Characterization of the necrotrophic effector ToxA in Kansas fungal leaf spot pathogen populations

by

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A.A.S., Community College of the Air Force, 2012
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Abstract

The Kansas fungal necrotrophic wheat leaf spot complex includes the pathogens *Bipolaris sorokiniana*, *Pyrenophora tritici-repentis*, and *Parastagonospora nodorum*. While all of these pathogens utilize multiple pathogenicity factors, in the last 20 years, all three pathogens were discovered to contain a highly conserved copy of the necrotrophic effector (NE) gene, *ToxA*. Evidence indicates that *ToxA* was likely transferred between the species through an interspecific gene-transfer event. This gene enables pathogenesis in susceptible hosts through an inverse gene-for-gene interaction with the wheat susceptibility gene, *Tsn1*. The primary research objectives of this study are to characterize the distribution and diversity of *ToxA* in the Kansas leaf spot pathogen population, and to determine the frequency of the genes *ToxA*, *ToxB*, and *ToxC* in the *P. tritici-repentis* population. A survey of Kansas cereals was conducted to collect isolates of leaf spot pathogens. Isolates of all three pathogens were molecularly characterized for presence of *ToxA* and their haplotype diversity was evaluated through comparison of *ToxA* sequences. Isolates of *P. tritici-repentis* were phenotypically screened against a wheat differential set, and molecularly characterized for presence of the genes *ToxA*, and *ToxB*. Results show that the NE gene *ToxA* was represented in 87% and 100% of *P. tritici-repentis* and *P. nodorum* isolates respectively, while only being represented in 44% of *B. sorokiniana* isolates. Additional evaluation of the *P. tritici-repentis* population showed that in addition to *ToxA*, only *ToxC* was common in the collected isolates. Characterization of the haplotype diversity in the three populations showed that the characterized isolates contain limited genetic diversity in all three backgrounds, but the most limited diversity is in the *P. tritici-repentis* population. The result that *ToxA* has now been found in three Kansas pathogen populations highlights the importance of *ToxA* and its susceptibility factor (*Tsn1*) in wheat. Knowledge about the rates of

ToxA in these populations will help pathologists in making management recommendations and will help breeders make selection decisions. Now that a baseline for *ToxA* has been established in the *B. sorokiniana* population in Kansas, future surveillance will be required to monitor for an increase of the NE gene. As *ToxA* likely increases pathogen fitness, selection in the population could potentially lead to a future in which *B. sorokiniana* is a more economically important pathogen.

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Dedication

This work is dedicated to my wife and our sons.

Chapter 1 - Literature review

Introduction

Wheat leaf spot pathogens have a long history of diminishing wheat yields. Just as growers and breeders have made selections on wheat over the millennia, the wheat host has, in turn, made passive selections on pathogens which continue to evolve to maintain their own populations. Three necrotrophic leaf spot pathogens of wheat have recently been shown to contain a highly conserved copy of a single necrotrophic effector gene, *ToxA* (Friesen et al., 2006; McDonald et al., 2018). This gene has been shown to be part of a transposon-mediated highly mobile pathogenicity element which appears to provide a significant fitness advantage (Friesen et al., 2018; McDonald et al., 2018, 2019). At least one of these transfers, to *Pyrenophora tritici-repentis*, is theorized to have occurred in modern time, approximately 80 years ago (Friesen et al., 2006). With the acquisition of this single gene, *P. tritici-repentis* may have emerged to become the economically important pathogen that wheat producers experience today. These transfers show that additional transfers of this or other pathogenicity factors can occur in modern time and could become an additional yield loss component to overcome.

Wheat

Wheat (*Triticum* spp.) provides a large portion of calories consumed by humans with an estimated average worldwide production of 886 million metric tons annually from 2015-2020 (FAOStat, https://www.fao.org/faostat/en/#data/QCL). In 2020, wheat was the most widely cultivated crop worldwide at 541 million acres (FAOStat). Its history is tied to the development of human society as it was one of the earliest domesticated crops and is tied to the change of humans from hunter-gatherers to agricultural societies (Charmet, 2011). Bread wheat (*T.*

aestivum) domestication dates back 7,000 to 9,000 years and its center of origin is near the mountainous regions around the Fertile Crescent in modern-day Egypt, Lebanon, Syria, Iraq, Jordan, Palestine, Israel, Kuwait, Turkey, and Iran (Braidwood et al., 1969; Nesbitt & Samuel, 1995).

Wheat is a grass from the Poaceae family that is grown worldwide, but primarily on more arid acreage due to its acceptable performance under water-limited conditions. To make the most in environments with limited water, winter wheat varieties are commonly planted in the fall, grow over the winter and the following spring before reaching maturity in the early summer. This practice is most common in areas with mild winters. Areas with more severe winters such as the North Central U.S. commonly plant 'spring wheat' varieties in the early spring, as winter wheat varieties would not survive the harsh conditions. These varieties typically mature slightly later than winter varieties.

Bread wheat has a complex genome structure. The genome is structured as an allohexaploid genome with three distinct species sources. The A genome is from *Triticum urartu*, the B genome was donated by an extinct relative of *Aegilops speltoides*, and the D genome was donated by *Aegilops tauschii*. The wheat genome size is approximately 17 Gb, and consists of more than 85% repetitive DNA and a predicted 107,891 high-confidence protein coding loci (International Wheat Genome Sequencing Consortium (IWGSC), 2018; Shi & Ling, 2018).

Wheat leaf spot pathogens

Several pathogens cause yield loss to wheat. Plant pathogenic fungi are a primary driver of yield loss to disease. A significant portion of this yield loss is cause by the necrotrophic leaf spot pathogens, *Pyrenophora tritici-repentis*, *Parastagonospora nodorum*, *Bipolaris*

sorokiniana, and *Zymoseptoria tritici*. These pathogens are all in the phylum Ascomycota and in the class Dothideomycetes. Together they make up the wheat leaf spot complex. These pathogens are easily observed sharing the same host niche and three of them have recently been reported to contain a necrotrophic effector that was likely shared through a horizontal gene transfer (HGT) event (McDonald et al., 2019).

Pyrenophora tritici-repentis

Taxonomy and symptoms

Pyrenophora tritici-repentis (Died.) Drechsler causes the disease tan spot (yellow spot) of wheat (*Triticum aestivum* L.). *P. tritici-repentis* is an ascomycete in the class Dothideomycetes, order Pleosporales, and the family Pleosporaceae. Tan spot is easily found in most of the world's wheat producing regions where it can cause yield losses up to 50% (Hosford, 1982; Rees & Platz, 1983). Over the last half century, management practices have shifted away from tillage and stubble burning and towards residue retention/no-till management, all practices which promote the retention of primary inoculum that overwinters on the stubble (Bockus, 1998; Lamari, McCallum, et al., 2005; Rees & Platz, 1992).

Typical tan spot symptoms present as oval or diamond-shaped tan lesions on the leaf which develop chlorotic halos around the margin and have a darker brown point at the initial infection site (De Wolf et al., 1998). Over time, the tan necrotic lesions expand and as the season progresses the growing leaf spots can coalesce if infection density is high enough. Yield loss in addition to reduced grain quality is caused by reduced test weight and kernel shriveling resulting from premature death of leaf area during grain fill (Cheong et al., 2004).

Life cycle

The sexual stage develops ascocarps called pseudothecia which contain ascospores in which the pathogen undergoes meiosis. The fungus overwinters in these melanized and durable structures on wheat residue. These pseudothecia develop and grow ascospores on wheat residue that remains in the field, where they can be observed with the naked eye. Ascospores serve as the primary inoculum when they are actively discharged onto the lower wheat leaves upon sensing the appropriate high moisture condition (Wolf & Hoffmann, 1993). Upon landing on the host, the ascospores germinate and grow germ tubes. The germ tubes develop appressoria on the leaf surface that penetrate the leaf epidermis using a penetration peg (Larez et al., 1986; Loughman & Deverall, 1986). Infection follows this process in both resistant and susceptible cultivars, however rapid intercellular growth is limited to susceptible cultivars (Lamari & Bernier, 1989a; Loughman & Deverall, 1986). After the initial infection, the pathogen begins to generate secondary inoculum. This secondary inoculum is the asexual conidia of the fungus, which is then blown or splashed further up the plant when conditions are conducive to infection (Platt & Morrall, 1980). The inoculum ultimately reaches the flag leaf where it causes a loss of productive leaf area and ultimately a decrease in the photosynthetic capacity. The flag leaf is widely accepted as the most important photosynthate source during grain fill, therefore causing the majority of yield loss (Kaul, 1974).

P. tritici-repentis is a homothallic ascomycete. In this mating system, individuals contain two mating type genes (*MAT1-1* and *MAT1-2*) consecutive in their genome (Lepoint et al., 2010). This allows individuals a greater chance to find a compatible isolate to complete sexual recombination.

The pathogen *P. tritici-repentis* has been found to vary in both chromosome number and genome size from 8 to 11+ chromosomes and from 25.5 to 48.0 Mb (Aboukhaddour et al., 2009).

Molecular basis of disease

The typical leaf spot symptoms of dark brown lesions which develop chlorotic halos are most common, but symptoms are dependent on the molecular interaction between the pathogen and host. These differences, based on the presence or absence of three necrotrophic effectors, make up the *P. tritici-repentis* race structure (Gamba et al., 1998; Lamari et al., 1995, 2003; Lamari & Bernier, 1989a; Strelkov & Lamari, 2003). Some pathogen races will present either the necrotic or chlorotic symptoms alone.

Tomas and Bockus first suggested that a toxin or toxins were involved in tan spot development which they concentrated by growing *P. tritici-repentis* in liquid culture (1987). A toxin from a *P. tritici-repentis* race causing only necrosis was then characterized as a protein. It was observed that denaturing conditions destroyed toxin activity (Ballance et al., 1989). Tomas et al., reported that 90 nM concentrations caused necrosis symptoms in susceptible cultivars (1990). Later research showed that concentrations of the protein as low as 10^{-13}-10^{-14} mol caused necrosis in susceptible cultivars (Dinglasan et al., 2018).

Segregation of F_2 populations from crosses of cultivars susceptible and resistant to the necrosis toxin found that sensitivity was controlled by one dominant gene, later identified to be *Tsn1* (Lamari & Bernier, 1989b).

P. tritici-repentis is a necrotrophic pathogen which uses effector toxins to cause the genetically independent symptoms of necrosis or chlorosis, depending on host susceptibility. There are three genetically independent loci, each controlling susceptibility to a single one of three effector toxins (Gamba et al., 1998). There are currently 8 races of the pathogen which are characterized from their ability to cause symptoms on three hexaploid wheat (*Triticum aestivum)* differential lines: ‘Glenlea’, ‘6B662’, and ‘6B365’ (Lamari et al., 1995, 2003). These toxins have

an inverse gene-for-gene relationship with susceptibility genes in susceptible wheat (Faris et al., 2010; Tan et al., 2010). Previously, *P. tritici-repentis* races 1-5 have been found in North America with the predominant races being those which carry *ToxA* (Lamari et al., 1998, 2003).

The overall trend from previous studies of the race structure from other regions of the world is high levels of race 1 (*ToxA* and *ToxC*) and race 2 (*ToxA*) in areas where there are low levels of *ToxB* in the population (Abdullah, Sehgal, & Ali, 2017; Abdullah, Sehgal, Ali, et al., 2017; Aboukhaddour et al., 2009, 2013; Ali et al., 2010; Ali & Francl, 2003; Antoni et al., 2010; Engle et al., 2006; F. M. Gamba et al., 2012; Lamari et al., 1998; Leisova-Svobodova et al., 2010; MacLean et al., 2017; Sarova et al., 2005; Singh et al., 2007). In populations where *ToxB* is present, including North Africa and Azerbaijan, the most common races are race 5 through race 8 (Aboukhaddour et al., 2009; Benslimane et al., 2011; Gamba et al., 2017; Kamel et al., 2019; Lamari, Strelkov, et al., 2005).

ToxA

Ptr ToxA was the first proteinaceous necrotrophic effector to be discovered (Tuori et al., 1995). Initially discovered by Tomás and Bockus in 1987, the 13.2-kDa effector was ultimately named ToxA by Ciuffetti et al. in 1998 (Ciuffetti et al., 1998; Tomas & Bockus, 1987). The effector was purified, and the protein sequence was used to clone the *ToxA* gene (Ballance et al., 1996; Ciuffetti et al., 1997). *ToxA* is a single copy gene in the *Pyrenophora tritici-repentis* genome (Ciuffetti et al., 1997).

Ptr ToxA has been shown to cause necrosis on wheat which contains the *Tsn1* gene located on the wheat chromosome 5BL (Ciuffetti et al., 1997; Faris et al., 1996; Stock et al., 1996). The *Tsn1* gene encodes a characteristic R-gene with nucleotide binding site leucine-rich repeat (NBS-LRR) and serine/threonine protein kinase (S/TPK) domain features (Faris et al.,

2010). The product has no trans-membrane domains, indicating it remains inside the cell (Faris et al., 2010). Manning and Ciuffetti showed that after expression of Ptr ToxA in the host's apoplastic space, Ptr ToxA is able to cross the plant plasma membrane and localize to cytoplasmic compartments and chloroplasts (2005). A yeast two-hybrid experiment showed that ToxA and *Tsn1* do not interact directly (Faris et al., 2010). Therefore, this relationship may follow a guard model (Faris et al., 2010).

ToxB

Much less is known about *P. tritici-repentis* NEs other than Ptr ToxA. *ToxB* encodes a small secreted protein with a mass of 6.61 kDa, that induces chlorosis on susceptible wheat varieties including the tan spot differential line '6B662' (Lamari et al., 1995; Strelkov et al., 1999). Although, no contributing functional motifs have been identified for *ToxB*, the NE does inhibit host photosynthesis and alter the proteome before the chlorosis phenotype develops (Ciuffetti et al., 2010; Kim et al., 2010). Multiple copies of *ToxB* are found in races 5, 6, 7, and 8 (Lamari et al., 1995, 2003). Some non-*ToxB* isolates have been found to contain a non-functional copy of *ToxB*, called *toxb* (Martinez et al., 2004).

The Ptr ToxB protein produces chlorosis at low minimum active concentration of 14 nM, and was shown to be heat stable for 1 hr at 55°C (Strelkov et al., 1999). Ptr ToxB susceptibility in wheat is dependent on the host containing the susceptibility factor *Tsc2* which is located on chromosome 2BS, unfortunately little is known about the interaction as *Tsc2* has not been cloned (Friesen & Faris, 2004; Orolaza et al., 1995; Strelkov et al., 1999).

ToxC

Ptr ToxC is a non-protein, polar, non-ionic, low molecular weight molecule (Effertz et al., 2002). Unlike Ptr ToxA and Ptr ToxB, host-selective toxins are primarily low molecular

weight secondary metabolites (Walton, 1996). Ptr ToxC, induces chlorosis on susceptible wheat varieties like the tan spot differential line '6B365' (Lamari et al., 1995). Susceptibility for this gene is linked to the wheat gene *Tsc1* which is located on the wheat chromosome 1AS, but little is known about the interaction as *Tsc1* has not been cloned. (Effertz et al., 2002; Faris et al., 1997).

Resistance sources

As susceptibility to *P. tritici-repentis* is based on susceptibility factors, resistance can be achieved through removal of these susceptibility factors. This response has been suggested previously and the removal of *Tsn1*, the most economically important susceptibility factor in this pathosystem, has been evaluated for deleterious effects (McDonald et al., 2018; Oliver et al., 2014; Virdi et al., 2016; Wu et al., 2020). These reports suggest that there have been no deleterious effects of *Tsn1* removal identified. However, the gene does remain at moderate levels in breeding populations which suggests a potential benefit. Ongoing research should continue to evaluate the effects of *Tsn1*.

Parastagonospora nodorum

Taxonomy and symptoms

Septoria nodorum blotch (SNB) of wheat is caused by the pathogen *Parastagonospora nodorum* (Berk). Quaedvl., Verkley & Crous. (telomorph: *Phaeosphaeria nodorum* (E. Müll.) Hedjar.). *P. nodorum* is an Ascomycete in the class Dothideomycetes, order Pleosporales, and the family Phaeosphaeriaceae.

Yield loss to this pathogen is common in all wheat growing regions and can reach 31% (Bhathal et al., 2003; Wiese, 1987). SNB leaf symptoms present as oval or diamond necrotic lesions accompanied by chlorosis on the margin and pycnidia embedded in the necrotic tissue

(Solomon et al., 2006). These symptoms are differentiated from other leaf spot pathogens based on the presence and pink or honey colored conidia that extrude conidial masses, cirrhi.

Life cycle

P. nodorum infects wheat from multiple inoculum sources including infected seed, infected residue present in the field or nearby, volunteer wheat, and Poaceae weeds (Arseniuk et al., 1998; Bathgate & Loughman, 2001; Shah et al., 1995; Shah & Bergstrom, 2000). *P. nodorum* undergoes sexual recombination during the winter in ascocarps on wheat residue (Bathgate & Loughman, 2001). After overwintering on residue in the field, springtime environmental conditions of high moisture and temperatures above 10°C enable ascospore release (Arseniuk et al., 1998; Bathgate & Loughman, 2001; Wolf & Hoffmann, 1994). These spores can be dispersed by wind or rain (Bathgate & Loughman, 2001).

Upon reaching the plant, infection occurs when the germinating spore sets multiple germ tubes which use appressoria to penetrate the leaf where they are able to spread intercellularly through the mesophyll (Bird & Ride, 1981). Once the primary inoculum has established itself on the wheat leaf surface, it begins to produce conidia which serve as the secondary inoculum. These conidia are produced in the pycnidia and then extruded in cirrhi, which are then splashed onto nearby wheat leaves where they begin successive infections (Shah et al., 2001). This asexual cycle can occur multiple times during the vegetative phase of wheat development. As the wheat plant begins to senesce, the pathogen begins to produce pseudothecia on the wheat stems which will grow ascospores that contribute to the following season's primary inoculum (Bathgate & Loughman, 2001; Hafez et al., 2020).

P. nodorum is a heterothallic fungus requiring two mating type genes (*MAT1-1* and *MAT1-2*). In this mating system, two individuals each with one of the mating type genes are

required to undergo sexual recombination. (Halama & Lacoste, 1991). The U.S. population contains both mating types and its sexual stage can be observed which indicates that recombination of isolates is occurring (Bennett et al., 2003; Cunfer, 1998).

Molecular basis of disease

The molecular basis of SNB pathogenesis is also based on the use of necrotrophic effectors that interact with host defense mechanisms to trigger a hypersensitive response that leads to host cell necrosis (Friesen et al., 2012; Liu et al., 2012). Nine interactions between NEs and host sensitivity genes have been identified in the pathosystem: SnTox1-*Snn1*, SnTox2-*Snn2*, SnTox3-*Snn3*, SnTox4-*Snn4*, SnTox5-*Snn5*, SnTox6-*Snn6*, SnTox7-*Snn7*, and SnToxA-*Tsn1* (Faris et al., 2010; Gao et al., 2015; Shi et al., 2015; Zhang et al., 2011). The three primary necrotrophic effector genes, *SnTox1*, *SnTox3*, and *SnToxA* have been cloned and characterized (Friesen et al., 2006; Liu et al., 2009, 2012). The greatest amount of genetic diversity in these genes has been observed in isolates from the Fertile Crescent which indicates that these NEs originated in that region likely alongside the host (Ghaderi et al., 2020).

Sn Tox1

SnTox1 is a small secreted necrotrophic effector which interacts with the wheat sensitivity gene product of *Snn1* (Friesen et al., 2008; Liu et al., 2012). The protein has been shown to share homology with plant chitin-binding proteins, indicating SnTox1 can provide some protection from host chitinases (Liu et al., 2016). The NE interacts with the protein product of *Snn1* which is found on chromosome 1BS (Liu et al., 2004).

Snn1 is a wall-associated kinase receptor which interacts directly with the SnTox1 extracellular domain to trigger a plant hypersensitive response that leads to programmed cell death (Liu et al., 2016; Shi et al., 2016). This interaction has been shown to induce epidermal cell

death, facilitating penetration into the host and resulting in disease (Liu et al., 2012; Shi et al., 2016).

Sn Tox3

SnTox3 is a proteinaceous necrotrophic effector that interacts with the wheat sensitivity gene product of *Snn3* (Liu et al., 2009). The protein, SnTox3, is estimated to be 25.8 kDa and has been shown to interact with products from genes on 5BS and 5D, indicating homologous copies of *Snn3* (Friesen et al., 2008; Liu et al., 2009; Zhang et al., 2011). Expression of SnTox3 in the host elicits a plant defense that results in host cell death (Winterberg et al., 2014). This interaction explains more than 51% of SNB phenotypic variation in seedling screening (Ruud et al., 2017).

Sn ToxA

P. nodorum ToxA is a homolog of the gene *ToxA* in *P. tritici-repentis* (Faris et al., 1996). This gene has been shown to interact indirectly with products of the host susceptibility factor *Tsn1* which is located on chromosome 5BL (Faris et al., 2010). *Tsn1* is a primary susceptibility factor for *P. nodorum* in wheat (Liu et al., 2006). This interaction has been shown to account for up to 68% of SNB phenotypic variation (Friesen et al., 2006; Liu et al., 2006).

Resistance sources

Wheat varieties are available containing a wide range of resistance (Aguilar et al., 2005). Foliar response QTLs of high confidence not associated with NE-susceptibility factor interactions have been found on chromosomes 1BS, 2AS, 2AL, and 7DL (Czembor et al., 2019; Francki et al., 2011; Francki et al., 2021; Lin et al., 2020). Additionally, the removal of NE susceptibility factors like *Snn1*/*Snn3*/*Tsn1* is a potential method of increasing resistance to necrotrophic pathogens taking advantage of host defense mechanisms (Lorang, 2019).

Bipolaris sorokiniana

Taxonomy and symptoms

Spot blotch is cause by the wheat pathogen *Bipolaris sorokiniana* (Sacc.) Shoemaker (telomorph: *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur). *B. sorokiniana* is an ascomycete in the class Dothideomycetes, order Pleosporales, and the family Pleosporaceae. *B. sorokokiniana* and *P. tritici-repentis* are more closely related to each other than they are to *P. nodorum* which is in a different family. *B. sorokiniana* is a hemibiotrophic fungus which has a short biotrophic phase before transitioning to necrotrophic phase.

Spot blotch foliar symptoms are similar in appearance to tan spot. Symptoms start as small spots at the infection point and grow to oblong light brown lesions with brown margins. The leaf spots will continue to elongate, ultimately coalescing if leaf spot density is high enough. Yield loss to this pathogen is common and minimal in most wheat growing regions but it has been reported to reach 40-70% on susceptible varieties in warm humid regions like South Asia (Badaruddin et al., 1994).

Life cycle

The primary inoculum sources for the pathogen *B. sorokiniana* are infected seed and fungal spores from the pathogen which overwinter on wheat residue (Chand et al., 2002; Pandey et al., 2005). Primary conidia inoculum is typically splashed or blown from residue onto the bottom leaves of the wheat plant (Acharya et al., 2011). Once conidia have reached the leaf, they will infect in a similar manner to *P. tritici-repentis* or *P. nodorum*, the spores will germinate and germ tubes will grow along the surface before developing an appressorium to penetrate the epidermis and begin to grow new conidia (Jansson & Åkesson, 2003). Conidia from the lower leaves are blown or splashed up the plant where they begin the infection process again (Acharya

et al., 2011; Duveiller et al., 2005). Spot blotch symptoms typically show up later in the season as warmer temperatures (18°C to 32°C) are preferred by the pathogen (Acharya et al., 2011; Chaurasia et al., 2000).

B. sorokiniana is a heterothallic fungi (Gupta et al., 2018). In this mating system, the population is regulated by two mating alleles (*MAT1-1* or *MAT1-2*) (Ahmadpour et al., 2018). However, despite having mating types and both present, the pathogens sexual stage has not been observed infecting wheat and the pathogen population appeared to operate asexually (Reis & Wunsche, 1984; Wen & Lu, 1991). The lack of a sexual stage could be a disadvantage for the pathogen in some situations because it is limited in its ability to undergo genetic recombination.

Molecular basis of disease

The molecular basis of spot blotch has been researched less than other leaf spot pathogens. In 2018, the NE gene *ToxA* was discovered in isolates of *B. sorokiniana*.

Bs ToxA

B. sorokiniana ToxA is a homolog of the gene *ToxA* in *P. tritici-repentis* and *P. nodorum.* (Friesen et al., 2018; McDonald et al., 2018). While the gene was only recently discovered in the *B. sorokiniana* population, it has already been identified in populations in Australia, India, Mexico, and the U.S. (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020; Wu et al., 2020). Isolates of the pathogen which contain the NE have been shown to be more virulent or to have a greater necrosis-causing ability on wheat which contains the susceptibility factor *Tsn1* (Friesen et al., 2018; McDonald et al., 2018; Wu et al., 2020). While *Tsn1* as a causal factor was not implicated directly, multiple previous QTL studies on *B. sorokiniana's* disease phenotype in wheat have identified QTLs on chromosome 5BL that explained over 50% of the spot blotch

phenotypic variation (Gurung et al., 2014; S. Kumar et al., 2016; U. Kumar et al., 2010; Sharma et al., 2007).

