BREEDING FOR ROOT LESION NEMATODE RESISTANCE IN MONTANA WINTER WHEAT

by

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ABSTRACT

Root lesion nematodes (RLN; *Pratylenchus* spp.) present a serious challenge to dryland wheat production worldwide. Development of resistant cultivars would provide great economic benefit to growers. From 2012-2013, a set of backcross lines (MT08185//MT08184/Persia 20) was screened twice for resistance to *P. neglectus*. Progeny and parent lines were grown in infested soil for 16 to 18 weeks. Nematodes were then extracted from roots of individual plants and counted to obtain per plant final populations. ANOVA results from the 2013 screen showed significant differences in mean *P. neglectus* populations among lines ($p < 0.01$). The median final population of susceptible parent MT08184 was an estimated 4.9 times greater than that of resistant parent Persia 20. A 2013 field trial in the absence of root lesion nematodes indicated reductions in grain yield, volume weight, and protein were not associated with resistance. Seven RLN-resistant lines were identified in field evaluations with agronomic phenotypes for yield, volume weight and protein comparable to those of five widely-grown checks. Identification of quantitative trait loci (QTL) for resistance to RLN will facilitate marker-assisted introgression of resistance genes in a backcross-breeding program. Single-marker analysis of 218 genome-wide single nucleotide polymorphism markers (SNPs) was performed to identify genomic regions associated with resistance to *P. neglectus*. The analysis identified putative marker-trait associations on chromosomes 1AL, 1DS, 2BL, 5BL, 5DL, 7AL and 7DL (all $p < 0.05$). Overall, phenotypic screens as applied were inadequate to consistently characterize wheat lines for RLN resistance. As such, RLN resistance phenotypes and putative QTL effects identified in the study must be verified in future experiments.

CHAPTER 1 – LITERATURE REVIEW

Background: Evolving Dryland Cropping Practices and Emerging Pest Problems

Each year in Montana, over four million acres of non-irrigated farmland are planted with spring and winter wheat (*Triticum aestivum* L.) varieties (NASS, 2013), producing over 160 million bushels of grain valued at over 1.3 billion dollars (according to 2012 statistics). The leading production areas are located in the northeastern portion of the state for spring wheat, and the north-central portion for winter wheat (NASS, 2013). Both regions experience a semi-arid climate and receive between 6 and 16 inches (<400 mm) of precipitation annually (NRIS, 2004). As water is a limiting factor for crop production in these regions, fallow has traditionally been utilized as part of a two-year rotation that has been practiced to build up sufficient soil moisture to produce crops on stored water plus precipitation during the growing season (Mielke *et al.,* 1984; Mielke and Wilhelm, 1998).

Today, the majority of wheat in Montana is still grown following fallow. However, with the emergence of no-till production systems, the continuous cropping of farmland has become a more common practice. While field pea (*Pisum sativum* subsp. *arvense* L. Asch.), field mustard (*Brassica rapa* L. var. *rapa*), camelina (*Camelina sativa* L. Crantz) and canola (*Brassica napus* L.) are recommended rotation options for regional farms employing continuous cropping

systems (McVay *et al*., 2010), some growers have opted for continually-cropped wheat due to the potential price advantage when compared to the lower-value alternative crops. According to the most recent estimates, continually cropped winter wheat accounts for 24 percent of the total winter wheat acreage harvested in the state (NASS, 2008). Such a monoculture system lacks diversity, which provides opportunity for the survival and increase of weed populations, diseases and insect pressure.

Previous studies have provided evidence that such changes in farming practices can increase the potential for damage by plant-parasitic nematodes. The root lesion nematode (RLN, *Pratylenchus* spp.) has, for many years, been recognized as a significant pathogen of irrigated high-value crops in the Pacific Northwest (Jensen, 1961); however, its impact on non-irrigated agriculture has become more widely acknowledged over the years as well. Gair *et al*. (1969) provided one of the earliest accounts of RLN affecting dryland crop production, reporting that RLN populations substantially increased when cropping frequency intensified in non-irrigated fields. Early studies analyzing the relationship between RLN and tillage systems, conducted by Thomas (1978) and Thompson *et al*. (1983), both found higher populations of *Pratylenchus* in fields managed as no-till compared to fields managed with conventional tillage. More recently, Smiley *et al*. (2004) reported that a substantial proportion of fields in the Pacific Northwest with high cropping frequency had potentially damaging populations of

RLN. The study also found that population density of *Pratylenchus neglectus* in winter wheat roots was negatively correlated with yield, providing the first field-based evidence that RLN were an economically important pathogen of dryland cropping systems in the region.

Root Lesion Nematodes (*Pratylenchus* spp.): A Nematode of Global Importance for Wheat Production

Today, *Pratylenchus* species are recognized as the third-most important plant-parasitic nematodes in the world, behind the root-knot (*Meloidogyne* spp.) and cereal cyst (*Heterodera* and *Globodera* spp.) nematodes, both in terms of their effect on global agriculture as well as their contribution to the further development of molecular plant pathology (Castillo and Volvas, 2007; Jones *et al*., 2013). *Pratylenchus thornei* Sher and Allen, and *P. neglectus* (Rensch) Filipjev, Schuurmans, and Stekhoven are the two species most commonly associated with yield loss in wheat, and both can be found in temperate cereal producing regions around the world (Smiley and Nicol, 2009). Of the two, *P. thornei* is more destructive, causing estimated yield losses of up to 85% in Australia, 70% in Israel, 37% in Mexico and 50% in the United States (Armstrong *et al.,* 1993; Nicol and Ortiz-Monasterio, 2004; Smiley *et al.,* 2005a). *Pratylenchus neglectus* has been reported to cause estimated yield losses of up to 30% for intolerant cereal cultivars in southern and western Australia (Vanstone *et al.,* 2008), and up to 37% in the Pacific Northwest region of the United States

(Smiley *et al.,* 2005b). In 2012, an estimated total loss of 10.4 million bushels due to RLN was reported in Kansas, the top wheat producing area in the United States (Appel *et al*., 2012).

Pratylenchus spp. are polyphagous, migratory, intracellular root endoparasites with a life cycle lasting three to nine weeks, depending on the species and conditions (Jones *et al.,* 2013; Jones and Fosu-Nyarko, 2014), and therefore pass through several generations during the life of one host crop. Young nematodes undergo development to a first juvenile stage within an egg, then molt to a second juvenile stage, which hatches from the egg. All subsequent juvenile and adult stages are vermiform and motile, and capable of both entering and leaving plant roots or storage organs (Jones and Fosu-Nyarko, 2014).

Using a protractible syringe-like structure called a stylet, *Pratylenchus* spp. pierce and enter host root cells. A mixture of effectors and plant cell wall degrading enzymes are secreted from pharyngeal glands and emitted from the stylet, to aid in migration and feeding (Jones *et al.,* 2013). Most of the life cycle is spent protected within the root systems of host plants, but nematodes may also be found at the root surface and in the adjacent soil (Jones and Fosu-Nyarko, 2014). When mature, females lay eggs within infested roots or in nearby soil (Jones and Fosu-Nyarko, 2014). Reproduction is typically achieved by parthenogenesis, and males are generally rare or absent in populations of *P.*

neglectus and *P. thornei* (Smiley and Nicol, 2009).

Both egg and mature stages are able to overwinter to ensure long-term survival under unfavorable environmental conditions. Mature *Pratylenchus* are able to survive in an inactive dehydrated state known as anhydrobiosis for periods of up to several years (Glazer and Orion, 1983; Talavera and Vanstone, 2001). Nematodes infesting plant roots after the overwintering stage have been found to reproduce more rapidly than those that did not undergo dormancy (Smiley and Nicol, 2009). Typically, populations have been found to decline after long periods of fallow (Smiley and Nicol, 2009), but high rates of survival have been reported (Orion *et al.,* 1984; Talavera and Vanstone, 2001). Nematodes inhabiting loose soil can be transported from field to field on farm equipment, shoes, animals, and by wind (Johnson *et al.,* 2008).

Infection results in reduced root growth, along with the formation of dark necrotic lesions that can serve as secondary infection sites for root-rotting fungi (Jones *et al.,* 2013). Negative synergistic effects on wheat growth and yield have been observed when roots were co-inoculated with *Pratylenchus thornei* and *Fusarium culmorum* (Hajihassani *et al.,* 2013). The root damage that results from *Pratylenchus* infection hinders a plant's ability to absorb water and nutrients. Crops infested while growing under moisture stress are the most likely to experience yield loss (Nicol and Ortiz-Monasterio, 2004). Above ground symptoms are not specific to root lesion nematode infection, and are often

confused with nutrient deficiencies or drought stress (Van Gundy *et al.,* 1974; Orion *et al.,* 1984; Doyle *et al.,* 1987; Thompson *et al.,* 1995; Smiley *et al.,* 2005a,b). These include poor vigor, yellowing and premature death of lower leaves, stunting, reduced tillering, and reduced grain yield and quality (Smiley and Nicol, 2009).

Management Strategies and Prospects

Management options for wheat growers in the United States are currently limited to crop rotations and the deployment of tolerant wheat cultivars. Chemical control is possible with the nematicide Aldicarb (Temik 15G), but because of its toxicity, persistence and cost, it is not utilized in commercial small grains production (Kimpinsky *et al.,* 1987). At present, Bayer CropScience, the manufacturer of Aldicarb, has phased out all production and reached an agreement with the EPA to terminate usage of the pesticide (Erickson, 2010).

Rotations to non-host crops such as safflower, flax, triticale, barley and lentil have been shown to restrict nematode reproduction in the soil (Smiley *et al.,* 2004; Zuck, 2010). However, results from these studies suggest that hosting ability is species and cultivar-specific, underscoring the need for further inquiry into the host or non-host status of local cultivars (Smiley and Nicol, 2009). Tolerant wheat varieties, which retain their yield potential under RLN infestation, have been successfully identified and utilized in both Australia and the United

States (Thompson *et al.*, 2008 – 2b; Vanstone *et al.*, 2008; Johnson *et al.*, 2008). Nematode reproduction is not necessarily inhibited by tolerant cultivars, however, and their widespread deployment may in fact mitigate the yield potential of future crops. Therefore, tolerance on its own is not considered an effective long-term management strategy.

Resistance, a term describing a plant's ability to inhibit a pathogen's reproductive rates, will be the most important and economical tool in working toward long-term management of RLN. Resistance and tolerance are independent phenotypes (Trudgill, 1991) and pyramiding both characteristics into a single cultivar would be a highly desirable result for growers experiencing yield loss due to *Pratylenchus*.

RLN resistance has been shown to be largely species-specific, meaning that resistance genes that are effective for *P. thornei* are not necessarily effective for *P. neglectus*, and vice versa (Farsi *et al.,* 1995). Problematically, mixed populations of both species are often found in the same field (Smiley *et al.,* 2004; Thompson *et al.,* 2008 – 2a). The development of cultivars with dual resistance would therefore eliminate the need for farmers to identify *Pratylenchus* to the species level prior to selecting an appropriate resistant cultivar.

Several sources of resistance have been identified so far: in commercial wheat varieties, in Middle East landrace lines, and in diploid and tetraploid progenitors of wheat, such as *Aegilops speltoides* and *Triticum dicoccoides*

(Thompson and Haak, 1997; Thompson *et al.,* 1999; Nicol *et al.,* 1999, 2001, 2003; Nombela and Romero, 1999; Hollaway *et al.,* 2000; Zwart *et al.,* 2004, 2005; Tokay *et al.,* 2006; Sheedy *et al.,* 2008, 2012). Certain lines are particularly interesting in that they have demonstrated excellent levels of resistance to both *P. thornei* and *P. neglectus* (Zwart *et al.,* 2005; Nicol *et al.,* 2007; Sheedy *et al*., 2007, 2008; Thompson, 2013). However, root lesion nematode-resistant lines often have poor agronomic qualities or lack the appropriate adapted phenotype to be utilized as-is, necessitating the incorporation of their resistance traits into local cultivars. Unfortunately, selecting resistant wheat lines in a conventional breeding program involves the tedious and time-consuming process of inoculating plants in the greenhouse and subsequently extracting and counting the nematodes that have multiplied in the plant roots (Zwart *et al.,* 2005). Application of marker-assisted breeding technologies will greatly accelerate the development of superior cultivars with RLN resistance.

Many experiments involving marker-trait associations for disease resistance in bread wheat have been conducted in the past, and have successfully identified associations for numerous resistance traits in bread wheat (Gupta *et al.,* 2010), including some for *Pratylenchus* species. Overall, these studies have demonstrated that resistance to RLN is polygenic and additive, and that quantitative trait loci (QTL) for resistance are present on all three genomes

(A, B and D) of bread wheat (Zwart *et al.,* 2010). QTL linked to resistance to *P. thornei* have been identified on chromosomes 1B, 2B, 3B, 4D, 6D and 7A (Schmidt *et al.*, 2005; Zwart *et al.,* 2005, 2006; Tokay *et al.,* 2006). Markers linked to the *P. neglectus* resistance gene *Rlnn1*, located on the long arm of chromosome 7A, have been identified and successfully implemented in both Australian and CIMMYT breeding programs (Williams *et al.,* 2002; Gupta *et al.,* 2010). Additional QTL for resistance to *P. neglectus* have been reported on chromosomes 2B, 4D, 5B, 6B and 7B (Zwart *et al.,* 2005, 2010; Mulki *et al.,* 2013). A more recent study utilized a recombinant inbred line population derived from the dual-resistant landrace accession AUS28451 and a susceptible USA wheat cultivar, and identified resistance-associated QTL on chromosomes 1A, 2B, 4A, 5A, 5B, 6B and 7A using phenotyping methods for both *P. thornei* and *P. neglectus* (Thompson, 2013).

Other potential management strategies include transgenic control of RLN, as well as environmentally-friendly nematicides. Transgenic control strategies would hypothetically involve gene silencing via RNA interference (RNAi) of a target nematode gene, as RNAi silencing of genes involved in nematode movement has been shown to reduce *P. thornei* reproduction by 81% (Tan *et al.*, 2013). Further applications of RNAi technology have identified candidate genes unique to plant-parasitic nematodes with lethal RNAi phenotypes, which may be utilized in development of new chemistries for nematode control (Danchin *et al.,*

2013). However, issues such as cost, safety, containment, trade regulation and lack of public education remain large obstacles to the widespread deployment of transgenic wheat varieties and novel pesticides. The integration of resistance breeding and further research into the efficacy of various crop rotations remains the most practical and sustainable approach to attaining long-term control of RLN.

