Quantifying Infection of Wheat by Races of *Pyrenophora tritici-repentis* Producing Different Host-Selective Toxins

by

Xinyi Ma

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Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Pyrenophora tritici-repentis, the cause of tan spot of wheat, produces several host-selective toxins (HSTs), including Ptr ToxA and Ptr ToxB. Ptr ToxA-producing isolates are predominant in Canada, while Ptr ToxB-producing isolates are extremely rare. The reason for the scarcity of $ToxB^+$ isolates is not clear, since about a quarter of current and historical Canadian wheat genotypes are sensitive to Ptr ToxB. Experiments were undertaken to determine if $ToxA^+$ isolates can outcompete $ToxB^+$ isolates when they occur together on wheat sensitive to both HSTs. Four wheat genotypes including 'Katepwa' (sensitive to Ptr ToxA and Ptr ToxB) were inoculated with isolates 86-124 ($ToxA^+$), Ptf3 ($ToxA^+$, $ToxB^+$), Alg3-24 ($ToxB^+$) and 90-2 (avirulent, no HSTs produced) of *P. tritici-repentis*, alone or in various combinations. The most severe tan spot symptoms were observed after inoculation of 'Katepwa' with 86-124 or Ptf3, followed by Alg3-24. Inoculation with 90-2 produced few if any symptoms. Symptom severity was reduced when 86-124 was co-inoculated with Alg3-24 or 90-2, or when Alg3-24 was co-inoculated with 90-2. Fluorescence microcopy analysis indicated more extensive colonization of inoculated leaf tissue by 86-124 and Ptf3 than by Alg3-24 at 120 hours post-inoculation. The avirulent isolate 90-2 rarely penetrated the leaves. Quantitative PCR analysis with isolate-specific probes and primers, and with an isolate-non-specific probe and primer set, also suggested significantly greater proliferation of 86-124 and Ptf3 than Alg3-24 in inoculated leaf tissue. Collectively, the results indicate that $ToxA^+$ isolates may outcompete $ToxB^+$ isolates, likely contributing to their greater prevalence in Canada.

Preface

This thesis is an original work by Ms. Xinyi Ma. No part of this thesis has been published previously. Ms. Ma conducted all of the experiments described in this document, with training and advice from Dr. Reem Aboukhaddour, Dr. Alireza Akhavan, and Ms. Ileana Strelkov (U of Alberta). Dr. Akhavan and Ms. Strelkov also provided advice on data interpretation and statistical analysis. Ms. Ma wrote the first drafts of all chapters, which were examined and edited by Dr. Akhavan and Ms. Strelkov prior to submission to Dr. Stephen Strelkov, Professor and Dr. Sheau-Fang Hwang, Adjunct Professor, who were Ms. Ma's supervisor and co-supervisor, respectively. Dr. Strelkov developed the research concept as part of his NSERC Discovery Grant-funded program, and provided editorial revisions and suggestions on the thesis.

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Table of Contents

List of Figures

Chapter 1. Introduction and Literature Review

1.1 Tan spot of wheat

1.1.1 History and nomenclature

Tan spot is an economically important foliar disease of wheat caused by the ascomycete fungus *Pyrenophora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) (Hosford, 1982). The fungus was first reported in Germany from couch grass (*Agropyron repens* (L.) Beauv.) and given the name *Pleospora trichostoma* (Diedicke, 1902). The teleomorph was later renamed *Pleospora tritici-repentis* Died. and the anamorph was termed *Helminthosporium tritici-repentis* (Rab. ex Schlecht) Died. (Diedicke, 1903, 1904; Hosford, 1982). Drechsler (1923) identified the fungus in the United States in the early 1920s and named it *Pyrenophora tritici-repentis*. It was also reported from Japan and India as *Drechslera tritici-vulgaris* (Nisikado, 1928; Mitra, 1934). In Canada, tan spot disease was first found on the wheat cultivar 'Marquis' in the 1930s, with the causal agent identified as *Helminthosporium tritici-repentis* (Conners, 1939). The pathogen also was found and identified as *H. tritici-repentis* in Australia, where tan spot disease is often referred to as yellow spot (Valder & Shaw, 1953). The nomenclature of the tan spot fungus remained inconsistent until Shoemaker (1962) proposed the names *Pyrenophora tritici-repentis* and *Drechslera tritici-repentis* for the teleomorph and anamorph, respectively. These are now the widely accepted names for the pathogen.