Mobile pathogenicity unit *ToxA*

ToxA was initially discovered in *P. tritici-repentis* however, in 2006 a highly conserved copy was identified in *P. nodorum* (Friesen et al., 2006; Tomas & Bockus, 1987). This transfer appeared to occur in part of an 11 kb region that was identified in common between the two pathogens (Friesen et al., 2006). Despite its initial identification in *P. tritici-repentis*, *ToxA* was hypothesized to have a longer history in the pathogen *Parastagonospora nodorum* due to a greater quantity of genetic diversity in *P. nodorum* populations (Friesen et al., 2006). *P. nodorum* also has a greater history of pathogenesis, dating back to the 1890's while *P. tritici-repentis* underwent a name change from 'yellow spot' to 'tan spot' that was speculated to be associated with an increase in virulence sometime around 1940 (Friesen et al., 2006). Global evaluation of *P. nodorum* isolates identified the greatest amount of genetic diversity in the *ToxA* gene at the center of origin for both wheat and the pathogen; this finding supports the hypothesis that *ToxA* evolved in *P. nodorum* alongside wheat (Ghaderi et al., 2020). This indicates that the original source of the NE gene *ToxA* is likely *P. nodorum*.

ToxA was also discovered in *Phaeosophaeria avenaria* f.sp. *tritici*, a sister species of *P. nodorum*, and there was evidence that this gene transfer occurred through a interspecific hybridization with *P. nodorum* (McDonald et al., 2012, 2013). Nearly 100% of *P. tritici-repentis* and *P. nodorum* isolates evaluated across Australia contain *ToxA* (Antoni et al., 2010; McDonald et al., 2013).

In 2018, *ToxA* was found in the genome of another fungal pathogen, *B. sorokiniana.* McDonald et al., sequenced the genomes of three Australian *B. sorokiniana* isolates from wheat

or barley and did a screen for known virulence genes. This search identified *ToxA* in one of the genomes (2018). This gene was later shown to be part of a transposon-mediated highly mobile pathogenicity element, which is highly conserved in all species currently identified to contain *ToxA.* This element also appears to provide a significant fitness advantage (Friesen et al., 2018; McDonald et al., 2018, 2019).

Virulence between the isolate identified to contain *ToxA* was compared with another sequenced isolate that did not contain *ToxA* on lines both with and without the wheat susceptibility gene *Tsn1*. These results showed that *B. sorokiniana* with *ToxA* is more virulent than isolates without *ToxA* on *Tsn1* wheat lines (McDonald et al., 2018). Thirty-five additional isolates from across Australia were sequenced by McDonald et al., and *ToxA* was discovered in 34.2% of the isolates from across the sampled area. Subsequent studies have found *ToxA* in *B. sorokiniana* populations on three continents (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020; Wu et al., 2020). Estimates of *ToxA* frequency in the North American *B. sorokiniana* population range from 10.2% to 86.7% (Friesen et al., 2018; Wu et al., 2020).

Rationale for study

Necrotrophic effectors play a large role in the wheat pathosystem. One weakness of these NEs is their dependence on an inverse gene-for-gene based susceptibility in the host genome. This weakness can be targeted through the removal of these susceptibility genes like *Tsn1* from breeding populations. The first step in realizing this goal is determining which NEs are present in a pathogen population. This research aims to determine which NEs are being used by the *P. tritici-repentis* population to cause disease in the state. This will be done by characterizing the pathogen's race structure. These results will be used to provide selection recommendations to breeders.

One of the most economically important NEs is ToxA. With high levels of the susceptibility gene *Tsn1* in many wheat populations, and a potentially growing *ToxA* presence in necrotrophic fungal pathogens, the risk to wheat producers could be increasing. To date, few isolates of *B. sorokiniana* containing *ToxA* have been characterized. Since the discovery of *ToxA* now in four different wheat pathogens, and with the gene being found in a mobile pathogenicity element, research to help estimate the distribution and genetic diversity of the *ToxA* gene is required.

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Chapter 2 - Race structure of *Pyrenophora tritici-repentis* in the Kansas wheat pathogen population

Abstract

Pyrenophora tritici-repentis (Ptr) carries at least three necrotrophic effectors (NE) as part of its host invasion repertoire. These three genes, *ToxA*, *ToxB*, and *ToxC*, exist in 7 possible combinations. These combinations, in addition to an 8th possibility of no necrotrophic effectors, make up a race nomenclature system that characterizes the disease phenotypes observed on susceptible wheat cultivars. The primary NE, Ptr ToxA, enables pathogenesis through an interaction with the wheat susceptibility gene, *Tsn1*. The research objective of this project was to determine the race structure of *P. tritici-repentis* population in Kansas. To complete this objective, a survey was conducted to collect isolates of the pathogen from the Kansas wheat production region. In addition, 16 isolates from an historic collection were also included. These were phenotypically scored on a wheat differential set, and molecularly characterized for presence of the genes *ToxA*, and *ToxB,* and a non-functioning *ToxB* haplotype called *toxb*. The survey identified 63 *P. tritici-repentis* isolates. Genotype and racing typing experiments identified that the primary Ptr race found in Kansas was race 1 which contains *ToxA* and *ToxC*. However, we also found isolates of race 2, 3 and 4. Some isolates caused atypical symptoms which did not match the current race structure. The results of molecular assays matched the screening results for Ptr ToxA and Ptr ToxB in most cases. Knowledge about the distribution and race structure of the *P. tritici-repentis* population in Kansas can help pathologists and breeders make management and breeding decisions. The high level of *ToxA* in the Kansas *P. tritici-repentis* population leads to the conclusion that *Tsn1* should be selected against in future wheat varieties under development.

Introduction

Pyrenophora tritici-repentis (Died.) Drechsler causes the disease tan spot (yellow spot) of wheat (*Triticum aestivum* L.). Tan spot is easily found in most of the world's wheat producing regions where it can cause yield losses up to 50% (Hosford, 1982; Rees & Platz, 1983). Yield loss to this disease has increased over the last half century due to shifts in management practices away from tillage and stubble burning and towards residue retention/no-till management, all practices which promote the retention of primary inoculum that overwinters on the stubble (Bockus, 1998; Lamari, McCallum, et al., 2005; Rees & Platz, 1992).

Typical tan spot symptoms present as dark brown diamond or oval-shaped lesions on the leaf which develop chlorotic halos and expand over time to oblong necrotic regions with a darker brown spot at the infection site (De Wolf et al., 1998). However, tan spot symptoms may vary depending on the presence or absence of at least three necrotrophic effectors (NE), previously referred to as host-selective toxins (HSTs), Ptr ToxA, Ptr ToxB, and Ptr ToxC (Ballance et al., 1989; Effertz et al., 2002; Orolaza et al., 1995; Tomas et al., 1990). These NEs operate using an inverse gene-for-gene interaction model and are each dependent on the respective host sensitivity genes in wheat: *Tsn1*, *Tsc2*, and *Tsc1* (Effertz et al., 2002; Faris et al., 1996; Friesen & Faris, 2004). Each of the NEs contribute to the phenotype by causing necrosis in the case of Ptr ToxA, or unique chlorosis phenotypes in the case of Ptr ToxB and Ptr ToxC (Ciuffetti et al., 1997; Effertz et al., 2002; Orolaza et al., 1995).

The primary determinant of virulence on wheat is Ptr ToxA, which in conjunction with the wheat susceptibility gene *Tsn1*, causes the necrosis phenotype (Ciuffetti et al., 2010; Faris et al., 2013; Lamari & Bernier, 1989c; Wolpert et al., 2002). *Tsn1* is located on the wheat chromosome 5BL. Its structure contains S/TPK and NBS-LRR domains. This structure suggests

that necrotrophic pathogens expressing *ToxA* are taking advantage of a resistance type gene which was evolved for defense against a hypothesized biotrophic pathogen (Faris et al., 2010).

A characterization system of 8 possible races, called race 1 (R1) through race 8 (R8), was developed to describe pathogen isolates, containing all possible combinations of the three NEs plus a single race which contains no NEs (Figure 2.1) (Gamba et al., 1998; Lamari et al., 1995, 2003; Lamari & Bernier, 1989b; Strelkov & Lamari, 2003). Ptr ToxA is expressed by the races 1, 2, 7, 8; Ptr ToxB is expressed by the races 5, 6, 7, 8, and Ptr ToxC is expressed by the races 1, 3, 6, and 8 (Strelkov & Lamari, 2003).

The race of a given isolate can be determined by screening it using a set of three wheat differential lines which are each only susceptible to a single NE. The cultivar 'Glenlea', and the lines '6B662', and '6B365' are susceptible to Ptr ToxA, Ptr ToxB, and Ptr ToxC respectively (Evans et al., 1972; Lamari et al., 1998, 2003; Strelkov et al., 2002). The variety 'Salamouni' was also added to the differential set due to its insensitivity to all three NEs of *P. tritici-repentis* (Ptr) and can act as a control (Lamari et al., 2003). In addition, isolates can also be characterized by NE specific primers for *ToxA*, and *ToxB* (Andrie et al., 2007; Friesen et al., 2006; Martinez et al., 2004). Diagnostic assays of *ToxC* are unavailable currently.

Previous evaluations of the Ptr race structure have been completed for many regions around the world. The primary race identified globally is race 1, containing *ToxA* and *ToxC*. However, the composition of NEs differ regionally. The southern cone of South America was reported to contain only race 1 (52.4%) and race 2 isolates (47.6%) (Gamba et al., 2012). Australian isolates were found to belong to race 1 (98.3%) and race 2 (1.7%) (Antoni et al., 2010). Reports from the European countries, Latvia, Lithuania, Romania, and the Czech Republic showed that isolates belong primarily to race 1 (75.9%), race 2 (5.2%), race 3 (8.7%),

and race 4 (8.5%) (Abdullah, Sehgal, Ali, et al., 2017; Leisova-Svobodova et al., 2010; Sarova et al., 2005). Reports from Western and Central Asia showed that isolates were mostly race 1 (70.5%), there were also moderate levels of race 5 (8.2%), race 7 (6.4%), and race 8 (9.3%) and low levels of race 2 (2.8%) and race 3 (2.8%) (Aboukhaddour et al., 2009; Lamari, Strelkov, et al., 2005). The trend of isolates primarily expressing Ptr ToxA and Ptr ToxC was broken in the north African countries of Algeria, Morocco and Tunisia, where the most common NE is Ptr ToxB, therefore making the primary races present, race 5 (27.7%), race 6 (23.9%), and race 7 (35.2%). There are also moderate levels of race 1 isolates (11.4%) (Aboukhaddour et al., 2009; Benslimane et al., 2011; Gamba et al., 2017; Kamel et al., 2019).

In North America, previous studies have evaluated the race structure of Ptr populations in South Dakota, Ohio, the Northern Great Plains, the Southern Great Plains, Arkansas, as well as the Canadian provinces of Alberta and Saskatchewan. The studies on Canadian regions conclude that these populations are characterized by high levels of the NEs Ptr ToxA (97.1%) and Ptr ToxC (51%), with the majority of isolates being either race 1 (49.6%) or race 2 (47.6%) (Aboukhaddour et al., 2009, 2013; Lamari et al., 1998; MacLean et al., 2017; Singh et al., 2007). The U.S. reports differ, showing a higher representation of race 1 (77.7%), indicating higher levels of Ptr ToxC (85.8%) on average, while maintaining high levels of Ptr ToxA (84.3%) (Abdullah, Sehgal, & Ali, 2017; Ali et al., 2010; Ali & Francl, 2003; Engle et al., 2006). There have also been reports of low levels of Ptr ToxB (0.5%) in the United States, from the report of 2 race 5 isolates from South Dakota (Abdullah, Sehgal, & Ali, 2017; Ali & Francl, 2003).

To decrease the occurrence of tan spot, we need to understand the pathogen's relationship with the host. While the race structure of Ptr has been determined in other regions of North America, the race structure of the Kansas pathogen population has not recently been thoroughly

evaluated. Breeders require this information to develop strategies that will improve cultivar response to the pathogen. Knowledge of the pathogen population can also help producers through the release of wheat varieties that are not susceptible to common local NEs. This information will also allow researchers to focus efforts on the primary NEs found in this population.

The objective of this research is to determine the race structure of the Kansas *P. tritici-repentis* population. This was completed by surveying the Kansas wheat production area for diseased wheat plants to collect a sample of *P. tritici-repentis* isolates. These isolates were then characterized using the wheat differential set and molecularly for presence of the *ToxA* and *ToxB* genes. In addition, the haplotype diversity of the *ToxA* gene was evaluated through sequence comparison. With this knowledge, breeders and pathologists can update the challenge isolates in our breeding nurseries to better select against isolates representative of the Kansas pathogen, *P. tritici-repentis*, population.

Methods

Isolate collection

Isolates of Ptr were collected from Kansas wheat producing counties during 2019-2021. Leaf tissue samples were collected from the lower canopy of production fields and wheat demonstration plots during the months of May and June. Only symptomatic tissue showing typical leaf spot necrosis or chlorosis or typical fungal signs were collected. Samples were transported to a laboratory and stored at 4°C. Isolates from an historic collection were also considered in the analysis (Bockus, unpublished).

Sample isolation

Small samples of leaf tissue were excised from the margin of the suspected fungal leaf spot. Tissue samples were then surface sterilized in 5% bleach for three minutes before being double rinsed in sterilized water for 1.5 minutes each rinse. Sterilized leaf tissue was then placed in petri dishes on water agar (4 g granulated agar, 250 ml water), dishes were wrapped in parafilm, and incubated in the growth chamber for 24 hours. The growth chamber (Powers Scientific, Inc, Pipersville, PA) was on a 20°C/19°C day/night regime, with a supplemental light regime of 16 hr days using L9T8SE240-G 4000K lights (MaxLite, West Caldwell, NJ). This procedure created a humid environment around the leaf tissue to encourage fungal sporulation. After 24 hours, plates were observed for *P. tritici-repentis* conidia which were transferred onto a petri plate containing ¼ Potato Dextrose Agar (¼ PDA) (2.5 g granulated agar, 3.8 g potato dextrose agar, 500 ml water) and placed back in the growth chamber for five days.

Isolates were single-spored for genetic uniformity on V8-potato dextrose agar (V8-PDA) (150 ml V8 juice, 10 g PDA, 10 g agar, 3g $CaCO_3$, 850 ml water) using the protocol described by Lamari and Bernier with the following changes (Lamari & Bernier, 1989a). The isolates were grown in the dark at 21°C for 5 days to encourage mycelium development (Khan, 1971). Isolates were then flattened to induce conidial growth by lubricating the mycelial surface with 300 µl sterile water and dragging the tip of a flame sterilized test tube in a circular motion across the surface of the plate radiating from the center outward. These plates were then placed in the growth chamber under a supplemental Agrobrite 6400K fluorescent light bank (Hydrofarm, Petaluma, CA) for a continuous 48 hours at 21°C.

A conidial spore suspension in water was collected and diluted to 100 spores/mL and 10 µl of this suspension was transferred to a petri plate containing ¼ PDA where it was spread using

a sterilized bent glass rod. The transferred fungi were grown for 24 hours in a growth chamber using the initial sample isolation conditions previously described. After 24 hours, an individual conidium was identified under a stereo microscope and transferred onto a new plate of ¼ PDA. This pure isolate was then grown for seven days to ensure there was no contamination. The isolates were then stored at 4°C in slant tubes on ¼ PDA until required for conidiation.

Molecular characterization

Identification of isolates was done morphologically and verified through amplification and sequencing of the ribosomal internal transcribed spacer region (ITS) using the primers ITS1 and ITS4 (Table 2.1) (White et al., 1990). Primers were amplified using a thermocycler (Labnet, Multigene Optimax, Edison, NJ) with the conditions 6 minutes at 95°C, then 35 cycles of 30 seconds at 95°C, 40 seconds at 60°C, 40 seconds at 72°C, then a 5-minute final extension at 72°C (Table 2.1). ITS primer amplicons were sent for PCR clean-up and Sanger sequencing (MCLAB, South San Francisco, CA). Results were compared to previously published sequences.

Fungal tissue for DNA was grown by inoculating flasks of Difco potato dextrose broth (PDB) (Difco Laboratories Inc., Detroit, MI) from samples stored at 4°C on ¼ PDA slant tubes. After 10 days of growth on a shaker table set to 120 rpm, mycelium was sampled from the flask and pressed between sterile paper towels to remove excess liquid. Tissue was placed in a 1.7 mL micro centrifuge tube and ground with sterilized 4.5 mm galvanized steel pellets (Daisy Outdoor Products, Rogers, AR) using a bead mill tissue disruptor (TissueLyser II, Qiagen, Hilden, Germany). Fungal genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol with the following volumes and modifications (Murray & Thompson, 1980). 80-100 mg of tissue was placed in the bead mill with 700 µl of CTAB. 14 µl of 2-mercaptoethanol (Fisher Scientific, Waltham, MA) was added after the tissue disruption. The

mixture was then incubated at 60°C for 30 minutes. Soluble DNA was separated from debris and insoluble material through two 500 µl, 24:1 chloroform:isoamyl alcohol phase separation extractions. DNA was precipitated using 0.8 volumes of 2-propanol. DNA concentration was quantified with an Implen C40 nanophotometer (Implen, Munich, Germany). DNA was diluted to 20 ng/µl using Tris-EDTA (TE) buffer (5 mL 1M Tris, 1 mL 0.5M EDTA, 496 mL H_2O).

PCR was performed in 20 µl reactions for *ToxA* primers, to accommodate sequencing volumes, and 12 µl for *ToxB* and *toxb* primers. *ToxA* was amplified using the ToxA1 and ToxA2 primer set and conditions described by Friesen et al. (Friesen et al., 2006) using GoTaq Green Master Mix (Promega, Madison, WI). *ToxB* was amplified using the primers TB71F and TB60R (Andrie et al., 2007; Martinez et al., 2004). Amplification of t*oxb* was attempted using the primers TB71F and TB58R (Andrie et al., 2007; Martinez et al., 2004). Primers can be observed in Table 2.1. Regions of interest were amplified using a thermocycler (Labnet, Multigene Optimax, Edison, NJ) with the conditions 4 minutes at 94°C, then 35 cycles of 45 seconds at 94°C, 30 seconds at 60°C for the *ToxA* primers and 58°C for the *ToxB* and *toxb*, 60 seconds at 72°C, then a 7 minute final extension at 72°C (Andrie et al., 2007; Martinez et al., 2004). The known isolates ‘Pti2’ (race 1) and ‘Dw7’ (race 5), kindly provided by Dr. Shaukat Ali, South Dakota State University, were used as positive and negative controls. No positive control was available for the *toxb* primer set. DNA was run on 1% agarose (Fisher Scientific, Waltham, MA) gels, using an electrophoresis all in one DNA gel system (Accuris myGel Mini, Edison, NJ) for 25 minutes at 50 volts, to verify amplification. A 100 bp DNA ladder (Fisher Scientific, Waltham, MA) was used to compare amplicon length.

Phenotypic characterization

The differential line set consisted of the variety ‘Glenlea’ and the lines ‘6B662’ and ‘6B365’. These lines are susceptible to Ptr ToxA, Ptr ToxB, and Ptr ToxC respectively. The variety ‘Salamouni’ was also included as a negative check. Three seeds of each differential line were planted into a 9-cubic inch pot using BM1 all-purpose mix soil (Berger, Saint-Modeste, QC, CA). After germination, each pot was thinned to one seedling. Plants were grown under greenhouse conditions with a 21°C/17°C day/night regime with a supplemental light regime of 14 hour days using LU400/H/ECO, 400 Watt high pressure sodium lamps (General Electric, Boston, MA).

Conidia were grown using methods previously described. Conidia were harvested from V8-PDA plates by flooding the plate with 15mL of a 0.05% Tween-20 (Polyoxyethylene (20) sorbitan monolaurate) solution and lightly scraping the surface of the mycelium, focusing on the leading edge of the isolate where most conidia developed, with a flame sterilized loop. The liquid was then decanted and conidia were counted using a phase counting chamber (Hausser Scientific, Horsham, PA) and diluted to 3000 conidia/mL using 0.05% Tween-20 (Lamari & Bernier, 1989a).

Plants were inoculated ten days after planting, when plants were at the Feekes 1.3 growth stage (Large, 1954). Inoculations onto plant tissue were made by spraying leaves until accumulated mist began to run off of the surface, using a DeVilbiss model 152 atomizer at 5psi (DeVilbiss, Somerset, PA). Plants were allowed to dry for one hour on the greenhouse bench before being placed into a model I-36DL humidity chamber (Percival Scientific, Fontana, WI) for 24 hours in complete darkness. The chamber’s wall and water temperatures were set to 5°C

and 41°C respectively, to create a chamber temp of 23°C with 100% relative humidity. Plants were then returned to the greenhouse bench and placed under conditions previously stated.

Each screened isolate was included in two replicates on four individual plants. The experiment used an incomplete block design with three isolates being replicated in every block. The three isolates that were included in every block were 'MER-6' (race 4), 'Pti2' (race 1), and 'Dw7' (race 5). These isolates were included as it ensured there would be one isolate acting as a positive control and two negative controls for every block of the experiment. Data were analyzed and adjusted means were calculated using the augmented block design, DAU.test, function from the R package 'Agricolae' version 1.3-3 (Felipe de Mendiburu & Muhammad Yaseen, 2020).

The plants were scored for the presence of chlorosis or necrosis after 7 days on the bench, using a 1 to 5 scale developed by Lamari and Bernier, where 1 is highly resistant and 5 is highly susceptible (Lamari & Bernier, 1989a). Races were designated using the combined molecular and phenotypic results.

Results

Isolate survey

A survey of the Kansas wheat production region yielded 63 isolates of *P. tritici-repentis*. During the 2019 field season, 35 isolates were surveyed. In 2020, 25 isolates were identified. In 2021, 3 isolates were collected (Table 2.2). The historic isolate collection included in this study consisted of 16 isolates collected from the Kansas region before 2015 (Table 2.3).

Phenotypic characterization

51 of the 63 survey isolates, and 12 of the 16 historic isolates were phenotyped on the wheat differential set to identify isolate race, based on the ability of the isolate to consistently produce conidia under laboratory conditions. Phenotype ratings for the survey isolates indicate

that there are high levels of Ptr ToxA and Ptr ToxC expression in the population (Table 2.2). Phenotype ratings of the historic isolates matched the results of the survey population (Table 2.3).

On the differential line ‘Glenlea’, which is susceptible to Ptr ToxA, the negative control isolate, ‘Dw7’, had a rating of 1.94 and the positive control, ‘Pti2’, had a rating of 4.53. On ‘6B662’, which is susceptible to Ptr ToxB, the positive control isolate, ‘Dw7’, had a rating of 4.81 and the negative control, ‘Pti2’, had a rating of 1.27. On ‘6B365’, which is susceptible to Ptr ToxC, the negative control isolate, ‘Dw7’, had a rating of 1.78 and the positive control, ‘Pti2’, had a rating of 4.35 (Table 2.4).

Molecular characterization

Molecular characterization of the 63 survey isolates revealed that primers were able to amplify a band matching the size of the predicted product for *ToxA* in 55 isolates (87.3%), for *ToxB* in 0 isolates, and for *toxb* in 1 isolate (1.6%). Amplification of *ToxA* showed only minor differences between survey collection years. The *ToxA* rate was 91.4% in 2019, and 82.1% in 2020. Of the 16 historic isolates phenotyped, primers amplified a band matching the expected size for *ToxA* in 14 isolates (87.5%), for *ToxB* in 1 isolate (6.2%), and for *toxb* in 0 isolates. Sample sizes of the other NEs and for the year 2021 were too small to make meaningful comparisons.

Race characterization

The survey isolates primarily fell into 4 races. 45 of the 51 phenotyped isolates were race 1, there was also one isolate each estimated to be the races 2, 3, and 4. Three isolates caused phenotypes which did not fit the current race structure. The historic isolates fell into races 1, 2,

and 4. 8 of the 12 phenotyped isolates were race 1, there was one race 2, and 1 race 4 isolate. A single isolate caused an atypical phenotype which did not fit the race structure.

Discussion

The research objective of this study was to characterize the race structure of the Kansas Ptr population. This was completed through a survey of leaf spot pathogens in the wheat production region, followed by molecular and phenotypic characterization of these Ptr isolates. These phenotypic and molecular characterization results combined to determine the race of the current Kansas *P. tritici-repentis* population as well as the race structure of an historic isolate collection.

Differential characterization utilizes varieties with known reactions to *P. tritici-repentis* pathogenicity factors to estimate the genotype of the isolate. The results of this study found that the greatest phenotypic leaf necrosis and chlorosis response was observed when both the survey and historic isolates were inoculated onto the differential lines ‘Glenlea’ and ‘6B365’, the lines susceptible to Ptr ToxA and Ptr ToxC respectively. The differential line ‘6B662’ had a relatively low overall phenotypic response compared to the other two differential lines. These phenotypes indicate that there are high levels of *ToxA* and *ToxC* in the isolate collections.

The phenotypes differed slightly between the survey isolates and the historic collection. Historic isolates appeared to cause a lower disease rating on the differential isolates than the contemporary isolates. This result could have been due to changes in the pathogen population between collection dates, or it could have been due to degradation of the isolates while in storage. All had been collected before 2015 and the prolonged storage of the isolates could have been detrimental to virulence.

The two control isolates, ‘Pti2’ and ‘Dw7’, showed phenotypic responses consistent with their race description (Table 2.4). Phenotypic responses of the survey and historic collections were more variable than expected. This data made making some race designations difficult. This serves as a reminder that these NEs and their inverse gene-for-gene interaction are important for pathogenesis, but they are not acting alone. The pathogens are only one component in a complex interaction with the host and environment. For this reason, molecular characterization was also completed to bolster the results obtained above. Some isolates were not phenotyped because they failed to produce conidia or produced only low numbers of conidia under laboratory conditions. These isolates were characterized molecularly, however their races were not included in this study.

The results of the molecular characterization showed that *ToxA* was present in 87.3% of survey isolates and 87.5% of historic isolates. *ToxB* was not identified in any isolate from the survey. However, it was identified in one isolate (‘HV-00’) from the historic collection. This isolate, unfortunately, did not produce conidia under laboratory conditions so this genotype was unable to be verified phenotypically. The non-functioning *toxb* allele was found in one isolate from the 2019 survey. This isolate (‘19BA72’) had a ‘6B662’ differential phenotype rating of 1.19, which when compared with the average of 1.15, does not appear to have caused an increase in fitness for the isolate.

