**Genetic structure, virulence and fungicide sensitivity of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada**

by

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## Abstract

The fungi *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) cause net form net blotch (NFNB) and spot form net blotch (SFNB) of barley, respectively. The genetic structure of a collection of 128 *Ptt* and 92 *Ptm* isolates, representing fungal populations from western Canada, was studied by simple sequence repeat (SSR) marker analysis. Thirteen SSR loci were examined and found to be polymorphic within both *Ptt* and *Ptm* populations. Significant genetic differentiation (PhiPT = 0.230, P = 0.001) was found among all populations. Isolates clustered in two distinct groups conforming to *Ptt* or *Ptm*, with no intermediate cluster. PCR analysis with mating type (MAT)-specific primers indicated that the MAT1 and MAT2 idiomorphs of *Ptt* and *Ptm* could be identified within the same field and on the same plants. There was no significant departure from the expected 1:1 MAT1/MAT2 ratio for either form. The virulence of a subset of 39 *Ptt* and 27 *Ptm* isolates was tested by inoculating these isolates onto barley differential hosts. Cluster analysis revealed 16 and 13 pathotype groups, respectively, among the *Ptt* and *Ptm* isolates. The barley differentials CI 5791 and CI 9820 were resistant to all *Ptt* isolates except one, while the differential CI 9214 was resistant to all *Ptm* except two. These differentials may prove useful in resistance breeding efforts, especially since some isolates were found to be highly virulent on barley cultivars previously classified as having good or very good NFNB and/or SFNB resistance. The propiconazole and pyraclostrobin sensitivity of a subset of 39 *Ptt* and 27 *Ptm* isolates also was evaluated against discriminatory doses of 5 mg propiconazole $L^{-1}$ and 0.15 mg pyraclostrobin $L^{-1}$ in microtiter plate bioassays. Two *Ptt* isolates appeared to be insensitive to propiconazole (growth inhibition < 30%), while one *Ptm* isolate was insensitive to pyraclostrobin and also exhibited decreased sensitivity to propiconazole. Populations of *Ptt* and *Ptm* from western Canada appear to be genetically and pathogenically diverse, and farmers

should avoid planting the same resistant barley cultivars in short rotation, as well as the excessive application of the same fungicidal modes of action.

## Preface

This dissertation is submitted by Alireza Akhavan in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Mr. Akhavan conducted all of the experiments and prepared the first draft of all the chapters. The chapters were then examined by Mr. Akhavan's supervisors, Dr. Strelkov and Dr. Turkington, who provided comments, suggestions and editorial revisions for each chapter, which were then incorporated or addressed as needed by Mr. Akhavan.

A version of Chapter 2 of this dissertation has been published as:

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Mr. Akhavan helped to conceptualize and design the study, conducted the experiments, collected and analyzed the data, and wrote the first draft of the manuscript. Drs. Strelkov and Turkington developed the initial research plan and assisted with manuscript editing and interpretation of the results. Dr. Berisso Kebede provided technical expertise with SSR capillary electrophoresis and contributed to manuscript editing. Drs. Andy Tekauz, H. Randy Kutcher, Kequan Xi, Krishan Kumar, Mr. James R. Tucker, and Ms. Coleen Kirkham were involved in the development of the initial research plan, and/or were responsible for surveying for net blotch in farmers' fields. They collected barley leaf samples with (likely) symptoms of net blotch, and sent them to Mr. Akhavan for further processing. Drs. Tekauz and Kutcher also provided editorial comments on the manuscript.

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The roles of Mr. Akhavan, Mr. Tucker, and Drs. Strelkov, Turkington, Tekauz, Xi and Kutcher were similar to those explained above for Chapter 2. In addition, Ms. Homa Askarian provided assistance in conducting the experiments. Mr. Tucker also contributed to manuscript editing.

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The roles of Mr. Akhavan, Mr. Tucker, Ms. Askarian and Drs. Strelkov, Turkington and Tekauz were similar to those explained above for Chapter 4. Dr. Swaroop W. Kher provided expertise with the statistical analyses and also contributed to manuscript editing. Dr. Legge was involved in development of the initial research plan.

Chapter 6 of this dissertation has been submitted as a manuscript for publication in a refereed journal (Akhavan A, Strelkov SE, Askarian H, Kher SW, Fraser M, Kutcher HR, Turkington TK.

Sensitivity of western Canadian *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* isolates to propiconazole and pyraclostrobin). It is currently under review. The roles of Mr. Akhavan, Drs. Strelkov, Turkington, Kher, Kutcher and Ms. Askarian were similar to those explained above for the previous Chapters. Ms. Michelle Fraser contributed to identifying the required analyses and also provided editorial comments on the manuscript.

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# Chapter 1 Introduction and literature review

## 1.1 General introduction

The necrotrophic fungus *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.) has two morphologically similar but genetically distinct forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), which cause the net form of net blotch (NFNB) and spot form of net blotch (SFNB), respectively, on barley (*Hordeum vulgare* L.). Both NFNB and SFNB are economically important foliar diseases throughout the major barley growing regions of the world, including western Canada (McDonald 1963; Smedegard-Petersen 1978; Tekauz 1990; Rau et al. 2005; Liu et al. 2011). Both *Ptt* and *Ptm* are stubble-borne pathogens, producing asexual conidia and sexual pseudothecia, which produce ascospores on overwintered infected crop debris (van den Berg and Rossnagel 1991; Liu et al. 2011). Losses caused by *Ptt* and *Ptm* are variable depending on environmental factors, stubble management practices, crop and cultivar rotations, and timely application of fungicides (van den Berg and Rossnagel 1990, 1991; McLean et al. 2009; Turkington et al. 2004, 2005, 2006, 2011, 2012, 2015). Yield losses of 10% to 40% were reported as typical in severe cases of NFNB, but the pathogen has the potential to cause total yield loss (Mathre 1997; Murray and Brennan 2010). Similarly, yield losses of 4% to 44% were reported for SFNB (Jayasena et al. 2007; McLean et al. 2016). Net blotch development also can lead to reductions in kernel weight, plumpness and test weight, resulting in grade losses, a loss of malt status, or reduced acceptability as high quality feed grain. Financial losses due to reduced grain quality would be over and above those due to yield losses. Crop rotation away from barley is an effective mitigation strategy; however, on-farm feed

requirements, local market factors, and malt/feed price differentials often lead producers to shorten rotation intervals. Efforts to manage net blotch have thus focused on the deployment of resistant cultivars and the application of fungicides. However, most current cultivars have only intermediate levels of resistance at best, while the excessive use of fungicides could result in fungicide insensitivity in pathogen populations. This chapter mainly reviews studies conducted to determine the genetic and pathogenic diversity of the net blotch pathogen, its ability to overcome host resistance, and its sensitivity to propiconazole and pyraclostrobin.

## 1.2 Net blotch of barley: symptoms and causes

Both net and spot form net blotch can cause symptoms on all above ground parts of the barley plant. The NFNB pathogen, *Ptt*, directly penetrates the leaves, initially causing small circular to elliptical lesions. As the pathogen continues to colonize the leaves in both horizontal and vertical directions, it causes the distinct net-like symptoms with horizontal and vertical reticulations (Fig. 1-1) (Steffenson 1997; Liu et al. 2011). Smedegard-Petersen (1971, cited in Smedegard-Petersen 1977) suggested the differentiation of the net blotch pathogen into two forms according to the different types of symptoms it can cause, with spot form net blotch associated with the development of dark brown circular to ovoid lesions encircled by a chlorotic zone (Fig. 1-2). The form name designation for *Ptm*, the causal agent of SFNB, has been widely adopted by the barley research community (Liu et al. 2011). Both forms of the net blotch pathogen are microscopically identical and can only be distinguished based on symptom development or molecular characteristics.

Drechsler (1923) first described the sexual stage of *P. teres*, which is an ascomycete heterothallic fungus that requires two opposite mating type individuals to form the sexual fruiting bodies, pseudothecia (McDonald 1963; Smedegard-Petersen 1978; Rau et al. 2005). The pseudothecia are typically 1-2 mm in diameter (Steffenson 1997), globose to elongate and covered by dark septate setae. Asci typically contain eight ascospores each, 10- 13 µm long and 20- 23 µm wide (van den Berg 1988; Steffenson 1997). The asexual stage of the net blotch pathogen, now known as *Dreschlera teres* (Sacc.) Shoem, produces phragmospore conidia 25- 300 µm long and 7- 11 µm wide. The conidia are straight and cylindrical, smooth with rounded ends and typically with 4 to 6 pseudo-septa in most cases. The conidia are produced on a single (or groups of 2-3) conidiophore(s) that are usually swollen at the base. Conidia are often sub-hyaline to yellowish-brown, while conidiophores are darker and look mid- to olive-brown. Under a dissecting microscope, the conidia and conidiophores are easily visible on barley leaves and stubble.

Rau et al. (2005) reported that *Ptt* mating-type genes and their predicted proteins are similar to those of other Pleosporales, such as *Pleospora* sp., *Cochliobolus* sp., *Alternaria alternata*, *Leptosphaeria maculans*, and *Parastagonospora nodorum*. DNA markers and mating-type gene sequences suggested that the two forms of the net blotch pathogen are closely related; however, several studies indicated that the two forms are grouped in two divergent phylogenetic groups (Campbell et al. 2002; Rau et al. 2003, 2007; Leisova et al. 2005a, b; Serenius et al. 2005; Bakonyi and Justesen 2007; Lehmensiek et al. 2010). Williams et al. (2001) described a PCR test that can differentiate the two forms of *P. teres*. MAT-specific single nucleotide polymorphism (SNP) primers were developed for a PCR-based analysis, which can also discriminate the two forms by the amplification of distinct PCR products. These primers include

*Ptt*MAT1F/R and *Ptt*MAT2F/R for NFNB MAT1 and MAT2 isolates, and *Ptm*MAT1F/R and *Ptm*MAT2F/R for SFNB MAT1 and MAT2 isolates, respectively (Lu et al. 2010). Other sets of primers that can distinguish between the two forms of the pathogen include the 14 STM markers reported by Bogacki et al. (2010), nine STM markers described by Keiper et al. (2008), and the form-specific PCR primers developed by Leisova et al. (2005a).

## 1.3 Distribution

The net and spot forms of net blotch are among the most prevalent foliar diseases of barley worldwide. In western Canada, *Ptt* seems the more prevalent form, representing 82% of the isolates in a collection that came mainly from the Prairie Provinces (Alberta, Saskatchewan and Manitoba) (Tekauz 1990). Nevertheless, *Ptm* was shown to be important locally in some areas of Saskatchewan, with SFNB being the most prevalent foliar disease of spring barley and more important economically than NFNB in those areas (Weller and Rossnagel 1988; Tekauz 1990; van den Berg and Rossnagel 1991). Tekauz (1990), van den Berg and Rossnagel (1991), and Liu and Friesen (2010) have noted that the occurrence of *Ptm* appears to be increasing over time. The increasing incidence of *Ptm* likely reflects its natural potential for dispersal, changes in the barley cultivars grown in the region, the resistance of barley cultivars to *Ptt* and *Ptm*, and (or) perhaps even climate change (Tekauz, 1990; Louw et al. 1996). In contrast, Serenius et al. (2005) reported only the net form of *P. teres* in two areas of Finland, although both forms had been equally common in an earlier survey (Makela 1972 cited in Serenius et al. 2007), indicating a drastic change in the prevalence of the forms over a 30-40 year period. It was reported that while both forms are economically important in the Czech Republic, spring and winter barley

crops were infected at relatively higher levels by *Ptt* and *Ptm*, respectively (Minarikova and Polisenska 1999; Leisova et al. 2005b).

## 1.4 Pathogen life cycle

Both the net and spot forms of the net blotch pathogen are stubble-borne (Liu et al. 2011), and there are no obvious differences in the life cycles of *Ptt* and *Ptm* on barley. It is assumed that mature pseudothecia release ascospores early in the growing season, which can be disseminated by wind currents and act as the primary inoculum (Piening 1961, 1968; Liu et al. 2011). In Canada, Piening (1961) noted the general occurrence of mature ascocarps of *P. teres* on barley straw from fields in the Calgary and Edmonton regions of Alberta. Similarly, Duczek et al. (1999) found pseudothecia of *P. teres* in two fields near Dafoe and Churchbridge, Saskatchewan. The *P. teres* teleomorph was also identified in Quebec in 1940 (Crowell 1941 cited in Piening 1961). In another study, Piening (1968) indicated that ascospores of *P. teres* were responsible for almost 50% of all net blotch lesions examined on volunteer barley plants in a field at the Lacombe Research Station, Alberta. Asexual tissues like conidia and mycelial pieces formed on stubble were also reported to serve as primary inoculum in some cases (Shipton et al. 1973; Jordan and Allen 1984; Louw et al. 1996; McLean et al. 2009). Following primary infection, the fungus colonizes the host tissue and produces large numbers of conidiophores and conidia, which are also wind-dispersed and serve as the secondary inoculum (Liu et al. 2011). The production of conidia continues throughout the entire growing season, resulting in multiple cycles of infection. Dissemination, germination and infection by conidia is impacted by many environmental factors, including relative humidity, leaf wetness and temperature (Jordan 1981; van den Berg and Rossnagel 1990, 1991). At the end of the growing season, the fungus

colonizes the senescent tissues and forms the sexual stage, which overwinters and initiates infection in the following year (McLean et al. 2009; Liu et al. 2011).

## 1.5 Host infection

The infection of the host starts with conidia or ascospores landing on barley leaves. Germination of both *Ptt* and *Ptm* conidia occurs within a few hours under suitable environmental conditions (Kenneth 1962; Shipton et al. 1973; van den Berg and Rossnagel 1990). In most cases, one of the two terminal cells forms the germ tube; however, other cells can potentially germinate as well (Van Caeseele and Grumbles 1979). Germ tubes can be of varying length and form appressoria to penetrate the cuticle directly (Van Caeseele and Grumbles 1979). Keon and Hargreaves (1983) observed that following the penetration of the outer epidermal cell wall by *Ptt*, the hypha forms a large intracellular primary vesicle. Subsequently, a secondary intracellular vesicle develops inside the epidermal cell, causing the physiological disruption of the infected and adjacent cells. The secondary vesicle then produces intracellular hyphae, which grow through the lower cells and later into the mesophyll. Hyphal growth is mainly intercellular thereafter (Keon and Hargreaves 1983). In contrast, Lightfoot and Able (2010) reported that while *Ptt* usually infects the host and feeds as a typical necrotroph, *Ptm* behaves partly like a biotroph during the initial infection stages, forming an intracellular vesicle within epidermal cells. Nonetheless, it enters a necrotrophic phase quickly, with intercellular growth in the mesophyll (Lightfoot and Able 2010). Host tissues in contact with or in a close proximity to the intercellular hyphae look water soaked within two days of inoculation in a susceptible interaction. These lesions enlarge and soon become necrotic. In more advanced stages of

infection, chlorotic zones appear around the necrotic tissues, possibly as a result of toxins/effectors secreted by the pathogen (Keon and Hargreaves 1983).

## 1.6 Heterothallism and sexual reproduction

*Pyrenophora teres* can reproduce sexually and asexually; therefore, the genetic structure of the pathogen population is dependent on the relative importance of these two types of reproduction in the fungal life cycle (Liu et al. 2011). Information regarding the extent of asexual versus sexual reproduction is required to understand the evolutionary potential of pathogen populations, which in turn will help to assess the durability of the resistance present in existing and future cultivars (Sommerhalder et al. 2006). The sexually reproduced component of a pathogen population can potentially lead to the rapid generation of new genotypes within the population, helping it to evolve in response to environmental changes (Peltonen et al. 1996). It may influence the speed at which fungicide sensitivity develops, the durability of host resistance (via the emergence of new pathotypes), and may facilitate dispersal among populations (Sommerhalder et al. 2006). Some studies showed that sexual reproduction is important in *P. teres* populations (Peever and Milgroom 1994; Jonsson et al. 2000; Rau et al. 2003). In contrast, it has been reported that reproduction within some *P. teres* populations is mainly asexual (Campbell et al. 2002; Lehmensiek et al. 2010).

The conclusions obtained from mating experiments and *in vitro* crosses may be skewed by isolate infertility or the growth medium used (Sommerhalder et al. 2006). Moreover, it takes 7 to 15 months (Smedegard-Petersen, 1978) to evaluate completely the sexual compatibility of *P. teres* isolates with standard mating type tester strains under laboratory conditions. PCR-based

mating-type studies can be useful to assess the potential of *P. teres* for sexual recombination (Rau et al. 2005). In all heterothallic ascomycetes including *P. teres*, sexual compatibility and recombination are controlled by a single regulatory mating-type (MAT) locus (Kronstad and Staben 1997; Turgeon 1998). The two alleles present in the mating type locus occupy the same chromosomal position and are referred to as idiomorphs, since they consist of two different sequences and encode dissimilar transcripts (Metzenberg and Glass 1990; Kronstad and Staben 1997; Rau et al. 2005). The existence of two fungal strains of different idiomorphs in close proximity to each other is a prerequisite for the development of the teleomorph stage, with each mating type detecting the other through pheromones produced by the opposite type (Kronstad and Staben 1997; Turgeon 1998; Rau et al. 2005; Sommerhalder et al. 2006; Vail and Banniza 2009).

A 1:1 mating type ratio is assumed when regular random mating occurs within populations (Milgroom 1996). The hypothesis that regional populations of ascomycete pathogens proceed to the teleomorph stage could be examined by studying the occurrence, distribution, and frequencies of the two mating types (Serenius et al. 2005; Sommerhalder et al. 2006). If the net blotch pathogen can produce pseudothecia, then ascospores could initiate disease in barley fields as the primary source of inoculum. For ascospores to be considered the major cause of primary infections, however, the two mating types must occur in statistically equal frequencies (Rau et al. 2005; Sommerhalder et al. 2006; Bogacki et al. 2010). Departures from statistically equal frequencies of the two mating types would be associated with a likely predominance of asexual reproduction via conidia (Sommerhalder et al. 2006). Several studies of *P. teres* have successfully used this approach to assess the relative importance of sexual versus asexual

reproduction in fungal populations (Rau et al. 2005; Bogacki et al. 2010; McLean et al. 2010; Liu et al. 2012). For example, Serenius et al. (2005) and Liu et al. (2012) demonstrated the existence of both mating types in a 1:1 ratio in Finland and North Dakota, respectively, and concluded that sexual recombination is common in those regions. A ratio of almost 1:1 between mating types also has been reported in *Ptm* and *Ptt* populations collected from barley fields in Australia (Bogacki et al. 2010; McLean et al. 2010), indicating that sexual recombination is possible.

For the identification of *P. teres* mating types without considering forms, two specific primer pairs were developed: the MAT1 forward and reverse primers that generate an approximately 1300 bp product, and the MAT2 forward and reverse primers that generate an approximately 1150 bp product (Rau et al. 2005). As explained earlier, Lu et al. (2010) also developed SNP primers, which can discriminate the two forms and the two mating types simultaneously by the amplification of distinct PCR products.

## 1.7 Sexual hybridization between the two forms

Studies using analysis of molecular variance (AMOVA) showed that the two *P. teres* forms are classified in two divergent genetic groups (Rau et al. 2003; Leisova-Svobodova et al. 2014). Ellwood et al. (2012) also estimated that *Ptt* and *Ptm* diverged about 519,000 years ago. Rau et al. (2003, 2007) demonstrated that *Ptt* and *Ptm* isolates cluster in two genetically distinct clades and did not find any genetically intermediate isolates in an analysis of amplified fragment length polymorphisms, suggesting that *Ptt* and *Ptm* are genetically isolated. Furthermore, Serenius et al. (2005) showed that meiosis did not occur properly in crosses between isolates of *Ptm* and *Ptt*,

resulting in the production of ascocarps that lacked asci or contained abnormal, non-culturable ascospores. This finding led Serenius et al. (2005) to conclude that successful sexual reproduction between the two forms of *P. teres* is very unlikely in nature. In contrast, Campbell et al. (1999) showed that *Ptt* and *Ptm* are capable of hybridization under laboratory conditions (Campbell et al. 1999) by conducting an efficient crossing of the two forms, and demonstrated that most of the sexual offspring caused intermediate symptoms on barley leaves. The resulting sexual offspring were later shown to be genetically stable (Campbell and Crous 2003). Moreover, Campbell et al. (2002) concluded, based on a random amplified polymorphic DNA analysis, that sexual recombination between *Ptt* and *Ptm* isolates might be occurring under field conditions. Similarly, Leisova et al. (2005b) and McLean et al. (2014) suggested that hybridization between the two forms was possible based on the presence of an intermediate clade consisting of haplotypes with shared markers. These apparently contradicting results might reflect differences in the isolates studied, population structure, and (or) environmental conditions in different geographical regions and cropping systems.

## 1.8 Genetic diversity

### 1.8.1 Amplified fragment length polymorphisms and simple sequence repeats

Among all polymerase chain reaction-based markers, amplified fragment length polymorphisms (AFLPs) and microsatellites or simple sequence repeats (SSRs) can be considered as efficient tools to uncover the genetic diversity at the population level (Garoia et al. 2007). AFLPs are generated by selective amplification of restriction fragments obtained from the digestion of total genomic DNA, providing a large number of markers (Vos et al. 1995). The resulting markers are dominant and bi-allelic. SSRs are co-dominant DNA markers consisting of short (1-6 base

pairs) tandem repeats that appear to be dispersed throughout the genome of eukaryotes (Jarne and Lagoda 1996). Variation in the number of repeat units can produce different alleles in a single locus, which can be distinguished by molecular size (Garoia et al. 2007). The use of either AFLP- or SSR-based methods to assess population genetic diversity has both advantages and drawbacks. In contrast with SSRs, which are generally specific loci markers, the AFLP technique does not require any prior knowledge of the genome and a large number of markers can be available quickly. The main disadvantages of AFLP markers for the study of a haploid ascomycete are PCR non-specificity and the need for purified, high molecular weight DNA (Vos et al. 1995; Garoia et al. 2007). Simple sequence repeats have proven to be a powerful tool for population genetic studies because of their variability, reproducibility, multiallelic nature, abundance, and wide genome coverage (Goldstein and Schlotterer 1999; Garoia et al. 2007; Singh et al. 2011; Leisova-Svobodova et al. 2014). The polymorphism in SSRs generally is believed to be the result of DNA polymerase slippage and unequal recombination (Li et al. 2002). Due to their high rate of mutation, SSRs are likely to have a role in genome evolution by generating and maintaining genetic variation (Tautz et al. 1986).

A common approach for the development of SSR markers involves constructing enriched genomic DNA libraries, followed by DNA sequencing (Edwards et al. 1996). This method is labor-intensive and time-consuming, which are significant drawbacks of SSR analysis when adequate primer sequences for the species of interest are unavailable (Kumar et al. 2009). In recent years, with the establishment of several DNA sequencing projects, an abundance of sequence data has become available, making it more economical and efficient to use computational tools to identify SSR loci (Robinson et al. 2004; Singh et al. 2011). These

sequences for expressed sequence tags (ESTs), genes and cDNA clones can be downloaded from different public domain databases and then analyzed with computer programs to identify SSRs, known as EST-SSRs or genic microsatellites (Singh et al. 2011). Consequently, flanking DNA sequences can be analyzed for the presence of suitable forward and reverse PCR primers to assay the SSR loci. Several computational programs are available for the discovery of SSRs within downloaded sequence data, as well as for the design of PCR primers for the amplification of specific loci (Robinson et al. 2004). The SSR sequences obtained through this *in silico* mining have the same quality and potential and are comparable with those identified from a genomic library (Singh et al. 2011).

### 1.8.2 Genetic diversity in *Pyrenophora teres*

The population structure in fungal pathogens is associated with factors such as mutation, migration, gene flow, and selection, as well as the relative importance of sexual versus asexual stages in the pathogen life cycle. Significant genetic diversity in pathogen populations helps them to evolve in response to environmental changes (Peltonen et al. 1996), and may impact the stability of host resistance. Information regarding the genetic structure of pathogen populations has, therefore, vital implications for plant breeding and fungicide screening programs (Leisova et al. 2005a). The genetic diversity and population structure of *P. teres* populations in several parts of the world have been explored by employing random amplified polymorphic DNA (RAPD) (Peever and Milgroom 1994; Jonsson et al. 2000; Campbell et al. 2002), AFLP (Rau et al. 2003; Leisova et al. 2005a and 2005b; Serenius et al. 2007), and SSR analysis (Bogacki et al. 2010; McLean et al. 2010; Liu et al. 2012; Leisova-Svobodova et al. 2014). In most of these investigations, a significant level of variability within the *P. teres* populations was demonstrated,

even over relatively small sampling regions (Campbell et al. 2002; Lehmensiek et al. 2010). Moreover, populations from fields close to each other in terms of geographical distance showed low levels of genetic differentiation compared with a higher level of genetic differentiation found in fields separated by greater distances (Serenius et al. 2007). This might be explained by the occurrence of higher levels of gene flow among geographically close populations. Peever and Milgroom (1994) investigated genetic diversity among five geographically distant *Ptt* populations collected from Canada, Germany and the United States, and calculated a high Nei's GST of 0.46, concluding that 46% of the total genetic variability was related to differentiation among these populations.

Many SSR primers were developed and utilized in *P. teres* population studies (e.g., Bogacki et al. 2010; McLean et al. 2010; Austin et al. 2011; Liu et al. 2012; Leisova-Svobodova et al. 2014) and genetic linkage mapping (Ellwood et al. 2010). For example, Keiper et al. (2007) identified 25 sequence tagged microsatellite sites (STMs) which revealed 26 polymorphic loci. In their study, 13 STMs amplified 14 polymorphic loci in both *P. teres* forms, seven STMs revealed polymorphism in *Ptm* only, and five were polymorphic in *Ptt* only. Bogacki et al. (2010) found that of the 20 SSR loci tested in their populations, 17 (85 %) were polymorphic within *Ptt* and *Ptm*. Ellwood et al. (2010) also reported 68 polymorphic SSRs, 20 from STMs markers (Keiper et al. 2007), 44 from the genome assembly sequence of *Ptt*, and four from ESTs. Later, Liu et al. (2012) developed and screened 40 pairs of SSR primers using the *Ptt* genome sequence assembly, and found that 13 primer pairs could amplify high quality and polymorphic markers from different loci across the genome. These SSR primer pairs produced an average of 6.5 alleles per locus for a *Ptt* collection from North Dakota (Liu et al. 2012). McLean et al. (2010)

also reported 15 sequence-tagged SSR primers, which produced two to five alleles per locus for a *Ptm* collection from Victoria, Australia.

Based on a UPGMA cluster analysis, McLean et al. (2010) reported that the population of *Ptm* from Victoria, Australia, was genetically diverse, with no clones, and given the equal presence of both mating types, likely underwent sexual recombination. Such high levels of genotypic diversity also were reported previously among *Ptt* and/or *Ptm* field populations in Italy (Rau et al. 2003), Finland (Serenius et al. 2005, 2007), the Czech Republic (Leisova et al. 2005b), Russia (Serenius et al. 2007), Sweden (Jonsson et al. 2000), and Australia (Serenius et al. 2007; Bogacki et al. 2010). The diversity values from the studies in which SSR markers were employed (Bogacki et al. 2010; Liu et al. 2012) seem to be higher in general compared with values obtained using either RAPD or AFLP marker data (Peever and Milgroom 1994; Campbell et al. 2002; Rau et al. 2003). The higher number of alleles per locus and the co-dominant nature of the SSR markers may help to explain this observation (Bogacki et al. 2010; Liu et al. 2012).

The findings of several studies revealed no or very low genetic differentiation among geographical locations within a region or country (Peever and Milgroom 1994; Serenius et al. 2007; Lehmensiek et al. 2010; Liu et al. 2012). McLean et al. (2010) did not find any correlations with geographic origin in a cluster analysis of 44 *Ptm* isolates collected from Australia. For an outcrossing heterothallic species like *P. teres*, which is at least partly a windborne pathogen, a high gene flow is expected between and within populations (Perdereau et al. 2014). The potential for long distance transport of spores is likely limited given previous reports (Deadman and Cooke 1989; Piening 1968), although dispersal of conidia of the closely related species *P. tritici-repentis* may occur over tens to hundreds of kilometers (Francl 1997).

Moreover, seed-borne infections (Hampton 1980; Piening 1968) and the movement of seed between different regions also may potentially increase the gene flow.

## 1.9 Pathogenic variation

Barley cultivars with resistance to *Ptt* and *Ptm* represent effective and practical tools for the management of net blotch disease in western Canada. van den Berg and Rossnagel (1991) showed that even a moderately susceptible cultivar can provide adequate control of SFNB compared with a susceptible cultivar. However, the emergence of new pathotypes in local *Ptt* and *Ptm* populations is a significant threat to the durability of the currently used sources of resistance. Up to date knowledge of the extent of variability in the virulence of local pathogen populations is essential for the successful deployment of any resistant cultivar (Jalli 2011; Liu et al. 2012).

McDonald and Buchannon (1962) first reported physiologic specialization in *P. teres* in Canada. Steffenson and Webster (1992) found 13 pathotypes among 91 *Ptt* isolates from California using 22 differential barley genotypes. Wu et al. (2003) identified 15 pathotypes in a collection of 23 *Ptt* isolates collected from 12 different barley-growing regions of the world. Gupta and Loughman (2001) identified eight virulence groups among 74 *Ptt* isolates collected from different barley fields in Western Australia. Bouajila et al. (2012) found 23 pathotypes among 85 isolates of *Ptt* collected in Tunisia. Cromey and Parkes (2003) characterised 11 pathotypes among 29 New Zealand isolates of *Ptt* using 31 barley differential genotypes. Liu et al. (2012) showed significant differences in the virulence of isolates representing a population of *Ptt* collected from North Dakota, and reported that the host genotypes CI 5791, 'Algerian', and

'Heartland' showed a high level of seedling resistance to all of the isolates tested. Douiyssi et al. (1998) showed that each of 15 *Ptt* isolates from Morocco was unique in its virulence spectrum on 38 barley genotypes. Wallwork et al. (2016) also showed that almost all the *Ptt* isolates they tested varied in their virulence on seedlings and adult plants of barley varieties grown in South Australia. As noted by Liu et al. (2012), the pathogenic diversity and the virulence spectra reported in different studies are dependent mainly on the pathogen population structure, as well as the host genotypes included in each differential set. The use of distinct differential sets makes it challenging to compare pathotype composition in different regions directly.

Khan (1982), Bockelman et al. (1983), Karki and Sharp (1986), Wu et al. (2003) and Grewal et al. (2008) reported the occurrence of various degrees of pathogenic diversity among *Ptm* isolates. In contrast, McLean et al. (2010) reported low pathogenic variation among isolates in a local *Ptm* population collected from Victoria, Australia. More recently, however, McLean et al. (2014) identified 33 pathotypes among 60 isolates collected across Australia using 16 barley differential genotypes. McLean et al. (2009, 2012, 2014) also suggested the inclusion of five additional barley genotypes (CI 3576, CI 9776, CI 9819, CI 9831 and 'Haruna Nijo') in their differential set to develop a standard set of *Ptm* differentials for international use, as these genotypes could effectively differentiate pathotypes among non-Australian *Ptm* isolates. Gupta et al. (2012) and McLean et al. (2010) also previously documented differences in the virulence of isolates from the eastern and western states of Australia. Working with *Ptm* populations collected from the Mediterranean region and the United States, Karki and Sharp (1986) found that almost 10 of the 20 differential genotypes they used showed differential reactions. Gupta et al. (2012) also reported a high pathogenic diversity in *Ptm* populations collected in Australia. Tekauz (1990),

and Liu and Friesen (2010) reported that the occurrence of SFNB in Canada and in the Northern Great Plains of the United States has been increasing. Neupane et al. (2015) suggested that this increase resulted from changes in the virulence of local pathogen populations. In the last comprehensive assessment of pathogenic diversity among *Ptt* and *Ptm* populations from western Canada, Tekauz (1990) classified 182 *Ptt* isolates into 10 main pathotype groups (A – J), with one to nine subgroups in each group (45 distinct virulence profiles in total). Tekauz (1990) also classified 42 *Ptm* isolates into seven main pathotype groups (P-V), with one to five subgroups in each group (20 distinct virulence profiles in total). Liu et al. (2012) suggested that there is a higher percentage of virulent *Ptt* isolates in western Canada (Tekauz 1990) than in North Dakota, based on a comparison of the virulence of isolates on three differentials that were used in both studies. Tekauz (1990) found that the *Ptt* pathotypes Cl and Gl were common in western Canada, with the three most virulent pathotypes identified primarily from Manitoba and Saskatchewan. In the case of *Ptm*, pathotypes T1 and S1 were the most common, with the most virulent isolate coming from Athabasca, Alberta (Tekauz 1990).

## 1.10 Management of net blotch

Crop rotation, fungicide application, and the deployment of resistant cultivars can be used to manage net blotch of barley in western Canada (van den Berg and Rossnagel 1991; Turkington et al. 2004, 2005, 2006, 2011, 2012, 2015). Many western Canadian farmers have adopted a canola-cereal-canola rotation, which is not sufficient to manage net blotch or other barley leaf spot diseases adequately (Mathre 1997; Tekauz 2003; Turkington et al. 2011, 2015). Therefore, there is an increased interest in the use of resistant cultivars and fungicides for net blotch control. Currently, many cultivars of barley, especially malting types, are susceptible to net blotch

(Alberta Government 2016a), and very few NFNB-resistant cultivars are available (Alberta Government 2016b). Consequently, while crop rotation and resistance are considered the most ecofriendly measures to control net blotch, many farmers routinely apply fungicides to manage barley foliar diseases (Turkington et al. 2011, 2015; Poole and Arnaudin 2014). Most fungicides are registered for use from stem elongation (Z31) to head emergence (Z39), but also are registered for tank mixing with herbicides and may be applied at earlier crop growth stages (Turkington et al. 2015; Alberta Government 2016a). When weather conditions are conducive to disease development and the barley cultivar is susceptible, many farmers will follow an application of fungicide that is tank-mixed with herbicide(s) with a second fungicide-only application between flag leaf and head emergence (Turkington et al. 2015; Alberta Government 2016a). The dual application of foliar fungicide provides significant suppression of both NFNB and SFNB (Sutton and Steele 1983; McLean et al. 2016). This section of the literature review focuses on host resistance and fungicide application in the management of net blotch.

### 1.10.1 Host resistance

Some barley cultivars resistant to one form of net blotch are not resistant to the other form (Bockelman et al. 1983), suggesting that there are distinct host–pathogen interactions associated with each form of *P. teres*, and that resistance breeding efforts should focus on each form individually (Liu et al. 2011). This is currently the case in western Canada, where candidate cultivars are evaluated for resistance to each of the two forms independently (Prairie Recommending Committee for Oat and Barley 2015). Many barley breeding lines and commercial cultivars have been reported to be sources of resistance against *Ptt* and *Ptm*, and the inheritance mechanisms and chromosomal locations of the related genes have been investigated.

In the case of NFNB, dominant and recessive major genes and quantitative trait loci (QTL), many of which mapped to the centromeric region of chromosome 6H, were shown to be involved in conferring resistance or susceptibility to various *Ptt* isolates (reviewed in Liu et al. 2011, 2015; Konig et al. 2013, 2014; Afanasenko et al. 2015; Richards et al. 2016). Therefore, it has been hypothesized that the pathogen avirulence genes or necrotrophic effectors interact with the host genes, resulting in either a compatible or incompatible interaction (Ho et al. 1996; Steffenson et al. 1996; Raman et al. 2003; Cakir et al. 2003; Ma et al. 2004; Manninen et al. 2006; Friesen et al. 2006; Abu Qamar et al. 2008; St. Pierre et al. 2010; Richards et al. 2016). Ho et al. (1996) investigated the genetics of resistance to *Ptt* isolates WRS102 and WRS858 and *Ptm* isolate WRS857, which have been routinely employed for screening for net blotch resistance in Canada. They found that in a cross between 'Leger' and CI 9831, resistance to WRS102 was controlled by three recessive genes, resistance to WRS858 was controlled by one recessive gene, and resistance to WRS857 by either one dominant gene or two complementary genes. O'Boyle et al. (2011) reported single dominant genes for *Ptt* resistance in the resistant spring barley lines CIho 2291 and CIho 5098 and the winter barley 'Nomini'. Grewal et al. (2012) identified a major NFNB seedling and adult-plant resistance QTL named *QRpt6*, accounting for 32–61% of phenotypic variation for NFNB resistance in a CDC Bold/TR251 population. This confirmed earlier reports of a major QTL or gene on chromosome 6H (Cakir et al. 2003; Friesen et al. 2006; Grewal et al. 2008; Manninen et al. 2000; Steffenson et al. 1996). Afanasenko et al. (2007) investigated the segregation of host resistance and pathogen avirulence, and concluded that one or two genes control both traits, indicating a specific gene-for-gene interaction. Friesen et al. (2006) reported dominant resistance to three different *Ptt* isolates, also mapped to chromosome

6H, in a finding that is also consistent with the hypothesis that the *Ptt*-barley pathosystem follows the classical gene-for-gene model. Differences in isolate virulence may be one reason that host genotypes classified as resistant in one study may perform poorly in another study (Baergen et al. 1993).

Weiland et al. (1999) studied the genetics of avirulence in *Ptt* employing a *P. teres* mapping population resulting from a cross between isolate 0-1 with high virulence and isolate 15A with low virulence. They mapped a gene (*AvrHar)* conferring low virulence on 'Harbin', a barley cultivar possessing a single major gene conferring resistance to *P. teres* (Mode and Schaller 1958). Using the same pathogen population, two other genes (*AvrPra1* and *AvrPra2*) were later revealed to confer avirulence in the *Ptt* parental isolate 0–1 on the barley cultivar 'Prato' (Lai et al. 2007). Beattie et al (2007) also identified another avirulence gene, *Avrheartland*, in a *Ptt* population produced from a cross between two Canadian isolates segregating for avirulence on the barley cultivar 'Heartland'. In the case of NFNB resistance, Douiyssi et al. (1998) showed that selection programs that utilize single isolates of *Ptt* in the greenhouse can select for lines that are susceptible in the field. Douiyssi et al. (1998) also found a significant cultivar by isolate interaction and showed that lines ND B112 and CI 12034 exhibited both resistant and susceptible reactions depending on the isolate examined. The authors concluded that there was a specific rather than a generalized NFNB resistance mechanism. Shjerve et al. (2014) identified four virulence QTL in the pathogen. Two QTL, designated *VR1* and *VR2*, were identified in *Ptt* isolate 6A which accounted for 35% and 20% of the disease reaction on 'Rika'. Another two QTL, *VK1* and *VK2*, were identified in isolate 15A, which accounted for 26% and 19% of the disease reaction on 'Kombar' (Shjerve et al. 2014). Recently, Richards et al. (2016) described

the high-resolution mapping of a dominant susceptibility locus related to *Ptt* isolates 6A and 15A. It was also reported that sensitivity to a proteinaceous necrotrophic effector produced by *Ptt* isolate 0-1, designated *Ptt*NE1, which interacts with a single dominant susceptibility gene on chromosome 6H in barley cv. Hector, was responsible for 31% of the variation observed in disease (Liu et al. 2015). This sensitivity mapped to the barley chromosome 6H. Therefore, it was proposed that the barley–*Ptt* pathosystem follows, partially, a necrotrophic effector-triggered susceptibility (NETS) model (Shjerve et al. 2014; Liu et al. 2015; Richards et al. 2016).

Many studies investigated the reactions of barley genotypes against *Ptt* and *Ptm* at the seedling stage (Douiyssi et al. 1998; Tekauz 1990). It has been shown that seedling reactions are often consistent with adult plant reactions (Buchannon and McDonald 1965; Cakir et al. 2003; Grewal et al. 2008, 2012). Grewal et al. (2008) and Cakir et al. (2003) compared seedling and adult plant resistance within the same populations, and showed that the resistance reaction conferred by the 6H locus was effective at both the seedling and adult plant stages, indicating that seedling resistance is also effective at the adult plant stage in these cases. However, in other studies, host reactions differed at the seedling and adult stages (Douiyssi et al. 1998; Grewal et al. 2012). It was also shown that resistance in some Manchurian cultivars increased with age (Khan and Boyd 1969). Tekauz (1986) also suggested that older plants were more resistant to NFNB and less resistant to SFNB based on the isolates they tested. Recently, Wallwork et al. (2016) also showed that the same or increased NFNB host resistance occurred when an isolate was tested on adult vs. seedling plants, while no evidence for increased susceptibility was found in older plants. Grewal et al. (2012) identified that NFNB seedling resistance QTL, *QRpt6*, spanning an approximately 1-cM interval on chromosome 6H, was effective against *Ptt* isolates WRS858 and

WRS1607. They then identified the same QTL for adult-plant resistance. Nonetheless, Grewal et al. (2012) also identified three adult-plant resistance QTLs from their field data, indicating that some QTLs are effective only at the adult-plant stage. Gupta et al. (2003) screened 69 barley genotypes as seedlings and in the field against nine *Ptt* isolates from Australia, and found that the resistance expressed at the seedling stage was expressed frequently in adult plants under field conditions.

In the case of SFNB, one or more seedling and/or adult plant resistance genes were identified on chromosomes 3H, 4H, 5H, 6H and 7H in different barley lines (Molnar et al. 2000; Friesen et al. 2006; Gupta et al. 2006; Manninen et al. 2006; Grewal et al. 2007, 2012). McLean et al. (2009, 2010) also characterized 13 lines with combinations of major and minor genes, located on a single or multiple chromosomes (Williams et al. 1999, 2003; McLean et al. 2012), which confer seedling, adult or all-stage resistance against SFNB. Neupane et al. (2015) evaluated the SFNB reactions of a barley core collection of more than 2,000 barley accessions against four geographically diverse *Ptm* isolates and reported that only 15 barley accessions were resistant against all tested isolates, and also identified isolate-specific susceptibility in several of the accessions. Grewal et al. (2012) reported that QTLs identified for SFNB isolate WRS857 in CDC Bold/TR251 were different from those found for the CDC Dolly/TR251 population, with the same isolate suggesting the important role of the susceptible parent in breeding for SFNB resistance. Wang et al. (2015) also employed association mapping to study QTL conferring SFNB resistance at both seedling and adult plant growth stages and found 29 significant QTLs, with 22 of these involved in resistance at both plant growth stages. Using the same set of barley genotypes employed by Neupane et al. (2015), Tamang et al. (2014) showed a total of 10, 8, 13,

and 10 quantitative trait loci (QTL) associated with SFNB resistance against four geographically diverse isolates collected from the United States, New Zealand, Australia, and Denmark, respectively.