1.1.2 Disease symptoms

While the first report of tan spot of wheat in Canada is from 1939, the first extensive outbreak of the disease did not occur until 1974 (Tekauz, 1976). By the 1980s, tan spot had become an important foliar disease of wheat. The increasing prevalence of tan spot appears to have resulted from the widespread adoption of conservation tillage techniques by farmers, which allow the stubble-borne inoculum of *P. tritici-repentis* to survive on the soil surface (Bailey, 1996; Bockus & Shroyer, 1998). The fungus can overwinter on wheat residues, forming pseudothecia that produce ascospores in the spring, which serve as the primary inoculum (Rees & Platz, 1979). Nonetheless, other agricultural practices, such as short crop rotations and the planting of susceptible wheat, also have likely contributed to increased tan spot severity in recent decades (Bailey et al., 1992; Rees & Platz, 1992; Lamari et al., 1995). Yield losses of 3-15% are considered typical for tan spot infection, although losses exceeding 40% have been reported under conditions favorable for disease development (Rees et al., 1982; Rees & Platz, 1983).

Infection by *P. tritici-repentis* results in the development of oval-shaped spots or lesions on the leaves of susceptible wheat plants. Typically, the lesions consist of dark-brown centers surrounded by chlorotic or necrotic haloes. The necrosis or chlorosis can expand with the lesions coalescing, eventually resulting in the death of the entire leaf when symptoms are severe (Hosford, 1971; Lamari & Bernier, 1989b). The necrosis and chlorosis associated with tan spot result from highly specific interactions between fungal isolates and host genotypes (Lamari & Bernier, 1989a, 1989b), as will be discussed later in this review. In addition to foliar symptoms, *P. tritici-repentis* also causes a condition called ‘red smudge’ in infected wheat kernels (Francl & Jordahl, 1992; Fernandez et al.,

1994), wherein the kernels exhibit a reddish discoloration. Seeds with red smudge usually have a lower emergence rate and reduced seed quality (Fernandez et al., 1997).

1.1.3 Host range

The most economically important hosts of *P. tritici-repentis* are durum wheat (*Triticum turgidum* L. var. *durum*) and common wheat (*Triticum aestivum* L.) (Hosford, 1971). Nevertheless, the fungus also has a wide range of secondary hosts in the Poaceae including rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.) and many wild grasses (Hosford, 1971; Krupinsky, 1982, 1992). *Pyrenophora tritici-repentis* can survive and overwinter on these secondary hosts, which can serve as sources of fungal inoculum and may contribute to genetic diversity in the fungal population (De Wolf et al., 1998).

1.1.4 Disease cycle

As noted above, *P. tritici-repentis* survives in infected plant debris, where it can overwinter and produce sexual fruiting bodies called pseudothecia (Hosford, 1971). Under favorable conditions (6-8 hours of high humidity and temperatures of 10-18°C), the pseudothecia produce ascospores which serve as primary inoculum (Hosford, 1971; Reez & Platz, 1983; Summerell & Burgess, 1989). The ascospores are wind-dispersed and cause primary infections on the leaves of young wheat plants. The pathogen then colonizes the infected tissues and produces conidia (asexual spores) on the leaf surface during the growing season (Hosford, 1972; Schilder & Bergstrom, 1992). The conidia are generated sympodially at the apical region of conidiophores, which emerge from the foliar lesions (Ellis & Waller, 1976). At temperatures of around 21 °C, conidia are produced continually and can be spread by wind or rain onto new hosts, serving as the

secondary inoculum (Platt & Morrall, 1980; Francl, 1998). Infections may move vertically up the plant from older lesions on the lower leaves, to new lesions on the upper leaves. Infection of the upper canopy results in the greatest yield losses, since the upper leaves make the greatest contribution to grain fill. Following harvest, *P. tritici-repentis* survives in the infected crop residues, producing new pseudothecia to reinitiate the disease cycle (Conners, 1939; Hosford, 1971, 1972). Asexual structures such as conidiophores and mycelium also can develop in infected seeds, resulting in the emergence of infected seedlings (Schilder & Bergstron, 1994).

1.1.5 Disease development

Like many necrotrophic fungal pathogens, *P. tritici-repentis* invades host plants by direct penetration. After a conidium lands on the leaf surface, it germinates and produces several germ tubes (2-4 germ tubes on average). Germination rates can be as high as 65% or 95% at 3 or 6 hours, respectively, after the conidia land on the leaf surface (Larez et al., 1986; Dushnicky et al., 1996; Haueisen & Stukenbrock, 2016). The germ tubes grow along the leaf surface and form appressoria, in which a high turgor pressure unit called an infection peg is produced (Larez et al., 1986; Loughman & Deverall, 1986; Lamari & Bernier, 1989b; Dushnicky et al., 1996). Penetration by the infection peg occurs at leaf epidermal cells via release of cell wall degrading degrading enzymes, such as cutinases, pectinases and cellulases, and mechanical puncture through the periclinal cell wall (Dickman et al., 1989; Dushnicky et al., 1996). Penetration occurs on epidermal cells parallel to the veins, as well as on guard cells and trichomes (Larez et al., 1986; Loughman & Deverall, 1986; Lamari & Bernier, 1989b; Dushnicky et al., 1996). Following penetration, the infection peg forms an intracellular vesicle, where a secondary

