, differences in total numbers are not surprising. It is worth noting both methods arrived at comparable answers with regard to distribution of PPNs, which match previous reports as well (McSorley and Walter, 1991).



Figure 1. Map showing the Montana counties surveyed, in red, in both 2015 and 2016.



Figure 2. Nematodes of significance in Montana. Top row, left to right: *Pratylenchus neglectus*, *Heterodera* spp., and *Tylenchorhynchus* spp.
Middle row, left to right: *Helicotylenchus* spp., *Tylenchus* spp., and *Xiphinema* spp.
Bottom row, left to right: *Paratylenchus* spp., *Gracilacus* spp., and *Aphelenchoides* spp.

Table 1. Putative species identification by classical morphological trait and by molecular barcoding and BLAST searches of standard nucleotide BLAST

Genus	Putative Morphological Species	Putative Barcoding Species	Blast % Similarity
Pratylenchus	*Pratylenchus neglectus*	*Pratylenchus neglectus*	98
Heterodera	*Heterodera filipjevi*	*Heterodera filipjevi*	100
Heterodera	*Heterodera avenae*	*Heterodera avenae*	100
Tylenchorhynchus	*Tylenchorhynchus clarus*	*Tylenchorhynchus maximus*	96
Tylenchorhynchus	*Tylenchorhynchus clarus*	*Merlinius joctus*	98
Tylenchus	*Tylenchus parvus*	*Tylenchus arcuatus*	97
Helicotylenchus	*Helicotylenchus vulgaris*	*Helicotylenchus vulgaris*	99
Helicotylenchus	*Helicotylenchus vulgaris*	*Helicotylenchus varicaudatus*	99
Paratylenchus	*Paratylenchus nanus*	*Paratylenchus nanus*	94
Haplolaimus	*Haplolaimus galeatus*	*Haplolaimus galeatus*	96
Xiphinema	*Xiphinema elongatum*	*Xiphinema americanum*	99
Xiphinema	*Xiphinema elongatum*	*Xiphinema brevicolle*	99
Aphelenchoides	*Aphelenchoides fragariae*	*Aphelenchoides fragariae*	96
Aphelenchus	*Aphelenchus* spp.	*Aphelenchus* spp.	95
Scutellonema	*Scutellonema* spp.	*Scutellonema bradys*	94

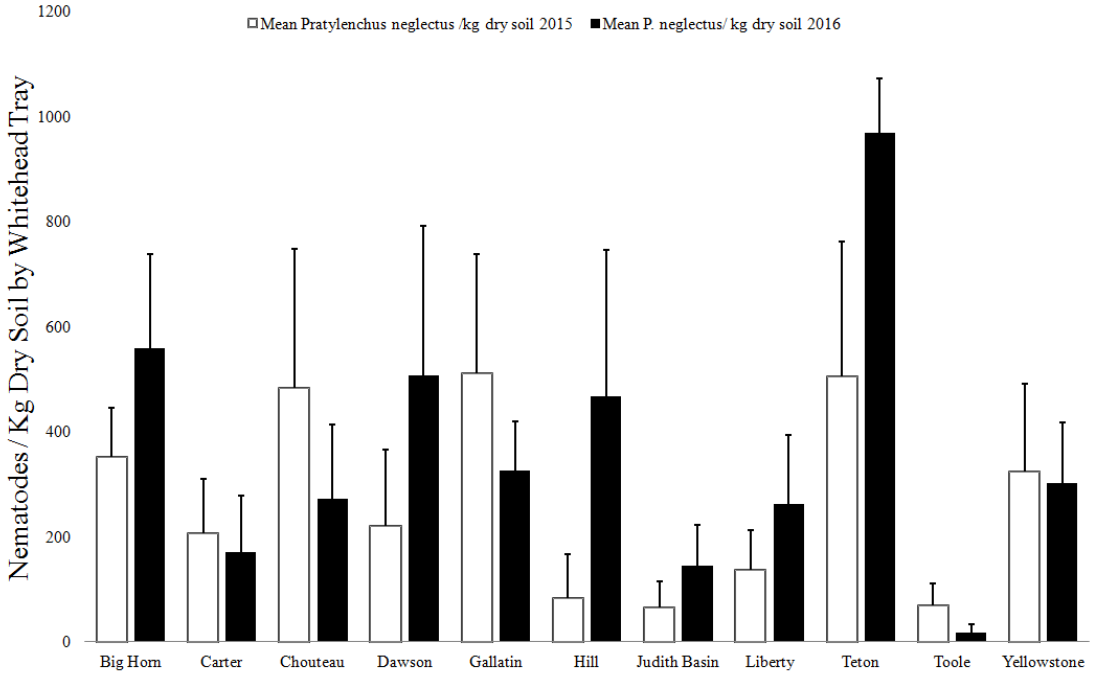


Figure 3. Mean of root lesion nematodes *Pratylenchus neglectus* densities per kg dry soil by Whitehead trays in Montana counties in 2015 and 2016