### 1.10.2 Threats to durability of host resistance

As discussed earlier, both forms of the net blotch pathogen likely go through regular cycles of sexual and asexual reproduction in western Canada (Piening 1961, 1968; Duczek et al. 1999, van den Berg and Rossnagel 1991; Peever and Milgroom 1994) and have an outcrossing mating system. Given these characteristics, both *Ptt* and *Ptm* populations are likely to be able to adapt rapidly to major host resistance genes and fungicides with single modes of action (McDonald and Linde 2002). The evolution of virulent pathotypes of *P. teres* is a primary challenge for the deployment of durable net blotch resistant barley cultivars (Tekauz 1990; McLean et al. 2014), since a particular resistance source may be effective only toward a limited number of pathotypes (McLean et al. 2012). Hence, up-to-date knowledge of the pathogenic diversity and virulence spectrum of local pathogen populations is essential for successful incorporation of resistance genes into commercial cultivars. For example, the *Rpt6* resistance gene was effective against *Ptm* isolates from Finland, but not to *Ptm* isolates from Australia or the Czech Republic (Manninen et al. 2006). Khan (1982) reported that resistance against *Ptm* in the barley cultivar 'Beecher' was overcome by the development of new virulent pathotypes in Western Australia. Different pathotypes, also called virulence phenotypes, have been observed in populations of both *Ptm* and *Ptt*, globally and within particular regions (Liu et al. 2011). The breeding lines CI 4976 and CI 9820, reported to be resistant to *Ptt* under both greenhouse and field conditions in Canada and Minnesota (Buchannon and McDonald 1965; Keeling and Banttari 1975; Suganda

and Wilcoxson 1993), were susceptible in the field in Morocco (Douiyssi et al. 1998). Furthermore, many isolates from Syria and Tunisia were virulent on CI 9820 at the seedling stage (Bouajila et al. 2012). Jonsson et al. (1997) also showed that European barley cultivars were susceptible to all the Swedish isolates tested, while some were resistant to the Canadian isolate WRS 1607. This underscores the importance of local pathotype composition in relation to the effectiveness of resistance sources, and further suggests that variability and shifts in the virulence profiles of local pathogen populations can play a role in overcoming host resistance.

### 1.10.3 Chemical control

The nature and concentration of a fungicide, the form of the pathogen and epidemiological factors can influence the effectiveness of chemical (fungicidal) disease control strategies. For instance, benomyl, sulphur and triadimephon were found to have either stimulating or inhibitory effects on *P. teres*, depending on the pathogen strain and treatment conditions (Toubia-Rahme et al. 1995). Most of the fungicides used for net blotch management in barley have the same active ingredient or similar modes of action. Therefore, there is a risk of the development of fungicide resistance in *P. teres*, in particular when and where producers apply fungicides routinely in short rotations. Triazoles or demethylation inhibitors (DMIs) and strobilurins or quinone outside inhibitors (QoIs) are the two main fungicide groups registered for the management of net blotch of barley and other cereal leaf diseases in western Canada (Alberta Government, 2016b).

### 1.10.4 Propiconazole

Propiconazole is a triazole or DMI that inhibits 14α–sterol demethylase, an enzyme that synthesizes ergosterol, which is essential for cell wall development (Dahl et al. 1987; reviewed

in Parker et al. 2014). In western Canada, propiconazole has been used on various crops, including barley, since the early 1990s as the foliar fungicide Tilt (propiconazole, 250 g L-1; Syngenta Canada Inc.) (Thomas 1997; Xue et al. 1994). Campbell and Crous (2002) found that isolates of *Ptm* were less sensitive to propiconazole than isolates of *Ptt*, and suggested that the former isolates may have developed greater insensitivity as a result of different evolutionary paths. Scott et al. (1992) also showed that two applications of propiconazole were required to control SFNB reliably, while only one application was sufficient for NFNB control. While DMIs are highly effective and have broad-spectrum activity (Parker et al. 2014), their overuse has selected for insensitivity in cereal pathogens such as *Ptt* (Mair et al. 2016), *Blumeria graminis* f. sp. *hordei* (Delye et al. 1998); *Mycosphaerella graminicola* (Leroux et al. 2007; Bean et al. 2009; Cools and Fraaije 2012) and *Fusarium graminearum* (Talas and McDonald 2015). Insensitivity to fungicides usually results from point mutations in the gene(s) encoding the protein targeted by each compound. Alterations in the *CYP51* gene are one of the major mechanisms resulting in reduced sensitivity towards DMIs (reviewed in Parker et al. 2014). Mair et al. (2016) reported the occurrence of insensitivity among *Ptt* isolates collected from Australia against multiple DMI compounds. This insensitivity was correlated with both a mutation in the *Cyp51A* gene and overexpression of both the *Cyp51A* and *Cyp51B* genes, which are paralogs of the *Cyp51* gene. Rallos and Baudoin (2016) also reported that *CYP51* over-expression and target-site mutation contributed to insensitivity in *Erysiphe necator*.

### 1.10.5 Pyraclostrobin

Pyraclostrobin is a strobilurin or QoI fungicide that has been used extensively in western Canada since the early 2000s, including in the management of net blotch of barley (BASF 2003;

Government of Canada: Health Canada Pest Management Regulatory Agency 2011). It is the active ingredient in the commercial formulation Headline 250 EC (BASF Canada, Mississauga, ON, Canada). Marzani (2011) investigated the efficiency of several QoI fungicides on *Ptt* isolates and reported that pyraclostrobin was the most active, based on growth inhibition. Strobilurins have a single site of activity and function by preventing mitochondrial respiration in fungi, binding to the Qo site of cytochrome *b* and interfering with electron transfer in the III complex (Sauter et al. 1999; Bartlett et al. 2002; Grasso et al. 2006). This halts the energy-generating reactions in sensitive fungi, as adenosine triphosphate (ATP) is not produced (Bartlett et al. 2002). The Fungicide Resistance Action Committee (FRAC 2016) labelled strobilurins as compounds at a high risk for fungicide insensitivity because of their high site-specificity, particularly if applied excessively. In western Canada, research into pyraclostrobin insensitivity in *Ascochyta rabiei* on chickpea and *Mycosphaerella pinodes* on field pea revealed isolates with qualitative insensitivity to this product (Bowness et al. 2016; Chang et al. 2007; Gossen and Anderson 2004; Thaher 2011; Wise et al. 2008, 2009). By contrast, Fraser et al. (2016) screened a large collection of *Leptosphaeria maculans* (blackleg of canola) isolates from Alberta and did not find any insensitivity in the pathogen population.

A G143A mutation in cytochrome *b* results in qualitative insensitivity to strobilurins, while a F129L mutation results in moderate insensitivity (Gisi et al. 2000; Sierotzki et al. 2007). In *P. teres*, only the F129L mutation has been identified (Semar et al. 2007; Sierotzki et al. 2007; Marzani et al. 2013). Under *in vitro* conditions, some strobilurin-sensitive fungal pathogens exhibit apparent insensitivity to these compounds (Joseph-Horne and Hollomon 2000). This results from the activity of an alternative respiratory pathway, which relies on alternative oxidase

1 (AOX) and allows cytochrome bc1 to be bypassed (Ziogas et al. 1997; Olaya and Koller 1999; Vincelli and Dixon 2002; Inoue et al. 2012). The AOX pathway is active only under *in vitro* conditions, since under field conditions it is blocked by flavonoid compounds produced by the host (Avila-Adame et al. 2003; Wise et al. 2008; Inoue et al. 2012). Salicylhydroxamic acid (SHAM) blocks alternative respiration, and therefore is often included in fungicide sensitivity studies (Miguez et al. 2003; Wood and Hollomon 2003; Wise et al. 2008; 2009). Marzani (2011) identified insensitivity to pyraclostrobin in *Ptt* isolates from the United Kingdom. However, Marzani et al. (2013) did not identify any qualitative or extreme insensitivity among the *Ptt* isolates evaluated. They found that the efficacy of QoI fungicides varied amongst a population of isolates with the F129L mutation, suggesting that some QoIs were compromised by the F129L mutation to some degree.

### 1.10.6 Threats to chemical control strategies

The routine application of site-specific fungicides on genetically diverse populations of *Ptt* and *Ptm* poses a high risk for resistance development, particularly since insensitivity to propiconazole was reported to have a high heritability rate (Talas and McDonald 2015), and there was no fitness penalty associated with increased strobilurin insensitivity in *Ptt* (Marzani 2011). Campbell and Crous (2002) also suggested that sexual recombination could increase the rate of resistance development in *Ptm* isolates toward DMI fungicides in South Africa. Insensitive isolates may multiply rapidly through asexual reproduction. In Europe, the frequency of *M. graminicola* haplotypes with resistance to QoI fungicides (due to the G143A mutation) increased quickly as a result of strong fungicide selection pressure, and the pathogen then spread in a west-to-east direction via wind dispersal of ascospores (Torriani et al. 2009). Recently,

Rehfus et al. (2016) reported mutations in the succinate dehydrogenase complex (*SdhB*, *SdhC*, and *SdhD*), which are associated with insensitivity in *Ptt* against modern generation fungicides of the succinate dehydrogenase inhibitor (SDHI) group.

## 1.11 Objectives of the research and null hypothesis

The main aim of this research was to characterize *Ptt* and *Ptm* populations from western Canada, in order to understand better the risks they pose to barley production in this region. The occurrence and frequency of *P. teres* mating types has not been studied in Canada. The genetic structure of the *Ptt* and *Ptm* populations is unknown, and the only information available on pathogenic diversity comes from a study conducted with isolates collected in 1985 (Tekauz 1990). Furthermore, there are no published reports on the propiconazole and pyraclostrobin sensitivity of *Ptt* and *Ptm* populations from Alberta, Saskatchewan and Manitoba.

Therefore, the specific objectives of my Ph.D. project were to: (1) assess the occurrence and frequency of pathogen mating types in western Canada; (2) evaluate the genetic structure of *Ptt* and *Ptm* populations from this region; (3) evaluate the pathotype composition and determine if there have been any changes over the past three decades; (4) assess the risk for resistance breakdown in commercial barley cultivars and breeding lines, and determine whether significant genotype × isolate interactions exist; and (5) evaluate the propiconazole and pyraclostrobin sensitivity of a set of representative *Ptt* and *Ptm* isolates.

The corresponding null hypotheses were that: (1) there is a consistent 1:1 ratio of the two mating-type genes for both pathogen forms; (2) there is no significant genetic diversity within and among the western Canadian populations of *Ptt* and/or *Ptm*; (3) there is no pathogenic

variation in western Canadian populations of these pathogens, and there have been no changes in their pathotype composition over the past three decades; (4) there is no risk for resistance breakdown in commercial cultivars and breeding lines previously identified as resistant to NFNB and/or SFNB, and there is no significant genotype × isolate interactions between barley genotypes and isolates of *Ptt* and *Ptm*; and (5) there is a lack of qualitative insensitivity in western Canadian *Ptt* and *Ptm* isolates to propiconazole and pyraclostrobin.

**Figure 1-1.** Symptoms of the net form of net blotch of barley. The bottom panel illustrates the increasing severity of foliar symptoms from left to right. (Photo credit: Alireza Akhavan)

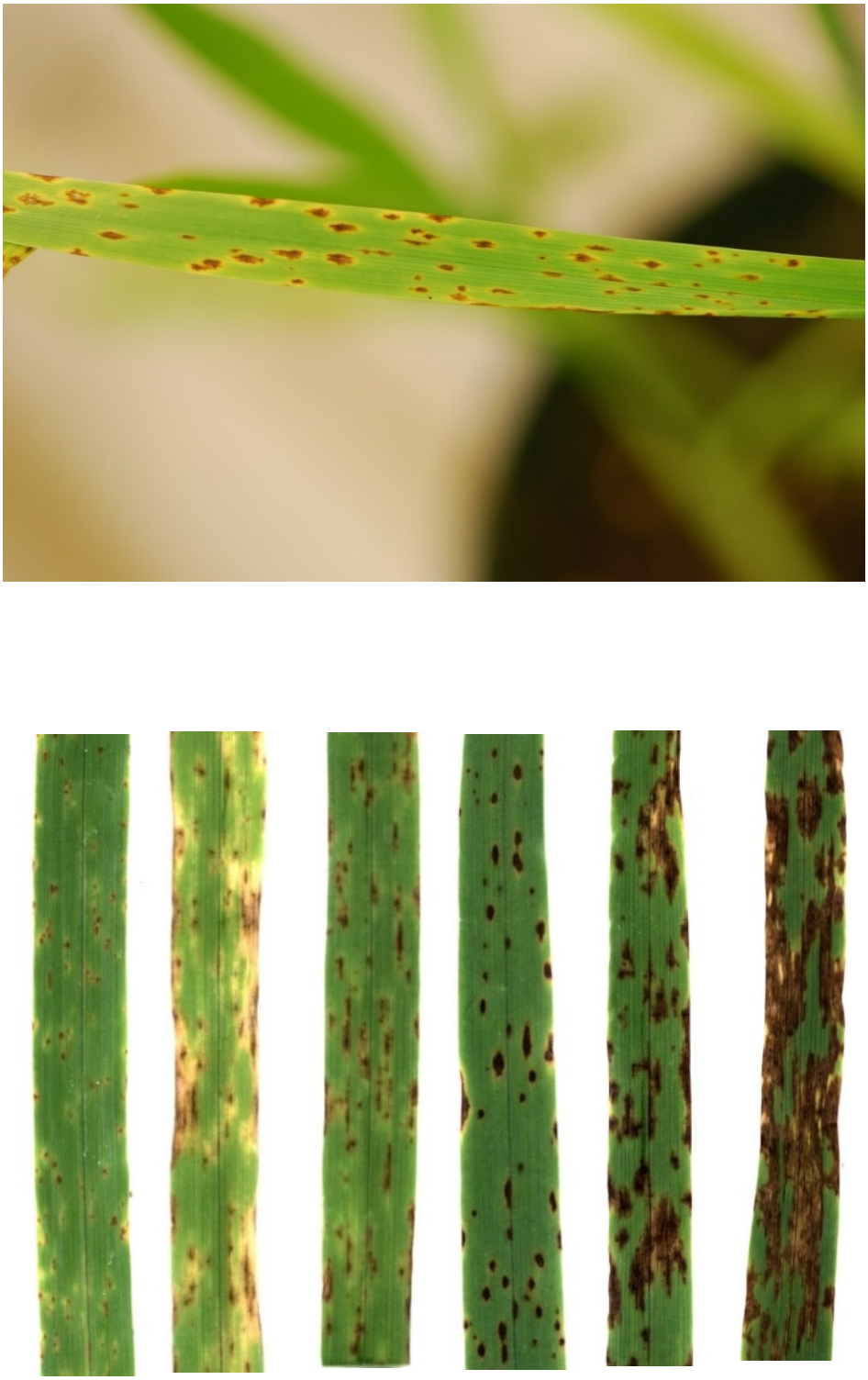

**Figure 2-2.** Symptoms of the spot form of net blotch of barley. The bottom panel illustrates the increasing severity of foliar symptoms from left to right. (Photo credit: Alireza Akhavan)

## 1.12 References


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# Chapter 2 Prevalence of mating type idiomorphs in *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada[1]

## 2.1 Introduction

The heterothallic fungus *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.) has two morphologically similar but genetically distinct forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), which cause the net form of net blotch (NFNB) and spot form of net blotch (SFNB), respectively, on barley (*Hordeum vulgare* L.). Both NFNB and SFNB are economically important foliar diseases throughout the major barley growing regions of the world, including western Canada (McDonald 1963; Smedegard-Petersen 1978; Tekauz 1990; Rau et al. 2005; Liu et al. 2011). Both forms of net blotch are stubble-borne diseases, producing asexual conidia and sexual pseudothecia, which produce ascospores on overwintered infected crop debris (van den Berg and Rossnagel 1991; Liu et al. 2011). Yield losses of 10% to 40% were reported as typical in severe cases of NFNB, but the pathogen has the potential to cause total yield loss (Mathre 1997; Murray and Brennan 2010). Similarly, yield losses of up to 44% were reported for SFNB (Jayasena et al. 2007). Crop rotation, fungicide application, and the use of resistant cultivars are all effective in managing net blotch and are components of an integrated management approach in barley (Tekauz 1990; Turkington et al. 2011).

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The last major study of variation in the populations of *P. teres* from western Canada was conducted in the 1980s, and revealed that *Ptt* was the dominant form of the fungus, representing 82% of the isolates in a collection that came mainly from the prairie provinces (Alberta, Saskatchewan and Manitoba) (Tekauz 1990). Nevertheless, *Ptm* was shown to be important locally in some areas of Saskatchewan, with SFNB being the most prevalent foliar disease of spring barley and more important economically than NFNB in those areas (Weller and Rossnagel 1988; Tekauz 1990; van den Berg and Rossnagel 1991).

*Pyrenophora teres* can reproduce sexually and asexually; therefore, the genetic structure of the pathogen population is dependent on the relative importance of these two types of reproduction in the fungal life-cycle (Liu et al. 2011). Some studies have shown that sexual reproduction is important in *P. teres* populations (Peever and Milgroom 1994; Jonsson et al. 2000; Rau et al. 2003). In contrast, it also has been reported that reproduction within some *P. teres* populations is mainly asexual (Campbell et al. 2002; Lehmensiek et al. 2010). Information regarding the extent of asexual versus sexual reproduction in the net blotch pathogen is required to understand its evolutionary potential, which in turn will help to assess the durability of the resistance present in existing and future cultivars (Sommerhalder et al. 2006).

In Canada, Piening (1961) noted the general occurrence of mature ascocarps of *P. teres* on barley straw from many fields in the Calgary and Edmonton regions of Alberta. Similarly, Duczek et al. (1999) found pseudothecia of *P. teres* in two fields near Dafoe and Churchbridge, Saskatchewan. The *P. teres* teleomorph also was identified in Quebec in 1940 (Crowell 1941 cited in Piening 1961). In another study, Piening (1968) indicated that ascospores of *P. teres* were responsible for almost 50 % of all net blotch lesions examined on volunteer barley plants in a field at the Lacombe Research Station in Alberta.

Polymerase chain reaction (PCR)-based mating-type studies can be useful to evaluate the potential of *P. teres* for sexual recombination (Rau et al. 2005). In all heterothallic ascomycetes including *P. teres*, sexual compatibility and recombination are controlled by a single regulatory mating-type (MAT) locus (Kronstad and Staben 1997; Turgeon 1998). The two alleles present in the mating type locus occupy the same chromosomal position and are referred to as idiomorphs, since they consist of two different sequences and encode dissimilar transcripts (Metzenberg and Glass 1990; Kronstad and Staben 1997; Rau et al. 2005). The existence of two fungal strains of different idiomorphs in close proximity to each other is a prerequisite for the development of the teleomorph stage, with each mating type detecting the other through pheromones produced by the opposite type (Kronstad and Staben 1997; Turgeon 1998; Rau et al. 2005; Sommerhalder et al. 2006; Vail and Banniza 2009).

A 1:1 mating type ratio is assumed when regular random mating occurs within populations (Milgroom 1996). The hypothesis that regional populations of ascomycete pathogens proceed to the teleomorph stage could be examined by studying the occurrence, distribution, and frequencies of the two pathogen mating types (Serenius et al. 2005; Sommerhalder et al. 2006). If the net blotch pathogen can produce pseudothecia, then ascospores could initiate disease in barley fields as the primary source of inoculum. For ascospores to be considered the major cause of primary infections, however, the two mating types must occur in statistically equal frequencies (Rau et al. 2005; Sommerhalder et al. 2006; Bogacki et al. 2010). Departures from statistically equal frequencies of the two mating types would be associated with a likely predominance of asexual reproduction via conidia (Sommerhalder et al. 2006). Several studies of *P. teres* have successfully used this approach to assess the relative importance of sexual versus

asexual reproduction in fungal populations (Rau et al. 2005; Bogacki et al. 2010; McLean et al. 2010; Liu et al. 2012).

Williams et al. (2001) described a PCR-based test that can differentiate the two forms of *P. teres*. For the identification of *P. teres* mating types without considering forms, two specific primer pairs were developed: MAT1 forward and reverse primers that generate an approximately 1300 bp product, and MAT2 forward and reverse primers that generate an approximately 1150 bp product (Rau et al. 2005). More recently, MAT-specific single nucleotide polymorphism (SNP) primers were developed for a PCR-based analysis, which can also discriminate the two forms by the amplification of distinct PCR products. These primers include *Ptt*MAT1F/R (1143 bp) and *Ptt*MAT2F/R (1421 bp) for NFNB MAT1 and MAT2 isolates, and *Ptm*MAT1F/R (194 bp) and *Ptm*MAT2F/R (939 bp) for SFNB MAT1 and MAT2 isolates, respectively (Lu et al. 2010).

Inconsistent results have been reported with respect to the frequencies of *P. teres* mating types in pathogen populations from different parts of the world (Liu et al. 2011). Rau et al. (2005) found that the two mating type genes occurred in equal frequencies in *P. teres* populations of both forms collected from the island of Sardinia, Italy, and concluded that sexual reproduction was the main source of primary inoculum. In contrast, Lehmensiek et al. (2010) observed a high level of genetic relatedness within *Ptt* and *Ptm* populations collected from the south-western Cape in South Africa and from across Australia, and suggested that asexual reproduction is predominant for both forms of the pathogen in those regions. The occurrence and frequencies of mating types of *P. teres* have not been studied in Canada. Therefore, this study was conducted to test the hypothesis that net blotch pathogen populations from the Canadian Prairies are reproducing mainly by sexual reproduction.

## 2.2 Materials and methods

### 2.2.1 Isolate collection

A total of 124 barley fields were sampled in Alberta, Saskatchewan and Manitoba from 2009 to 2011. Leaves with symptoms of NFNB and/or SFNB were collected, placed in paper envelopes, air-dried at room temperature, and stored at 4°C. Leaf sections (about 10 mm × 5 mm) were surface-sterilized in 50% ethanol for 15 s, and in 2% sodium hypochlorite for 30 s, then rinsed with sterile water and placed on moistened filter paper in 9 cm-diameter plastic Petri dishes (Tekauz 1990). Dishes were put into an incubator at 20 ± 0.5° C with a 12 h photoperiod under fluorescent and near-ultraviolet light at 368 nm to induce pathogen sporulation. After 3-5 days, single conidia of *P. teres* were transferred onto 10% V-8 juice agar (V-8 juice, 100 mL; $CaCO_3$ 3 g; Difco agar 20 g; distilled water 900 mL) supplemented with 50 mg $L^{-1}$ kanamycin, and incubated as above to produce new colonies (Tekauz 1990). Single-spore isolations were made from at least one leaf showing symptoms of NFNB and/or SFNB from each of the 124 fields. For collections in 2011, when applicable, two isolations were made from each infected plant, with an isolate obtained from an upper leaf (either the flag, penultimate, or antepenultimate leaf) and another from the lower canopy. In some cases, single-spore isolations were made from different lesions on the same leaf or from different areas on a single lesion. The resulting isolates were initially identified as *P. teres* based on the morphology of the conidia and conidiophores (McLean et al. 2009; Liu et al. 2011). Spore suspensions of single-spore isolates were prepared in 25% sterile glycerol, and placed in liquid nitrogen or at -80°C in an ultra-low temperature freezer for storage.

### 2.2.2 Genomic DNA extraction

Isolates chosen for genomic DNA extraction were removed from liquid nitrogen or a -80ºC freezer, and grown on fresh 10% V-8 juice agar. Mycelia were then transferred from 14 day old cultures into fresh potato dextrose broth containing 50 mg $L^{-1}$ kanamycin and 10 mg $L^{-1}$ streptomycin. The cultures were incubated for 10 days on a shaker rotating at 100 rpm at room temperature. Mycelia were harvested by transferring the cultures to plastic centrifuge tubes (50 mL), followed by centrifugation at 4000 rpm (Heraeus Megafuge 40R, Thermo Scientific Inc.) for 5 min at 4°C. The supernatant was discarded and mycelia were washed three times by filling the tubes with distilled water, shaking thoroughly and centrifuging at 4000 rpm for 3 min at 4°C. The mycelial pellets were lyophilized and kept at -20°C (or -80°C for long term storage) (Serenius et al. 2005). DNA was extracted from 20–30 mg of the lyophilized mycelium with a Wizard® Genomic DNA Extraction Kit (Promega Corp, Madison, WI) following the manufacturer's instructions. This was followed by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) (Abboukhaddour et al. 2011). To verify the quality and quantity of the extracted DNA, all samples were analysed with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The extracted DNA was then diluted to a final concentration of 10 ng $\mu L^{-1}$ prior to PCR analysis.

### 2.2.3 Species-, form-, mating type-, and mating type form-specific PCR analysis

Species-specific PCR analysis was performed in a reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 1 U Taq polymerase, 10 pmol of each of the OPF01F900 and OPF01R900 primers (Table 2-1), and 20 ng template DNA (Williams et al. 2001). Reaction conditions consisted of an initial denaturation step at 94˚C for 60 s, followed by 35 cycles of denaturation at 94˚C, annealing at 57˚C and extension at 72˚C

each for 30 s, and a final extension at 72 ˚C for 7 min. DNA extracted from an isolate of *Pyrenophora tritici-repentis* was included as a negative control. Amplicons were resolved on a 1.5% agarose gel and visualized with SYBR Safe (Invitrogen, Carlsbad, CA) stain.

PCR amplification with form-specific primers to differentiate the two forms of *P. teres* as either *Ptt* or *Ptm* was optimized as a duplex PCR protocol. The reaction mixture was the same as for the species-specific PCR analysis, except that 10 pmol of each of the form-specific *PTT*-F, *PTT*-R, *PTM*-F, and *PTM*-R primers (Table 2-1) was included (Williams et al. 2001). Reaction conditions consisted of an initial denaturation step at 94˚C for 60 s, followed by 35 cycles at 94˚C for 30 s, 53˚C for 30 s and 72˚C for 30 s, with a final extension of 72 ˚C for 7 min. PCR products were resolved and visualized as above.

Mating type-specific PCR analysis was optimized and conducted for all *P. teres* isolates as a duplex reaction, as per Rau et al. (2005), to determine mating type (MAT-1 or MAT-2) regardless of form. The reaction mixture was as described for the species- and form-specific PCR assays, except that the mating type-specific MAT-1 forward, MAT-1 reverse, MAT-2 forward, and MAT-2 reverse primers (Table 2-1) were substituted for the other primers. Reaction conditions consisted of an initial denaturation step at 94˚C for 60 s, followed by 35 cycles at 94˚C for 30 s, 55˚C for 30 s, 72˚C for 30 s, and a final extension at 72 ˚C for 7 min.

A mating type form-specific PCR assay was used to confirm form/type classification of the *P. teres* isolates (Lu et al. 2010). This assay can also discriminate between the two forms of the pathogen by the amplification of distinct PCR products. The primers *Ptt*MAT1F/R, *Ptt*MAT2F/R, *Ptm*MAT1F/R, and *Ptm*MAT2F/R (Table 2-1) were included in the analysis to confirm the identification of *Ptt* MAT1, *Ptt* MAT2, *Ptm* MAT1, and *Ptm* MAT2 isolates,

respectively. PCR assays were performed in a reaction mixture having the same composition as described for the species-specific PCR analysis, with thermal cycling conditions identical to those described by Lu et al. (2010). Amplification products were resolved on 2.25% agarose gels and visualized using SYBR Safe (Invitrogen).

### 2.2.4 Clone correction

Simple sequence repeat (SSR) polymorphisms were detected with 13 polymorphic SSR primers developed from the genome assembly of *P. teres* (Ellwood et al. 2010; Liu et al. 2012), in order to identify and eliminate repeated genotypes and uncover clonality (James et al. 2009) in the isolate collection. The PCR analysis was conducted as described by Liu et al. (2012), and the SSR-PCR products were separated by capillary electrophoresis on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA). For each locus, microsatellite allele sizes were determined by comparing the amplicons with a LIZ 500 internal size standard (Applied Biosystems) using GeneMapper software v3.7 (Applied Biosystems) (Bogacki et al. 2010).

### 2.2.5 Data analysis

Chi-square tests were conducted to determine if the observed mating type ratio for each of the populations of *P. teres* from each province and the cross province pool for western Canada departed significantly from the null hypothesis of a 1:1 MAT1:MAT2 ratio (Bogacki et al. 2010). Mating type frequencies were calculated using original and clone corrected data sets in which only one representative haplotype per clone was selected for further analysis (Sommerhalder et al. 2006; James et al. 2009). Chi-square values were calculated according to the formula: $x^2=\sum[(o-e)^2/e]$, where: o is the observed value of the mating type and e is the expected value (Moore and Novak Frazer 2002; Serenius et al. 2005; Sommerhalder et al. 2006). A 0.05 Type I error rate was applied to accept or reject the null hypothesis of a statistically equal mating type

ratio of 1:1 (Vail and Banniza 2009). Chi-square tests were not conducted on province-year populations with sample sizes of less than five because of a lack of statistical power (Sommerhalder et al. 2006). Alternatively, the pooled provincial populations were used to provide suitable sample sizes and the associated statistical power. For clone correction, similarity matrices representing all possible pairwise comparisons of the tested isolates were constructed based on the presence/absence data for each marker type. The similarity matrix was then used to perform cluster analysis by the unweighted pair-group method using arithmetic means (UPGMA) procedure. The software NTSYSpc version 2.2 (Exeter Software, New York, NY) was used to construct the similarity matrix and perform the UPGMA analysis (McLean et al. 2010).

## 2.3 Results

*Pyrenophora teres* f. *teres* was readily isolated from barley leaf lesions showing typical symptoms of the net form of net blotch. In contrast, both *Cochliobolus sativus* (Ito and Kuribayashi) Drechs. ex Dastur (anamorph *Bipolaris sorokiniana* (Sacc.) Shoemaker), the causal agent of spot blotch, and *P. teres* f. *maculata* were isolated from dark necrotic spots on green and senesced barley leaf tissue placed on wet filter paper. A total of 190 *P. teres* isolates were isolated from leaves exhibiting typical symptoms of NFNB or SFNB. Thirty isolates also were collected and identified from plants exhibiting intermediate symptoms that were not clearly distinguishable as NFNB or SFNB, but were identified as *P. teres* based on the morphological characteristics of the conidiophores and conidia. Thus, a total of 220 single spore isolates were derived and used in the analyses.

### 2.3.1 Species- and form-specific analyses

Species-specific primers amplified a 900bp band in each of the 220 isolates and confirmed them to be *P. teres*. No amplicon was produced from DNA of *P. tritici-repentis*, which was included as a negative control. There were a total of 128 isolates identified as *Ptt* and 92 as *Ptm*, based on form-specific markers (Table 2-2). The *Ptt* form-specific primers *PTT*-F and *PTT*-R amplified a ~380 bp fragment, while the *Ptm* form-specific primers *PTM*-F and *PTM*-R generated a ~410 bp fragment from isolates morphologically designated as *Ptt* and *Ptm*, respectively. It was common to find both forms in the same field, the same plant, and on the same leaf in very close proximity. Of the 30 *P. teres* isolates recovered from lesions that were not clearly distinguishable as NFNB or SFNB, 19 were *Ptt* and 11 were *Ptm*.

In all cases, morphological-based species identification of the isolates was confirmed by PCR analysis. A separate analysis was done to identify repeated genotypes among the isolates. This analysis revealed a clonal fraction of approximately 10% in the entire population. There were 198 distinct genotypes consisting of 115 *Ptt* and 83 *Ptm* isolates (Table 2-3). These included 49 *Ptt* and 43 *Ptm* isolates from Saskatchewan, 51 *Ptt* and 21 *Ptm* isolates from Alberta, and 15 *Ptt* and 19 *Ptm* isolates from Manitoba, respectively.

Cluster analysis of the SSR data was done using the UPGMA procedure and Jaccard's similarity coefficient. These results indicated that all isolates, including the 30 recovered from the indistinct lesions, clustered in two distinct divergent groups conforming to either *Ptt* or *Ptm*. There was no intermediate clade detected between the two forms.

### 2.3.2 Mating type-specific PCR analysis

Among the 128 *Ptt* isolates, 73 produced a single 1300 bp amplicon corresponding to MAT-1, while 55 isolates produced a single 1150 bp amplicon corresponding to the MAT-2 idiomorph (Table 2-2). Among the 92 *Ptm* isolates, 39 produced the amplicon corresponding to MAT-1, while 53 produced the amplicon corresponding to the MAT-2 idiomorph (Table 2-2). Using the clone correction step, 30 *Ptt* isolates from Saskatchewan were identified as MAT1 and 19 as MAT2, 28 *Ptt* isolates from Alberta were identified as MAT1 and 23 as MAT2, and 10 isolates from Manitoba were identified as MAT1 and five were MAT2 (Table 2-3). Similarly, 18 *Ptm* isolates from Saskatchewan were identified as MAT1 and 25 as MAT2. Twelve *Ptm* isolates from Alberta were identified as MAT1 and nine as MAT2, and seven isolates from Manitoba were identified as MAT1 and 12 as MAT 2 (Table 2-3). MAT1 and MAT2 idiomorphs of both forms were identified within the same crop, on the same plant, and on the same leaf. In three instances, both mating types were identified within a single lesion caused by *Ptt*; however, only one mating type was found within each single lesion caused by *Ptm* in all cases.

Chi-square analysis was conducted to test the null hypothesis of a 1:1 ratio between the two mating types for both *Ptt* and *Ptm*. With the exception of the 2011 *Ptm* population from Saskatchewan, no significant departure from a 1:1 mating type ratio was observed in any of the populations of *Ptt* or *Ptm* in the original data set (Table 2-2). Chi-square analysis of the dataset following the clone correction step revealed no statistical differences in mating type frequency for either *Ptt* or *Ptm* in all populations (Table 2-3).

Form and mating type classifications using species-, form-, and mating type-specific PCR assays were confirmed by analysis with MAT-specific SNP primers. In isolates originally classified as *Ptt* and MAT1, the primer set *Ptt*MAT1F/R amplified a 1143 bp fragment, while for those

classified as *Ptt* and MAT2, the primer set *Ptt*MAT2F/R amplified a 1421 bp fragment. Similarly, in isolates originally classified as *Ptm* and MAT1, the primer set *Ptm*MAT1F/R amplified a 194 bp band, while for those classified as *Ptm* and MAT2, the primer set *Ptm*MAT2F/R amplified a 939 bp band. These results confirm the form and mating type designations using the species-, form-, and mating type-specific PCR markers for all isolates.

## 2.4 Discussion

This is the first study of mating type frequencies in western Canadian *P. teres* f. *teres* and *P. teres* f. *maculata* populations. Given the 1:1 ratio of the two mating types for both forms of the pathogen, and the small number of clones identified in the isolate collections, it appears that *Ptt* and *Ptm* go through regular cycles of sexual reproduction in the Canadian prairie provinces. Overall, these results concur with the hypothesis that primary infection of barley fields is likely caused by ascospores discharged from pseudothecia on stubble.

The occurrence and frequency of mating types was assessed in a collection of *P. teres* isolates from 124 barley fields across Alberta, Saskatchewan and Manitoba. Analysis of both the original and clone corrected data sets revealed no significant departure, with the exception of Saskatchewan in 2011, from the null hypothesis of a 1:1 ratio between the mating types for either *Ptt* or *Ptm* in any of the three Prairie Provinces or across the entire Prairie region. This finding implies that there is no selection for mating types in either the *Ptt* or *Ptm* populations, supporting the likelihood of sexual hybridization within each form. Serenius et al. (2005) and Liu et al. (2012) also demonstrated the existence of both mating types in a 1:1 ratio in Finland and North Dakota, respectively, and concluded that sexual recombination is common in those regions. A ratio of almost 1:1 between mating types also has been reported in *Ptm* and *Ptt* populations

collected from barley fields in Australia (Bogacki et al. 2010; McLean et al. 2010), indicating that sexual recombination is possible.

A two-step process was conducted to ensure the accuracy of the mating type data presented in this report. The mating type of individual isolates was first assessed by PCR analysis with the mating type-specific primers of Rau et al. (2005), and then confirmed with a mating type locus-specific PCR assay developed by Lu et al. (2010). The data also were clone corrected to avoid over representation of clonal isolates (Sommerhalder et al. 2006; James et al. 2009). The identification of both mating types in both forms of *P. teres* in a statistically equal 1:1 ratio in all tested populations suggests that both *Ptt* and *Ptm* are capable of sexually reproducing. Therefore, it can be concluded that ascospore dispersal is likely to have a key role in the initiation of both NFNB and SFNB in each of the Prairie Provinces, likely followed by several cycles of conidia production, with the conidia serving as secondary inoculum during the growing season. Results from this study are in agreement with the earlier findings of Piening (1961, 1968), and Duczek et al. (1999), who identified the *P. teres* teleomorph in Alberta and Saskatchewan, respectively.

The two mating types of both *Ptt* and *Ptm* were occasionally recovered from the same leaf, different leaves of the same plant, and different plants in the same barley field, indicating that the mating types occurred in close proximity to each other. However, while both mating types could occasionally be recovered from single lesions caused by *Ptt*, single lesions caused by *Ptm* typically consisted of only one mating type. This may reflect the fact that what may appear to be single net form lesions are not necessarily caused by single-spores of *Ptt*. Instead, two or more spores of different mating types may initially cause small lesions in close proximity, which would then coalesce to form larger lesions with no distinct boundary. Such lesions may be

considered a single lesion at the time of single-spore isolation. In contrast, spot form lesions can be easily distinguished from one another under a dissecting microscope.

All isolates derived from leaves with symptoms that were not clearly distinguishable as NFNB or SFNB were confirmed as either *Ptt* or *Ptm* by PCR analysis. Cluster analysis using the UPGMA procedure revealed that all isolates, including those from indistinct lesions, clustered in two distinct *Ptt* and *Ptm* groups with no intermediate clade. Rau et al. (2003, 2007) also demonstrated that *Ptt* and *Ptm* isolates cluster in two genetically distinct clades and did not find any genetically intermediate isolates in an analysis of amplified fragment length polymorphisms, suggesting that *Ptt* and *Ptm* are genetically isolated. Furthermore, Serenius et al. (2005) showed that meiosis did not occur properly in crosses between isolates of *Ptm* and *Ptt*, resulting in the production of ascocarps that lacked asci or contained abnormal, non-culturable ascospores. This finding led Serenius et al. (2005) to conclude that successful sexual reproduction between the two forms of *P. teres* is very unlikely in nature. In contrast, Campbell et al. (1999) reported an efficient crossing of the two forms under laboratory conditions and demonstrated that most of the sexual offspring caused intermediate symptoms on barley leaves. The resulting sexual offspring were later shown to be genetically stable (Campbell and Crous 2003). Moreover, Campbell et al. (2002) concluded, based on a random amplified polymorphic DNA analysis, that sexual recombination between *Ptt* and *Ptm* isolates might be occurring under field conditions. Similarly, Leisova et al. (2005) suggested that hybridization between the two forms was possible based on the presence of an intermediate clade consisting of haplotypes with shared markers. These apparently contradicting results might reflect differences in the isolates studied, population structure, and (or) environmental conditions in different geographical regions and cropping systems. Nonetheless, the results from the current study suggest that no case of successful

hybridization between *Ptt* and *Ptm* was detected among the 220 studied isolates collected from western Canada.

## 2.5 Tables

**Table 2-1.** Sequences of species-, form-, mating type- and mating type locus-specific primers used to characterize *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada.

| Primer name | Primer sequence | Amplicon size | Reference |
|---|---|---|---|
| OPF01F900 | ACGGATCCTGGGTCCAGA | ~ 900 bp | Williams et al. (2001) |
| OPF01R900 | CGGATCCTGAGCAGAAAATG | | |
| *PTT*-F | CTCTGGCGAACCGTTC | ~ 380 bp | Williams et al. (2001) |
| *PTT*-R | ATGATGGAAAAGTAATTTGTA | | |
| *PTM*-F | TGCTGAAGCGTAAGTTTC | ~ 410 bp | Williams et al. (2001) |
| *PTM*-R | ATGATGGAAAAGTAATTTGTG | | |
| MAT-1 forward | AACAGACTCCTCTTGACAACCCG | ~ 1300 bp | Rau et al., (2005) |
| MAT-1 reverse | TGACGATGCATAGTTTGTAAGGGTC | | |
| MAT-2 forward | CAACTTTTCTCTACCACACGTATCCC | ~ 1150 bp | Rau et al., (2005) |
| MAT-2 reverse | TGTGGCGAT GCATAGTTCGTAC | | |
| *Ptt*MAT1F | ATGAGACGCTAGTTCAGAGTCT | 1143 bp | Lu et al. (2010) |
| *Ptt*MAT1R | GATGCCCAGCCAAGGACAA | | |
| *Ptt*MAT2F | TACGTTGATGCAGCTTTCTCAAT | 1421 bp | Lu et al. (2010) |
| *Ptt*MAT2R | AACACCGTCCAAAGCACCT | | |
| *Ptm*MAT1F | TGTTAGAGACCCCACCAGCGT | 194 bp | Lu et al. (2010) |
| *Ptm*MAT1R | CAGCTTTCTTGGCCTTCTGAA | | |
| *Ptm*MAT2F | ACGCAAGGTACTCTGTACGCA | 939 bp | Lu et al. (2010) |
| *Ptm*MAT2R | GACGTCGAGGGAGTCCATTT | | |

**Table 2-2.** Form and mating type frequencies in western Canadian populations of *Pyrenophora*. *teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) (original data set).

| Province | Year | Number of fields | *Ptt*1 | *Ptt*2 | *Ptt* $\chi^2$ (1:1) types | p | *Ptm*1 | *Ptm*2 | *Ptm* $\chi^2$ (1:1) types | p |
|---|---|---|---|---|---|---|---|---|---|---|
| Alberta | 2010 | 20 | 19 | 10 | 2.793 | 0.095 | 11 | 12 | 0.043 | 0.835 |
| Alberta | 2011 | 11 | 13 | 13 | 0.000 | 1.000 | 3 | 1 | _ | _ |
| Manitoba | 2009 | 10 | 4 | 0 | _ | _ | 1 | 5 | 2.667 | 0.102 |
| Manitoba | 2010 | 10 | 0 | 2 | _ | _ | 6 | 5 | 0.091 | 0.763 |
| Manitoba | 2011 | 9 | 6 | 3 | 1.000 | 0.317 | 0 | 2 | _ | _ |
| Saskatchewan | 2010 | 22 | 5 | 5 | 0.000 | 1.000 | 7 | 5 | 0.333 | 0.564 |
| Saskatchewan | 2011 | 42 | 26 | 22 | 0.333 | 0.564 | 11 | 23 | 4.235 | 0.040* |
| Alberta | pooled† | 31 | 32 | 23 | 1.473 | 0.225 | 14 | 13 | 0.037 | 0.847 |
| Manitoba | pooled† | 29 | 10 | 5 | 1.667 | 0.197 | 7 | 12 | 1.316 | 0.251 |
| Saskatchewan | pooled† | 64 | 31 | 27 | 0.276 | 0.599 | 18 | 28 | 2.174 | 0.140 |
| W Canada | pooled† | 124 | 73 | 55 | 2.531 | 0.112 | 39 | 53 | 2.130 | 0.144 |

* Denotes mating-type frequencies that are significantly different from a 1:1 ratio at P = 0.05.
- Indicates that $\chi^2$ analysis was not performed due to small sample size.
† Pooled data for each province are for the combined years; for the western Canada (W Canada) entry, pooled data include all years and provinces.