hypha is produced, with further growth occurring intercellularly in the mesophyll (Larez et al., 1986; Loughman & Deverall, 1986; Lamari & Bernier, 1989b). Both penetration and infection typically occur within 24 hours following spore contact with the leaf surface, although delays in the penetration process of up to 96 hours also have been reported (Dushnicky et al., 1996; Dushnicky et al., 1998).

There do not seem to be significant differences in the reaction of susceptible and resistant wheat genotypes during the initial stages of penetration and infection by *P. tritici-repentis* (Loughman & Deverall, 1986). Papillae or haloes are observed at the penetration site in both susceptible and resistant hosts, although more papillae appear to develop on resistant cultivars (Dushnicky et al., 1996; Dushnicky et al., 1998). Several studies also showed that leaf damage was reduced in resistant hosts due to the suppression of secondary hyphae formation in the leaf mesophyll (Larez et al., 1986; Dushnicky et al., 1996; Dushnicky et al., 1998). Nonetheless, it is not until about 72 hours after inoculation that significant differences in the reactions of resistant and susceptible hosts are observed (Lamari & Bernier, 1989b). In incompatible reactions, the fungus cannot colonize the mesophyll, and remains confined to the epidermis.

1.1.6 Disease management

The management of tan spot of wheat can be difficult, since *P. tritici-repentis* overwinters in both crop residues and susceptible grasses. Cultural control methods can include burning or plowing down the crop debris, and the rotation of wheat with non-hosts crops (Huber et al., 1987; Pfender et al., 1988; Bockus & Claasen, 1992; Bockus, 1998). Fungicides represent another tool to manage tan spot. A number of fungicides such as epoxyconazole, tebuconazole, prothioconazole and picoxystrobin can

effectively reduce yield losses caused by tan spot when applied as foliar treatments (Colson et al., 2003; Jørgensen & Olsen, 2007). The application of triadimenol as a seed treatment also was reported as effective for managing the pathogen (Luz & Bergstrom, 1986). Nevertheless, the application of fungicides increases input costs and raises environmental concerns (De Wolf & Francl, 1998; De Wolf et al., 1998).

Biological control and the planting of resistant cultivars represent other, potentially more environmentally friendly and cost-effective approaches to tan spot management. For example, inoculation of *P. tritici-repentis*-infected wheat straw with the bio-control agent *Limonomyces roseipellis* Stalpers & Loerakker reduced pathogen inoculum levels by 60-80% (Pfender et al., 1993). The use of biological control agents to manage tan spot, however, is not widespread. In contrast, there has been greater interest in the development and deployment of genetically resistant wheat cultivars to manage the disease. While several research groups have studied genetic resistance to *P. tritici-repentis*, only a few cultivars with a high level of resistance have been developed (De Wolf et al., 1998; Lamari et al., 2005a; Faris et al., 2013; Tran et al., 2017). Ideally, tan spot resistant cultivars should also possess good resistance to other common diseases of wheat, making resistance breeding more challenging. Chu et al. (2008) reported that wild emmer wheat seedlings, *Triticum dicoccum* Korn, showed resistance to both tan spot disease and Stagonospora leaf blotch, and suggested that wild emmer could serve as a resistance source for the development of wheat cultivars resistant to these foliar diseases.

1.1.7 Physiologic specialization and the races of *P. tritici-repentis*

As noted earlier in this review, *P. tritici-repentis* can cause two distinct symptoms on infected wheat leaves: tan necrosis and chlorosis (Lamari & Bernier, 1989a). Lamari &

Bernier (1989b) recognized that specific isolates of the fungus could differentially induce these symptoms on susceptible wheat genotypes, and initially grouped the isolates into pathotypes based on their ability to induce necrosis or chlorosis. Pathotype 1 isolates caused both necrosis and chlorosis on susceptible hosts (nec+ chl+); pathotype 2 isolates caused only cause necrosis (nec+ chl-); and pathotype 3 isolates caused only chlorosis (nec- chl+) (Lamari & Bernier, 1989b). Those isolates that could cause neither symptom (nec- chl-) were grouped into pathotype 4 (Lamari et al., 1991). While useful, this symptom-based classification system could distinguish a maximum of only four pathotypes, a limitation which was highlighted by the identification of isolates from Algeria that could cause only chlorosis (and hence were pathotype 3), but did so on different hosts than the original pathotype 3 isolates (Lamari et al., 1995).