There were minor differences in rates of *ToxA* between the survey years, but a statistical comparison was not made due to the small sample size.

While this research does provide a consistent representation of the Kansas Ptr population NE diversity, it does not have the population size required to identify NEs that are represented at low levels in the population. A larger sample size may have identified more isolates which

contain *ToxB*. Another structural problem with this survey is the sampling bias that comes with surveying diseased wheat. This likely kept some race four isolates or less virulent isolates from being collected. Care was taken to minimize this problem by surveying isolates with diverse disease phenotypes. However, seemingly healthy tissue was not sampled.

Race was determined using both the phenotypic response of differential lines as well as the genotypes for *ToxA* and *ToxB*. The most common race of 2019-2021 survey isolates and historic isolates was race 1. 88.2% of the survey isolates, and 66.6% of the historic isolates were race 1. Races 2, 3, and 4 had only minor representation. There were also 3 survey isolates and 1 historic isolate which failed to match the current race structure system. These isolates have been labeled "Atypical" (Table 2.2 & 2.3). The reason these isolates did not match the structure was because a product matching the size of *ToxA* was amplified but the phenotypic ratings did not show a strong response on the Ptr ToxA susceptible differential line 'Glenlea'. Evaluating these isolates to verify expression of *ToxA* could clarify these observations.

The results of this survey of Kansas isolates showed that the population has and has had high levels of *ToxA*. These results were expected as *ToxA* has previously been shown to be the primary determinant of disease in this pathosystem (Ciuffetti et al., 2010; Faris et al., 2013; Lamari & Bernier, 1989c; Wolpert et al., 2002). *ToxA* in our collections was represented in 87.3% of isolates. This result was similar to rates published from the Northern Great Plain (95%), South Dakota (79%), Ohio (95%), the Southern Great Plains (95%), and Arkansas (68%) (Abdullah, Sehgal, & Ali, 2017; Ali et al., 2010; Ali & Francl, 2003; Engle et al., 2006).

This report also found 1 isolate from which *ToxB* could be amplified. However, this isolate was unable to be phenotyped as it did not grow conidia under our experimental

conditions. This matches previous results which have indicated that *ToxB* is present at low levels (0.5%) in the US population (Abdullah, Sehgal, & Ali, 2017; Ali & Francl, 2003).

ToxC was present in high levels in our survey and historic collections. This matched previous research on U.S. populations which have reported an average of 85.8% of isolates contain *ToxC* (Abdullah, Sehgal, & Ali, 2017; Ali et al., 2010; Ali & Francl, 2003; Engle et al., 2006). Unfortunately, the population could not be genotyped for *ToxC* as the gene has yet to be molecularly characterized. This study determined that the driving pathogenicity factor in the Kansas *P. tritici-repentis* population, based on frequency and contribution to disease severity is the NE Ptr ToxA. In response to high levels of *ToxA* in the population, producers with a history of yield loss to tan spot should consider planting varieties that do not contain the susceptibility gene, *Tsn1*. Wheat varieties without the susceptibility factor are likely to have decreased loss to tan spot.

The removal of the susceptibility gene for Ptr ToxA, *Tsn1*, from current breeding populations has been recommended or suggested by many researchers (McDonald et al., 2018; Oliver et al., 2014; Virdi et al., 2016; Wu et al., 2020). Selecting against susceptibility components is an efficient method of decreasing susceptibility to necrotrophic pathogens that leverage host defenses to cause disease (Lorang, 2019). This is possible using the validated marker *Xfcp623* to select for insensitivity (*tsn1*) to *ToxA* (Faris et al., 2010; Kokhmetova et al., 2019). Research into the removal of this susceptibility allele has gone as far as verification of yield sustainability in the absence of *Tsn1* (Oliver et al., 2014). However, in the absence of a full understanding of *Tsn1*, removal of the gene should only be considered in areas with a high frequency of *ToxA* in the pathogen population, where there is a benefit to outweigh the risk of the

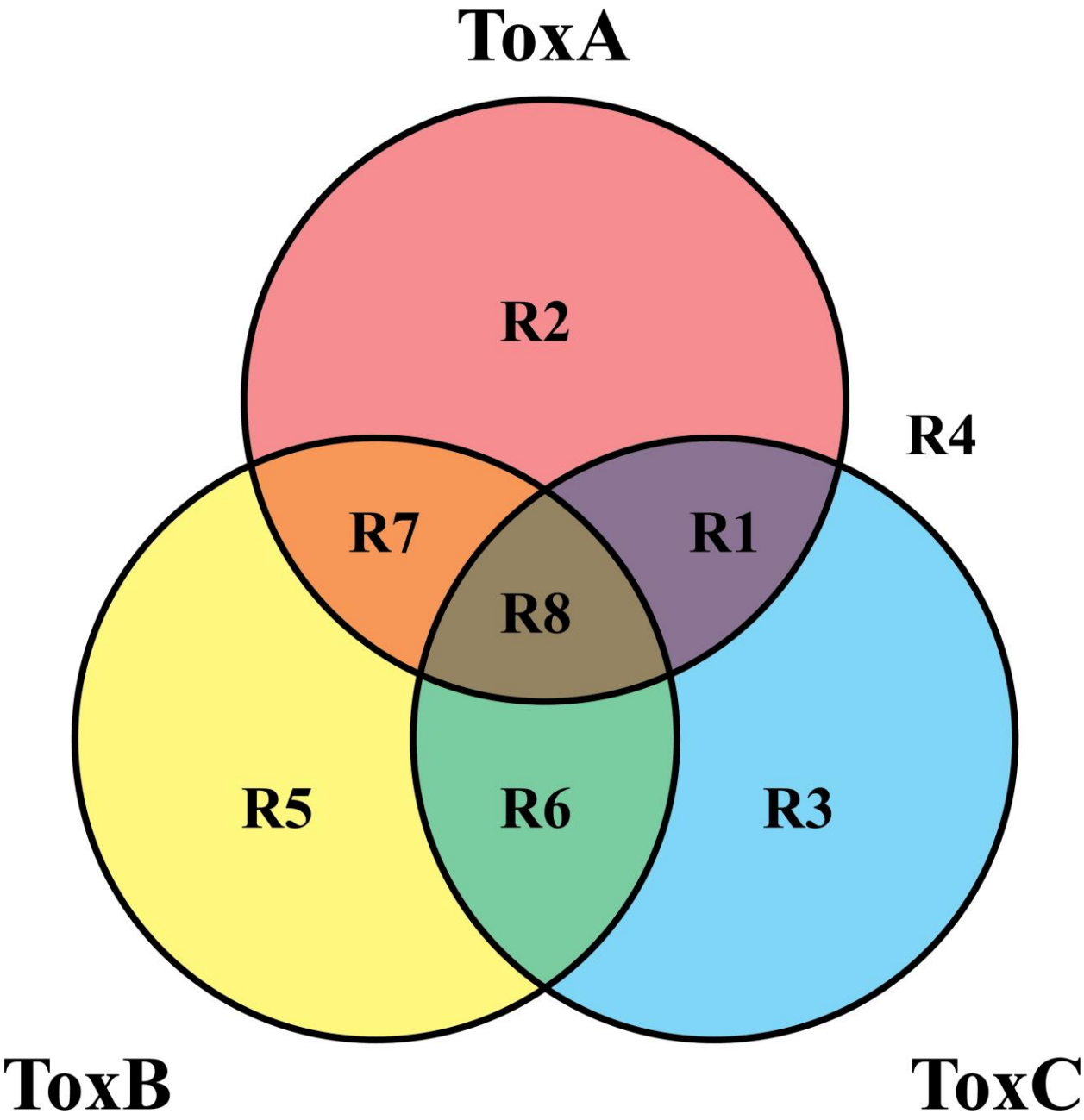
unknown. Kansas, with its high *ToxA* rates, is one of those areas. Future research should focus on evaluating any potential benefits from *Tsn1* in wheat.

While the importance of the susceptibility gene *Tsc1* is less understood, the presence of *ToxC* in the pathogen population indicates it is also important. Recent research has identified genetic markers for *Tsc1* and favorable alleles are available in adapted varieties for the Kansas production region (Running et al., 2022).

Overall breeding efforts should focus on improving the wheat response to *ToxA,* and *ToxC* but variation of wheat varieties with known susceptibility genes indicates that there are additional important factors in this pathosystem. With this knowledge, scientists can more effectively evaluate germplasm to stand up against the current Kansas *P. tritici-repentis* population.

Figure 2.1.

P. tritici-repentis Race Structure Diagram



Note. Three known necrotrophic effectors (NE) make up 8 possible race combinations of *P. tritici-repentis*. All races of the pathogen have either one, two, or three NEs with the exception of Race 4 which has no necrotrophic effectors. *ToxA* is expressed by the races: 1, 2, 7, 8; *ToxB* is expressed by the races: 5, 6, 7, 8, and *ToxC* is expressed by the races: 1, 3, 6, and 8. Race is

indicated by "R" in the figure followed by its race number. The differential lines 'Glenlea', '6B662', and '6B365' are respectively susceptible to the NEs Ptr ToxA, Ptr ToxB, and Ptr ToxC.

Table 2.1.

List of All PCR Primers Used in this Study

Purpose	Primer Name	Primer Sequence (5' to 3')	Tm (°C)	Expected Amplicon Length (bp)	Source
ToxA amplification for identification and sequence characterization	ToxA1	CGTCCGGCTACCTAGCAATA	60	964	Friesen et al., 2006
	ToxA2	TTGTGCTCCTCCTTCTCGA			
Amplification of *ToxB* and *ToxB*-like sequence (Race 3)	TB71F	GCTACTTGCTGTGGCTATC	58	885	Andrie et al., 2007
	TB60R	ACTAACAACGTCCTCCACTTTG			Martinez et al., 2004
toxb (race4) amplification	TB71F	GCTACTTGCTGTGGCTATC	58	204	Andrie et al., 2007
	TB58R	TATGAATGATTGACTGGGGTTA			Martinez et al., 2004
Species verification	ITS1	TCCGTAGGTGAACCTGCGG	60	600	White et al., 1990
	ITS4	TCCTCCGCTTATTGATATGC			

Table 2.2.

Genotype and Disease Phenotype Ratings of *P. tritici-repentis* Isolates Collected in a Kansas Wheat Survey.

	Genotype			Phenotype (1-5 scale)			
Isolate	***ToxA***	***ToxB***	***toxb***	**Glenlea (ToxA)**	**6B662 (ToxB)**	**6B365 (ToxC)**	**Race**
19BA72	-	-	+	3.39	1.19	3.71	R3
19BT49	+	-	-	1.50	0.88	1.25	Atypical
19CD94	+	-	-				
19CL147-6	+	-	-	4.73	1.16	4.32	R1
19CL147-7	+	-	-	4.98	1.11	3.99	R1
19CY93	+	-	-	4.48	1.11	4.24	R1
19CY93-8	-	-	-	2.88	1.00	3.13	R1
19CY93-9	+	-	-				
19DC107	+	-	-	4.88	1.13	5.00	R1
19DC130-5	+	-	-	0.88	0.50	0.50	Atypical
19FO55	+	-	-	3.00	1.00	3.88	R1
19GH135	+	-	-				
19KW78-1	+	-	-	3.88	1.63	4.08	R1
19NS155	+	-	-	3.25	1.00	4.25	R1
19NS156-1	+	-	-	4.00	2.25	4.00	R1
19OB98	+	-	-	4.13	1.00	4.25	R1
19OB98-2	+	-	-	2.75	1.13	1.83	R2
19PL125-1	+	-	-	4.38	1.00	4.75	R1
19PN68-1	+	-	-	4.25	1.50	4.00	R1
19PN68-3	+	-	-				
19RH167-1	+	-	-	4.13	1.88	4.33	R1
19RL170-11	+	-	-	3.88	1.63	3.92	R1
19RL170LS	+	-	-	4.75	1.00	4.50	R1
19RL170LS-3	+	-	-	4.63	1.25	3.25	R1
19RL171-13	+	-	-	4.25	1.38	3.58	R1
19RL172	+	-	-	3.08	0.92	2.58	R1
19RL37	+	-	-	3.75	1.63	2.75	R1
19RO144-1	+	-	-	4.14	0.94	4.21	R1
19RO150	+	-	-	4.50	1.88	3.50	R1
19SD102-2-2	-	-	-				
19SG149	+	-	-	3.88	1.00	3.50	R1
19SG149-1	+	-	-	4.13	1.00	4.67	R1
19SM126-1-1	+	-	-	4.13	1.00	3.75	R1
19SM126-1-2	+	-	-	2.88	1.00	2.63	R1

19TH133-2-2	+	-	-	4.00	1.00	4.50	R1
20CY269.21	+	-	-	0.50	0.38	0.75	Atypical
20CY269.3	+	-	-	4.38	1.00	4.33	R1
20EL264.1.1	+	-	-	3.75	1.00	4.21	R1
20EL264.1.2	+	-	-	3.75	1.00	5.00	R1
20EL264.2	+	-	-	2.63	1.00	4.00	R1
20HP266.1	+	-	-	4.25	1.00	4.88	R1
20HP266.2	+	-	-	3.38	1.00	2.63	R1
20LE252	+	-	-	3.64	0.94	4.46	R1
20LE255	+	-	-	3.75	1.63	4.08	R1
20MC222.2	+	-	-	2.88	0.88	3.33	R1
20MC223	-	-	-				
20MP195	+	-	-	4.63	1.00	4.38	R1
20NS243	-	-	-	0.52	1.04	0.33	R4
20NS244	+	-	-	4.00	2.00	4.25	R1
20OB213	+	-	-	4.39	1.19	5.71	R1
20OB213.1	+	-	-				
20OB213.2	+	-	-	3.25	1.00	4.25	R1
20OB214	+	-	-	3.38	1.25	4.13	R1
20OB216	+	-	-	3.50	1.00	3.96	R1
20OB217	+	-	-	2.88	1.00	3.38	R1
20OB218	+	-	-	3.13	1.00	2.50	R1
20RH248	+	-	-	3.50	1.13	3.38	R1
20SA271	-	-	-				
20SD263.1	-	-	-				
20SM208	+	-	-	4.13	1.13	3.88	R1
21GE295	-	-	-				
21GE296	+	-	-				
21RN313	+	-	-				

Note. Genotypes were determined using PCR primers for the host selective toxin genes: *ToxA, ToxB,* and the alternative *toxb* genotypes. "+" indicates amplification of a band matching a product of the expected size. "-" indicates no product was amplified. Phenotypes were determined by phenotyping isolates inoculated onto a wheat differential set consisting of lines susceptible to only one necrotrophic effector. Race judgments were made and verified using both the genotype and phenotype results. Isolates designated with an "Atypical" race are those with incongruent genotype and phenotype ratings. Mean isolates phenotypes were reported on a 1-5 scale where 1 is highly resistant and 5 is highly susceptible.

Table 2.3.

Genotype and Disease Phenotype Ratings of *P. tritici-repentis* Isolates from an Historic Kansas Isolate Collection.

	Genotype			Phenotype			
Isolate	***ToxA***	***ToxB***	***toxb***	**Glenlea (ToxA)**	**6B662 (ToxB)**	**6B365 (ToxC)**	**Race**
KO-08	+	-	-	3.88	1.25	2.42	R1
AZ-00	+	-	-	3.38	1.13	1.67	R2
MCS-I	-	-	-	3.25	1.13	3.21	R1
MCR-1	+	-	-				
SE-09	+	-	-	2.75	1.13	3.92	R1
WWB-36	+	-	-	1.77	1.04	1.33	R1
AUB-i	+	-	-	3.00	1.13	1.50	R2
HV-99-2	+	-	-	3.13	1.38	3.25	R1
LM-98	+	-	-	1.38	0.63	1.25	Atypical
MER-6	-	-	-	1.59	0.75	2.02	R4
PT-85-6	+	-	-				
HV-00	+	+	-				
FH-26	+	-	-				
FH-86	+	-	-	4.13	1.00	3.38	R1
RN-89	+	-	-	2.64	0.44	1.21	R1
HV-98	+	-	-	4.88	1.13	4.50	R1

Note. Genotypes were determined using PCR primers for the host selective toxin genes: *ToxA, ToxB,* and the alternative *toxb* genotypes. "+" indicates amplification of a band matching a product of the expected size. "-" indicates no product was amplified. Phenotypes were determined by phenotyping isolates inoculated onto a wheat differential set consisting of lines susceptible to only one necrotrophic effector. Race judgments were made and verified using both the genotype and phenotype results.

Table 2.4.

Genotype and Phenotype Ratings of *P. tritici-repentis* Control Isolates.

	Genotype			Phenotype			
Isolate	***ToxA***	***ToxB***	***toxb***	**Glenlea (ToxA)**	**6B662 (ToxB)**	**6B365 (ToxC)**	**Race**
Pti2	+	-	-	4.53	1.27	4.35	R1
Dw7	-	+	-	1.94	4.81	1.78	R5

Note. Genotypes were determined using PCR primers for the host selective toxin genes: *ToxA, ToxB,* and the alternative *toxb* genotypes. "+" indicates amplification of a band matching a product of the expected size. "-" indicates no product was amplified. Phenotypes were determined by phenotyping isolates inoculated onto a wheat differential set consisting of lines susceptible to only one necrotrophic effector. Race judgments were made and verified using both the genotype and phenotype results.

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Chapter 3 - First report of *ToxA* in *Bipolaris sorokiniana* in Kansas, and report of *ToxA* haplotype diversity in Kansas pathogens

Abstract

Bipolaris sorokiniana, the causal agent of the wheat disease spot blotch, was recently found to contain a homologue of the *Pyrenophora tritici-repentis* necrotrophic effector (NE) gene *ToxA* in Australia, India, Mexico, and the U.S. State of Texas. A homologue of this NE has also been previously found in the wheat pathogen *Parastagonospora nodorum*, and its sister species *Parastagonospora avenaria*. This NE has been shown to serve as a primary determinant of pathogenesis in both *P. tritici-repentis* and *P. nodorum*. While the Kansas production region currently experiences yield loss from spot blotch, the status of the gene in the pathogen *B. sorokiniana* is unknown. The introduction of a new necrotrophic effector, called Bs ToxA, to this population could enable the pathogen to become an emergent economic threat. The objectives of this research were to determine if Kansas isolates of *B. sorokiniana* contain the NE gene *ToxA*, to determine the distribution of the NE gene in the population, and to evaluate the genetic diversity of *ToxA* in populations which contain the gene. This research also sought to determine if isolates of *B. sorokiniana* that contain the NE gene *ToxA* had a greater necrosis-inducing ability on susceptible wheat varieties than isolates which do not contain the NE gene. Diseased wheat leaves were sampled across wheat production regions of Kansas, and isolated fungal leaf spot pathogens were characterized molecularly for presence of *ToxA*. In addition, 16 isolates of *P. tritici-repentis* from an historic collection were also included for comparison. Amplified *ToxA* sequences were compared with publicly available sequence data. Additionally, a phenotypic comparison was made between isolates containing the NE and those without to determine if Bs ToxA increases virulence on susceptible wheat varieties. The survey identified 63 isolates of *P.*

tritici-repentis, 14 isolates of *P. nodorum*, and 18 isolates of *B. sorokiniana*. 87.3% *P. tritici-repentis* survey isolates contained *ToxA*. 85.7% of historic *P. tritici-repentis* isolates contained *ToxA*. All surveyed *P. nodorum* isolates contained *ToxA*. 44% of the *B. sorokiniana* isolates contained *ToxA*. Haplotype analysis of the three populations showed that all surveyed haplotypes matched previously identified haplotypes of the given species. Through comparison of culture filtrate infiltration assays, isolates containing *ToxA* were shown to have a greater necrosis-inducing ability on susceptible wheat varieties than isolates which did not contain *ToxA*. These results show that the Kansas *B. sorokiniana* population contains the NE gene *ToxA* and that the gene is likely playing a significant role in pathogenesis. Comparisons of *ToxA* haplotype diversity showed that the greatest amount of genetic diversity continues to be found in *P. nodorum*, supporting the hypothesis that it is the source of the NE in the wheat leaf spot pathogen populations. *ToxA* is only found at a relatively low rate in the *B. sorokiniana* population, as compared to the near ubiquity of the gene in *P. tritici-repentis* and *P. nodorum* populations in the region. *ToxA* appears to provide a fitness advantage. The potential exists for this gene to become more common in the *B. sorokiniana* population, leading to greater future losses from the pathogen.

Introduction

Plant pathogen colonization is often thwarted when plants mount a hypersensitive defense response based on a gene-for-gene interaction (Flor, 1956). This relationship takes on new meaning when a fungal necrotrophic pathogen expresses a necrotrophic effector to trigger and take advantage of this defense response by using it to enable susceptibility in what is called an inverse gene-for-gene interaction (Friesen & Faris, 2010; Wolpert et al., 2002). An example of this relationship is the oat Victoria blight pathosystem, in which *Cochliobolus victoriae*

expresses a NE that triggers susceptibility in lines which contain *Vb* for sensitivity (Lorang et al., 2007). A more closely related example is *Pyrenophora tritici-repentis*' use of the NE Ptr ToxA to enable pathogenesis in wheat varieties which carry the susceptibility factor, *Tsn1* (Faris et al., 2010).

ToxA was initially discovered in *P. tritici-repentis*, the causal agent of the disease tan spot in wheat (Tomas & Bockus, 1987; Tuori et al., 1995). In 2006, a highly conserved copy of *ToxA* was discovered in the genome of a second fungal necrotrophic pathogen, *Parastagonospora nodorum*, and the source was theorized to be a potential interspecific gene transfer event of a highly mobile pathogenicity element from *P. nodorum* to *P. tritici-repentis* (Friesen et al., 2006; McDonald et al., 2018, 2019). This gene transfer event is believed to have caused the emergence of *P. tritici-repentis* as an important pathogen of wheat in the 1940s (Friesen et al., 2006).

In 2012, *ToxA* was identified in *Parastagonospora avenaria* f. sp. *triticae* (genus previously known as *Phaeosphaeria*), a species closely related to *P. nodorum* (McDonald et al., 2012). This transfer was postulated to be due to an interspecific hybridization event with *P. nodorum* (McDonald et al., 2012, 2013). In 2018, *ToxA* was discovered in *B. sorokiniana* isolates from Australia and Texas, USA, and in the years following it was found in isolates from India and Mexico (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020; Wu et al., 2020).

With reports of the NE in *B. sorokiniana*, as far north as Texas, the status of *ToxA* in the Kansas *B. sorokiniana* population and the role it may be playing in the disease spot blotch is unknown. This information is important to determine if the disease may become more economically important based on the potential recent acquisition of the NE Bs ToxA. Several differences between the genetic element in the three backgrounds provide evidence that the

copies in *P. tritici-repentis*, and *B. sorokiniana* diverged more recently than from *P. nodorum* (McDonald et al., 2018).

Since *ToxA* was identified in *B. sorokiniana*, researchers have reported its presence ranges from 10.2% to 86.7% in different populations around the world (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020; Wu et al., 2020). Research has also been conducted to determine if the necrosis-inducing ability of *ToxA*-containing *B. sorokiniana* isolates is greater than those that do not contain the gene. This has been tested using both inoculation and culture filtrate infiltrations on both wheat lines differing for *Tsn1* allelic state and *Tsn1* mutants (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020; Wu et al., 2020). Friesen et al., also verified that sensitivity to culture filtrates was caused by the ToxA/*Tsn1* inverse gene-for-gene interaction by mapping the necrotic phenotype to the *Tsn1* locus in a mapping population (Friesen et al., 2018).

ToxA is a powerful pathogenic tool for a necrotrophic pathogen, therefore there is likely a high selection pressure for this gene (Friesen et al., 2018; McDonald et al., 2018). The aim of this research is to determine the role that Bs ToxA is playing in the Kansas *B. sorokiniana* population and to evaluate additional *ToxA* haplotypes to determine the extent of diversity in populations of *P. tritici-repentis, B. sorokiniana*, and *P. nodorum*. In addition, this study seeks to compare the necrosis-inducing ability of *ToxA*+ and *ToxA*- isolates.

To address this research gap, a survey for leaf spot pathogens was conducted on wheat (*Triticum aestivum* L.) and the intermediate wheatgrass (*Thinopyrum intermedium*), Kernza® (The Land Institute). Samples were molecularly characterized for presence of *ToxA*, and amplicons were sequenced for comparison with existing public haplotypes. Pathogenicity was characterized on susceptible and non-susceptible wheat varieties using culture filtrate infiltration.

ToxA has been shown to be in a highly mobile pathogenicity element with the power to enable fungal necrotrophic pathogens to cause severe yield loss (McDonald et al., 2018). There are very few reported isolates of *B. sorokiniana* containing *ToxA* in the U.S. A better understanding of this gene's distribution and diversity in pathogen populations will inform breeding decisions and help increase understanding of how this NE has moved through fungal populations.

Methods

Isolate collection

A survey of Kansas leaf spot pathogens was conducted by sampling diseased wheat plants from May to June of 2019, 2020, and 2021. Samples were also collected from the intermediate wheatgrass Kernza®. After collection, samples were stored in paper envelopes in a cool dark container until returned to the laboratory where they were stored at 4°C until fungal isolation. Isolates from an historic collection that was stored on ¼ PDA in slant tubes at 4°C were also used (Bockus, unpublished) (Table 3.1).