**Table 2-3.** Form and mating type frequencies in western Canadian populations of *Pyrenophora*. *teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) (clone corrected data set).

| Province | Year | Number of fields | *Ptt*1 | *Ptt*2 | *Ptt* $\chi^2$ (1:1) | p | *Ptm*1 | *Ptm*2 | *Ptm* $\chi^2$ (1:1) | p |
|---|---|---|---|---|---|---|---|---|---|---|
| Alberta | 2010 | 20 | 16 | 10 | 1.385 | 0.239 | 9 | 8 | 0.059 | 0.808 |
| Alberta | 2011 | 11 | 12 | 13 | 0.040 | 0.841 | 3 | 1 | _ | _ |
| Manitoba | 2009 | 10 | 4 | 0 | _ | _ | 1 | 5 | 2.667 | 0.102 |
| Manitoba | 2010 | 10 | 0 | 2 | _ | _ | 6 | 5 | 0.091 | 0.763 |
| Manitoba | 2011 | 9 | 6 | 3 | 1.000 | 0.317 | 0 | 2 | _ | _ |
| Saskatchewan | 2010 | 22 | 5 | 3 | 0.500 | 0.480 | 7 | 5 | 0.333 | 0.564 |
| Saskatchewan | 2011 | 42 | 25 | 16 | 1.976 | 0.1599 | 11 | 20 | 2.613 | 0.106 |
| Alberta | pooled† | 31 | 28 | 23 | 0.490 | 0.484 | 12 | 9 | 0.429 | 0.513 |
| Manitoba | pooled† | 29 | 10 | 5 | 1.667 | 0.197 | 7 | 12 | 1.316 | 0.251 |
| Saskatchewan | pooled† | 64 | 30 | 19 | 2.469 | 0.1161 | 18 | 25 | 1.140 | 0.286 |
| W Canada | pooled† | 124 | 68 | 47 | 3.835 | 0.0502 | 37 | 46 | 0.976 | 0.323 |

* Denotes form and mating-type frequencies that are significantly different from a 1:1 ratio at P = 0.05.
- Indicates that $\chi^2$ analysis was not performed due to small sample size.
† Pooled data for each province are for the combined years; for the western Canada (W Canada) entry, pooled data include all years and provinces.

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# Chapter 3 Genetic structure of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada[2]

## 3.1 Introduction

Net blotch, caused by the heterothallic ascomycete *Pyrenophora teres* Drechsler (anamorph *Drechslera teres* [Sacc.] Shoem.) (Smedegard-Petersen 1978), is an important foliar disease of barley (*Hordeum vulgare* L.) in western Canada (Wallace 1960; Tekauz 1990). The pathogen consists of two morphologically similar, but genetically distinct forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), which cause the net form of net blotch (NFNB) and spot form of net blotch (SFNB), respectively (Rau et al. 2007; Liu et al. 2011; Ellwood et al. 2012). Yield losses of 10 - 40% (Steffenson et al. 1991; Mathre 1997; Murray and Brennan 2010) and up to 44% (Jayasena et al. 2007) were reported for severe cases of NFNB and SFNB, respectively. Losses can be reduced by crop rotation, fungicide application and the deployment of resistant cultivars, which are the three main components of integrated net blotch management in barley (Tekauz 1990; Turkington et al. 2011).

Despite the fact that the two forms of *P. teres* are morphologically the same species, it was shown that they are likely two different phylogenetic species (Rau et al. 2007; Serenius et al. 2007; Ellwood et al. 2012). *Ptt* seems the more prevalent form in western Canada (Tekauz 1990; Chapter 2; Akhavan et al. 2015), but in some areas of Saskatchewan, SFNB caused by *Ptm* was

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reported as the most prevalent foliar disease of spring barley, and was considered more economically important than NFNB (Weller and Rossnagel 1988; Tekauz 1990; van den Berg and Rossnagel 1991). It also was reported that while both forms are considered to be economically important in the Czech Republic, spring and winter barley crops were infected at relatively higher levels by *Ptt* and *Ptm*, respectively (Minarikova and Polisenska 1999; Leisova et al. 2005a).

Both forms likely go through regular cycles of sexual reproduction on the Canadian prairies, with sexual hybridization occurring on overwintering crop residues (Piening 1961, 1968; Duczek et al. 1999) followed by multiple cycles of asexual reproduction during the growing season (Peever and Milgroom 1994). Therefore, both *Ptt* and *Ptm* have mixed reproduction and an outcrossing mating system. Given these characteristics, both forms fall into the category of pathogens with a relatively high risk of being able to adapt rapidly to major host resistance genes and fungicides with single modes of action (McDonald and Linde 2002). It was demonstrated that *Ptt* and *Ptm* are capable of hybridization under laboratory conditions (Campbell et al. 1999); however, many studies using analysis of molecular variance (AMOVA) showed that these two forms are classified in two divergent genetic groups (Rau et al. 2003; Leisova-Svobodova et al. 2014). Ellwood et al. (2012) estimated that *Ptt* and *Ptm* diverged about 519,000 years ago.

The population structure in fungal pathogens is associated with factors such as mutation, migration, gene flow, and selection, as well as the relative importance of sexual versus asexual stages in the pathogen life cycle. Significant genetic diversity in pathogen populations helps them to evolve in response to environmental changes (Peltonen et al. 1996), and may impact the stability of host resistance. Information regarding the genetic structure of pathogen populations has, therefore, vital implications for plant breeding and fungicide screening programs (Leisova et

al. 2005a). The genetic diversity and population structure of *P. teres* populations in several parts of the world were explored by employing random amplified polymorphic DNA (RAPD) (Peever and Milgroom 1994; Jonsson et al. 2000; Campbell et al. 2002), amplified fragment length polymorphism (AFLP) (Rau et al. 2003; Leisova et al. 2005a and 2005b; Serenius et al. 2007), and simple sequence repeat (SSR) analysis (Bogacki et al. 2010; Mclean et al. 2010; Liu et al. 2012; Leisova-Svobodova et al. 2014). In most of these investigations, a significant level of variability within the *P. teres* populations has been demonstrated, even over relatively small sampling regions (Campbell et al. 2002; Lehmensiek et al. 2010). Moreover, populations from fields close to each other in terms of geographical distance showed low levels of genetic differentiation compared with a higher level of genetic differentiation found in fields separated by greater distances (Serenius et al. 2007). This can be explained by the potential occurrence of higher levels of gene flow among geographically close populations. Peever and Milgroom (1994) investigated genetic diversity among five geographically distant *Ptt* populations collected from Canada, Germany and the United States, and calculated a high Nei's GST of 0.46, concluding that 46% of the total genetic variability was related to differentiation among these populations.

Among all polymerase chain reaction (PCR)-based markers, SSRs or microsatellites have proven to be a powerful tool for population genetic studies because of their variability, reproducibility, multiallelic nature, abundance, and wide genome coverage (Goldstein and Schlotterer 1999; Garoia et al. 2007; Singh et al. 2011; Leisova-Svobodova et al. 2014). Many SSR primers were developed and utilized in *P. teres* population studies (e.g., Bogacki et al. 2010; McLean et al. 2010; Austin et al. 2011; Liu et al. 2012; Leisova-Svobodova et al. 2014) and genetic linkage mapping (Ellwood et al. 2010). For example, Keiper et al. (2007) identified 25 sequence tagged

microsatellite sites (STMs) which revealed 26 polymorphic loci. In their study, 13 STMs amplified 14 polymorphic loci in both *P. teres* forms, seven STMs revealed polymorphism in *Ptm* only, and five were polymorphic in *Ptt* only. Bogacki et al. (2010) found that out of the 20 SSR loci tested on their populations, 17 (85 %) were polymorphic within the *Ptt* and *Ptm* populations. Ellwood et al. (2010) also reported a total of 68 polymorphic SSRs, 20 from STM markers (Keiper et al. 2007), 44 from the genome assembly sequence of *Ptt*, and four from expressed sequence tags (ESTs). Later, Liu et al. (2012) developed and screened 40 pairs of SSR primers using the *Ptt* genome sequence assembly, and found that 13 primer pairs could amplify high quality and polymorphic markers from different loci across the genome. These SSR primer pairs produced an average of 6.5 alleles per locus for a *Ptt* collection from North Dakota (Liu et al. 2012). McLean et al. (2010) also reported 15 sequence-tagged SSR primers which produced two to five alleles per locus for a *Ptm* collection from Victoria, Australia.

There is limited knowledge of the genetic structure of the *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations in western Canada, a major barley growing region. In addition, the nature and diversity of these populations have not been studied using molecular genetics techniques. Therefore, this study was conducted to explore the genetic structure of *Ptt* and *Ptm* populations collected from this region, and to test the null hypothesis of a lack of significant genetic differentiation among and within these populations.

## 3.2 Materials and methods

### 3.2.1 Fungal isolates

Barley leaves showing typical and/or intermediate symptoms of NFNB and SFNB were collected from each of the western Canadian provinces of Saskatchewan, Alberta and Manitoba (Fig. 3-1)

in 2009, 2010 and 2011. For single-spore isolations, leaf sections (about 10 mm × 5 mm) were surface sterilized in 50% ethanol for 15 s and in 2% sodium hypochlorite for 30 s, rinsed with sterile water and placed on moistened filter paper in 9 cm-diameter plastic Petri dishes (Tekauz 1990). To induce pathogen sporulation, the dishes were then incubated at 20° C ± 0.5° C with a 12 h photoperiod under a mixture of light from a common fluorescent lamp and a black light blue fluorescent lamp emitting near ultraviolet light at 368 nm. After 3-5 days, single conidia of *P. teres* were transferred onto 10% V-8 juice agar (V-8 juice, 100 mL; $CaC0_3$ 3g; Difco agar 20 g; distilled water 900 mL) supplemented with 50 mg $L^{-1}$ kanamycin, and incubated as above to produce new colonies (Tekauz 1990). Single-spore isolates were stored in liquid nitrogen or at -80$^{o}$C in an ultra-low temperature freezer, either as 0.5 cm-diameter mycelial plugs in 50% sterile glycerol or as conidial suspensions in 25% sterile glycerol. A total of 190 *P. teres* isolates, representing one to five single-spore isolates per form per each sampled field/plot, were obtained from leaves exhibiting typical symptoms of NFNB or SFNB. Thirty isolates also were recovered and identified from plants exhibiting intermediate symptoms that were not clearly distinguishable as NFNB or SFNB, but were identified as *P. teres* based on the morphological characteristics of conidiophores and conidia. Thus, a total of 220 single-spore isolates were obtained and used in the SSR analysis (Table 3-1). To prevent over-representation of alleles in frequently occurring clones, redundant haplotypes with identical alleles collected from the same region in the same year were removed prior to the final analysis of population genetic statistics (Zaffarano et al. 2006).

### 3.2.2 Species, form, mating type, and molecular marker analyses

Genomic DNA extraction, species-, form-, mating type-specific primers, PCR conditions and electrophoresis were the same as those described in Chapter 2 (Akhavan et al. 2015). To validate

the form-specific PCR, a subset of isolates including 39 isolates of *Ptt* and 27 of *Ptm* were inoculated onto the barley cultivar 'Steptoe' as described by Tekauz (1990). For population genotyping, PCR analysis was conducted using 13 SSR primer pairs to assess variation among western Canadian *Ptt* and *Ptm* populations. These primer pairs were developed from the *P. teres* f. *teres* genome assembly (Ellwood et al. 2010; Liu et al. 2012) and reported by Liu et al. (2012) as being capable of generating quality polymorphic markers from loci across the genome. The expected allele size, primer sequence, and linkage group location for each SSR locus are listed in Liu et al. (2012). All forward primers were appended with the universal M13 primer sequence '5-CACGACGTTGTAAAACGAC-3' following the M13-tailing technique as described by Schuelke (2000). To facilitate detection of the PCR products, a FAM labeled M13 primer (Applied Biosystems, Foster City, CA) was used.

The final 12.5 µL reaction volume was comprised of a mixture containing 1X PCR Buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM of each forward and reverse SSR primer, 0.5mM of FAM labeled M13 primer, 0.6 U of Platinum Taq DNA polymerase (Invitrogen), and 2 µL (20 ng) of genomic fungal DNA as template (Bogacki et al. 2010). The PCR was run under the following cycling conditions: 7 min at 95°C, followed by 3 cycles of 30 s at 95°C, 30 s at 50°C, and 80 s at 72°C, then 33 cycles of 15 s at 94°C, 15 s at 52°C, and 45 s at 72°C, and a final extension step of 10 min at 72°C (Liu et al. 2012; Zhong et al. 2009). The SSR-PCR products were separated by capillary electrophoresis on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA). For each locus, SSR allele sizes were determined by comparing the amplicons with a LIZ 500 internal size standard (Applied Biosystems) using GeneMapper software v3.7 (Applied Biosystems) (Bogacki et al. 2010), and for each SSR primer pair, amplicons of the same size were considered to be the same allele (Liu et al. 2012). In SSR genotyping, one *P.*

*tritici-repentis* reference isolate, and one *Sclerotinia sclerotiorum* isolate (courtesy of B. Ziesman, University of Alberta), also were included as controls.

### 3.2.3 Data analysis

Similarity matrices representing all possible pairwise comparisons of the tested isolates were constructed using the presence/absence of peaks of the expected band sizes for each marker type. All redundant haplotypes with identical alleles collected from the same region in the same year were removed before further analysis. Two *Ptt* isolates and one *Ptm* isolate also were excluded from further analysis due to poor SSR amplification. A similarity matrix was then used to perform cluster analysis using Jaccard's similarity coefficient and employing the unweighted pair-group method using the arithmetic means (UPGMA) procedure with NTSYSpc v. 2.1 (Exeter Software, New York, NY, USA). The stability of the clustering was evaluated by calculation of the consensus index using the Consensus program of the same software. Consensus index values range from 0 (highly dissimilar) to 1 (highly similar). The molecular analysis of variance (AMOVA) and the degrees of genetic differentiation between western Canadian *P. teres* populations were determined using the PhiPT formula: PhiPT = AP / (WP + AP) = AP / TOT, where AP = estimated variance among populations, WP = estimated variance within populations, and TOT = total estimated variance, employing the GenAlEx 6.501 program (Peakall and Smouse 2006, 2012). Overall gene diversity (h = Diversity = 1 - Sum $pi^2$, where pi is the frequency of the ith allele for the population and Sum $pi^2$ is the sum of the squared population allele frequencies), unbiased diversity (Uh = [N / (N-1)] * h), the Nei's measure of genetic identity, and the mean percentage of polymorphic loci within populations for each form also were calculated using GenAlEx v6.501 (Peakall and Smouse 2006, 2012). The GST coefficient of gene differentiation, total genetic diversity (Ht), the genetic diversity within

population (Hs), gene flow (Nm = 0.5(1 - GST)/GST) and the Shannon's information index (I) were calculated using the POPGENE program, v. 1.31 (Yeh et al. 1999). Chi-square test values also were used to determine if the populations departed significantly from the expected 1:1 ratio of mating type frequencies (Bogacki et al. 2010; Dastkar et al. 2013; Liu et al. 2012).

## 3.3 Results

Species-, form-, and mating type-specific PCR primers confirmed 73, 55, 39 and 53 isolates as *Ptt* mating type 1, *Ptt* mating type 2, *Ptm* mating type 1 and *Ptm* mating type 2, respectively. In terms of the differentiation of the two forms of the pathogen, the results obtained through form-specific PCR (Williams et al. 2001) were consistent with those obtained through the inoculation method for all 39 *Ptt* and 27 *Ptm* isolates tested.

All 13 SSR loci employed in this study amplified successfully, and were polymorphic within both *Ptt* and *Ptm* populations from western Canada. The mean percentage of polymorphic loci over three provincial populations was calculated as 74.1% for *Ptt* and 75.2% for *Ptm* (Table 3-2). In total, 110 distinct alleles were identified, of which 19 (17%) were shared between *Ptt* and *Ptm*, 75 (68%) were specific to *Ptt* and 16 (15%) were specific to *Ptm*. Among the *Ptt* isolates, a total of 94 alleles was detected with an average of 7.2 alleles per locus and a range of 3 to 11 alleles per locus, while for the *Ptm* isolates, 35 alleles were detected with an average of 2.7 and a range of 1 to 5. High levels of genotypic diversity were found among isolates of both forms. The observed polymorphism at the 13 loci revealed 113 and 82 different genotypes among the 126 and 91 *Ptt* and *Ptm* isolates tested, respectively, indicating a likely clonal fraction of approximately 10% in each of the entire *Ptt* and *Ptm* populations. In one case for *Ptt*, the same multilocus haplotype was found once in Alberta in 2011 and twice again in Saskatchewan in

2010 and 2011. For *Ptm*, in three cases, one multilocus haplotype was found in more than one province, and in four cases, one haplotype was shared between different sampling years.

The level of genetic differentiation was high when all six *Ptt* and *Ptm* populations were included together in the same analysis. Following clone correction, significant genetic differentiation (PhiPT = 0.230, P = 0.001) was detected among the six *Ptt* and *Ptm* populations, with 77% of the genetic variation occurring within populations and 23% between populations (Table 3-3). Low but still significant genetic differentiation (PhiPT = 0.038, P = 0.001) was detected among *Ptt* populations, rejecting the null hypothesis that no differentiation exists among the *Ptt* provincial populations; however, 96% of the genetic variation was still occurring within populations and only 4% between populations (Table 3-4). No significant genetic differentiation (PhiPT = 0.010, P = 0.177) was observed among *Ptm* populations, with 99% of the total genetic diversity found within populations and only 1% between populations (Table 3-5). Likewise, no significant genetic differentiation was observed among *Ptt* (PhiPT = 0.003, P = 0.299) and *Ptm* (PhiPT = 0.029, P = 0.075) populations sampled in different years. A high and significant coefficient of genetic differentiation (PhiPT = 0.316, P = 0.001) was obtained when considering the *Ptt* and *Ptm* isolates as two populations at the form level, with 68% of the total genetic diversity found within populations and only 32% between populations. Cluster analysis using Jaccard's similarity coefficient and the UPGMA procedure indicated that all tested isolates clustered in two distinct groups conforming to either *Ptt* or *Ptm* (Fig. 3-2). The *Ptt* isolates further clustered in two main groups, with 69% of isolates collected from Alberta in the first group and 67% of isolates collected from Saskatchewan in the second. Isolates collected from Manitoba grouped in either group in statistically equal numbers. No clear clustering based on geographical origin was

observed within *Ptm* populations. A high consensus index (CIc = 0.94) confirmed the stability of the clustering.

Overall diversity, as calculated by GenAlEx 6.501, was 0.176 and 0.217 for *Ptt* and *Ptm*, respectively. Unbiased diversity (Uh) values of 0.185 for *Ptt* and 0.227 for *Ptm* also were calculated using the same software. Likewise, total genetic diversity (Ht), calculated by POPGENE 1.31, was 0.18 for the *Ptt* and 0.22 for the *Ptm* populations. The genetic diversity within populations (Hs) was calculated as 0.14 and 0.21 for *Ptt* and *Ptm*, respectively, indicating that the major source of variation in western Canadian populations of *Ptt* and *Ptm* comes from within populations. Shannon's information index was 0.3031 and 0.3521 for *Ptt* and *Ptm*, respectively. Low GST coefficients of gene differentiation (proportion of total genetic diversity partitioned among populations) of 0.043 and 0.041 were found for the *Ptt* and *Ptm* populations, respectively. Overall, this confirms that provincial populations of the same form of *P. teres* have similar genetic variation, while further supporting the observation that most of the genetic diversity for both forms is within populations. Gene flow, Nm = 0.5(1 - GST)/GST, was 10.99 and 11.59 for *Ptt* and *Ptm* populations, respectively, indicating high rates of migration.

Nei's average gene diversity values were very similar for each individual *Ptt* population (h = 0.181), with the average gene diversity across all loci combined being 0.186 for the entire western Canadian population. For the *Ptm* populations, Nei's average gene diversity values also were similar for each individual population (h = 0.247, 0.202, and 0.202 for Alberta, Manitoba, and Saskatchewan, respectively), with the average gene diversity across all loci combined being 0.222. Nei's measure of genetic identity further supported the close relatedness of *Ptt* (I = 0.98-0.99) and *Ptm* (I = 0.97-0.99) populations, indicating a minimum chance of 98% for *Ptt*, and of 97% for *Ptm*, of finding the same allele at any locus in two randomly selected individuals from

these populations (Bogacki et al. 2010). A lower but still quite high Nei's measure of genetic identity (I =0.89) also was found between the two *Ptt* and *Ptm* populations at the form level, underscoring the close relatedness of the two forms of the net blotch pathogen.

## 3.4 Discussion

Using SSR markers, the genetic structure of *Ptt* and *Ptm* populations collected from western Canada was studied. Cluster analysis using the SSR data and UPGMA procedure revealed that all tested isolates clustered in two distinct (*Ptt* and *Ptm*) groups. This is consistent with other studies that suggested that *Ptt* and *Ptm* are genetically differentiated (Williams et al. 2001; Leisova et al. 2005a; Bakonyi and Justesen 2007; Keiper et al. 2008; Bogacki et al. 2010). Rau et al. (2003, 2007) also demonstrated that *Ptt* and *Ptm* isolates cluster in two genetically distinct clades and did not find any genetically intermediate isolates in an analysis of amplified fragment length polymorphisms, suggesting that *Ptt* and *Ptm* are genetically isolated. In contrast, Campbell et al. (2002) concluded, based on a random amplified polymorphic DNA analysis, that sexual recombination between *Ptt* and *Ptm* isolates might be occurring under field conditions. Similarly, Leisova et al. (2005a; 2014) and McLean et al. (2014) also suggested hybridization between the two forms was possible based on the presence of an intermediate cluster consisting of haplotypes with shared markers. These contradictory results might reflect differences in the isolates studied and the structure of pathogen populations. The set of primers used in this study proved a strong tool to differentiate the two forms of the net blotch pathogen in western Canada. These primers add to the 14 STM markers reported by Bogacki et al. (2010), nine STM markers described by Keiper et al. (2008), and the form-specific PCR primers developed by Leisova et al. (2005b) and Williams et al. (2001).

In this study, the SSR markers developed by Elwood et al. (2010) and screened and found to be highly polymorphic by Liu et al. (2012), were employed to explore the genetic structure of *Ptt* and *Ptm* populations sampled from three western Canadian provinces. Bogacki et al. (2010) found that out of the 20 SSR loci tested on their populations, 17 (85%) were polymorphic within the *Ptt* and *Ptm* populations. In the current study, all 13 SSR loci examined were polymorphic within both *Ptt* and *Ptm* populations, indicating that the primers used were suitable to study variability in populations of both forms of the net blotch pathogen (Liu et al. 2012). Using these SSR loci, high genotype diversity in populations of both forms of *P. teres* also was detected. In an earlier study using the same collection of isolates (Chapter 2; Akhavan et al. 2015), no significant departure was detected from the expected 1:1 MAT1/MAT2 ratio for either form of *P. teres* in each of the three provinces in western Canada. The high number of unique multilocus haplotypes (almost 90%) observed within the *Ptt* and *Ptm* populations, combined with an equal mating type ratio for both forms in each of the three provinces, suggests that both forms of *P. teres* are capable of going through regular cycles of sexual recombination in western Canada. Based on a UPGMA cluster analysis, McLean et al. (2010) reported that the population of *P. teres* f. *maculata* from Victoria, Australia, was genetically diverse, with no clones, and, given the equal presence of both mating types, likely underwent sexual recombination. Such high levels of genotypic diversity also were reported previously among *Ptt* and/or *Ptm* field populations in Italy (Rau et al. 2003), Finland (Serenius et al. 2005, 2007), the Czech Republic (Leisova et al. 2005b), Russia (Serenius et al. 2007), Sweden (Jonsson et al. 2000), and Australia (Serenius et al. 2007; Bogacki et al. 2010).

A relatively high and significant coefficient of genetic differentiation (PhiPT = 0.316, P = 0.001) was obtained when considering the *Ptt* and *Ptm* isolates as two populations, which provides

additional support for the conclusion that the *Ptt* and *Ptm* isolates are genetically different (Bogacki et al. 2010). A small PhiPT (PhiPT = 0.038, P = 0.001 for *Ptt*, and PhiPT = 0.010, P = 0.177 for *Ptm*) was obtained when the *Ptt* and *Ptm* isolates collected from Alberta, Manitoba and Saskatchewan were compared, showing that a large fraction of the genetic differentiation occurred within the three provinces rather than between them. Nonetheless, the statistical analysis revealed that *Ptt* isolates from the three provinces were slightly, but significantly, differentiated; in contrast, for *Ptm*, the low and insignificant coefficient of genetic differentiation suggested that the *Ptm* populations collected from the three provinces are genetically similar. This is consistent with the absence of clear clustering based on geographical origin that was observed within *Ptm* populations using the UPGMA procedure. Similarly, Liu et al. (2012) calculated a Wright's FST value of 0.11, indicating a relatively low but significant differentiation between the Fargo and Langdon *Ptt* populations in North Dakota. For *Ptm*, McLean et al. (2010) also did not find any correlations with geographic origin in a cluster analysis of 44 isolates collected from Australia. Nm, the estimated gene flow, was 10.99 and 11.59 for *Ptt* and *Ptm*, respectively, indicating the occurrence of a high level of gene flow between populations of both forms, which in turn would result in less differentiation between these local *Ptt* and *Ptm* populations. For an outcrossing heterothallic species like *P. teres*, which is at least partly a windborne pathogen, a high gene flow is expected between and within populations (Perdereau et al. 2014). The potential for long distance transport of spores is likely limited given previous reports (Deadman and Cooke 1989; Piening 1968), although for the related species *P. tritici-repentis* it has been reported that dispersal of conidia may occur over tens to hundreds of kilometers (Francl 1997). Moreover, seed-borne infections (Hampton 1980; Piening 1968) and the movement of seed between areas and provinces also can potentially increase the gene flow.

For *Ptm*, this high level of gene flow, combined with a lack of significant differentiation among the provincial populations, suggests the occurrence of one singular panmictic population on the Canadian prairies. However, this is preliminary and needs to be further confirmed by studying more populations and employing additional molecular markers. The results from the current study are consistent with the findings of many others that revealed no or very low genetic differentiation among geographical locations within a region or country (Peever and Milgroom 1994; Serenius et al. 2007; Lehmensiek et al. 2010; Liu et al. 2012). In this study, an average of 2.7 alleles was identified in *Ptm*, which was lower than the average of 7.2 alleles identified in *Ptt*. This likely reflects the fact that the SSR markers used were derived from the *Ptt* genome. As such, even though these markers may have been adequate for *Ptm*, they probably represent conserved regions between the two forms of the fungus. Therefore, the results obtained for *Ptt* and *Ptm* were not directly compared, and instead the diversity indices were discussed independently for each form. Hence, the conclusions remain free of ascertainment bias for each of the net and spot form pathogen populations.

Using SSR markers, relatively high mean gene diversity values of h = 0.181 for *Ptt* and 0.222 for *Ptm* were observed over all isolates and all loci for the three *Ptt* and three *Ptm* populations. These values were lower than those reported by Bogacki et al. (2010) (0.38 for *Ptt* and 0.40 for *Ptm*) and by Liu et al. (2012) (0.62 for *Ptt*); however, they were higher than the values reported by Rau et al. (2003) (0.046 for *Ptt* and 0.045 for *Ptm*) for populations from Italy, or by Campbell et al. (2002) (0.062 for *Ptt* and 0.082 for *Ptm*) for populations from South Africa. Gene diversity values of 0.182 and 0.216 for two Swedish *Ptt* populations also were reported previously by Jonsson et al. (2000). Indices ranging from 0.08 to 0.17 were reported for five *P. teres* populations from North America and Germany (Peever and Milgroom 1994). The diversity

values from the current and other studies in which SSR markers were employed (Bogacki et al. 2010; Liu et al. 2012) seem to be generally higher compared with values obtained using either RAPD or AFLP marker data (Peever and Milgroom 1994; Campbell et al. 2002; Rau et al. 2003). The higher number of alleles per locus and the co-dominant nature of the SSR markers might be a reason for this observation (Bogacki et al. 2010; Liu et al. 2012). No significant differentiation was observed among the collection years for the *Ptt* and *Ptm* populations. This suggests that the structure of western Canadian populations of *Ptt* and *Ptm* did not significantly change between 2009 and 2011. This is in contrast with the findings of Leisova-Svobodova et al. (2014) and Liu et al. (2012), who concluded that diversity is likely to be affected more by sampling year than the origin of the isolates.

In this first systematic study of *Ptt* and *Ptm* populations from western Canada using SSR analysis, a large number of pathogen genotypes was detected, indicating that these populations are diverse and undergo regular sexual recombination. Given these characteristics, the *Ptt* and *Ptm* populations appear to be at a relatively high risk of being able to adapt to major host resistance genes when deployed individually in barley cultivars (McDonald and Linde 2002). In addition, in the absence of suitable crop rotations and/or resistant cultivars, these characteristics also would increase the risk of fungicide resistance if the same fungicide mode of action was used repeatedly within or between growing seasons. Therefore, breeding for NFNB and SFNB resistance in western Canada should employ strategies such as pyramiding as many effective resistance genes as possible, while encouraging prudent use and management of fungicides.

## 3.5 Tables

**Table 3-1**. Location and year of collection for 220 isolates used to study the genetic structure of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) populations from western Canada.

| Province | Total number of isolates | *Ptt* | *Ptm* | Year of collection |
|---|---|---|---|---|
| Alberta | 52 | 29 | 23 | 2010 |
| Alberta | 30 | 26 | 4 | 2011 |
| Manitoba | 10 | 4 | 6 | 2009 |
| Manitoba | 13 | 2 | 11 | 2010 |
| Manitoba | 11 | 9 | 2 | 2011 |
| Saskatchewan | 22 | 10 | 12 | 2010 |
| Saskatchewan | 82 | 48 | 34 | 2011 |
| Total | 220 | 128 | 92 | 2009-2011 |

**Table 3-2.** Percentage of polymorphic loci in western Canadian collections of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*).

| Location | %P [a] (*Pyrenophora teres* f. *teres*) | %P (*Pyrenophora teres* f. *maculata*) |
|---|---|---|
| Alberta | 80.85% | 80.00% |
| Manitoba | 61.70% | 60.00% |
| Saskatchewan | 79.79% | 85.71% |
| Mean | 74.11% | 75.24% |
| SE | 6.21% | 7.80% |

[a] %P = Percentage of polymorphic loci

**Table 3-3.** Summary of the analysis of molecular variance (AMOVA) results for three populations of *Pyrenophora teres* f. *teres* and three populations of *P. teres* f. *maculata* from western Canada (one population of each form from each of the provinces of Alberta, Saskatchewan, and Manitoba).

| Source | df | SS | MS | Estimated Variance | Percentage % | PhiPT [a] | P |
|---|---|---|---|---|---|---|---|
| Among Populations | 5 | 603.88 | 120.776 | 3.501 | 23% | 0.23 | 0.001 |
| Within Populations | 189 | 2210.49 | 11.696 | 11.696 | 77% | | |
| Total | 194 | 2814.37 | | 15.196 | 100% | | |

[a] PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, AP = estimated variance among populations, WP = estimated variance within populations) denotes the proportion of the total genetic variance that is due to the variance among populations (P=0.05).

**Table 3-4.** Summary of the analysis of molecular variance (AMOVA) results for three populations of *Pyrenophora teres* f. *teres* collected from western Canada (one population from each of the provinces of Alberta, Saskatchewan, and Manitoba).

| Source | df | SS | MS | Estimated Variance | Percentage % | PhiPT [a] | P |
|---|---|---|---|---|---|---|---|
| Among Populations | 2 | 44.753 | 22.377 | 0.375 | 4% | 0.038 | 0.001 |
| Within Populations | 110 | 1048.16 | 9.529 | 9.529 | 96% | | |
| Total | 112 | 1092.91 | | 9.904 | 100% | | |

[a] PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, AP = estimated variance among populations, WP = estimated variance within populations) denotes the proportion of the total genetic variance that is due to the variance among populations (P=0.05).

**Table 3-5.** Summary of the analysis of molecular variance (AMOVA) results for three populations of *Pyrenophora teres* f. *maculata* collected from western Canada (one population from each of the provinces of Alberta, Saskatchewan, and Manitoba).

| Source | df | SS | MS | Estimated Variance | Percentage % | PhiPT [a] | P |
|---|---|---|---|---|---|---|---|
| Among Populations | 2 | 11.874 | 5.93699 | 0.046903108 | 1% | 0.00975 | 0.177 |
| Within Populations | 79 | 376.163 | 4.76155 | 4.761551976 | 99% | | |
| Total | 81 | 388.037 | | 4.808455084 | 100% | | |

[a] PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, AP = estimated variance among populations, WP = estimated variance within populations) denotes the proportion of the total genetic variance that is due to the variance among populations (P=0.05).

## 3.6 Figures

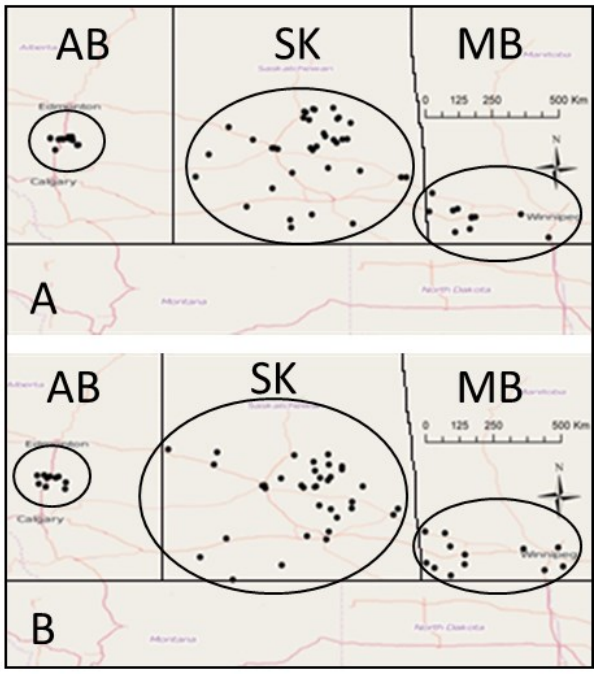


**Figure 3-1.** Map of the western Canadian provinces of Alberta (AB), Saskatchewan (SK) and Manitoba (MB), showing the collection sites (black points) for the (A) *Pyrenophora teres* f. *teres* and (B) *Pyrenophora teres* f. *maculata* isolates analysed in this study.

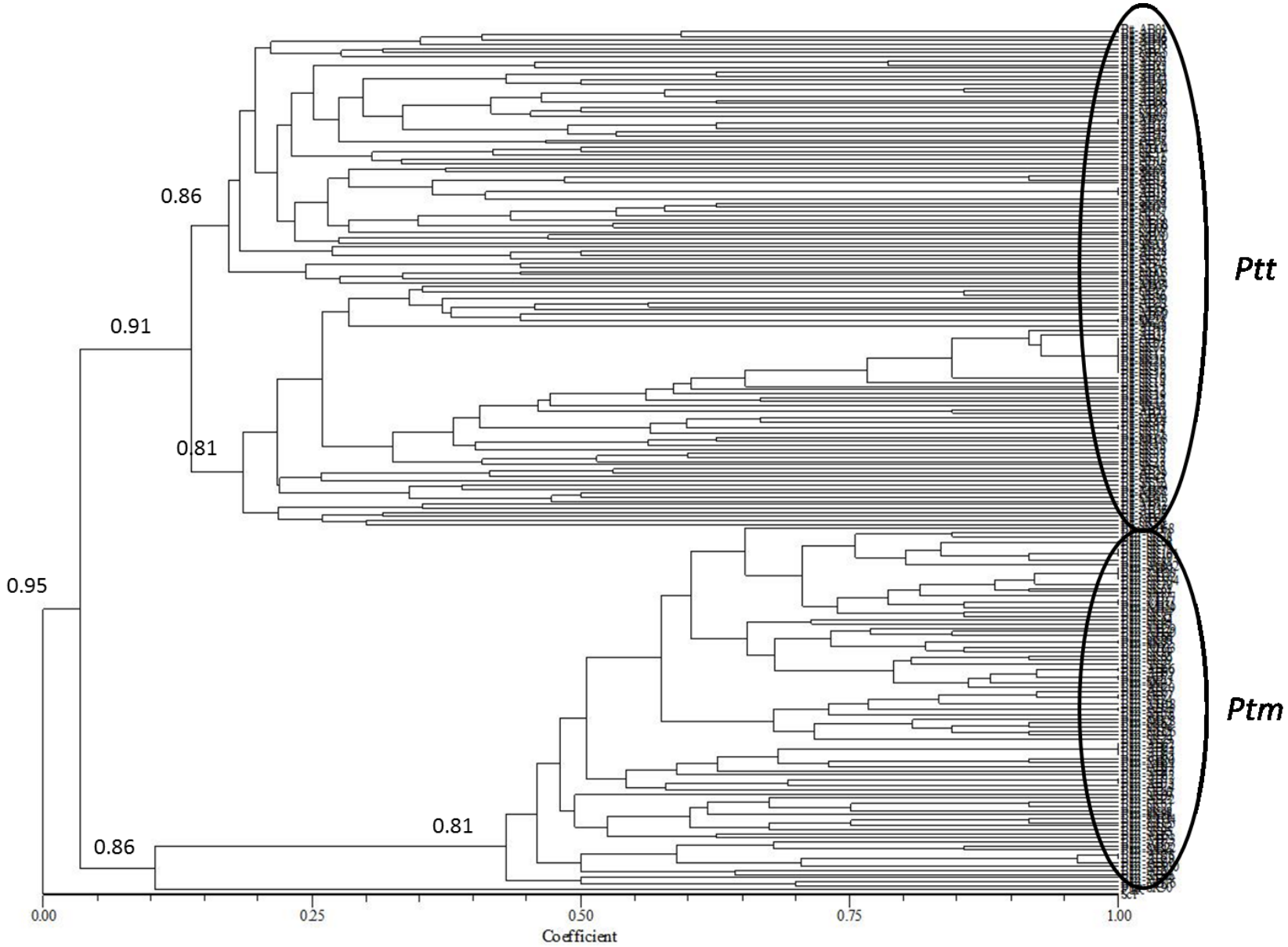
0.86
0.91
0.81
0.95
0.81
0.86
Ptt
Ptm
0.00
0.25
0.50
0.75
1.00
Coefficient

**Figure 3-2.** Genetic similarity of a large collection of western Canadian isolates of *Pyrenophora teres*. The size of the figure precludes listing each of the individual isolates in a larger font, but groups of isolates identified as *P. teres* f. *teres* or *P. teres* f. *maculata* have been circled and labeled *Ptt* or *Ptm*, respectively. Cluster analysis revealed that all isolates clustered in two distinct divergent groups conforming to either *P. teres* f. *teres* or *P. teres* f. *maculata*, with no intermediate cluster between the two forms. The two isolates at the bottom of the dendrogram are *Pyrenophora tritici-repentis* and *Sclerotinia sclerotiorum*, which were included as controls. The dendrogram was produced with NTSYSpc ver. 2.2, using UPGMA cluster analysis based on Jaccard's similarity coefficient. The stability of the clustering was confirmed by calculation of a high consensus index (CIc = 0.94) using the same software. Consensus index values for major nodes also are indicated on the dendrogram.

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# Chapter 4 Virulence of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations in western Canada[3]

## 4.1 Introduction

Net blotch, caused by the heterothallic ascomycete *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.) (McDonald 1963; Smedegard-Petersen 1978), is an economically important disease of barley (*Hordeum vulgare* L.) across western Canada (Tekauz 1990; Xi et al. 1999). *P. teres* has two morphologically similar but genetically distinct forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), which cause the net (NFNB) and spot (SFNB) forms of net blotch, respectively (Rau et al. 2007; Liu et al. 2011; Ellwood et al. 2012). Both *Ptt* and *Ptm* are stubble-borne pathogens and likely go through regular cycles of sexual reproduction in western Canada (Chapter 2; Akhavan et al. 2015), with sexual hybridization occurring on the stubble (Piening 1961, 1968; Duczek et al. 1999) followed by multiple cycles of asexual reproduction during the growing season (van den Berg and Rossnagel 1991; Peever and Milgroom 1994). Therefore, both forms of the pathogen have mixed reproduction and an outcrossing mating system, falling into the category of pathogens with a relatively high risk of being able to rapidly adapt to major host resistance genes and fungicides with single modes of action (McDonald and Linde 2002). It was demonstrated that *Ptt* and *Ptm* are capable of hybridization under laboratory conditions (Campbell et al. 1999, 2003; Jalli 2011). However, many studies have shown that these two forms are classified in two divergent groups (Rau et al.