In order to accommodate these new isolates, Lamari et al. (1995) proposed a race-based classification scheme for *P. tritici-repentis*. Under this system, isolates are classified into races based on their virulence patterns on a set of differential hosts consisting of five hexaploid wheats ('Glenlea', 'Katepwa', 6B662, 6B365, 'Erik') and two durum wheats (4B1149, 'Coulter'). A total of eight races have been identified to date and numbered 1 to 8 (Lamari et al., 2003); all of the races are virulent on at least one of the differential hosts with the exception of race 4, which represents avirulent isolates. The differential reaction of the various races on the differential hosts is mediated by the action of host-selective toxins (≡ necrotrophic effectors) as will be discussed below.

1.2 The involvement of host-selective toxins in tan spot of wheat

Host-selective toxins (HSTs) are plant pathogen-produced molecules that have negative biological effects only on the hosts of that pathogen (Pringle & Scheffer, 1964).

In recent years, the term ‘necrotrophic effector’ is often used instead of HST, to reflect the fact that these are molecules released by necrotrophic pathogens to serve as positive agents of virulence. Most HSTs are secondary metabolites, but over the past few decades a number of proteinaceous HSTs also have been identified, including Ptr ToxA and Ptr ToxB produced by *P. tritici-repentis*. Sensitivity to HSTs is generally conferred by dominant genes in the host, and pathosystems involving HSTs follow an ‘inverse’ gene-for-gene model (Wolpert et al., 2002). In such systems, which include tan spot of wheat, susceptibility results from the unique interaction between HSTs produced by the pathogen and specific targets or receptors in the host (Strelkov & Lamari, 2003).

Pyrenophora tritici-repentis produces at least three HSTs, termed Ptr ToxA, Ptr ToxB and Ptr ToxC. The reaction to each of these HSTs in the host is controlled by single dominant and independently inherited genes (Strelkov & Lamari, 2003; Lamari & Strelkov, 2010). The eight races of *P. tritici-repentis* are defined by their capacity to differentially produce these HSTs, which determine their virulence on the hosts of the tan spot differential set. Race 1 produces Ptr ToxA + Ptr ToxC; race 2 produces Ptr ToxA; race 3 produces Ptr ToxC; race 4 produces no known HSTs and is avirulent; race 5 produces Ptr ToxB; race 6 produces Ptr ToxB + Ptr ToxC; race 7 produces Ptr ToxA + Ptr ToxB; and race 8 produces Ptr ToxA + Ptr ToxB + Ptr ToxC (Lamari et al., 2003). These HSTs are discussed in more detail in the section below.

1.2.1 Ptr ToxA

Ptr ToxA is a 13.2 kDa-protein encoded by the single-copy gene *ToxA* (Ballance et al., 1989, 1996; Ciuffetti et al., 1997). The first of the HSTs to be identified from *P. tritici-repentis*, Ptr ToxA induces necrosis on sensitive host genotypes (Ballance et al.,

1989; Ciuffetti et al., 1997). Sarma et al. (2005) found that the protein has a β-sandwich-fold-structure with a solvent-exposed loop containing an arginyl-glycyl-aspartic acid (RGD) motif. The solvent-exposed loop is hypothesized to play an important role in toxin recognition, with the RGD motif interacting with an extracellular receptor on the host membrane (Sarma et al., 2005; Manning et al., 2008). Ptr ToxA is internalized in the mesophyll cells of Ptr ToxA-sensitive but not Ptr ToxA-insensitive wheat genotypes (Ciuffetti et al., 2010). While the exact mechanism of internalization is unknown, it appears to involve endocytosis in endosomes. Following release from the endosomes, Ptr ToxA targets the chloroplasts, where it has been demonstrated to interact with a chloroplast-localized protein called ToxA Binding Protein 1 (ToxABP1) (Manning & Ciuffetti, 2005; Manning et al., 2007). The activity of Ptr ToxA is light-dependent and appears to result from the accumulation of reactive oxygen species (ROS), eventually leading to tissue necrosis (Manning et al., 2009). Sensitivity in the host to Ptr ToxA is controlled by the dominant gene *Tsn1* (Faris et al., 1996; Gamba et al., 1998), and it has been postulated that the *Tsn1* locus is involved in Ptr ToxA uptake (Ciuffetti et al., 2010).