Isolation

Fungi were isolated from leaf samples by excising a small piece of leaf tissue from the margin of a potential fungal leaf spot. The leaf tissue was then surface sterilized in 5% bleach for 3 minutes, before being rinsed twice in two dishes of sterilized water for 1.5 minutes each. Sterilized leaf tissue was then placed on water agar (4 g granulated agar, 250 ml autoclaved water) petri dishes, sealed with parafilm, and incubated in a growth chamber for 24 hours. The growth chamber (Powers Scientific, Inc, Pipersville, PA) was kept on a day/night temperature regime of a 20°C/19°C with 16 hour days under supplemental L9T8SE240-G 4000K lights (MaxLite, West Caldwell, NJ). After 24-hours, water agar plates were observed under the stereo

microscope (Zeiss Microscopy, Jena, Germany) for signs of fungi growing from the leaf sample. *P. tritici-repentis* and *B. sorokiniana* isolates produced conidia readily on leaf samples. *P. nodorum* conidia were collected from pycnidia in the leaf's epidermis after humidification on water agar plates. Spores of each were transferred onto a petri plate containing ¼ Potato Dextrose Agar (¼ PDA) (2.5 g granulated agar, 3.8 g potato dextrose agar, 500 ml water) and placed back in the growth chamber for five days.

Single spores of each isolate were collected through sporulation and dilution for genetic uniformity. *P. tritici-repentis* isolate conidia were grown on V8-potato dextrose agar (V8-PDA) (150 ml V8 juice, 10 g PDA, 10 g agar, 3 g $CaCO_3$, 850 ml water) in accordance with the Lamari and Bernier protocol with the following changes (Lamari & Bernier, 1989). Isolates were grown in the dark for 5 days at 21°C. The isolates leading edge mycelium was then flattened by adding 300 µl of water and using the flame sterilized tip of a test tube to mat the mycelium. Plates were then wrapped with parafilm and placed under supplemental light from Agrobrite 6400K fluorescent light bank (Hydrofarm, Petaluma, CA) for a continuous 48 hours at 21°C. *B. sorokiniana* isolates were sporulated from isolates stored in slant tubes at 4°C. A small sample of mycelium was transferred from each slant tube on to a plate of ¼ PDA. Plates were then wrapped in parafilm to maintain humidity at the medium surface, and placed in the growth chamber as previously described day/night temperature regime with 16-hour days for two weeks. *P. nodorum* isolates were purified by selecting and transferring a small mycelium sample from the margin, identified under the stereo microscope.

Molecular characterization

Isolate species level identification was conducted morphologically and verified molecularly. Fungal tissue was collected from isolates of *B. sorokiniana* and *P. nodorum* by

scraping surface mycelium from 10-day old plates of ¼ PDA that had been inoculated from fungal samples stored at 4°C on ¼ PDA slant tubes. Tissue was collected from *P. tritici-repentis* isolates by growing mycelium in flasks of Difco potato dextrose broth (PDB) (Difco Laboratories Inc., Detroit, MI) from samples stored at 4°C on ¼ PDA slant tubes. Mycelium was collected from the flasks after 10 days and pressed between paper towels to remove excess liquid.

80-100 mg of fungal tissue was placed in a 1.7 mL micro centrifuge tube and ground with sterilized 4.5 mm galvanized steel pellets (Daisy Outdoor Products, Rogers, AR) using a bead mill tissue disruptor (TissueLyser II, Qiagen, Hilden, Germany). Fungal genomic DNA was extracted from tissue using a cetyltrimethylammonium bromide (CTAB) protocol with the changes and modifications described in chapter 2 (Murray & Thompson, 1980). Tissue was ground in a bead mill with 700 µl of CTAB. 14 µl of 2-mercaptoethanol (Fisher Scientific, Waltham, MA) was added after the tissue disruption. The mixture was then incubated at 60°C for 30 minutes. Soluble DNA was separated from debris and insoluble material through two 500 µl, 24:1 chloroform:isoamyl alcohol phase separation extractions. DNA was precipitated using 0.8 volumes of 2-propanol. DNA concentration was quantified using an Implen C40 nanophotometer (Implen, Munich, Germany). DNA was diluted to 20 ng/µl using Tris-EDTA (TE) buffer (5 mL 1M Tris, 1 mL 0.5M EDTA, 496mL water).

Molecular species verification was conducted by amplifying and sequencing the ribosomal internal transcribed spacer (ITS) region using primers ITS1 and ITS4 (White et al., 1990). ITS fragments were amplified using a thermocycler (Labnet, Multigene Optimax, Edison, NJ) with the conditions: 6 minutes at 95°C, then 35 cycles of 30 seconds at 95°C, 40 seconds at 60°C, 40 seconds at 72°C, then a 5-minute final extension at 72°C (Table 3.2). ITS amplicons

were sent for PCR clean-up and Sanger sequencing (MCLAB, South San Francisco, CA). Sequences were compared to previously published sequences reported on the National Center for Biotechnology Information (NCBI).

Isolates were genotyped for the *ToxA* locus using the ToxA1 and ToxA2 primer set and conditions described by Friesen et al. (Table 3.2) (Friesen et al., 2006) using GoTaq Green Master Mix (Promega, Madison, WI). PCR was performed in 20 µl reactions. *P. tritici-repentis* isolates of known race, 'Pti2' (Race 1), and 'Dw7' (Race 5), were used as positive and negative controls for PCR. PCR conditions were as follows: 4 minutes at 94°C, then 35 cycles of 45 seconds at 94°C, 30 seconds at 60°C, 60 seconds at 72°C, followed by a final 7-minute extension at 72°C.

ToxA primer amplicons underwent PCR clean-up and Sanger sequencing (MCLAB, South San Francisco, CA). Each sample sequence was replicated with three forward and three reverse replicate reactions. Consensus sequences were generated using the Pregap4 and Gap4 programs in Staden Package (Staden et al., 2000).

Haplotype comparison

Sequence alignment, using the MUSCLE function, and phylogenetic trees were generated using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar et al., 2018). A single 55-nucleotide intron was removed, and the sequences were trimmed to the 534-nucleotide ORF. Public sequences with greater than 12.5% missing data were removed from the dataset. The remaining sequences were annotated with missing data and used in the haplotype comparison to try and identify as many rare mutations as possible.

All publicly available *ToxA* sequences on NCBI were collected to conduct a broader analysis of haplotype diversity (Table 3.3). These sequences were then aligned with novel

sequences using MEGA X as previously described. Haplotypes were then compared using the Population Analysis with Reticulate Trees (PopART) software to generate a minimum spanning network figure (Bandelt et al., 1999; Leigh & Bryant, 2015).

A representation alignment of predicted amino acids from representative haplotypes was generated. MegaX was used to run a nucleotide alignment of the representative haplotype sequences followed by conversion to amino acids. The BioEdit Sequence Alignment Editor was used to generate an alignment figure (Hall, 1999).

Comparison of necrosis-inducing ability

A virulence comparison between *B. sorokiniana* isolates containing *ToxA* and isolates that did not contain *ToxA* was conducted by infiltrating culture filtrates into susceptible and non-susceptible wheat varieties. The ToxA susceptible varieties used, characterized by containing *Tsn1*, were 'Glenlea' (Accession: CI- 17272) and 'Jagger' (Accession: PI-593688) and the non-susceptible varieties, which did not contain *Tsn1*, used were 'Salamouni' (Accession: PI-182673) and 'Everest' (Accession: PI-659807) (Evans et al., 1972; Friesen et al., 2018; Sears et al., 1997). Culture filtrates from 17 *B. sorokiniana* isolates were produced and infiltrated in accordance with Wu et al., using the following volumes and modifications (Wu et al., 2020). Flasks with 100 ml of Fries medium (5 g ammonium tartrate, 1 g ammonium nitrate, 0.5 g magnesium sulfate, 1.3 g potassium phosphate [dibasic], 2.6 g potassium phosphate [monobasic], 30 g sucrose, 1 g yeast extract, 1000 ml water) were inoculated with each *B. sorokiniana* isolate and grown on a shaker table at 130 rpm for three days, followed by three weeks of stationary growth in the dark at room temperature to encourage expression of Bs ToxA (Liu et al., 2004). Mycelium was removed using two layers of cheesecloth and culture filtrates were then filtered through a 0.22 µm filter

(RephiLe Bioscience, Boston, US). Remaining solid material was removed through centrifugation at 12,000 RPM for 5 minutes (Eppendorf 5424 Centrifuge, Hamburg, Germany).

Culture filtrates were infiltrated into each of the four wheat varieties in two separate replicates. Infiltration sites were scored as sensitive (necrosis response) or insensitive (no necrosis response) for response 72 hours after infiltration. In addition to the culture filtrates, a water treatment and a Fries culture medium treatment were also included as negative checks to ensure the culture media was not causing necrosis.

Results

Isolate collection

The survey of diseased cereals from across the Kansas wheat production region yielded 18 isolates of *B. sorokiniana*, 16 of these isolates were isolated from wheat, and 2 of these isolates were collected from Kernza®. The survey identified 14 isolates of *P. nodorum*, 13 isolates from wheat, and 1 isolate from Kernza®. The survey identified 63 isolates of *P. tritici-repentis*, all of which were isolated from wheat. 16 historic isolates of *P. tritici-repentis* isolated from wheat, were also characterized.

Molecular characterization

Molecular characterization of the novel isolates amplified a product the expected size of *ToxA* in all populations (Figure 3.1). 44% of *B. sorokiniana* isolates amplified a product the expected size of *ToxA*. This is the first report of *ToxA* in the *B. sorokiniana* population in Kansas. 100% of the *P. nodorum* isolates surveyed and characterized in this study amplified a product of the expected size. 87.3% of *P. tritici-repentis* survey isolates amplified a product of the expected size. 85.7% of historic *P. tritici-repentis* isolates amplified a product of the expected size.

Haplotype comparison

The *P. tritici-repentis* survey and historic isolates all consisted of one single haplotype that was shared with the publicly available *B. sorokiniana* NCBI accession CM017963.1 (Table 3.1) (McDonald et al., 2019). The survey *B. sorokiniana* survey isolates fell into two haplotype groups; 2 isolates matched the haplotype of all Kansas *P. tritici-repentis* isolates, and 6 isolates matched the publicly available *B. sorokiniana* NCBI accession KX816409.1 (McDonald et al., 2018). The *P. nodorum* survey isolates fell into two haplotype groups; 9 isolates matched the publicly available *P. nodorum* NCBI accession EF108454.1, and 5 matched the publicly available *P. nodorum* NCBI accession EF108451.1 (Stukenbrock & McDonald, 2007).

A collection of publicly available *ToxA* sequence from the four fungal species reported to contain *ToxA* shows that there are 22 known unique sequence haplotypes of the gene (Figure 3.2). The greatest diversity is in *P. nodorum* with 18 previously reported haplotypes. *B. sorokiniana*, has much less genetic diversity with only 2 previously reported haplotypes. The least amount of genetic diversity is found in the *P. tritici-repentis* population with all but 1 publicly available sequence matching a single haplotype. The single differing sequence (Accession: U79662.1) represented a closely related haplotype differing by one single nucleotide polymorphism (SNP) (Ballance et al., 1996).

Comparison of necrosis-inducing ability

Seventeen *B. sorokiniana* isolates were characterized for presence or absence of a *ToxA* genomic region and infiltrated into two replicates of four differential lines each (Table 3.4). Isolates were rated as either sensitive or insensitive based on the differential plant's response to the infiltrated culture filtrate. Plant ratings are shown in table 3.4 and leaf images are shown in Figure 3.3. In two replicates, isolates that were determined to contain *ToxA* had a greater necrosis

rating on the susceptible (*Tsn1*) wheat varieties ‘Glenlea’ and ‘Jagger’, than they did on the non-susceptible (*tsn1*) wheat varieties ‘Salamouni’ and ‘Everest’, which showed no necrosis response. The isolates that did not contain *ToxA* did not have a necrosis phenotype response on either the susceptible or non-susceptible varieties.

Discussion

Isolates in this study were molecularly characterized to determine if they contain *ToxA*. The *ToxA* sequence was amplified using PCR and if a band matching the expected size of *ToxA* was amplified, the product was sequenced to determine if it was the expected sequence (Figure 3.1).

P. tritici-repentis molecular characterization showed that 87.3% survey isolates contained *ToxA*, and 85.7% of historic *P. tritici-repentis* isolates contained *ToxA*. Unfortunately, records do not exist showing the age of the historic isolates used in this study, but an average age of the collection is estimated to be at least 20 years old. This lack of information and the small sample size of the historic collection allows only a basic comparison of *ToxA* rates; however, these rates do appear to be comparable with the survey isolates which suggests that the rate has not changed drastically in this population. These results are also in line with previously published U.S. rates of *ToxA* in the population at rates from 68% to 95% (Abdullah et al., 2017; Ali et al., 2010; Ali & Francl, 2003; Engle et al., 2006).

Of the *P. nodorum* isolates collected in the study, 100% were found to contain *ToxA*. Previous studies have shown that *P. nodorum ToxA* rates range from 100% in Australia to 5% in China (Friesen et al., 2006). Results on North American isolates have previously only shown 25.5% to have *ToxA* (Stukenbrock & McDonald, 2007).

The results obtained in this study show that some of the *B. sorokiniana* survey isolates contained the necrotrophic effector gene *ToxA*. This is the first report of *ToxA* in the *B. sorokiniana* population in Kansas. Of the 18 isolates collected, 44% (n=8) contained *ToxA*. The distribution of *B. sorokiniana ToxA* isolates throughout the state appears to be random, and populations in these areas appear to be mixed with both *ToxA* positive and negative isolates being found near each other.

Identification of *ToxA* in the Kansas *B. sorokiniana* population was expected as the first report of *B. sorokiniana ToxA* in North America was found in Texas, on the southern end of the same wheat production region (Friesen et al., 2018). There is also a long history of pathogens moving easily from areas like Texas as far as the northern Great Plains. For this reason, it is likely that the pathogen contains this NE throughout the Great Plains wheat production region. With so few isolates previously reported and evaluated, it is difficult to generalize about the frequency of *ToxA* in the *B. sorokiniana* population. However, the frequency in the small sample reported here does fall within previously reported ranges. Of the *B. sorokiniana* isolates collected from Texas in 2016, 13 of 15 isolates collected from 15 distinct necrotic lesions on wheat leaves collected in Castroville, TX were found to also contain *ToxA* (Friesen et al., 2018). However, these isolates likely do not accurately represent the entire population as they were collected from a single location.

The best estimation of *B. sorokiniana ToxA* population distribution from North America available was undertaken across nine states in Mexico between 1992 and 2017. This research identified 196 *B. sorokiniana* isolates and found that only 20 (10.2%) of them contained *ToxA* (Wu et al., 2020). This study did not address the change in *ToxA* frequency over time or provide a breakdown of isolate age distribution, so a collection heavily weighted with older isolates

could underestimate the *ToxA* frequency if the population was undergoing a change during the timeframe of sample collection.

These findings are similar to reports from other continents which also report a less than ubiquitous rate of *ToxA* in the *B. sorokiniana* population. McDonald et al., sampled 35 Australian isolates and found 34% to contain *ToxA*, with the *ToxA* isolates being found in a random distribution around the country (McDonald et al., 2018). Samples from two different Indian megaenvironments found that 70% (n=77/110) contained *ToxA* (Navathe et al., 2020). All populations sampled to date show that *ToxA* is not ubiquitous in the population.

This survey focused on collecting all necrotic or chlorotic diseased tissue. Based on this survey, the sample collected provides a good estimate of the true pathogen frequency and frequency of *ToxA* in the populations. The side-by-side population level survey comparison between *B. sorokiniana, P. nodorum*, and *P. tritici-repentis* populations, provided by this study, shows good estimation of the difference in *ToxA* frequency in the three populations.

Haplotype diversity of *ToxA* has been of great interest around the globe. This diversity is important to understanding the history of *ToxA* in diverse backgrounds, as greater diversity can be an indication of the length of time a gene has had to undergo genetic mutation. This mutation rate can be used as an estimation for when a given species gained this gene. For this reason, this study sought to increase our understanding about genetic diversity in the *P. tritici-repentis*, *P. nodorum*, and the *B. sorokiniana* populations by evaluating novel isolates in conjunction with recently reported *P. tritici-repentis* isolates and publicly available isolates of *ToxA* in all backgrounds.

This research has identified 14 isolates of *P. nodorum*, and *ToxA* was able to be amplified from each isolate. The haplotypes represented matched the two most common *P. nodorum* haplotypes that were publicly available. (Table 3.1, 3.3, Figure 3.1).

Of the 9 *B. sorokiniana* isolates identified in this survey from which *ToxA* was amplified, two different haplotypes were represented. Both of these haplotypes were previously reported (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020).

P. tritici-repentis haplotype diversity was limited. All novel survey isolates fell into a single haplotype group, haplotype 1 (Figure 3.1). Analysis of publicly available haplotypes identified 50 isolates and 49 (98%) fell into haplotype group 1. A single sequence (Accession: U79662.1) was identified which different by a single SNP. Due to the age and unknown confidence in the sequence, this may be a sequencing mistake. There is likely only a single haplotype of *ToxA* in *P. tritici-repentis* which indicates that the species has the least amount of genetic diversity of any containing this gene. This result could be due to the limited amount of time that the gene has had to accumulate mutation, or it could be due to a greater fitness advantage from an individual haplotype that swept the population. These results fit the hypothesis that *P. tritici-repentis* was the most recent species to obtain *ToxA* (Friesen et al., 2006).

Previously surveyed isolates show that the greatest amount of genetic diversity is found in the *P. nodorum* (Friesen et al., 2006). This study was unable to identify novel haplotypes or a wide range of previously identified haplotypes. This is likely due to the small number of isolates sampled or the relatively small region in which these samples were collected. However, another explanation could be that some haplotypes provide a greater fitness advantage to the pathogen and are therefore represented at higher levels.

The evidence here adds to the knowledge on diversity of the *ToxA* gene, which is important in understanding the genetic history of this pathosystem. It provides greater evidence that the species with the least genetic diversity in the *ToxA* gene is *P. tritici-repentis*. *B. sorokiniana*, which is feared to have acquired *ToxA* more recently, has greater genetic diversity in the *ToxA* region but has *ToxA* represented in far less of its population. This could indicate two transfer events, or it could indicate that the gene contributes less to the fitness of *B. sorokiniana* than other species which may have gained it around the same time. This is a hopeful explanation as it provides an explanation where *B. sorokiniana* may not be emerging to become a more economically important pathogen.

This research also aimed to compare the necrosis-inducing ability of *B. sorokiniana* isolates that differed for *ToxA* status from the Kansas production region. Our results show that isolates containing *ToxA* were able to induce a greater necrosis phenotype when culture filtrates were infiltrated into the susceptible (*Tsn1*) wheat varieties 'Glenlea' and 'Jagger' than they were when infiltrated into the non-susceptible (*tsn1*) wheat varieties 'Salamouni' and 'Everest' (Table 3.4).

Previous work using conidial inoculations have shown that isolates containing *ToxA* produce more severe symptoms than isolates which do not contain the NE on susceptible wheat varieties (*Tsn1*) and comparable symptoms on non-susceptible (*tsn1*) wheat varieties (Friesen et al., 2018; McDonald et al., 2018; Navathe et al., 2020). Assays have also verified that isolates containing *ToxA* produced the necrosis phenotype when culture filtrates were infiltrated into 'Glenlea' (*Tsn1*) and not when infiltrated into 'Erick' (*tsn1*) (Wu et al., 2020). Friesen et al., conducted the same verification using *Tsn1* wheat varieties and corresponding mutants to further verify expression of the NE (Friesen et al., 2018).

These results add to previous evidence that *ToxA* increases the virulence of *B. sorokiniana*. For this reason, it is puzzling why *ToxA* has not worked its way through the *B. sorokiniana* population as it had with the *P. tritici-repentis* and *P. nodorum* pathogen populations. One potential explanation for this difference are the differences between the lifecycles of the three pathogens.

The most worrisome potential explanation for the low rate of *ToxA* in the *B. sorokiniana* population would be that the pathogen only recently gained the gene, and it is currently sweeping through the population. This explanation would mean that the disease could become more severe in future years and losses to spot blotch would increase. The oldest *B. sorokiniana* isolate identified which contains *ToxA* was collected from wheat in 1966 (McDonald et al., 2018). Establishing baseline *ToxA* frequencies is integral for monitoring future changes in this population.

There are alternative explanations for the low rate of *ToxA* in the *B. sorokiniana* population. One of these explanations could be a fitness cost caused by or linked to *ToxA* in *B. sorokiniana*, or the lack of a fitness advantage from the gene. While research has shown that isolates containing the gene appear to be more virulent, it is hard to determine true fitness under laboratory conditions.

Another explanation could be lifecycle differences between the pathogens. *P. tritici-repentis* is a homothallic pathogen, which is able to undergo sexual recombination (Lepoint et al., 2010). The benefit of this ability is a likely increase in the amount of outcrossing which will increase recombination to ensure that *ToxA* is placed with the most efficient combination of genes. *Bipolaris* is a heterothallic pathogen for which it is theoretically more difficult to undergo sexual recombination. This population could be more dependent on asexual reproduction and in

combination with an unfortunate initial placement of the *ToxA* mobile pathogenicity element in the genome could lead to a smaller fitness advantage of the gene due to linkage drag (McDonald et al., 2018; Wen & Lu, 1991).

Going forward, greater surveillance of the *B. sorokiniana* population will be required to determine if this pathogen is becoming a more significant threat to wheat production. With low rates of a gene that likely increases the fitness of the pathogen, there is a good chance that this will occur.

The research objective of this study was to determine if Kansas isolates of *B. sorokiniana* contain the NE *ToxA*, to determine the distribution of the NE in the population, and to evaluate the genetic diversity of *ToxA* in the *P tritici-repentis*, *P. nodorum*, and *B. sorokiniana* populations. This research also sought to determine if isolates of *B. sorokiniana* that contain *ToxA* had a greater necrosis-inducing ability on susceptible wheat varieties than isolates which do not contain the NE.

This study showed that *ToxA* is present in the Kansas *B. sorokiniana* population, and that the gene does appear to increase virulence on susceptible wheat varieties. A survey of the state's wheat leaf spot pathogen population showed that *ToxA* was present in *P. tritici-repentis*, *P. nodorum*, and *B. sorokiniana*, at a rate of 87.3%, 100%, and 44% respectively. The genetic diversity of this gene matched previously reported haplotypes (Table 3.3). *P. tritici-repentis* and *B. sorokiniana* have limited genetic diversity in the *ToxA* gene. This could indicate a recent acquisition of the gene in these populations (Friesen et al., 2006; McDonald et al., 2018). The greatest amount of genetic diversity observed was in the *P. nodorum* population.