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2003; Leisova-Svobodova et al. 2014). Yield losses of 10% to 40% for NFNB, and up to 44% for SFNB, are typical in severe outbreaks, but *P. teres* has the potential to cause a total loss in susceptible cultivars under conditions that favour disease development (Steffenson et al. 1991; Mathre 1997; Jayasena et al. 2007; Murray and Brennan 2010). Losses can be reduced by crop rotation, fungicide application and the deployment of resistant cultivars, which are the three main components of integrated net blotch management in barley (Tekauz 1990; Turkington et al. 2011; McLean et al. 2012). However, the application of fungicides increases input costs, and long crop rotation is not always practical on many farms in western Canada, resulting in an increased interest in the use of resistant cultivars for net blotch management. Currently, very few cultivars exhibiting NFNB resistance are available, while most cultivars have intermediate to moderately resistant reactions to SFNB (Alberta Agriculture and Rural Development 2015). Therefore, incorporating resistance to the net blotch pathogen in barley cultivars remains a priority for barley breeding programs in western Canada.

Tekauz (1990) and Liu and Friesen (2010) reported that the occurrence of SFNB in Canada and in the Northern Great Plains of the United States has been increasing. Neupane et al. (2015) suggested that this increase likely results from changes in the virulence of local pathogen populations. Khan (1982), Bockelman et al. (1983), Karki and Sharp (1986), Wu et al. (2003) and Grewal et al. (2008) also reported the occurrence of various degrees of pathogenic diversity among *Ptm* isolates. In contrast, McLean et al. (2010) reported low pathogenic variation among isolates in a local *Ptm* population collected from Victoria, Australia. More recently, however, McLean et al. (2014) identified 33 pathotypes among 60 isolates collected across Australia using 16 barley differential genotypes.

The evolution of virulent pathotypes of *P. teres* is a primary challenge for the deployment of

durable net blotch resistant barley cultivars (Tekauz 1990; McLean et al. 2014), and an updated knowledge of the pathogenic diversity and virulence spectrum of local pathogen populations is essential for successful incorporation of resistance genes into commercial cultivars. The pathogenic diversity present in a local pathogen population represents a challenge to barley breeders, since a particular resistance source may be effective only toward a limited number of pathotypes (McLean et al. 2012). For example, it was shown that barley lines with the *Rpt6* resistance gene resulted in a resistant reaction against *Ptm* isolates collected from Finland, but not to *Ptm* isolates from Australia or the Czech Republic (Manninen et al. 2006). Moreover, since *P. teres* has the potential to undergo sexual recombination in western Canada (Chapter 2; Akhavan et al. 2015), there is a likelihood that new virulent pathotypes may emerge and overcome the currently used sources of resistance (Jayasena et al. 2004). Khan (1982) reported that resistance against *Ptm* in the barley cultivar 'Beecher' was overcome by the development of new virulent pathotypes in Western Australia. The virulence of *Ptt* and *Ptm* isolates varies on differential host lines, and has been examined in many studies. Different pathotypes, also called virulence phenotypes, have been observed in the populations of both forms, both globally and within particular areas (Liu et al. 2011).

In the last comprehensive assessment of pathogenic diversity among *Ptt* and *Ptm* populations from western Canada, Tekauz (1990) classified 182 *Ptt* isolates into 10 main pathotype groups (A – J) with one to nine subgroups in each group (45 distinct virulence profiles in total). Tekauz (1990) also classified 42 *Ptm* isolates into seven main pathotype groups (P-V) with one to five subgroups in each group (20 distinct virulence profiles in total). Liu et al. (2012) suggested that western Canada has a higher percentage of virulent *Ptt* isolates (Tekauz 1990) than North Dakota

by comparing the virulence of isolates on three differentials, CI 5791, 'Heartland', and CI 9214, shared between the two studies.

Canadian barley is produced mainly in the provinces of Alberta, Saskatchewan and Manitoba (Statistics Canada 2015), and both NFNB and SFNB are among the most important foliar diseases of barley in this region (Turkington et al. 2011). However, there has been only one comprehensive study that assessed pathogenic diversity of *Ptt* and *Ptm* populations collected from western Canada, which was conducted using a population collected in 1985 (Tekauz 1990). In the current study, the virulence of a set of isolates collected from 2009-2012 was evaluated on the same differential sets previously developed and used to characterize the *Ptt* and *Ptm* isolates collected in 1985 (Tekauz 1990), enabling a direct comparison of pathogenic diversity with the earlier collection. The objective of this study was to evaluate the virulence and pathotype composition of western Canadian populations of *Ptt* and *Ptm*, and to determine if there have been any changes over the past three decades.

## 4.2 Materials and methods

### 4.2.1 Fungal isolation and preparation of inoculum

Previously, a cluster analysis using simple sequence repeat (SSR) DNA markers was performed using the methods and primers described by Liu et al. (2012) on a collection of 128 *Ptt* and 92 *Ptm* isolates collected from across western Canada (Alberta, Saskatchewan and Manitoba) (Chapter 3; Akhavan et al. 2016). In the current study, 36 *Ptt* and 21 *Ptm* isolates were selected as representatives of these different clades to assess pathogenic variation. Three *Ptt* and six *Ptm* isolates were added from another collection made in 2012. The origins of the *Ptt* isolates included the provinces of Alberta (17 isolates), Saskatchewan (12), and Manitoba (10). The *Ptm*

isolates originated from Alberta (8 isolates), Saskatchewan (10), and Manitoba (9). Reference isolates of *Ptt* (WRS102) and *Ptm* (WRS857) also were included for comparison (Table 4-1).

To obtain single-spore isolates from barley leaves, leaf sections (about 10 mm × 5 mm) were surface-sterilized in 50% ethanol for 15 s and in 2% sodium hypochlorite for 30 s, flushed with sterile water and placed on moistened filter paper in 9 cm-diameter plastic Petri dishes (Tekauz 1990). To induce fungal sporulation on the leaves, the dishes were incubated at 20°C ± 0.5°C with a 12 h photoperiod under a mixture of common fluorescent lamp light and a black light blue fluorescent lamp emitting near ultraviolet light at 368 nm. After 3-5 days, single conidia of *P. teres* were transferred onto 10% V-8 juice agar (V-8 juice, 100 mL; CaC03 3g; Difco agar 20 g; distilled water 900 mL) supplemented with 50 mg $L^{-1}$ kanamycin, and incubated as above to produce new colonies (Tekauz 1990). Isolates were stored as mycelial plugs (0.5 cm in diameter) in liquid nitrogen or at -80ºC in an ultra-low temperature freezer. Identification of the isolates as *P. teres* was based on the morphological characteristics of the conidiophores and conidia, and species-specific PCR analysis. The form of *P. teres* (*Ptt* or *Ptm*) was confirmed by form-specific PCR as described in Chapter 2 (Akhavan et al. 2015).

To produce conidia for use as inoculum, the protocol described by Lamari and Bernier (1989) as modified by Aboukhaddour et al. (2013) for the closely related fungus *Pyrenophora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) was used. Briefly, isolates were removed from storage and the mycelial plugs were placed on fresh 10% V-8 juice agar in 9 cm-diameter Petri dishes, with one plug (isolate) per dish. The Petri dishes were then stored in darkness at room temperature for 5-7 days, until the colonies reached 4-5 cm in diameter. Approximately 10 mL of sterile distilled water was then added to each Petri dish, and the fungal colonies were flattened with a sterile glass tube. The water was decanted and the Petri

dishes were incubated overnight under fluorescent light at room temperature, followed by 24 h incubation in darkness at 15°C to induce sporulation. Spore suspensions were prepared by adding 4-5 mL of sterile distilled water to the sporulating colonies and gently removing the conidia from the conidiophores with a sterile inoculation loop or an art brush.

### 4.2.2 Virulence assessments

The virulence of the *Ptt* isolates was evaluated on seedlings of nine barley differential genotypes, including the two-rowed 'Norbert', TR 473, CI 5791, and CI 9820, and the six-rowed 'Heartland', 'OAC 21', 'Steptoe', BT 201, and CI 9214. Similarly, the virulence of the *Ptm* isolates was evaluated on seedlings of 11 barley differential genotypes, including the two-rowed 'Betzes', 'Herta', 'Norbert', TR 473, CI 5791, and CI 9820, and the six-rowed 'Bonanza', 'Heartland', 'OAC 21', 'Steptoe', and CI 9214. These differential sets were developed by Tekauz (1990) to evaluate collections of *Ptt* and *Ptm* isolates made in the 1980s. For each isolate-host genotype combination, two replicates (each consisting of three barley plants in 10 cm-diameter plastic pots,) were included. The entire test was conducted twice on two different occasions. If the results were inconsistent between the two runs of the experiment for a particular isolate or host genotype, the inoculations were repeated as necessary to clarify the host reaction.

Plants were grown in growth cabinets maintained at 20°C ± 2°C with 18 h light and 6 h dark for 2 weeks, and were inoculated at the two to three leaf stage. Fungal spore concentrations were measured with a Fuchs Rosenthal Counting Chamber (Hausser Scientific, Blue Bell, PA), and manually adjusted to $1 \times 10^4$ conidia per mL for *Ptt*, and $2 \times 10^3$ conidia per mL for *Ptm*. The spore suspensions were applied at a rate of 10 mL per pot (Tekauz 1990) with a pressurized atomizer connected to an air-line. The inoculated plants were covered with plastic bags for 24 h

and maintained at almost 100% relative humidity, following which the bags were removed and the plants returned to the previous growth conditions. One week following inoculation, the second and third leaves of each seedling were rated on the 1–10 and 1–9 disease severity scales developed by Tekauz (1985) for *Ptt* and *Ptm*, respectively.

### 4.2.3 Analysis of data

For each barley differential-isolate interaction, the average disease severity was calculated by averaging the arithmetic mean of reactions in the two experiments (two leaves per plant, three plants per replicate, and two replicates per experiment). Barley differentials rated as <5 and ≥5, for *Ptt,* and 1–3 and >3, for *Ptm*, were scored as resistant and susceptible, respectively. Data were transformed into a 0-1 matrix with 0 as resistant and 1 as susceptible. The similarity matrix was then used to perform cluster analysis by the unweighted pair-group method using the arithmetic means (UPGMA) procedure and simple similarity coefficient. The qualitative data program of NTSYSpc v. 2.2 (Exeter Software, New York, NY) was used to construct the similarity matrix. The SAHN program of NTSYS-pc was employed to perform the UPGMA analysis, and dendrograms were produced using the Tree Plot program of the same software package (McLean et al. 2014). A SSR dendrogram also was produced using the same procedure and programs.

For ease of comparison between the results from the current study and those of Tekauz (1990), the isolates were grouped based on the relative number of resistant (r) and susceptible (s) reactions on the nine *Ptt* and 11 *Ptm* differentials as described by Tekauz (1990). For the *Ptt* population, these groups ranged from 9r/0s, designated as group A, to 1r/8s, designated as group J, and each isolate was further designated with a number (1-11) based on the combination of specific susceptible or resistance reactions on each of the nine barley genotypes. For the *Ptm*

population, groups ranged from 11r/0s, designated as group L, to 0r/11s, designated as group W, and each isolate was further designated with a number (1-6) according to the combination of specific reactions of each of the 11 barley genotypes (Tekauz 1990).

## 4.3 Results

### 4.3.1 Virulence of *Pyrenophora teres* f. *teres*

The reaction of each of the nine differential genotypes to each of the 39 *Ptt* isolates, along with their pathotype designations and relative virulence, is summarized in Table 4-2. Based on the reaction of the nine barley lines, the UPGMA cluster analysis using simple similarity coefficient revealed 16 pathotype groups among the 39 *Ptt* isolates, with a mean of 0.41 pathotypes per isolate (Fig. 4-1). Two pathotype groups (C04 and E09), which comprised 43% of the isolates, were found to be predominant in the collection, while nine isolates (22.5%) had virulence profiles distinct from all other isolates. The average disease severity for the 39 isolates on the nine differential genotypes was 4.5. Variation in virulence ranged from that of an isolate (MB14) which was virulent on eight of the nine differentials, with an average disease severity rating of 6.5, to an avirulent isolate (MB11) with an average rating of 2.8. Among the host genotypes tested, BT 201 and 'OAC 21' were the most susceptible to *Ptt*, with average disease ratings of almost 8.0 and 7.5, respectively. With the exception of the avirulent isolate MB11, all *Ptt* isolates examined in this study were virulent on BT 201. None of the barley differential hosts were resistant to all 39 isolates, although CI 5791 and CI 9820 were resistant to all isolates except MB14, with average ratings of 2.6 and 2.7, respectively. MB14 from Manitoba, which was the most virulent isolate, could overcome the resistance and cause mild symptoms of disease on the most resistant differential CI 5791. The percentage of *Ptt* isolates virulent on each differential genotype is shown in Fig. 4-2A. Seven pathotypes (B2, C6, D9, D10, E10, E11, and

I3) were identified for the first time in the *Ptt* population from western Canada, while pathotypes G, H and J, previously reported by Tekauz (1990), were not identified among the *Ptt* isolates studied (Fig. 4-3A).

### 4.3.2 Virulence of *Pyrenophora teres* f. *maculata*

In the case of *Ptm*, the reaction of the 11 differential host genotypes to each of the 27 isolates, along with their pathotype designations and relative virulence, is summarized in Table 4-3. The UPGMA cluster analysis revealed 13 pathotype groups (Fig. 4-4), with two of these groups (V1 and T1) comprising 52% of the isolates and therefore predominant among the *Ptm* isolates examined; nine isolates (33%) had distinct virulence profiles. Variation in virulence ranged from a Manitoba isolate (MBV25) that was virulent on 10 of the 11 differentials (with an average disease rating of 6.1) and a Saskatchewan isolate (SK60) that was virulent on all 11 differentials (and caused an average disease rating of 5.7) to an avirulent isolate from Alberta (AB57) with an average disease rating of 2.2. The percentage of virulent *Ptm* isolates on each differential genotype is shown in Fig. 4-2B. While pathotypes Q and R, which were previously reported by Tekauz (1990), were not identified in the current study, eight new *Ptm* pathotypes (L1, N1, O1, P3, T6, U3, U4 and W1) were found for the first time in western Canada (Fig. 4-3B). Among the differential genotypes tested, 'Herta', 'Betzes', 'Norbert', and TR 473 were the most susceptible, while the differential CI 9214 was resistant to all but two isolates, SK60 from Saskatchewan and MB22 from Manitoba. For both *Ptt* and *Ptm*, no clear trends were observed with respect to the geographic distribution of pathotypes in Alberta, Saskatchewan and Manitoba.

### 4.3.3 Comparative virulence and molecular polymorphism

Previously, a cluster analysis using simple sequence repeat (SSR) DNA markers was performed on a collection of 128 *Ptt* and 92 *Ptm* isolates from across western Canada (Alberta,

Saskatchewan and Manitoba) (Chapter 3; Akhavan et al. 2016). Among the isolates tested in the current study, 36 *Ptt* and 21 *Ptm* isolates were selected as representatives of the different clusters to assess variation in virulence. For these isolates, a UPGMA analysis was conducted using simple similarity coefficient on the combined *Ptt* and *Ptm* virulence data sets (Fig. 4-5). The isolates clustered in two distinct groups, with 35 *Ptt* and 6 *Ptm* isolates in the first group and 1 *Ptt* and 15 *Ptm* isolates in the second group. The results obtained through a similar analysis using SSR data (Fig. 4-6), however, indicated that the *Ptt* and *Ptm* isolates clustered into two divergent groups conforming to each form. Moreover, the dendrograms produced for these 36 *Ptt* and 21 *Ptm* isolates using the SSR and virulence data sets had a very low consensus index (CIc = 0.07).

## 4.4 Discussion

Barley cultivars with resistance to *Ptt* and *Ptm* represent effective and practical tools for the management of net blotch disease in western Canada. van den Berg and Rossnagel (1991) showed that even a moderately susceptible cultivar can provide adequate control of SFNB compared with a susceptible cultivar. However, the emergence of new pathotypes in local *Ptt* and *Ptm* populations is a great threat to the durability of the currently used sources of resistance. Up to date knowledge of the extent of variability in the virulence of pathogen populations is essential for successful deployment of any resistant cultivar. McDonald and Buchannon (1962) first reported physiologic specialization in *P. teres* in Canada. Steffenson and Webster (1992) found 13 pathotypes among 91 *Ptt* isolates from California using 22 differential barley genotypes. Wu et al. (2003) identified 15 pathotypes from a collection of 23 *Ptt* isolates collected from 12 different barley-growing regions of the world. In Australia, Gupta and Loughman (2001) identified eight virulence groups among 74 *Ptt* isolates collected from

different barley fields in Western Australia. Bouajila et al. (2012) found 23 pathotypes among 85 isolates of *Ptt* collected in Tunisia. Cromey and Parkes (2003) characterised 11 pathotypes among 29 New Zealand isolates of *Ptt* using 31 barley differential genotypes. Liu et al. (2012) showed significant differences in the virulence of isolates representing a population of *Ptt* collected from North Dakota, and also reported that the host genotypes CI 5791, 'Algerian', and 'Heartland' showed a high level of seedling resistance to all of the isolates tested. Working with *Ptm* populations collected from the Mediterranean region and the United States, Karki and Sharp (1986) found that almost 10 of the 20 differential genotypes they used showed differential reactions. Gupta et al. (2012) also reported a high pathogenic diversity in *Ptm* populations collected in Australia.

In the present study, 39 *Ptt* and 27 *Ptm* isolates were selected as representatives of *Ptt* and *Ptm* populations from across western Canada and assessed for virulence on sets of differential hosts, revealing relatively high levels of pathogenic diversity. In all, 16 *Ptt* and 13 *Ptm* pathotypes were identified. For *Ptt*, this level of pathogenic diversity (0.41 pathotype/isolate) was higher than that reported by Tekauz (1990) in western Canada (0.24 pathotype/isolate), Steffenson and Webster (1992) in California (0.14 pathotype/isolate), or by Cromey and Parkes (2003) in New Zealand (0.38 pathotype/isolate), but lower than that reported by Liu et al. (2012) in North Dakota (0.65 pathotype/isolate), Wu et al. (2003) in a global collection of *Ptt* (0.65 pathotype/isolate), and Jonsson et al. (1997) in Sweden (0.48 pathotype/isolate). Douiyssi et al. (1998) showed that each of 15 *Ptt* isolates from Morocco was unique in its virulence spectrum on 38 barley genotypes. For *Ptm*, the level of pathogenic diversity identified in the current study (0.48 pathotype/isolate) was equal to that reported by Tekauz (1990) in western Canada, but slightly lower than reported by McLean et al. (2014) for Australian isolates (0.55

pathotype/isolate). As noted by Liu et al. (2012), the pathogenic diversity and the virulence spectrum reported in different studies are dependent mainly on the pathogen population structure, as well as the genotypes included in each differential set and their differential capacity. The use of distinct differential sets makes it challenging to compare the specific pathotype composition in different regions.

The development of resistant cultivars is an effective strategy for net blotch management, and is a high priority for all western Canadian barley-breeding programs. However, a thorough knowledge of the variation in the local populations of a pathogen is required for the development of such resistant cultivars (Jalli 2011; Liu et al. 2012). It also was shown that some cultivars resistant to one form of net blotch are not resistant to the other form (Bockelman et al. 1983), which suggests that there are distinct host–pathogen interactions associated with each form of *P. teres*, and that resistance breeding efforts should focus on each form individually (Liu et al. 2011). This is currently the case in western Canada, where candidate cultivars are evaluated for each of the two forms independently (Prairie Recommending Committee for Oat and Barley 2015). Resistance against *Ptt* was reported in many barley genotypes and the inheritance and chromosomal location of the related genes were studied. Dominant and recessive major genes and quantitative trait loci (QTLs) on different barley chromosomes were shown to be involved in conferring resistance to various isolates of *Ptt* (Ho et al. 1996; Friesen et al. 2006; Abu Qamar et al. 2008; O'Boyle et al. 2011; Liu et al. 2011; Grewal et al. 2012; König et al. 2013; Liu et al. 2015). Recently, Liu et al. (2015) showed that the sensitivity to a *Ptt* proteinaceous necrotrophic effector (NE) which mapped to barley chromosome 6H accounted for 31% of the disease variation, and suggested that the barley–*Ptt* pathosystem follows, at least partially, a necrotrophic effector-triggered susceptibility (NETS) model. For *Ptm*, McLean et al. (2009, 2010)

characterized 13 lines with combinations of major and minor genes controlling seedling, adult, or all-stage resistance against SFNB. In these sources, SFNB resistance is related mostly to genes with major or minor effects, located on a single or multiple chromosomes (Williams et al. 1999, 2003; McLean et al. 2012). Wang et al. (2015) used association mapping to investigate for quantitative trait loci (QTL) controlling SFNB resistance at both the seedling and adult plant growth stages, and found 29 significant QTL; 22 of the QTL influenced resistance at both plant growth stages, while 2 and 5 QTL controlled resistance only at the seedling or adult growth stages, respectively. Neupane et al. (2015) assessed the resistance of more than 2,000 barley accessions against four geographically diverse *Ptm* isolates and found only 15 resistant barley accessions against all isolates tested. In addition, Neupane et al. (2015) reported isolate-specific susceptibility in several of the barley accessions they tested. Employing the same set of barley genotypes used by Neupane et al. (2015), Tamang et al. (2015) reported a total of 8 to 13 quantitative trait loci (QTL) associated with SFNB resistance against four different isolates from the United States, New Zealand, Australia, and Denmark.

The *Ptt* isolates in this study caused average disease severities that ranged from 2.8 to 6.5 on the barley differentials, which was similar to the range of severities reported by Liu et al. (2012) for a collection of *Ptt* isolates from North Dakota (2.6 to 6.2). Disease severities caused by the *Ptm* isolates ranged from 2.2 to 6.1. Among the genotypes included in the *Ptt* differential set, BT 201 and 'OAC 21' were the most susceptible, with average disease ratings of almost 8.0 and 7.5, respectively. In contrast, the genotypes CI 5791 and CI 9820 were resistant to all *Ptt* isolates except one, with average ratings of 2.6 and 2.7, respectively. Line CI 5791, with resistance mapped to chromosome 6 H (Friesen et al. 2014), also was found to be the most resistant among a set of barley differentials assessed against *Ptt* isolates from North Dakota (Liu et al. 2012).

Using the pathotyping procedure reported by Tekauz (1990), two *Ptt* pathotype groups (C4 and E9) comprising 43% of the isolates were predominant in western Canada. Tekauz (1990) previously found that pathotypes Cl and Gl were common in this region. The four most virulent pathotypes identified in this study included five isolates in total: three from Manitoba, one from Saskatchewan and one from central Alberta. Similarly, Tekauz (1990) found that the *Ptt* isolates in the three most virulent pathotypes were primarily from Manitoba and Saskatchewan, with only one from Alberta. MB14, an isolate from Manitoba, was the most virulent and the only isolate of *Ptt* pathotype I identified in the current study. Comparison of the present results with those of Tekauz (1990) suggests that the virulence profile of *Ptt* in western Canada has changed in the past three decades (Fig. 4-3A). Pathotypes G, H and J, which were previously reported by Tekauz (1990), were no longer identified, while seven new pathotypes (B2, C6, D9, D10, E10, E11, and I3) not reported by Tekauz (1990) were found. Nevertheless, the sources of resistance in CI 5791 and CI 9820, which have been used extensively in western Canadian breeding programs (Tekauz 1990), are still effective against all current pathotypes excluding pathotype I of *Ptt*.

For *Ptm,* two *Ptm* pathotype groups (V1 and T1) comprising 52% of the isolates were found to be predominant in western Canada. In contrast, Tekauz (1990) previously reported that pathotypes T1 and S1 were the most common in this region. The two most virulent *Ptm* pathotypes identified in this study included 11 isolates in total, five from Manitoba, four from Saskatchewan, and two from central Alberta. Tekauz (1990) reported that the most virulent *Ptm* pathotypes in his study originated from Athabasca, Alberta, followed by isolates from all three provinces. As was the case for *Ptt*, it seems that the virulence profile of *Ptm* in western Canada also has changed over the past three decades (Fig. 4-3B). While pathotypes Q and R (previously

reported by Tekauz 1990) were not identified, eight new *Ptm* pathotypes (L1, N1, O1, P3, T6, U3, U4 and W1) were found for the first time in western Canada. The differential CI 9214, with the *Rpt4* gene mapped to chromosome 7H (Williams et al. 2003), was resistant to all but two isolates, indicating that the resistance in this genotype, which has been employed for a long time in western Canadian breeding programs (Tekauz 1990), is still for the most part effective against *Ptm*. Gupta et al. (2012) and McLean et al. (2010) previously documented differences in the virulence of isolates between the eastern and western states of Australia. In the current study, however, no clear geographic trends were observed with respect to the pathotype distribution of either *Ptt* or *Ptm* in western Canada.

The differential sets developed by Tekauz (1990) for both *Ptt* and *Ptm* still seem effective for assessing variability in the virulence of western Canadian isolates of these pathogens. Afanasenko et al. (2009) developed an international barley differential set (IBDS) for *Ptt*, and proposed it for global use to profile virulence patterns in *Ptt*. Tekauz et al. (2011) assessed the utility of the IBDS in evaluating the virulence of nine Canadian *Ptt* isolates and found this differential set was effective in uncovering pathogenic variability in this collection. However, five barley genotypes had similar reaction profiles to the Canadian isolates, suggesting some redundancy. More recently, Liu et al. (2012) also developed a set of 22 barley differential lines and identified 0.65 pathotype/isolate, which is higher than found in the current study (0.41 pathotype/isolate). Therefore, in future assessments of the virulence of western Canadian *Ptt* populations, it may be useful to add the genotypes with the highest differentiating capacity from the IBDS differential set (Afanasenko et al. 2009) or those of Liu et al. (2012) to the differentials of Tekauz (1990). McLean et al. (2014) assessed the ability of 16 barley genotypes to uncover the extent of pathogenicity in an Australian *Ptm* population and characterized 33 pathotypes

among 60 isolates, but also found that some of the differentials exhibited similar reactions. McLean et al. (2009, 2012, 2014) also suggested the inclusion of five additional barley genotypes (CI 3576, CI 9776, CI 9819, CI 9831 and 'Haruna Nijo') in their differential set to develop a standard *Ptm* differential set for international use, as these genotypes could effectively differentiate pathotypes among non-Australian *Ptm* isolates. Thus, adding the genotypes with the highest differentiating capacity from the differential set proposed by McLean et al. (2012) to that of Tekauz (1990) also may result in better pathotype discrimination among western Canadian *Ptm* populations. Among the differentials used in this study, the resistance against *Ptt* has been characterised in CI5791 (Friesen et al. 2014), 'Steptoe' (Steffenson et al. 1996) and CI 9214 (Gupta et al. 2004), and against *Ptm* in CI 9214 (Williams et al. 2003). Differential sets that include genotypes that are well characterized with respect to their genetic background would lead to a more informative assessment of virulence diversity among the pathogen populations. This reinforces the need to study the genetic basis of resistance in all genotypes employed in a differential set, such as the one used in the current study or those suggested by other researchers (Liu et al. 2012; McLean et al. 2014).

The *P. teres* isolates examined in this study clustered into mixed groups based on UPGMA analysis of the combined *Ptt* and *Ptm* virulence data sets (Fig. 4-5), but into groups conforming to each form of the fungus based on the SSR data (Fig. 4-6). The dendrograms produced with the virulence and SSR data sets also had a very low consensus index. Collectively, these observations suggest that variability in the SSR sites is not likely an indication of variability in virulence, and that these two types of variation are independent. Therefore, the selection of a subset of isolates based on their SSR variability for the purposes of assessing variation in virulence may not be any more effective than a random selection of representative isolates.

The fairly high level of variability in the virulence of *Ptt* and *Ptm* populations from western Canada, combined with the fact that considerably greater diversity was found in *Ptt* relative to an earlier study (Tekauz 1990), suggests the need for the ongoing incorporation of novel sources of resistance into commercial barley cultivars in this region, and continued monitoring of pathogen populations. Resistance sources should be evaluated at both the adult plant and seedling stages (Liu et al. 2012). Moreover, the current findings confirm the conclusion of Tekauz (1990) that the virulence spectrum of *P. teres* in western Canada is broad and changes rapidly. The observation that the differentials CI 5791 and CI 9820 were resistant to all isolates of *Ptt* except one, while the differential CI 9214 was resistant to all isolates of *Ptm* except two, suggests that the resistance in these genotypes is still (for the most part) effective. Nonetheless, the identification of a *Ptt* isolate virulent on CI 5791 and CI 9820, and two *Ptm* isolates virulent on CI 9214, indicates that producers should not rely exclusively on host resistance as the sole strategy for net blotch management, but rather use resistance together with other strategies such as crop rotation and the judicious application of fungicides.

## 4.5 Tables

**Table 4-1.** Isolate number, location and year for a collection of 39 *Pyrenophora teres* f. *teres* (*Ptt*) and 27 *P. teres* f. *maculata* (*Ptm*) isolates used to study pathogenic variability of *P. teres* populations from western Canada.

| Isolate number | Location collected | Year collected |
|---|---|---|
| *Ptt*-AB01 | Lacombe, AB | 2010 |
| *Ptt*-AB04 | Bentley, AB | 2010 |
| *Ptt*-AB06 | Bentley, AB | 2010 |
| *Ptt*-AB11 | East Gull Lake, AB | 2010 |
| *Ptt*-AB12 | Clive, AB | 2010 |
| *Ptt*-AB16 | Bentley, AB | 2010 |
| *Ptt*-AB28 | Lacombe, AB | 2010 |
| *Ptt*-AB32 | Clive, AB | 2011 |
| *Ptt*-AB34 | Bentley, AB | 2011 |
| *Ptt*-AB35 | Bentley, AB | 2011 |
| *Ptt*-AB38 | Clive, AB | 2011 |
| *Ptt*-AB48 | Lacombe, AB | 2011 |
| *Ptt*-AB51 | Lacombe, AB | 2011 |
| *Ptt*-AB53 | Lacombe, AB | 2011 |
| *Ptt*-ABV01 | Lacombe, AB | 2012 |
| *Ptt*-ABV18 | Lacombe, AB | 2012 |
| *Ptt*-ABV28 | Lacombe, AB | 2012 |
| *Ptt*-MB01 | Binscarth, MB | 2009 |
| *Ptt*-MB02 | Kirkella, MB | 2009 |
| *Ptt*-MB03 | Oak River, MB | 2009 |
| *Ptt*-MB04 | Montcalm, MB | 2009 |
| *Ptt*-MB05 | Unknown, MB | 2010 |
| *Ptt*-MB06 | Minto, MB | 2010 |
| *Ptt*-MB10 | Chater, MB | 2011 |
| *Ptt*-MB11 | Oakner, MB | 2011 |
| *Ptt*-MB14 | Portage La Prairie, MB | 2011 |

| | | |
|---|---|---|
| *Ptt*-MB15 | Brandon, MB | 2011 |
| *Ptt*-SK01 | Weirdale, SK | 2010 |
| *Ptt*-SK03 | Mcmahon, SK | 2010 |
| *Ptt*-SK05 | Ridgedale, SK | 2010 |
| *Ptt*-SK07 | Rokeby, SK | 2010 |
| *Ptt*-SK08 | McTaggart, SK | 2010 |
| *Ptt*-SK16 | Semans, SK | 2011 |
| *Ptt*-SK24 | Naicam, SK | 2011 |
| *Ptt*-SK26 | St Brieux, SK | 2011 |
| *Ptt*-SK33 | Gronlid, SK | 2011 |
| *Ptt*-SK41 | Albertville, SK | 2011 |
| *Ptt*-SK52 | Naicam, SK | 2011 |
| *Ptt*-SK53 | Naicam, SK | 2011 |
| *Ptm*-AB57 | Park St Rimbey, AB | 2010 |
| *Ptm*-AB58 | Bentley, AB | 2010 |
| *Ptm*-AB61 | Penhold, AB | 2010 |
| *Ptm*-AB68 | Lousana, AB | 2010 |
| *Ptm*-AB74 | Clive, AB | 2010 |
| *Ptm*-AB79 | Edmonton, AB | 2010 |
| *Ptm*-AB82 | Lacombe, AB | 2011 |
| *Ptm*-ABV14 | Lacombe, AB | 2012 |
| *Ptm*-MB16 | Selkirk, MB | 2009 |
| *Ptm*-MB21 | Sinclair, MB | 2009 |
| *Ptm*-MB22 | Pipestone, MB | 2010 |
| *Ptm*-MB23 | Binscarth, MB | 2010 |
| *Ptm*-MB24 | Oak River, MB | 2010 |
| *Ptm*-MB26 | Oak River, MB | 2010 |
| *Ptm*-MB32 | Deloraine, MB | 2010 |
| *Ptm*-MBV25 | Roblin, MB | 2012 |
| *Ptm*-MBV37 | Portage la Prairie, MB | 2012 |
| *Ptm*-SK60 | Stony Beach, SK | 2010 |
| *Ptm*-SK64 | Dollard, SK | 2010 |

| | | |
|---|---|---|
| *Ptm*-SK69 | Richardson, SK | 2010 |
| *Ptm*-SK73 | Assiniboia, SK | 2011 |
| *Ptm*-SK87 | Sylvania, SK | 2011 |
| *Ptm*-SK88 | Sylvania, SK | 2011 |
| *Ptm*-SK100 | Naicam, SK | 2011 |
| *Ptm*-SKV08 | Melfort, SK | 2012 |
| *Ptm*-SKV10 | Melfort, SK | 2012 |
| *Ptm*-SKV12 | Melfort, SK | 2012 |

**Table 4-2.** Pathogenic variation of 39 Canadian isolates of *Pyrenophora teres* f. *teres* (net form net blotch of barley) on nine barley differentials based on a 1 – 10 scale. Regular and boldface numbers denote resistant (<5) and susceptible (≥5) phenotypes, respectively.

| Isolate | CI5791 | CI9820 | TR473 | Norbert | BT201 | Heartland | Steptoe | CI9214 | OAC21 | Average | Pathotype |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MB11 | 2.0 | 3.4 | 3.0 | 2.8 | 3.9 | 2.3 | 3.4 | 2.2 | 2.7 | 2.8 | A01 |
| AB11 | 2.0 | 2.3 | 3.3 | 2.7 | **7.3** | 2.0 | 2.1 | 2.5 | 3.7 | 3.1 | B02 |
| MB10 | 3.0 | 3.0 | 3.7 | 3.7 | **9.1** | 3.7 | 4.0 | 4.9 | **9.5** | 4.9 | C04 |
| AB53 | 3.0 | 3.8 | 2.9 | 3.8 | **8.8** | 3.8 | 3.0 | 4.0 | **9.3** | 4.7 | C04 |
| ABV28 | 3.0 | 3.0 | 2.0 | 3.0 | **9.0** | 3.5 | 4.0 | 4.9 | **8.5** | 4.5 | C04 |
| SK33 | 2.5 | 3.2 | 3.3 | 2.9 | **7.9** | 3.8 | 3.3 | 3.3 | **7.3** | 4.2 | C04 |
| AB04 | 1.7 | 1.9 | 2.0 | 2.1 | **9.3** | 2.8 | 4.0 | 3.9 | **9.0** | 4.1 | C04 |
| MB04 | 2.5 | 2.6 | 2.3 | 2.4 | **8.8** | 2.9 | 2.8 | 3.8 | **8.3** | 4.0 | C04 |
| AB01 | 2.0 | 2.7 | 2.2 | 2.3 | **7.4** | 2.1 | 2.2 | 3.9 | **8.5** | 3.7 | C04 |
| ABV01 | 3.0 | 3.0 | 3.0 | 3.0 | **7.0** | 2.5 | 3.0 | 2.0 | **6.0** | 3.6 | C04 |
| SK52 | 2.1 | 1.8 | 3.8 | **5.6** | **6.6** | 1.5 | 3.7 | 1.4 | 1.5 | 3.1 | C06 |
| SK07 | 2.1 | 3.4 | 2.5 | 3.7 | **9.3** | 2.5 | 3.6 | **5.3** | **9.8** | 4.7 | D04 |
| AB28 | 2.3 | 2.6 | 1.9 | 2.1 | **8.5** | 2.9 | 2.6 | **6.8** | **9.0** | 4.3 | D04 |
| SK08 | 1.7 | 1.9 | 1.5 | 2.5 | **9.1** | 2.3 | 2.7 | **5.8** | **9.2** | 4.1 | D04 |
| AB06 | 1.5 | 2.1 | 2.0 | 1.7 | **7.5** | 2.0 | 2.7 | **7.0** | **8.4** | 3.9 | D04 |
| AB51 | 4.0 | 3.6 | 3.0 | 3.5 | **8.3** | 3.8 | **7.4** | 4.0 | **7.5** | **5.0** | D05 |
| AB35 | 3.0 | 2.6 | 2.4 | 2.7 | **8.9** | 4.2 | **5.8** | 4.2 | **8.7** | 4.7 | D05 |
| SK53 | 3.1 | 2.4 | 4.0 | 4.9 | **8.0** | 2.9 | **5.0** | 3.4 | **8.3** | 4.7 | D05 |
| AB32 | 1.8 | 1.5 | 1.8 | 1.5 | **8.3** | 2.4 | **8.2** | 3.2 | **8.9** | 4.2 | D05 |
| SK03 | 3.8 | 3.3 | **6.6** | **7.0** | **8.3** | 2.5 | 3.8 | 3.3 | 2.7 | 4.6 | D09 |
| SK24 | 2.0 | 2.4 | **5.6** | 4.0 | **8.0** | 2.5 | **5.5** | 1.5 | 2.2 | 3.7 | D10 |
| ABV18 | 3.0 | 3.0 | 3.0 | 3.5 | **9.0** | 3.0 | **8.0** | **5.5** | **5.5** | 4.8 | E02 |
| MB15 | 1.8 | 2.2 | 2.0 | 2.0 | **9.0** | 2.4 | **6.3** | **5.8** | **9.0** | 4.5 | E02 |
| AB16 | 3.5 | 3.3 | 3.3 | 3.0 | **9.3** | **7.8** | 3.3 | **7.3** | **9.3** | **5.5** | E03 |
| AB38 | 2.5 | 2.1 | 2.2 | 2.9 | **9.3** | **5.8** | **6.5** | 3.0 | **9.1** | 4.8 | E07 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MB01 | 2.4 | 2.6 | **7.5** | **7.3** | **7.5** | 2.1 | 2.9 | 4.9 | **8.5** | **5.1** | E09 |
| SK26 | 2.9 | 2.8 | **7.8** | **8.1** | **7.7** | 1.8 | 2.3 | 2.6 | **8.1** | 4.9 | E09 |
| MB03 | 2.0 | 2.3 | **8.3** | **7.1** | **7.8** | 2.4 | 3.0 | 2.6 | **7.4** | 4.8 | E09 |
| AB12 | 2.3 | 2.1 | **7.3** | **7.7** | **8.1** | 1.3 | 2.7 | 1.3 | **7.8** | 4.5 | E09 |
| SK16 | 3.0 | 2.9 | **5.7** | **7.1** | **6.4** | 1.7 | 4.5 | 2.3 | **6.0** | 4.4 | E09 |
| MB06 | 2.3 | 2.2 | **5.7** | **6.6** | **6.3** | 1.4 | 3.8 | 2.0 | **8.3** | 4.3 | E09 |
| SK05 | 2.1 | 2.2 | **5.6** | **6.2** | **7.3** | 1.0 | 2.8 | 2.6 | **7.8** | 4.2 | E09 |
| AB48 | 2.0 | 2.0 | **6.0** | **7.1** | **6.9** | 1.1 | 2.1 | 1.3 | **7.8** | 4.0 | E09 |
| SK01 | 2.1 | 1.8 | **6.0** | **5.8** | **6.4** | 1.2 | 1.8 | 1.0 | **7.0** | 3.7 | E09 |
| WRS102 | 3.4 | 3.3 | 3.5 | 3.9 | **9.3** | **5.1** | **7.3** | 4.5 | **9.5** | **5.5** | E10 |
| MB02 | 3.7 | 3.3 | **7.8** | **7.8** | **8.8** | 3.6 | 3.8 | **5.6** | 4.3 | **5.4** | E11 |
| MB05 | 3.0 | 2.8 | 3.3 | 4.3 | **9.0** | **6.2** | **7.6** | **7.6** | **9.3** | **5.9** | F01 |
| AB34 | 2.8 | 2.7 | 3.5 | 3.6 | **9.0** | **6.3** | **7.6** | **6.9** | **9.7** | **5.8** | F01 |
| SK41 | 2.9 | 3.7 | **7.3** | **6.9** | **6.2** | 2.8 | **8.2** | 3.3 | **8.7** | **5.6** | F06 |
| MB14 | **5.3** | **6.7** | 3.9 | **6.5** | **9.0** | **6.2** | **6.0** | **5.8** | **9.3** | **6.5** | I03 |
| Average | 2.6 | 2.8 | 4.1 | 4.4 | **8.0** | 3.0 | 4.3 | 3.9 | **7.5** | 4.5 | N/A |

**Table 4-3.** Pathogenic variation of 27 Canadian isolates of *Pyrenophora teres* f. *maculata* (spot form net blotch of barley) on 11 barley differentials based on a 1 – 9 scale. Regular and boldface numbers denote resistant (1-3) and susceptible (>3) phenotypes, respectively.