1.2.2 Ptr ToxB

Ptr ToxB is another proteinaceous HST produced by *P. tritici-repentis* (Orolaza et al., 1995; Strelkov et al., 1999). This small protein of 6.5 kDa mass induces chlorosis in sensitive hosts (Strelkov et al., 1999; Martinez et al., 2001) and is encoded by the multi-copy *ToxB* gene (Martinez et al., 2004; Strelkov et al., 2006). The severity of symptoms caused by Ptr ToxB-producing isolates of *P. tritici-repentis* is correlated with *ToxB* copy number, and isolates having the greatest number of copies cause the most

severe chlorosis (Strelkov et al., 2006). Moreover, RNA-mediated gene silencing of *ToxB* showed that the most strongly silenced strains of the fungus caused the mildest chlorosis and also formed fewer appressoria, suggesting that the amount of Ptr ToxB produced by *P. tritici-repentis* plays a role in quantitative variation in fungal virulence (Aboukhaddour et al., 2012).

The mode of action of Ptr ToxB appears to involve the light-dependent photooxidation of chlorophyll, likely through a direct or indirect inhibition of photosynthesis (Strelkov et al., 1998; Kim et al., 2010). However, the exact mechanism of this inhibition is unknown. Localization studies with fluorescently labelled Ptr ToxB suggested that it remains in the apoplast of both toxin-sensitive and insensitive wheat genotypes, indicating that it may act as an extracellular effector (Figueroa et al., 2015). In the wheat host, sensitivity to Ptr ToxB is controlled by the *Tsc2* gene (Friesen & Faris, 2004).

In addition to the 'wild-type' *ToxB* found in isolates of *P. tritici-repentis* possessing Ptr ToxB activity, an inactive form of the gene (*toxb*) is found in avirulent race 4 isolates and race 3 isolates that lack ToxB activity (Martinez et al., 2004; Strelkov et al., 2006). The *toxb* homolog in race 4 shares 86% similarity with *ToxB* in virulent isolates (Strelkov & Lamari, 2003; Kim & Strelkov, 2007), and appears to be expressed only at very low levels (Amaike et al., 2008). Furthermore, the heterologously expressed protein product (Ptr toxb) possesses only trace levels of chlorosis-inducing activity, versus the strong chlorosis induced by the wild-type Ptr ToxB (Kim & Strelkov, 2007). Although both Ptr ToxB and Ptr toxb have 78% similarity at the amino acid level, and both have the same β-sandwich fold structure, Nyarko et al. (2014) identified differences between the two

forms in one half of this sandwich, which may contribute to the absence of chlorosis-inducing activity by Ptr toxb.

1.2.3 Ptr ToxC and other HSTs from *P. tritici-repentis*

Ptr ToxC is another HST produced by some races of *P. tritici-repentis* (Strelkov & Lamari, 2003; Lamari & Strelkov, 2010). Like Ptr ToxB, Ptr ToxC also induces chlorosis, but on different host genotypes. Sensitivity to Ptr ToxC in the host is controlled by a single gene, *Tsc1*, although there are conflicting reports on whether insensitivity to chlorosis induction is dominant or recessive (Gamba & Lamari, 1998; Effertz et al., 2002; Faris et al., 2013). Ptr ToxC has not been purified to homogeneity, although characterization of the partially purified molecule suggests that it is a low-molecular-mass compound with non-ionic and polar properties (Effertz et al., 2002). Ptr ToxC activity was reported recently in spore germination fluids of a *P. tritici-repentis* race 3 isolate (Tran et al., 2017).

In addition to Ptr ToxA, Ptr ToxB and Ptr ToxC, there have been suggestions in the literature regarding the existence of other HSTs produced by *P. tritici-repentis*. For example, Gamba & Lamari (1998) suggested that isolates of races 3 and 5 produce toxic constituents that cause a highly specific foliar necrosis on a durum wheat line. There were also preliminary reports of other putative necrosis and chlorosis-inducing HSTs from *P. tritici-repentis* (Ciuffetti et al., 2003; Meinhardt et al., 2003). None of these toxins, however, has been characterized fully or described in the refereed literature. It is clear that there is much to discover with respect to the HSTs produced by the tan spot fungus.

1.3 Resistance and susceptibility to tan spot of wheat

As noted above, the wheat/*P. tritici-repentis* pathosystem appears to follow the inverse gene-for-gene model of host-parasite interactions (Lamari et al*.,* 2003), wherein pathogen-produced toxins interact with specific receptors or targets in the host to result in disease (Ptr ToxA/*Tsn1*; Ptr ToxB/*Tsc2*; Ptr ToxC/*Tsc1*). In this model, resistance is passive, since it is based on the absence of sensitivity to an HST. Nonetheless, while sensitivity to the Ptr toxins can be sufficient to confer disease (Lamari & Strelkov, 2010), there also have been reports of qualitative genes (*Tsr1-Tsr5*) for resistance to *P. tritici-repentis* (Singh et al., 2006; Singh et al., 2008a, 2008b) and of quantitative trait loci (QTLs) for race non-specific tan spot resistance (Faris & Friesen, 2005).