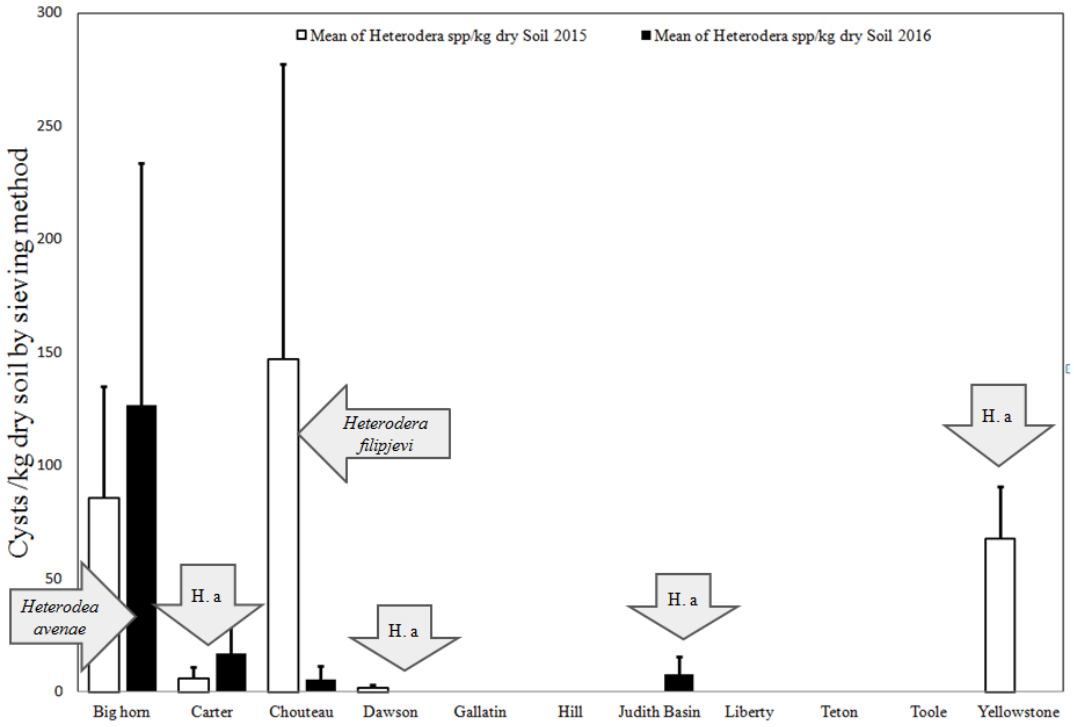


Figure 4. Mean cereal cyst nematode (*Heterodera filipjevi* and *Heterodera avenae)* densities per kg dry soil in Montana wheat and barley in 2015 and 2016, as extracted by the sieving method.

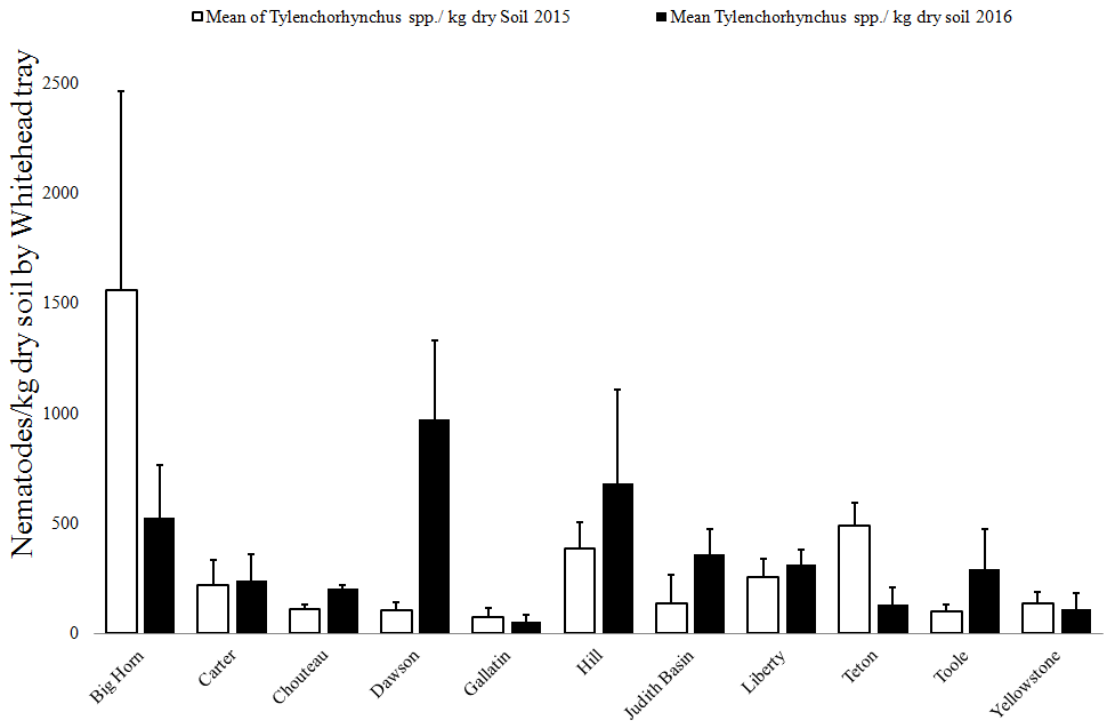


Figure 5. Mean number of stunt nematodes (*Tylenchorhynchus* spp.) per kg dry soil in Montana wheat and barley in 2015 and 2016 as extracted by Whitehead trays.

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CHAPTER THREE

DETERMINING VARIATION IN VIRULENCE AMONG POPULATIONS OF *PRATYLENCHUS NEGLECTUS* COLLECTED FROM MONTANA

Abstract

The root lesion nematode, *Pratylenchus neglectus*, is one of the most damaging nematodes to affect wheat worldwide. In Montana, the nematode is widely distributed, primarily affecting winter wheat within the state. Tools available for managing the nematode include rotations to resistant and moderately resistant crops (peas, lentils, and barley), and the incorporation of resistances from six CIMMYT wheat lines into locally adapted cultivars. In order to assess the broad applicability of these controls for Montana, greenhouse trials were conducted to challenge these controls using 8 populations of *P. neglectus* collected from geographically diverse locations across the state. In two trials conducted with resistant crops species, significant interaction was detected between crops and populations of nematodes (ANOVA $P < 0.001$ and $P = 0.01$). In the first trial, populations collected from Hill, Dawson, and Chouteau counties were found to be virulent on barley, with a mean reproductive factor (Rf) of 10.9. Populations from other counties were either non-virulent on barley or their inoculations were ineffective. In the second trial, the population from Hill county was again virulent on barley (mean Rf= 4.4), as was a population from Carter county (mean Rf= 10.7). Trials examining virulence of populations across resistant CIMMYT lines found no interactions between populations and wheat lines (ANOVA $P = 0.60$ and $P = 0.93$). While significant variation

in nematode reactions to the CIMMYT lines were detected, none of the lines appeared particularly resistant to the Montana nematode populations, with mean Rf values of 13.1 and 15.4 for trials 1 and trials 2, respectively. Variation in body length, stylet length, and vulva position were observed, but these did not coincide with differences in virulence. Male nematodes were detected in all 8 of the *P. neglectus* pot cultures, and these were confirmed to be *P. neglectus* by morphological and molecular methods. Males were most prevalent in poorly performing pot cultures. Results suggest a re-evaluation of barley and resistant wheat germplasms may be needed.