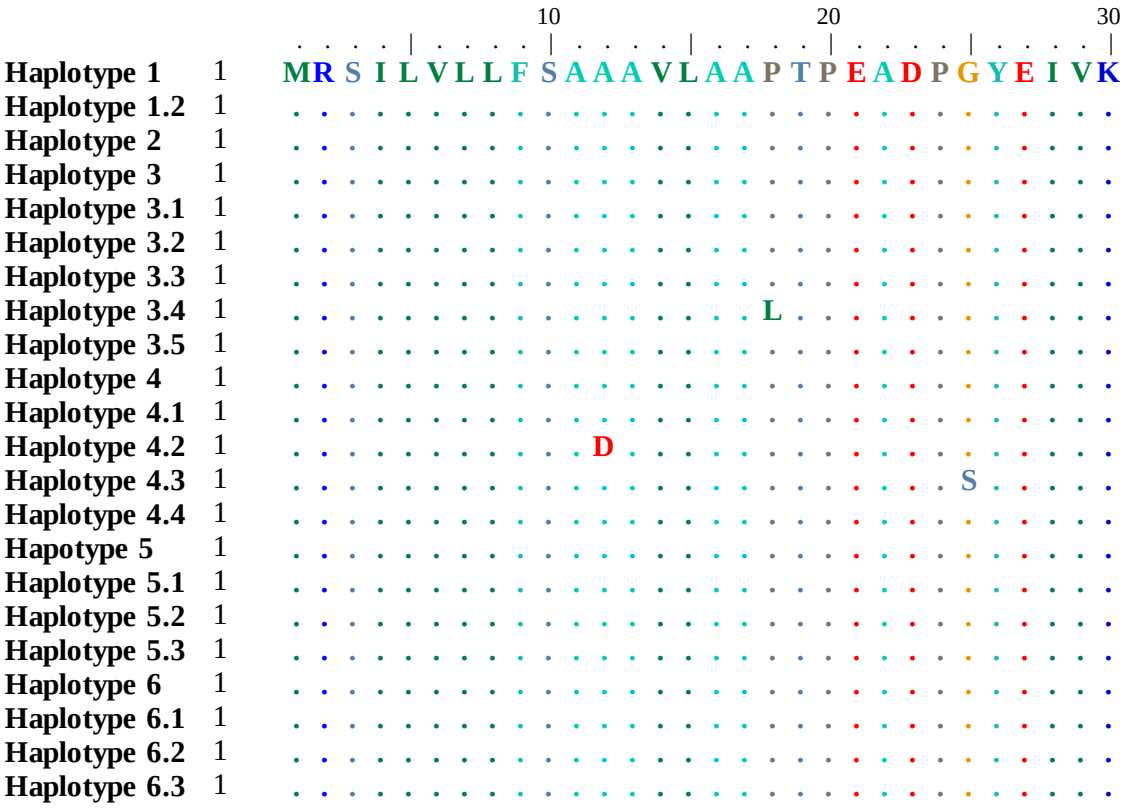
As the *P. tritici-repentis* and *P. nodorum* population contains the NE in a large majority of the isolates collected, an increase in disease enabled by an increase of the frequency of the

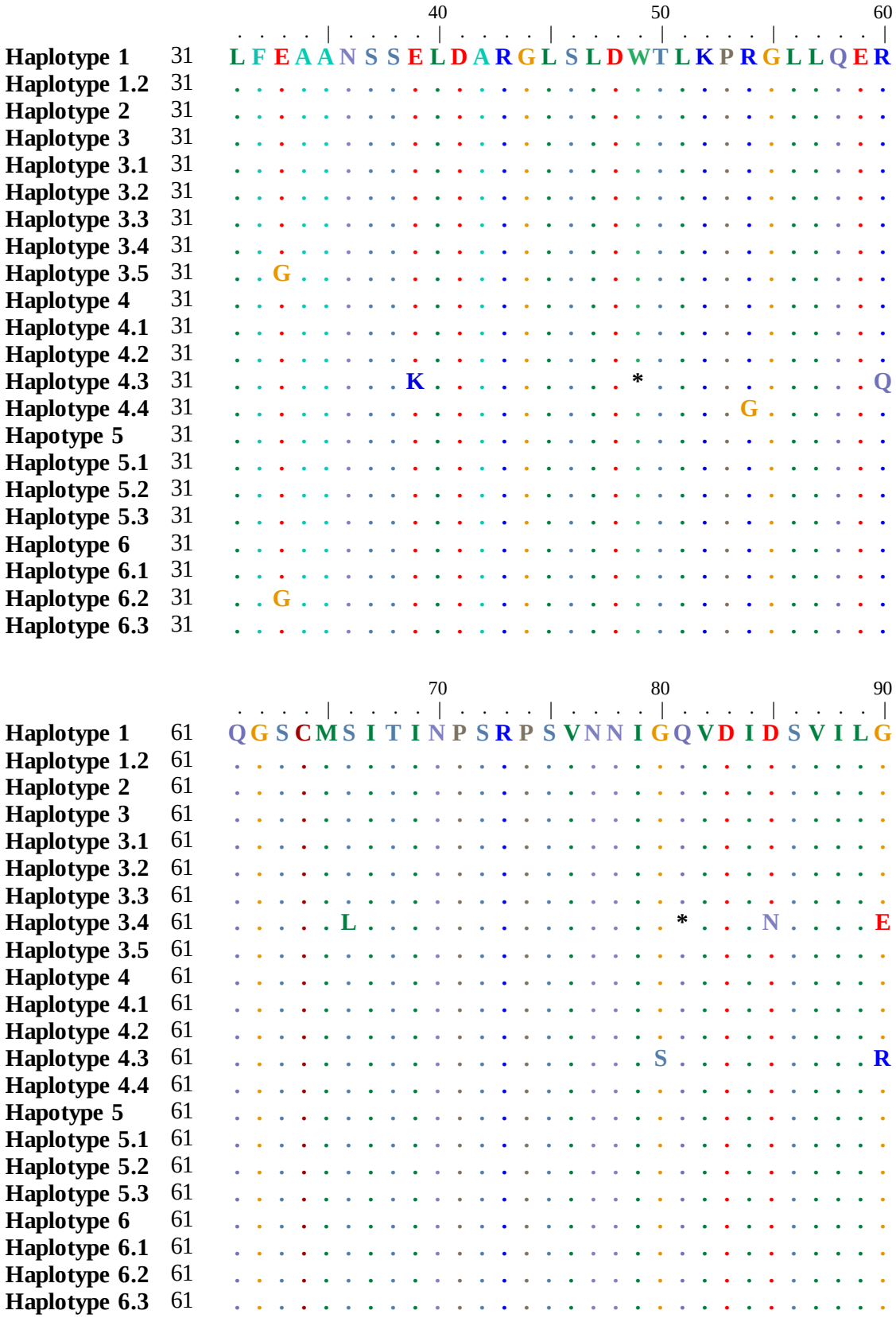
gene in these populations is limited. This study showed that *B. sorokiniana* has *ToxA* in only 44% of isolates examined. This rate, along with the knowledge that *ToxA* appears to increase the fitness of the necrotrophic pathogen, indicates that the population could benefit from an increase in the frequency of *ToxA* in wheat production systems where the susceptibility gene *Tsn1* is present at a high frequency. Therefore, the economic importance of the disease spot blotch could increase as this population gains the NE. While this threat looms, there is evidence that this problem has been emerging relatively slowly when compared to *P. tritici-repentis*, which appeared to gain the gene in the 1940's when a change in its phenotype and virulence was quickly noticed (Friesen et al., 2006). The oldest identified example of *ToxA* in *B. sorokiniana* was in the isolate BRIP10943, which was collected in 1966 (McDonald et al., 2018). In the years since its presence in the population has been verified, the gene is spreading slowly.

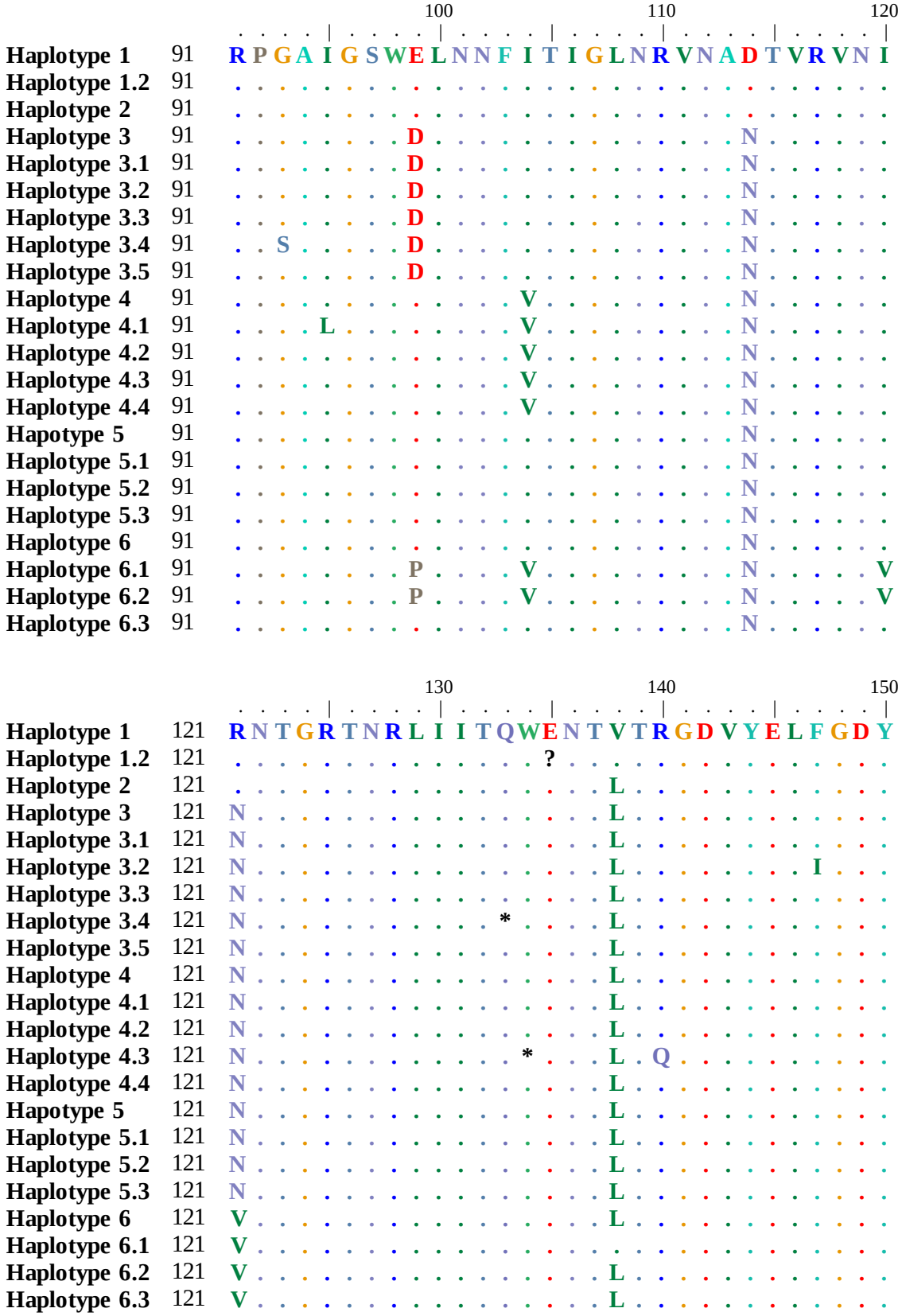
Based on the presence of the *ToxA* NE in at least three pathogen populations, and the knowledge that the NE is being used to cause disease in this region, the identified susceptibility factor (*Tsn1*) should continue to be selected against in wheat varieties under development. Continued surveillance should monitor the *B. sorokiniana* population for changes in *ToxA* frequency.

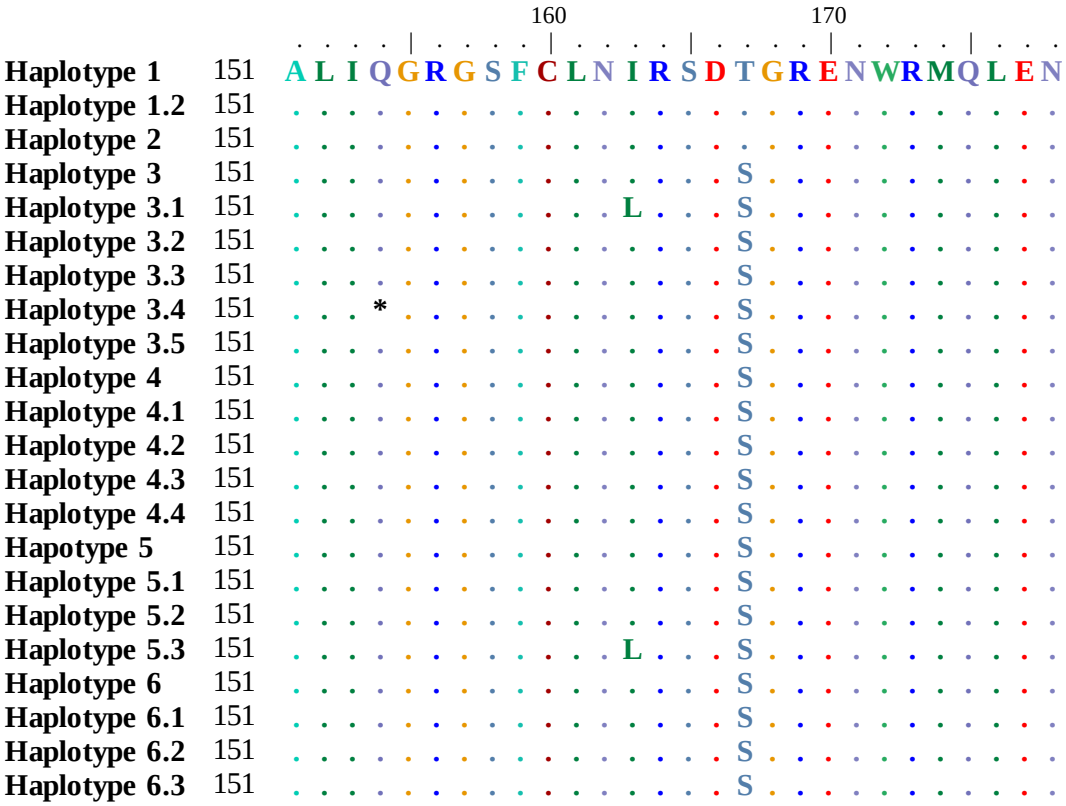
Figure 3.1.

Alignment of Representative *ToxA* Haplotypes




40
50
60
Haplotype 1 31 L F E A A N S S E L D A R G L S L D W T L K P R G L L Q E R
Haplotype 1.2 31
Haplotype 2 31
Haplotype 3 31
Haplotype 3.1 31
Haplotype 3.2 31
Haplotype 3.3 31
Haplotype 3.4 31
Haplotype 3.5 31 G
Haplotype 4 31
Haplotype 4.1 31
Haplotype 4.2 31
Haplotype 4.3 31 K * Q
Haplotype 4.4 31 G
Hapotype 5 31
Haplotype 5.1 31
Haplotype 5.2 31
Haplotype 5.3 31
Haplotype 6 31
Haplotype 6.1 31
Haplotype 6.2 31 G
Haplotype 6.3 31
70
80
90
Haplotype 1 61 Q G S C M S I T I N P S R P S V N N I G Q V D I D S V I L G
Haplotype 1.2 61
Haplotype 2 61
Haplotype 3 61
Haplotype 3.1 61
Haplotype 3.2 61
Haplotype 3.3 61
Haplotype 3.4 61 L * N E
Haplotype 3.5 61
Haplotype 4 61
Haplotype 4.1 61
Haplotype 4.2 61
Haplotype 4.3 61 S R
Haplotype 4.4 61
Hapotype 5 61
Haplotype 5.1 61
Haplotype 5.2 61
Haplotype 5.3 61
Haplotype 6 61
Haplotype 6.1 61
Haplotype 6.2 61
Haplotype 6.3 61

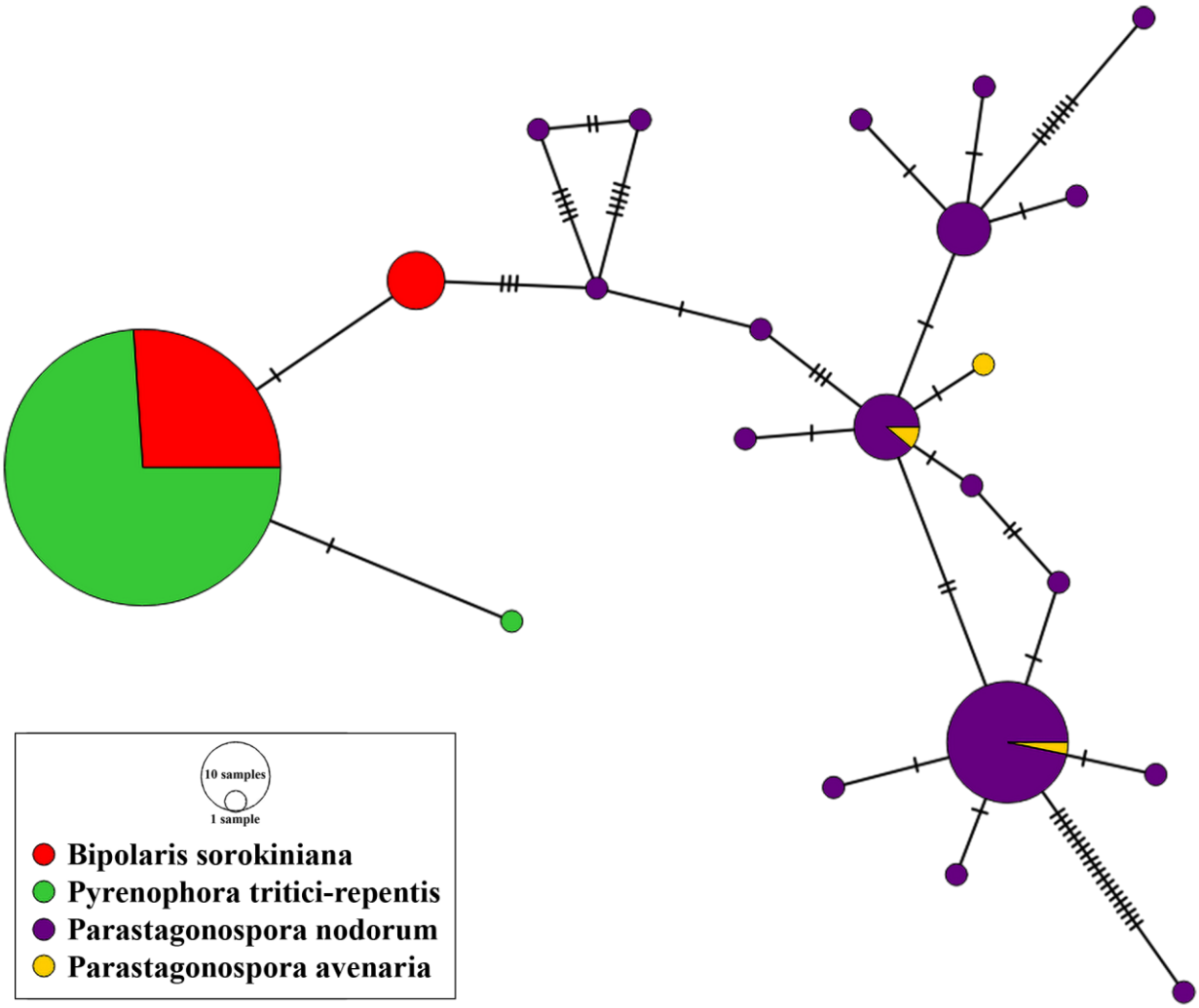

100
110
120
Haplotype 1 91 R P G A I G S W E L N N F I T I G L N R V N A D T V R V N I
Haplotype 1.2 91
Haplotype 2 91
Haplotype 3 91 D N
Haplotype 3.1 91 D N
Haplotype 3.2 91 D N
Haplotype 3.3 91 D N
Haplotype 3.4 91 S D N
Haplotype 3.5 91 D N
Haplotype 4 91 V N
Haplotype 4.1 91 L V N
Haplotype 4.2 91 V N
Haplotype 4.3 91 V N
Haplotype 4.4 91 V N
Hapotype 5 91 N
Haplotype 5.1 91 N
Haplotype 5.2 91 N
Haplotype 5.3 91 N
Haplotype 6 91 N
Haplotype 6.1 91 P V N V
Haplotype 6.2 91 P V N V
Haplotype 6.3 91 N
130
140
150
Haplotype 1 121 R N T G R T N R L I I T Q W E N T V T R G D V Y E L F G D Y
Haplotype 1.2 121 ?
Haplotype 2 121 L
Haplotype 3 121 N L
Haplotype 3.1 121 N L
Haplotype 3.2 121 N L I
Haplotype 3.3 121 N L
Haplotype 3.4 121 N * L
Haplotype 3.5 121 N L
Haplotype 4 121 N L
Haplotype 4.1 121 N L
Haplotype 4.2 121 N L
Haplotype 4.3 121 N * L Q
Haplotype 4.4 121 N L
Hapotype 5 121 N L
Haplotype 5.1 121 N L
Haplotype 5.2 121 N L
Haplotype 5.3 121 N L
Haplotype 6 121 V L
Haplotype 6.1 121 V
Haplotype 6.2 121 V L
Haplotype 6.3 121 V L



Note. This figure represents the 22 reported *ToxA* haplotypes. Amino acids that match the haplotype 1 are represented by a period in the rows below. Unknown amino acid residues, due to missing data in the only known example of haplotype 1.2, are represented by a question mark. Nonsense codons are represented on the figure as an asterisk.

Figure 3.2.

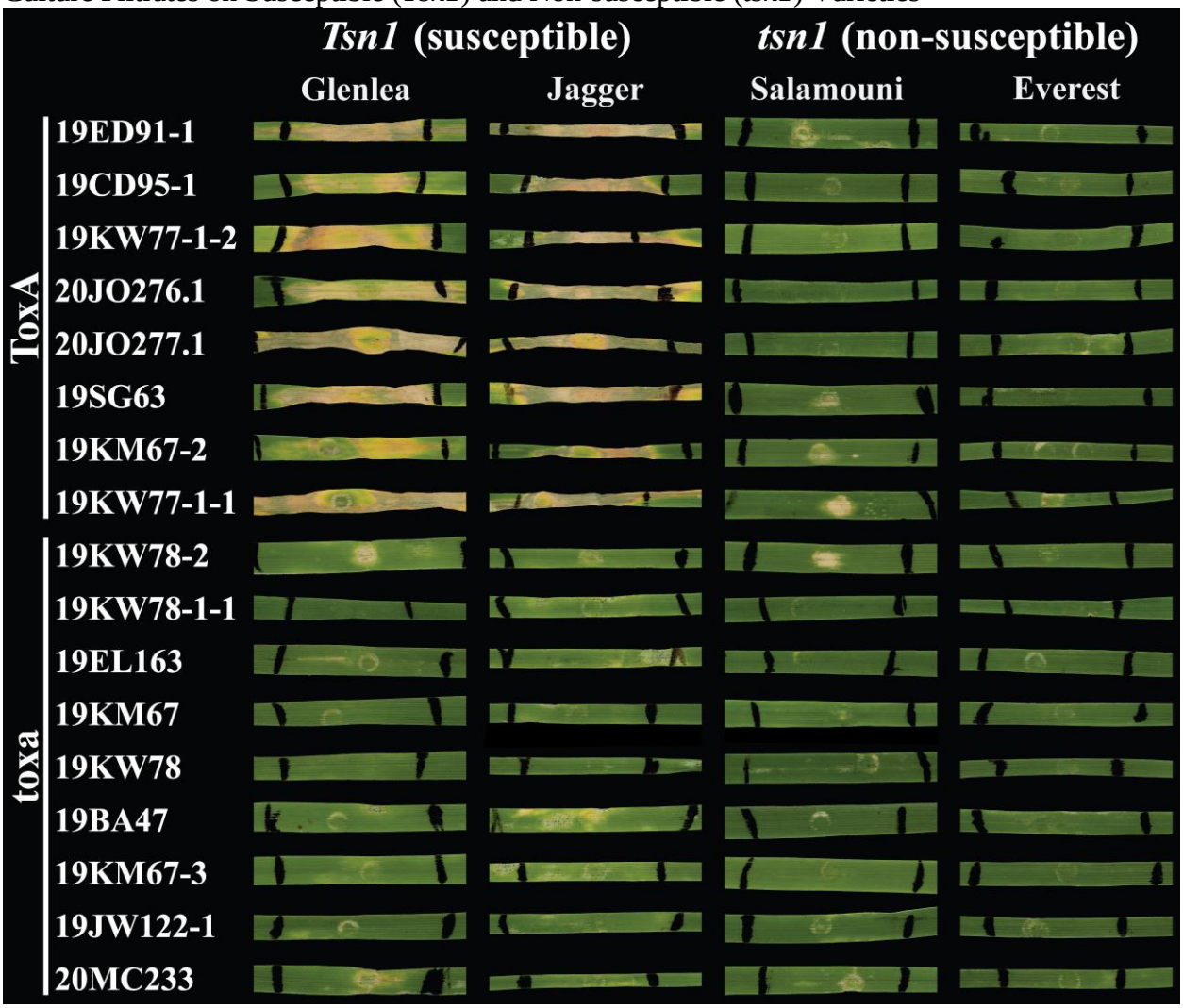
Minimum Spanning Network of Public and Novel Necrotrophic Effector ToxA Sequences



Note. This figure represents the genetic diversity of the *ToxA* open reading frame (ORF) gene sequence from both publicly available data previously reported on NCBI and novel isolates collected as part of this study. The size of the circle represents the quantity of isolates which share that haplotype. The hash marks on lines connecting the circles indicate the number of single nucleotide polymorphs (SNPs) that differentiate the haplotypes.

Figure 3.3.

Images Showing Wheat Seedling Necrosis Reactions After Infiltration with *B. sorokiniana* Culture Filtrates on Susceptible (*Tsn1*) and Non-susceptible (*tsn1*) Varieties



Note. Infiltration of *B. sorokiniana* isolates differing for their *ToxA* status on susceptible and non-susceptible wheat varieties. The susceptible wheat varieties used were 'Glenlea' and 'Jagger'. The non-susceptible varieties used were 'Salamouni' and 'Everest'.

Table 3.1.