1.4 Occurrence of the races of *P. tritici-repentis* in Canada

While eight races of *P. tritici-repentis* have been reported to date, nearly 90% of all isolates collected from Canada have been classified as race 1 (Ptr ToxA + Ptr ToxC) or race 2 (Ptr ToxA) (Strelkov & Lamari, 2003; Aboukhaddour et al., 2013; MacLean et al., 2017). In contrast, other races of the fungus are fairly rare in Canada. For example, recent studies found that race 3 (Ptr ToxC) comprised only 2-3% of all *P. tritici-repentis* isolates evaluated from Alberta and Saskatchewan (Aboukhaddour et al., 2013; MacLean et al., 2017). Race 5 (Ptr ToxB) appears to be even more rare, since its occurrence in Canada is known from a single isolate found on the Manitoba-Saskatchewan border in 1991 (Strelkov et al., 2002). Unlike other race 5 isolates from Central Asia and North Africa, however, the Canadian race 5 isolate is weakly virulent (Strelkov et al., 2002). This reduced virulence appears to be related to a dosage effect, with Canadian race 5 having only 2 copies of *ToxB* versus 8-10 copies in other ('wild-type') isolates, and producing

significantly less *ToxB* transcript and Ptr ToxB protein (Strelkov et al., 2002; Amaike et al., 2008; Strelkov et al., 2006). Other virulent races, including races 6 (Ptr ToxB + Ptr ToxC), 7 (Ptr ToxA + Ptr ToxB) and 8 (Ptr ToxA + Ptr ToxB + Ptr ToxC), have not been identified from Canada and appear to be restricted to North Africa and/or the wheat center of diversity (Lamari et al., 2003; Lamari et al., 2005b). Race 4 is also very rare in surveys of Canadian wheat (Lamari et al., 1998), as might be expected for a race that appears to be avirulent and likely survives mainly as a saprophyte.

1.5 Canadian wheat and sensitivity to the Ptr toxins

Spring hexaploid wheat (*Triticum aestivum* L.) is grown extensively in Canada. There are many classes of Canadian wheat, including Canadian Western Amber Durum (CWAD), Canadian Prairie Spring (CPS), Canadian Western Red Spring (CWRS), Canadian Western Extra Strong (CWES), and Canadian Western Soft White Spring (CWSWS) (McCallum & Depauw, 2008). Each class has its own defined characteristics and end-uses. The CWRS class accounts for 86% of total wheat production (Canadian Grain Commission, 2017). The main wheat cultivars grown in Canada have changed over time, as new cultivars have been bred for traits such as improved disease resistance, yield and grain quality.

Unfortunately, sensitivity to the Ptr toxins is widespread in Canadian wheat cultivars (Lamari et al., 2005a; Tran et al., 2017). In a study focused on CWRS wheat, Lamari et al. (2005) found that sensitivity to Ptr ToxA was introduced to Canadian cultivars via the first major variety, ‘Red Fife’, in the late 1800s and was then passed on to other cultivars unknowingly through extensive backcrossing. Sensitivity to Ptr ToxB appears to have been introduced in 1934 with the release of ‘Thatcher’ (Lamari et al., 2005a). In a more

recent study, the sensitivity of a more diverse collection of Canadian cultivars developed over the past century and representing all wheat classes was assessed for sensitivity to Ptr ToxA, Ptr ToxB and Ptr ToxC (Tran et al., 2017). This study found that 92 of the 100 wheat cultivars tested were sensitive to at least one HST, with 68% sensitive to Ptr ToxA, 63% sensitive to Ptr ToxC, and 24% sensitive to Ptr ToxB (Tran et al., 2017). Given that sensitivity to the Ptr toxins is well-correlated with susceptibility to the fungal isolates which produce them (Lamari et al., 2003), the results of these studies indicate that susceptibility to tan spot is also extensive in Canadian wheat cultivars (Lamari et al., 2003; Tran et al., 2017).

While sensitivity to Ptr ToxB appears to be less common than sensitivity to Ptr ToxA, it is nonetheless widespread and found in nearly a quarter of Canadian cultivars (Tran et al., 2017). In this context, it is not clear why Ptr ToxB-producing isolates are so rare. There are a couple of possibilities (S.E. Strelkov, *personal communication*). One is that this rarity is a consequence of the absence of the wild-type *ToxB* gene from Canadian populations of *P. tritici-repentis*. The other is that the selective advantage provided by *ToxB* is less than that from *ToxA*. It is important to distinguish between these possibilities, because if the former is true, then the introduction of isolates with wild-type *ToxB* to Canada could represent a threat to Canadian wheat.