Introduction

Wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) are important staple food and feed crops globally. Wheat is planted and grown on more land area around the world than any other crop (FAOSTAT, 2011; Urueña, 2009; McMullen et al, 2012). In 2014, wheat production in the United States US was 7.5 % (55,395,400 metric tons) of the world's wheat production. Barley production in US was 2.7 % (3,849,230 metric tons) of the world's barley production (FAOSTAT, 2016). Wheat and barley production in US are both for domestic consumption, and for export trade. In the United States, wheat is grown on approximately 22.5 million hectares per year (FAOSTAT, 2011), and barley is grown on approximately 0.4 million hectares (USDA, 2016). Consumption of wheat per capita in the United States exceeds that of any other food staple, and is the third largest staple crop worldwide (USDA, 2016; FAOSTAT, 2011).

There are more than 4000 species of plant-parasitic nematodes (PPNs) (Decraemer and Hunt, 2006) and they play an important role in the constraint of global food security. In the USA, a survey across different crops showed nematode-related losses as high as 25% (Koenning et al. 1999). Worldwide losses due to PPN have been estimated at $US80 billion per year (Handoo, 1998; Nicol et al., 2011). This is likely an underestimate as estimates for developing nations such Iraq, where available management options are limited, have not been assessed.

Root lesion nematodes (RLN) (*Pratylenchus* spp.) are serious nematode pests that infest wheat roots and limit crop productivity (Smiley et al. 2005; Smiley 2004a; Thompson et al, 2008). The primary species affecting wheat are *P. crenatus* Loof, 1960, Syn *P. pratensis* auctt. (prior to 1960) nec (de Man, 1880); *P. neglectus* (Rensch, 1924) Filipjev & Schuurmanns Stekhoven, 1941; *P. penetrans* (Cobb, 1917) and *P. thornei* Sher and Allen, 1953 (Nickle, 1991, Smiley et al, 2008). Among these, *P. neglectus* and *P. thornei* are the primary species affecting wheat in the western United States. They are both migratory endoparasites (Thorne, 1961; Smiley et al. 2005) that are capable of completing their entire lifecycle within the root system of the host plant (Williams et al. 2002). Yield losses for *P. neglectus* have been reported as high as 37% in the Pacific Northwest region of the United States, while those for *P. thornei* have been reported as high as 50% (Smiley et al. 2005b). In Montana, a survey completed in 2006-2007 (May et al, 2016) found damaging populations of *P. neglectus* primarily occurring in winter wheat production areas of the state. The survey did not detect *P. thornei* (May et al 2016).

A few studies have reported races of RLNs (Griffin and Gray, 1990; Olthof, 1968; France et al., 1996). These involved *P. neglectus* on alfalfa and *P. penetrans* on tobacco and potato. They describe clear differences in the virulence reactions of RLN populations to variation in a host species resistance. Although several sources of resistance in wheat to *P. neglectus* have recently been reported (Thompson et al., 2016; May et al, 2016) to date there has been no reports of variation in the pathogen's virulence amongst them. It is unclear whether this is due to there being no variation within the species, or simply due to a lack of experimental testing. While the incorporation of resistance to RLN in wheat is uncommon, the use of non-host rotational crops to manage RLN has been highly successful (Smiley et al. 2004a, 2005a; Taylor et al. 2000). Root lesion nematodes can be managed through select crop rotations and fallowing of affected fields (Florini and Loria, 1986; May et al, 2016), but the literature is conflicted on their efficacy (Gair et al, 1969; Riga et al, 2008; Smiley, 2009). This may be due to mis-identification of RLN species or due to variation in genetic resistance of the cultivars used (Keil et al 2009). To date, no research has been conducted to identify variation in the virulence of different RLN populations to different hosts as the possible source of the conflicting reports. Since the original survey in Montana was conducted in 2006 to 2007, variation in response of field populations to rotation have been observed (data not presented). These reports suggest the use of crop rotation is a more complex story that has been previously reported in the literature. The variation in host genetics of rotation crops (May et al, 2016; Keil et al., 2009) as well as variation in nematode virulence needs to be examined. This variation may carry over to differences in virulence across resistant wheat germplasm. In a survey

conducted in 2015, male nematodes were discovered amongst *P. neglectus* populations. Males have been previously reported for *P. neglectus* but they are extremely rare (Sher and Allen, 1953; Loof, 1960; Handoo and Golden; 1989; Nickle, 1991; Mahran et al, 2010). The presence of males suggests that sexual recombination is possibly occurring in Montana. Sexual recombination tremendously increases the chances for genetic variation and mutation over mitotic asexual reproduction, and may represent a significant source of variation in virulence among Montana's *P. neglectus* populations. The purpose of this work is to: (1) assess variation in virulence among *P. neglectus* populations to 6 sources of resistance in wheat to determine those resistances most useful for incorporation into locally adapted germplasm, and (2) assess the variation in virulence among Montana's *P. neglectus* populations to the common rotational crops grown is the state. In the process, the program seeks to document the production of male nematodes within the collected populations.

Materials and Methods

A set of commonly grown rotation crops and RLN resistant wheat lines were used to determine the variation in virulence of *Pratylenchus neglectus* from different counties throughout Montana (Sasser, 1972; Baker et.al, 1985). Eight root lesion nematodes populations collected from different geographical regions in Montana: Carter County, Chouteau County, Dawson County, Gallatin County (P1 and P2), Hill County, Pondera County, and Yellowstone County. These populations were used to assess RLN variation within the state. Each population was collected from a field where the RLN populations

where high and geographically separated from the other collection sites. The populations were maintained in pot cultures in the greenhouse using the winter wheat cultivar "Yellowstone (Bruckner and Berg, 2016)". Sub-cultures were produced as needed to provide sufficient numbers to conduct greenhouse trials and to assess nematode morphology. For virulence trials described below, cone-tainer (with height 20.7 cm and diameter 4 cm) planting tubes were used. These tubes were filled with sufficient soil from the appropriate pot cultures to achieve between 200 and 500 nematode per cone-tainer. This soil was supplemented in MSU mix soil to fill the cone-tainers to the rim (a steam-pasteurized 1:1:1 mixture of topsoil, peat moss, and concrete sand, augmented with the wetting agent Aqua-Gro 2000G). Initial population densities (Pi) were determined for each trial by producing 6 additional cone-tainers per nematode population from which nematodes populations were immediately extracted and enumerated using the Whitehead tray method and a light microscope.