| Isolate | CI5791 | CI9820 | TR473 | Norbert | Heartland | Steptoe | CI9214 | OAC21 | Bonanza | Herta | Betzes | Average | Pathotype |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AB57 | 1.90 | 1.80 | 2.40 | 2.40 | 1.40 | 1.80 | 1.70 | 3.00 | 2.60 | 2.50 | 3.00 | 2.20 | L01 |
| AB68 | 2.50 | 2.50 | 2.20 | 2.10 | 2.00 | 2.60 | 2.30 | 2.10 | 2.80 | 2.60 | 2.90 | 2.40 | L01 |
| AB82 | 2.40 | 2.80 | 2.80 | 2.60 | 1.80 | **3.80** | 1.60 | 2.40 | 2.80 | 3.00 | **4.00** | 2.70 | N01 |
| MB32 | 3.00 | 3.00 | **4.00** | **4.30** | 2.50 | 3.00 | 2.50 | 3.00 | 3.00 | **4.00** | **4.00** | **3.30** | P03 |
| AB61 | 3.00 | 3.00 | 3.00 | 3.00 | 2.80 | 3.00 | 3.00 | 3.00 | **5.00** | **4.00** | **5.50** | **3.50** | O01 |
| WRS857 | **3.70** | 3.00 | **3.90** | **3.50** | **4.00** | 3.00 | **4.00** | 3.00 | 3.00 | **3.80** | **5.00** | **3.60** | T06 |
| MB26 | **3.90** | **4.80** | **4.80** | **5.30** | 3.00 | **4.80** | 3.00 | 3.00 | **4.00** | **4.50** | **5.00** | **4.20** | T01 |
| SK88 | 3.00 | **4.00** | **4.50** | **5.20** | 2.50 | **5.00** | 3.00 | 3.00 | **4.40** | **6.80** | **6.50** | **4.40** | S01 |
| SK87 | 3.00 | 3.00 | **5.80** | **6.50** | **4.50** | **4.50** | 2.50 | **4.00** | **6.30** | **5.00** | **5.00** | **4.60** | T04 |
| SK69 | **4.00** | **4.30** | **4.80** | **6.80** | 3.00 | **5.40** | 2.80 | 2.80 | **5.50** | **5.60** | **5.10** | **4.60** | T01 |
| MB22 | **4.00** | **4.00** | **6.00** | **6.50** | 3.00 | **4.50** | **4.00** | 3.00 | **5.00** | **5.00** | **6.00** | **4.60** | U04 |
| AB58 | 3.00 | **5.20** | **6.10** | **5.30** | **3.80** | **4.90** | 3.00 | 3.00 | **5.60** | **6.90** | **4.90** | **4.70** | U02 |
| AB74 | **4.50** | **5.40** | **6.00** | **4.80** | 3.00 | **5.50** | 3.00 | **4.00** | **4.60** | **6.80** | **5.70** | **4.80** | U01 |
| SK73 | **4.30** | **5.50** | **5.30** | **5.70** | 2.90 | **5.10** | 2.50 | **5.30** | **6.70** | **4.10** | **6.00** | **4.90** | U01 |
| SKV08 | **4.00** | **5.00** | **7.00** | **6.00** | 3.00 | **5.00** | 2.00 | 3.00 | **5.50** | **7.00** | **6.00** | **4.90** | T01 |
| SK100 | **4.00** | **5.00** | **7.00** | **7.00** | 3.00 | **4.00** | 3.00 | 3.00 | **5.00** | **6.00** | **7.00** | **4.90** | T01 |
| MB23 | **3.60** | **5.00** | **5.80** | **7.10** | **3.60** | 3.00 | 2.70 | **3.70** | **6.20** | **7.10** | **6.30** | **4.90** | U03 |
| MB24 | **4.80** | **5.00** | **5.80** | **6.80** | **4.30** | **5.10** | 3.00 | **3.90** | **5.40** | **5.20** | **5.30** | **5.00** | V01 |
| SK64 | **5.00** | **5.00** | **6.00** | **5.60** | **4.00** | **3.70** | 3.00 | **3.80** | **6.30** | **6.40** | **6.80** | **5.10** | V01 |
| AB79 | **4.00** | **4.80** | **5.80** | **6.10** | **3.80** | **5.00** | 2.90 | **3.90** | **5.80** | **6.80** | **6.70** | **5.10** | V01 |
| MB16 | **4.80** | **5.00** | **6.40** | **6.30** | **4.50** | **4.50** | 2.50 | **4.50** | **6.00** | **6.80** | **5.30** | **5.10** | V01 |
| SKV12 | **4.00** | **5.00** | **7.50** | **8.00** | **4.00** | **5.00** | 2.00 | **4.00** | **5.50** | **7.00** | **6.00** | **5.30** | V01 |
| ABV14 | **5.00** | **5.00** | **6.50** | **7.50** | **4.30** | **4.50** | 3.00 | **3.80** | **4.00** | **8.50** | **8.00** | **5.50** | V01 |
| SKV10 | **4.50** | **4.50** | **8.00** | **8.00** | **4.50** | **4.50** | 3.00 | **4.00** | **6.00** | **8.00** | **6.50** | **5.60** | V01 |
| MB21 | **4.00** | **4.00** | **7.50** | **8.00** | **4.50** | **6.00** | 3.00 | **4.50** | **6.50** | **7.00** | **7.00** | **5.60** | V01 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SK60 | **4.50** | **5.50** | **6.00** | **5.50** | **5.30** | **5.40** | **4.50** | **4.50** | **7.50** | **7.20** | **6.80** | **5.70** | W01 |
| MBV37 | **5.50** | **5.50** | **7.30** | **7.50** | **4.00** | **4.00** | 3.00 | **4.00** | **5.50** | **9.00** | **8.30** | **5.80** | V01 |
| MBV25 | **5.00** | **6.00** | **8.00** | **7.30** | **5.40** | **4.00** | 3.00 | **4.00** | **8.00** | **9.00** | **7.50** | **6.10** | V01 |
| Average | **3.90** | **4.40** | **5.60** | **5.70** | **3.50** | **4.30** | 2.80 | **3.50** | **5.20** | **5.90** | **5.80** | **4.60** | N/A |

## 4.6 Figures

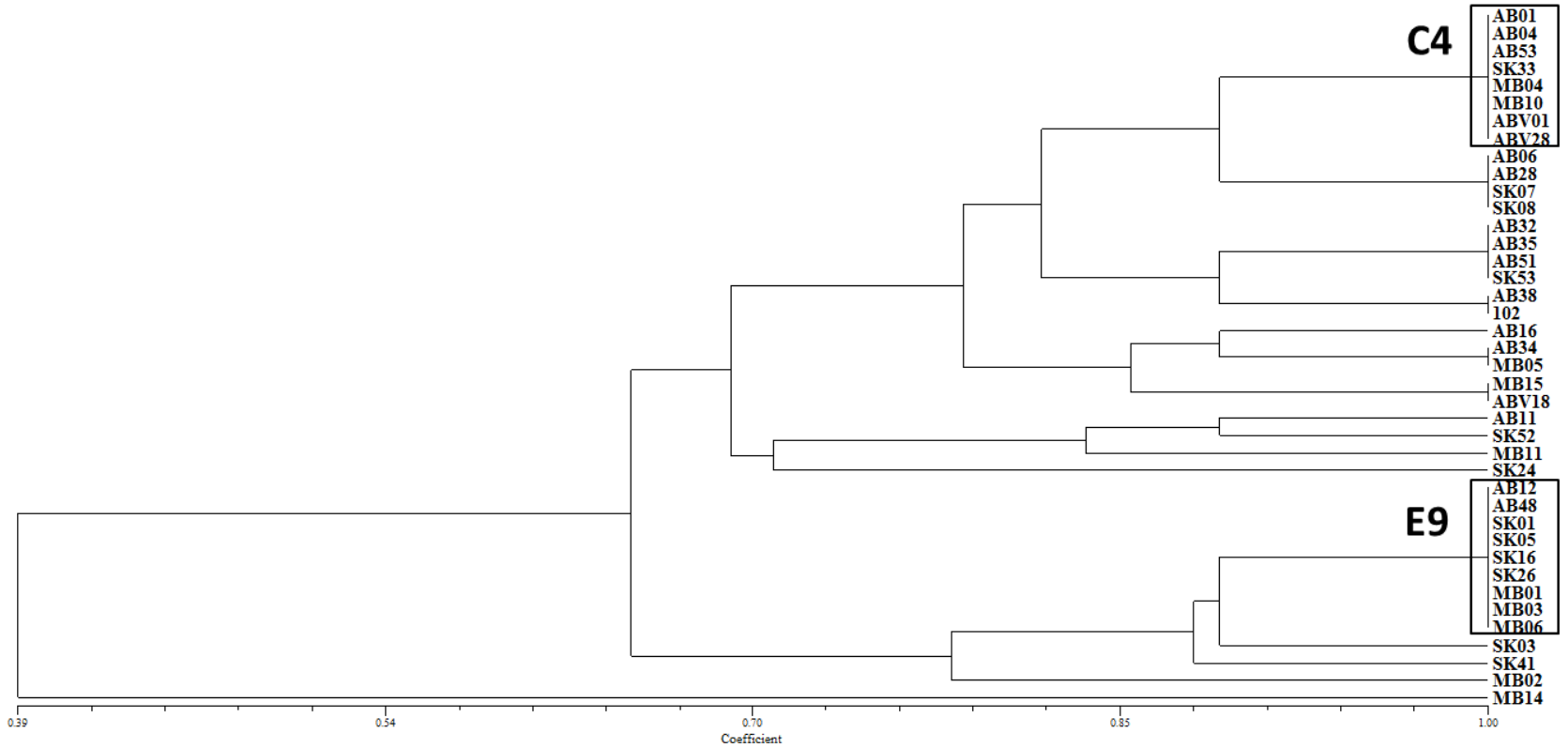


**Figure 4-1.** Pathogenic similarity of 39 *Pyrenophora teres* f. *teres* (net form net blotch of barley) isolates recovered from barley crops in western Canada; two pathotype groups, highlighted in boxes, were found to be predominant. The dendrogram was produced using the unweighted pair-group method using the arithmetic means (UPGMA) procedure and simple similarity coefficient with NTSYSpc ver. 2.2.

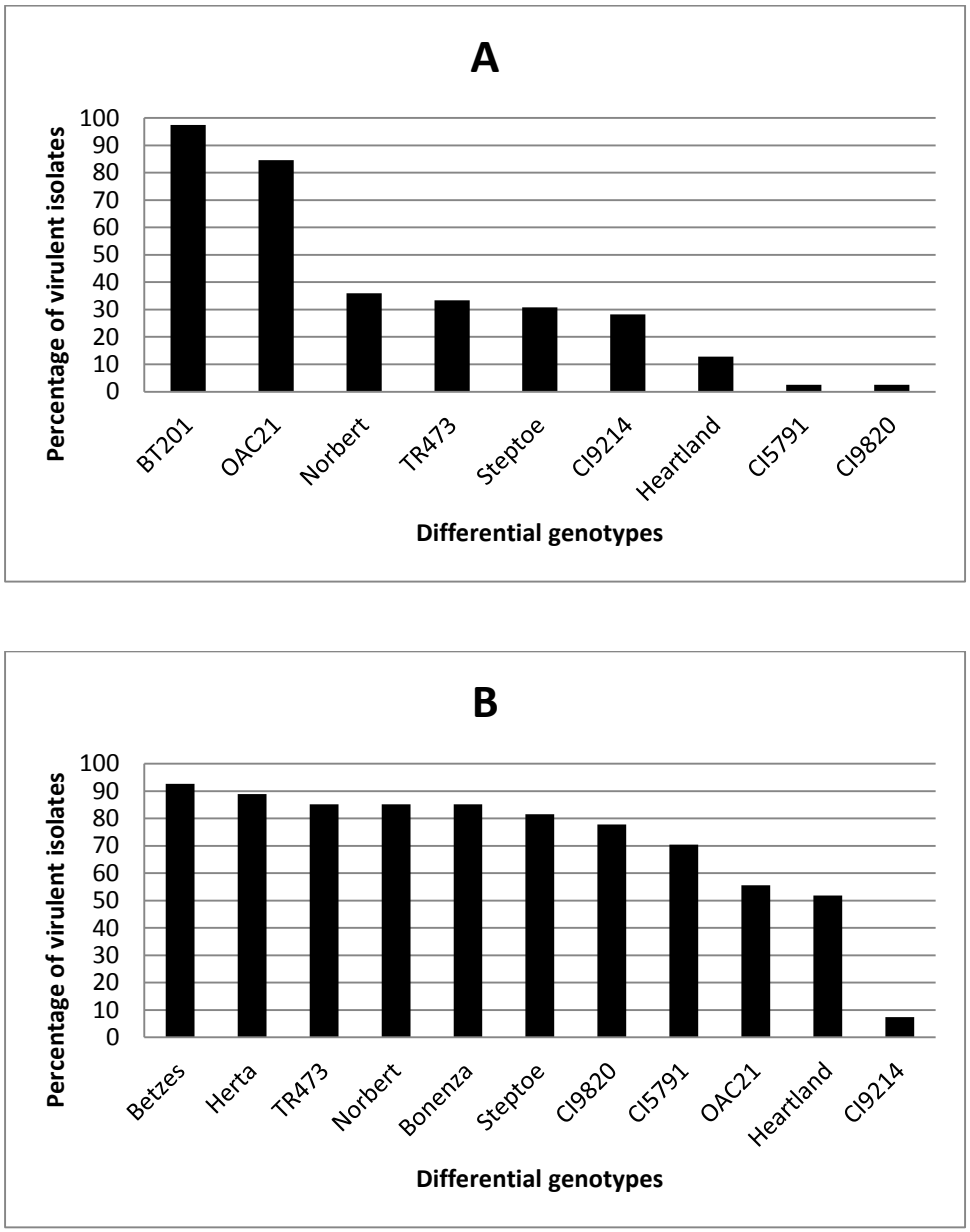


**Figure 4-2. (A)** Percentage of western Canadian *Pyrenophora teres* f. *teres* isolates virulent on nine barley differential genotypes; BT 201 (97.4%, 38 isolates), 'OAC 21' (84.6%, 33 isolates), 'Norbert' (35.9%, 14 isolates), TR 473 (33.3%, 13 isolates), 'Steptoe' (30.8%, 12 isolates), CI 9214 (28.2%, 11 isolates), 'Heartland' (12.8%, 5 isolates), CI 5791 (2.6%, 1 isolate), CI 9820 (2.6%, 1 isolate). **(B)** Percentage of western Canadian *Pyrenophora teres* f. *maculata* isolates virulent on 11 barley differential genotypes; 'Betzes' (92.6%, 25 isolates), 'Herta' (88.9%, 24 isolates), TR 473 (85.2%, 23 isolates), 'Norbert' (85.2%, 23 isolates), 'Bonanza' (85.2%, 23 isolates), 'Steptoe' (81.5%, 22 isolates), CI 9820 (77.8%, 21 isolates), CI 5791 (70.4%, 19 isolates), 'OAC 21' (55.6%, 15 isolates), 'Heartland' (51.9%, 14 isolates), CI 9214 (7.4%, 2 isolates).

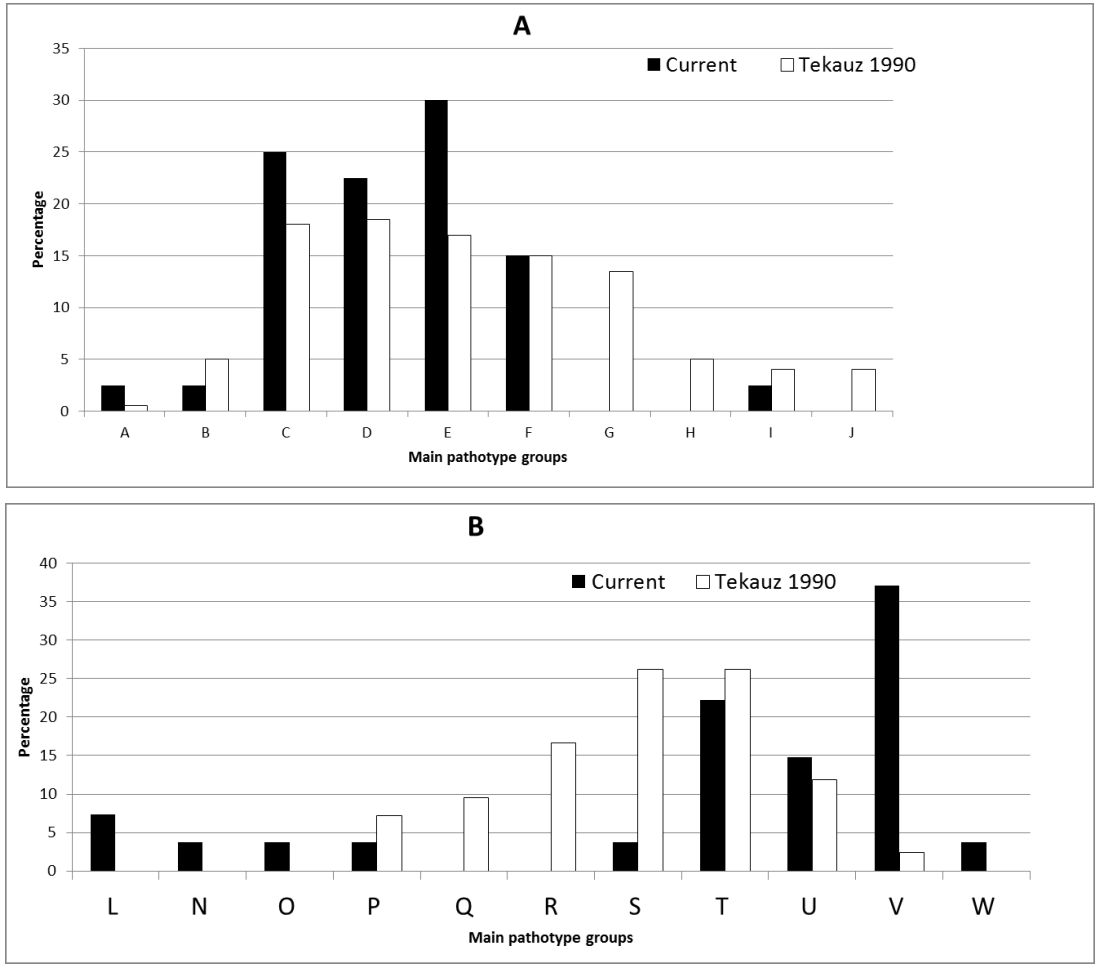


**Figure 4-3. (A)** Comparison of the main pathotype groups (A – J) found among *Pyrenophora teres* f. *teres* (net form net blotch of barley) populations from western Canada in 1985 (Tekauz, 1990) versus 2009-2011 (current study). **(B)** Comparison of the main pathotype groups (L - W) found among the *Pyrenophora teres* f. *maculata* (spot form net blotch of barley) populations from western Canada in 1985 (Tekauz, 1990) versus 2009-2011 (current study).

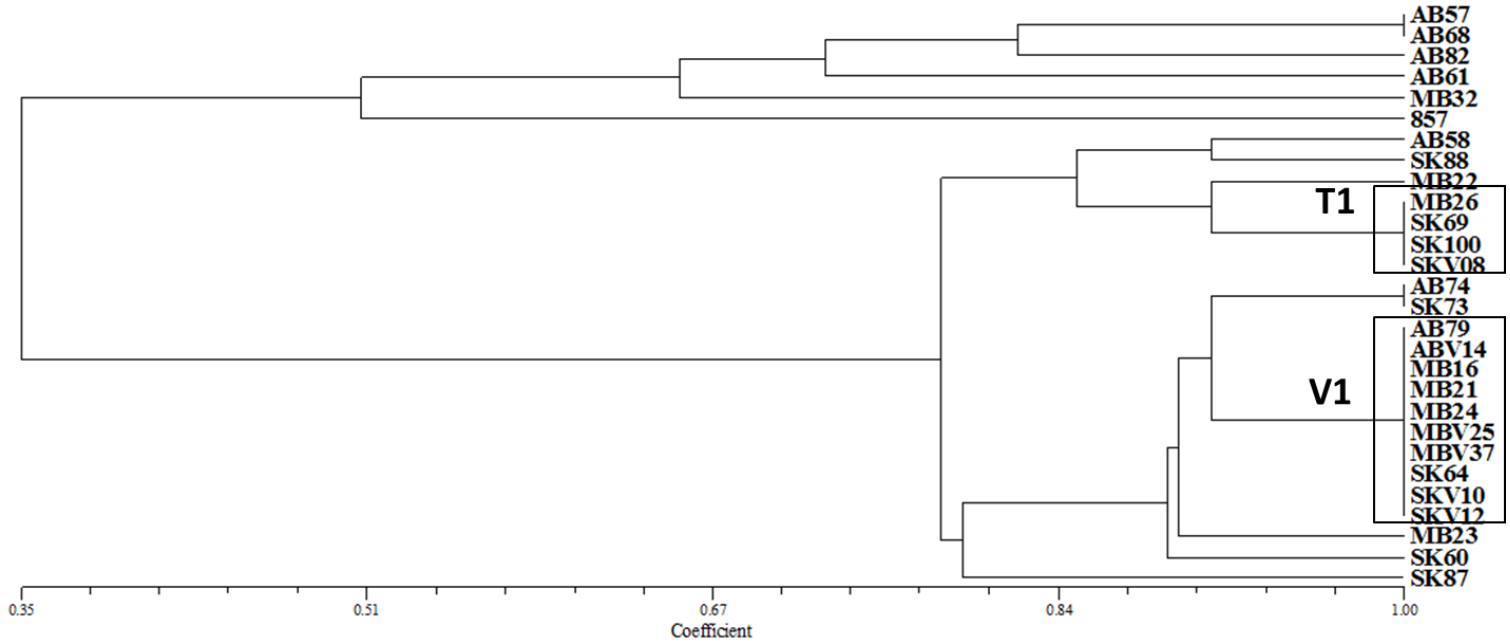


**Figure 4-4.** Pathogenic similarity of *Pyrenophora teres* f. *maculata* (spot form net blotch of barley) isolates recovered from barley crops in western Canada; two pathotype groups, highlighted in boxes, were found to be predominant. The dendrogram was produced using the unweighted pair-group method using the arithmetic means (UPGMA) procedure and simple similarity coefficient with NTSYSpc ver. 2.2.

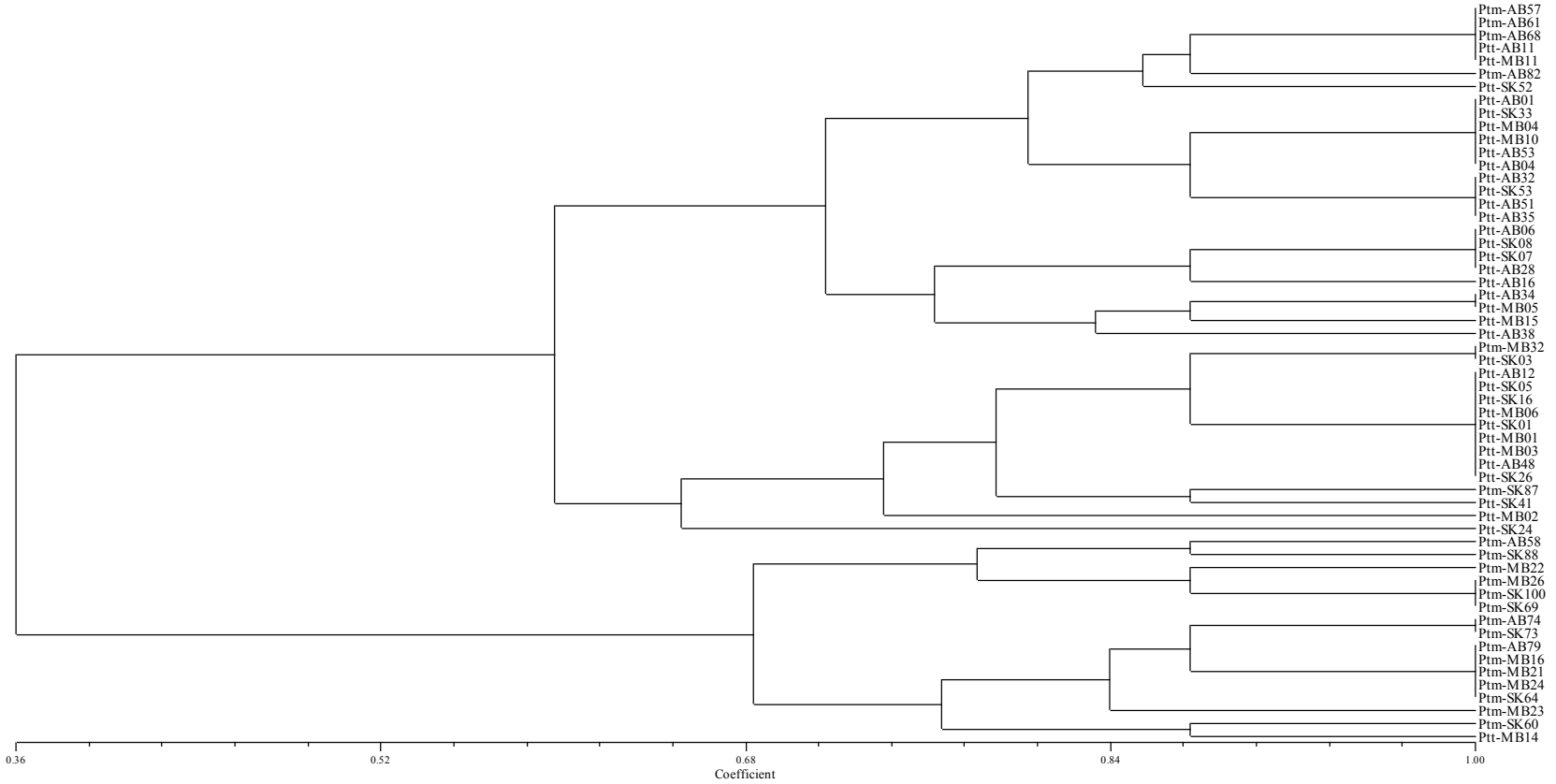


**Figure 4-5.** Pathogenic similarity of 36 *P. teres* f. *teres* (*Ptt*) and 21 *P. teres* f. *maculata* (*Ptm*) isolates collected from western Canada. Isolates clustered in two distinct groups with 35 *Ptt* and 6 *Ptm* isolates in the first group and 1 *Ptt* and 15 *Ptm* isolates in the second group. The dendrogram was produced using the unweighted pair-group method using the arithmetic means (UPGMA) procedure and simple similarity coefficient with NTSYSpc ver. 2.2.

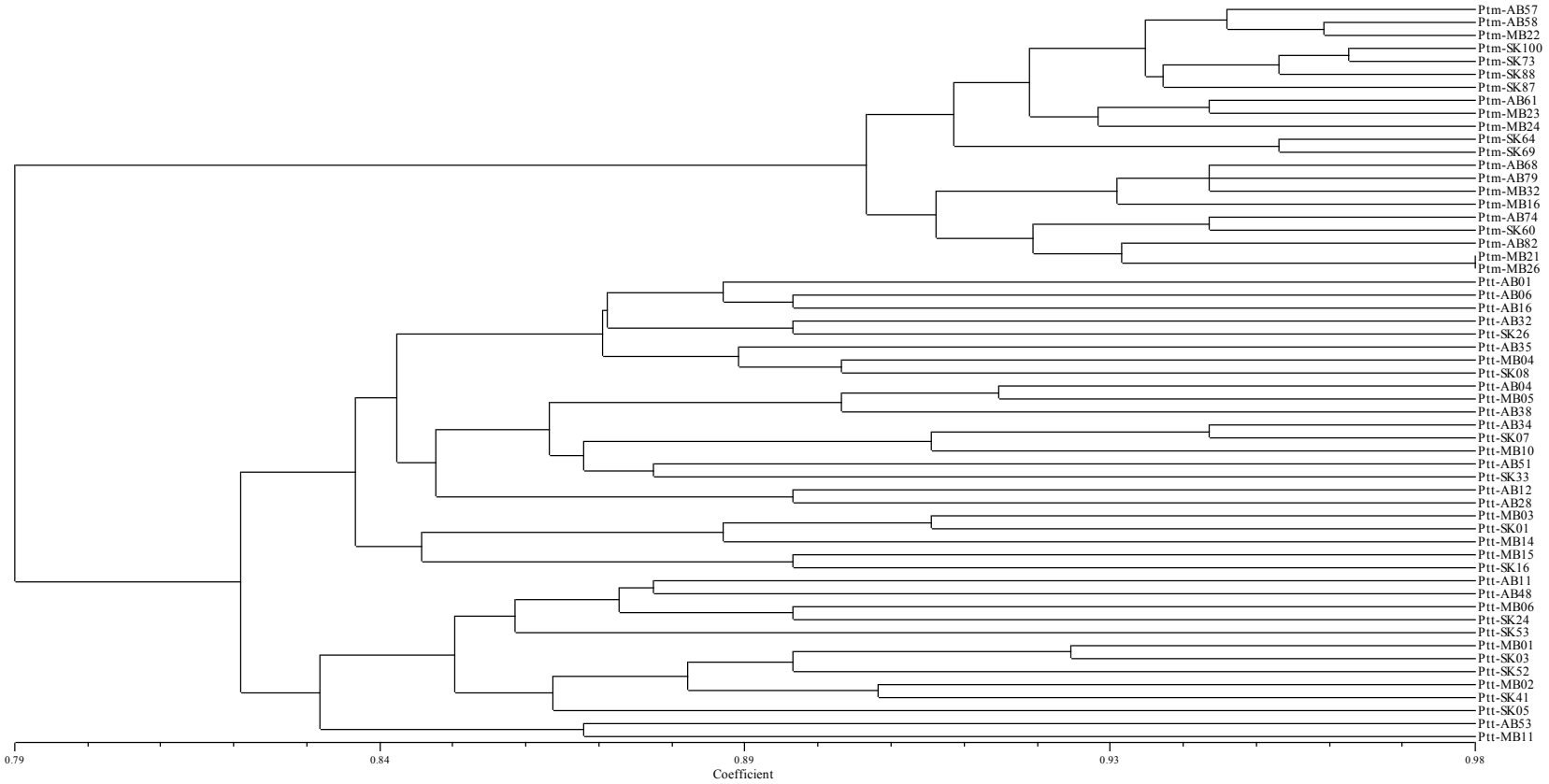


**Figure 4-6.** Genetic similarity of 36 *P. teres* f. *teres* (*Ptt*) and 21 *P. teres* f. *maculata* (*Ptm*) isolates collected from western Canada. Cluster analysis revealed that all isolates clustered in two distinct divergent groups conforming to either *Ptt* or *Ptm*, with no intermediate cluster between the two forms. The dendrogram was produced using the unweighted pair-group method using the arithmetic means (UPGMA) procedure and simple similarity coefficient with NTSYSpc ver. 2.2.

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# Chapter 5 Resistance to *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* in Canadian Barley Genotypes[4]

## 5.1 Introduction

*Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.) is a destructive foliar pathogen of barley (*Hordeum vulgare* L.) in western Canada (Tekauz 1990; Xi et al. 1999; Grewal et al. 2012) and worldwide (Steffenson 1997). *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) are the two genetically distinct forms of *P. teres*, which incite the net form of net blotch (NFNB) and the spot form of net blotch (SFNB), respectively (Tekauz 1990; Rau et al. 2007). Yield losses of 10% to 40% for NFNB and up to 44% for SFNB are typical for infected barley crops, but this disease has the potential to cause total loss in susceptible cultivars under conditions conducive for disease development (Steffenson et al. 1991; Jayasena et al. 2007; Murray and Brennan 2010). In western Canada, both forms of *P. teres* appear to have mixed sexual and asexual reproduction and an outcrossing mating system, leading to the occurrence of genetically diverse populations in this region (Chapters 2-4; Akhavan et al. 2015; 2016b; 2016a). As a consequence, *Ptt* and *Ptm* fall into the category of pathogens with a high risk of being able to develop populations with abilities to overcome host resistance genes and fungicides, particularly those with single modes of action (McDonald and Linde 2002).

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Many barley breeding lines and commercial cultivars have been reported as sources of resistance against *Ptt* and *Ptm*, and the inheritance mechanisms and chromosomal locations of the related genes have been investigated. In the case of NFNB, dominant and recessive major genes and quantitative trait loci (QTLs), many of which mapped to chromosome 6H, were shown to be involved in conferring resistance or susceptibility to various *Ptt* isolates (reviewed in Liu et al. 2011, 2015; Konig et al. 2014; Afanasenko et al. 2015). For SFNB, one or more seedling and/or adult plant resistance genes were identified on chromosomes 3H, 4H, 5H, 6H and 7H in different barley lines (Molnar et al. 2000; Friesen et al. 2006; Gupta et al. 2006; Manninen et al. 2006; Grewal et al. 2007, 2012). McLean et al. (2009, 2010) also characterized 13 lines with combinations of major and minor genes, located on a single or multiple chromosomes (Williams et al. 1999, 2003; McLean et al. 2012), which confer seedling, adult or all-stage resistance against SFNB.

Many studies investigated the reactions of barley genotypes against *Ptt* and *Ptm* at the seedling stage (Tekauz 1990; Douiyssi et al. 1998). It has been shown that seedling reactions are often consistent with adult plant reactions (Buchannon and McDonald 1965; Cakir et al. 2003; Grewal et al. 2008, 2012). Grewal et al. (2008) and Cakir et al. (2003) compared seedling and adult plant resistance within the same populations, and showed that the resistance reaction conferred by the 6H locus was effective at both the seedling and adult plant stages, indicating that seedling resistance is also effective at the adult plant stage in these cases. However, in other studies, host reactions differed at the seedling and adult stages (Douiyssi et al. 1998; Grewal et al. 2012). It was also shown that resistance in some Manchurian cultivars increased with age (Khan and Boyd 1969). Tekauz (1986) also suggested that older plants were more resistant to NFNB and less resistant to SFNB based on the isolates they tested.

Variability and associated shifts in the virulence profiles of local pathogen populations can play a role in overcoming the resistance in host genotypes. It was shown that the breeding lines CI 4976 and CI 9820, reported to be resistant under both greenhouse and field conditions in Canada and Minnesota (Buchannon and McDonald 1965; Keeling and Banttari 1975; Suganda and Wilcoxson 1993), were susceptible in the field in Morocco (Douiyssi et al. 1998). Furthermore, many isolates from Syria and Tunisia were reported as virulent on CI 9820 at the seedling stage (Bouajila et al. 2012). Jonsson et al. (1997) also showed that European barley cultivars were susceptible to all the Swedish isolates tested, while some were resistant to the Canadian isolate WRS 1607. This suggests the importance of local pathotype composition of the net blotch pathogen in relation to the effectiveness of sources of resistance. In addition, many cultivars resistant to one form of net blotch were not resistant to the other form (Bockelman et al. 1983; Liu et al. 2011; Grewal et al. 2012), indicating the possibility of distinct host–pathogen interactions associated with each form of *P. teres*, and that resistance breeding efforts should focus on each form independently (Liu et al. 2011). This is the practice in western Canada, where candidate barley cultivars are screened for each of the two forms independently (Prairie Recommending Committee for Oat and Barley 2015).

Canadian barley is grown mainly in the prairie provinces of Alberta, Saskatchewan and Manitoba (Statistics Canada 2015), and both NFNB and SFNB are among the most important foliar diseases of barley in this region (Turkington et al. 2011). The objective of this study was to assess how commercial cultivars and breeding lines previously identified as resistant to NFNB and/or SFNB in western Canada respond to recently collected isolates of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata*. These breeding lines and cultivars were evaluated at the seedling stage against 12 recently characterized *Ptt* and *Ptm* isolates collected from the provinces of

Alberta, Manitoba and Saskatchewan. Finally, this study considered whether significant genotype × isolate interactions could be detected between barley genotypes and isolates of *Ptt* and *Ptm*.

## 5.2 Materials and methods

### 5.2.1 Fungal isolates and preparation of inoculum

Seven *Ptt* and seven *Ptm* isolates (six test isolates and one reference isolate) were used to screen the barley genotypes for NFNB and SFNB resistance, respectively (Table 5-1). These isolates were collected from commercial fields across western Canada, and were previously characterized for pathotype designation on host differential sets (Chapter 4; Akhavan et al. 2016a). To induce sporulation and prepare inoculum, a procedure described by Lamari and Bernier (1989) as modified by Aboukhaddour et al. (2013) for the closely related pathogen *Pyrenophora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) was employed. Briefly, representative isolates were taken from storage in a -80ºC freezer, and re-grown on fresh 10% V-8 juice agar in the dark at room temperature for 5-7 days until colonies were about 5 cm in diameter. Then, approximately 10 ml of sterile distilled water was added to each 9 cm Petri dish and the fungal colony was flattened with a sterile glass tube. The solution was decanted and the Petri dishes were incubated overnight under fluorescent light at room temperature, followed by 24 hours incubation in darkness at 15°C to induce sporulation. Spore suspensions were prepared by adding approximately 5 ml of sterile distilled water to sporulating colonies and gently dislodging the conidia from conidiophores with a sterile art brush or an inoculation loop. Two isolates, WRS102 and WRS857, which have been frequently used in barley breeding programs in western Canada, were obtained from Dr. A. Tekauz and also included in the *Ptt* and *Ptm* experiments, respectively. Isolate WRS102 was collected from plants growing in

Saskatchewan (Metcalfe et al. 1970), while WRS857 was collected from plants growing in Manitoba (Tekauz and Mills 1974; Ho et al. 1996).

### 5.2.2 Resistance screening procedure

Independent experiments were conducted for each of the net and spot form pathogens, using a randomized complete block design with four replicates and four plants per replicate, under controlled conditions in growth chambers using a factorial treatment structure, with chambers considered as blocks. The entire experiment for each form was repeated on two different occasions and data were pooled. Barley genotypes were chosen for evaluation based on an initial screening (A. Tekauz, unpublished data, 2012) or based on previous reports of their resistance to each form of the net blotch pathogen (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013).

Ten barley genotypes CI 9819, CIho 11976, CN 3729, TR 236, TR 253, 'Vivar' (Helm et al., 2003), 'AAC Synergy' (Legge et al. 2014), 'CDC Helgason' (Rossnagel 2002), 'Major' (Legge et al. 2013b) and the susceptible control 'Xena' with very poor (VP) resistance were evaluated against seven *Ptt* isolates at the seedling stage. At the time the current study was initiated, 'Vivar' was the only commercial cultivar identified as having very good (VG) NFNB resistance, while 'AAC Synergy' and 'CDC Helgason' were the only cultivars identified with good resistance (G). The remaining genotypes were classified as having fair (F), poor (P) or very poor (VP) resistance against NFNB (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013), among which 'Major', with a fair (F) rating for NFNB resistance (Manitoba Agriculture, Food and Rural Initiatives 2013), was also included in the NFNB experiments.

For inoculations with the seven *Ptm* isolates, the 10 genotypes used included CI 9819, CIho 11976, CN 3729, TR 236, TR 253, 'CDC Meredith'(VG) (Rossnagel 2008), 'AAC Synergy'(VG), 'Major' (G), 'Cerveza' (G) (Legge et al. 2013a), and a susceptible control 'Harrington' (Harvey and Rossnagel 1984) (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013).

Plants were grown in growth cabinets set at 20° C ± 2° C with an 18 h light and 6 h dark photoperiod for two weeks, and then inoculated at the two to three leaf stage. Plants were watered on alternate days and never fertilized or sprayed during the study; however, the Sunshine potting mix (W.R. Grace and Co., Fogelsville, PA) used contained sources of the main required plant nutrients. Pathogen inoculum was manually adjusted to $1 \times 10^4$ conidia per mL for *Ptt* and to $2 \times 10^3$ conidia per mL for *Ptm*, using a Fuchs Rosenthal Counting Chamber (Hausser Scientific, Blue Bell, PA), and inoculated at a rate of 10 mL per pot (Tekauz 1990) using a pressurized atomizer connected to an air source. Inoculated plants were covered with sterile plastic bags for 24 h and maintained at approximately 100% relative humidity at room temperature in the inoculation room. Following this period, the bags were removed, and plants were returned to the previous growth conditions.

One week following inoculation, the second and third leaves of each seedling were independently rated on 1–10 and 1–9 disease reaction scales developed by Tekauz (1985) for *Ptt* and *Ptm*, respectively. For each barley differential-isolate interaction, the average disease reaction was calculated by averaging the arithmetic mean of reactions in the two experiments (two leaves per plant, four plants per replicate, and four replicates per experiment). For both forms, barley genotypes were placed into five resistance classes: very good resistant (VG) when

the reactions ranged from 1 to ≤2; good (G) from >2 to ≤4; fair (F) from >4 to ≤6; poor (P) from >6 to ≤7, and very poor (VP) >7 or more (Douiyssi et al. 1998; McLean et al. 2014; Tekauz et al. 2014).

### 5.2.3 Statistical analyses

Mean disease reaction data from both the experiments involving *Ptt* and *Ptm* isolates were tested for normality and variance homogeneity using Shapiro-Wilk and Kolmogorov-Smirnov tests, and Leven's test, respectively. However, the data did not conform to the assumptions of the Gaussian distribution. As a result, Generalized Estimating Equations (GEEs) with a negative binomial distribution function were fitted to the data using PROC GLIMMIX with LOG as the link function (SAS Institute 2008; Shah and Madden 2004). Because disease reaction ratings were based on an ordinal scale, the data were rank transformed (Conover and Iman 1981) using PROC RANK (SAS Institute 2010) before fitting GEEs. In both analyses, genotype, isolate and their interaction were treated as fixed effects while block was treated as a random effect. The initial model indicated an absence of block effect and hence, the model was re-run without a block effect. Mean disease reaction ratings among treatments for the host genotype x pathogen isolate interaction were sliced by genotypes using SLICE option in PROC GLIMMIX (SAS Institute 2008), and compared using Tukey's *post hoc* test. The comparisons were conducted at $\alpha=0.05$.