Discovery and Distribution of RLN in Montana, and Rationale for Project

In 2005, RLN were identified in soil samples taken from unthrifty wheat fields within Montana. In the following years, a team of Montana State University researchers conducted a statewide survey of RLN populations. Seventeen Montana counties, accounting for 82% of the state's total wheat acreage, were chosen for the survey (Johnson, 2007). Populations of *P. neglectus* found in soil samples collected from wheat farms in Fergus, Choteau, and Cascade counties averaged over 3,200 RLN / kg soil (Johnson, 2007), a number in excess of the damage threshold of 2,500 *P. neglectus* / kg soil defined by researchers in the Pacific Northwest (Smiley *et al*., 2005a). Choteau, Fergus, and Cascade counties currently rank as the first, third, and fourth-highest winter wheat producing counties in Montana, respectively (NASS, 2013). Furthermore, the assessment showed damaging populations of *P. neglectus* occurring primarily in winter wheat fields and fields managed as no-till (Johnson *et al.,* 2008). *P.*

thornei was not found in the survey. As rotation to non-host crops has proven to be either impracticable or undesirable for growers, the development of agronomically competitive varieties with resistance to RLN might provide an attractive option to Montana's winter wheat producers.

The objective of this research project was to further breeding efforts toward the release of nematode-resistant winter wheat cultivars for Montana growers. Our initial experiments were designed in hopes of identifying single nucleotide polymorphisms (SNPs) linked to QTL for resistance to *P. neglectus*, for use in marker-assisted introgression of resistance genes in a backcross breeding program. Secondly, through replicated field trials, we sought to evaluate the agronomic performance of our breeding lines compared to a set of widely grown checks. The designs and results of these experiments are described in detail in the pages that follow.

CHAPTER 2 - IDENTIFICATION OF *PRATYLENCHUS NEGLECTUS*–RESISTANT LINES IN A BACKCROSS BREEDING POPULATION (MT08185//MT08184/PERSIA 20)

Introduction

As soil-inhabiting parasites of plant roots, RLN represent a hidden enemy for crop production in nearly every part of the world. At a length of half the thickness of a dime, and a diameter one-quarter of a human hair, they are too small to see with the naked eye. Nevertheless, RLN are capable of inflicting considerable damage on crop plants, although the symptoms of infection are often confused with drought and nutrient deficiencies. Yield losses due to RLN can reach a level of 37% in winter wheat (*Triticum aestivum* L.) crops in low precipitation environments, at a level of 10,000 *Pratylenchus neglectus* / kg soil (Johnson *et al.*, 2008).

In 2006 and 2007, a team of MSU researchers conducted a statewide survey of RLN populations. The assessment showed damaging populations of *P. neglectus* occurring primarily in winter wheat fields and fields managed as no-till (Johnson *et al.*, 2008). As both nematicide treatments and rotation to non-host crops have proven to be either impracticable or undesirable for growers, the development of agronomically competitive varieties with genetic resistance to *P. neglectus* might provide an attractive option to Montana's winter wheat

producers.

Resistance is a term describing a plant's ability to inhibit a nematode's reproductive rate. The utilization of resistance genetics will be the most important and economical step in the long-term management of root lesion nematodes. Pyramiding both tolerance (the ability to maintain yield while under infestation) and resistance traits into a single cultivar would be a highly desirable outcome for growers experiencing yield loss due to *Pratylenchus*.

Several sources of resistance to *Pratylenchus* have been identified so far. These include commercial wheat varieties, Middle East landrace lines, and diploid and tetraploid progenitors of wheat, such as *Aegilops speltoides* and *Triticum dicoccoides* (Thompson and Haak, 1997; Thompson *et al.,* 1999; Nicol *et al.,* 1999, 2001, 2003; Nombela and Romero, 1999; Hollaway *et al.,* 2000; Zwart *et al.,* 2004, 2005; Tokay *et al.,* 2006; Sheedy *et al.,* 2008, 2012). Some of these lines are of particular interest to breeders, because they are resistant to both *P. thornei* and *P. neglectus* (Zwart *et al.,* 2005; Nicol *et al.,* 2007; Sheedy *et al*., 2007, 2008; Thompson, 2013). However, RLN-resistant lines often have poor agronomic qualities or lack the appropriate adapted phenotype to be utilized as-is, necessitating the incorporation of their resistance traits into adapted local cultivars. Unfortunately, selecting resistant wheat lines using conventional breeding methods involves the tedious and time-consuming process of inoculating plants in the greenhouse and subsequently extracting and counting

the nematodes that have multiplied in the plant roots (Zwart *et al.,* 2005). Application of marker-assisted breeding technologies will greatly accelerate the development of superior cultivars with RLN resistance.

Previous studies have indicated that the underlying genetic control of root lesion nematode resistance in wheat is polygenic and additive, and that resistance-associated loci are present on all three of its genomes (A, B and D) (Zwart *et al*., 2010). The mapping of quantitative trait loci (QTL) associated with nematode resistance will provide the framework for marker-assisted introgression of such genes into Montana winter wheat cultivars. QTL mapping, as with any genetic study, is only as accurate as its phenotypic screening procedure. In studies of polygenic disease resistance, factors ranging from the quality and quantity of inoculum to difficulties in quantitative assessment of resistance make QTL mapping more challenging (Bai and Shaner, 1994; Young, 1996).

This study utilized a backcross breeding population derived from an initial cross of a low polyphenol oxidase (PPO) plant selection of susceptible Montana winter wheat cultivar “Yellowstone” to the resistant Iranian landrace accession “Persia 20”. Resistance to *P. neglectus* was assessed in independent replicated greenhouse trials repeated over the course of a two-year period. These trials were designed to obtain accurate phenotypic information to confidently proceed to locating and tagging QTL associated with resistance to *P. neglectus,* for their potential use in marker-assisted selection. Concurrently, data generated in the

trials were to be used to select backcross lines that stably expressed a resistant phenotype, for further crosses to adapted winter wheat lines and advancement in the breeding program.

Materials and Methods

Population Development

Initial screening began with a population of 200 BC_1F_3 (MT08185//MT08184/Persia 20) lines. MT08185 and MT08184 are low PPO single-plant selections of the Montana winter wheat cultivar Yellowstone. Low PPO activity is a desired trait in Montana winter wheat, as a large portion of the state's crop is exported for the production of Asian noodles – a product for which the enzymatic activity of PPO causes unsightly brown discoloration. These lines were identified using the low-PPO STS marker *PPO18*, located on chromosome 2AL (Sun *et al.*, 2005). Yellowstone was selected as the recurrent parent in developing the population, because this particular cultivar is the most widely planted and highest-yielding winter wheat variety in Montana (NASS, 2013). Yellowstone is an awned, white chaffed, medium maturity, intermediate height hard red winter wheat with good winter hardiness, high yield potential, and excellent milling and baking quality (Bruckner *et al.*, 2007). The Iranian landrace accession Persia 20 was chosen as the donor parent, since it was shown to have excellent levels of resistance to both *P. neglectus* and *P. thornei* (Sheedy *et al.,*

2007, 2008). Persia 20 seed was obtained from Dr. Richard Smiley of the Columbia Basin Agricultural Research Center in Pendleton, OR.

The initial cross of MT08184 and Persia 20 was made in Montana State University's Plant Growth Center in Bozeman, MT in 2008. The backcross of the F_1 to MT08185 followed in 2009, and the resulting BC_1F_1 population was planted that fall at the Arthur H. Post farm in Bozeman, MT and harvested in bulk in the summer of 2010. The BC_1F_2 population was space-planted at the Fort Ellis (Bozeman, MT) site for the 2010 - 2011 field season. At the conclusion of the growth period, seed was harvested from individual plants and given a line designation.

Experimental Design and Setup

2012 Trial: Plant Growth Center, Bozeman, MT. The first resistance screening trial was planted in February 2012, and consisted of 200 BC_1F_3 (MT08185//MT08184/Persia 20) lines along with Persia 20 and MT08184 as the resistant and susceptible controls, respectively. The 2012 experiment utilized a randomized complete block design consisting of five blocks, with 210 entries per block (the 200 BC_1F_3 lines, and five replications each for Persia 20 and MT08184). A block consisted of a rack holding a total of 294 SC10 cone-tainer pots with 3.8 cm cell diameters and 21 cm cell depths. Each block was 21 cells long and 14 cells wide.

The outermost 66 cells were filled with a greenhouse mix and planted to Yellowstone winter wheat. These plants acted as a border zone to prevent excessive drying of the peripheral inner cells. The inner 228 cells were each filled with 200 grams of a mixture of 80% *P. neglectus*-infested soil and 20% perlite by volume. Inoculum was obtained from pot cultures derived from populations of *P. neglectus* found in soils at the Arthur H. Post Farm in Bozeman, MT. For each block, the 210 entries were planted one seed per cell. Five of the remaining 18 cell positions were randomly selected from each block and were subjected to the nematode extraction and quantification protocol described in the section that follows. Using the calculation explained below, it was determined that plants were inoculated with an average of 70 *P. neglectus* per cone-tainer pot. The 18 cell positions without entries assigned to them were planted with Yellowstone winter wheat and were not subjected to further analysis.

Plants were grown in the greenhouse for a period of 18 weeks. This included a six-week period in a 4° C vernalization chamber, beginning at the three-leaf stage. Water was applied to all plants at a rate sufficient to maintain moisture at the soil surface. Plants were fertilized using J.R. Peters (Allentown, PA) General Purpose 20-20-20 N-P-K water-soluble fertilizer. Fertilizer was applied twice weekly during watering at a rate of 79 ppm N, using a 15:1 siphon fertilizer injector.

2013 Trial: Plant Growth Center, Bozeman, MT. The design of the second greenhouse screen was similar to the first, with several modifications. It was decided upon the conclusion of the 2012 disease assessment that improved separation between entries, as well as reduced variance within progeny lines, could be achieved by selecting a subset of the 200 lines, based on similar performance and variance compared to the resistant and susceptible controls. More advanced BC_1F_5 lines would be screened in the repeat experiment, which would contain 25% of the genetic heterogeneity present in the BC_1F_3 lines.

After a thorough analysis of the performance of each line in the previous phenotypic screen, 28 lines with performance similar to MT08184 (RLN susceptible) and 27 lines similar to Persia 20 (RLN resistant) were chosen for a second screening trial. Seed from each of the selected 55 BC_1F_5 lines was derived from a single BC_1F_4 head, and along with two seeds each of the control lines MT08184 and Persia 20, was planted one seed per cell in a randomized complete block design consisting of nine blocks. Each block in the 2013 trial consisted of a rack holding a total of 98 SC10 cone-tainer pots. The outer 38 cells of the block acted as a border zone, as described for the 2012 experiment. Inoculum, again obtained from field soils in Bozeman, MT, averaged 50 *P. neglectus* per cone-tainer pot.

Plantings of blocks began in May 2013, and were staggered at one block planting per week, starting in May 2013 and ending in August 2013. This was

done in order to extract and quantify all samples from one block soon after the growth period was complete, to minimize degradation due to storage. Plants were grown for 16 weeks. The six-week vernalization period was omitted from this trial to ensure plentiful nematode numbers on susceptible lines after extraction, as cold storage temperatures (1-5° C) have been shown to significantly reduce populations of *Pratylenchus* spp. (Olthof and Yu, 1999). Watering rates remained identical to the 2012 trial. Fertilizer was provided to each experimental cell as a single application of 2.5 g (½ tsp.) Osmocote (Scotts MiracleGro, Marysville, OH) 19-6-12 N-P-K Outdoor & Indoor Smart Release Plant Food incorporated into cell soil.

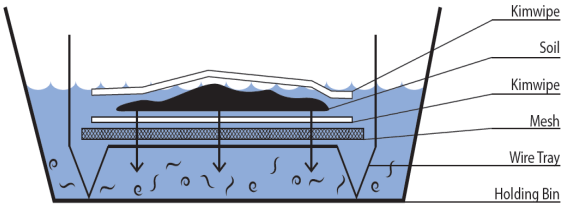
Nematode Extraction Protocol
and Quantification of Resistance

At the conclusion of the growth period, plants were cut back and blocks were stored at 4° C until further processing. Storage periods for blocks ranged from one to eight weeks in the 2012 trial, and one to four weeks for the 2013 trial.

A modified Whitehead tray method was used to extract nematodes from the samples (Whitehead and Hemming 1965). For each 200 g soil sample, nematodes were extracted over the duration of 48 hours, using 2 L of tap water (Figure 1). At the conclusion of the 48-hour period, the extraction solution was passed through a 20 µm mesh sieve. Nematodes were then rinsed from the

(a)

(b)



(c)

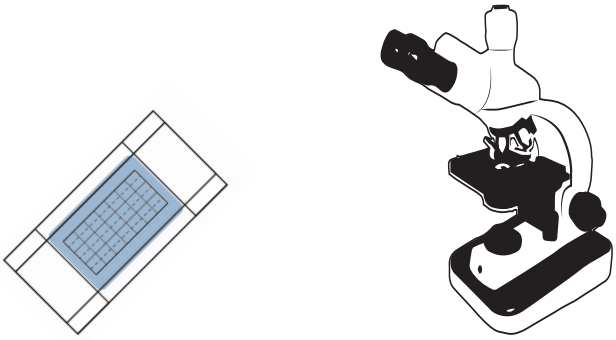


Figure 1. Nematode extraction protocol: (a) Each line in the screen is planted in soil inoculated with RLN and grown for a period of 16-18 weeks; (b) At the conclusion of the growth period, soil and roots from each entry are assembled in the configuration below and remain undisturbed for a 48-hour period; (c) RLN extracted from each sample are sieved and pipetted into 2 mL counting slides for quantification. (Illustrations courtesy of Mina Talajoor)

sieve surface into 50 mL vials, and the volume of extraction solution in each vial was recorded. Extracted samples were stored at 4° C until microscopic examination could be performed. Storage periods for extracted samples ranged from one to two weeks for both trials.