1.6 Research objectives

The main objective of this thesis project was to explore the epidemiological significance of *ToxB* in *P. tritici-repentis*, by comparing the competitive advantage provided by Ptr ToxA and Ptr ToxB in joint inoculation experiments. Specifically, we wished to determine if Ptr ToxB-producing isolates were competitive with the

predominant Ptr ToxA-producing isolates, when these were inoculated together on wheat hosts sensitive to both HSTs. This goal was accomplished by: (1) monitoring the proliferation of fungal biomass in the inoculated hosts via quantitative-PCR analysis and histological observation, and (2) measuring disease severity and development over a time-course.

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Chapter 2. Quantification of Infection by Races 2, 4 and 5 of *Pyrenophora tritici-repentis*

2.1 Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) causes tan spot of wheat. Tan spot became a major problem on the Canadian prairies in the 1970s, likely because of the introduction of zero-tillage practices that enable the stubble-borne inoculum of *P. tritici-repentis* to survive on the soil surface (Tekauz, 1976; Krupinsky, 1982). Tan spot is associated with the development of necrotic and/or chlorotic lesions on the leaves of susceptible wheat (Lamari & Bernier, 1989a, 1989b). These lesions can expand and coalesce, eventually covering most of the infected leaf, reducing photosynthetic capacity and yield when the upper canopy is affected. The pathogen overwinters in plant debris and produces ascospores and conidia under cool and dry conditions (Hosford, 1971, 1972; Schilder & Bergstrom, 1992). While both spore types function as primary inoculum to initiate the infection cycle, the conidia also serve as secondary inoculum, disseminating the pathogen up and through the canopy. After landing on the leaf surface, conidia germinate from the basal and intercalary cells to form germ tubes (Larez et al., 1986; Dushnicky et al., 1996). The germ tubes produce appressoria, with fungal penetration of the host epidermal cells occurring via a penetration peg (Larez et al., 1986; Dickman et al., 1989). Most infections occur between 6 and 24 h following inoculation (Larez et al., 1986; Loughman & Deverall, 1986; Dushnicky et al., 1998a), with the fungus forming intracellular vesicles in the infected epidermal cells. Fungal growth proceeds from the vesicle by the formation of

intercellular hyphae in the leaf mesophyll (Lamari & Bernier, 1989b; Dushnicky et al., 1998a).

P. tritici-repentis produces at least three different host-selective toxins (HSTs), namely, Ptr ToxA, Ptr ToxB, and Ptr ToxC, which serve as fungal pathogenicity factors (Strelkov & Lamari, 2003; Lamari & Strelkov, 2010). Ptr ToxA and Ptr ToxB selectively induce necrosis and chlorosis, respectively, on sensitive wheat genotypes. Both are small proteins, with Ptr ToxA (13.2 kDa) encoded by the single-copy gene *ToxA* (Ballance et al., 1996; Ciuffetti et al., 1997), and Ptr ToxB (~6.5 kDa) encoded by the multi-copy gene *ToxB* (Martinez et al., 2004; Strelkov et al., 2006). An inactive homolog of *ToxB*, designated *toxb*, is found in some isolates that lack Ptr ToxB activity (Martinez et al., 2004; Strelkov et al., 2006). The third HST produced by *P. tritici-repentis*, Ptr ToxC, also induces chlorosis, but on different wheat genotypes than Ptr ToxB (Lamari & Bernier 1991). Unlike Ptr ToxA and Ptr ToxB, however, Ptr ToxC does not appear to be proteinaceous, and instead is a low-molecular mass, non-ionic, polar molecule (Effertz et al., 2002). The gene(s) responsible for its biosynthesis are unknown. The production of additional HSTs by *P. tritici-repentis* has been reported by various groups, but these compounds remain to be characterized (Gamba & Lamari, 1998; Meinhardt et al. 2003).