Virulence of *Pratylenchus neglectus* on Montana crops

Four crops and a fallow treatment were used in this experiment to determine variation in virulence of *P. neglectus* populations: Three cultivars of barley (Harrington, Haxby, and Merit), one pea cultivar (Delta), one lentil cultivar (Richlia), one wheat cultivar (Yellowstone, positive control), and a unseeded fallow treatment (negative control) were used as the crop treatments. These were challenged with each of the 8 RLN populations. The trial was run as a randomized complete block design with 36 replicates, blocked across 3 time points planted 1 month apart (6 replicates at each time point). A

thermo-hygrometer was used to record the temperature and relative humidity in greenhouse at 21.1 °C day and 18.3 °C night; with a 16/8 hour day/night photoperiod.

Virulence of RLN on six sources of resistance in wheat

Seven wheat lines were used in this experiment, six of which were sources of *P. neglectus* resistance: Aus 28451, Bez/HAWK/ES14, BILINMIYEN 96.7, GS50a, Persia 20, Suzen 97, and Yellowstone (susceptible control) (Smiley et al., 2014; Smiley et al., 2004b). An additional unseeded fallow treatment was included as negative control. The trial was run as a randomized complete block design with 18 replicates, blocked across 3 time points planted 1 month apart (6 replicates at each time point). The experiment was conducted twice. These lines reportedly displayed significant resistance to *P. neglectus* and come from divergent genetic backgrounds.

Nematode extraction: Whitehead-Hemming method

A coarse Sieve (4.76 mm) was used to remove large debris such as stones, rocks, gravel, and organic matter from soil samples. The soil from each cone-tainer was spread out on a Kimwipe lab tissue (Kimtech, 30 x 30 cm.) over a plastic screen mesh (1 mm openings) on a metal frame (phosphor-bronze gauze with 25 mm openings), resting in a shallow pool of water in plastic trays (dimensions: 43w x 34l x 10d cm). The extractions were carried out over 48 hours at 20 °C. The suspension was then concentrated by passing it through 20 μm mesh sieve, then decanting and washing the nematodes into a 50 ml container using 35 - 40 ml of sterile tap water. A McMaster Counting Slide (Chalex Corporation, Wallowa, OR) was used to count nematodes. The nematode

solution (2ml) was placed into the counting slide and the nematode were then counted using a Nikon Eclipse 50i microscope. Resulting counts were multiplied by total extraction volume to get a yield per cone (May et al, 2016).

The resulting value from these counts was referred to as the reproduction factor (heretofore referred to as Rf). This value represents the ratio between the number of nematodes that a cone-tainer started with compared to the number of nematodes present at the end of the experiment. An Rf value of 1.0 indicates that RLN populations were maintained throughout the duration of the experiment; a value of less than one indicates a reduction in nematode populations, and values above 1.0 indicate that nematode populations increased during the experiment. A plant capable of hosting *P. neglectus* is a plant upon which the nematodes can reproduce; thus, Rf values substantially above 1.0 indicate that the host is a susceptible host.

Morphological Measurements

Classical identification was used to identify nematodes to genera by using 4x, 10x & 40x magnification on a Nikon Eclipse 50i microscope (Kent, WA), and using "Manual of Agricultural Nematology" as a reference to identify each genus to species (Nickle, 1991), supplemented with other sources (Handoo et al, 2014; Loof and Luc, 1990; Siddiqi, 1963; Van den Berg et al, 2014; and Wouts and Knight, 1993). A Leica 10445929 0.5x dissecting microscope was used to check up samples for cereal cysts, other samples were assessed using a Nikon Eclipse 50i microscope. Nematodes were physically moved using a dental pick (K-Files, size #08, 25mm, [07-0871558] Patterson Brand) and the dissecting microscope. To assess morphological variation among

populations, two trials were conducted in May and October 2017. Nematodes were examined up from each population separately, one population per day, to prevent cross-contamination. The dental pick was used to isolate each individual nematode and place it in a drop of water on a slide, which was then covered with a cover slip. Photos were taken of the nematodes on the compound microscope, and measurements were calculated on the screen using a Nikon electronic scaling feature in μm. Body length, distance of vulva from postior, V % (distance of vulva from anterior), overlap of esophagus to intestine length, and stylet length were measured and then data analyzed statistically. Female and males identifications were carried out based on length, width (for males and females), and vulva placement in relationship to percent body length for (females); diagnositic vulva placement for *P. negelctus* is between 80 and 87 % of the body length (Nickle, 1991; Handoo and Golden, 1989). Finally, 12 typical samples were sent Columbia Basin Agricultural Research Station (Pendleton, OR) for confirmation of results to species.

Single Nematode DNA Extraction

To confirm the species of male nematodes found in each population, the males were barcoded using a single nematode DNA extraction method and PCR. The protocol DNA extractions was adapted from Kelley Thomas' Lab (Department of Molecular Cellular and Biomedical Sciences and Program in Genetics University of New Hampshire): Nematodes were individually isolated using a dental pick K-Files, size#08, 25mm, 07-0871558, Lot 0501001674, Patterson Brand, Stainless steel, Color coded plastic handles, made in Germany, Pkg.6 and Leica 10445929 0.5 x dissecting

microscope. Five replications from each genus were picked up per field. Glass slides were sterilized by ETOH 70% and DNA Away solution. Slides were used to relatively clean up the surface parts of nematode by dip it in 5 µl deionized water. Air flow pipe was used to dry out this water or it dry by room temperature. Nematode cut out into two halves by a pipette tip which will be using to get 5 µl Worm Lysis Buffer: 1M Tris (10 µl), 1M $MgCl_2$ (2.5 µl), 1M KCl (50 µl), Tween 20 (4.5 µl), 1% (w/v) gelatin (50 µl), 20mg/ml Proteinase K (3.3 µl) and ddH2O (879.7 µl), aliquoted and stored in -80 °C. By pipette tip, all nematode tissues moved from the slide. Sucking this WLB with a single nematode into 15 µl of lysis buffer in one eppendorf tube, kept (-80 °C) for 10 minutes, incubated at 60C for 1 hour, heated to 95 °C for 15 minutes, cooled to 4 °C. From the resulting extract, 2 µl of DNA template was used for PCR testing.