To examine final *P. neglectus* populations per plant, 2 mL of the extraction solution was taken from each sample and injected into a McMaster Counting Slide (Chalex Corporation, Wallowa, OR). Nematodes within the slide's 1 mL grid were counted under 10X magnification on a Nikon Eclipse 50i microscope (Kent, WA). The resulting per mL *P. neglectus* counts were multiplied by the corresponding sample volumes to obtain final populations per plant. *P. neglectus* was identified in samples based on a set of morphological features specific to the species: a strong stylet, sclerotized labia, esophageal overlap with the intestine, and a vulval position 80-87% down the length of the body (Handoo and Golden, 1989).

Statistical Procedures Used

Due to complete lack of emergence in any of the blocks, entries 174 and 198 were omitted from statistical analysis in the 2013 screening assessment. In both years, all entries with final *P. neglectus* populations of 0 were assumed to have no quantifiable disease development due to errors in inoculation and/or extraction, and were therefore omitted from further analysis. Forty-six out of 1,050 total observations (< 5%) were therefore omitted from the 2012 analysis,

and 132 out of 513 total observations (> 20%) were omitted from the 2013 analysis. Assumptions of normality and constant variance were evaluated prior to statistical analysis. Residual plots for both trials revealed a trend of increasing variance with increasing mean final *P. neglectus* population. Data were log-transformed in order to account for this perceived multiplicative effect.

To compare mean final nematode populations among the progeny lines, data from the two trials were independently subjected to a two-way analysis of variance (ANOVA) with terms for "Block" and "Line ID". When the overall ANOVA resulted in a *p*-value of less than 0.05, Fisher's least significant difference (LSD) tests between the entries and each of the controls were performed, in order to discern lines with mean final *P. neglectus* populations that were lower than that of Yellowstone, and also equal to or lower than that of Persia 20. Both ANOVA and *post hoc* comparisons were performed using PROC GLM in SAS v. 9.3 statistical software (SAS Institute Inc., Cary, NC). PROC CORR in SAS was used to test for evidence of correlation between the results of the two trials.

Results

2012 Disease Screening Assessment

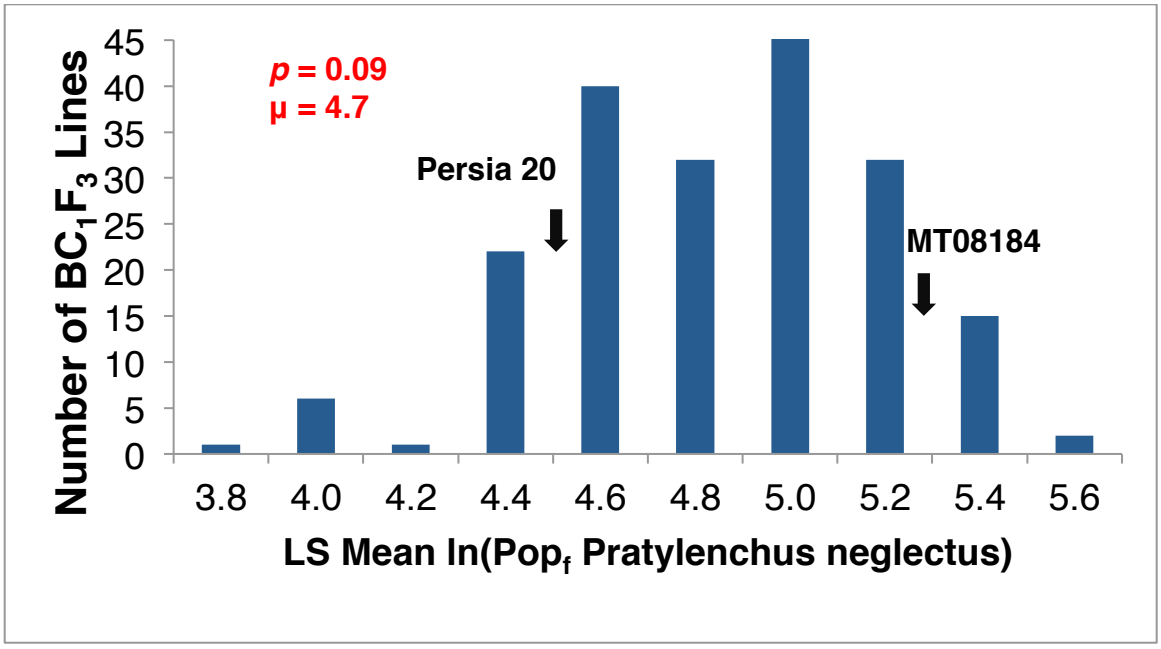
Least squares means for final *Pratylenchus neglectus* populations on the scale of the natural logarithm ranged from 3.8 to 5.6 (44 to 267 *P. neglectus* per

plant after back-transformation) for the 2012 assessment, with an overall population mean of 4.7 (122 *P. neglectus* per plant after back-transformation). The BC_1F_3 population showed a continuous distribution (Figure 2a) of resistance phenotypes, lending further credence to previous reports of the polygenic nature of wheat resistance to *Pratylenchus* (Zwart *et al.,* 2010).

ANOVA results indicated no significant differences among lines in mean final *P. neglectus* population ($p = 0.09$). As the overall ANOVA for the 2012 screening resulted in a *p*-value over 0.05, a protected Fisher's LSD test was not performed. Without adjusting for multiple comparisons, the individual pairwise comparison (paired *t*-test) of susceptible control MT08184 and resistant control Persia 20 indicated ($p < 0.01$) that the mean difference in final *P. neglectus* population between the two entries was not equal to zero. Least squares mean final *P. neglectus* populations on the scale of the natural logarithm for MT08184 and Persia 20 were 5.3 and 4.5, respectively (Figure 2b). Exponentiation of the log-scale values revealed the least squares mean final *P. neglectus* population to be 196 for MT08184, and 96 for Persia 20. It is estimated that the median final *P. neglectus* population for MT08184 was 2.1 times greater than that of Persia 20, with an associated 95% confidence interval from 1.4 to 3.3 times greater.

This result indicated that while the overall experimental error was too large for the ANOVA to provide conclusive evidence of mean differences between entries, the *P. neglectus* inoculum utilized in the experiment behaved as

(a)



(b)

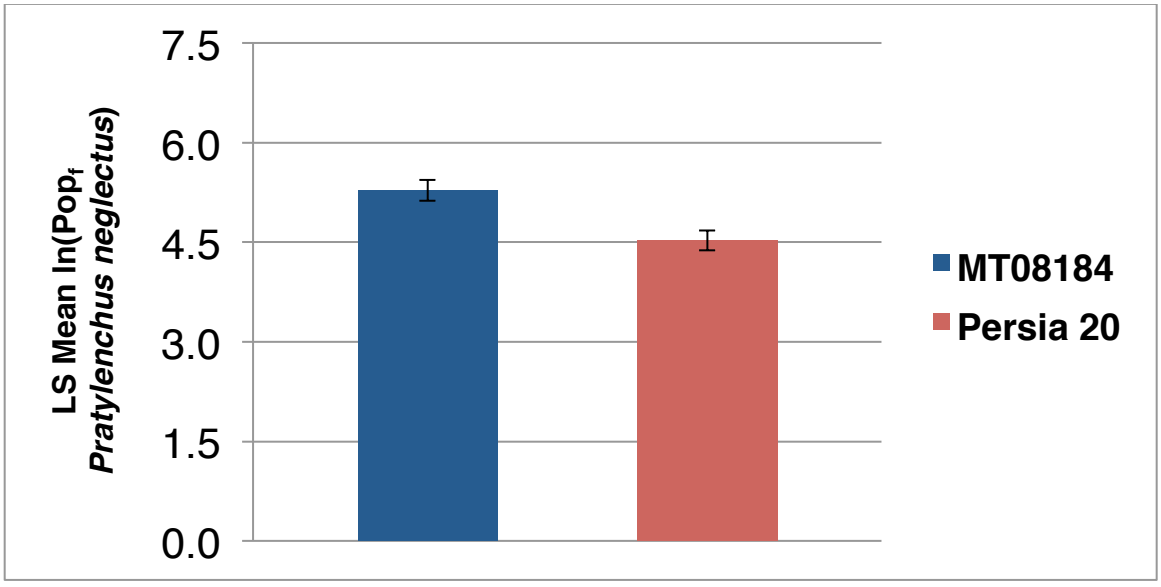


Figure 2. 2012 Disease screen results for 200 progeny lines and parental controls screened for RLN (*P. neglectus*) resistance; (a) distribution of progeny lines, (b) comparison of parental genotypes. Error bars indicate standard errors.

expected, with MT08184 accumulating substantially higher final populations of the nematode than Persia 20.

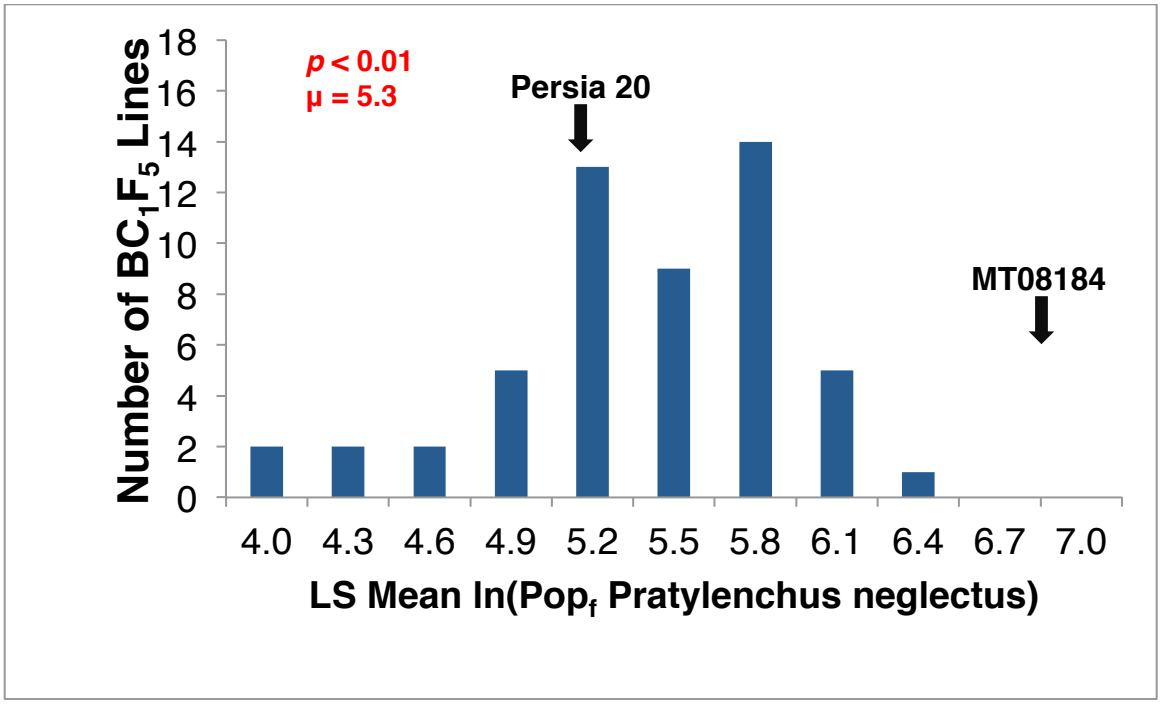
Least squares mean final *P. neglectus* populations for entries and their standard deviations were then used to select lines with similar performance to the resistant and susceptible controls and low standard deviation, in the hopes of identifying homozygous resistant and susceptible lines. The strategy of eliminating supposed heterozygotes would presumably result in a reduction of variance due to segregation within the lines for the RLN resistance trait, and also increase the separation between resistant and susceptible entries, thereby increasing the likelihood of obtaining evidence for a difference in means among the entries from the statistical tests.

2013 Disease Screening Assessment

Final *P. neglectus* populations for entries were higher in the 2013 disease screening than in the 2012 trial. Least squares mean final *P. neglectus* populations ranged from 3.9 to 6.8 on the scale of the natural logarithm (50 to 505 nematodes per plant after back-transformation), with an overall population mean of 5.3 (229 *P. neglectus* per plant after back-transformation). A histogram of least squares mean final *P. neglectus* populations for the BC_1F_5 lines again showed a continuous distribution (Figure 3a).

ANOVA provided convincing evidence of at least one difference in mean final *P. neglectus* population among the lines tested ($p < 0.01$). A protected

(a)



(b)

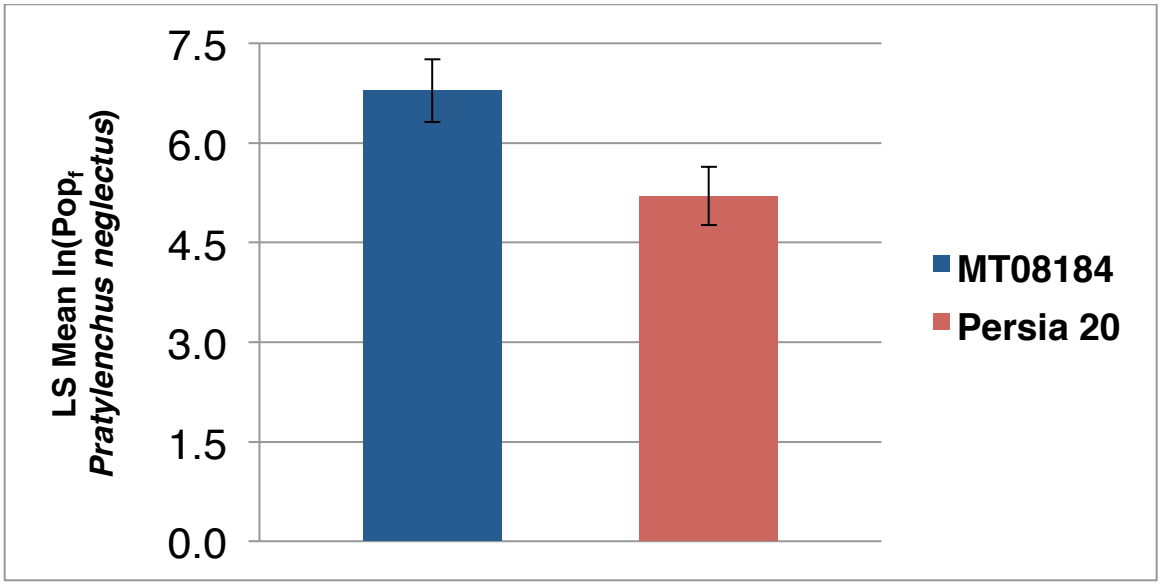


Figure 3. 2013 Disease screen results for 53 progeny lines and parental controls screened for RLN (*P. neglectus*); (a) distribution of progeny lines, (b) comparison of parental genotypes. Error bars indicate standard errors.