## 5.3 Results

### 5.3.1 *Pyrenophora teres* f. *teres*

Significant effects of pathogen isolate ($F=53.08$, $P<0.0001$), host genotype ($F=76.35$, $P<0.0001$), and host genotype by pathogen isolate interaction ($F=18.97$, $P<0.0001$) for disease reaction were

detected. Seedling responses were variable for the seven isolates of *Ptt*, ranging from 3.0 for TR 253 to 7.5 for 'Xena' (Table 5-2). For *Ptt*, each host genotype tested, excluding the susceptible control 'Xena', was rated as VG or G against at least one of the examined isolates. Among the commercial genotypes tested, 'AAC Synergy' exhibited the best overall resistance with reactions ranging from 2.3 to 5.0, and showed significantly lower disease reaction values than 'Vivar' ($P = 0.0250$), 'CDC Helgason' ($P < 0.0001$) and 'Major' ($P < 0.0001$). However, isolates MB01 from Manitoba and SK26 from Saskatchewan, both belonging to pathotype E09 (Chapter 4; Akhavan et al. 2016a), produced disease reaction values of 5 and 4.6, respectively, on 'AAC Synergy'. The resistance in 'Vivar', which had reactions that ranged from 2.6 to 7.0, was clearly overcome by isolates AB34 and MB14 from Alberta and Manitoba, respectively. However, 'Vivar' did exhibit a resistant type reaction for the remaining four isolates, SK26 and SK41 from Saskatchewan, ABV28 from Alberta, and MB01 from Manitoba. Given its overall reaction, 'Vivar' was ranked as a cultivar with fair resistance against the *Ptt* isolates tested in this experiment, with an average disease reaction score of 4.1. Overall, the reaction of 'Vivar' to the *Ptt* isolates was not significantly different from that of Ciho 11976 ($P = 1.0000$) or CN 3729 ($P = 0.9959$), which had average disease reaction scores of 3.7 and 3.6, respectively, and which were classified as G for resistance using the disease reaction scale cut-offs indicated previously. Moreover, 'Vivar' exhibited significantly lower disease reaction values than 'CDC Helgason' ($P < 0.0001$) and 'Major' ($P = 0.0006$). For 'Major', *Ptt* reactions ranged from 2.0 to 9.0, and this cultivar exhibited a fair reaction as reported previously (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013).

Among the commercial cultivars, 'CDC Helgason' was the most susceptible cultivar with reactions that ranged from 2.1 to 8.3 with an average of 6.1. Therefore, using the disease reaction scale cut-offs, 'CDC Helgason' ranked as a cultivar with poor NFNB seedling resistance; however, its reaction was not significantly different from 'Major' ($P = 0.7537$), which had an average rating of 6.0 and was classified as having fair resistance to NFNB. The resistance in 'CDC Helgason' was clearly overcome by MB01 and MB14 from Manitoba, and SK26 and SK41 from Saskatchewan, while this cultivar was resistant to both representative isolates from Alberta, AB34 and ABV28. Disease reaction on the susceptible check 'Xena' was significantly greater than the overall average ratings obtained for the other genotypes. The reaction of 'Xena' ranged from 4.4 to 9.0 against individual isolates. Among the breeding lines tested for NFNB resistance, TR 253, CI 9819, and TR 236 had disease reaction values classified as good resistance, with average severities of 3.0 for TR 253, and 3.1 for CI 9819 and TR 236. For TR 253, disease reactions ranged from 2.5 to 3.5, while for CI 9819 and TR 236, the reactions ranged from 2.9 to 3.3 and from 2.0 to 4.3, respectively. These three lines developed significantly lower disease severities than CN 3729 and CIho 11976. The reactions of CN 3729 and CIho 11976 did not differ significantly ($P = 0.9997$), ranging from 2.5 to 4.8 and from 2.5 to 6.0, respectively.

Overall, all of the breeding lines (i.e., TR 253, CI 9818, TR 236, CN 3729, and CIho 11976) exhibited good levels of resistance against all seven *Ptt* isolates tested. Among the breeding lines, the most susceptible reaction occurred when CIho 11976 was inoculated with isolate ABV28 from Alberta, resulting in a disease reaction rating of 6.0. The isolates SK26, MB01, and MB14 had statistically equal mean disease reactions; however, MB01 and MB14 were the most virulent, as they produced susceptible reactions on four of the barley genotypes using a

disease reaction cut-off value of 5 (Chapter 4; Akhavan et al. 2016a); in contrast, isolate ABV28 was the least virulent.

In the case of inoculations with *Ptt*, significant isolate by cultivar interactions were detected in the analysis of variance. Examples of host genotype by pathogen isolate interactions included the reactions of the genotypes 'Major' and 'CDC Helgason' against two isolates from Alberta. While these cultivars were resistant to inoculation with ABV28 and AB34, they exhibited susceptible reactions against all of the other isolates. Increased disease reaction and a more susceptible reaction were observed for 'Vivar' following inoculation with isolates AB38 and MB14 (disease severities of 7.0 and 6.4, respectively), while this cultivar was resistant to all of the other isolates. Therefore, 'Major', 'CDC Helgason' and 'Vivar' expressed both resistant and susceptible reactions depending on isolate.

### 5.3.2 *Pyrenophora teres* f. *maculata*

Significant effects for isolate ($F= 124.32$, $P < 0.0001$), host genotype ($F = 351.04$, $P < 0.0001$), and host genotype by isolate interaction ($F = 10.58$, $P < 0.0001$) were also detected for disease reaction in response to inoculation with *Ptm*. The responses of the host cultivars to the seven *Ptm* isolates were variable, ranging from 1.9 for 'Cerveza' to 6.9 for the susceptible check 'Harrington'. With the exception of 'CDC Meredith', TR 253 and the susceptible check 'Harrington', the other host genotypes were found to be VG or G against at least one *Ptm* isolate in addition to the reference isolate WRS857, which was avirulent on all but 'Harrington'. The resistance in 'CDC Meredith' was clearly overcome by all tested *Ptm* isolates excluding the reference isolate WRS857. In contrast, 'Cerveza', 'Major' and 'AAC Synergy' exhibited resistance against all isolates. The disease reaction of 'Cerveza' was significantly lower than that of 'Major' ($P < 0.0001$) and 'AAC Synergy' ($P < 0.0001$) (Table 5-3). Although 'Major'

had slightly lower disease severities than 'AAC Synergy', this difference was not statistically different (P = 0.0659). For 'Cerveza', reactions ranged from 1.5 to 2.0 with an average of 1.9 (VG), while for both 'Major' (G) and 'AAC Synergy' (G), the reactions ranged from 2.0 to 3.0 with averages of 2.6 and 2.8, respectively. Disease reaction on 'Harrington' was significantly greater than on all of the other genotypes, with reactions ranging from 5.0 to 7.5 (Table 5-3). In addition to the susceptible control 'Harrington', 'CDC Meredith' was the most susceptible among the cultivars tested, with an average disease reaction of 6.0. Among the breeding lines tested against *Ptm*, TR 236 had the lowest disease reaction values, with reactions ranging from 3.0 to 4.5 and an average rating of 3.9 (G). The line TR 236 also had significantly lower ratings than CN 3729 (P = 0.0004), CIho 11976 (P = 0.0132) and CI 9819 (P <0.0001). The reaction of CN 3729 was not significantly different from that of CIho 11976 (P = 0.9979) or CI 9819 (P = 0.9502), while TR 253 was the most susceptible among the breeding lines. In CN 3729, CIho 11976 and CI 9819, the disease ratings ranged from 3.0 to 5.0, while in TR 253 and 'CDC Meredith' the reactions ranged from 2.0 to 7.5.

Among the *Ptm* isolates tested, MBV25, AB58 and SKV10 caused the highest average disease reaction values (Table 5-3). The reference isolate WRS857 was the least virulent, and isolate ABV14 from Alberta had the second lowest virulence. Despite a significant host genotype by pathogen isolate interaction, no obvious specificity was observed in the host–pathogen relationships, and overall, excluding the reference isolate WRS857, all other isolates exhibited similar virulence profiles on the tested genotypes, with only one exception. The line TR253 was fairly resistant to ABV14, but had poor resistance to the other recently collected isolates.

## 5.4 Discussion

The development and deployment of resistant cultivars is an environmentally and economically preferred measure for net blotch management. Therefore, information on the reaction of barley genotypes previously identified as resistant to isolates representing local populations of *Ptt* and *Ptm* is required to ensure their continued utility. The isolates used in the current study were recently characterized on a differential set previously developed in western Canada (Tekauz 1990; Chapter 4; Akhavan et al. 2016a). The seven *Ptt* and *Ptm* isolates examined represented six and five different pathotype groups, respectively, including the predominant pathotype groups C4 and E9, for *Ptt*, and V1 and T1, for *Ptm* (Table 5-1). Akhavan et al. (2016a; Chapter 4) reported that *Ptt* pathotype groups C4 and E9 comprised 43% of the *Ptt* isolates in a collection from western Canada, while the *Ptm* pathotype groups V1 and T1 comprised 52% of the *Ptm* isolates. No clear pattern was observed in a recent study with respect to the geographic distribution of *Ptt* and *Ptm* pathotypes across the Canadian prairies (Chapter 4; Akhavan et al. 2016a). Pathotype distribution in each region is difficult to assess, particularly on a regular basis. Moreover, the pathogen and host genotype interaction in this pathosystem is very complex and still not well understood (Liu et al. 2015; Richards et al. 2016). Therefore, we used the overall mean disease reactions for each host genotype and isolate to compare the relative virulence and resistance of isolates and host genotypes, respectively, as well as specific interactions.

In the current study, the overall reactions of TR 253 and CI 9819 were similar, ranging from 2.5 to 3.5 and 2.9 to 3.3, respectively, suggesting that these two breeding lines still represent reliable sources of *Ptt* resistance for western Canadian barley breeding programs, at least relative to the other lines examined. Afanasenko et al. (2009) reported that the lowest mean frequency of

virulent isolates across *Ptt* populations tested was observed on the barley genotypes CI 9819, c-8755, CI 5791 and CI 9825. Manninen et al. (2000, 2006) studied the genetic background of CI 9819 and reported a major gene (*Rpt5*) on chromosome 6H responsible for 60-88% of the resistance in this line, and five other minor genes on different chromosomes. Grewal et al. (2012) used TR 253 as a resistant check and found that it was consistently resistant to the *Ptt* isolates WRS858 and WRS1607. In the current study, TR 236 also exhibited a mean disease reaction very similar to TR 253 and CI 9819, although one isolate from Saskatchewan (SK26) produced an intermediate level of disease (4.3) on this line. CN 3729 exhibited significantly lower resistance against the tested isolates, ranging from 2.5 to 4.8. Using Chi-squared analysis, Grewal et al. (2012) found single gene segregation for resistance to the *Ptt* isolate WRS858 and two-gene segregation for resistance to the *Ptm* isolate WRS857 at the seedling stage in line TR 251. Cromey et al. (2003) reported that none of the *Ptt* isolates they collected from New Zealand were virulent on CI 5791, CI 9819 or CI 9820. Similarly, Steffenson and Webster (1992) reported that the barley genotypes Cl 9819 and CI 5791 were resistant to a collection of 91 *Ptt* isolates from California. The barley differentials CI 5791 and CI 9820 were also found to be resistant to almost all isolates of *Ptt* in a collection from western Canada (Chapter 4; Akhavan et al. 2016a). Thus, CI 5791 and CI 9820 may also represent effective sources of NFNB resistance in addition to TR 253, CI 9819 and TR 236.

The relatively high disease reaction (average rating = 6.1) on 'CDC Helgason' was unexpected, since this cultivar is classified as resistant to NFNB (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013). The resistance in 'CDC Helgason' was overcome by all isolates tested except ABV28 and AB34, suggesting poor seedling resistance against representative *Ptt* isolates

collected from Saskatchewan and Manitoba. 'CDC Helgason' is the result of a cross between TR 236/TR 327 (Grewal et al. 2010). However, TR 236 exhibited good NFNB resistance against the *Ptt* isolates in this study, which suggests the presence of additional resistance genes and/or QTLs in TR 236 that are absent in 'CDC Helgason'. Disease reactions of 7.0 and 6.4 on 'Vivar' following inoculation with isolates from Alberta (AB34) and Manitoba (MB14) also were not expected, since 'Vivar' is the only cultivar with very good resistance (VG) against NFNB in western Canada (Alberta Agriculture and Rural Development 2015). Nonetheless, 'Vivar' may still be considered as having fair resistance, given its overall performance against *Ptt* in this study (reactions ranging from 2.6 to 7.0). The most resistant cultivar was 'AAC Synergy', with disease reactions of 2.3 to 5.0. Its performance was consistent with the previous classification of this variety as having good NFNB resistance (Manitoba Agriculture, Food and Rural Initiatives 2013). However, there appears to be some adaptation to the *Ptt* resistance in this cultivar, since isolate MB01 produced a disease reaction rating of almost 5.0. 'Xena', the susceptible check, had reactions that ranged from 4.4 to 9.0, resulting in a susceptible rating as expected from previous classifications.

The mean disease reactions induced by the various *Ptt* isolates ranged from 3.6 for ABV28 from Alberta to 4.9 for SK26 from Saskatchewan. MB14, an isolate from Manitoba causing a mean disease reaction of 4.8, was the only isolate that could overcome the resistance in both 'Vivar' and 'CDC Helgason'. Differences in isolate virulence may be one reason that host genotypes classified as resistant in one study may perform poorly in another study (Baergen et al. 1993). Results from the current study emphasize the importance of employing diverse representative pathogen isolates rather than a single isolate when screening for improved *Ptt* resistance. In the

case of NFNB resistance, Douiyssi et al. (1998) showed that selection programs that utilize single isolates of *Ptt* in the greenhouse may select for lines that are susceptible in the field.

The significant isolate by cultivar interactions found in the current study suggest the occurrence of specific host-pathogen interactions. Douiyssi et al. (1998) also found a significant cultivar by isolate interaction and showed that lines ND B112 and CI 12034 exhibited both resistant and susceptible reactions depending on the isolate examined. The authors concluded that there was a specific rather than a generalized NFNB resistance mechanism. It was reported that sensitivity to a proteinaceous necrotrophic effector produced by *Ptt* and mapped to barley chromosome 6H was responsible for 31% of the variation observed in disease (Liu et al. 2015). As a result of this finding, it was proposed that the barley–*Ptt* pathosystem follows, at least partially, a necrotrophic effector-triggered susceptibility (NETS) model (Liu et al., 2015). It is possible that differential production of this necrotrophic effector contributes to the virulence of different isolates of *Ptt*. Very recently, Richards et al. (2016) also mapped a dominant susceptibility locus close to the centromere of chromosome 6H, which presumably can be targeted by *Ptt* necrotrophic effectors. These findings collectively emphasize the complexity of the host–parasite genetic interactions in this pathosystem, and suggest that barley breeding programs should also consider eliminating dominant susceptibility genes or genes inducing NETS in order to develop *Ptt* resistant cultivars (Liu et al. 2015; Richards et al. 2016). In the case of *Ptm*, the average disease reactions ranged from 1.9 for 'Cerveza' to 6.9 for the susceptible control 'Harrington'. The average reaction for 'CDC Meredith' (6.0) was higher than expected, since this cultivar was previously classified as having very good SFNB resistance (Alberta Agriculture and Rural Development 2013). Similarly, TR 253 is a breeding line considered by barley breeders and pathologists to be resistant to *Ptm*, and yet developed an average disease reaction of 5.9. TR 253 was used as a

resistant check in studies by Grewal et al. (2012), and was found to be consistently resistant to the *Ptm* isolates WRS857 and LO233. However, while TR 253 also exhibited a resistant reaction to WRS857 in the current study, it was highly susceptible to the more recently collected isolates of *Ptm*. In contrast, 'Cerveza', 'Major' and 'AAC Synergy' were resistant to all *Ptm* isolates, with 'Cerveza' exhibiting the best overall resistance among all tested genotypes, with an average disease reaction of 1.9. The overall reactions of 'Major' and 'AAC Synergy' against the seven *Ptm* isolates were similar, ranging from 2.0 to 3.0, suggesting that these two cultivars are also reliable sources of *Ptm* resistance. The observation that the commercial cultivars 'Cerveza', 'Major' and 'AAC Synergy' were all more resistant to *Ptm* than the breeding lines examined suggests that the resistance in these cultivars may be derived from more than a single resistant breeding line. Grewal et al. (2012) found that even a susceptible parent can be a source of additional QTLs, conferring more resistance to a barley population derived from the cross between susceptible and resistant parents.

Among the breeding lines tested, TR 236 exhibited the best overall reaction to the *Ptm* isolates, with an average rating of 3.9, followed by CN 3729, CIho 11976 and CI 9819. CI 9819 developed significantly higher levels of disease against the *Ptm* isolates than did TR 236. Karki and Sharp (1986) reported that CI 9819 exhibited a differential reaction to *Ptm* isolates from Montana, Morocco, Tunisia, and Turkey, while all isolates were avirulent on CI 9214. It was shown that CI 9214 is also resistant to almost all of the representative *Ptm* isolates recently collected from western Canada (Chapter 4; Akhavan et. al. 2016a). Among the breeding lines tested in the current study, TR 253 showed the lowest level of resistance, i.e., highest disease reactions, which ranged from 4.5 to 7.5 in response to the six recently collected *Ptm* isolates. This finding is in contrast to a report by Grewal et al. (2012), who used TR 253 as a resistant

check and found that it was consistently resistant throughout their experiments. In the current study, 'CDC Meredith' exhibited reactions ranging from 6.0 to 7.5, and 'Harrington' (the susceptible control) developed disease reactions ranging from 7.0 to 7.5. Resistance to SFNB in 'CDC Meredith', which was previously identified as having very good resistance (Alberta Agriculture and Rural Development 2013), was overcome by all of the isolates tested.

Neupane et al. (2015) evaluated the SFNB reactions of a barley core collection and identified isolate-specific susceptibility in several of the accessions. In the current study, there was a statistically significant host genotype by pathogen isolate interaction, but this appeared to be due mainly to the influence of the reference isolate WRS 857. The six recently collected *Ptm* isolates induced disease reactions of 4.2 to 4.9 when averaged over all of the hosts, while the mean reaction induced by WRS 857 was only 2.7. This isolate was avirulent on all barley genotypes except the susceptible check 'Harrington'. While WRS 857 is used commonly for SFNB resistance screening in barley breeding programs, it appears that it is no longer representative of western Canadian *Ptm* populations. As such, it should be replaced with other isolates, or at least used in conjunction with more recent collections of the pathogen. The correlation between seedling stage reactions induced by different isolates of *Ptm* was reported to be very low (r = 0.10) (Grewal et al. 2012), highlighting the need to use multiple isolates when screening for SFNB resistance.

The current study examined the *Ptt* and *Ptm* resistance of barley genotypes only at the seedling stage, but did not examine adult plant resistance. Previously, Grewal et al. (2012) identified a NFNB seedling resistance QTL, *QRpt6*, and reported the same QTL for adult-plant resistance. Similarly, Gupta et al. (2003) screened 69 barley genotypes against nine *Ptt* isolates, and found that the seedling resistance also was frequently expressed in adult plants. A strong correlation (r

= 0.65–0.71) also was calculated between seedling and adult plant reactions, suggesting that seedling screening could be effective for the selection of resistant lines (Grewal et al. 2012). However, Grewal et al. (2012) also reported three additional adult-plant resistance QTLs, concluding that some QTLs are effective only at the adult-plant stage. Moreover, Douiyssi et al. (1998) found that adult plant resistance in nine of the barley lines they tested was undetected at the seedling stage. Given these reports, the barley cultivars and lines examined in the current study should also be evaluated for adult plant resistance.

The current study and previous research suggest that *Ptt* and *Ptm* populations are diverse in western Canada (Chapters 2-4; Akhavan 2015, 2016b, 2016a; Tekauz 1990). Unfortunately, this diversity may increase the potential for rapid adaptation of both *Ptt* and *Ptm* to particular barley cultivars, ultimately resulting in the loss of net blotch resistance (Douiyssi et al. 1998). As a consequence, the currently used sources of host resistance should be managed cautiously by farmers and breeders. Producers should avoid relying exclusively on resistant cultivars, and avoid repeatedly growing the same resistant barley cultivar in short rotations, while using resistance together with other strategies such as rotation to non-hosts and the judicious application of fungicides to reduce overall disease pressure over time. The present study showed that some barley cultivars classified as having good or very good resistance to net blotch may be vulnerable to current populations of *Ptt* and *Ptm*. The identification of isolates with increased virulence on resistant barley underscores the importance of ongoing efforts to incorporate new sources of net blotch resistance in Canadian cultivars.

## 5.5 Tables

**Table 5-1.** Form, isolate number, location collected, year collected, and pathotype designation (Chapter 4; Akhavan et al. 2016a) of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) isolates used in the current study.

| Form | Isolate | Location collected | Year collected | Pathotype |
|---|---|---|---|---|
| *Ptt* | AB34 | Bentley, AB | 2011 | F01 |
| *Ptt* | ABV28 | Lacombe, AB | 2012 | C04 |
| *Ptt* | MB01 | Binscarth, MB | 2009 | E09 |
| *Ptt* | MB14 | Portage La Prairie, MB | 2011 | I03 |
| *Ptt* | SK26 | St Brieux, SK | 2011 | E09 |
| *Ptt* | SK41 | Albertville, SK | 2011 | F06 |
| *Ptt* | WRS102* | Indian Head, SK | Before 1970 | E10 |
| *Ptm* | AB58 | Bentley, AB | 2010 | U02 |
| *Ptm* | ABV14 | Lacombe, AB | 2012 | V01 |
| *Ptm* | MB22 | Pipestone, MB | 2010 | U04 |
| *Ptm* | MBV25 | Roblin, MB | 2012 | V01 |
| *Ptm* | SK69 | Richardson, SK | 2010 | T01 |
| *Ptm* | SKV10 | Melfort, SK | 2012 | V01 |
| *Ptm* | WRS857* | Oakbank, MB | 1973 | T06 |

*Reference isolates, WRS102 and WRS857, which have been frequently used in barley breeding programs in western Canada, were obtained from Dr. A. Tekauz and included in the *Ptt* and *Ptm* experiments, respectively. Isolate WRS102 was collected from plants growing in Saskatchewan (Metcalfe et al. 1970), while WRS857 was collected from plants growing in Manitoba (Ho et al. 1996; Tekauz and Mills 1974).

**Table 5-2.** Net form net blotch disease reaction values on barley genotypes following inoculation with different isolates of *Pyrenophora teres* f. *teres* from western Canada.

| Genotype | Isolate | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **ABV28** | **AB34** | **MB01** | **MB14** | **SK26** | **SK41** | **WRS858** | **Genotype Average** |
| **TR 253** | 3.0 [A; a, b ‡] | 2.5 [A; a] | 3.5 [A, B; b] | 2.9 [A, B; a, b] | 3.3 [A, B; a, b] | 3.0 [A; a, b] | 3.0 [A; a, b] | 3.0 [a] |
| **CI 9819** | 2.9 [A; a] | 3.3 [A, B; a] | 3.0 [A; a] | 3.0 [A, B; a] | 3.1 [A, B; a] | 3.0 [A; a] | 3.1 [A; a] | 3.1 [a] |
| **TR 236** | 2.0 [A; a] | 3.0 [A, B; b] | 3.6 [A , B; b, c] | 2.6 [A; b] | 4.3 [B, C; c] | 3.0 [A; b] | 3.0 [A; b] | 3.1 [a] |
| **AAC Synergy** | 2.3 [A; a] | 2.5 [A; a] | 5.0 [B, C; d] | 3.8 [B, C; b, c, d] | 4.6 [B, C,D ; c, d] | 3.0 [A; b] | 3.0 [A; b] | 3.5 [b, a] |
| **CN 3729** | 4.8 [B; a] | 4.5 [B, C, D; a] | 4.1 [B, C; a] | 2.9 [A, B; b, c] | 3.6 [B; c] | 2.9 [A; b, c] | 2.5 [A; b] | 3.6 [b, c] |
| **CIho 11976** | 6.0 [C; a] | 4.0 [B, C; a, b] | 3.0 [A; b] | 3.4 [B; b] | 3.9 [B, C; b] | 3.3 [A, B; b] | 2.5 [A; c] | 3.7 [b, c] |
| **Vivar** | 2.6 [A; a] | 7.0 [C, D; c] | 3.3 [A, B; a, b] | 6.4 [C,D; c] | 2.8 [A; a] | 3.0 [A, B; a, b] | 4.0 [B, C; b, c] | 4.1 [c] |
| **Major** | 2.0 [A; a] | 3.4 [A, B; b] | 7.5 [C; c] | 6.8 [C, D; c] | 9.0 [C, D; c] | 6.5 [C; c] | 6.9 [C, D; c] | 6.0 [d] |
| **CDC Helgason** | 2.1 [A; a] | 4.4 [B, C, D; b] | 7.4 [C; b] | 7.0 [C, D; b] | 8.3 [C, D; b] | 6.9 [C; b] | 6.6 [C,D; b] | 6.1 [d] |
| **Xena** | 8.0 [D; a, b] | 9.0 [D; a] | 7.0 [C; a, b] | 9.0 [D; a] | 5.8 [B, C, D; a, b] | 4.4 [B, C; b] | 9.0 [D; a] | 7.5 [e] |
| **Isolate Average** | 3.6 [a] | 4.4 [b] | 4.7 [c] | 4.8 [c] | 4.9[c] | 3.9 [b] | 4.4 [b] | 4.4 [NA] |

Disease reaction was assessed on a scale of 1-10 at 7 days after inoculation.
CI 9819, CIho 11976, CN 3729, TR 236, TR 253 are breeding lines, while 'Vivar' (Helm et al., 2003), 'AAC Synergy' (Legge et al., 2014), 'CDC Helgason' (Rossnagel, 2002), 'Major' (Legge et al., 2013b) and the susceptible control 'Xena' are commercial cultivars (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013).
‡Means sharing identical upper-case letters within columns are not significantly different for the genotype* isolate interaction sliced by isolate.
‡Means sharing identical lower-case letters within rows are not significantly different for the genotype* isolate interaction sliced by genotype.

**Table 5-3.** Spot form net blotch disease reaction values on barley genotypes following inoculation with different isolates of *Pyrenophora teres* f. *maculata* from western Canada.

| Genotype | Isolate | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **ABV14** | **AB58** | **SKV10** | **SK69** | **MB22** | **MBV25** | **WRS857** | **Genotype Average** |
| **Cerveza** | 2.0 [A; a‡] | 2.0 [A; a] | 2.0 [A; a] | 1.5 [A; a] | 2.0 [A; a] | 2.0 [A; a] | 2.0 [A; a] | 1.9 [a] |
| **Major** | 2.5 [A; a] | 3.0 [B; a] | 3.0 [B; a] | 2.0 [B; b] | 3.0 [B; a] | 3.0 [B; a] | 2.0 [A; b] | 2.6 [b] |
| **AAC Synergy** | 3.0 [A; a] | 3.0 [B; a] | 3.0 [B; a] | 2.5 [B; a] | 3.0 [B; a] | 3.0 [B; a] | 2.0 [A; b] | 2.8 [b] |
| **TR 236** | 4.0 [B; a] | 4.0 [C;a] | 4.0 [C; a] | 4.0 [C; a] | 4.0 [C; a] | 4.5 [C; a] | 3.0 [B; b] | 3.9 [c] |
| **CN 3729** | 4.0 [B; a] | 5.0 [D,C; a] | 5.0 [D,C; a] | 4.5 [C; a] | 4.5 [C; a] | 4.5 [C; a] | 3.0 [B; b] | 4.4 [d] |
| **CIho 11976** | 5.0 [B,C,D; a] | 5.0 [D,C; a] | 5.0 [D,C; a] | 4.5 [C;a] | 3.0 [B; b] | 5.0 [D; a] | 3.0 [B; b] | 4.4 [d] |
| **CI 9819** | 4.0 [B; a] | 5.0 [D,C; a] | 5.0 [D,C; a] | 4.5 [C; a] | 5.0 [D; a] | 5.0 [D; a] | 3.0 [B; b] | 4.5 [d] |
| **TR 253** | 4.5 [B,C; a] | 7.0 [D; b] | 7.0 [E;b] | 6.5 [D; b] | 7.5 [E; b] | 7.0 [E; b] | 2.0 [A; c] | 5.9 [e] |
| **CDC Meredith** | 6.0 [C,D; a] | 7.0 [D; a] | 6.0 [D, E; a] | 6.5 [D; a] | 7.0 [E, D; a] | 7.5 [E; a] | 2.0 [A; b] | 6.0 [e] |
| **Harrington** | 7.0 [D; a, b] | 7.0 [D; a, b] | 7.0 [E; a, b] | 7.0 [D; a, b] | 7.5 [E; a] | 7.5 [E; a] | 5.0 [C; b] | 6.9 [f] |
| **Isolate Average** | 4.2 [a] | 4.8 [b, c] | 4.7 [b, c] | 4.4 [a] | 4.7 [b] | 4.9 [c] | 2.7 [d] | 4.3 [NA] |

Disease reaction was assessed on a scale of 1-9 at 7 days after inoculation.

CI 9819, CIho 11976, CN 3729, TR 236, and TR 253 are breeding lines, while 'CDC Meredith' (Rossnagel, 2008), 'AAC Synergy' (Legge et al., 2014), 'Major' (Legge et al., 2013b), 'Cerveza' (Legge et al., 2013a), and the susceptible control 'Harrington' (Harvey and Rossnagel, 1984) are commercial cultivars (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013).

‡Means sharing identical upper-case letters within columns are not significantly different for the genotype* isolate interaction sliced by isolate.

‡Means sharing identical lower-case letters within rows are not significantly different for the genotype* isolate interaction sliced by genotype.

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# Chapter 6 Sensitivity of *Pyrenophora teres* to propiconazole and pyraclostrobin in western Canada[5]

## 6.1 Introduction

The necrotrophic fungus *Pyrenophora teres* Drechs. (anamorph: *Drechslera teres* [Sacc.] Shoem.) has two microscopically identical but genetically distinct forms: *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*), which incite the net form of net blotch (NFNB) and spot form of net blotch (SFNB) of barley (*Hordeum vulgare* L.), respectively (McDonald 1963; Smedegard-Petersen 1978). These are among the most economically important foliar diseases across the major barley growing regions of the world, including western Canada with 2.5 million hectares planted with barley in 2016 (Tekauz 1990; Steffenson 1997; Liu et al. 2011; Statistics Canada 2016). Yield losses of 10-40% are typical in severe cases of NFNB, although *Ptt* has the potential to cause total yield loss (Steffenson et al. 1991; Steffenson 1997; Murray and Brennan 2010). Likewise, yield losses of up to 44% were reported for SFNB (Jayasena et al. 2007). Crop rotation, fungicide application, and the deployment of resistant cultivars can be used to manage net blotch of barley in western Canada (van den Berg and Rossnagel 1991; Turkington et al. 2004, 2005, 2006, 2011, 2012, 2015). However, many western Canadian farmers have adopted a canola-cereal-canola rotation, which is not sufficient to manage barley leaf spot diseases such as net blotch (Mathre 1997; Tekauz 2003; Turkington et al. 2011, 2015). Moreover, many two and

[5] A version of this Chapter has been submitted as a manuscript for publication in a refereed journal (Akhavan A, Strelkov SE, Askarian H, Kher SW, Fraser M, Kutcher HR, Turkington TK. Sensitivity of western Canadian *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* isolates to propiconazole and pyraclostrobin). It is currently under review.

six row barley cultivars, especially malting types, are susceptible to net blotch (Alberta Government 2016a). As a consequence, many farmers routinely use fungicides as their main strategy for managing barley leaf diseases (Turkington et al. 2011, 2015; Poole and Arnaudin 2014). Most barley fungicides are registered for use from stem elongation to head emergence, but also are registered for tank mixing with herbicides and may be applied at earlier crop growth stages (Turkington et al. 2015; Alberta Government 2016a). When weather conditions are conducive to disease development and the barley cultivar is susceptible, many farmers will follow an application of fungicide that is tank-mixed with herbicide(s) with a second fungicide-only application between flag leaf and head emergence (Turkington et al. 2015; Alberta Government 2016a).

Both *Ptt* and *Ptm* are stubble-borne pathogens, producing asexual conidia and sexual pseudothecia, which produce ascospores on overwintered infected crop debris (Piening 1961, 1968; van den Berg and Rossnagel 1991; Peever and Milgroom 1994; Duczek et al. 1999). Based on a 1:1 ratio of the two mating types for *Ptt* and *Ptm* in western Canada (Chapter 2; Akhavan et al. 2015), it is likely that both forms of the net blotch pathogen go through regular cycles of sexual reproduction in this region. Moreover, *Ptt* and *Ptm* populations in western Canada are genetically diverse, with almost 90 percent of the entire population consisting of distinct haplotypes (Chapter 3; Akhavan et al. 2016b). These diverse *Ptt* and *Ptm* populations with mixed reproduction and an outcrossing mating system fall into the category of pathogens that have a greater likelihood of quickly adapting to fungicides with a single mode of action (McDonald and Linde 2002), ultimately resulting in a reduction or loss of fungicide effectiveness.

The fungicides propiconazole and pyraclostrobin are commonly applied to manage net blotch on barley in western Canada (Alberta Government 2016b). Propiconazole has been used on various crops including barley since the early 1990s as the foliar fungicide Tilt (propiconazole, 250 g $L^{-1}$; Syngenta Canada Inc.) (Xue et al. 1994; Thomas 1997). Propiconazole is a triazole belonging to the demethylation inhibitors (DMIs) group. This group of fungicides inhibits 14α–sterol demethylase which is used by fungi to synthesize ergosterol, which is essential for fungal cell wall development (Dahl et al. 1987; reviewed in Parker et al. 2014). Excessive use of DMIs has been reported to select for insensitivity in cereal pathogens such as *Ptt* (Mair et al. 2016), *Blumeria graminis* f. sp. *hordei* (Delye et al. 1998), *Mycosphaerella graminicola* (Leroux et al. 2007; Bean et al. 2009; Cools and Fraaije 2012), and *Fusarium graminearum* (Talas and McDonald 2015), increasing the level of insensitivity within these pathogen populations (Kuck et al. 2012). Insensitivity to fungicides usually results from point mutations in the gene(s) encoding the proteins targeted by specific fungicides. Alterations in the *CYP51* gene in plant pathogens were reported to be one of the major mechanisms resulting in reduced sensitivity towards DMIs (reviewed in Parker et al. 2014). Mair et al. (2016) reported the occurrence of insensitivity among *Ptt* isolates collected from Australia against multiple DMI compounds. This insensitivity was correlated with both a mutation in the *Cyp51A* gene and overexpression of both the *Cyp51A* and *Cyp51B* genes, which are paralogs of the *Cyp51* gene. Rallos and Baudoin (2016) also reported that *CYP51* over-expression and target-site mutation contributed to insensitivity in *Erysiphe necator*.

Pyraclostrobin is classified within the strobilurin or quinine outside inhibitors (Qol) group of fungicides, and is the active ingredient in the fungicide Headline 250 EC (BASF Canada, Mississauga, ON, Canada). Products containing pyraclostrobin have been registered and utilized

by western Canadian farmers for cereal leaf spot management since the early 2000's (BASF 2003; Government of Canada: Health Canada Pest Management Regulatory Agency 2011). Marzani (2011) investigated the efficiency of several QoI fungicides on *Ptt* isolates and reported that pyraclostrobin was the most active QoI, resulting in a high growth inhibition. Strobilurins have a single site of activity and prevent mitochondrial respiration in fungi by binding to the Qo site of cytochrome *bc1*, interfering with electron transfer in the III complex (Sauter et al. 1999; Bartlett et al. 2002; Grasso et al. 2006). The Fungicide Resistance Action Committee (FRAC 2016) has labeled strobilurins as compounds at a high risk of selecting for insensitivity in pathogen populations.

In the current study, a microtiter bioassay was used to address the objectives. This method is more efficient relative to other more conventional techniques, such as the agar radial growth bioassay (Tremblay et al. 2003). The microtiter bioassay is an approved method of assessing fungicide sensitivity for *P. teres* (FRAC 2006a, 2009) and other species of *Pyrenophora* (FRAC 2006b) to DMI, QoI and succinate dehydrogenase inhibitor (SDHI) fungicides. It involves measurement of the optical density of fungal tissues against different concentrations of chemicals in a 96-well microplate, followed by comparison of the growth of each isolate with the growth of the same isolate in non-amended controls (Pijls et al. 1994; Tremblay et al. 2003). For DMIs, discriminatory doses from near the half-maximal effective concentration ($EC_{50}$) value (Smith et al. 1991; Peever and Milgroom 1992, 1993) to 100 times greater than the baseline $EC_{50}$ (Villani and Cox 2011) have been employed. The inhibition of fungal growth for a single fungicide concentration close to the $EC_{50}$ value was shown to be an appropriate response to measure with DMI fungicides (Smith et al. 1991) and was also shown in *P. teres* to be highly correlated with $EC_{50}$ values determined with a series of concentrations (Peever and Milgroom 1992, 1993).

However, Romero and Sutton (1997) suggested that a discriminatory dose slightly higher than both the mean $EC_{50}$ value and the highest detected $EC_{50}$ is more useful for monitoring sensitivity to DMI fungicides. For QoIs, discriminatory doses from near the $EC_{50}$ value (Mondal et al.2005) to almost 70 times greater than the baseline $EC_{50}$ (Fraser et al. 2016) have been typically employed.

Information on the sensitivity of local isolates of *P. teres* to fungicides used commercially is required to help in the formulation of integrated net blotch management strategies. Although both propiconazole and pyraclostrobin fungicides have been applied extensively in barley fields in western Canada, no information is available on the fungicide sensitivity of *P. teres* populations to these compounds. The objectives of the current study were to: 1) determine the $EC_{50}$ of propiconazole (DMI) and pyraclostrobin (QoI) for a set of *Ptt* and *Ptm* isolates from western Canada; 2) test the null hypothesis of a lack of significant differences between the $EC_{50}$ values obtained for *Ptt* and *Ptm*; 3) quantify the degree of sensitivity to propiconazole and pyraclostrobin in a representative collection of *Ptt* and *Ptm* isolates from commercial barley crops across western Canada; and 4) test the null hypothesis of a lack of significant differences in propiconazole and pyraclostrobin sensitivity among *Ptt* and *Ptm* isolates from each the three western Canadian provinces of Alberta, Saskatchewan and Manitoba.

## 6.2 Materials and methods

### 6.2.1 Fungal isolation and preparation of inoculum

A total of 39 *Ptt* and 27 *Ptm* isolates collected from across western Canada (Alberta, Saskatchewan and Manitoba) were studied. These included 36 *Ptt* and 21 *Ptm* isolates representing different clades identified in earlier genetic analyses (Chapters 2, 3; Akhavan et al.

2015, 2016b), plus an additional three isolates of *Ptt* and six isolates of *Ptm* from another collection made in 2012 (Chapter 4; Akhavan et al. 2016a). All 39 *Ptt* and 27 *Ptm* isolates also were previously characterized for their virulence on a selection of barley genotypes and differentials (Chapter 4; Akhavan et al. 2016a). The collection included 17 *Ptt* isolates from Alberta, 12 from Saskatchewan, and 10 from Manitoba, as well as 8 *Ptm* isolates from Alberta, 10 from Saskatchewan, and 9 from Manitoba (the isolates were same as those presented previously in Table 4-1). Two previously collected isolates of *Ptt* (WRS858 and WRS102) and one of *Ptm* (WRS857), which were obtained from Dr. A. Tekauz (Agriculture and Agri-Food Canada, Winnipeg, MB), were also included for comparison in experiments to test the sensitivity of *Ptt* and *Ptm* isolates to propiconazole and pyraclostrobin. These three isolates were collected prior to registration of both propiconazole and pyraclostrobin. Isolate WRS102 was originally collected from Saskatchewan (Metcalfe et al. 1970), while WRS858 and WRS857 were collected from Manitoba (Tekauz and Mills 1974; Ho et al. 1996).

To obtain single-spore isolates, segments (10 mm × 5 mm) were cut from barley leaves with symptoms of NFNB or SFNB, and soaked in 50% ethanol for 15 s and in 2% sodium hypochlorite for 30 s, flushed with sterile water and placed on moistened filter paper in 9 cm-diameter plastic Petri dishes (Tekauz 1990). To boost fungal sporulation on the leaves, the Petri dishes were incubated at 20°C ± 0.5°C with a 12 h photoperiod under a mixture of common fluorescent lamp light and a black light blue fluorescent lamp emitting near ultraviolet light at 368 nm. Following 3-5 days of incubation, single conidia of *P. teres* were transferred onto 10% V-8 juice agar (V-8 juice, 100 mL; CaC03 3g; Difco agar 20 g; distilled water 900 mL) amended with 50 mg $L^{-1}$ kanamycin, and incubated as above to form new colonies (Tekauz 1990). Single-spore isolates were preserved at -80°C in an ultra-low temperature freezer, either as 0.5 cm-

diameter mycelial plugs in 50% sterile glycerol or as conidial suspensions in 25% sterile glycerol. Identification of the isolates as *P. teres* was based on the morphological features of the conidiophores and conidia and species-specific PCR analysis. The form of *P. teres* (*Ptt* or *Ptm*) was confirmed by form-specific PCR as described in Chapter 2 (Akhavan et al. 2015). To validate the results of the form-specific PCR, isolates were inoculated onto the barley cultivar 'Steptoe' as described previously by Tekauz (1990).

Conidial suspensions were produced as per the technique developed by Lamari and Bernier (1989) for *Pyrenophora tritici-repentis* (Died.) Drechs., as modified by Aboukhaddour et al. (2013). Briefly, the isolates were removed from a -80ºC ultra-low temperature freezer, and the mycelial plugs were transferred onto fresh 10% V-8 juice agar supplemented with 50 mg $L^{-1}$ kanamycin in 9 cm-diameter Petri dishes, with one plug at the center of each Petri dish. Alternatively, for those isolates preserved as a frozen conidial suspension, approximately 100 µL of thawed spore suspension was transferred onto the same medium. The Petri dishes were incubated in darkness at room temperature for 5-7 days, until the colonies were 4-5 cm in diameter. Approximately 10 mL of sterile distilled water was then added to each Petri dish, and the fungal tissues were flattened with the bottom of a sterile glass tube. The water was decanted and the Petri dishes were incubated overnight under fluorescent light at room temperature, followed by 24 h incubation in darkness at 15°C to encourage sporulation. The spore suspensions were prepared by adding 4-5 mL of sterile distilled water to the fungal colonies, and then gently dislodging the conidia from the conidiophores with the help of a sterile inoculation loop or an art brush.