Molecular Assessment

A protocol from Al-Banna et al, 2004 was used. DNA sequence for the 26S rDNA D3 expansion region from *P. neglectus* was used. Specific-specific *P. neglectus* F (5'-ATG AAA GTG AAC ATG TCC TC-3') was used, whereas D3B R primer (5'-TCG GAA GGAACC AGC TAC TAC TA-3') to identify females and males.

Master Mix (25 µl) for PCR was made by mixing together each H_2O (13.38 µl), flexi 5x buffer (5 µl), MgCl2 (2 µl), 10mM dntp (0.5 µl), 10µM F (1 µl), 10µM R (1 µl), and go taq (0.125 µl). 2 µl DNA/tube will be taken. The thermal cycling was conducted in a Eppendorf Ag 22331 Hamburg Thermocycler as follows: a hot start at 95 °C for 3 minutes; 35 amplification cycles at 95 °C for 1 minute; 62, 63, or 68 °C, for 1 minute; 72 °C for 1 minute; and a final extension step for 7 minutes at 72 °C.

Statistical Analysis

Analysis of variance were conducted using linear models, which included crop, RLN population, interaction between crop and RLN population, and replicate for the trial assessing virulence of *P. neglectus* on Montana crops. Models for the trial assessing virulence of *P. neglectus* across six sources of resistance in wheat included wheat line, RLN population, interaction between line and RLN population, and replicate. Reproduction factor (Rf) was natural-log transformed after adding 1 to the values, as the data was non-normal. Data were natural log-transformed to generate a normal dataset, and further analyses were conducted on these ln-transformed data. Where the analysis of variance indicated treatment differences, a Fisher's protected least significant difference (LSD) ($\alpha = 0.05$) was generated to compare variable means (Devore and Peck 1997).

Quantities of male nematodes in the RLN populations were evaluated via single-factor ANOVA. Similarly, analysis of nematode morphological measurements for the eight populations was conducted via single-factor ANOVA. The data for these analyses were not transformed.

Analysis was conducted for all trials using RStudio version 3.2.3 R (RStudio Team, 2016); linear modelling used the lm function in R (R Core Team, 2017 with the Integrated Development Environment).

Results

Variation in Virulence across Rotational Crops

Analysis of variance (ANOVA) showed that were significant differences for nematode reproductive factor across *P. neglectus* populations (P <0.001, Table 1), and across crop species (P<0.001, Table 1). A significant interaction between nematode populations and host crops was detected (P < 0.001, Table 1). Average reproductive factor on the wheat positive control was 13.9. On wheat, there was particularly strong reproduction among populations from Hill (Rf = 44.3), Carter (Rf = 11.2), Chouteau (Rf = 16.1), and Gallatin (P2) (Rf = 13.6) counties. Reproduction was poor for the other Gallatin County population (P1) (Table 1). The highest average reproductive factor across all treatments was recorded for the populations from Hill county (Rf = 14.1). Among rotational crops, barley recorded the highest average reproductive factor (Rf = 4.2); in particular, the cultivar Haxby was the most susceptible barley cultivar tested (Rf = 5.2). The strongest interactions were observed between populations from Hill, Chouteau, and Dawson counties, where populations from Hill and Chouteau reproduced relatively well on the barley cultivars and the wheat positive control, while populations from Dawson only reproduced well on the wheat positive control and the Haxby barley cultivar (Table 1).

Nematode Population Response to Resistant Wheat Lines

Analysis of results from the first wheat trial identified significant differences between nematode reproductive factors of *P. neglectus* on the different wheat lines (*P*

<0.001), and a significant difference between nematode reproductive factors for the different nematode populations ($P < 0.001$), but no significant interaction between nematode populations and hosts ($P = 0.39$) (ANOVA, Table 2). The second trial's ANOVA showed a significant difference between nematode reproductive factors of *P. neglectus* among hosts ($P < 0.001$), a significant difference between nematode reproductive factors among the populations ($P < 0.001$), and no significant interaction between nematode populations and wheat lines ($P = 0.93$) (Table 3). While there was significant difference among nematode susceptibility of the wheat lines, none of the lines displayed strong resistance as all had large reproductive factors (mean Rf= 14.5 in trial 1 and 17.1 in trial 2) significantly greater than 1. Significant differences among populations were recorded for both trials but no discernable patterns were observed between the two trials, except that all populations had robust reproduction on the susceptible and putative resistant wheat lines.

Other Observations for RLN Populations

Significant differences in body length, vulva position, vulva distance from posterior, esophageal overlap, and stylet sizes were observed (Table 4). The most notable of these was the difference in body length, wherein the populations appeared to segregate into those around 500 µm in length (Dawson, Carter, Gallatin (P1)), and those around 600 µm in length (Chouteau, Hill, Gallatin (P2), and Yellowstone). Male nematodes were observed in all of the pot cultures except for the population from Pondera County (Figures 1 and 2). Their identity as *P. neglectus* was confirmed using morphology (Figure 3) and by individual nematode extractions along with species specific PCR assays (Figure

4) and through comparisons of barcode sequences. Male populations in some cases achieved high populations, exceeding 10% of the total RLN populations.