Fisher's LSD test revealed considerable evidence of a difference in mean final *P. neglectus* population between the resistant and susceptible controls ($p = 0.01$). MT08184 exhibited the highest mean final pathogen population of any line in the trial at 6.8 on the logarithmic scale (887 *P. neglectus* per plant after back-transformation). Persia 20 had a mean final pathogen population of 5.2 on the logarithmic scale (181 *P. neglectus* per plant after back-transformation), which was intermediate to those of the BC_1F_5 lines tested in the 2013 trial. The median final *P. neglectus* population of MT08184 was estimated to be 4.9 times greater than that of Persia 20, with an associated 95% confidence interval from 1.4 to 17.5 times greater.

Protected Fisher's LSD tests between the BC_1F_5 lines and the controls identified two lines (145 and 84) with significantly ($p < 0.05$) lower mean final *P. neglectus* populations than both susceptible control MT08184 and resistant control Persia 20 (Table 1). An additional 22 lines were identified in *post hoc* analysis whose mean final pathogen populations were significantly lower than MT08184. These 22 lines exhibited least squares mean final *P. neglectus* populations equal to or lower than resistant check Persia 20, and the LSD test provided no evidence (all $p > 0.10$) of a difference in mean final pathogen population between these lines and Persia 20. Of the 24 RLN-resistant lines identified in 2013, three (137, 77 and 155) ranked among the top 10% in terms of resistance in the 2012 disease screening assessment.

Table 1. A comparison of RLN least square means of the 2013 RLN-resistant lines to susceptible check MT08184 and resistant check Persia 20. 2012 RLN least square means are included in the far right columns as a reference.

Entry	LS Mean ln(Pop$_f$ *P. neglectus*) 2013	LS Mean Pop$_f$ *P. neglectus* 2013	LSD test *p*-value H_0: LS Mean=MT08184	LSD test *p*-value H_0: LS Mean=Persia 20	LS Mean ln(Pop$_f$ *P. neglectus*) 2012	LS Mean Pop$_f$ *P. neglectus* 2012
145	3.9	50	<.0001	0.0339	5.4	218
84	4.0	53	<.0001	0.0430	4.4	78
119	4.2	66	<.0001	0.1072	5.4	219
137	4.3	72	0.0002	0.1582	4.2	69
193	4.4	83	0.0002	0.1933	4.3	74
77	4.5	86	0.0010	0.2770	4.2	68
130	4.7	111	0.0014	0.4315	5.3	191
155	4.7	115	0.0017	0.4660	3.8	44
57	4.8	123	0.0053	0.5773	5.2	180
30	4.9	128	0.0029	0.5787	5.1	163
172	4.9	131	0.0034	0.6077	5.0	154
103	5.0	144	0.0040	0.7081	4.5	91
191	5.0	155	0.0058	0.8013	5.3	202
13	5.0	156	0.0098	0.8167	4.4	80
107	5.0	156	0.0059	0.8057	4.5	89
153	5.1	156	0.0076	0.8132	4.5	93
154	5.1	157	0.0105	0.8286	5.3	195
32	5.1	160	0.0086	0.8466	5.2	184
96	5.1	161	0.0055	0.8401	5.3	195
79	5.2	175	0.0124	0.9580	5.3	207
78	5.2	175	0.0083	0.9577	5.1	160
44	5.2	177	0.0129	0.9705	4.6	95
19	5.2	179	0.0109	0.9854	4.5	93
38	5.2	179	0.0138	0.9867	5.6	267
Persia 20	5.2	181	0.0144	-	4.5	92
MT 08184	6.8	887	-	0.0144	5.3	196

Correlations

Simple linear regression analysis provided no evidence of correlation between least squares mean final *P. neglectus* populations for entries obtained in 2012 and least squares means for entries obtained in 2013 ($p = 1.00$, $R^2 < 0.0001$).

Discussion

Several factors may have contributed to the high proportion of unexplained variance in the 2012 phenotypic screen, which resulted in the high *p*-value obtained by the ANOVA *F*-test. These include within-line genetic heterogeneity in the BC_1F_3 population, as well as degradation of samples due to the large number of entries and longer assessment period. Additionally, secondary infection by *Fusarium pseudograminearum* was detected in stem tissues of severely diseased plants in each of the blocks. Compounding the problem of the high amount of variance unexplained by the statistical model was the low number of replications utilized in the trial. Lastly, due to the slowing of biological activity caused by the six-week vernalization period, RLN multiplication was insufficient to efficiently differentiate the wheat lines in the 2012 phenotypic screen. For these reasons, the 2012 disease screening assessment was not satisfactory and should be considered only preliminary data, which were used to select potential resistant and susceptible lines for use in subsequent trials.

Moreover, phenotypic data obtained from the 2012 disease screening are too variable to confidently proceed with QTL analysis. The lack of concordance among screening runs could lead to identification of spurious marker-trait associations from a QTL analysis using phenotypic data from the 2013 disease screening. As a result, the effects of any putative QTL tagged in subsequent marker analyses using the 2013 phenotypic data would require validation in

future experiments.

The phenotypic screening methods utilized in these experiments were prone to error and did not completely account for the complexity of the biological system. The increase in replication, augmentation of the backcross population, and omission of the vernalization stage in the 2013 trial did result in increased separation between entries and much stronger evidence of a difference between the entries. However, the exorbitant number of data omitted from the 2013 assessment due to lack of quantifiable nematode reproduction points to issues with equal allocation, quantity and quality of the inoculum used.

Reports from other research groups have detailed the use of carrot callus cultures to obtain pure, plentiful and healthy inoculum for use in controlled resistance screenings (Moody *et al*., 1973). Pipetting a fixed volume of a prepared nematode suspension derived from carrot callus culture into each entry's growth medium can result in higher and more equitable levels of *Pratylenchus* inoculum (Williams *et al*., 2002). Still, the throughput of this phenotypic screening method remains very low, as the higher levels of inoculum result in higher final pathogen populations that must be extracted and counted at the conclusion of the growth period. Highly susceptible entries may have over 10,000 *Pratylenchus* per 1 mL sample to be counted under the microscope (Dr. Richard Smiley, personal correspondence). Additionally, if seed is desired at the conclusion of assessment for advance, the use of populations with winter wheat

genetic backgrounds, which require a vernalization period, further inhibits the throughput of RLN resistance screening experiments.

The low-throughput nature of traditional *Pratylenchus* resistance screening procedures limits the size of populations utilized for QTL experiments. The most prominent QTL studies of *P. neglectus* resistance in wheat have used plant populations ranging from 300 to 600 plants in number (Williams *et al.*, 2002; Zwart *et al.*, 2005; Mulki *et al.*, 2013). A phenomenon known as the Beavis effect describes the overestimation of QTL effects and lack of detection power at smaller sample sizes. In a well-known simulation study, crop geneticist William D. Beavis (1998) found that estimates of phenotypic variances associated with correctly identified QTL were significantly overestimated if 100 progeny were evaluated, slightly overestimated if 500 progeny were evaluated, and approached the actual value when 1000 progeny were evaluated. In order to optimize the power and precision of the statistical models used in QTL analyses, it will be necessary to develop high-throughput phenotyping methods for RLN resistance that can accurately obtain data from larger populations. High-throughput nematode resistance phenotyping, when used in conjunction with the high-throughput genotyping resources widely available today, will result in greater detection power for small effect QTL, along with more accurate estimates of QTL phenotypic effects. These benefits will greatly improve the success of marker-assisted selection for resistance to *Pratylenchus* species.

In recent years, several studies have been conducted, directed at developing phenotypic screening methods for *Pratylenchus* resistance with improved throughput. The technique of real-time quantitative polymerase chain reaction (qPCR) using species-specific primers has been shown to be effective in detecting and quantifying *P. neglectus* populations from DNA extracts of soil (Yan *et al*., 2013). Research conducted by Thompson (2013) attempted to utilize canopy temperature, photosynthetic yield and root lignin content as indirect measures of *Pratylenchus* resistance; however, QTL associated with these phenotypes were not always identical to those identified using traditional resistance screening methods.

Furthermore, the identification of a resistance mechanism at a specific stage of invasion, plant growth, or within a particular tissue might enhance screening procedures by reducing costs associated with DNA-based detection methods, or the amount of time associated with traditional resistance screening techniques (Linsell *et al*., 2014). Results of *in vivo* greenhouse experiments conducted by Linsell *et al*. showed suppression of *P. thornei* migration, juvenile maturation and reproduction inside and near resistant wheat roots, suggesting that the resistance mechanism occurs after nematodes have penetrated host tissues. Unfortunately, inhibitory compounds identified in the Linsell study had no effect on *P. neglectus*. However, years earlier, Farsi (1996) observed equal root penetration of *P. neglectus* in both resistant and susceptible wheat lines. *In vivo*

studies similar to those of Linsell *et al*. can and should be conducted in populations containing contrasting resistance phenotypes to *P. neglectus*, in order to gain better understanding of the time frame and effects of wheat resistance on *P. neglectus* biology, for the development of improved phenotyping methods.

Initial resistance screenings of Pacific Northwest spring wheat, winter wheat and barley cultivars reported that the resistant donor parent used to develop our backcross population, Persia 20, exhibited high levels of resistance to both *P. neglectus* and *P. thornei* (Sheedy *et al.*, 2007, 2008; Smiley and Nicol, 2009). In the intervening period, however, studies have shown Persia 20 to be highly susceptible to *P. thornei* (Thompson *et al.*, 2008 – 1) and moderately susceptible to *P. neglectus* (Smiley *et al.*, 2014). Results obtained in our study indicate that Persia 20 has only partial resistance to *P. neglectus*, with an observed multiplicative rate of 1.3 in the 2012 trial and 3.9 for the 2013 trial. While partial resistance is still superior to complete susceptibility, pyramiding resistance traits from an alternate resistance source in addition to those provided by Persia 20 may be necessary to achieve an overall level of resistance acceptable to Montana's winter wheat growers. The dual-resistant landrace accession AUS28451 has become the resistance source of choice in disease screening assessments conducted by other research groups in the Pacific Northwest, and other landraces with resistance and even better agronomic

characteristics than AUS28451 have recently been identified (Thompson, 2013; Smiley *et al*., 2014). If the development of Montana wheat cultivars resistant to *Pratylenchus* is to truly become a priority, then this germplasm should be included in future crossing blocks by the Montana State wheat breeding projects.

Lastly, the results of the 2013 phenotypic screen identified 24 BC_1F_5 lines with a level of resistance equal to or superior than Persia 20. *Post hoc* analysis provided strong evidence that the RLN least squares means of two of these lines (145 and 84) were less than that of Persia 20. This result, while promising, is complicated by the lack of correlation between the 2012 and 2013 phenotypic screens. Certain lines, such as 77, 137, and 155, exhibited high levels of resistance in both years' trials. Conversely, line 145, the most resistant line tested in the 2013 screen, was among the most susceptible lines screened in 2012. Greater credence should be given to the results of the 2013 assessment, where adjustments to the phenotypic screening procedure resulted in higher levels of RLN reproduction. Corroboration of the results obtained in 2013 in a subsequent phenotypic screen utilizing the procedural adjustments should be a prerequisite for selection of 2013 RLN-resistant lines 145 and 84 for further development in the breeding program.

CHAPTER 3 - IDENTIFICATION OF GENOME-WIDE SINGLE NUCLEOTIDE POLYMORPHISM (SNP) MARKERS ASSOCIATED WITH RESISTANCE TO *P. NEGLECTUS*

Introduction

Phenotypic selection for quantitative disease resistance typically involves the laborious and time-consuming process of allowing inoculated plant populations to grow under controlled conditions, and quantifying pathogen populations at the conclusion of the growth period (Zwart *et al.,* 2005). Many of the most valuable sources of disease resistance genes are landrace accessions and progenitors of modern bread wheat (*Triticum aestivum* L.) (Thompson and Haak, 1997; Thompson *et al.,* 1999; Nicol *et al.,* 1999, 2001, 2003; Nombela and Romero, 1999; Hollaway *et al.,* 2000; Zwart *et al.,* 2004, 2005; Tokay *et al.,* 2006; Sheedy *et al.,* 2008, 2012). Such germplasm often carries various unfavorable agronomic characteristics that must be selected against during the breeding process (Thompson, 2013; Smiley *et al*., 2014). Further complicating matters, without a tool to track the inheritance of targeted resistance loci, important genes could potentially be lost over the course of multiple backcrosses to adapted susceptible cultivars. Marker-assisted breeding technologies have enabled plant breeders to identify lines containing the genes controlling traits of interest, thereby improving both the precision and duration of selection for both qualitative

and simpler quantitative traits.

Many experiments involving marker-trait associations for disease resistance in bread wheat have been conducted in the past, and have successfully identified associations for numerous resistance traits (Gupta *et al.,* 2010), including some for *Pratylenchus* species. Overall, these studies have demonstrated that resistance to the root lesion nematode is polygenic and additive, and that quantitative trait loci (QTL) for resistance are present on all three genomes (A, B and D) of bread wheat (Zwart *et al.,* 2010). QTL linked to resistance to *P. thornei* have been identified on chromosomes 1B, 2B, 3B, 4D, 6D and 7A (Schmidt *et al.*, 2005; Zwart *et al.,* 2005, 2006; Tokay *et al.,* 2006). Markers linked to the *P. neglectus* resistance gene *Rlnn1*, located on the long arm of chromosome 7A, have been identified and successfully implemented in both Australian and CIMMYT breeding programs (Williams *et al.,* 2002; Gupta *et al.,* 2010). Additional QTL for resistance to *P. neglectus* have been reported on chromosomes 2B, 4D, 5B, 6B and 7B (Zwart *et al.,* 2005, 2010; Mulki *et al.,* 2013). A recent study utilized a recombinant inbred line population derived from the dual-resistant landrace accession AUS28451 and a susceptible USA wheat cultivar, and identified resistance-associated QTL on chromosomes 1A, 2B, 4A, 5A, 5B, 6B and 7A using phenotyping methods for both *P. thornei* and *P. neglectus* (Thompson, 2013).