### 6.2.2 Calculation of the mean $EC_{50}$

Independent experiments were run to determine $EC_{50}$(s) for propiconazole and pyraclostrobin. Experiments were conducted using a completely randomized design (CRD) with 3 replicate wells assigned for each treatment. There were 3 replicate control wells per isolate that were not amended with fungicide. All experiments were repeated.

Fungal spore concentrations were estimated using a Fuchs Rosenthal Counting Chamber (Hausser Scientific, Blue Bell, PA) and manually adjusted to $1 \times 10^4$ conidia per mL for both *Ptt* and *Ptm*. An aliquot of 100 µL of the spore suspension, containing approximately 1000 spores, was transferred into each well of a 96-well microtiter plate (Thermo Scientific, Waltham, MA, Catalog # 167008), which contained 100 µL of the fungicide solution prepared in 2x concentrated potato dextrose broth (PDB; Difco Laboratories, Detroit, Michigan). The combined solution was mixed thoroughly. Kanamycin (100 mg $L^{-1}$) and streptomycin (20 mg $L^{-1}$) were added to the concentrated media to avoid bacterial contamination. Technical grade propiconazole and pyraclostrobin fungicides were purchased from Sigma-Aldrich Corporation (USA) and were diluted to achieve end concentrations. The $EC_{50}$ of propiconazole was determined based on eight different concentrations of the fungicide (0, 0.25, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 mg $L^{-1}$) and eight isolates each of *Ptt* and *Ptm*. These isolates were randomly selected from the full collection of 39 *Ptt* and 27 *Ptm* isolates. The $EC_{50}$ value of pyraclostrobin was determined based on eight different concentrations of the fungicide (0, 0.0015, 0.0075, 0.0150, 0.0750, 0.1500, 0.4500, and 1.050 mg $L^{-1}$) and 12 isolates each of *Ptt* and *Ptm*, with four isolates of each form randomly selected from each province.

The endpoint optical density (OD) of samples in the 96-well microtiter plates was measured at 405 nm, on a Molecular Devices SpectraMax M3 microtiter plate reader (Sunnyvale, CA),

immediately after filling the plates. Plates were then incubated in darkness at 20°C for 7 days. Following incubation, the OD was measured and final values for absorbance were obtained by subtracting the values of the initial reading from those of the final reading. Subsequently, for each isolate-concentration replicate, growth inhibition was calculated using the equation: 1 - [(absorption for each treatment / absorption of non-amended control)], and then presented as a percentage.

Since some fungi exhibit *in vitro* insensitivity to the QoIs based on an alternative respiratory pathway using alternative oxidase 1 (AOX), a preliminary assessment was conducted to determine if the addition of salicylhydroxamic acid (SHAM), which blocks this pathway (Miguez et al. 2003; Wood and Hollomon 2003), was required. The pyraclostrobin sensitivity of three isolates each of *Ptt* and *Ptm* was assessed in the presence or absence of 100 mg $L^{-1}$ SHAM. Although a paired t-test indicated no significant difference for the results obtained in the presence or absence of this chemical (*results not shown*), all pyraclostrobin bioassays were conducted in the presence of 100 mg $L^{-1}$ SHAM (SHAM, 99%; Sigma-Aldrich, St. Louis, MO) to avoid any potential alternative respiration, which could confound sensitivity assessments.

### 6.2.3 Evaluation of the sensitivity of *Ptt* and *Ptm* isolates

As was the case for the $EC_{50}$ assessment, independent experiments were performed for each fungicide and for each pathogen form, employing a completely randomized design (CRD) with 3 replicate wells per isolate-concentration combination. All experiments were repeated. There were 3 replicate control wells per isolate with no fungicide amendment. Each of the 39 *Ptt* and 27 *Ptm* isolates was tested using the microtiter bioassay procedure, described previously, at a discriminatory dose of 5.0 mg $L^{-1}$ for propiconazole, and 0.15 mg $L^{-1}$ for pyraclostrobin. For both fungicides, these concentrations provided overall growth inhibitions of almost 90% for *Ptt*

and 86% for *Ptm* when averaged over the representative isolates used in the calculation of mean $EC_{50}$ values (Fig 6-1). The dose response curve was not, however, used directly to calculate the average $EC_{50}$ values, so as to not violate the assumptions of normality and homogeneity of variance required to pool the data from individual isolates. Therefore, average $EC_{50}$ values were estimated as described in the data analysis section below. The discriminatory dose of propiconazole (5 mg $L^{-1}$) was 2.6× greater than the average $EC_{50}$ values calculated for the *Ptt* and *Ptm* isolates, and was 1.4 times greater than the highest calculated $EC_{50}$ value among the 16 examined *P. teres* isolates which was 3.687 mg $L^{-1}$ for *Ptm* isolate SKV12. The discriminatory dose of pyraclostrobin (0.15 mg $L^{-1}$) was 7.7× greater than the average $EC_{50}$ values calculated for *Ptt* and *Ptm*, and was 2.5 times greater than the highest calculated $EC_{50}$ value among the 24 examined *P. teres* isolates which was 0.061 mg $L^{-1}$ for *Ptm* isolate MBV37.

Fungal isolates were grouped as sensitive to propiconazole and pyraclostrobin if growth was reduced by >70%, intermediate if growth was reduced by 30-70%, and insensitive if growth was reduced <30% (Thaher 2011; Bowness et al. 2016). For each isolate replicate, percent growth inhibition was calculated as described above and averaged over the three replicates and the two experiments. All isolates for which reduced sensitivity was observed were examined further by treatment with higher discriminatory doses of each fungicide. In the case of propiconazole, all isolates with growth inhibition values of <50% at 5.0 mg $L^{-1}$ were also evaluated against discriminatory doses of 10 mg $L^{-1}$, 20 mg $L^{-1}$, and 100 mg $L^{-1}$ of the fungicide. In the case of pyraclostrobin, isolates with growth inhibition values of <50% at 0.15 mg $L^{-1}$ were further tested against a new discriminatory dose of 0.45 mg $L^{-1}$.

### 6.2.4 Data analysis

For each isolate, the $EC_{50}$ value was independently estimated by probit analysis (Finney 1971; Stammler et al. 2012) with SPSS statistical software (IBM SPSS Statistics for Windows, Version 23.0, IBM Corporation, Armonk, NY, USA). The maximum likelihood procedure was used for linear regression analysis to fit the regression of the response versus the concentration (Marzani 2011). Briefly, a $log_{10}$-transformed fungicide concentration was used to linearize fungal growth inhibition (as a proportion of the control) and a regression line was fitted. The resulting linear regression equation was used to calculate the fungicide concentration at which fungal growth was inhibited by 50% ($EC_{50}$) for each isolate. Paired t-tests (PROC TTEST) were performed with SAS 9.3 (SAS Institute Inc., Cary, NC) to determine if the $EC_{50}$ values calculated for a set of isolates in an experiment were significantly ($P<0.05$) different from the $EC_{50}$ values calculated for the same set when the entire test was repeated. Since there was no significant difference between the estimated values, the final $EC_{50}$ values of the eight *Ptt* and eight *Ptm* isolates, for propiconazole, and 12 *Ptt* and 12 *Ptm* isolates, for pyraclostrobin, were calculated by averaging the arithmetic means of the calculated $EC_{50}$ values from the two experiments. For analysis of the fungal growth inhibition data, the assumptions of normality and variance homogeneity of the residuals were tested using Shapiro-Wilk and Kolmogorov-Smirnov tests, and Leaven's test, respectively (PROC UNIVARIATE) in SAS 9.3 (SAS Institute). The growth inhibition data were then subjected to a one-way analysis of variance (PROC GLM) to test the null hypothesis of a lack of significant difference in the propiconazole and pyraclostrobin sensitivities of *Ptt* and *Ptm* isolates from Alberta, Saskatchewan and Manitoba.

## 6.3 Results

### 6.3.1 Determination of the $EC_{50}$ of propiconazole for the *Ptt* and *Ptm* isolates

The mean $EC_{50}$ of propiconazole for the *Ptt* isolates was 1.5 mg $L^{-1}$ and ranged from 0.8 mg $L^{-1}$ for isolate MB15 from Brandon, Manitoba, to 3.0 mg $L^{-1}$ for isolate AB38 from Clive, Alberta. Similarly, the mean $EC_{50}$ of propiconazole for the *Ptm* isolates was 2.3 mg $L^{-1}$, and ranged from 1.1 mg $L^{-1}$ for isolate MB22 from Pipestone, Manitoba, to 3.7 mg $L^{-1}$ for isolate SKV12 from Melfort, Saskatchewan (Table 6-1). A two-tailed t-test indicated that the difference between the $EC_{50}$ values obtained for *Ptt* and *Ptm* was just above the P = 0.05 level of significance (P = 0.07).

### 6.3.2 Assessing the propiconazole sensitivity of the *Ptt* and *Ptm* isolates

Growth inhibition values in response to propiconazole (5 mg $L^{-1}$) among the 39 *Ptt* isolates assessed ranged from 12.4% for isolate AB48 to 95.2% for isolate AB28. A mean growth inhibition of 78.7% was obtained over all of the *Ptt* isolates examined. Two isolates, AB48 and AB11, collected from the Lacombe and Bentley areas in central Alberta, had a growth inhibition of <30%. Another four isolates had growth inhibition values of 30-70%, while the growth inhibition of the remaining 33 isolates ranged from 70-100% (Table 6-2, Fig. 6-2). Growth inhibition for the *Ptt* reference isolates WRS102 and WRS858 was 84.8% and 92.0%, respectively. The isolates AB48 and AB11 were designated as insensitive to the fungicide, based on the low growth inhibition observed, and also were assessed at discriminatory doses of 10 mg $L^{-1}$, 20 mg $L^{-1}$ and 100 mg $L^{-1}$ propiconazole. Both isolates had a growth inhibition of <50% at the 10 mg $L^{-1}$ dose, but growth inhibition at 20 mg $L^{-1}$ was >50%. Neither AB48 nor AB11 grew at a dose of 100 mg $L^{-1}$ propiconazole.

For the *Ptm* isolates, growth inhibition in response to propiconazole (5 mg $L^{-1}$) ranged from 48.2% for isolate SK60 to 92.1% for isolate ABV14. The mean growth inhibition was 75.0% over all of the *Ptm* isolates examined. No isolates had growth inhibition values of <30%, while growth inhibition ranged from 30-70% for seven isolates and from 70-100% for the remaining 20 (Table 6-2, Fig. 6-2). Growth inhibition for the *Ptm* reference isolate WRS857 was 73.2%. Three isolates with growth inhibition values of 48-49% (MB26, SK64 and SK60) also were evaluated at discriminatory doses of 10 mg $L^{-1}$, 20 mg $L^{-1}$ and 100 mg $L^{-1}$ propiconazole. Only SK64 from Dollard, Saskatchewan, had a growth inhibition of <50% at 10 mg $L^{-1}$ or 20 mg $L^{-1}$ propiconazole, but did not grow at a dose of 100 mg $L^{-1}$.

No significant differences were observed in the propiconazole sensitivity of *Ptt* or *Ptm* isolates collected from the western Canadian provinces of Alberta, Saskatchewan or Manitoba (P = 0.25 for *Ptt*, and P = 0.15 for *Ptm*).

### 6.3.3 Determination of the $EC_{50}$ of pyraclostrobin for the *Ptt* and *Ptm* isolates

The mean $EC_{50}$ of pyraclostrobin for the *Ptt* isolates was 0.015 mg $L^{-1}$, and ranged from 0.002 mg $L^{-1}$ for isolate MB03 from Oak River, Manitoba, to 0.059 mg $L^{-1}$ for isolate SK24 from Naicam, Saskatchewan. In the case of the *Ptm* isolates, the mean $EC_{50}$ of pyraclostrobin was 0.024 mg $L^{-1}$, ranging from 0.008 mg $L^{-1}$ for isolate SK69 from Richardson, Saskatchewan, to 0.061 mg $L^{-1}$ for isolate MBV37 from Portage la Prairie, Manitoba (Table 6-3). A two-tailed t-test indicated that the difference between the $EC_{50}$ values obtained for *Ptt* and *Ptm* was just above the P = 0.05 level of significance (P = 0.06).

### 6.3.4 Assessing the pyraclostrobin sensitivity of *Ptt* and *Ptm* isolates

Growth inhibition values in response to pyraclostrobin (0.15 mg $L^{-1}$) for the 39 *Ptt* isolates ranged from 40.5% for isolate ABV01 to 100% for isolates ABV28, SK01 and MB05. No isolates had a growth inhibition of <30%, four had a growth inhibition of 30-70%, and 35 had a growth inhibition of 70-100%. The mean growth inhibition across all of the *Ptt* isolates was 89.0%. The *Ptt* reference isolates WRS102 and WRS858 had growth inhibition values of 84% and 99%, respectively. The sensitivity of two isolates with growth inhibition values of 40.5% and 44.9% (ABV01 and SK24, respectively) also was evaluated at a discriminatory dose of 0.45 mg $L^{-1}$ pyraclostrobin, but growth of both was inhibited >50% at this concentration of fungicide.

In the case of the 27 *Ptm* isolates, growth inhibition ranged from 23.8% for isolate SK64 to 100% for isolate AB79. One isolate (SK64) had a growth inhibition value of <30%, 5 isolates had a growth inhibition of 30-70%, and 21 isolates had a growth inhibition of 70-100% (Table 6-4, Fig. 6-3). The mean growth inhibition over all of the *Ptm* isolates was 84.6%. Growth of the *Ptm* reference isolate WRS857 was completely inhibited (100%). The isolate SK64 was designated as insensitive to pyraclostrobin based on the low inhibition of its growth, and this isolate, along with three others (SK100, MB16 and MB22) that had growth inhibition values of 38-49%, were evaluated under a higher discriminatory dose of 0.45 mg $L^{-1}$ pyraclostrobin. At this concentration the growth of none of the isolates was inhibited by <50%. Isolate SK64 also showed decreased sensitivity to propiconazole (see above).

No significant differences were detected in the pyraclostrobin sensitivity of *Ptt* or *Ptm* originating from Alberta, Saskatchewan or Manitoba ($P = 0.11$ for *Ptt*, and $P = 0.20$ for *Ptm*).

## 6.4 Discussion

Propiconazole and pyraclostrobin are commonly used to manage net blotch of barley and other cereal leaf diseases in western Canada. To evaluate the sensitivity of *Ptt* and *Ptm* to these fungicides, the first step was to establish an $EC_{50}$ for each compound. In fungicide sensitivity screening, isolates with no history of exposure to the fungicidal compounds are typically preferred to calculate a baseline $EC_{50}$. However, since no *P. teres* collections made before the registration of propiconazole or pyraclostrobin were available for this study, randomly selected *Ptt* and *Ptm* isolates from across western Canada were used to estimate the baseline $EC_{50}$ of these fungicides. The $EC_{50}$ values calculated based on the sensitivity of these isolates, which may have been exposed to propiconazole or pyraclostrobin in the past, could be higher than the $EC_{50}$ values obtained for isolates that had never been exposed to these products. Nonetheless, this study provides important information as to the current fungicide sensitivity status of *P. teres* populations in western Canada, serving as a baseline against which to compare future assessments. To our knowledge, this is the first evaluation of the propiconazole and pyraclostrobin sensitivity of *Ptt* and *Ptm* in this important barley growing region.

Ascospores and conidia are the most important primary inocula in the disease cycle of both *Ptt* and *Ptm* in western Canada, and are responsible for primary infection of barley tissues (Piening 1961, 1968; van den Berg and Rossnagel 1991; Duczek et al. 1999). Conidia are also the main secondary inoculum and are responsible for most secondary infections. Therefore, in this study, conidia were used to calculate the $EC_{50}$ values, and also to assess the sensitivity of a collection of *P. teres* isolates to propiconazole and pyraclostrobin.

The $EC_{50}$ of propiconazole for the *Ptt* isolates ranged from 0.8 to 3.0 mg $L^{-1}$ with a mean value of 1.5 mg $L^{-1}$, while the $EC_{50}$ for the *Ptm* isolates ranged from 1.1 to 3.7 mg $L^{-1}$ with a mean of 2.3 mg $L^{-1}$ (Table 6-1). Using an agar radial growth procedure, Campbell and Crous (2002) calculated mean $EC_{50}$ values of 1.2 and 0.4 mg $L^{-1}$ propiconazole for two *Ptt* populations (with a range of 0.004 to 7.693 mg $L^{-1}$ for individual isolates), and of 3.0 and 1.4 mg $L^{-1}$ for two *Ptm* populations (with a range of 0.138 to 5.772 mg $L^{-1}$) collected from four separate fields in South Africa. The wider range of $EC_{50}$ values reported by Campbell and Crous (2002) suggests the occurrence of more variability in the propiconazole sensitivity of South African versus Canadian isolates of *P. teres*. It is difficult to make direct comparisons however, since the $EC_{50}$ values in the current study were determined in a microtitre plate bioassay using conidial suspensions, as opposed to the mycelial growth that was measured by Campbell and Crous (2002).

In the case of pyraclostrobin, the $EC_{50}$ values for the individual *Ptt* isolates ranged from 0.002 to 0.059 mg $L^{-1}$ with a mean of 0.015 mg $L^{-1}$. The $EC_{50}$ values for the *Ptm* isolates ranged from 0.008 to 0.061 g $L^{-1}$ pyraclostrobin with a mean of 0.024 mg $L^{-1}$ (Table 6-3). Marzani (2011) calculated a mean $EC_{50}$ of 0.1 to 0.22 mg $L^{-1}$ pyraclostrobin for wild-type *Ptt* isolates, and of 0.28 to 0.69 mg $L^{-1}$ for mutant *Ptt* isolates with increased pyraclostrobin insensitivity. They observed similar $EC_{50}$ values for some of the mutant and wild-type isolates, suggesting that both types of the fungus were highly sensitive to pyraclostrobin. While the $EC_{50}$ values reported by Marzani (2011) were substantially higher than those reported here, their estimates were based on a modified radial growth bioassay instead of the microtiter procedure employed in the current study, making direct comparisons difficult.

Only two of the 39 *Ptt* isolates, AB48 and AB11, both of which were collected in central Alberta, had a growth inhibition of <30% and thus were regarded as propiconazole insensitive

when tested at a discriminatory dose of 5 mg $L^{-1}$ propiconazole. These two isolates were closely related, as revealed previously by simple sequence repeat marker analysis (Chapter 4; Akhavan et al. 2016a). The growth inhibition of both isolates remained <50% even when the discriminatory dose was doubled to 10 mg $L^{-1}$ propiconazole. While no *Ptm* isolates were designated as insensitive to propiconazole, the growth inhibition of isolates SK60 and SK64 was only 48.2% and 48.4%, respectively. Further testing with a discriminatory dose of 10 mg $L^{-1}$ propiconazole showed that inhibition of the growth of SK64 remained <50%. When AB48, AB11 and SK64 were evaluated at an even higher discriminatory dose of 20 mg $L^{-1}$ propiconazole, only SK64 had a growth inhibition of <50%. On the basis of these results, isolate SK64 appears to be the most insensitive to propiconazole among all *Ptt* and *Ptm* isolates evaluated. No isolate could grow in 100 mg $L^{-1}$ propiconazole, a concentration almost 53× greater than the mean $EC_{50}$ values calculated for *Ptt* and *Ptm*. This suggests that it is unlikely that any of the *P. teres* isolates examined possess extreme or qualitative resistance to propiconazole. Alterations in the *CYP51* gene in many plant pathogens (reviewed by Parker et al. 2014), including *Ptt* (Mair et al. 2016), have been reported to be the main mechanism for increased insensitivity towards DMIs. Azole insensitivity is increasing in pathogen populations as a result of the selection pressure imposed by the excessive application of triazole fungicides (Parker et al 2014). Interestingly, isolate SK60 was previously reported to be one of two *Ptm* isolates virulent on barley breeding line CI 9214, which is classified as highly resistant to SFNB (Chapter 4; Akhavan et al. 2016a).

No *Ptt* isolate out of the 39 examined had a growth inhibition of <30% when tested at a discriminatory dose of 0.15 mg $L^{-1}$ pyraclostrobin (Table 6-4, Fig. 6-3). Marzani (2011) found that among the QoI fungicides they tested on a collection of *Ptt* isolates, pyraclostrobin and

picoxystrobin were highly inhibitory, whilst the efficacy of other compounds was less pronounced. In the current study, only one isolate of *Ptm* (SK64) had a growth inhibition of <30% in response to 0.15 mg $L^{-1}$ pyraclostrobin (Table 6-4, Fig. 6-3). This is the same isolate that also exhibited increased insensitivity to propiconazole, suggesting that this isolate is tolerant to both products. However, when evaluated against a higher discriminatory dose of 0.45 mg $L^{-1}$ pyraclostrobin, growth of all isolates was inhibited by >50%. Since this dose was only 23× the average $EC_{50}$, it appears that no isolates in the current study had qualitative insensitivity to pyraclostrobin. In contrast, such extreme insensitivity to pyraclostrobin has been reported previously in western Canada for both *Ascochyta rabiei* on chickpea and *Mycosphaerella pinodes* on field pea (Gossen and Anderson 2004; Chang et al. 2007; Wise et al. 2008, 2009; Thaher 2011; Bowness et al. 2016). Two isolates of *A. rabiei* had an $EC_{50}$ value almost 704× greater than that of baseline isolates (Wise et al. 2009). Similarly, the mean $EC_{50}$ of *M. pinodes* pyraclostrobin-insensitive isolates was nearly 1,500× than that of sensitive controls (Bowness et al. 2016). Mutations (G143A and F129L) in the mitochondrial target gene cytochrome *b* have been found to be associated with the insensitivity of many plant pathogens to QoIs (Grasso et al. 2006; Sierotzki et al. 2007). The G143A mutation, which is stronger and more frequent, results in qualitative insensitivity to the fungicide, while the F129 L mutation results in moderate insensitivity (Gisi et al. 2000; Sierotzki et al. 2007). In *P. teres*, only the F129L mutation has been reported to confer pyraclostrobin insensitivity (Semar et al. 2007; Sierotzki et al. 2007; Marzani et al. 2013). The current findings are consistent with those of other studies (Sierotzki et al. 2007; Marzani et al. 2013) that found no qualitative or extreme insensitivity to pyraclostrobin in the net blotch fungus.

No significant differences were observed in the propiconazole or pyraclostrobin sensitivity of the *Ptt* and *Ptm* isolates from Alberta, Saskatchewan and Manitoba. Recently, Akhavan et al (2016b; Chapter 3) reported that *Ptt* and *Ptm* isolates from these provinces are similar genetically, with a large fraction of the genetic differentiation occurring within as opposed to between provinces.

Campbell and Crous (2002) found that *Ptm* isolates were less sensitive to propiconazole, tebuconazole, triadimenol, bromuconazole and flusilazole than were *Ptt* isolates, and concluded that *Ptm* may have developed more insensitivity as a result of a different evolutionary path. Scott et al. (1992) reported that two applications of propiconazole were required to reliably control SFNB caused by *Ptm*, while only one application was sufficient for control of NFNB caused by *Ptt*. Similar, but weaker trends ($P < 0.10$) were found in the current study, for propiconazole or pyraclostrobin sensitivity where slightly higher $EC_{50}$ values were observed for *Ptm* versus *Ptt*.

Propiconazole has been used for the control of diseases of cereals and other crops in western Canada since the early 1990s (Xue et al. 1994; Thomas 1997), while pyraclostrobin has been applied commercially to cereals only since the early 2000s (BASF 2003; Government of Canada: Health Canada Pest Management Regulatory Agency 2011). In western Canada, barley stubble likely contains both the sexual and asexual stages of the net blotch pathogens, and given fungicide use by barley farmers, fungal populations are frequently exposed to these products. Therefore, the routine application of these site-specific compounds on the genetically diverse populations of *Ptt* and *Ptm* poses a high risk for the development of insensitivity to both fungicides, particularly since the sensitivity to fungicides such as propiconazole has a high heritability rate (Talas and McDonald 2015). Moreover, no fitness penalty was observed for

mutant *Ptt* isolates with increased insensitivity to strobilurins (Marzani 2011). Campbell and Crous (2002) also suggested that sexual recombination could increase the rate of insensitivity of *Ptm* isolates toward DMI fungicides in South Africa. If the pathogen develops insensitivity, then with many asexual cycles during the following growing season, the insensitive individual may multiply and constitute a significant portion of a whole new emerging *P. teres* population. The high gene flow reported among the provincial populations of both forms of the net blotch pathogen in western Canada (Chapter 3; Akhavan et al. 2016b) suggests there is a risk of the transfer of fungicide insensitivity from one population to another. In Europe, it was shown that the frequency of *M. graminicola* haplotypes with insensitivity to QoI fungicides (due to the G143A mutation) increased rapidly via strong fungicide selection pressure, and that the pathogen then spread in a west-to-east direction via wind dispersal of ascospores (Torriani et al. 2009).

The identification of *Ptt* and *Ptm* isolates insensitive to propiconazole, and of a *Ptm* isolate with increased insensitivity to both propiconazole and pyraclostrobin, suggest the need for increased fungicide stewardship in western Canada. Farmers should avoid relying exclusively on a single or a few fungicide active ingredients, and employ other strategies to reduce the risk of fungicide insensitivity in *Ptt* and *Ptm* populations. Fungicide mixtures, comprising QoIs and DMIs or the novel SDHI formulations, were found to have great efficacy for net blotch disease management (Marzani 2011). Ideally, a broad combination of control methods, in addition to the application of fungicides, should be used to manage net blotch of barley.

In the current study, we assessed the sensitivity of *Ptt* and *Ptm* for the DMI fungicide propiconazole and the QoI fungicide pyraclostrobin. Our research mainly focused on detecting insensitivity and comparing differences between the two forms of the pathogen. Given the occurrence of insensitive isolates, our results underscore the need for continued monitoring of

the fungicide sensitivity of *P. teres* populations in western Canada. In addition, future studies are needed to investigate the genetic mechanisms underlying these insensitivities, and to also evaluate the relative fitness of insensitive isolates; this research will help with the early detection of fungicide insensitivity, and allow for more extensive evaluation of insensitivity development in western Canadian populations of *Ptt* and *Ptm*.

## 6.5 Tables

**Table 6-1.** The effective concentration of propiconazole (mg $L^{-1}$) required to inhibit fungal growth by 50% ($EC_{50}$) in eight isolates each of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*).

| ***Ptt*** | **Experiment 1** | **Experiment 2** | **Average** | ***Ptm*** | **Experiment 1** | **Experiment 2** | **Average** |
|---|---|---|---|---|---|---|---|
| MB15 | 0.842 | 0.780 | 0.811 | MB22 | 1.182 | 0.993 | 1.088 |
| SK26 | 1.011 | 0.763 | 0.887 | SK88 | 1.956 | 2.289 | 2.123 |
| ABV01 | 1.236 | 0.786 | 1.011 | SK69 | 1.367 | 3.052 | 2.210 |
| SK24 | 1.340 | 0.876 | 1.108 | AB82 | 2.064 | 2.453 | 2.259 |
| ABV28 | 1.383 | 1.622 | 1.503 | MB23 | 2.705 | 1.821 | 2.263 |
| MB10 | 1.862 | 1.717 | 1.790 | AB61 | 1.726 | 2.375 | 2.051 |
| AB01 | 2.334 | 2.194 | 2.264 | MBV37 | 2.116 | 2.809 | 2.463 |
| AB38 | 3.043 | 2.935 | 2.989 | SKV12 | 4.322 | 3.052 | 3.687 |
| Average | 1.632 | 1.459 | 1.545 | Average | 2.180 | 2.356 | 2.268 |

These isolates were randomly selected from across western Canada to establish a baseline sensitivity to propiconazole. A paired t-test indicated that there was no significant difference between $EC_{50}$ values calculated in each of the two independent experiments conducted for *Ptt* ($P = 0.07$) and *Ptm* ($P = 0.61$). Therefore, the two $EC_{50}$ values for each isolate were averaged. A log10-transformed fungicide concentration was used to linearize fungal growth inhibition (as a proportion of the control) to fit a regression line to estimate the $EC_{50}$ using probit analysis.

**Table 6-2.** Propiconazole sensitivity of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) isolates collected in 2009-2012 from Alberta, Saskatchewan and Manitoba, Canada.

| ***Ptt*** | **Percent growth inhibition** | **Sensitivity** | ***Ptm*** | **Percent growth inhibition** | **Sensitivity** |
|---|---|---|---|---|---|
| AB28 | 95.2 | Sensitive | ABV14 | 92.1 | Sensitive |
| MB10 | 93.3 | Sensitive | MB22 | 91.7 | Sensitive |
| MB14 | 93.3 | Sensitive | AB61 | 91.6 | Sensitive |
| ABV01 | 91.4 | Sensitive | SK100 | 90.5 | Sensitive |
| SK16 | 90.7 | Sensitive | SK87 | 89.8 | Sensitive |
| SK41 | 90.2 | Sensitive | AB57 | 89.2 | Sensitive |
| MB02 | 90.0 | Sensitive | AB68 | 86.1 | Sensitive |
| SK53 | 89.3 | Sensitive | AB82 | 82.6 | Sensitive |
| ABV28 | 89.1 | Sensitive | MB16 | 81.8 | Sensitive |
| AB53 | 88.3 | Sensitive | AB58 | 80.9 | Sensitive |
| SK03 | 88.1 | Sensitive | MB32 | 80.6 | Sensitive |
| AB01 | 87.9 | Sensitive | MB24 | 79.3 | Sensitive |
| MB05 | 87.6 | Sensitive | SK73 | 77.2 | Sensitive |
| ABV18 | 87.1 | Sensitive | SK88 | 75.5 | Sensitive |
| AB04 | 86.1 | Sensitive | SKV08 | 74.2 | Sensitive |
| AB35 | 86.0 | Sensitive | SKV10 | 72.4 | Sensitive |
| SK05 | 85.9 | Sensitive | SKV12 | 72.3 | Sensitive |
| SK26 | 85.8 | Sensitive | AB74 | 72.0 | Sensitive |
| AB06 | 85.6 | Sensitive | MB23 | 71.4 | Sensitive |
| MB04 | 85.6 | Sensitive | SK69 | 70.4 | Sensitive |
| SK24 | 84.9 | Sensitive | MB21 | 67.3 | Intermediate |
| SK52 | 81.3 | Sensitive | AB79 | 66.1 | Intermediate |
| MB11 | 79.4 | Sensitive | MBV25 | 64.7 | Intermediate |
| SK08 | 78.9 | Sensitive | MBV37 | 58.7 | Intermediate |
| MB01 | 78.5 | Sensitive | MB26 | 49.2 | Intermediate |
| AB51 | 78.0 | Sensitive | SK64 | 48.4 | Intermediate |

**Table 6-2.** continued.

| *Ptt* | Percent growth inhibition | Sensitivity | *Ptm* | Percent growth inhibition | Sensitivity |
|---|---|---|---|---|---|
| MB06 | 77.7 | Sensitive | SK60 | 48.2 | Intermediate |
| MB03 | 76.4 | Sensitive | | | |
| SK33 | 76.3 | Sensitive | | | |
| AB38 | 74.7 | Sensitive | | | |
| AB34 | 72.7 | Sensitive | | | |
| SK01 | 71.4 | Sensitive | | | |
| SK07 | 70.2 | Sensitive | | | |
| AB12 | 68.6 | Intermediate | | | |
| MB15 | 64.2 | Intermediate | | | |
| AB16 | 63.2 | Intermediate | | | |
| AB32 | 62.6 | Intermediate | | | |
| AB11 | 22.1 | Insensitive | | | |
| AB48 | 12.4 | Insensitive | | | |

Sensitivity to propiconazole was assessed based on the reduction in fungal growth in a microtiter plate bioassay at a discriminatory dose of 5 mg $L^{-1}$. The endpoint optical density (OD) of samples in the 96-well microtiter plates was measured at 405 nm immediately after filling the plates. Plates were then incubated in darkness at 20°C for 7 days. Following incubation, the OD was measured and final values for absorbance were obtained by subtracting the values of the initial reading from those of the final reading. Subsequently, for each of the three isolate replicates, growth inhibition was calculated using the equation: 1 - [(absorption for the treatment / absorption of non-amended control)], and then averaged. The experiments were then repeated and the mean of the two growth inhibition values for each isolate was presented as a percentage.

**Table 6-3.** The effective concentration of pyraclostrobin (mg $L^{-1}$) required to inhibit fungal growth by 50% ($EC_{50}$) in 12 isolates each of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata*.

| ***Ptt*** | **Experiment 1** | **Experiment 2** | **Average** | ***Ptm*** | **Experiment 1** | **Experiment 2** | **Average** |
|---|---|---|---|---|---|---|---|
| MB03 | 0.002 | 0.003 | 0.002 | SK69 | 0.004 | 0.012 | 0.008 |
| MB10 | 0.003 | 0.001 | 0.002 | AB74 | 0.009 | 0.01 | 0.009 |
| MB01 | 0.007 | 0.001 | 0.004 | AB58 | 0.005 | 0.014 | 0.01 |
| AB38 | 0.002 | 0.006 | 0.004 | MBV25 | 0.011 | 0.013 | 0.012 |
| SK01 | 0.006 | 0.009 | 0.008 | AB82 | 0.007 | 0.024 | 0.016 |
| ABV28 | 0.01 | 0.007 | 0.009 | MB23 | 0.013 | 0.019 | 0.016 |
| SK53 | 0.009 | 0.01 | 0.01 | MB22 | 0.023 | 0.026 | 0.024 |
| MB15 | 0.002 | 0.018 | 0.01 | SKV12 | 0.036 | 0.015 | 0.026 |
| AB01 | 0.014 | 0.012 | 0.013 | AB61 | 0.04 | 0.026 | 0.033 |
| ABV01 | 0.021 | 0.026 | 0.024 | SK88 | 0.026 | 0.042 | 0.034 |
| SK26 | 0.026 | 0.043 | 0.034 | SK60 | 0.025 | 0.062 | 0.043 |
| SK24 | 0.056 | 0.062 | 0.059 | MBV37 | 0.059 | 0.063 | 0.061 |
| Average | 0.013 | 0.017 | 0.015 | Average | 0.021 | 0.027 | 0.024 |

Four isolates from each of the three provinces of Alberta, Saskatchewan and Manitoba, Canada, were selected randomly to establish the baseline sensitivity to pyraclostrobin. A paired t-test indicated that there was no significant difference between $EC_{50}$ values calculated in each of two independent experiments conducted for *Ptt* ($P = 0.13$) and *Ptm* ($P = 0.20$). Therefore, the two $EC_{50}$ values for each isolate were averaged. A log10-transformed fungicide concentration was used to linearize fungal growth inhibition (as a proportion of the control) to fit a regression line to estimate the $EC_{50}$ using probit analysis.

**Table 6-4.** Pyraclostrobin sensitivity of *Pyrenophora teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*) collected in 2009-2012 from Alberta, Saskatchewan and Manitoba, Canada.

| ***Ptt*** | **Percent growth inhibition** | **Sensitivity** | ***Ptm*** | **Percent growth inhibition** | **Sensitivity** |
|---|---|---|---|---|---|
| ABV28 | 100 | Sensitive | AB79 | 100 | Sensitive |
| SK01 | 100 | Sensitive | SKV10 | 99.9 | Sensitive |
| MB05 | 100 | Sensitive | SKV12 | 99.6 | Sensitive |
| MB04 | 99.8 | Sensitive | MB32 | 99.2 | Sensitive |
| AB53 | 99.5 | Sensitive | AB58 | 99.2 | Sensitive |
| MB06 | 99.4 | Sensitive | MB24 | 98.6 | Sensitive |
| MB15 | 99.2 | Sensitive | AB68 | 98.1 | Sensitive |
| AB51 | 99.1 | Sensitive | ABV14 | 97.9 | Sensitive |
| MB10 | 98.4 | Sensitive | AB74 | 97.0 | Sensitive |
| SK08 | 98.3 | Sensitive | SK69 | 96.0 | Sensitive |
| MB02 | 98.3 | Sensitive | SK88 | 95.8 | Sensitive |
| AB34 | 97.9 | Sensitive | MBV25 | 95.8 | Sensitive |
| AB38 | 97.6 | Sensitive | AB61 | 95.7 | Sensitive |
| AB04 | 97.4 | Sensitive | SK60 | 95.0 | Sensitive |
| SK41 | 97.2 | Sensitive | MB26 | 94.5 | Sensitive |
| SK03 | 96.8 | Sensitive | MB23 | 94.2 | Sensitive |
| SK05 | 96.6 | Sensitive | MB21 | 93.7 | Sensitive |
| MB11 | 96.1 | Sensitive | MBV37 | 92.6 | Sensitive |
| AB16 | 96.0 | Sensitive | SK73 | 89.2 | Sensitive |
| SK33 | 94.9 | Sensitive | AB57 | 85.5 | Sensitive |
| MB01 | 94.7 | Sensitive | AB82 | 83.5 | Sensitive |
| MB14 | 94.5 | Sensitive | SK87 | 64.3 | Intermediate |
| MB03 | 93.2 | Sensitive | SKV08 | 58.8 | Intermediate |
| AB01 | 91.9 | Sensitive | MB16 | 49.6 | Intermediate |
| AB48 | 90.3 | Sensitive | MB22 | 48.8 | Intermediate |
| SK53 | 90.3 | Sensitive | SK100 | 38.3 | Intermediate |

**Table 6-4.** continued.

| *Ptt* | Percent growth inhibition | Sensitivity | *Ptm* | Percent growth inhibition | Sensitivity |
|---|---|---|---|---|---|
| AB12 | 89.9 | Sensitive | SK64 | 23.8 | Insensitive |
| AB35 | 86.1 | Sensitive | | | |
| SK52 | 85.4 | Sensitive | | | |
| SK26 | 85.4 | Sensitive | | | |
| AB06 | 85.3 | Sensitive | | | |
| ABV18 | 84.4 | Sensitive | | | |
| AB11 | 81.1 | Sensitive | | | |
| SK07 | 75.3 | Sensitive | | | |
| AB32 | 72.3 | Sensitive | | | |
| AB28 | 63.2 | Intermediate | | | |
| SK16 | 61.7 | Intermediate | | | |
| SK24 | 44.9 | Intermediate | | | |
| ABV01 | 40.5 | Intermediate | | | |

Sensitivity to pyraclostrobin fungicide was assessed based on the reduction in fungal growth in a microtiter plate bioassay at a discriminatory dose of 0.15 mg $L^{-1}$. The endpoint optical density (OD) of samples in the 96-well microtiter plates was measured at 405 nm immediately after filling the plates. Plates were then incubated in darkness at 20°C for 7 days. Following incubation, the OD was measured and final values for absorbance were obtained by subtracting the values of the initial reading from those of the final reading. Subsequently, for each of the three isolate replicates, growth inhibition was calculated using the equation: 1 - [(absorption for the treatment / absorption of non-amended control)], and then averaged. The experiments were then repeated and the mean of the two growth inhibition values for each isolate was presented as a percentage.

## 6.6 Figures

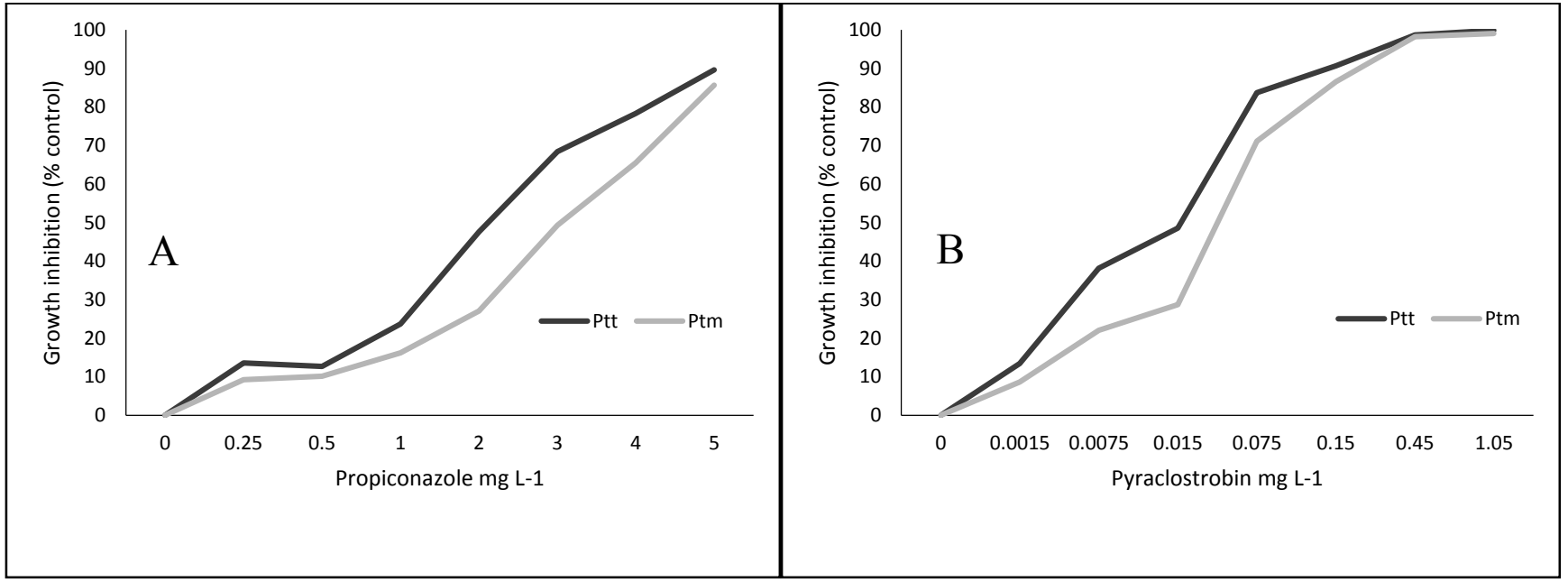


**Figure 6-1.** Dose response curve of the mean fungal growth inhibition of representative *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* isolates from western Canada in response to propiconazole **(A)** and pyraclostrobin **(B)**. Eight and 12 isolates each of *Ptt* and *Ptm*, respectively, were employed in the experiments with propiconazole (16 isolates in total) and pyraclostrobin (24 isolates in total). Three replicate wells were included for each combination of isolate and concentration. Experiments were conducted twice, and the mean fungal growth inhibitions were averaged. Growth inhibition is relative to non-amended control treatments.