Survey Isolates Containing the Gene *ToxA*

Isolate	Species	Host	Location	Collection Date[a]	*ToxA* Haplotype
19ED91-1	*B. sorokiniana*	*Triticum aestivum*	Edwards County, Kansas, USA	5/28/2019	2
19CD95-1	*B. sorokiniana*	*Triticum aestivum*	Cloud County, Kansas, USA	6/4/2019	1
19KW77-1-2	*B. sorokiniana*	*Triticum aestivum*	Kiowa County, Kansas, USA	5/28/2019	2
20JO276.1	*B. sorokiniana*	*Thinopyrum intermedium*	Johnson County, Kansas, USA	6/10/2020	2
20JO277.1	*B. sorokiniana*	*Thinopyrum intermedium*	Johnson County, Kansas, USA	6/10/2020	2
19SG63	*B. sorokiniana*	*Triticum aestivum*	Sedgwick County, Kansas, USA	5/28/2019	1
19KM67-2	*B. sorokiniana*	*Triticum aestivum*	Kingman County, Kansas, USA	5/28/2019	2
19KW77-1-1	*B. sorokiniana*	*Triticum aestivum*	Kiowa County, Kansas, USA	5/28/2019	2
19KM92-1	*P. nodorum*	*Triticum aestivum*	Kingman County, Kansas, USA	5/28/2019	5
19KM92	*P. nodorum*	*Triticum aestivum*	Kingman County, Kansas, USA	5/28/2019	3
19RL170LS-2	*P. nodorum*	*Triticum aestivum*	Riley County, Kansas, USA	6/14/2019	5
19CY143-3	*P. nodorum*	*Triticum aestivum*	Clay County, Kansas, USA	6/4/2019	3
19DC130-2	*P. nodorum*	*Triticum aestivum*	Decatur County, Kansas, USA	6/3/2019	3
19BA72-3	*P. nodorum*	*Triticum aestivum*	Barber County, Kansas, USA	5/28/2019	3
19HV148-2	*P. nodorum*	*Triticum aestivum*	Harvey County, Kansas, USA	5/22/2019	3
19RL154LS-1	*P. nodorum*	*Triticum aestivum*	Riley County, Kansas, USA	6/6/2019	3
19ST88-1-2	*P. nodorum*	*Triticum aestivum*	Stanton County, Kansas, USA	5/28/2019	3
19MT64-2-3	*P. nodorum*	*Triticum aestivum*	Morton County, Kansas, USA	5/28/2019	3
19DC129-1-1	*P. nodorum*	*Triticum aestivum*	Decatur County, Kansas, USA	6/3/2019	5
19BT49-2	*P. nodorum*	*Triticum aestivum*	Barton County, Kansas, USA	5/29/2019	5
20JO237.3.1	*P. nodorum*	*Thinopyrum intermedium*	Johnson County, Kansas, USA	6/10/2020	5
19NS158LS-3	*P. nodorum*	*Triticum aestivum*	Ness County, Kansas, USA	5/28/2019	3
KO-08	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
AZ-00	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1

MCR-1	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
SE-09	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
WWB-36	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
AUB-i	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	< 2015	1
HV-99-2	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
LM-98	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
PT-85-6	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
HV-00	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
FH-26	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
FH-86	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
RN-89	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
HV-98	*P. tritici-repentis*	*Triticum aestivum*	-	< 2015	1
20OB213.1	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
19RO144-1	*P. tritici-repentis*	*Triticum aestivum*	Rooks County, Kansas, USA	5/23/2019	1
20LE252	*P. tritici-repentis*	*Triticum aestivum*	Lane County, Kansas, USA	6/5/2020	1
20LE255	*P. tritici-repentis*	*Triticum aestivum*	Lane County, Kansas, USA	6/5/2020	1
20NS244	*P. tritici-repentis*	*Triticum aestivum*	Ness County, Kansas, USA	6/5/2020	1
20OB213	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
19FO55	*P. tritici-repentis*	*Triticum aestivum*	Ford County, Kansas, USA	5/29/2019	1
19BT49	*P. tritici-repentis*	*Triticum aestivum*	Barton County, Kansas, USA	5/29/2019	1
19CD94	*P. tritici-repentis*	*Triticum aestivum*	Cloud County, Kansas, USA	6/4/2019	1
19CL147-6	*P. tritici-repentis*	*Triticum aestivum*	Cowley County, Kansas, USA	5/22/2019	1
19CL147-7	*P. tritici-repentis*	*Triticum aestivum*	Cowley County, Kansas, USA	5/22/2019	1
19CY93	*P. tritici-repentis*	*Triticum aestivum*	Clay County, Kansas, USA	6/4/2019	1
19CY93-9	*P. tritici-repentis*	*Triticum aestivum*	Clay County, Kansas, USA	6/4/2019	1
19DC107	*P. tritici-repentis*	*Triticum aestivum*	Decatur County, Kansas, USA	6/3/2019	1
19DC130-5	*P. tritici-repentis*	*Triticum aestivum*	Decatur County, Kansas, USA	6/3/2019	1
19GH135	*P. tritici-repentis*	*Triticum aestivum*	Graham County, Kansas, USA	6/4/2019	1
19KW78-1	*P. tritici-repentis*	*Triticum aestivum*	Kiowa County, Kansas, USA	5/28/2019	1

19NS155	*P. tritici-repentis*	*Triticum aestivum*	Ness County, Kansas, USA	6/4/2019	1
19NS156-1	*P. tritici-repentis*	*Triticum aestivum*	Ness County, Kansas, USA	6/4/2019	1
19OB98	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/4/2019	1
19OB98-2	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/4/2019	1
19PL125-1	*P. tritici-repentis*	*Triticum aestivum*	Phillips County, Kansas, USA	6/3/2019	1
19PN68-1	*P. tritici-repentis*	*Triticum aestivum*	Pawnee County, Kansas, USA	5/28/2019	1
19PN68-3	*P. tritici-repentis*	*Triticum aestivum*	Pawnee County, Kansas, USA	5/28/2019	1
19RH167-1	*P. tritici-repentis*	*Triticum aestivum*	Rush County, Kansas, USA	6/14/2019	1
19RL170-11	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	6/14/2019	1
19RL170LS	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	6/14/2019	1
19RL170LS-3	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	6/14/2019	1
19RL171-13	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	6/14/2019	1
19RL172	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	6/14/2019	1
19RL37	*P. tritici-repentis*	*Triticum aestivum*	Riley County, Kansas, USA	5/13/2019	1
19RO150	*P. tritici-repentis*	*Triticum aestivum*	Rooks County, Kansas, USA	5/23/2019	1
19SG149	*P. tritici-repentis*	*Triticum aestivum*	Sedgwick County, Kansas, USA	5/22/2019	1
19SG149-1	*P. tritici-repentis*	*Triticum aestivum*	Sedgwick County, Kansas, USA	5/22/2019	1
19SM126-1-1	*P. tritici-repentis*	*Triticum aestivum*	Smith County, Kansas, USA	6/3/2019	1
19SM126-1-2	*P. tritici-repentis*	*Triticum aestivum*	Smith County, Kansas, USA	6/3/2019	1
19TH133-2-2	*P. tritici-repentis*	*Triticum aestivum*	Thomas County, Kansas, USA	6/3/2019	1
20CY269.21	*P. tritici-repentis*	*Triticum aestivum*	Clay County, Kansas, USA	6/8/2020	1
20CY269.3	*P. tritici-repentis*	*Triticum aestivum*	Clay County, Kansas, USA	6/8/2020	1
20EL264.1.1	*P. tritici-repentis*	*Triticum aestivum*	Ellis County, Kansas, USA	6/4/2020	1
20EL264.1.2	*P. tritici-repentis*	*Triticum aestivum*	Ellis County, Kansas, USA	6/4/2020	1
20EL264.2	*P. tritici-repentis*	*Triticum aestivum*	Ellis County, Kansas, USA	6/4/2020	1
20HP266.1	*P. tritici-repentis*	*Triticum aestivum*	Harper County, Kansas, USA	6/2/2020	1
20HP266.2	*P. tritici-repentis*	*Triticum aestivum*	Harper County, Kansas, USA	6/2/2020	1
20MC222.2	*P. tritici-repentis*	*Triticum aestivum*	Mitchell County, Kansas, USA	6/3/2020	1
20MP195	*P. tritici-repentis*	*Triticum aestivum*	McPherson County, Kansas, USA	4/30/2020	1

20OB213.2	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
20OB214	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
20OB216	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
20OB217	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
20OB218	*P. tritici-repentis*	*Triticum aestivum*	Osborne County, Kansas, USA	6/3/2020	1
20RH248	*P. tritici-repentis*	*Triticum aestivum*	Rush County, Kansas, USA	6/5/2020	1
20SM208	*P. tritici-repentis*	*Triticum aestivum*	Smith County, Kansas, USA	6/3/2020	1
21GE296	*P. tritici-repentis*	*Triticum aestivum*	Geary County, Kansas, USA	3/25/2021	1
21RN313	*P. tritici-repentis*	*Triticum aestivum*	Reno County, Kansas, USA	4/9/2021	1

Note. This table contains isolates of *P. tritici-repentis, B. sorokiniana*, and *P. nodorum* from a 2019-2021 Kansas field survey, and isolates of *P. tritici-repentis* isolates from an historic collection.

[a] All *P. tritici-repentis* isolates collected before 2015 are historic isolates.

Table 3.2.

List of all PCR Primers Used in this Study

Purpose	Primer Name	Primer Sequence (5' to 3')	Tm (°C)	Expected Amplicon Length (bp)	Source
Species verification	ITS1	TCCGTAGGTGAACCTGCGG	62	600	White et al., 1990
	ITS4	TCCTCCGCTTATTGATATGC			
ToxA amplification for identification and sequence characterization	ToxA1	CGTCCGGCTACCTAGCAATA	60	964	Friesen et al., 2006
	ToxA2	TTGTGCTCCTCCTTCTCGA			

Table 3.3.

Public Necrotrophic Effector *ToxA* Sequences

Isolate	Accession	Species	Location	Collection Date	*ToxA* Haplotype
Sn2000	CP022834.1	*P. nodorum*	North Dakota	-	3
LDN03-Sn4	CP022808.1	*P. nodorum*	Langdon, ND	2007	3
SN15	CP069026.1	*P. nodorum*	Western Austrailia	2001	4
BRIP10943	CM017963.1	*B. sorokiniana*	-	1966	1
WAI2674	KX816409.1	*B. sorokiniana*	-	-	2
SnTJ1-3	EF108463.1	*P. nodorum*	-	-	6
SnKZ30-5	EF108462.1	*P. nodorum*	-	-	6.3
SNNDKXE02-1	EF108454.1	*P. nodorum*	-	-	3
Sn01Aus.A1	EF108451.1	*P. nodorum*	-	-	5
SnCA1-3	EF108461.1	*P. nodorum*	-	-	3.1
SnKZ3-1-6	EF108460.1	*P. nodorum*	-	-	5.3
SnSA95.134	EF108457.1	*P. nodorum*	-	-	3.3
Sn95SA.103	EF108455.1	*P. nodorum*	-	-	3.5
Sn01AUS.A2	EF108452.1	*P. nodorum*	-	-	4
SN15	XM_001806615.1	*P. nodorum*	-	-	4
SnSA95.23	EF108459.1	*P. nodorum*	-	-	4.4
SnSa95.8	EF108458.1	*P. nodorum*	-	-	4.2
Sn01AUS.B2	EF108453.1	*P. nodorum*	-	-	4
SnSA95.113	EF108456.1	*P. nodorum*	-	-	4.3
BS140	MN601396.1	*B. sorokiniana*	India	-	1
BS139	MN601395.1	*B. sorokiniana*	India	-	1
BS138	MN601394.1	*B. sorokiniana*	India	-	1
BS137	MN601393.1	*B. sorokiniana*	India	-	1
BS136	MN601392.1	*B. sorokiniana*	India	-	1
BS135	MN601391.1	*B. sorokiniana*	India	-	1

BS134	MN601390.1	*B. sorokiniana*	India	-	1
BS133	MN601389.1	*B. sorokiniana*	India	-	1
BS132	MN601388.1	*B. sorokiniana*	India	-	1
BS131	MN601387.1	*B. sorokiniana*	India	-	1
BS130	MN601386.1	*B. sorokiniana*	India	-	1
BS129	MN601385.1	*B. sorokiniana*	India	-	1
BS128	MN601384.1	*B. sorokiniana*	India	-	1
BS127	MN601383.1	*B. sorokiniana*	India	-	1
BS126	MN601382.1	*B. sorokiniana*	India	-	1
BS125	MN601381.1	*B. sorokiniana*	India	-	1
BS124	MN601380.1	*B. sorokiniana*	India	-	1
BS112	MN601379.1	*B. sorokiniana*	India	-	1
BS94	MN601378.1	*B. sorokiniana*	India	-	1
BS91	MN601377.1	*B. sorokiniana*	India	-	1
BS88	MN601376.1	*B. sorokiniana*	India	-	1
BS75	MN601375.1	*B. sorokiniana*	India	-	1
BS72	MN601374.1	*B. sorokiniana*	India	-	1
BS69	MN601373.1	*B. sorokiniana*	India	-	1
BS68	MN601372.1	*B. sorokiniana*	India	-	1
BS66	MN601371.1	*B. sorokiniana*	India	-	1
BS65	MN601370.1	*B. sorokiniana*	India	-	1
BS54	MN601369.1	*B. sorokiniana*	India	-	1
BS50	MN601368.1	*B. sorokiniana*	India	-	1
BS32	MN601367.1	*B. sorokiniana*	India	-	1
BS30	MN601366.1	*B. sorokiniana*	India	-	1
BS29	MN601365.1	*B. sorokiniana*	India	-	1
BS27	MN601364.1	*B. sorokiniana*	India	-	1
BS14	MN601363.1	*B. sorokiniana*	India	-	1
BS11	MN601362.1	*B. sorokiniana*	India	-	1

BS6	MN601361.1	*B. sorokiniana*	India	-	1
BS4	MN601360.1	*B. sorokiniana*	India	-	1
BS3	MN601359.1	*B. sorokiniana*	India	-	1
BS1	MN601358.1	*B. sorokiniana*	India	-	1
AP1156	JX997421.1	*P. avenaria f. sp. tritici*	-	-	3
AI829	JX997420.1	*P. avenaria f. sp. tritici*	-	-	5
AS1298	JX997419.1	*P. nodorum*	-	-	5
WAC13666	MH511822.1	*P. nodorum*	-	-	4
AD260	JX997418.1	*P. nodorum*	-	-	5.2
AI825	JX997416.1	*P. avenaria f. sp. tritici*	-	-	5.1
WAC2285	MH511825.1	*P. nodorum*	-	-	4.1
Fr15_02	MH511823.1	*P. nodorum*	-	-	6.1
Fr15_21	MH511824.1	*P. nodorum*	-	-	6.2
AF385	JX997417.1	*P. nodorum*	-	-	3.4
Pt-1C-BFP	NW_001939247.1	*P. tritici-repentis*	-	-	1
PTR-42	HM234160.1	*P. tritici-repentis*	-	-	1
PTR-43	HM234161.1	*P. tritici-repentis*	-	-	1
PTR-01	HM234155.1	*P. tritici-repentis*	-	-	1
AB42	MN062700.1	*P. tritici-repentis*	Canada	2010	1
AB40	MN062699.1	*P. tritici-repentis*	Canada	2010	1
AB38	MN062698.1	*P. tritici-repentis*	Canada	2010	1
AB35	MN062697.1	*P. tritici-repentis*	Canada	2010	1
AB32	MN062696.1	*P. tritici-repentis*	Canada	2010	1
AB29	MN062695.1	*P. tritici-repentis*	Canada	2010	1
AB28	MN062694.1	*P. tritici-repentis*	Canada	2010	1
AB25	MN062693.1	*P. tritici-repentis*	Canada	2010	1
AB22	MN062692.1	*P. tritici-repentis*	Canada	2010	1
AB20	MN062691.1	*P. tritici-repentis*	Canada	2010	1
AB15	MN062690.1	*P. tritici-repentis*	Canada	2010	1

AB11	MN062689.1	*P. tritici-repentis*	Canada	2010	1
AB09	MN062688.1	*P. tritici-repentis*	Canada	2010	1
AB05	MN062687.1	*P. tritici-repentis*	Canada	2010	1
AB02	MN062686.1	*P. tritici-repentis*	Canada	2010	1
AB01	MN062685.1	*P. tritici-repentis*	Canada	2010	1
S415-4	MT052956.1	*P. nodorum*	Scott, Saskatchewan, Canada	2018	3
S415-3	MT052955.1	*P. nodorum*	Scott, Saskatchewan, Canada	2018	3
S203-5	MT052954.1	*P. nodorum*	Scott, Saskatchewan, Canada	2018	3
S203-4	MT052953.1	*P. nodorum*	Scott, Saskatchewan, Canada	2018	3
G313-2	MT052952.1	*P. nodorum*	Lethbridge, Alberta, Canada	2018	3
G215-6	MT052951.1	*P. nodorum*	Lethbridge, Alberta, Canada	2018	3
G213-5	MT052950.1	*P. nodorum*	Lethbridge, Alberta, Canada	2018	3
G211-5	MT052949.1	*P. nodorum*	Lethbridge, Alberta, Canada	2018	3.2
G206-9	MT052948.1	*P. nodorum*	Lethbridge, Alberta, Canada	2018	3
G104-7	MT052947.1	*P. nodorum*	Lethbridge, Alberta, Canada	2018	3
C419-1	MT052946.1	*P. nodorum*	Lacombe, Alberta, Canada	2018	3
C314-8	MT052945.1	*P. nodorum*	Lacombe, Alberta, Canada	2018	3
C215-7	MT052944.1	*P. nodorum*	Lacombe, Alberta, Canada	2018	3
B418-11	MT052943.1	*P. nodorum*	Beaverlodge, Alberta, Canada	2018	3
B313-13	MT052942.1	*P. nodorum*	Beaverlodge, Alberta, Canada	2018	3
B304-5	MT052941.1	*P. nodorum*	Beaverlodge, Alberta, Canada	2018	3
B201-3	MT052940.1	*P. nodorum*	Beaverlodge, Alberta, Canada	2018	5
B106-16	MT052939.1	*P. nodorum*	Beaverlodge, Alberta, Canada	2018	3
T103-1	MN052896.1	*P. tritici-repentis*	Tunisia	2018	1
T168-8	MN052895.1	*P. tritici-repentis*	Tunisia	2018	1
T173-5	MN052894.1	*P. tritici-repentis*	Tunisia	2018	1
T75-1	MN052893.1	*P. tritici-repentis*	Tunisia	2018	1
T103-2	MN052892.1	*P. tritici-repentis*	Tunisia	2018	1
T176-1	MN052891.1	*P. tritici-repentis*	Tunisia	2018	1

T132-2	MN052890.1	*P. tritici-repentis*	Tunisia	2018	1
T168-3	MN052889.1	*P. tritici-repentis*	Tunisia	2018	1
T44-4	MN052888.1	*P. tritici-repentis*	Tunisia	2018	1
T44-1	MN052887.1	*P. tritici-repentis*	Tunisia	2018	1
T173-9	MN052886.1	*P. tritici-repentis*	Tunisia	2018	1
T17-2	MN052885.1	*P. tritici-repentis*	Tunisia	2018	1
T176-2	MN052884.1	*P. tritici-repentis*	Tunisia	2018	1
T178-1	MN052883.1	*P. tritici-repentis*	Tunisia	2018	1
T178-2	MN052882.1	*P. tritici-repentis*	Tunisia	2018	1
T25-7	MN052881.1	*P. tritici-repentis*	Tunisia	2018	1
T168-2	MN052880.1	*P. tritici-repentis*	Tunisia	2018	1
T168-7	MN052879.1	*P. tritici-repentis*	Tunisia	2018	1
T168-1	MN052878.1	*P. tritici-repentis*	Tunisia	2018	1
T25-4	MN052877.1	*P. tritici-repentis*	Tunisia	2018	1
T168-4	MN052876.1	*P. tritici-repentis*	Tunisia	2018	1
T176-3	MN052875.1	*P. tritici-repentis*	Tunisia	2018	1
86-124	U79662.1	*P. tritici-repentis*	-	-	1.2
-	AF004369.1	*P. tritici-repentis*	-	-	1
Sn4	HM191251.1	*P. nodorum*	-	-	3
Sn5	HM191250.1	*P. nodorum*	-	-	3
unk	DQ423483.1	*P. nodorum*	-	-	4
NZ1	MH017419.1	*P. tritici-repentis*	New Zealand	2017	1
SN002B	MH017416.1	*P. tritici-repentis*	Germany	2017	1
EW13061_2_1	MH017415.1	*P. tritici-repentis*	Denmark	2017	1
SN001C	MH017418.1	*P. tritici-repentis*	Germany	2017	1
EW4_4	MH017417.1	*P. tritici-repentis*	Denmark	2017	1
CC142	MH017414.1	*P. tritici-repentis*	United Kingdom	2017	1

Note. This table contains all publicly available sequence of the *ToxA* ORF from the species: *P. tritici-repentis*, *P. nodorum*, *P. avenaria* f. sp. *tritici*, and *B. sorokiniana*. Sequences were downloaded from the National Center for Biotechnology Information (NCBI).

Table 3.4.

Wheat Seedling Necrosis Reactions after Infiltration with *B. sorokiniana* Culture Filtrates on Susceptible (*Tsn1*) and Non-susceptible (*tsn1*) Varieties.

		Susceptible (*Tsn1*)		Non-susceptible (*tsn1*)	
		Glenlea	Jagger	Salamouni	Everest
ToxA+	19ED91-1	Sensitive	Sensitive	Insensitive	Insensitive
	19CD95-1	Sensitive	Sensitive	Insensitive	Insensitive
	19KW77-1-2	Sensitive	Sensitive	Insensitive	Insensitive
	20JO276.1	Sensitive	Sensitive	Insensitive	Insensitive
	20JO277.1	Sensitive	Sensitive	Insensitive	Insensitive
	19SG63	Sensitive	Sensitive	Insensitive	Insensitive
	19KM67-2	Sensitive	Sensitive	Insensitive	Insensitive
	19KW77-1-1	Sensitive	Sensitive	Insensitive	Insensitive
ToxA-	19KW78-2	Insensitive	Insensitive	Insensitive	Insensitive
	19KW78-1-1	Insensitive	Insensitive	Insensitive	Insensitive
	19EL163	Insensitive	Insensitive	Insensitive	Insensitive
	19KM67	Insensitive	Insensitive	Insensitive	Insensitive
	19KW78	Insensitive	Insensitive	Insensitive	Insensitive
	19BA47	Insensitive	Insensitive	Insensitive	Insensitive
	19KM67-3	Insensitive	Insensitive	Insensitive	Insensitive
	19JW122-1	Insensitive	Insensitive	Insensitive	Insensitive
	20MC233	Insensitive	Insensitive	Insensitive	Insensitive

Note. Infiltration of *B. sorokiniana* isolates differing for their *ToxA* status on susceptible and non-susceptible wheat varieties. Isolates from which *ToxA* was amplified are shown below as *ToxA+*, and isolates without *ToxA* are shown as *ToxA-* isolates. Plants were rated as sensitive (necrosis response) or insensitive (no necrosis response) for response 72 hours after infiltration.

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Chapter 4 - Wheat bacterial leaf blight susceptibility is tightly linked to *Sr2*

Abstract

Bacterial leaf blight (BLB) of wheat has been attributed to the pathogen *Pseudomonas syringae* pv. *syringae* (Pss) van Hall. Disease development occurs under conditions of high humidity and cool temperatures during the boot stage of wheat development. The symptoms of BLB are large gray-green lesions or blotches on the upper leaves that quickly become necrotic and bleached to gray or white. However, the symptoms are sporadic under field conditions, which has led to equally sporadic research on the disease. Historic ratings on the wheat BLB phenotype have had a variety specific reaction, suggesting a strong genetic component. Recently, a correlation was identified between the BLB phenotype and the *Sr2* phenotypes pseudo-black chaff and stem melanism. This suggested that the resistance gene *Sr2* may play a role in BLB pathogenesis. A backcross recombinant inbred line population (RIL), developed from the *Sr2*-containing parent 'Kingbird' and the non-*Sr2* parent 'KS05HW14', was genotyped and rated for the BLB phenotype over two field seasons. In addition, the population was also rated for the known *Sr2* phenotype, pseudo-black chaff (PBC) over two field seasons. The population's phenotype segregated for severity of BLB and PBC symptoms in the F5 generation. QTL analysis of the population identified three QTLs related to the BLB phenotype. QTLs associated with increased BLB severity for the allele from 'Kingbird' were tightly linked to markers for *Sr2* (3BS) and *Lr34* (7DS). A QTL was identified on chromosome 5DS which was associated with increased BLB severity for the allele from 'KS05HW14'. The PBC phenotype was also tightly linked to the *Sr2* marker and identified secondary QTLs in the same locations on chromosomes 5DS and 7DS. This evidence confirms the correlation of *Sr2* and the BLB phenotype and shows

that the phenotype is tightly linked with the *Sr2* marker on chromosome 3BS. Ongoing research intends to determine if Pss is required for symptom development, or if this is strictly an additional negative phenotype associated with *Sr2*.

Introduction

Bacterial leaf blight (BLB) of wheat (*Triticum aestivum* L.) is characterized by very small water-soaked spots on the upper leaves that show up during the boot or heading stage of development. These spots develop into large gray-green lesions or blotches that quickly become necrotic and collapse before bleaching to gray or tan (Otta, 1974).

This disease, which dates to at least 1965 in South Dakota, was attributed to *Pseudomonas syringae* pv. s*yringae* van Hall, a gram-negative, mono-flagellate, rod-shaped bacterium (Otta, 1972). The Northern Great Plains experienced reports of the phenotype from 1968 to at least 1975 on winter wheat and for the first time on spring wheat in 1974 (Otta, 1974; Scharen et al., 1976; Sellam & Wilcoxson, 1976). This phenotype appears to be more prevalent in years with higher rates of wind-driven rain during the boot to heading stage of development (Otta, 1974).

While epidemic years were common, the phenotype also persisted in non-epidemic years, and its appearance was sporadic due to a narrow range of environmental conditions required for disease development (Otta, 1974). As a complicating factor, Hosford presented evidence that *Bacillus megaterium* pv. *cerealis* de Bary was causing a highly similar phenotype to BLB throughout the 1960's and 1970's in North Dakota (Hosford, 1982). Differences noted between the two phenotypes were limited to the blotches often being broader than Otta's descriptions, and that there was no water-soaking period caused by *B. megaterium* pv. *cerealis* (Hosford, 1982). Hosford specifically ruled out Pss in his evaluations but acknowledged the phenotype similarities

(Hosford, 1982). Both Otta and Hosford rated varieties and indicated that many of the same varieties were susceptible (Hosford, 1982; Otta, 1974).

In the years following the phenotype's initial report, researchers have had difficulty replicating disease symptoms when inoculating with Pss, often concluding that Pss is a weak pathogen (Shane & Baumer, 1987; J. Smith & Hattingh, 1991). In addition to this difficulty, it is also widely accepted that Pss is a common wheat epiphyte that can be easily isolated from most wheat plants, indicating that environmental conditions are more important than the presence of Pss for disease development (Kazempour et al., 2010). Reports indicate that symptom development, in addition to environmental specificity, is highly dependent on variety (Hosford, 1982; Otta, 1974; Shane & Baumer, 1987). The variety-specific nature of this phenotype suggests that there is a strong genetic component in this interaction.

Recently, a correlation between the BLB phenotype and the wheat phenotypes; pseudo-black chaff (PBC), and stem melanism (SM), was observed (Robert Bowden, personal communication). PBC and SM are physiological necrosis responses associated with the stem rust resistance gene, *Sr2* (E. McFadden, 1939; Tabe et al., 2019). These responses are characterized by the easily observable brown and purple "melanization" or "pigmentation" of the chaff (PBC) or stem (SM) (E. McFadden, 1939).

S*r2* was moved into wheat as part of an introgression from tetraploid 'Yaroslav' emmer (*Triticum turgidum* ssp. *dicoccum*) that was crossed into hexaploid 'Marquis' wheat (*Triticum aestivum*) (E. S. McFadden, 1930) The linkage between these phenotypes and stem rust resistance (*Sr2*) was quickly noticed and they were used as phenotypic markers for the stem rust resistance gene, *Sr2* (E. McFadden, 1939). The gene *PBC1* has been named for the PBC phenotype susceptibility locus and the linkage between PBC and *Sr2*, has been extensively tested

and with almost complete consensus (Mishra et al., 2005) is unable to be broken (Kota et al., 2006; Mago, Tabe, et al., 2011).