Discussion

This study shows that populations of *P. neglectus* from different geographical regions displayed differential virulence on barley. In particular, the populations from Hill and Choteau counties readily and repeatedly reproduced on barley. This reproduction was less than that observed on the winter wheat cultivar Yellowstone but was comparable to results previously observed for spring wheat cultivars (May et al, 2016). The results for the Hill county population were not surprising as the field from where is it was collected has displayed a history of unusual population dynamics relative to crop rotations (data not presented). A previous report has detailed differential virulence for *P. neglectus* on alfalfa (Griffin, 1991), and two previous reports have found differential virulence for *P. penetrans* on potato (Olthof 1968; France and Brodie 1996). To date there has been no other reports of differential virulence for *P. neglectus* on barley. The relative susceptibility of barley cultivars reported in these trials matches those of our previous report (May et al 2016). In those reports as well as this one, the barley cultivar Haxby was considered the most susceptible while the other two barley cultivars (Harrington and Merit) were the most resistant (May et al, 2016). The original barley testing was conducted using the Gallatin population (P1) also used for this trial. It is apparent based on these trials that barley may act as a significant host for *P. neglectus* depending on location and that significant variation in susceptibility occurs within the crop. The consistently high reproduction for the Hill and Chouteau populations on barley and the

relative lack of reproduction for these populations on other rotational crops suggest races for *P. neglectus* on barley may exist.

Based on recent survey results, RLN populations in Montana are currently low unlike those reported in 2006 and 2007. Based on this report, the differences in populations between the two surveys is most likely due to the widespread incorporation of legumes (Nass, 2016, Long et al, 2014; Nagy, 2001) in rotation. Legumes were consistently resistant to RLN populations collected from across Montana and their resistance to *P. neglectus* is well established in the literature (Taylor et al., 2000). The relative susceptibility of barley cultivars suggests their value in managing *P. neglectus* is somewhat less than previously supposed (May et al. 2016).

The high reproduction of all *P. neglectus* populations across the putative resistant wheat lines was surprising. Our previous work suggested Persia 20, AUS 28451 and Bez/Hawk/ES14 displayed strong resistances to *P. neglectus* (data not published). Those earlier results confirmed work conducted in the Pacific Northwest (Smiley et al, 2014; Smiley et al, 2004b). The lack of resistance among the lines in the current testing is unexplainable. Seed lots were double and triple checked and *P. neglectus* species identities were checked on multiple occasions using both morphological characters (Nicol 1991) as well as molecular tools (Al-Bana 2006). Until results can be independently confirmed, considerable concerns surround the use of these lines for breeding efforts by our program.

Variation in morphological features was observed among the RLN populations used in these trials. Significant variation was observed for body length, vulva position,

esophageal overlap and stylet length. The most dramatic of these was for body length where populations appeared to favor one of two sizes. The population from Hill County was one of the larger nematodes observed. This along with its unusual virulence instigated the current study. Morphological differences were not association with particular virulence behaviors and the species has been previously reported as displaying a great deal of plasticity (Nicol 1991). For those reasons, the biological relevance for the variation reported here may be negligible.

We observed unusually high male populations within our pot cultures relative to other reports (Sher and Allen, 1953; Loof, 1960; Handoo and Golden; 1989; Nickle, 1991; Mahran et al, 2010). The morphological and molecular results confirm their identity as *P. neglectus*. The highest relative populations appear associated with the least robust pot cultures, which matches reports of stress induced male production. Recent reports have detected *Wolbachia* bacterial parasites among *Pratylenchus* species (Tortora et al, 2004; Werren 1997; Juchault & Mocquard 1993; Rigaud 1997). These bacteria have been reported to influence sex ratios in insects, and may be affect nematodes similarly. At this time samples from these nematode populations are being tested for the bacteria's presence.

Table 1. Variation in reproduction factors across crops and RLN populations. Reproduction factors (Rf) were assessed for populations of *P. neglectus* collected from different locations across Montana, and grown in pot culture on wheat, pea, lentil, and barely crops. Mean Rf across blocks is listed in the table, with ln-transformed mean italicized below. A significant interaction was detected between populations and crops (ANOVA, F= 2.88, P <0.001)[3]

	Crop						
Population	Wheat (Yellowstone)	Pea (Delta)	Barley (Harrington)	Barley (Haxby)	Barley (Merit)	Lentil (Richlia)	Population Mean[1]
Hill	44.3	0.5	11.6	13.6	13.6	0.8	14.1
	2.39	*0.31*	*1.30*	*2.05*	*1.74*	*0.45*	*1.37*
Carter	11.2	0.1	1.1	1.9	1.5	0.9	2.8
	1.89	*0.08*	*0.56*	*0.71*	*0.63.*	*0.46*	*0.72*
Dawson	7.7	0.7	2.9	6.8	1.9	4.9	4.2
	1.53	*0.37*	*1.13*	*1.45*	*0.81*	*1.30*	*1.10*
Yellowstone	8.6	0.2	0.7	0.9	0.7	1.0	2.0
	1.67	*0.14*	*0.45*	*0.47*	*0.40*	*0.53*	*0.61*
Gallatin (P2)	13.6	0.4	0.8	1.0	0.8	0.4	2.8
	1.27	*0.22*	*0.42*	*0.41*	*0.44*	*0.23*	*0.50*
Chouteau	16.1	0.6	7.0	11.1	6.4	1.7	7.2
	1.56	*0.39*	*1.49*	*2.07*	*1.69*	*0.81*	*1.34*
Pondera	7.1	0.7	2.2	2.8	3.5	3.0	3.2
	1.08	*0.39*	*0.86*	*0.87*	*0.99*	*0.99*	*0.86*
Gallatin (P1)	2.8	0.7	1.9	3.8	1.3	3.4	2.3
	0.63	*0.42*	*0.94*	*1.29*	*0.76*	*1.21*	*0.88*
	13.9	0.5	3.5	5.2	3.7	2.0	4.8
Crop Mean[2]	*1.50*	*0.29*	*0.89*	*1.16*	*0.77*	*0.93*	*0.92*

[1] Fisher's protected LSD ($\alpha = 0.05$) on Ln-transformed data for RLN population means = 0.25

[2] Fisher's protected LSD ($\alpha = 0.05$) on Ln-transformed data for crop means = 0.22

[3] Fisher's protected LSD ($\alpha = 0.05$) on Ln-transformed data for the statistically significant RLN populations x crop interaction = 0.61

Table 2. Reproductive factors for different wheat lines across 8 populations of *P neglectus* for trial 1. Significant differences were detected among wheat lines and among nematodes populations (ANOVA, both P< 0.001), but there was no evidence of a population x wheat line interaction (*P* = 0.39)