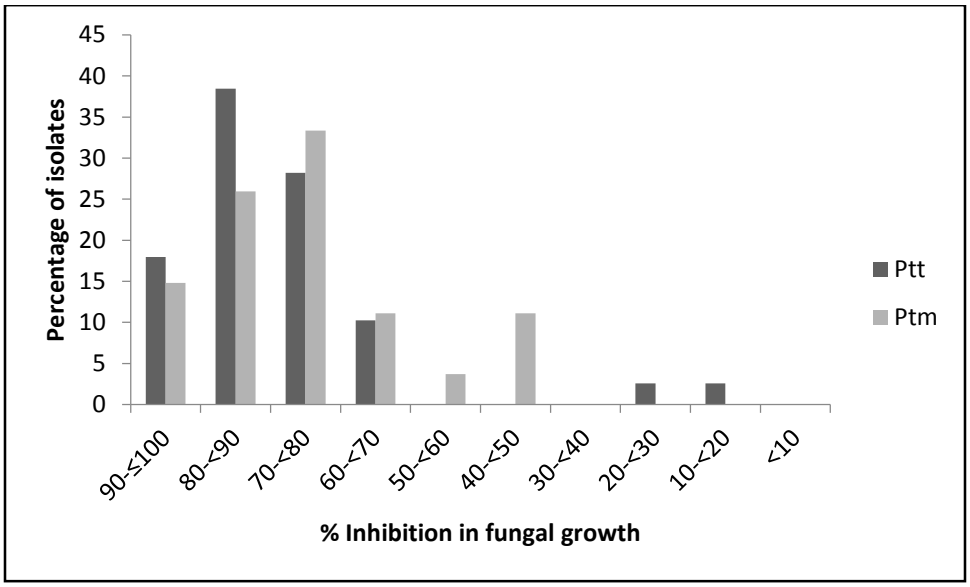


**Figure 6-2.** Frequency distribution of inhibition of fungal growth in 39 *Pyrenophora teres* f. *teres* and 27 *P. teres* f. *maculata* isolates from western Canada in response to 5 mg $L^{-1}$ propiconazole in potato dextrose broth. Inhibition of fungal growth is expressed as a percentage relative to a control treatment in which no propiconazole was included.

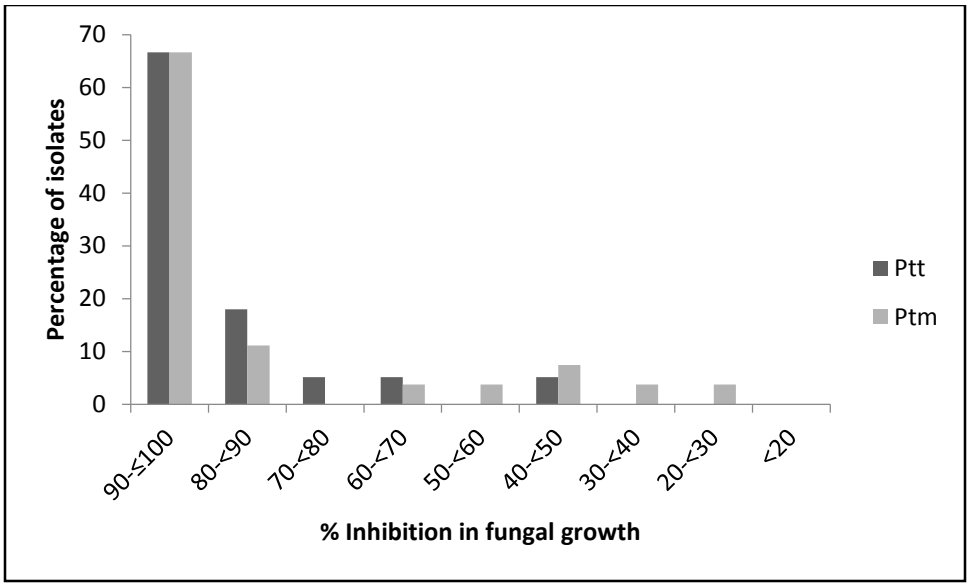


**Figure 6-3.** Frequency distribution of inhibition of fungal growth in 39 *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* isolates from western Canada in response to 0.15 mg $L^{-1}$ pyraclostrobin in potato dextrose broth. Inhibition of fungal growth is expressed as a percentage relative to a control treatment in which no pyraclostrobin was included. Experiments were performed in the presence of 100 mg $L^{-1}$ salicylhydroxamic acid (SHAM).

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# Chapter 7 General discussion

The last comprehensive assessment of *Pyrenophora teres* populations in western Canada was conducted in the 1980s, when Tekauz (1990) documented extensive variation in pathogen virulence. The importance of ongoing pathogen surveillance is emphasized by shifts in cropping practices, changes in cultivars and acreages, and the appearance of new disease issues. Knowledge regarding the occurrence and frequency of pathogen mating types and population structure is critical for predicting the evolutionary potential of the net blotch pathogen, and thus the longevity of resistance and fungicide effectiveness (Tekauz 1990; McDonald and Linde 2002). In this dissertation, I presented a number of studies aimed at improving our understanding of the genetic and virulence structure of *P. teres* populations in western Canada. Moreover, I assessed how some commercial cultivars and breeding lines previously classified as resistant to net form net blotch (NFNB) and/or spot form net blotch (SFNB) responded to more recent collections of *P. teres* f. *teres* (*Ptt*) and *P. teres* f. *maculata* (*Ptm*). Finally, I also looked for any increased insensitivity in *Ptt* and *Ptm* populations to propiconazole and pyraclostrobin, two fungicides that are routinely used in western Canada. At this point, it is beneficial to briefly reflect on the various studies included in this dissertation, and to suggest a few areas for future research to advance the current findings.

## 7.1 Prevalence of mating type idiomorphs in *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada

Departures from statistically equal frequencies of the two mating types for heterothallic ascomycetes such as *Ptt* and *Ptm* would most likely be associated with a predominance of asexual reproduction via conidia (Sommerhalder et al. 2006). However, for ascospores to be

considered the major source of primary inoculum, the two mating types must occur in statistically equal frequencies (Rau et al. 2005; Sommerhalder et al. 2006; Bogacki et al. 2010). In this study, with the exception of a *Ptm* population collected in 2011 from Saskatchewan, we observed no significant departure from a 1:1 mating type ratio in any of the *Ptt* or *Ptm* populations in the original data set. Subsequently and following a clone correction step, we observed no statistical differences in mating type frequency for either *Ptt* or *Ptm* in all populations. In western Canada, Piening (1961) previously noted the general occurrence of mature ascocarps of *P. teres* on barley straw from fields in the Calgary and Edmonton regions of Alberta. Similarly, Duczek et al. (1999) found pseudothecia of *P. teres* in two fields near Dafoe and Churchbridge, Saskatchewan. Piening (1968) also indicated that ascospores of *P. teres* were responsible for almost 50 % of all net blotch lesions examined on volunteer barley plants in a field at the Lacombe Research Station in Alberta. Considering all of the observations and results from the current and previous studies, I conclude that *Ptt* and *Ptm* go through regular cycles of sexual reproduction in western Canada. Overall, these results concur with the hypothesis that primary infection of barley fields in this region is caused mainly by ascospores discharged from pseudothecia on stubble. Another potential source of primary infection for net blotch is seed-borne inoculum (Hampton 1980; Jordan 1981; Sheridan et al. 1983; Shipton et al. 1973; Singh and Chand 1985; Turkington et al. 2011). Seed-to-seedling transmission of the net blotch pathogen would result in net blotch lesions on developing barley seedlings, which then produce successive generations of secondary inoculum via conidia. Over a three-year period in Alberta, Turkington et al. (2002) reported average levels of seed infection with net blotch ranging from 7.1-22.6%, with some grain samples showing up to 81-89% infection. However, given the relatively short rotation intervals between barley crops, infested crop residues would represent

the main source of inoculum initiating primary infections. Further studies are needed to provide direct evidence for the extensive occurrence of sexual reproduction and the development of the mature pseudothecia on barley stubble in western Canada. The relative importance of the role of ascospores, relative to conidia, as primary inoculum, and their capacity for dispersal should also be assessed using classical and molecular epidemiology techniques.

## 7.2 Genetic structure of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada

The genetic structure of *Ptt* and *Ptm* populations from western Canada had not been studied previously, despite the fact that Canadian barley is grown mainly in Alberta, Saskatchewan and Manitoba (Statistics Canada 2015), and that both NFNB and SFNB are among the most important foliar diseases of barley in this region (Turkington et al. 2011). In the current work, we employed simple sequence repeat (SSR) markers first developed by Ellwood et al. (2010), which were screened and found to be highly polymorphic by Liu et al. (2012), to explore the genetic structure of *Ptt* and *Ptm* populations sampled from the three western Canadian provinces. Cluster analysis using the SSR data and UPGMA procedure revealed that all isolates tested clustered in two distinct (*Ptt* and *Ptm*) groups, with no intermediate clade, suggesting that recombination between the two pathogen forms is either very unlikely in western Canada or, at least, was not present in our collection. We calculated a relatively high and significant coefficient of genetic differentiation (PhiPT = 0.316, P = 0.001) when considering the *Ptt* and *Ptm* isolates as two populations, providing additional support for the conclusion that the *Ptt* and *Ptm* isolates are genetically close yet distinct. This is consistent with other studies that have suggested that *Ptt* and *Ptm* are genetically differentiated (Williams et al. 2001; Leisova et al. 2005; Bakonyi and Justesen 2007; Keiper et al. 2008; Bogacki et al. 2010). Small PhiPTs

(PhiPT = 0.038, P = 0.001 for *Ptt*, and PhiPT = 0.010, P = 0.177 for *Ptm*) were obtained when the *Ptt* and *Ptm* isolates collected from Alberta, Manitoba and Saskatchewan were compared, showing that a large fraction of the genetic differentiation occurred within the three provinces rather than between them. This suggests that for the purposes of resistance breeding, we can consider pathogen populations from these Prairie Provinces either as genetically very close populations (i.e., for *Ptt*), or even as a single panmictic (i.e., for *Ptm*) population.

Overall, in this first systematic study of *P. teres* populations from western Canada, a large number of distinct pathogen genotypes with a clonal fraction of approximately 10% detected within both *Ptt* and *Ptm* populations, suggesting that these populations are generally diverse, and further strengthening the conclusion (Chapter 2) that they undergo regular sexual recombination. Given these characteristics, the *Ptt* and *Ptm* populations appear to be at a relatively high risk of being able to overcome major host resistance genes, if these genes are deployed individually in barley cultivars (McDonald and Linde 2002). In addition, these characteristics also would increase the risk of fungicide resistance if the same fungicidal mode of action was used repeatedly within or between growing seasons. Therefore, barley breeders should aim to pyramid as many effective NFNB and SFNB resistance genes as possible within the same host genotypes, in addition to removing any susceptibility genes that may be present (Richards et al. 2016). The prudent use and stewardship of fungicides also should be encouraged. In the current study, I used one or a couple of isolates from each of a large number of fields across western Canada to assess the genetic structure of *Ptt* and *Ptm*. It would be useful to compare my results with those that would be obtained from an assessment of many isolates from just a few distantly located fields, to determine if the type of sampling (extensive versus intensive) has any impact

on the final conclusions that can be made with respect to genetic differentiation among and within pathogen populations.

## 7.3 Virulence of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations in western Canada

In the last comprehensive assessment of pathogenic diversity among *Ptt* and *Ptm* populations from western Canada, Tekauz (1990) classified 182 *Ptt* isolates into 10 main pathotype groups (A – J), with one to nine subgroups in each group (45 distinct virulence profiles in total). Tekauz (1990) also classified 42 *Ptm* isolates into seven main pathotype groups (P-V), with one to five subgroups in each group (20 distinct virulence profiles in total). In the current work, we evaluated the virulence of a collection of 39 *Ptt* and 27 *Ptm* isolates collected from western Canada by inoculating these isolates onto the same sets of barley differential hosts developed by Tekauz (1990). Cluster analysis revealed 16 and 13 distinct pathotype groups, respectively, among the 39 and 27 representative *Ptt* and *Ptm* isolates. In the case of *Ptt*, this level of pathogenic diversity (0.41 pathotype/isolate) was higher than that reported by Tekauz (1990) (0.24 pathotype/isolate), while for *Ptm*, the level of pathogenic diversity identified in the current study (0.48 pathotype/isolate) was equal to that reported by Tekauz (1990). We found that *Ptt* pathotype groups C4 and E9 comprised 43% of the *Ptt* isolates in a collection from western Canada, while the *Ptm* pathotype groups V1 and T1 comprised 52% of the *Ptm* isolates. The barley differentials CI 5791 and CI 9820 were resistant to all isolates of *Ptt* we tested except one, whilst the differential CI 9214 was resistant to all isolates of *Ptm* except two. Therefore, the differential lines CI 5791 and CI 9820, for *Ptt*, and CI 9214, for *Ptm*, can still be considered as potentially useful sources of resistance for Canadian barley breeding programs. Overall, the current findings, discussed in Chapter 4, confirm the conclusion of Tekauz (1990) that the

virulence spectrum of *P. teres* in western Canada is broad and also suggest that this virulence has changed in the past three decades. Ideal differential sets for characterizing the pathotypes present in any pathogen population should include genotypes with well characterized resistance and/or susceptibility. Pathotyping of isolates on such differentials would provide a more informative assessment of virulence diversity and pathotype composition. Therefore, there is a need to study the genetic basis of resistance/susceptibility in all host genotypes employed in the *Ptt* and *Ptm* differential sets, including the ones used in the current study or those suggested by other researchers (Liu et al. 2012; McLean et al. 2014).

## 7.4 Resistance to *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* in Canadian barley genotypes

Current (Chapters 2 and 3) and previous (Tekauz 1990) studies suggest that *Ptt* and *Ptm* populations are generally diverse in western Canada. Unfortunately, this diversity may increase the potential for rapid population-level adaptation (i.e., evolution) of both *Ptt* and *Ptm* to particular barley cultivars, ultimately resulting in the loss of net blotch resistance. We assessed the reaction of a collection of barley genotypes which had been previously classified as resistant to NFNB and/or SFNB, to seven isolates each of *Ptt* and *Ptm*. At the time we initiated this work, 'Vivar' was the only commercial cultivar rated as having very good (VG) NFNB resistance, while 'AAC Synergy' and 'CDC Helgason' were the only cultivars identified as having good resistance (G). The other barley genotypes were classified as having fair (F), poor (P) or very poor (VP) resistance against NFNB (Alberta Agriculture and Rural Development 2013; Manitoba Agriculture, Food and Rural Initiatives 2013; Saskatchewan Ministry of Agriculture 2013). The NFNB resistance in 'Vivar' and 'CDC Helgason' was overcome by two and four of the examined *Ptt* isolates, respectively. 'AAC Synergy' was resistant to all tested *Ptt* isolates,

although two isolates caused disease severities of 4.6 and 5.0, suggesting some adaptation to the resistance in this cultivar. The breeding lines TR 253, CI 9819 and TR 236 were the most resistant to *Ptt*, with average disease severities of almost 3.0. We used overall mean disease reactions for each host genotype and isolate to compare the relative virulence and resistance of isolates and host genotypes, respectively, as no clear pattern was observed in Chapter 4 with respect to the geographic distribution of *Ptt* and *Ptm* pathotypes across the Canadian prairies. Pathotype distribution in each region is difficult to assess, particularly on a regular basis, and also, the pathogen and host genotype interaction in this pathosystem is very complex and still not well understood (Liu et al. 2015; Richards et al. 2016). However, we still explained and discussed some of the interesting interactions observed in the study described in Chapter 5 of this dissertation.

In the case of SFNB, resistance in 'CDC Meredith' was overcome by all *Ptm* isolates tested, while 'Cerveza', 'Major' and 'AAC Synergy' were resistant to all of the isolates. Among the breeding lines, TR 236 was the most resistant to *Ptm*, with an average rating of 3.9. There was a statistically significant host genotype by pathogen isolate interaction, but this appeared to be due mainly to the influence of the reference isolate WRS 857. The six recently collected *Ptm* isolates induced disease reactions of 4.2 to 4.9 when averaged over all of the hosts, while the mean reaction induced by WRS 857 was only 2.7. Overall, this isolate was avirulent on all barley genotypes except the susceptible check 'Harrington'. While WRS 857 is used commonly for SFNB resistance screening in barley breeding programs, it appears that it is no longer representative of western Canadian *Ptm* populations. The identification of isolates virulent on cultivars classified as 'resistant' highlights the need for integrated disease management approaches to avoid net blotch resistance breakdown in western Canada. As a consequence, the

currently used sources of host resistance should be managed cautiously by farmers and breeders. Producers should avoid relying exclusively on resistant cultivars, and avoid repeatedly growing the same resistant barley cultivar in short rotations, while using resistance together with other strategies such as rotation to non-hosts and the judicious application of fungicides to reduce overall disease pressure. Unfortunately, the genetic basis of the resistance and susceptibility in many of the commercial cultivars and breeding lines in western Canada is either not known or well understood. Such information is important for the sustainable deployment of resistance sources.

## 7.5 Sensitivity of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* to propiconazole and pyraclostrobin in western Canada

The fungicides propiconazole and pyraclostrobin are commonly applied to manage net blotch on barley in western Canada (Alberta Government 2016). We used microtiter plate bioassays to evaluate the sensitivity of *Ptt* and *Ptm* isolates, collected from this region, to these two compounds. We calculated the effective concentration of propiconazole and pyraclostrobin needed to inhibit fungal growth by 50% ($EC_{50}$) in isolates of *Ptt* and *Ptm* (Chapter 6). Subsequently, to identify isolates with increased insensitivity to these fungicides, the *Ptt* and *Ptm* isolates were screened with discriminatory doses of 5 mg propiconazole $L^{-1}$ and 0.15 mg pyraclostrobin $L^{-1}$. Growth inhibition values in response to propiconazole ranged from 12-95% for *Ptt* and from 48-92% for *Ptm*, and from 40-100% and 24-100% in response to pyraclostrobin, respectively. Two *Ptt* isolates were found to be insensitive to propiconazole, and one isolate of *Ptm* was found to be insensitive to pyraclostrobin. The latter isolate also showed decreased sensitivity to propiconazole. The routine application of fungicides with site-specific activity on genetically diverse populations of *Ptt* and *Ptm* poses a high risk for the development of

insensitivity (Talas and McDonald 2015). This risk is magnified if one considers that no fitness penalty was observed for mutant *Ptt* isolates with increased insensitivity to strobilurins (Marzani 2011). The high gene flow we calculated among the provincial populations of both forms of the net blotch pathogen in western Canada (Chapter 3) also suggests that there is a risk of the transfer of fungicide insensitivity from one population to another in this region. The identification of *Ptt* and *Ptm* isolates insensitive to propiconazole and pyraclostrobin, and of a *Ptm* isolate with increased insensitivity to both compounds, suggest the need for increased fungicide stewardship in western Canada.

Considering the relatively small number of isolates examined in our study, the identification of a *Ptm* isolate (SK64) with insensitivity to both propiconazole and pyraclostrobin is a cause for concern. This finding underscores the need for fungicide stewardship and the use of an integrated approach for net blotch management in barley. Farmers should avoid relying exclusively on a single or a few fungicide active ingredients, and employ other strategies to reduce the risk of fungicide insensitivity in *Ptt* and *Ptm* populations. Fungicide mixtures, comprising QoIs and DMIs or the novel SDHI formulations, were found to have great efficacy for net blotch disease management (Marzani 2011). Ideally, a broad combination of control methods, in addition to the application of fungicides, should be used to control net blotch of barley. Finally, the identification of fungicide-insensitive isolates emphasizes the importance of continued monitoring of *P. teres* populations in western Canada, and also suggests a need to characterize the genetic mutations/changes associated with increased insensitivity.

## 7.6 Overall conclusions and impact

The results of this work provide strong evidence that both the net and spot forms of the net blotch pathogen go through regular cycles of sexual reproduction in western Canada, resulting in a genetically diverse population. Therefore, both *Ptt* and *Ptm* fall into the category of pathogens at high risk of being capable to quickly adapt, at the population level, to major host resistance genes and fungicides with single modes of action. We also showed that *Ptt* and *Ptm* populations are pathogenically diverse, and, in general, capable of overcoming the resistance in the barley cultivars previously identified as having very good or good resistance. The identification of fungal isolates virulent on resistant cultivars, and isolates insensitive to commonly used fungicides suggests that producers avoid growing the same resistant barley variety in short rotation, and excessively applying the same fungicidal mode of action. Judicious use of fungicides, coupled with rotations of at least two years between barley crops and the planting of diverse varieties of barley, will promote the effective and sustainable management of net blotch. Barley genotypes with resistance that is still effective, as well as the prevalent pathotypes of *Ptt* and *Ptm* in western Canada, also were identified. Knowledge gained from the current project will be used by breeders, pathologists, farmers, and industry to effectively and sustainably manage net blotch of barley. This work also helped to develop expertise and knowledge in barley pathology, pathogen variation, pathogen population genetics, and host-pathogen interactions, which will have long-term benefits for barley producers, breeders, and the barley industry and research community in western Canada.

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# Appendix

Identification number, year of collection, and location collected for 220 isolates used to study the prevalence of mating type idiomorphs and genetic structure of *Pyrenophora teres* f. *teres* and *P. teres* f. *maculata* populations from western Canada

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-AB01 | 2010 | Lacombe, AB | Same lesion as AB02, AB03 | AB02 |
| *Ptt*-AB02 | 2010 | Lacombe, AB | Same lesion as AB01, AB03 | AB01 |
| *Ptt*-AB03 | 2010 | Lacombe, AB | Same lesion as AB01, AB02 | |
| *Ptt*-AB04 | 2010 | Bentley, AB | Same lesion as AB05, AB06, AB07 | |
| *Ptt*-AB05 | 2010 | Bentley, AB | Same lesion as AB04, AB06, AB07 | |
| *Ptt*-AB06 | 2010 | Bentley, AB | Same lesion as AB04, AB05, AB07 | |
| *Ptt*-AB07 | 2010 | Bentley, AB | Same lesion as AB04, AB05, AB06 | |
| *Ptt*-AB08 | 2010 | Bentley, AB | | |
| *Ptt*-AB09 | 2010 | Lacombe, AB | | |
| *Ptt*-AB10 | 2010 | Bentley, AB | | |
| *Ptt*-AB11 | 2010 | East Gull Lake, AB | | |
| *Ptt*-AB12 | 2010 | Clive, AB | Same lesion as AB13, AB14, AB15 | |
| *Ptt*-AB13 | 2010 | Clive, AB | Same lesion as AB12, AB14, AB15 | |
| *Ptt*-AB14 | 2010 | Clive, AB | Same lesion as AB12, AB13, AB15 | |
| *Ptt*-AB15 | 2010 | Clive, AB | Same lesion as AB12, AB13, AB14 | |
| *Ptt*-AB16 | 2010 | Bentley, AB | Same lesion as AB17, AB18 | AB17, AB18 |
| *Ptt*-AB17 | 2010 | Bentley, AB | Same lesion as AB16, AB18 | AB16, AB18 |
| *Ptt*-AB18 | 2010 | Bentley, AB | Same lesion as AB16, AB17 | AB16, AB17 |
| *Ptt*-AB19 | 2010 | Bentley, AB | | |
| *Ptt*-AB20 | 2010 | Penhold, AB | | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-AB21 | 2010 | Delburne, AB | Same lesion as AB22 | |
| *Ptt*-AB22 | 2010 | Delburne, AB | Same lesion as AB21 | |
| *Ptt*-AB23 | 2010 | Alix, AB | | |
| *Ptt*-AB24 | 2010 | Lacombe, AB | Same leaf as AB25, AB26, AB27, AB28 | |
| *Ptt*-AB25 | 2010 | Lacombe, AB | Same leaf as AB24, AB26, AB27, AB28 | |
| *Ptt*-AB26 | 2010 | Lacombe, AB | Same leaf as AB24, AB25, AB27, AB28 | |
| *Ptt*-AB27 | 2010 | Lacombe, AB | Same leaf as AB24, AB25, AB26, AB28 | |
| *Ptt*-AB28 | 2010 | Lacombe, AB | Same leaf as AB24, AB25, AB26, AB27 | |
| *Ptt*-AB29 | 2010 | Lacombe, AB | | |
| *Ptt*-AB30 | 2011 | Clive, AB | Same plant as AB31 | |
| *Ptt*-AB31 | 2011 | Clive, AB | Same plant as AB30 | |
| *Ptt*-AB32 | 2011 | Clive, AB | Same plant as AB33 | AB33 |
| *Ptt*-AB33 | 2011 | Clive, AB | Same plant as AB32 | AB32 |
| *Ptt*-AB34 | 2011 | Bentley, AB | Same plant as AB35 | |
| *Ptt*-AB35 | 2011 | Bentley, AB | Same plant as AB34 | |
| *Ptt*-AB36 | 2011 | Lacombe, AB | | |
| *Ptt*-AB37 | 2011 | Bentley, AB | | |
| *Ptt*-AB38 | 2011 | Clive, AB | | |
| *Ptt*-AB39 | 2011 | Clive, AB | | |
| *Ptt*-AB41 | 2011 | Lacombe, AB | | SK05, SK06, SK13, SK17, SK20, SK28, SK30, SK55, SK56 |
| *Ptt*-AB42 | 2011 | Lacombe, AB | | |
| *Ptt*-AB43 | 2011 | Lacombe, AB | Same plant as AB44 | |
| *Ptt*-AB44 | 2011 | Lacombe, AB | Same plant as AB43 | |
| *Ptt*-AB45 | 2011 | Lacombe, AB | | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-AB46 | 2011 | Lacombe, AB | | |
| *Ptt*-AB47 | 2011 | Lacombe, AB | | |
| *Ptt*-AB48 | 2011 | Lacombe, AB | | |
| *Ptt*-AB49 | 2011 | Lacombe, AB | | |
| *Ptt*-AB50 | 2011 | Lacombe, AB | | |
| *Ptt*-AB51 | 2011 | Lacombe, AB | Same plant as AB52 | |
| *Ptt*-AB52 | 2011 | Lacombe, AB | Same plant as AB51 | |
| *Ptt*-AB53 | 2011 | Lacombe, AB | Same plant as AB54 | |
| *Ptt*-AB54 | 2011 | Lacombe, AB | Same plant as AB53 | |
| *Ptt*-AB55 | 2011 | Lacombe, AB | | |
| *Ptt*-AB56 | 2011 | Lacombe, AB | | |
| *Ptm*-AB57 | 2010 | Park St Rimbey, AB | | |
| *Ptm*-AB58 | 2010 | Bentley, AB | Same lesion as AB59, AB60 | AB59 |
| *Ptm*-AB59 | 2010 | Bentley, AB | Same lesion as AB58, AB60 | AB58 |
| *Ptm*-AB60 | 2010 | Bentley, AB | Same lesion as AB58, AB59 | |
| *Ptm*-AB61 | 2010 | Penhold, AB | | |
| *Ptm*-AB62 | 2010 | Markerville, AB | Same lesion as AB63, AB64, AB65 | AB63, AB64, AB65 |
| *Ptm*-AB63 | 2010 | Markerville, AB | Same lesion as AB62, AB64, AB65 | AB62, AB64, AB65 |
| *Ptm*-AB64 | 2010 | Markerville, AB | Same lesion as AB62, AB63, AB65 | AB62, AB63, AB65 |
| *Ptm*-AB65 | 2010 | Markerville, AB | Same lesion as AB62, AB63, AB64 | AB62, AB63, AB64 |
| *Ptm*-AB66 | 2010 | Markerville, AB | | |
| *Ptm*-AB67 | 2010 | Lousana, AB | Same lesion as AB68 | |
| *Ptm*-AB68 | 2010 | Lousana, AB | Same lesion as AB67 | |
| *Ptm*-AB69 | 2010 | Delburne, AB | Same lesion as AB70, AB71 | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptm*-AB70 | 2010 | Delburne, AB | Same lesion as AB69, AB71 | |
| *Ptm*-AB71 | 2010 | Delburne, AB | Same lesion as AB69, AB70 | SK75 |
| *Ptm*-AB72 | 2010 | Clive, AB | | |
| *Ptm*-AB73 | 2010 | Clive, AB | Same lesion as AB74, AB75 | AB75 |
| *Ptm*-AB74 | 2010 | Clive, AB | Same lesion as AB73, AB75 | |
| *Ptm*-AB75 | 2010 | Clive, AB | Same lesion as AB73, AB74 | AB73 |
| *Ptm*-AB76 | 2010 | Edmonton, AB | | SK59 |
| *Ptm*-AB77 | 2010 | Edmonton, AB | | |
| *Ptm*-AB78 | 2010 | Edmonton, AB | | |
| *Ptm*-AB79 | 2010 | Edmonton, AB | | |
| *Ptm*-AB80 | 2011 | Lacombe, AB | | |
| *Ptm*-AB81 | 2011 | Blackfalds, AB | | MB20, MB32, SK104 (SK104 belonged to a different mating type) |
| *Ptm*-AB82 | 2011 | Lacombe, AB | | |
| *Ptm*-AB83 | 2011 | Lacombe, AB | | |
| *Ptt*-MB01 | 2009 | Binscarth, MB | | |
| *Ptt*-MB02 | 2009 | Kirkella, MB | | |
| *Ptt*-MB03 | 2009 | Oak River, MB | | |
| *Ptt*-MB04 | 2009 | Montcalm, MB | | |
| *Ptt*-MB05 | 2010 | Unknown, MB | | |
| *Ptt*-MB06 | 2010 | Minto, MB | | |
| *Ptt*-MB07 | 2011 | Deloraine, MB | | |
| *Ptt*-MB08 | 2011 | Deloraine, MB | | |
| *Ptt*-MB09 | 2011 | Brandon, MB | | |
| *Ptt*-MB10 | 2011 | Chater, MB | | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-MB11 | 2011 | Oakner, MB | | |
| *Ptt*-MB12 | 2011 | Oakner, MB | | |
| *Ptt*-MB13 | 2011 | Chater, MB | | |
| *Ptt*-MB14 | 2011 | Portage La Prairie, MB | | |
| *Ptt*-MB15 | 2011 | Brandon, MB | | |
| *Ptm*-MB16 | 2009 | Selkirk, MB | | |
| *Ptm*-MB17 | 2009 | Mom's Way Sarto, MB | | MB31 (belonged to a different mating type) |
| *Ptm*-MB18 | 2009 | Carroll, MB | | |
| *Ptm*-MB19 | 2009 | Morris, MB | | |
| *Ptm*-MB20 | 2009 | Oakburn, MB | | AB81, MB32, SK104 (SK104 belonged to a different mating type) |
| *Ptm*-MB21 | 2009 | Sinclair, MB | | |
| *Ptm*-MB22 | 2010 | Pipestone, MB | | |
| *Ptm*-MB23 | 2010 | Binscarth, MB | | |
| *Ptm*-MB24 | 2010 | Oak River, MB | Same leaf as MB25, MB26 | |
| *Ptm*-MB25 | 2010 | Oak River, MB | Same leaf as MB24, M26 | |
| *Ptm*-MB26 | 2010 | Oak River, MB | Same leaf as MB24, MB25 | |
| *Ptm*-MB27 | 2010 | Unknown, MB | | |
| *Ptm*-MB28 | 2010 | Mom's Way Sarto, MB | Same leaf as MB29 | |
| *Ptm*-MB29 | 2010 | Mom's Way Sarto, MB | Same leaf as MB28 | |
| *Ptm*-MB30 | 2010 | Unknown, MB | | |
| *Ptm*-MB31 | 2010 | Poplar Point, MB | | MB17 (belonged to a different mating type) |
| *Ptm*-MB32 | 2010 | Deloraine, MB | | AB81, MB20, SK104 (SK104 belonged to a different mating type) |
| *Ptm*-MB33 | 2010 | Brandon, MB | Same plant as MB34 | |
| *Ptm*-MB34 | 2010 | Brandon, MB | Same plant as MB33 | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-SK01 | 2010 | Weirdale, SK | | |
| *Ptt*-SK02 | 2010 | Humboldt, SK | | |
| *Ptt*-SK03 | 2010 | Mcmahon, SK | | SK04 |
| *Ptt*-SK04 | 2010 | Unknown, SK | | SK03 |
| *Ptt*-SK05 | 2010 | Ridgedale, SK | | AB41, SK06, SK13, SK17, SK20, SK28, SK30, SK55, SK56 |
| *Ptt*-SK06 | 2010 | Henribourg, SK | | AB41, SK05, SK13, SK17, SK20, SK28, SK30, SK55, SK56 |
| *Ptt*-SK07 | 2010 | Rokeby, SK | | |
| *Ptt*-SK08 | 2010 | Mctaggart, SK | | |
| *Ptt*-SK09 | 2010 | Davidson, SK | | |
| *Ptt*-SK10 | 2010 | Congress, SK | | |
| *Ptt*-SK11 | 2011 | Spring Valley, SK | | |
| *Ptt*-SK12 | 2011 | Battleford, SK | | |
| *Ptt*-SK13 | 2011 | Eatonia, SK | | AB41, SK05, SK06, SK17, SK20, SK28, SK30, SK55, SK56 |
| *Ptt*-SK14 | 2011 | Assiniboia, SK | | |
| *Ptt*-SK15 | 2011 | Central Butte, SK | | |
| *Ptt*-SK16 | 2011 | Semans, SK | | |
| *Ptt*-SK17 | 2011 | West Bend, SK | | AB41, SK05, SK06, SK13, SK20, SK28, SK30, SK55, SK56 |
| *Ptt*-SK18 | 2011 | Burr, SK | | |
| *Ptt*-SK19 | 2011 | Rokeby, SK | | |
| *Ptt*-SK20 | 2011 | Carmel, SK | | AB41, SK05, SK06, SK13, SK17, SK28, SK30, SK55, SK56 |
| *Ptt*-SK21 | 2011 | Carmel, SK | | |
| *Ptt*-SK22 | 2011 | Archerwill, SK | | |
| *Ptt*-SK23 | 2011 | Naicam, SK | Same plant as SK51 | |
| *Ptt*-SK24 | 2011 | Naicam, SK | Same plant as SK25 | SK25 |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-SK25 | 2011 | Naicam, SK | Same plant as SK24 | SK24 |
| *Ptt*-SK26 | 2011 | St Brieux, SK | Same leaf as SK27/Same plant as SK28, SK29 | |
| *Ptt*-SK27 | 2011 | St Brieux, SK | Same leaf as SK26/Same plant as SK28, SK29 | |
| *Ptt*-SK28 | 2011 | St Brieux, SK | Same leaf as SK29/Same plant as SK26, SK27 | AB41, SK05, SK06, SK13, SK17, SK20, SK30, SK55, SK56 |
| *Ptt*-SK29 | 2011 | St Brieux, SK | Same leaf as SK28/Same plant as SK26, SK27 | |
| *Ptt*-SK30 | 2011 | St Brieux, SK | | AB41, SK05, SK06, SK13, SK17, SK20, SK28, SK55, SK56 |
| *Ptt*-SK31 | 2011 | Gronlid, SK | Same plant as SK32 | |
| *Ptt*-SK32 | 2011 | Gronlid, SK | Same plant as SK31 | |
| *Ptt*-SK33 | 2011 | Gronlid, SK | Same leaf as SK34 | |
| *Ptt*-SK34 | 2011 | Gronlid, SK | Same leaf as SK33 | |
| *Ptt*-SK35 | 2011 | Snowden, SK | Same leaf as SK36 | |
| *Ptt*-SK36 | 2011 | Snowden, SK | Same leaf as SK35 | |
| *Ptt*-SK37 | 2011 | Meath Park, SK | Same leaf as SK39/Same plant as SK38, SK40 | |
| *Ptt*-SK38 | 2011 | Meath Park, SK | Same leaf as SK40/Same plant as SK37, SK39 | |
| *Ptt*-SK39 | 2011 | Meath Park, SK | Same leaf as SK37/Same plant as SK38, SK40 | |
| *Ptt*-SK40 | 2011 | Meath Park, SK | Same leaf as SK38/Same plant as SK37, SK39 | |
| *Ptt*-SK41 | 2011 | Albertville, SK | Same plant as SK42 | |
| *Ptt*-SK42 | 2011 | Albertville, SK | Same plant as SK41 | |
| *Ptt*-SK43 | 2011 | Albertville, SK | Same plant as SK44 | |
| *Ptt*-SK44 | 2011 | Albertville, SK | Same plant as SK43 | |
| *Ptt*-SK45 | 2011 | Prince Albert, SK | Same leaf as SK46 | |
| *Ptt*-SK46 | 2011 | Prince Albert, SK | Same leaf as SK45 | |
| *Ptt*-SK47 | 2011 | Weldon, SK | Same plant as SK48 | |
| *Ptt*-SK48 | 2011 | Weldon, SK | Same plant as SK47 | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptt*-SK49 | 2011 | Weldon, SK | Same leaf as SK50 | |
| *Ptt*-SK50 | 2011 | Weldon, SK | Same leaf as SK49 | |
| *Ptt*-SK51 | 2011 | Naicam, SK | Same plant as SK23 | |
| *Ptt*-SK52 | 2011 | Naicam, SK | Same plant as SK53 | |
| *Ptt*-SK53 | 2011 | Naicam, SK | Same plant as SK52 | |
| *Ptt*-SK54 | 2011 | Saskatoon, SK | | |
| *Ptt*-SK55 | 2011 | Saskatoon, SK | Same leaf as SK56 | AB41, SK05, SK06, SK13, SK17, SK20, SK28, SK30, SK56 |
| *Ptt*-SK56 | 2011 | Saskatoon, SK | Same leaf as SK55 | AB41, SK05, SK06, SK13, SK17, SK20, SK28, SK30, SK55 |
| *Ptt*-SK57 | 2011 | Dodsland, SK | | |
| *Ptt*-SK58 | 2011 | Borden, SK | | |
| *Ptm*-SK59 | 2010 | Border, SK | | AB76 |
| *Ptm*-SK60 | 2010 | Stony Beach, SK | | |
| *Ptm*-SK61 | 2010 | Cupar, SK | | |
| *Ptm*-SK62 | 2010 | Rhein, SK | | |
| *Ptm*-SK63 | 2010 | Unknown, SK | | |
| *Ptm*-SK64 | 2010 | Dollard, SK | | |
| *Ptm*-SK65 | 2010 | Marshall, SK | | |
| *Ptm*-SK66 | 2010 | Clavet, SK | | |
| *Ptm*-SK67 | 2010 | Battleford, SK | | |
| *Ptm*-SK68 | 2010 | St Gregor, SK | | SK76 (belonged to a different mating type) |
| *Ptm*-SK69 | 2010 | Richardson, SK | | |
| *Ptm*-SK70 | 2010 | Quinton, SK | | |
| *Ptm*-SK71 | 2011 | Medstead, SK | | |
| *Ptm*-SK72 | 2011 | Swift Current, SK | | SK101, SK103 |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptm*-SK73 | 2011 | Assiniboia, SK | | |
| *Ptm*-SK74 | 2011 | Gronlid, SK | | |
| *Ptm*-SK75 | 2011 | Semans, SK | | AB71 |
| *Ptm*-SK76 | 2011 | West Bend, SK | | SK68 (belonged to a different mating type) |
| *Ptm*-SK77 | 2011 | Leslie, SK | | SK84 |
| *Ptm*-SK78 | 2011 | Nut Mountain, SK | | |
| *Ptm*-SK79 | 2011 | Burr, SK | | |
| *Ptm*-SK80 | 2011 | Muenster, SK | | SK92 (belonged to a different mating type) |
| *Ptm*-SK81 | 2011 | Zehner, SK | | |
| *Ptm*-SK82 | 2011 | Zehner, SK | | |
| *Ptm*-SK83 | 2011 | Rokeby, SK | | |
| *Ptm*-SK84 | 2011 | Carmel, SK | | SK77 |
| *Ptm*-SK85 | 2011 | Melfort, SK | | |
| *Ptm*-SK86 | 2011 | Sylvania, SK | Same plant as SK87 | |
| *Ptm*-SK87 | 2011 | Sylvania, SK | Same plant as SK86 | |
| *Ptm*-SK88 | 2011 | Sylvania, SK | | |
| *Ptm*-SK89 | 2011 | Naicam, SK | | |
| *Ptm*-SK90 | 2011 | Annaheim, SK | | |
| *Ptm*-SK91 | 2011 | St Brieux, SK | | |
| *Ptm*-SK92 | 2011 | Prince Albert, SK | | SK80 (belonged to a different mating type) |
| *Ptm*-SK93 | 2011 | Kinistino, SK | Same plant as SK94 | |
| *Ptm*-SK94 | 2011 | Kinistino, SK | Same plant as SK93 | |
| *Ptm*-SK95 | 2011 | Unknown, SK | | |
| *Ptm*-SK96 | 2011 | Alvena, SK | Same plant as SK97, SK 98 | |

| Isolate identification number | Year collected | Location collected | Comments | Isolates with identical SSR profile* |
|---|---|---|---|---|
| *Ptm*-SK97 | 2011 | Alvena, SK | Same leaf as SK98/Same plant as SK96 | |
| *Ptm*-SK98 | 2011 | Alvena, SK | Same leaf as SK97/Same plant as SK96 | |
| *Ptm*-SK99 | 2011 | Naicam, SK | Same plant as SK99 | |
| *Ptm*-SK100 | 2011 | Naicam, SK | Same plant as SK100 | |
| *Ptm*-SK101 | 2011 | Saskatoon SK | Same lesion as SK102, SK103 | SK72, SK103 |
| *Ptm*-SK102 | 2011 | Saskatoon, SK | Same lesion as SK101, SK103 | |
| *Ptm*-SK103 | 2011 | Saskatoon, SK | Same lesion as SK101, SK102 | SK72, SK101 |
| *Ptm*-SK104 | 2011 | Saskatoon, SK | | AB81, MB20, MB32 (SK104 belonged to a different mating type) |

*Isolates that were found to have identical simple sequence repeat (SSR) profiles are indicated