The correlation between the BLB phenotype and the *Sr2*-linked phenotypes led to the hypothesis that genetic susceptibility may be a pleiotropic effect of the resistance gene *Sr2*. To address this research gap, a Bc_1F_5 generation backcross recombinant inbred line (RIL) population, segregating for the *Sr2* gene, was phenotyped for the bacterial leaf blight phenotype, and the known *Sr2* phenotype, pseudo-black chaff, over two field seasons to conduct QTL analysis. Identifying the genetic components associated with this disease/phenotype will allow breeders to utilize the identified markers when making breeding decisions.

Methods

Population selection

A backcross recombinant inbred line (RIL) population (KS05HW14*2/Kingbird), developed from the *Sr2*-containing parent 'Kingbird' and the non-*Sr2* parent 'KS05HW14', was selected for use in this study. The population consisted of 376 offspring. The population was advanced to the BC_1F_5 generation by single-seed descent and thereafter increased as bulks for phenotyping.

Genotyping

The KS05HW14*2/Kingbird population was genotyped using the Illumina iSelect 90K SNP array (Liu et al., 2016; Wang et al., 2014). In addition to the array, gene-specific markers were also included for the adult plant resistance genes *Sr2* (*csSr2-CAP*), *Lr34* (*Lr34Exon-11*), *Lr68* (*Lr68-csGS*), *Lr46* (*csLV46G22*), and *Lr78* (*IWA6289*) (Gambone, 2016). The *Sr2*, *Lr68*, and *Lr46* markers were cleaved amplified polymorphic sequence (CAPS) markers (Gambone, 2016; Herrera-Foessel et al., 2012; Mago et al., 2011, Laguda unpublished). The *Lr78* and *Lr34*

markers were kompetitive allele specific PCR (KASP) markers (Lagudah et al., 2009; Rasheed et al., 2016).

Phenotyping

The KS05HW14*2/Kingbird RIL population was phenotyped under dryland field nursery conditions in 2014 and 2016 for the bacterial leaf blight phenotype, and for the PBC phenotype in 2014 and 2015. Severity ratings for the BLB and PBC phenotypes were assigned using a 0 to 3 scale (0 = no symptoms, 1 = weak or ambiguous symptoms, 2 = clear minor symptoms, 3 = clear significant symptoms). The population segregated for both phenotypes during all field seasons observed.

Boxplots of the observed phenotypes were plotted using the R package R/ggplot2, (Wickham, 2016). Correlation rates between the four phenotypes were calculated using the R package R/corrplot (Wei & Simko, 2021). Phenotypic symptoms were evaluated for heritability using a genotype-means basis using the R/lme4 package (Bates et al., 2015) using the genotype covariance (σ_g^2), the residual covariance (σ_e^2), and the number of replicates (r):

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{\mathrm{r}}}$$

Linkage map generation

The linkage map for the mapping population was previously generated using JoinMap 4.1 (Gambone, 2016; Van Ooijen, 2006, 2011) (Kyazma B.V., Wageningen, Netherlands). The final linkage map for the population contained 3,339 SNP markers, including the gene-specific markers for *Lr34* and *Sr2* (Gambone, 2016). Missing data were imputed using Beagle 4.1 (https://faculty.washington.edu/browning/beagle/beagle.html) (Gambone, 2016).

QTL analysis

Quantitative trait locus (QTL) mapping analysis was conducted using the R-package, R/qtl (Arends et al., 2010; Broman et al., 2003; R Core Team, 2021). QTL analysis was conducted individually on the four phenotype datasets: 2014 PBC, 2014 BLB, 2015 PBC, and 2016 BLB.

Simple interval mapping (SIM) using Haley-Knot (HK) regression was conducted to identify preliminary QTLs for use in model development. These initial results were then used with the composite interval mapping (CIM) function to identify weaker QTLs by including the strongest previously identified QTL as a covariate. HK regression was also used for the CIM procedures. CIM permutation was used to identify 90% and 95% logarithm of odds (LOD) thresholds.

QTLs identified using the preliminary SIM and CIM models were included in a forward model selection using multiple QTL mapping (MQM) functions. The data were evaluated for additional QTLs and all possible QTL by QTL interactions. After the final model was selected, the Bayesian 95% marker confidence intervals were calculated to estimate marker locations for each model using R/qtl. LOD thresholds were calculated using 1000 permutations and a Type 1 error of 0.05 for the four individual datasets.

Single QTL effects plots and interaction effects plots were fit to calculate QTL effects. Additive QTL effects were calculated using comparison of allele means. QTL interaction effects were estimated by comparing the change in phenotype between alleles at two levels of the interacting allele.

Results

Genotyping

The 376 offspring and the two parent lines were genotyped which generated 3,337 polymorphic SNP markers (Figure 4.1). A plot of pairwise recombination frequency showed the greatest rates of recombination were on linked chromosomal regions (Figure 4.2). These markers were supplemented with two additional gene-specific markers for a total of 3,339 markers. The gene-specific markers for *Sr2*, *Lr34,* and *Lr78* were found to be present in the 'Kingbird' and not present in the 'KS05HW14' parent (Table 4.1) (Gambone, 2016).

Phenotypic response

The wheat RIL population segregated for both the PBC and BLB phenotypes in each of the two years in which phenotypes were recorded (Figure 4.3). A comparison of the correlation between the four phenotypes indicates that the phenotypes are highly correlated (Figure 4.4). The broad sense heritability (H^2) of the traits, PBC and BLB, were 0.831 and 0.914 respectively (Table 4.2).

QTL mapping results

QTL mapping of the two traits repeatedly revealed three high confidence QTLs (Table 4.3). The primary QTL was located on chromosome 3B short (3BS), which showed up in all four datasets. Secondary QTLs included a chromosome 5DS QTL and 7DS QTL that showed up intermittently throughout the datasets.

Both of the PBC datasets identified the 3BS QTL. The 2014 PBC dataset placed the 3BS QTL at 28.105 cM with a Bayesian 95% confidence interval (CI) from 28 to 28.105 cM (Table 4.3 & Figure 4.5). The 2015 PBC dataset identified the 3BS QTL at 24.5 cM with a Bayesian 95% CI from 23 to 25 cM (Figure 4.6). The phenotypic variance explained by this QTL over the

2014 and 2015 field seasons was 62.3% and 60.6% respectively. In addition to the 3BS QTL, the 2014 PBC dataset included a QTL on chromosome 7DS at 37 cM with a Bayesian 95% confidence interval from 35 to 136.8 cM. This QTL explained only 3.6% of phenotypic variance, however it was also involved in an interaction with the 3BS QTL that explained an additional 3.7% of phenotypic variance (Table 4.4). The 2015 PBC dataset included a QTL on chromosome 5DS at 48.4 cM with a Bayesian 95% CI from 41 to 56 cM. This QTL explained 5.1% of phenotypic variance, and there was also an interaction with the 3BS QTL that explained an additional 3.3% of phenotypic variance.

The BLB datasets from 2014 and 2016 both identified QTLs on chromosome 3BS and 7DS. In the 2014 dataset, the 3BS QTL mapped to 20.3 cM with a Bayesian 95% CI from 19.9 to 27 cM (Figure 4.7). The 7DS QTL mapped to 59.1 cM with a Bayesian 95% CI from 56 to 61 cM. In the 2016 dataset, the 3BS QTL mapped to 31 cM with a Bayesian 95% CI from 21 to 31 cM (Figure 4.8). The 7DS QTL mapped to 56 cM with a Bayesian 95% CI from 52 to 59 cM. The 3BS QTL explained the majority of phenotypic variance, accounting for 41.3% in the 2014 dataset and for 61.7% in the 2016 dataset. The 7DS QTL explained 16.5% of phenotypic variance in 2014 and 5.8% in 2016 (Table 4.3). There was a significant interaction between these two QTLs in both field seasons (Table 4.4). In the 2014 dataset, this interaction explained 10.95% of the phenotypic variation and the interaction effect was estimated to be a greater than additive 0.465. In the 2016 dataset, this interaction explained 2.58% of the phenotypic variation and had an estimated interaction effect of 0.326.

In addition to the 3BS, and 7DS QTLs, the 2016 dataset identified an additional QTL on chromosome 5DS at 51 cM with a Bayesian 95% CI from 49 to 52 cM. This QTL accounted for

11.99% of the phenotypic variance. This QTL also interacted with the primary 3BS QTL, accounting for 8.64% of the phenotypic variance and having a less than additive effect of -0.308.

Discussion

The bacterial leaf blight phenotype has had a sporadic history, showing up both periodically and regionally. Its distribution is intrinsically linked with specific wheat varieties and environments. This distribution indicates that there is a strong genetic component to the disease. The observed correlation between the PBC and BLB phenotypes across multiple field seasons suggests that they may have a shared genetic source. The PBC phenotype is known to be associated with the stem rust resistance gene, *Sr2*. Because of the observed correlation between the BLB and PBC phenotypes, we hypothesized that *Sr2* was related to the BLB phenotype. To test this hypothesis, we conducted a QTL analysis on two years of field data each for the PBC and BLB phenotypes.

Analysis of the field data had a broad sense heritability of 0.914 for BLB and 0.831 for PBC (Table 4.2). This high level of heritability indicates that a large component of the variation in these phenotypes was under genetic control. The QTL models generated accounted for an average of 63.29% and 61.87% of the variation in the PBC and BLB phenotypes respectively. These levels verify that these traits are under a high level of genetic control (Table 4.3).

The results of this study identified three QTLs related to both the PBC and the BLB phenotypes. The strongest QTLs were found near the same location on 3BS in all four datasets. These QTLs were all tightly linked to the gene-specific *Sr2* marker, *csSr2-Cap*, mapped to chromosome 3BS at 28.105 cM. This QTL result, in conjunction with the known link between PBC and *Sr2*, provides strong evidence that the QTL is mapping to the gene, or is tightly linked to, *Sr2*.

The genetic link between PBC and *Sr2* has been previously established so this result was expected for the PBC phenotype. The previously observed correlation between the PBC and BLB traits also indicated that *Sr2* may be playing a role in the BLB phenotype. The 3BS QTL was the primary QTL in these models and it accounted for an average 61.5% of the variation in PBC phenotype, and 51.5% of the variation in the BLB phenotype (Table 4.3).

In addition to the 3BS QTL, both BLB datasets and the 2014 PBC dataset mapped a QTL on chromosome 7DS. The 7DS QTLs, mapped to a region of the chromosome near the leaf rust resistance gene, *Lr34*. In this RIL population linkage map, the gene-specific *Lr34* marker, *Lr34Exon-11*, mapped to chromosome 7DS at 59.09 cM. *Lr34* has previously been reported along with *Sr2* to control the PBC trait (Kaur et al., 2009). This provides evidence that the QTL identified on 7DS is *Lr34*.

Lr34 has previously been shown to interact with other genes to enhance resistance to leaf rust (German & Kolmer, 1992; Silva et al., 2015). In one study it was also observed that the *Sr2* locus is also involved in the leaf tip necrosis phenotype which is associated with *Lr34* indicating an association in the genetic basis of the responses (Juliana et al., 2015). It is possible that *Lr34* is working as a *Sr2* modifier to increase sensitivity to biotic or abiotic stress, which ultimately leads to the BLB phenotype.

The 5DS QTL was identified in the 2015 PBC dataset and the 2016 BLB dataset. The QTL mapped between 41 and 56 cM for both traits. A previous study identified a PBC QTL located on chromosome 5DL and concluded its location corresponded to the seedling stem rust resistance gene *Sr30* (Kaur et al., 2009). However, *Sr30* was not considered a candidate because it is not on the same arm of 5D as the QTL identified in this study (Knott & McIntosh, 1978). In

addition, a previous analysis for stem rust QTLs on this population failed to identify a QTL on 5DS (Gambone, 2016).

Several rust resistance genes are located on chromosome 5DS. These include *Lr57*/*Yr40*, *Lr76*/*Yr70*, *Lr70*, *Lr78* (Bansal et al., 2017; Hiebert et al., 2014; Kolmer et al., 2018; Kuraparthy et al., 2007). The *Lr57/Yr40* resistance locus comes from an introgression from *Aegilops geniculata* (Kuraparthy et al., 2007). The *Lr76/Yr70* resistance locus comes from an *Aegilops umbellulata* introgression (Bansal et al., 2017). Both of these genes can be ruled out as they were introgressed after the development of lines used in this population.

The leaf rust resistance gene, *Lr70* is located on the terminal region of 5DS (Hiebert et al., 2014). This resistance source provides seedling resistance to leaf rust and its response is based on a hypersensitive reaction (Hiebert et al., 2014). *Lr70* is located 5.6 cM distal the marker '*barc130*' and 7.4 cM distal the marker '*wmc233'*, placing its physical distance at approximately 6 Mb on 5DS (Hiebert et al., 2014; International Wheat Genome Sequencing Consortium (IWGSC), 2018). By referencing our linkage map's most significant markers with the reference genome (IWGSC RefSeq V1.0), the physical position of our 5DS QTL is approximately 22 Mb. This gene is a potential candidate for our 5DS QTL.

The resistance gene *Lr78* is located on 5DS in a position proximal to *Lr70*, mapping 2.2 cM distal the marker 'IWA6289' (Kolmer et al., 2018). In the IWGSC reference genome version 1, the marker, 'IWA6289', maps to a physical position of 33 Mb (International Wheat Genome Sequencing Consortium (IWGSC), 2018; Kolmer et al., 2018). This position is also near the 5DS QTL, at approximately 22 Mb.

The parent 'Kingbird' has the marker, 'IWA6289', for *Lr78*, and the 'KS05HW14' parent does not. A comparison of phenotypes for the datasets which mapped the 5DS QTL

showed that lines which had the ‘KS05HW14’ allele had a 2015 PBC rating that was 19.34% higher than lines with the ‘Kingbird’ allele, and that lines which had the ‘KS05HW14’ allele had a 2016 BLB rating that was 140.8% higher than lines with the ‘Kingbird’ allele. These differences were also similar in the datasets which did not map the 5DS QTL. These differences match the effects size predicted for the 5DS QTL (Table 4.3). Based on these findings, from these genes, the most likely source of the 5DS QTL we identified is *Lr78* or near *Lr78.*

Sr2 has been known to provide broad spectrum adult plant resistance for stem rust since its introduction into breeding germplasm about 100 years ago (Ellis et al., 2014; Singh et al., 2011). In addition, the locus has also been shown to provide resistance for leaf rust (*P. triticina*), stripe rust (*P. striiformis* f.sp *tritici*), and powdery mildew (*Blumeria graminis* f. sp. *tritici*) (Mago, Tabe, et al., 2011; Singh & McIntosh, 1984a, 1984b; Suenaga et al., 2003; Yang et al., 2013).

Sr2 has yet to be cloned and its molecular function has been difficult to establish. However, Tabe et al., showed that its defense response to *P. graminis* f. sp. *tritici* and *B. graminis* f. sp. *tritici* were associated with the death and collapse of plant tissue surrounding the infection site (2019). Tabe et al., also showed that the physiological PBC and SM phenotypes were associated with necrosis at the site of the “melanized” or “pigmented” tissue. It is also well established that PBC and SM show up in response to abiotic stress, heat and humidity (Johnson & Hagborg, 1944; E. McFadden, 1939; Sheen et al., 1968). A final important revelation shown by Tabe et al., was that *Sr2* wheat varieties can undergo leaf tissue necrosis following exposure to biotic or abiotic stress alone (2019). These observations match our field observation that this phenotype develops during high humidity or wet conditions.

Several inoculation studies have attempted to replicate BLB symptoms with limited success. Fryda & Otta inoculated plants in both the growth chamber and field but did not observe BLB symptoms in either case (1978). Shane and Baumer attempted to investigate resistance of spring wheat varieties but had trouble finding inoculation methods which led to a successful infection of the host, ultimately determining that Pss was a weak pathogen as it did not increase to significant concentrations from the initial inoculation (1987). Smith and Hattingh confirmed the findings by Shane and Baumer that Pss is a weak pathogen on wheat based on its inability to cause disease unless the tissue was pretreated with a mist chamber and infiltrated with a high number of bacterial cells (Smith & Hattingh, 1991). As a side note, Smith and Hattingh reported that many of the isolates they collected for their study were isolated from the variety Palmiet, which is known to carry adult plant stem rust resistance, possibly from *Sr2* (1991). These results show that attempts to complete Koch's postulate of recreating BLB symptoms in healthy plants by inoculating with Pss from pure culture are met with inconsistent success, and that pathogenesis or symptom development is overwhelmingly dependent on environmental conditions and host genotype (Koch, 1890).

Since its introgression into wheat, *Sr2* has been cherished but misunderstood. The *Sr2*-linked PBC phenotype was initially believed to be caused by the pathogen *Xanthomonas translucens* pv. *undulosa*, and susceptibility to the pathogen was believed to be in genetic linkage with the Hope/H-44 (*Sr2*) derived stem rust resistance (Ausemus, 1934; Bamberg, 1936; Hayes et al., 1934). While *X. translucens* pv. *undulosa* does cause true black chaff in wheat, it is easily confused with the pseudo-black chaff phenotype which was often found in absence of a biotic explanation (Broadfoot & Robertson, 1933; Hagborg, 1936; E. F. Smith, 1917). It was ultimately

established that PBC was caused by high temperature, humidity, and a genetic component in complete linkage with *Sr2* (Johnson & Hagborg, 1944; Sheen et al., 1968; Tabe et al., 2019).

It is possible that the BLB phenotype is a response to abiotic stress, and the symptoms have been associated with epiphytic or opportunistic bacteria that are often isolated from the lesion. This revelation would mirror the nuance observed when differentiating the *Sr2*-linked PBC phenotype from the true black chaff phenotype. This could also explain the parallel finding that a highly similar phenotype was also associated with *B. megaterium* pv. *cerealis* (Hosford, 1982).

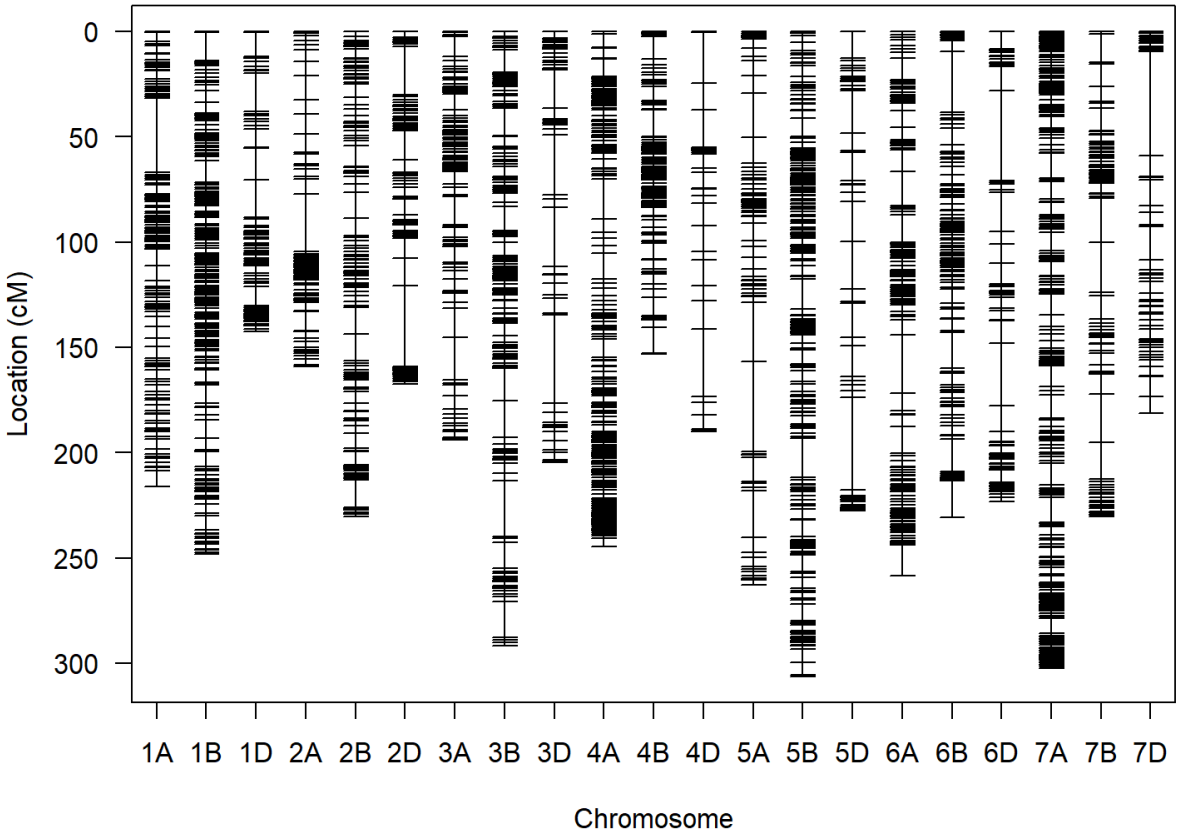
An explanation for the confusion surrounding *Sr2* is that there may be several modifier genes impacting the BLB phenotype. The PBC phenotype has been shown to have several modifier genes, and its appearance on different varieties will often appear quantitative (Bhowal & Narkhede, 1981; Brown, 1997). These modifier genes may have obscured its sources and may have led to confusion around the ability to break the linkage between *PBC1* and *Sr2*.

The variety-specific nature of the disease phenotype bacterial leaf blight of wheat indicates that there is a strong genetic component to this pathosystem. This study conducted a QTL analysis on two field seasons each of the *Sr2*-associated phenotype, PBC, and a suspected *Sr2*-associated phenotype, BLB to investigate its genetic basis. This study identified three QTLs. A chromosome 3BS QTL, which showed up in all BLB and PBC datasets, maps tightly to the *Sr2* marker (*csSr2-CAP*). A chromosome 7DS QTL which was found in both BLB datasets and the 2014 PBC dataset, maps tightly to the *Lr34* marker (*Lr34Exon-11*). A third QTL was mapped in the 2015 PBC and 2016 BLB populations is found on chromosome 5DS. The primary QTL in these models, tightly linked to *Sr2*, accounts for over 50% of the variation in both phenotypes.

Sr2 has a long history of confusion around the identification of its linked or pleiotropic effects like SM, PBC, and seedling chlorosis (SC). Developments in the understanding of the function of *Sr2* show that there is a potential abiotic explanation of the BLB phenotype (Tabe et al., 2019). Previous research on the Pss pathosystem has concluded that Pss is a weak pathogen, due to difficulty replicating the disease phenotype in inoculated plants. This prompts the hypothesis that the BLB phenotype is due to abiotic stress and that Pss is not required for symptom development. A competing theory is that *Sr2* or a linked gene is a susceptibility gene for Pss. To evaluate these hypotheses, ongoing research will need to conduct population level isolation studies, as well as replication of the phenotype in absence of Pss.

Figure 4.1.

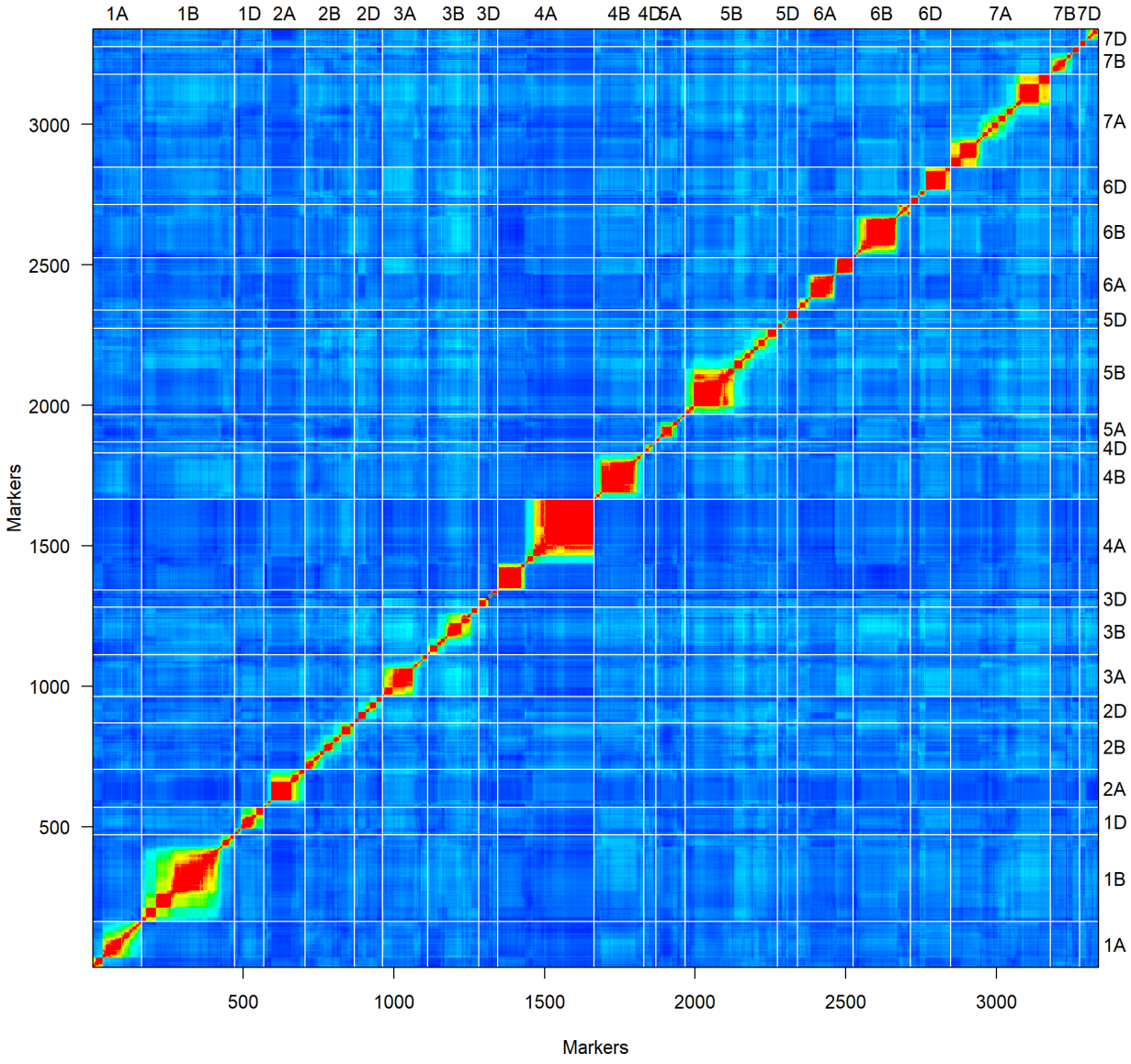
Genetic Map



Note. Marks show the placement of 3339 markers on 21 linkage groups.