	Nematode Resistant Wheat Lines									
Population	Yellowstone	Aus 28451	Bez/ HAWK/ES14	BILINMIYEN 96.7	GS50a	Machete	Persia 20	Suzen 97	Fallow	Population Mean[1]
Carter	12.3	21	11.2	16.4	9.3	8.6	27.7	25.0	0.4	14.6 $_{D}$
Chouteau	12.2	7.7	12.5	12	20.4	26.9	16.3	35.2	1.6	16.0 $_{B}$
Gallatin (P2)	7.9	4.2	3.2	6.1	5.0	8.9	6.0	11.9	1.4	6.0 $_{B C}$
Yellowstone	7.5	12.6	4.9	9.7	8.2	15.2	7.5	15.1	1.9	9.1 $_{B C}$
Dawson	7.2	12.7	13.4	8.1	17.0	18.4	15.9	12.2	0.4	11.7 $_{C D}$
Gallatin(P1)	6.8	12.6	11.9	16	23.7	27.9	13.5	25.1	0.6	15.3 $_{A}$
Hill	6.8	7.3	2.3	11.2	19.8	40	37.2	29.9	2.1	17.4 $_{C D}$
Pondera	3.4	14.1	10.8	12.9	13.7	41.7	12.9	20.6	2.0	14.6 $_{B C}$
Line Mean[2]	8.0 $_{B}$	11.5 $_{B C}$	8.7 $_{A}$	11.5 $_{B C}$	14.6 $_{C D}$	23.4 $_{E}$	17.1 $_{D E}$	21.8 $_{E}$	1.3 (N/A)	13.1

[1]Letters were assigned based on Fisher's protected LSD (α = 0.05) on Ln-transformed data (LSD for lines = 0.27). Lines that share a letter are not significantly different.

[2] Letters were assigned based on Fisher's protected LSD (α = 0.05) on Ln-transformed data (LSD for RLN populations = 0.28). Lines that share a letter are not significantly different.

Table 3. Reproductive factors for different wheat lines, averaged across 8 populations of *P neglectus* for trial 2. Significant differences were detected among wheat lines and among nematodes populations (ANOVA, both P< 0.001), but there was no evidence of a population x wheat line interaction (*P* = 0.93)

	Nematode Resistant Wheat Lines									
Population	Yellowstone	Aus 28451	Bez/ HAWK/ES14	BILINMIYEN 96.7	GS50a	Machete	Persia 20	Suzen 97	Fallow	Population Mean[1]
Carter	2.2	3.4	1.5	9.6	4.8	5.6	4.5	8.6	0.6	4.5 A
Chouteau	17.5	22.1	32.8	38.4	25.2	36	31.4	33.1	2.8	26.5 E
Gallatin (P2)	7.3	14.5	8.9	9.8	10.0	16.5	9.6	9.2	2.4	9.8 C
Yellowstone	13.5	11.8	9.3	7.9	14.5	21.5	11.8	27.6	0.7	13.1 C
Dawson	6.5	5.5	11.4	7.0	7.2	14.0	6.7	13.2	0.2	7.9 B C
Gallatin (P1)	10.5	11.7	17.9	24.1	22.9	27.2	18.9	23.1	2.0	17.5 D
Hill	5.9	9.4	14.8	12.2	12.9	17.1	11	15.8	0.9	11.1 B
Pondera	13.1	19.5	20.2	24.8	41.7	66.5	62.4	45.9	3.0	33.0 E
Line Mean[2]	9.5 A	12.2 A B	14.6 A	16.7 B C	17.4 C D	25.5 D	19.5 C D	22.0 D	1.5 (N/A)	15.4

[1]Letters were assigned based on Fisher's protected LSD (α = 0.05) on Ln-transformed data (LSD for lines = 0.25). Lines that share a letter are not significantly different.

[2] Letters were assigned based on Fisher's protected LSD (α = 0.05) on Ln-transformed data (LSD for RLN populations = 0.25). Lines that share a letter are not significantly different.

Table 4. Variation in physical characteristics among *P. neglectus* populations from different origins in Montana in 2017 at time point 1 (December 2016-March 2017) and time point 2 (March 2017-June 2017)

	Body Length Mean (μm)		V % (Distance of Vulva From Anterior) Mean		Distance of Vulva from posterior) (μm)		Overlap of Esophagus to Intestine Length Mean (μm)		Stylet length Mean (μm)	
	1	2	1	2	1	2	1	2	1	2
Population										
Chouteau	613.7	495.3	85.4	82.9	89.5	84.1	21.6	36.1	18	17.9
Hill	587.6	481.6	84.5	83.7	91	78.8	21.37	31.7	18.6	18.7
Gallatin (P1)	583.5	482.8	84.5	79.6	90.4	97.7	34.4	31.9	19.7	18.8
Yellowstone	576.5	523.5	84.0	81.6	91.8	96.2	28.3	31.3	18.2	18.8
Pondera	572.5	518.5	81.9	80.9	103.1	98.1	36.2	35.9	17.9	19.2
Gallatin (P2)	507.5	485.8	82.7	82.7	87.6	83.6	27.2	33.7	18.2	18.9
Carter	484.6	485.4	81.5	81.8	89.5	87.9	24.5	36.1	18.5	18.4
Dawson	466.6	478.3	80.6	81.3	90.2	88.6	35.9	34.2	18.6	18.7
Mean	549	493.9	83.1	81.8	91.6	89.3	28.6	33.8	18.4	18.6
Tukey HSD	68.9		2.5			12	9.9		1.13	
ANOVA *p*.value	< 0.001	0.172	<0.001	0.262	0.247	0.05	< 0.001	0.449	0.007	0.076