In the past, restriction fragment length polymorphisms (RFLPs), amplified

fragment length polymorphisms (AFLPs), and more recently, simple sequence repeats (SSRs, microsatellites) were the most important tools in the implementation of marker-assisted selection in plant breeding programs. However, due to limitations in throughput and marker density they are no longer the preferred systems for screening genetic backgrounds. Today, array-based high-throughput low-cost marker systems such as single nucleotide polymorphisms (SNPs) have become the markers of choice for whole genome profiling (Gupta *et al*., 2010).

First discovered in the human genome, SNPs have proven to be the most abundant forms of genetic variation among individuals of the same species, for all forms of life (Rafalski, 2002). Although they are less polymorphic than SSR markers due to their biallelic nature, SNPs easily compensate for this shortcoming by being abundant, ubiquitous and amenable to high-throughput automation (Mammadov *et al*., 2012). Characteristics such as these make SNPs particularly appealing for marker-assisted selection in wheat breeding programs, where low levels of marker polymorphism are often a problem due to wheat's narrow gene pool (Langridge *et al*., 2001). These low levels of polymorphism are particularly significant in the D genome, the result of a relatively recent polyploidization event with the diploid grass species *Aegilops tauschii* around 10,000 years ago (Marcussen *et al*., 2014; Gupta *et al*., 2010).

One of the primary goals of this research project was to identify SNPs

linked to QTL for resistance to *P. neglectus*, for use in a marker-assisted backcross-breeding regime. Phenotypic data compiled in the experiments detailed in Chapter 2 was paired with genotypic data (consisting of a set of genome-wide SNPs polymorphic between MT08184/MT08185 and Persia 20) for use in association analysis.

Materials and Methods

Plant Tissue Collection

In February 2013, tissue was collected from four flag leaf stage plants from each of the 55 BC_1F_4 (MT08185//MT08184/Persia 20) progeny lines along with five flag leaf stage plants each of MT08184, MT08185 and Persia 20, for a total of 235 samples. In 2014, tissue was collected from a 3-week seedling of each of the 200 BC_1F_3 progeny lines, each of the 55 BC_1F_5 (MT08185//MT08184/Persia 20) progeny lines, as well as five 3-week seedlings each of MT08184, MT08185 and Persia 20, for a total of 270 samples.

To ensure consistent and adequate DNA yields, five 3 cm pieces (15 cm total) of leaf tissue from each plant were placed in individual wells of a VWR (Radnor, PA) 96-Well Deep Well Microplate. Microplates were fitted with flexible mat lids and stored at -80° C until shipment. Upon completion of tissue collection, samples were packed in dry ice (frozen CO_2) and overnighted to Dr. Deven See of the USDA-ARS Western Regional Small Grains Genotyping

Laboratory for further processing.

DNA Extraction and Genotyping Assays

2013 Assay. Samples were received in February 2013 and stored at -80° C until further processing. DNA was extracted from each sample and DNA concentrations were adjusted to 20 ng/µL. Based on sequencing data obtained from a prior Illumina (San Diego, CA) iSelect 9K SNP assay (Cavanagh *et al.*, 2013) of parent lines Yellowstone and Persia 20, a set of polymorphic genome-wide SNPs were selected and primers were designed in-house to genotype the 55 BC_1F_4 lines along with the parents. A spreadsheet containing genotypic information for 142 genome-wide SNPs was sent back to Bozeman in October 2013 for further analysis.

2014 Assays**.** To confirm the effects of putative resistance QTL identified in a prior association analysis, DNA was extracted in May 2014 from the 200 BC_1F_3 lines that were phenotyped in the fall of 2012, and the concentration adjusted to 20 ng/µL. These 200 lines, along with the controls, were genotyped with markers that initially showed statistically significant ($p < 0.05$) association with resistance to *P. neglectus*.

In addition, 20 ng/µL DNA extracted from the 55 BC_1F_5 lines, along with the controls, was sent to Dr. Shiaoman Chao of the North Central Regional Small Grains Genotyping Laboratory in Fargo, ND. These lines were then genotyped

using the Illumina (San Diego, CA) iSelect 90K SNP assay (Wang *et al.*, 2014). Results from both assays were compiled in spreadsheets and sent to Bozeman in July 2014 for analysis.

Marker Evaluation and Recoding

Upon receipt of genotypic data, markers were evaluated for polymorphism between MT08184/MT08185 and Persia 20. Markers that showed no polymorphism between the susceptible and resistant controls were discarded. Additionally, markers that showed segregation within parental and progeny lines, as well as those that showed heterogeneous genotypes between MT08184 and MT08185, were discarded prior to association analysis. At the conclusion of marker evaluations, the initial 142 genome-wide SNPs used to genotype the 55 BC_1F_4 (MT08185//MT08184/Persia 20) lines in 2013 was reduced to 104 SNPs covering 19 of the 21 chromosomes of wheat. No marker genotypes were obtained for chromosomes 4D and 6D. Map positions of SNPs utilized in the 9,000 SNP assay were obtained from the Triticeae Coordinated Agricultural Project (TCAP) group.

Marker genotypes acquired from the 2014 90,000 SNP array assay of the 55 BC_1F_5 (MT08185//MT08184/Persia 20) progeny lines and controls were used to obtain data for chromosomes 4D and 6D. Using map information from the TCAP group, all SNPs other than those mapped to chromosomes 4D and 6D were culled from the data set. The remaining 4D and 6D data from the 2014

90,000 SNP array assay was subjected to a clustering analysis using Illumina (San Diego, CA) GenomeStudio 2011.1 software. Markers that formed two distinct nucleotide clusters, where MT08184/MT08185 and Persia 20 were in separate nucleotide groups, were selected for association analysis. At the conclusion of the 2014 marker evaluation, there were 18 polymorphic SNPs mapped to chromosome 4D, along with 96 polymorphic SNPs mapped to chromosome 6D, which fit the selection criteria.

For each of the association analyses described in this chapter, marker genotypes were recoded as “p” for those that shared the corresponding Persia 20 genotype, and “y” for those sharing the corresponding MT08184/MT08185 (Yellowstone) genotype.

Statistical Procedures Used

Markers were assessed for evidence of an association with least squares mean ln(Pop_{final} *P. neglectus*) using a one-way ANOVA with a term for Marker ID. All single-marker analyses were performed using RStudio statistical Software (R Foundation for Statistical Computing, Vienna, Austria).

Results

Single-marker Analysis

Recoded single nucleotide polymorphism genotypes, from a total of 218 genome-wide SNPs, for 53 of the 55 BC_1F_4 lines assayed, were utilized in the

initial single-marker analysis. Significant differences ($p < 0.10$) in mean ln(Pop_{final} *P. neglectus*) between entries with the Persia 20 ("p") genotype and Yellowstone ("y") genotype were detected for 12 of the 218 markers tested: IWA2607, IWA5081, IWA3128, IWA6721, IWB35058, IWA6246, IWA2314, IWA4716, IWB45037, IWA4898, IWA2395, and IWB42351, suggesting potential linkages with QTL for resistance to *P. neglectus*. Consensus map information located these SNPs on chromosomes 1AL, 1DS, 2BL, 4DL, 5BL, 5DL, 7AL and 7BL. For a complete list of information for these markers, including consensus chromosome positions (cM), R-square values, estimates of phenotypic effects and associated 95% confidence intervals, see Table 2 below. Sequence

Table 2. Single-marker analysis identified SNP genotypes exhibiting statistically significant ($p < 0.10$) association with resistance to *P. neglectus*. * = Map location according to the 9,000 SNP consensus map provided by the TCAP group.

SNP ID	Location	cM	*p*-value	R^2 (%)	Resistant Genotype (p = Persia 20; y = Yellowstone)	Ratio of Median Pop_f *P. neglectus* (susceptible/resistant)	LB 95% CI	UB 95% CI
IWA2314	1AL	91.8	0.0372	12.2	p	1.5	1.0	2.3
IWA4898	1AL	137.2	0.0585	7.2	y	1.4	1.0	2.1
IWA4716	1DS	25.7*	0.0480	8.8	y	1.5	1.0	2.2
IWA5081	2BL	130.6	0.0078	15.0	p	1.8	1.2	2.7
IWB42351	4DL	85.1	0.0935	5.5	y	1.4	1.0	2.0
IWB35058	4DL	102.8	0.0268	9.4	y	1.4	1.0	1.9
IWA2395	4DL	118.1	0.0672	6.7	y	1.3	1.0	1.8
IWB45037	4DL	118.1	0.0581	7.3	y	1.3	1.0	1.8
IWA6721	5BL	76.9	0.0144	13.4	y	1.6	1.1	2.2
IWA2607	5DL	67.5	0.0076	15.1	p	1.6	1.1	2.1
IWA3128	7AL	164.3	0.0113	14.6	y	1.6	1.1	2.2
IWA6246	7BL	133.6	0.0361	11.9	p	1.5	1.0	2.2

information for RLN resistance-associated SNPs, for reproduction of these markers using the Kompetitive Allele-Specific Polymerase Chain Reaction

(KASP) method (LGC Genomics, Teddington, UK), is available in Appendix A.

R-square values for RLN resistance-associated markers ranged from 5.5 to 15.1%, and estimates of ratios of median final *P. neglectus* populations for the susceptible genotype to those for the resistant genotype ranged from 1.3 to 1.8. These results indicated that the effects of each putative RLN resistance-associated QTL tagged in the single-marker analysis, if valid, had only a small overall effect on the entries' responses to *P. neglectus*. Persia 20 genotypes at markers IWA2607, IWA5081, IWA6246 and IWA2314 were associated with resistance to *P. neglectus,* while Yellowstone genotypes at markers IWA3128, IWA6721, IWB35058, IWA4716, IWB45037, IWA4898, IWA2395 and IWB42351 were associated with an RLN-resistant phenotype.

Marker Validation Using the BC_1F_3 Population

No data was obtained for markers IWA2607 and IWB35058 for the 200 BC_1F_3 lines. Single-marker analysis failed to identify putative markers with significant associations (all $p > 0.20$) with resistance to *P. neglectus* when genotypic and phenotypic data from 200 BC_1F_3 (MT08185//MT08184/Persia 20) progeny lines were used (Table 3).

Discussion

The lack of concordance between phenotypic screenings of the BC_1F_3 and

Table 3. Significant associations found using BC_1F_5 phenotypic data were not found when single marker analysis was conducted using genotypic and phenotypic data from the BC_1F_3 generation.

SNP ID	*p*-value
IWA5081	0.8511
IWA3128	0.2105
IWA6721	0.5191
IWA6246	0.8422
IWA2314	0.4211
IWA4716	0.8469

BC_1F_5 generations makes identification of QTL associated with RLN resistance problematic. SNPs that showed significant associations with resistance to *P. neglectus* will not be suitable for use in marker-assisted breeding until their effects are confirmed in repeat disease screen experiments conducted both in the greenhouse, as well as in the field under pressure from indigenous RLN populations.

Previous research on the genetic control of resistance to *P. neglectus* in bread wheat has identified QTL with similar map locations to putative RLN resistance-associated QTL tagged in this study. The *Rlnn.1* locus, the first published resistance gene for *P. neglectus*, was mapped to chromosome arm 7AL in an initial study (Williams *et al.*, 2002). More recent experiments conducted by Mulki *et al.* (2013) and Thompson (2013) identified diversity arrays technology (DArT) and SNP markers associated with resistance to *P. neglectus* that also mapped to chromosome 7A. Markers identified in the Mulki and Thompson experiments, along with the RLN resistance-associated SNP IWA3128 identified in this study, may potentially be linked to the *Rlnn.1* locus

described by Williams *et al*. (2002).

Furthermore, the *P. neglectus*-resistant SNP genotypes at chromosome regions 5BL and 7BL identified in this study have previously been reported to harbor loci that confer RLN resistance. An association mapping study conducted by Mulki *et al*. (2013) also identified *P. neglectus* resistance-associated DArT markers that mapped to chromosomes 5BL (59.8 cM) and 7BL (146.5 cM), based on a consensus genetic map of wheat consisting of 5,000 DArT markers (Detering *et al*., 2010). Concordance between results reported in this study and those of Williams *et al*. (2002), Mulki *et al*. (2013), and Thompson (2013) does not provide the experimental validation necessary to proceed with the deployment of RLN resistance-associated SNPs IWA3128, IWA6721 or IWA6246 in the Montana State University Winter Wheat Breeding Program. It does, however, lend credence to these researchers' suggestions that chromosomal regions 5BL, 7AL and 7BL contain loci that play an important role in the polygenic inheritance of resistance to *P. neglectus*.

Also, notably, four SNPs exhibiting moderately significant association ($0.10 < p < 0.05$) with RLN resistance were identified, which are located in the same chromosomal regions as two of the RLN resistance-associated SNPs tagged in single marker analysis. Markers IWB42351, IWB45037 and IWA2395 showed moderate association with the *P. neglectus* resistance phenotype, and flanked RLN resistance-associated SNP IWB35058 on chromosome 4DL,

forming a putative QTL region 33 cM in size. IWA4898 showed moderate association with the RLN resistance phenotype and mapped to chromosome 1AL, along with RLN resistance-associated SNP IWA2314, resulting in a region 45.4 cM in size. These results, more so than any other putative QTL identified in our single marker analysis, suggest that chromosomal regions 1AL and 4DL may harbor loci that confer a partial *P. neglectus*-resistance phenotype. Neither 1AL nor 4DL are among the chromosomal regions associated with resistance to *P. neglectus* that have been reported in the literature. If confirmed in a future resistance assessment utilizing the experimental adjustments employed in the 2013 greenhouse screening, these would represent novel QTL present in resistant parent Persia 20 – a line for which no QTL studies of RLN resistance have currently been published.