Figure 4.2.

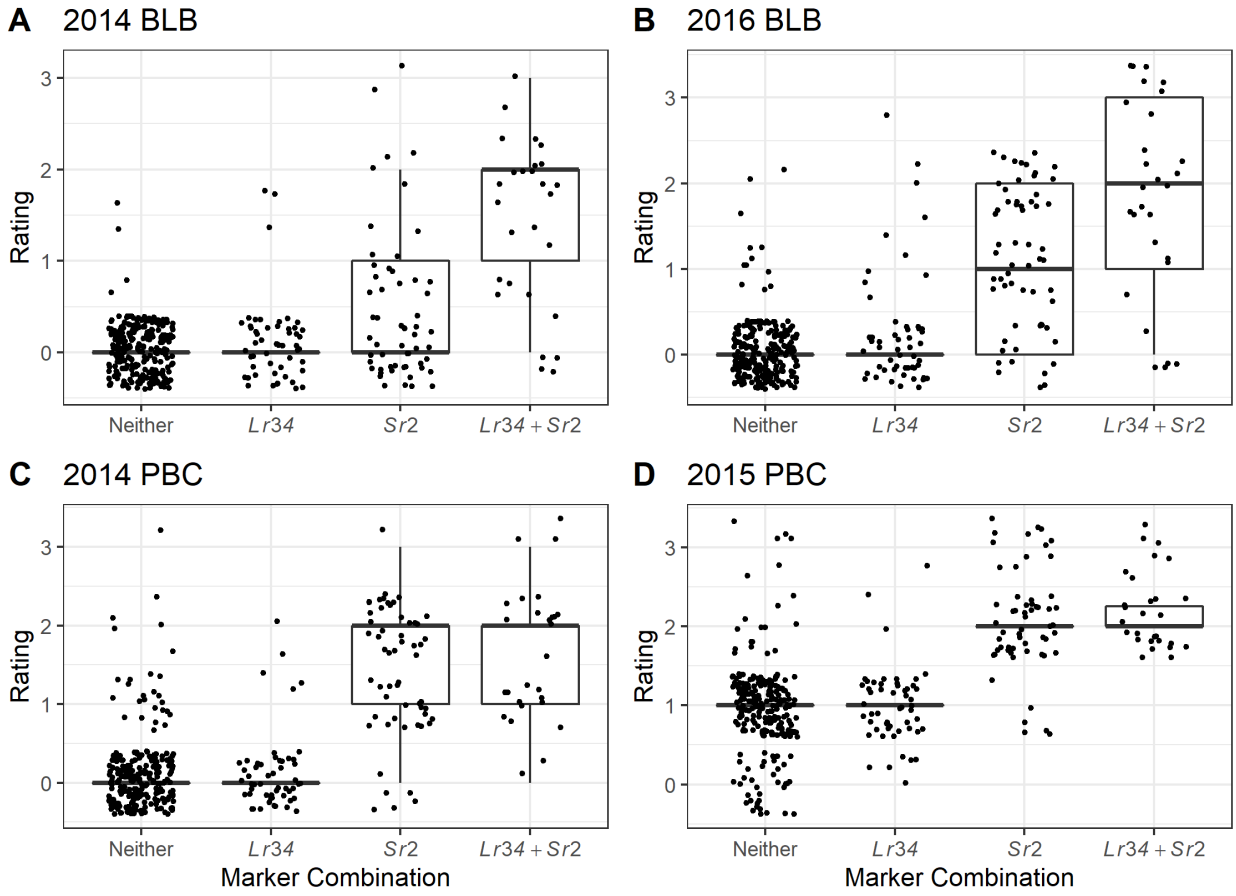
Pairwise Recombination Frequency LOD Scores



Note. Pairwise recombination frequency across 21 chromosomes. Shows that areas of high recombination frequency (Red) are proximally located across the genome. Low recombination frequency (Blue) is found between distant chromosomal regions. This shows the location of genetic linkage on the 21 chromosomes.

Figure 4.3.

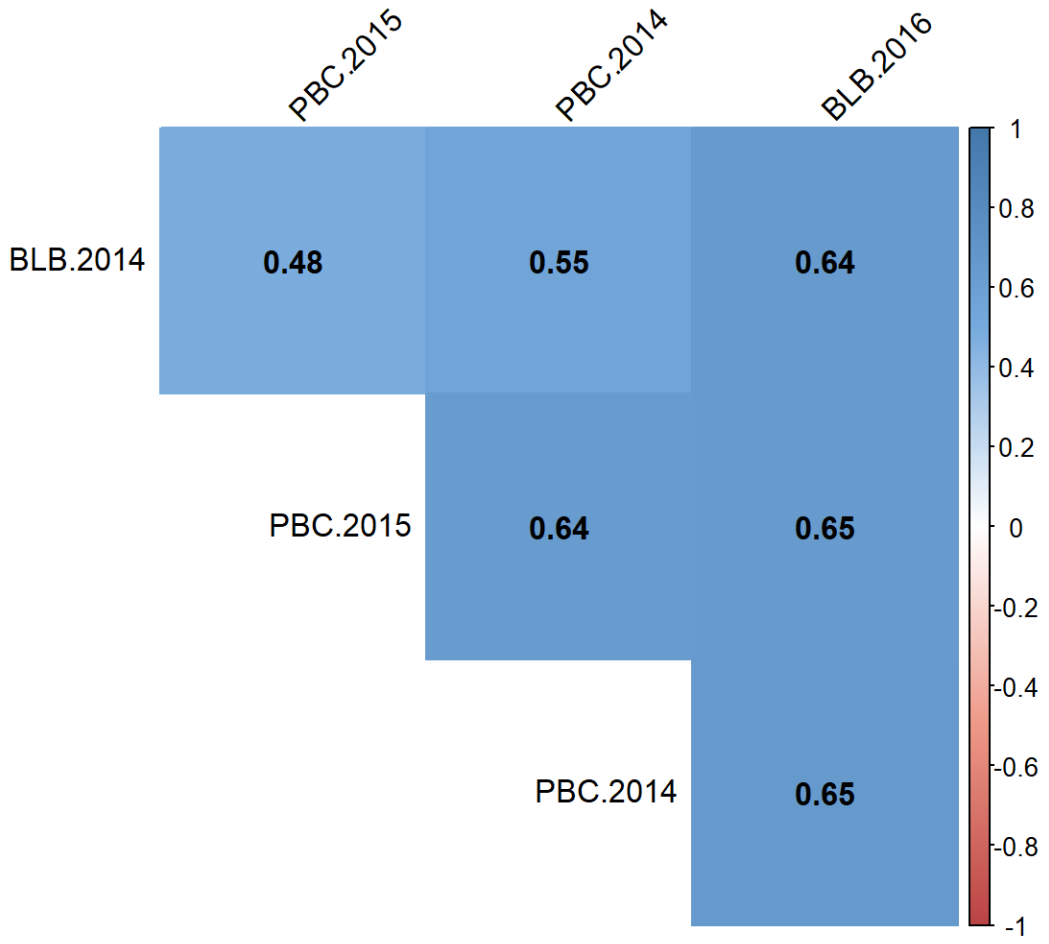
Observed Phenotypes for Polymorphic Gene-specific Supplemental Markers



Note. Phenotype rating observations for two field seasons each for each trait. Traits included are 2014 bacterial leaf blight (BLB) (A) 2016 BLB (B), 2014 pseudo-black chaff (PBC) (C), and 2015 PBC (D). Box plot components represent the median, the first and third quartiles, and the whiskers which are 1.5 times the inter-quartile range.

Figure 4.4.

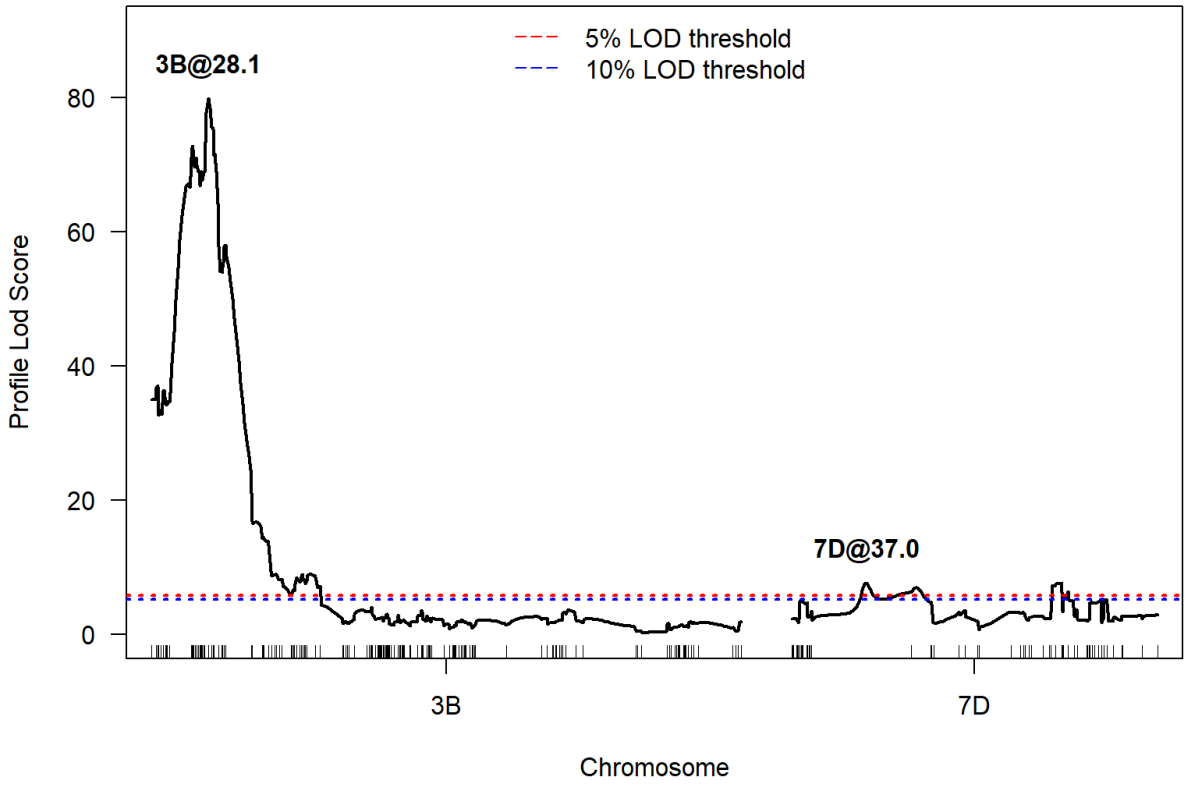
Phenotypic Correlation Matrix



Note. The correlation between phenotypic score data from the u6380 population. The ratings PBC are for the pseudo-black chaff phenotype which was scored in 2014 and 2015. The BLB ratings are for bacterial leaf blight scores which were recorded in 2014 and 2016. Positive correlations are indicated by positive values, and the color blue. Inverse correlations are indicated by negative values and the color red.

Figure 4.5.

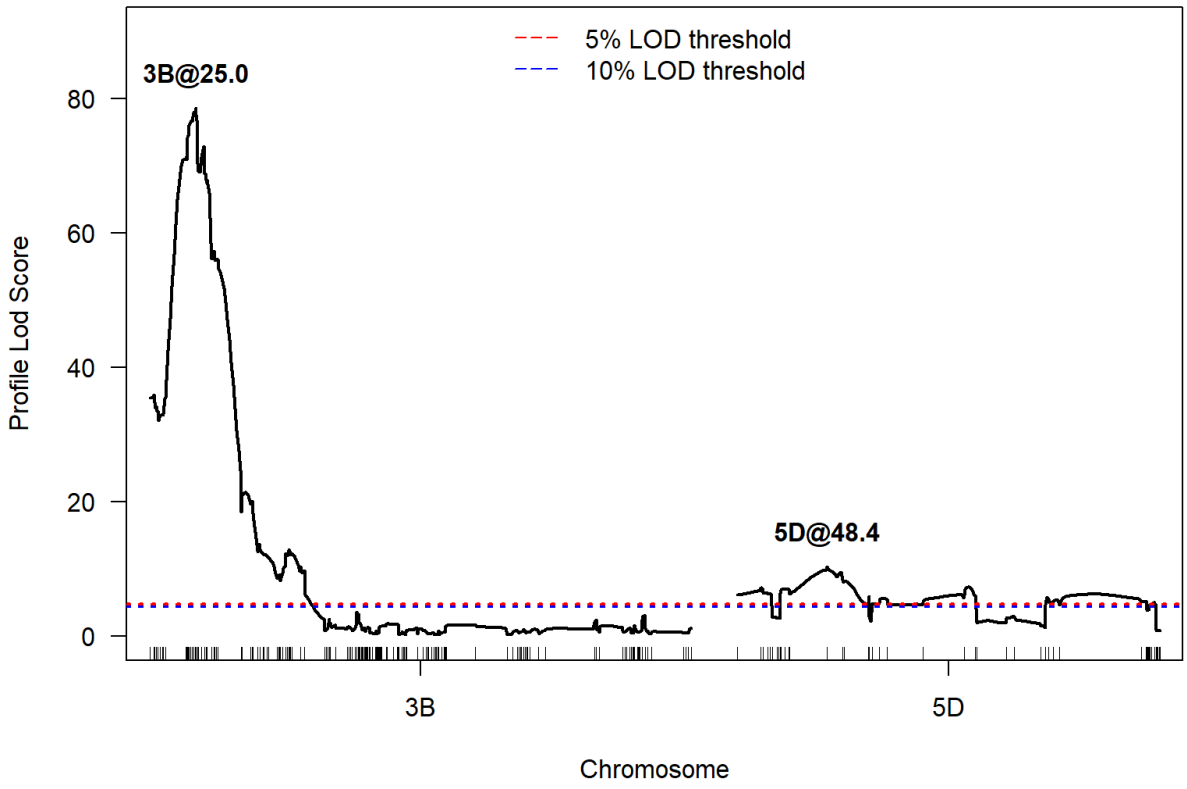
MQM LOD Profile of 2014 PBC Phenotypes



Note. The MQM model identified three QTL on the chromosomes 3B, and 7D. The 5% LOD and 10% LOD thresholds are indicated by the red and blue dashed lined respectively.

Figure 4.6.

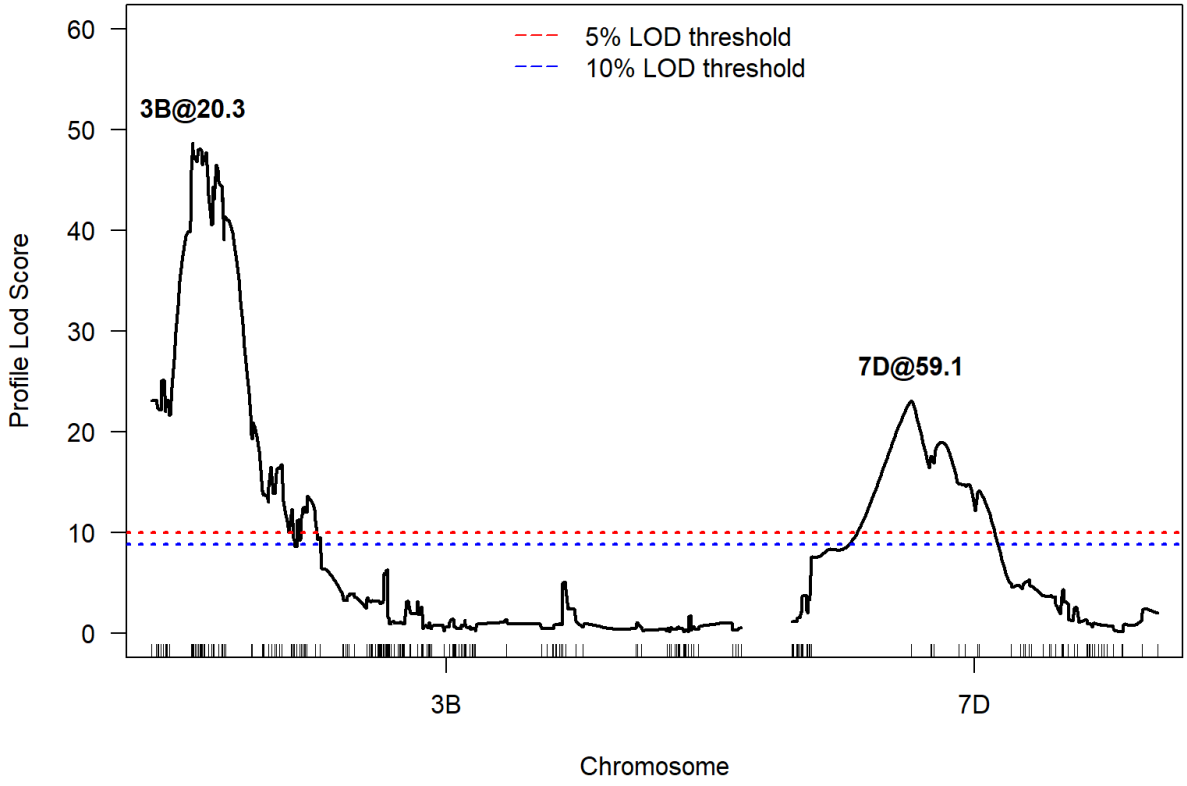
MQM LOD Profile of 2015 PBC Phenotypes



Note. The MQM model identified three QTL on the chromosomes 3B, and 5D. The 5% LOD and 10% LOD thresholds are indicated by the red and blue dashed lined respectively.

Figure 4.7.

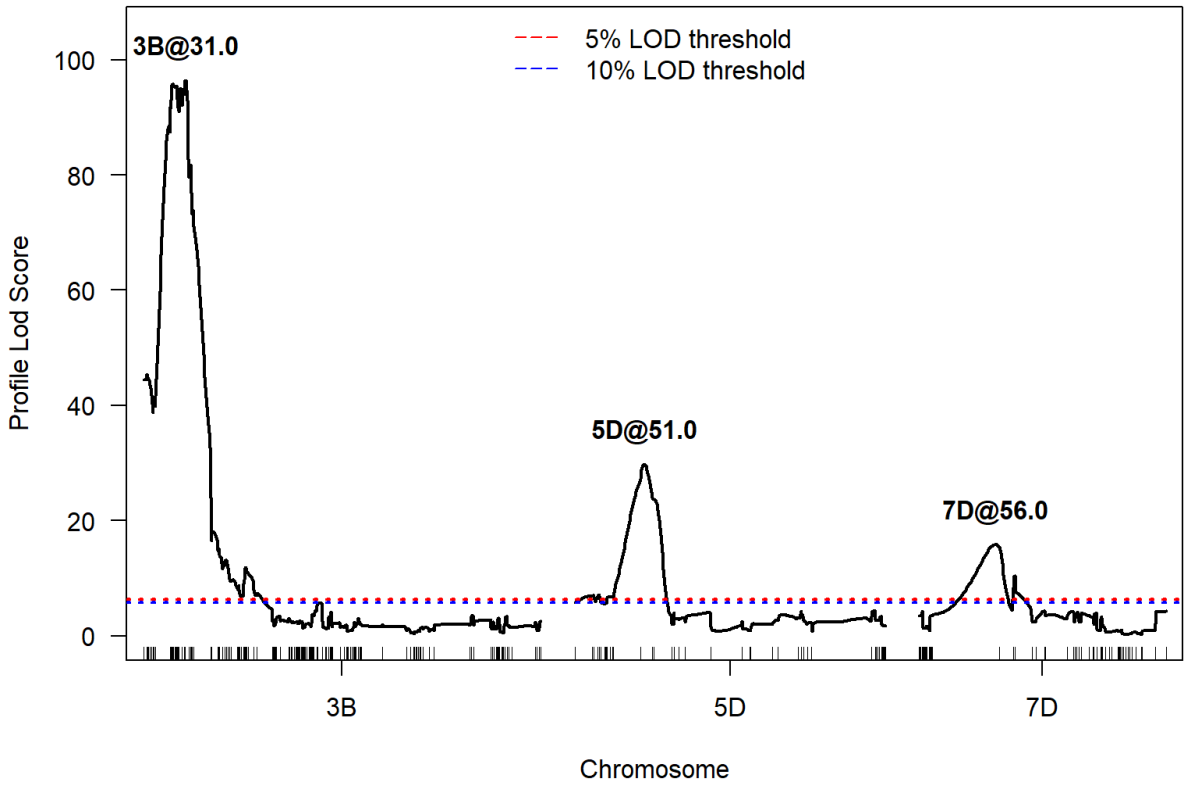
MQM LOD Profile of 2014 BLB Phenotypes



Note. The MQM model identified three QTL on the chromosomes 3B, and 7D. The 5% LOD and 10% LOD thresholds are indicated by the red and blue dashed lined respectively.

Figure 4.8.

MQM LOD Profile of 2016 BLB Phenotypes



Note. The MQM model identified three QTL on the chromosomes 3B, 5D, and 7D. The 5% LOD and 10% LOD thresholds are indicated by the red and blue dashed lined respectively.

Table 4.1.

Supplemental Gene-specific Screening Markers

Gene	Marker Name	Marker Type	KS05HW14	Kingbird
Sr2	*csSr2-CAP*	CAPS[a]	-	+
Lr34	*Lr34Exon-11*	KASP[b]	-	+
Lr68	*Lr68-csgs*	CAPS	-	-
Lr46	*csLV46G22*	CAPS	+	+
Lr78	*IWA6289*	KASP	-	+

Note. Results of supplemental gene-specific marker genotyping.

[a] Cleaved Amplified Polymorphic Sequences marker

[b] Kompetitive Allele Specific PCR marker

Table 4.2.

Trait Heritability Estimates

Trait	Genotype Covariance	Residual Covariance	Replicates	Broad Sense Heritability (H^2)[a]
PBC[b]	0.3598	0.5121	2	0.831
BLB[c]	0.29676	0.19453	2	0.914

Note. Estimations of heritability for PBC and BLB phenotype ratings.

[a] Broad sense heritability calculated on a genotype means basis.

[b] Pseudo-black chaff

[c] Bacterial leaf blight

Table 4.3.

MQM QTL Models

Year	Trait[a]	Significance Threshold	Chromosome	Linkage Map Position (cM)	LOD	95% Bayes Interval, Low	95% Bayes Interval, High	90K Map Consensus Location (Mb)[b]	Phenotypic Variance (R^2)	Estimated QTL Effect[c]
2014	PBC	5%: 5.71, 10%: 5.17	3B	28.105	79.893	28	28.1053	6.2	62.271	1.40
			7D	37	7.647	35	136.8142	50	3.635	0.23
2014	BLB	5%: 10.0, 10%: 8.77	3B	20.3	48.66	19.93	27	6.2	41.26	0.76
			7D	59.089	23.07	56	61	50	16.47	0.44
2015	PBC	5%: 4.68, 10%: 4.36	3B	24.5	78.566	23.81	25.09502	6.2	60.641	1.26
			5D	48.4	10.332	41	56	20	5.051	-0.24
2016	BLB	5%: 6.31, 10%: 5.82	3B	31	96.45	21	31	6.2	61.67	1.31
			7D	56	15.84	52	59.08918	50	5.827	0.64
			5D	51	29.79	49	52	22	11.991	-0.40

Note. MQM models for each individual dataset indicate that at least three QTLs are involved in the bacterial leaf blight phenotype, and that three QTLs are involved in the PBC phenotype. Not all included QTL are significant.

[a] BLB=Bacterial Leaf Blight phenotype, PBC=Pseudo-Black Chaff phenotype

[b] Relative 90k consensus map position

[c] Estimated additive effect of QTL. Positive values indicate the phenotype rating in lines with the Kingbird allele were higher, negative values indicate that the phenotype rating in lines with the KS05HW14 allele were higher.

Table 4.4.

MQM Model QTL Interactions

Year	Trait[a]	Significance Threshold	Chromosome Interactions	LOD	Phenotypic Variance (R^2)(%)	Estimated Interaction Effect[b]	Full model phenotypic variation (%)
2014	PBC	5%: 5.73, 10%: 5.34	3B@28.105 x 7D@37	7.517	3.57	0.097	63.90
2014	BLB	5%: 10.0, 10%: 8.77	3B@20.3 x 7D@59.089	16.06	10.95	0.465	50.79
2015	PBC	5%: 4.68, 10%: 4.36	3B@24.5 x 5D@48.417	6.791	3.25	-0.182	62.67
2016	BLB	5%: 6.31, 10%: 5.82	3B@31 x 7D@56 3B@31 x 5D@51	7.39 22.51	2.58 8.64	0.326 -0.308	72.94

Note. [a] BLB=Bacterial Leaf Blight phenotype, PBC=Pseudo-Black Chaff phenotype

[b] Estimated interaction effect of the QTL. Values greater than zero indicate a greater than additive effect while values less than zero indicate a less than additive effect.

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