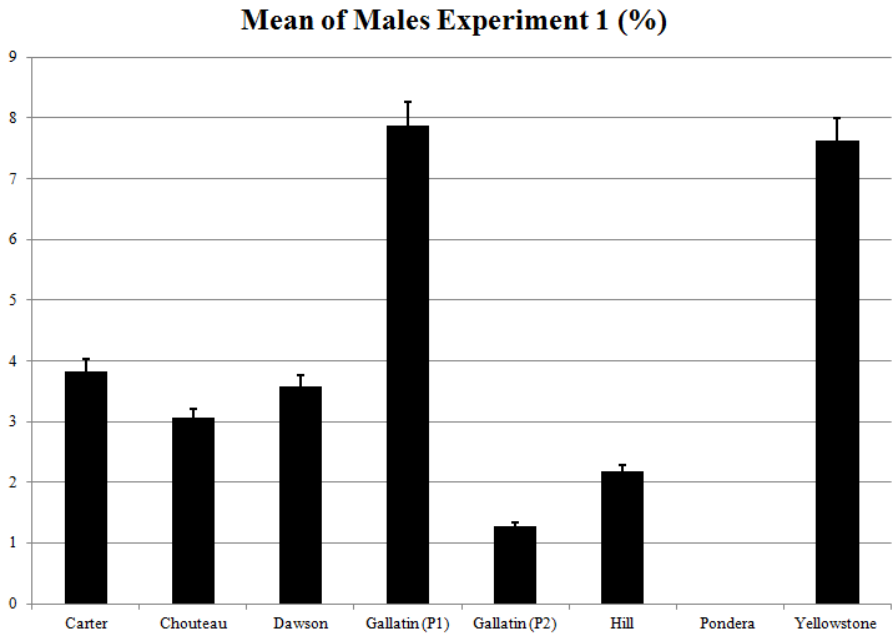


Figure 1. Percentage of *Pratylenchus neglectus* nematodes sampled from each population that were male, from wheat line experiment 1 (in greenhouse from December 2016 to March 2017).

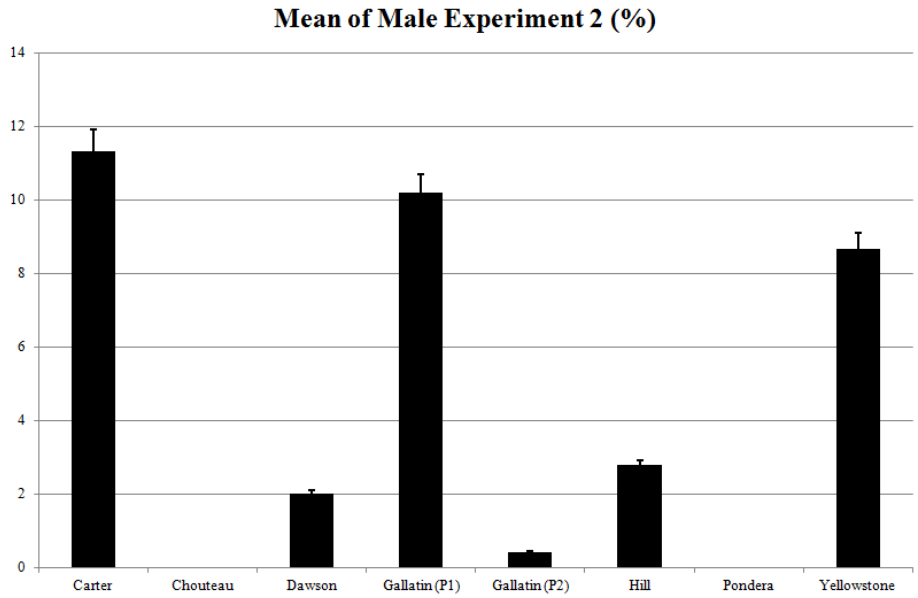


Figure 2. Percentage of *Pratylenchus neglectus* nematodes sampled from each population that were male, from wheat line experiment 2 (in greenhouse for March to June 2016).



Figure 3. (A) A female of *P. neglectus* with an egg inside the reproductive system, and (B) A male of *P. neglectus* with spicule on the reproductive system.

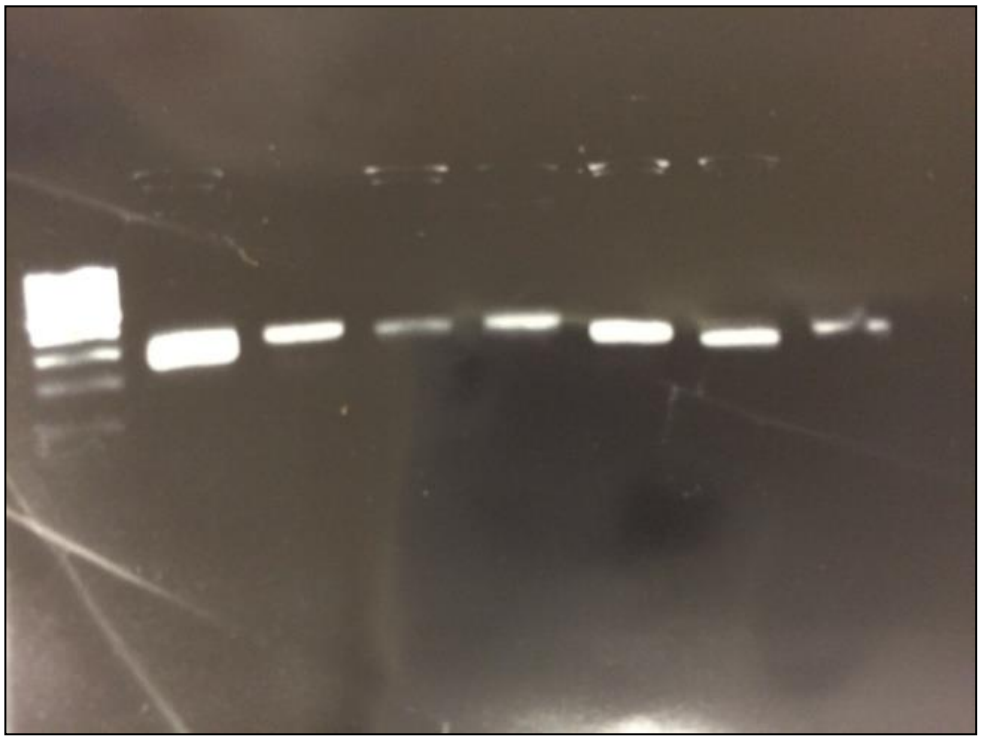


Figure 4. Amplification products for PCR reactions using *Pratylenchus neglectus* specific primers. Column 2 is the positive control; while columns 3-8 are from individual males identified as *P. neglectus* based on morphological characteristics.

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