Ultimately, RLN-resistant SNP markers identified in this study whose effects are confirmed in future studies may be inadequate predictors of a *P. neglectus*-resistant phenotype on their own, and would therefore be inappropriate choices for deployment in a wheat breeding program, due to the low level of marker density utilized in the experiment. This would mitigate the efficacy of marker-assisted selection for resistance to *P. neglectus*, as some lines carrying the desired marker genotype may not simultaneously carry the true RLN resistance locus. Fine-mapping of putative QTL regions associated with RLN resistance would identify markers consistently linked to the true RLN resistance

genes.

Contributing only four of the 12 RLN resistance-associated SNP genotypes, and with only a partial RLN resistance phenotype, Persia 20 has questionable suitability as the sole donor of RLN resistance genetics in a wheat-breeding program. RLN-resistant SNP genotypes contributed by Persia 20 were generally associated with larger RLN resistance effects and explained a larger proportion of the phenotypic variation when compared with RLN-resistant genotypes contributed by Yellowstone. The phenotypic effects of putative Persia 20-derived QTL for RLN resistance identified in our study were similar to other putative resistance-associated QTL previously reported in the literature (Zwart *et al*., 2005; Mulki *et al*., 2013; Thompson, 2013). Further crosses of selected lines identified in our work to the synthetic hexaploids and landrace accessions utilized in other studies of *P. neglectus* resistance may be necessary to obtain an effective overall level of resistance.

Future experiments at Montana State evaluating RLN resistance in wheat should be conducted if further progress is to be made in developing an RLN-resistant wheat cultivar. These studies ought to employ multiple sources of resistance, more accurate and higher-throughput phenotyping methods, higher marker densities, and larger population sizes in order to obtain a higher levels of detection power for QTL, higher mapping resolution and better estimates of phenotypic effects for the QTL identified. These measures will greatly improve

the efficiency and precision of marker-assisted selection for resistance to *P. neglectus*. As always, the true value of resistance phenotypes and genotypes identified in the greenhouse must be validated in the field.

CHAPTER 4 - FIELD EVALUATION OF AGRONOMIC TRAITS OF THE BC_1F_4 (MT08185//MT08184/PERSIA 20) LINES

Introduction

While landrace accessions such as Persia 20 carry *Pratylenchus* resistance traits that are potentially valuable to Montana wheat production, recent studies have shown that RLN-resistant landraces also exhibit several unfavorable agronomic characteristics (Thompson, 2013, Smiley *et al*., 2014). These traits would need to be selected against while introgressing *Pratylenchus* resistance into lines that could eventually be developed into commercial cultivars.

This chapter details preliminary work evaluating the agronomic performance of BC_1F_4 (MT08185//MT08184/Persia 20) progeny lines compared to a set of widely grown checks. Both classical and molecular breeding techniques were utilized in our efforts to develop a winter wheat cultivar that is resistant to *P. neglectus*. Using the agronomic data generated in our field evaluations in conjunction with the disease screen data from 2012 and 2013, we hoped to identify lines with both superior agronomic performance as well as resistance to *P. neglectus*. Additionally, the SNP marker data described in Chapter 3, along with our field evaluation data, were analyzed to identify any marker-trait associations between putative SNP resistance markers and the agronomic traits measured. In this way, potential issues with linkage drag

associated with RLN resistance markers could be detected and addressed.

Materials and Methods

Site Specifics and Field Procedures

A 10.4 x 21.3 m (221.5 m^2) portion of field located at the Arthur H. Post Farm in Bozeman, MT was selected as the site for the 2012 - 2013 field evaluation. Winter wheat yields at this location are typically high, often achieving levels of 6,725 kg/ha. Crop rotation is practiced to prevent the harmful buildup of soilborne disease and insect populations. During the 2010 growing season the field was fallowed, followed by a planting of spring wheat in 2011 and field pea (*Pisum sativum* subsp. *arvense*) in the 2012 growing season. Under environmental conditions such as these, in the absence of any significant nematode pressure, the lines were expected to reach their full yield potential.

Prior to planting, a soil test was performed at a depth of 0 to 9.4 cm, and based on the results of the test the soil was adjusted to 0.04 kg N per expected kg per ha. As 6,725 kg/ha was the expected yield level, the soil fertility was adjusted to 280 kg N/ha. Area soils were dry and hard that year, due to sparse precipitation, and a pre-planting application of water was required to facilitate planting and seedling establishment.

The experiment was planted on 9 October 2012, using a randomized complete block design consisting of four blocks. Each block contained 60 single

row plots, each 1.8 m long and spaced at 0.3 m. The total planted area for the trial was 131 m^2. The entries consisted of 54 BC_1F_4 (MT08185//MT08184/Persia 20) lines selected at the conclusion of the 2012 disease screen, the resistant donor parent Persia 20, and five widely planted Montana winter wheat cultivars: Yellowstone, Decade, Colter, CDC Falcon and Jagalene. Five grams of seed was allotted to each plot for planting, which was performed using a six-row Wintersteiger Hege (Salt Lake City, UT, USA) experimental drill.

Heading dates were recorded in May and June of 2013, and plant heights were measured in August prior to harvest. Each row was individually cut and bundled in August 2013, using a Suzue (Japan) rice binder. Grain from each single-row plot was threshed with a “Vogel” stationary thresher manufactured by Bill’s Welding (Pullman, WA). Seed from plots were cleaned before measuring grain yield, test weight and grain protein content. Plot weights were converted to kg/ha prior to statistical analysis. Volume weight was measured from a bulk of two blocks, with a Seedburo (Des Plaines, IL) pint cup. Grain protein was also measured from a bulk of two blocks, using the DICKEY-john (Minneapolis, MN) Instalab 700 NIR Analyzer.

Single-marker Analysis

Single nucleotide polymorphism marker data on the BC_1F_4 lines were obtained from the USDA-ARS Western Regional Small Grains Genotyping Laboratory. For information about DNA extraction protocols and genotyping

assays, refer to Chapter 3 materials and methods.

Statistical Procedures Used

Residual plots of data from both trials were analyzed for violations of the assumptions of normality and constant variance prior to applying the ANOVA tests. Using PROC GLM in SAS statistical software, a two-way ANOVA with terms for “Block” and “Line ID” was applied to the agronomic data, to test for evidence of a difference in mean performance for all of the traits measured. To test for a relationship between a line’s disease rating and its agronomic performance, a simple linear regression (SLR) model was fit using least squares mean 2013 final *P. neglectus* populations as the explanatory variable and least squares means for each of the agronomic traits measured as the response. SLR was performed using the *lm.* function in RStudio statistical software. Putative resistance markers (at $p < 0.05$) were assessed for evidence of an association with the agronomic traits using a one-way ANOVA with a term for Marker ID. The single-marker analysis was performed using RStudio statistical software.

Results

Replicated Yield Trial

Means of progeny lines, parents, and check cultivars varied (all $p < .0001$) for grain yield and for each of the other agronomic traits measured (Table 4). Parental lines Yellowstone and Persia 20 were among the poorest yielding lines

Table 4. Means for grain yield and major agronomic traits of 60 wheat lines at Arthur H. Post Farm (Bozeman, MT) in 2013. 2013 RLN least squares means are included in the right column as a reference.

Entry	Pedigree	Yield (kg/ha) RCB	Volume Weight (kg/hL) 2x2 rep Bulk	Heading Date (Julian) RCB	Plant Height (cm) RCB	Grain Protein (%) 2x2 rep Bulk	RLN LS Mean 2013
172	MT08185//MT08184/Persia 20	6305	73	170	105	14.2	131
38	MT08185//MT08184/Persia 20	6041	75	171	109	14.8	179
145	MT08185//MT08184/Persia 20	5715	72	169	92	14.7	50
19	MT08185//MT08184/Persia 20	5714	72	171	93	15.0	179
107	MT08185//MT08184/Persia 20	5692	74	169	108	14.9	156
114	MT08185//MT08184/Persia 20	5691	75	173	98	14.9	228
50	MT08185//MT08184/Persia 20	5636	71	169	103	15.4	213
54	MT08185//MT08184/Persia 20	5615	74	171	89	15.3	296
194	MT08185//MT08184/Persia 20	5615	75	169	109	15.2	341
6	MT08185//MT08184/Persia 20	5572	73	170	103	14.9	291
44	MT08185//MT08184/Persia 20	5550	75	170	104	14.6	177
150	MT08185//MT08184/Persia 20	5538	76	172	105	14.9	387
Colter	check	5520	72	170	85	15.4	-
155	MT08185//MT08184/Persia 20	5475	70	170	94	15.1	115
84	MT08185//MT08184/Persia 20	5470	74	171	103	14.6	53
63	MT08185//MT08184/Persia 20	5436	74	170	108	15.0	227
Decade	check	5410	72	168	80	15.6	-
118	MT08185//MT08184/Persia 20	5398	75	170	103	15.0	276
30	MT08185//MT08184/Persia 20	5377	75	170	108	14.8	128
Jagalene	check	5369	73	166	79	14.9	-
82	MT08185//MT08184/Persia 20	5338	72	168	105	14.9	198
174	MT08185//MT08184/Persia 20	5330	73	173	93	15.4	-
170	MT08185//MT08184/Persia 20	5315	76	173	106	15.3	323
32	MT08185//MT08184/Persia 20	5310	75	169	107	14.8	160
78	MT08185//MT08184/Persia 20	5304	71	171	88	14.8	175
103	MT08185//MT08184/Persia 20	5304	73	168	96	15.3	144
132	MT08185//MT08184/Persia 20	5290	73	170	92	15.3	295
69	MT08185//MT08184/Persia 20	5269	73	171	89	15.2	264
153	MT08185//MT08184/Persia 20	5198	72	169	95	15.7	156
154	MT08185//MT08184/Persia 20	5196	76	169	108	14.9	157
167	MT08185//MT08184/Persia 20	5137	76	172	102	14.9	269
121	MT08185//MT08184/Persia 20	5128	74	169	109	14.5	260
119	MT08185//MT08184/Persia 20	5117	72	171	104	15.4	66
96	MT08185//MT08184/Persia 20	5040	74	171	97	15.8	161
137	MT08185//MT08184/Persia 20	5002	74	169	103	15.4	72
47	MT08185//MT08184/Persia 20	4985	72	168	91	15.5	421
57	MT08185//MT08184/Persia 20	4982	73	170	83	15.2	123
79	MT08185//MT08184/Persia 20	4978	74	169	110	15.1	175
27	MT08185//MT08184/Persia 20	4966	71	170	102	15.7	417
130	MT08185//MT08184/Persia 20	4958	74	171	107	14.9	111
22	MT08185//MT08184/Persia 20	4931	72	169	98	15.3	254
77	MT08185//MT08184/Persia 20	4910	74	171	100	15.2	86
40	MT08185//MT08184/Persia 20	4899	76	169	115	14.7	217
16	MT08185//MT08184/Persia 20	4894	72	171	88	15.4	271
36	MT08185//MT08184/Persia 20	4875	70	170	90	15.5	277
180	MT08185//MT08184/Persia 20	4872	72	170	102	15.2	224
4	MT08185//MT08184/Persia 20	4846	74	170	104	15.7	316
193	MT08185//MT08184/Persia 20	4829	74	173	103	15.1	83
175	MT08185//MT08184/Persia 20	4813	71	169	85	15.5	277
66	MT08185//MT08184/Persia 20	4743	73	167	96	15.5	505
Yellowstone	check	4720	69	170	85	15.8	887
198	MT08185//MT08184/Persia 20	4716	73	170	88	15.7	-
115	MT08185//MT08184/Persia 20	4642	73	169	101	15.7	189
13	MT08185//MT08184/Persia 20	4608	73	171	101	15.0	156
177	MT08185//MT08184/Persia 20	4540	73	169	93	15.4	225
15	MT08185//MT08184/Persia 20	4443	72	172	101	15.7	433
CDC Falcon	check	4422	69	167	84	16.2	-
10	MT08185//MT08184/Persia 20	4175	72	171	94	15.8	283
Persia 20	check	4100	74	168	105	16.1	181
191	MT08185//MT08184/Persia 20	3887	72	170	95	15.5	155
Average		5131	73.1	169.7	98.1	15.2	
LSD (0.05)		760	1.3	1.6	7.3	0.6	
C.V. (%)		10.6	0.8	0.7	5.3	2.0	
***p*-value**		<.0001	<.0001	<.0001	<.0001	<.0001	

tested, averaging 4720 and 4100 kg/ha respectively. The relatively low yield of

susceptible parent Yellowstone is lower than expected based on previous data. Hard red winter wheat comparison trials conducted in Bozeman in 2009, 2011 and 2012 reported a 3-year average yield of 6,443 kg/ha for Yellowstone (Berg *et al.*, 2013). Montana winter wheat cultivar Colter was the highest yielding check in the 2013 field evaluation of the RLN lines, averaging 5,520 kg/ha. Several of the BC_1F_4 progeny lines, which exhibited levels of resistance equal or superior to resistant control Persia 20 in the 2013 resistance screening, yielded at levels similar to or significantly higher than Colter. Line 145, the most resistant progeny line screened in the 2013 greenhouse study, yielded an average of 5,715 kg/ha, a quantity significantly larger than two of the five checks, and exhibited an estimated mean volume weight and protein content that was statistically indistinguishable from least one of the five checks tested. Additionally, RLN-resistant line 84, which also exhibited a level of resistance superior to Persia 20 in 2013, had an estimated mean yield, volume weight and protein content that was statistically equivalent to at least one of the checks. Lines 77, 119, 137, 155 and 193, which showed levels of RLN resistance equivalent to Persia 20 in 2013, were all within the same statistical grouping as at least one of the five checks for yield, volume weight and protein content. However, these seven RLN-resistant progeny lines were significantly taller than all of the five checks (LSD = 7.3 cm). Five of these seven lines exceeded an average height of 100 cm.

Simple Linear Regression Analysis

No linear relationship between agronomic performance and RLN resistance rating of progeny lines is evident from the scatter plots (Figure 4), with the exception of grain protein (Figure 4e). The analysis provided convincing evidence that an increase in the mean susceptibility (higher final population *P. neglectus*) of the progeny line was associated with higher mean protein content, explaining 12 % of the phenotypic variation seen for grain protein content (p = 0.01, R^2 = 0.12).

(a)

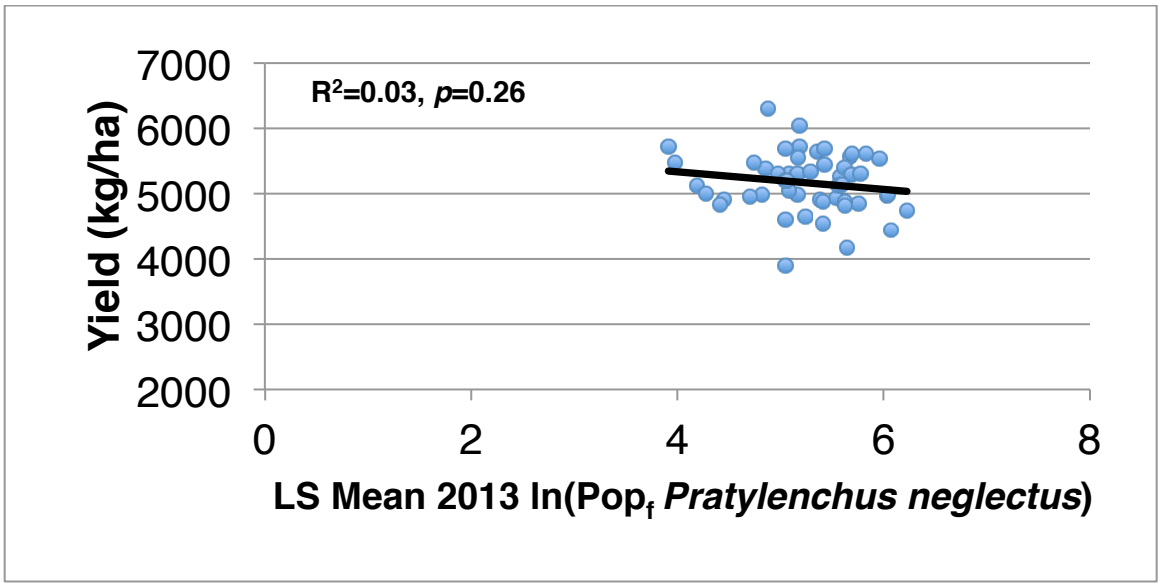
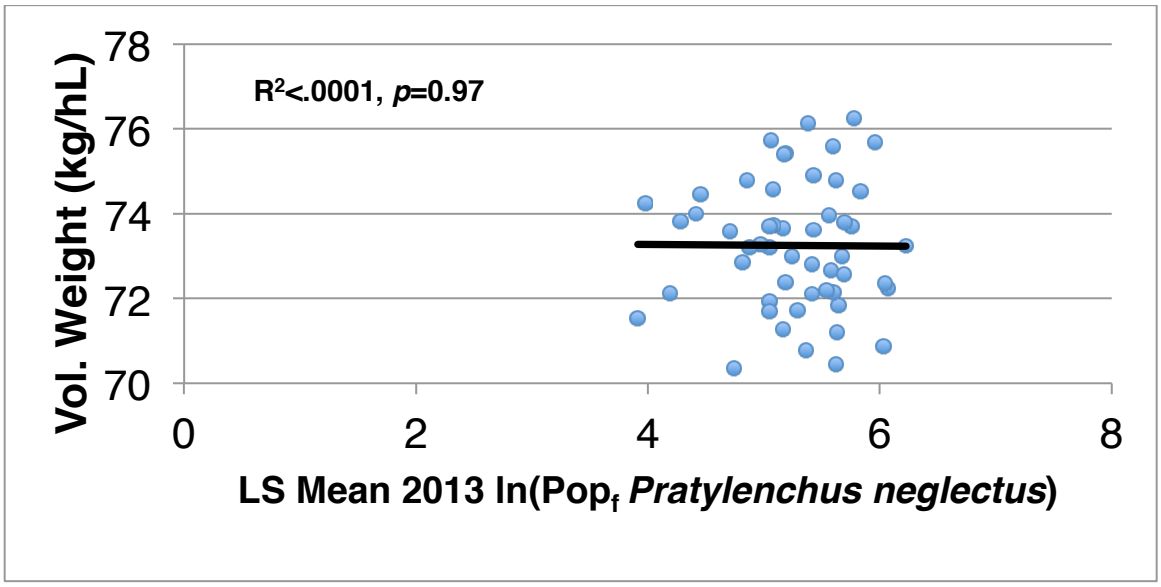


Figure 4. Simple linear regression analyses between RLN resistance and various agronomic traits: (a) grain yield, (b) grain volume weight, (c) heading date, (d) plant height, and (e) grain protein content. Significance code: '*' = 0.05.

Figure 4 – Continued

(b)



(c)

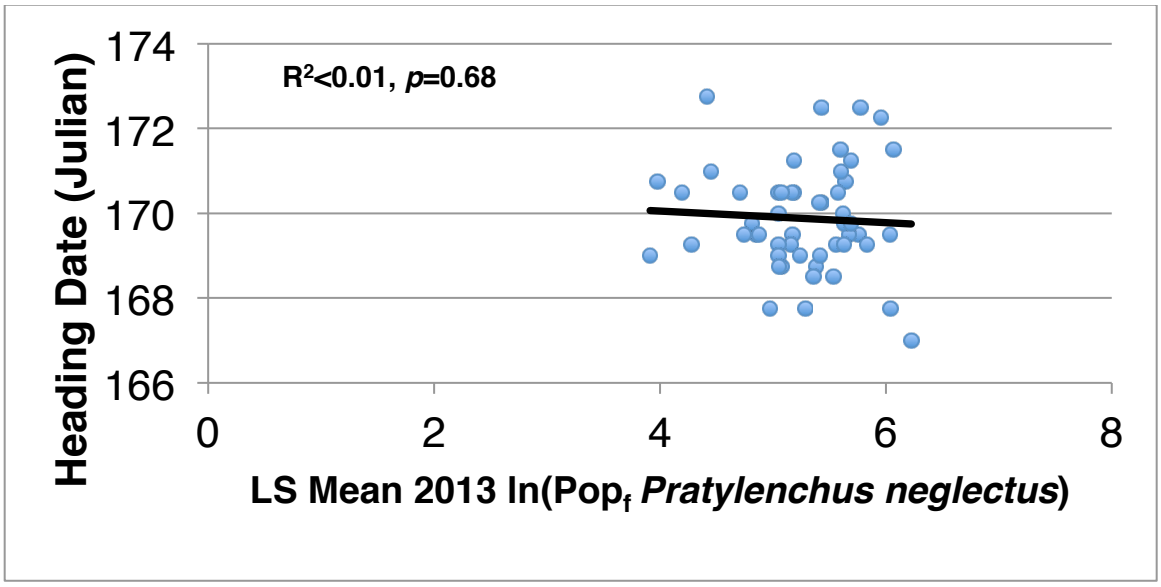
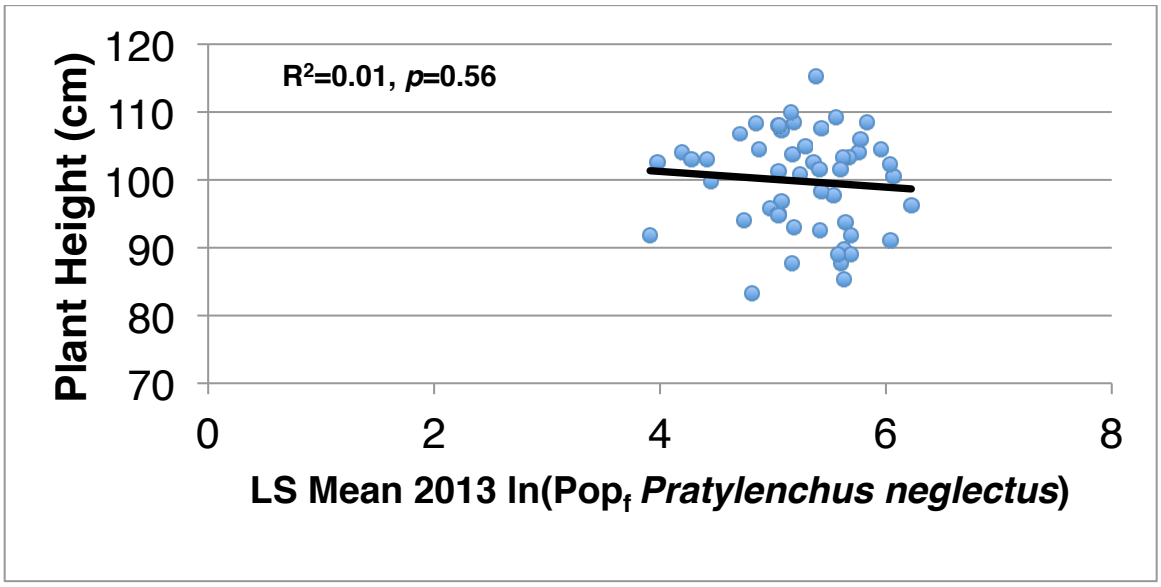
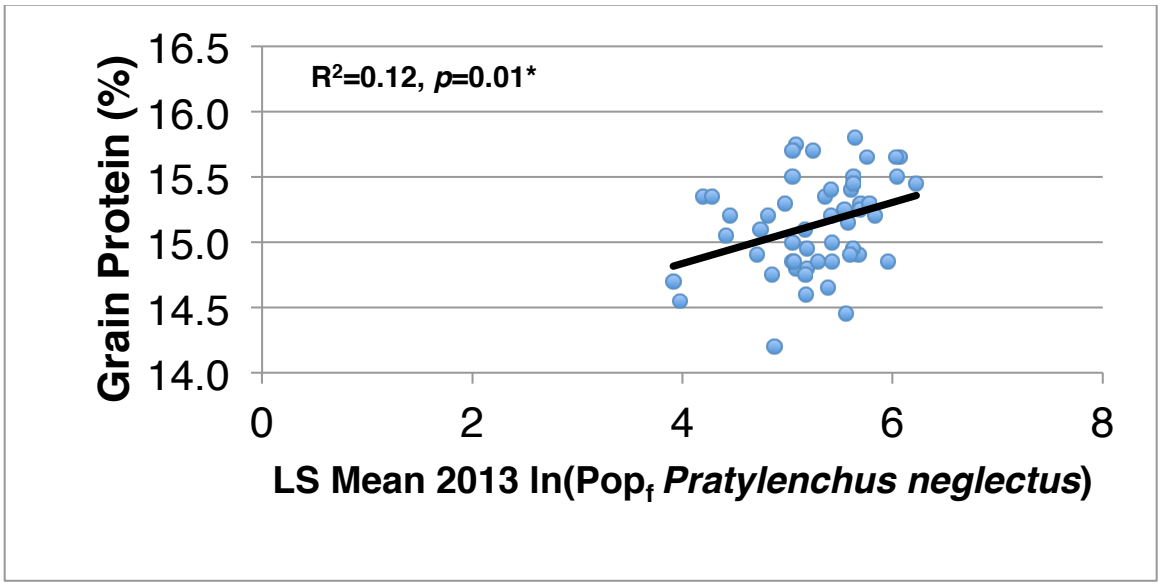


Figure 4 – Continued

(d)



(e)



Single-marker Analysis

Putative *P. neglectus* resistance markers IWA2314 and IWB35058 were

found to have statistically significant associations with grain yield, volume weight, heading date, and grain protein content (Table 5). Progeny lines with a MT08184/MT08185 (RLN-resistant) allele for IWA35058 had a higher estimated mean grain yield than progeny lines with the Persia 20 (RLN-susceptible) allele. For IWB35058, an RLN-resistant allele was associated with an estimated additive treatment effect (EATE) of 349 kg/ha compared to the RLN-susceptible allele (95% CI: 100 – 598 kg/ha greater). A similar trend was seen in the volume weight data for MT08184/MT08185 alleles compared to Persia 20 alleles at IWB35058 (EATE=0.9 kg/hL greater, 95% CI: 0.1 – 1.8 kg/hL greater). However, MT08184/MT08185 (RLN-susceptible) alleles at IWA2314 were associated with higher mean volume weight than the Persia 20 (RLN-resistant) alleles at IWA2314 (EATE=1.3 kg/hL greater, 95% CI: 0.2 – 2.5 kg/hL greater).

Table 5. *P. neglectus*-resistant alleles at SNP markers IWA2314 and IWB35058 showed statistically significant ($p < 0.05$) associations with grain yield, grain volume weight and grain protein content. Significance codes: '**' = 0.01, '*' = 0.05.

Marker ID	Yield *p* - value	Volume Weight *p* - value	Heading Date *p* - value	Plant Height *p* - value	Grain Protein *p* - value
IWA5081	0.16	0.35	0.41	0.17	0.85
IWA3128	0.17	0.07	0.85	0.35	0.48
IWA6721	0.22	0.07	0.17	0.71	0.85
IWA2607	0.62	0.82	0.81	0.43	0.88
IWA6246	0.79	0.57	0.65	0.14	0.93
IWA2314	0.23	0.03*	<.01**	0.74	0.99
IWA4716	0.73	0.95	0.09	0.82	0.34
IWB35058	<.01**	0.04*	0.37	0.18	<.01**

In addition, an RLN-susceptible allele at IWA2314 was associated with an estimated mean increase in heading date of 1.5 days compared to progeny

lines with the RLN-resistant allele (95% CI: 0.6 – 2.4 days later). For grain protein content, an RLN-susceptible genotype at marker IWA35058 had an EATE of +0.3% compared to lines with the RLN-resistant genotype (95% CI: 0.1 – 0.5% greater).

Discussion

Typically, breeding experiments conducted in the field are replicated at multiple geographic locations over a number of years, in order to gauge potential cultivars' yield prospects over a variety of environments and year-to-year weather conditions. The experiment described in this chapter was limited to one high-yield environment in a single year, in the absence of any significant pathogen pressure. Our scope of inference is therefore limited to the 2012-2013 growing season in Bozeman, MT.

The results obtained from the trial suggest that entries 145 and 84, the most resistant progeny lines in the 2013 *P. neglectus* screening, are capable of achieving a grain yield, volume weight and grain protein content equal or superior to at least one of the five check cultivars tested in the trial. Five additional lines identified as RLN-resistant in 2013 also attained mean yields, volume weights and grain protein contents that were competitive with the check varieties in the field evaluation. However, these seven lines all exhibited a height that is significantly taller than all of the recommended winter wheat cultivars that were

tested in the trial. Excessive plant height is an undesirable phenotype for winter wheat growers, not only because of the potential for yield loss due to lodging, but also because the excessive amount of straw produced by taller plants can hamper the efficiency of harvesting machinery. Further backcrosses to Yellowstone, or topcrosses to even shorter Montana winter wheat cultivars such as Bearpaw and Warhorse, would be expected to further reduce the height of these lines.

Single-marker analysis demonstrated that a Persia 20 (RLN-susceptible) allele at IWB35058 was associated with lower yield and volume weight. This is an encouraging result, as it may suggest that there is no agronomic penalty for a resistant genotype at this locus. A Persia 20 allele at marker IWA35058 was also associated with a higher mean protein content. This result is not surprising, as this particular marker genotype was also associated with lower mean yield and volume weight. Seed from the low-yielding resistant parent Persia 20, as well as the lowest yielding progeny lines, was extremely shriveled.

Conversely, a Persia 20 (RLN-resistant) allele at SNP marker IWA2314 was found to be associated with a lower mean volume weight. This result underscores the trade-offs inherent in marker-assisted selection versus phenotypic selection for quantitative traits. Marker genotypes and agronomic phenotypes must be analyzed in conjunction with one another, in order to arrive at the appropriate combination of resistance markers and agronomic phenotypes

that will satisfy the needs of growers. It is important to remember, however, that the QTL experiments were not able to confirm the validity of any of the putative resistance markers. Therefore any assertions about the effects of RLN resistance on agronomic phenotypes, whether they be positive or deleterious, are tenuous, and require verification.

Despite the fact that the results of this preliminary evaluation are merely suggestive and inconclusive at best, it did identify seven resistant lines with superior agronomic performance for the year tested. Furthermore, no evidence was found to indicate that increased RLN resistance need be associated with lower grain yield or poorer agronomic performance. The true value of the progeny lines will be established through replicated trials conducted in a variety of environments over the course of several years, as well as in the presence of *P. neglectus* pressure.

CHAPTER 5 - CONCLUSIONS

During the course of this study, over 1,700 plants have been carefully evaluated in three separate experiments for disease resistance, novel genetics and agronomic characteristics. Traditional plant breeding methods were employed in conjunction with the most current high-throughput genomic technologies, in the attempt to identify RLN-resistant phenotypes and genotypes that will satisfy the needs of Montana's wheat producers. Venues for our research ranged from the greenhouse, to the laboratory, to the field.

We thus conclude our description of the past years' proceedings with three main findings: First, phenotypic screens as applied were inadequate to consistently characterize wheat lines for RLN resistance. However, the more authoritative 2013 trial identified two backcross lines (84 and 145) with mean final *P. neglectus* populations that were significantly lower than both susceptible parent MT08184 and resistant parent Persia 20. Second, single-marker analysis of a panel of genome-wide SNPs revealed associations with RLN resistance for chromosomal regions 1AL, 1DS, 2BL, 4DL, 5BL, 5DL, 7AL and 7BL. Finally, no evidence was found from the agronomic data to indicate that increased RLN resistance need be associated with lower grain yield or poorer agronomic performance. Field trials identified seven RLN-resistant lines (77, 84, 119, 137, 145, 155 and 193) with competitive agronomic phenotypes for yield, volume weight and grain protein, although this result, like that of the QTL analysis, is

conditional based on the validity of RLN-resistant phenotypes identified in the 2013 screening assessment. These outcomes have important implications, not only for the development of RLN-resistant cultivars through the Montana State Winter Wheat Breeding Program, but also for the global effort in identifying and deploying genetics that confer resistance to *Pratylenchus*.

The 2012 phenotypic screening assessment had a high proportion of variance that was unexplained by the statistical model. This was likely due to heterozygosity present within BC_1F_3 lines, the long storage period and degradation of samples, and secondary disease presence. Furthermore, the low level of replication and low overall level of RLN reproduction made it difficult to efficiently differentiate wheat lines. For these reasons, the 2012 trial was unsatisfactory and should be considered preliminary data that was used to select potential resistant and susceptible entries for testing in subsequent trials. The 2013 trial utilized procedural adjustments that lowered the proportion of unexplained variance and considerably increased RLN reproduction, which allowed for statistical tests to effectively differentiate the resistant and susceptible controls, as well as distinguish a set of RLN-resistant lines. The 2013 trial should therefore be considered the authoritative phenotypic screening assessment described in the study. Verification of RLN-resistant phenotypes in subsequent trials should utilize the 2013 procedural adjustments.

The identification of entries 84 and 145 in our breeding population, lines

with superior resistance and agronomic phenotypes, marks a significant step toward the release of a *P. neglectus*-resistant winter wheat variety to Montana growers. These lines, along with entries 77, 119, 137, 155 and 193 have either already received further backcrosses to Yellowstone in previous crossing blocks, or will be included in future crossing blocks, to further improve their agronomic potential. Because the traditional resistance screening procedure we utilize requires four to five months to process, the application of phenotypic selection to identify resistant progeny after each backcross is too inefficient of a breeding strategy to develop RLN-resistant cultivars in a timely fashion. In this case, using molecular markers linked to QTL associated with resistance to *P. neglectus* to indirectly select resistant backcross progeny would be an ideal strategy. Yet, the incongruity between results of phenotypic screens in 2012 and 2013 makes the use of RLN resistance-associated SNPs identified in our QTL analysis an unreliable prospect at present.

Confirmation of these SNPs' association with the *P. neglectus*-resistant phenotype in future experiments will be necessary to confidently proceed with MAS of backcross progeny. Doubled haploid (DH) technology could then be employed to achieve instant homozygosity in selected backcross progeny lines. The use of DH in conjunction with MAS has been successfully implemented in European and Australian breeding programs (Tuvesson *et al.*, 2007; Mago *et al.*, 2011). A subsequent application of MAS would be required to select DH lines

with a resistant genotype for each RLN resistance-associated SNP. Finally, requisite field evaluations of agronomic performance and disease resistance (including resistance to *P. neglectus*) of selected lines, over multiple locations and years, could lead to the identification of lines suitable for release to Montana farmers. Obviously, the release of an RLN-resistant cultivar appropriate for Montana wheat production may be several years away; despite this, our efforts over the past several years have laid the foundation for future successes in resistance breeding for *Pratylenchus* by the Montana State Winter Wheat Breeding Program.

The identification of RLN resistance-associated SNPs mapped to chromosomes 5BL, 7AL and 7BL in our single marker analysis, is consistent with previous research showing these genomic regions are associated with resistance to *P. neglectus* (Williams *et al*., 2002; Mulki *et al*., 2013; Thompson, 2013). Pending validation in future experiments, putative QTL identified at chromosomal regions 1AL, 1DS, 2BL, 4DL and 5DL may represent areas harboring novel loci for resistance to *P. neglectus*. To develop an RLN resistance marker useful across wheat breeding programs, any QTL region associated with RLN resistance in our breeding population, if confirmed, should be fine-mapped to ensure marker linkage with the true resistance gene. In addition, QTL effects confirmed in the genetic background of our breeding population should be validated in an alternate genetic background in order to account for epistatic

effects.

The partial RLN resistance phenotype of resistant parent Persia 20, along with its undesirable tall height, might mean that resultant progeny lines will never exhibit the appropriate combination of RLN resistance and agronomic phenotypes that meet the criteria for cultivar release. Further backcrosses to Yellowstone, or topcrosses to even shorter Montana winter wheat varieties such as Bearpaw or Warhorse, may help selected backcross progeny lines obtain a height appropriate for Montana production. Additionally, subsequent studies have shown Persia 20 to be susceptible to *P. thornei* (Thompson *et al*., 2008 – 1), despite previous studies showing evidence to the contrary (Sheedy *et al*., 2007, 2008), and moderately susceptible to *P. neglectus* (Smiley *et al*., 2014). Persia 20's susceptibility to *P. thornei* is particularly problematic. Although no *P. thornei* was discovered in samples during a statewide survey of RLN populations conducted from 2006 to 2007 (Johnson *et al*., 2008), *P. thornei* is a major pest of wheat in the neighboring states of Utah, Idaho, Oregon and Washington, and RLN are readily transported from field to field on farm equipment, animals, shoes, and by wind (Johnson *et al*., 2008). The dual-resistant landrace AUS28451 has become the resistance source of choice for other breeding projects in the Pacific Northwest (Thompson, 2013; Smiley *et al*., 2014), and other dual-resistant accessions with even better agronomic characteristics than AUS28451 have been identified in recent studies (Thompson, 2013). Resistance traits from

Persia 20 may need to be pyramided with those from alternate sources such as AUS28451, in order to obtain a level of resistance acceptable for Montana growers.

Whether these seemingly contradictory results invalidate the data obtained in our proceedings, or suggest the existence of pathotypes within *P. thornei* and *P. neglectus*, remains unclear. Biological diversity among populations of *P. neglectus* has been observed on potato (Hafez *et al.*, 1999), although the potential impact of heterogeneity or pathotypes among *P. neglectus* on wheat has not yet been reported (Smiley and Nicol, 2009). All resistance assessments of sources of resistance to *Pratylenchus* species – including Persia 20 – should henceforth be conducted using inoculum obtained from geographically diverse locations. This would ensure that RLN resistance breeding efforts would result in cultivars with durable, sustainable resistance to both *P. neglectus* and *P. thornei*, should pathotypes and/or mixed populations of *Pratylenchus* become an issue in the future. In the meantime, however, an RLN-resistant cultivar developed from our breeding population may provide a stopgap relief measure for some Montana growers experiencing yield loss due to *P. neglectus*.

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APPENDIX A

INFORMATION FOR REPRODUCTION OF SNP MARKERS SIGNIFICANTLY ($P < 0.05$) ASSOCIATED WITH RESISTANCE TO *P. NEGLECTUS*

RLN Resistance SNP ID	Putative RLN resistance-associated SNPs and surrounding sequence as reported by Cavanagh *et al.* (2013) and Wang *et al.* (2014). SNPs are bracketed within the sequence. N = indeterminate base.	Chromosome
IWA2314	TTGCTGAATGAGCAAAATGCGAGATGCAGATTGAATGCATCAGAGCTGCGGAA CAAGATGCAGGCTGATATGTTCAGCTTTGCCGACAGGCTTGTATCGA[T/C]TGT CAAGGAGCACTGCAGGTGCTGCGCTAGGCGTGGACTAAAAAGATGCATCACA CTTGTTGAACAGGTGCGGCCAGTGAAGGTGTGCCAGGCATGTTTA	1A
IWA4716	TTCTTTTTTCAATGGAGAAGCTGCTGCAGGTGCTTGTGGATTACATATGATACT CCCACCAGTGCAAGTTGTCTGGAGGTTTTTGAAGTCAACAACGGAA[A/G]GTTT CTGCGCTCAGCAAAGTCAAATCCCCAGATACCATCATGCCAAAGAAATTGTTC ATAAGAAGTGCATATTTTCTTTCTATTTCTCCCCTTTTTTGTA	1D
IWA5081	CATGAATAAAGCTCTTGTACTTTGTGGTAAGTAGCTGAACAACACTCCTAGCTG ATTCAACGGTGTCGACCACCAGAACTTTGTCGTAGATGGTAGCAAG[A/G]GCCT CCCGTGCATCTTTAGCCTCCTTGGACAAGGGGGCTTTAGCTGGAGGAGGCTG TACCGCTGTCTTGGCTGTGCATTCATGCTCAGCTTCATTATAAC	2B
IWB35058	AAGGGCAAATATCCAGCCATAAGGACAAATAAGATTATGCCACAGGACCACAC ATCAG[A/C]AGCCATACCATCATAACCTTTATCAGCAAGCACCTCAGGTGCAAC NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNN	4D
IWA6721	CTAGGTGTGAAAATTTCAAGCTTGAAAGAAGTCACAAGGCGGCAGAGTTTGCA TCAAATAAATCTGCAGGAACAAGGCTTGATATTCGTTGCACCACATG[T/C]AAA ACGCCACTTGGATTACAGAAGGATGGCTTTCTTGTTTCATGTTCATTGGCATCG CCCTCAAAATTGTACTTGACATGTCTTTTAAGACATGGGCTAT	5B
IWA2607	GTATCCAAGTCAGTAGCACATACACCACGGTCGAATGTTGTGCGAACTTTTTT GGATATCCCATCTACCCCACCATGCATCTTTAAAGCTTTCAAGTCAT[A/G]CTTG CTTGTTATTGATGCCAGTTCTTCGGGATTGATGGAAAACCCAGCTTTGATTATA TAATCTGTTAGCTGGTACTCATTCTTCTTGGCGCCTTGAGCA	5D
IWA3128	TGCAAATGGAAATAAGAGTAGGAGTACAAACAGAAATGCGATCAGGAGTACAA CAGATGGAAATGCGAGGAAAAGTACAACCAGAAGTTCTGCAACTTTT[A/G]AAG ATATTTCGCCCCAGGCACTTATTGTTCCTTCAGATGGAGAAGTTTCAAAATCTG AAACAAACATTGTTGAATATACAAAAAAATATAAAGATACCGC	7A
IWA6246	GCCATTCGACAGGTTTGGACYAGAGTGTCTTGGGGGTTCTATTGTGCCTGAAG ATGGATGATGATGATGACGACGACGATGATGATGAATCTTGTATGCA[T/C]ATT GCAGATTCCATGTCTTGAATTTTCAATGGGAGCTACCCTTATCATTCTGTAGGT GATTGGATGGATGCAATATGACATTGTGTGGATTATTTTTTTG	7B