The *P. tritici-repentis*-wheat system follows an inverse gene-for-gene model (Strelkov & Lamari, 2003), in which the specific interaction between pathogen-produced HSTs and the products of host 'susceptibility' genes mediates compatibility (Strelkov & Lamari, 2003; Faris et al., 2013). In addition to host-HST interactions, several quantitative trait loci (QTLs) also have been found to contribute to broad-spectrum resistance to *P. tritici-repentis* in wheat (Faris & Friesen, 2005; Chu et al., 2008, 2010; Faris et al., 2012). Isolates of the fungus are classified into races based on their ability to produce the HSTs

Ptr ToxA, Ptr ToxB and/or Ptr ToxC, which results in unique virulence patterns on a host differential set (Lamari et al., 2003). Eight races of *P. tritici-repentis* have been identified: race 1 (Ptr ToxA + Ptr ToxC), race 2 (Ptr ToxA), race 3 (Ptr ToxC), race 4 (no known HSTs), race 5 (Ptr ToxB), race 6 (Ptr ToxB + Ptr ToxC), race 7 (Ptr ToxA + Ptr ToxB), and race 8 (Ptr ToxA + Ptr ToxB + Ptr ToxC) (reviewed in Strelkov & Lamari, 2003; Lamari & Strelkov, 2010). Race 4 is avirulent, since it produces no known HSTs or pathogenicity factors, although it carries the *ToxB* homolog, *toxb.*

The majority of *P. tritici-repentis* isolates characterized from western Canada belong to the Ptr ToxA-producing races 1 and 2 (Lamari et al., 1998; Aboukhaddour et al., 2013; MacLean et al., 2017). In contrast, while Ptr ToxB-producing isolates of the fungus are common in some regions (Lamari et al., 1995; Lamari et al., 2005b), they are extremely rare on the Canadian prairies (Lamari & Strelkov, 2010; Aboukhaddour et al., 2013; MacLean et al., 2017). The only race 5 (Ptr ToxB-producing) isolate of *P. tritici-repentis* identified from Canada to date is weakly virulent and possesses fewer copies of *ToxB* than 'wild-type' (highly virulent) race 5 isolates from other regions (Strelkov et al., 2002; Strelkov et al., 2006). The prevalence of Ptr ToxA-producing isolates in Canada could reflect the fact that most historical and current Canadian wheat cultivars are sensitive to Ptr ToxA (Tran et al., 2017), and that sensitivity to Ptr ToxA in Canadian wheat bread cultivars is more common than sensitivity to Ptr ToxB (Lamari et al., 2005a). Nevertheless, 24% of cultivars are sensitive to Ptr ToxB (Tran et al., 2017), and this sensitivity has been present continuously in Canadian wheat for over 70 years (Lamari et al., 2005a). Thus, while sensitivity to Ptr ToxB is less common than sensitivity to Ptr ToxA, it is nonetheless widespread and could represent a risk to Canadian wheat production. In this context, it is important to evaluate whether the scarcity of wild-type,

Ptr ToxB-producing isolates of *P. tritici-repentis* reflects their absence in Canadian populations of the pathogen, or whether the selective advantage conferred by Ptr ToxA is greater than that provided by Ptr ToxB.

In order to distinguish between these possibilities, a quantitative-PCR-based method was developed to measure the abundance of *ToxA*, *ToxB* and *toxb* in wheat leaves inoculated with races 2, 4 and 5 in various combinations. By evaluating the differential abundance of these genes, and fungal biomass in each separate or combined treatment, the virulence and competitiveness of each isolate was compared. In addition, mycelial growth and distribution within inoculated plant tissues were examined by fluorescence microscopy, and foliar symptom development was monitored. The aim of the study was to explore the possible reasons for the rarity of Ptr ToxB-producing isolates in Canada, and to determine if this reflects the existence of a stronger competitor, i.e., Ptr ToxA-producing isolates of *P. tritici-repentis*.

2.2 Materials and methods

2.2.1 Fungal isolates

The *P. tritici-repentis* (Ptr) isolates in this study included 86-124 (race 2), Alg3-24 (race 5), 90-2 (race 4), and Ptf3 (transgenic race 2, as described below). Inoculations were performed using each isolate on its own and with the following combinations of isolates: 86-124 and Alg3-24 (races 2 and 5), 86-124 and 90-2 (races 2 and 4), Alg3-24 and 90-2 (races 5 and 4), 86-124, Alg3-24 and 90-2 (races 2, 5 and 4). Isolates 86-124 and 90-2 were collected from western Canada (Lamari & Bernier, 1989b), whereas Alg3-24 was collected in Algeria (Lamari et al., 1998).

Ptf3 corresponds to the race 2 (86-124) isolate transfected with Ptr ToxB with its encoding gene from race 5 (Alg3-24) (Kim et al., 2014). All isolates were grown on V8-potato dextrose agar (V8-PDA) in 9-cm-diameter Petri dishes, except for Ptf3, which was grown on V8-PDA supplemented with hygromycin (150 µg/mL). Fungal cultures were incubated in darkness at room temperature for 5 days until the colonies reached 4-5 cm in diameter. The cultures were then flooded with sterile distilled (sd)-H_2O, and gently flattened using the bottom of a sterilized glass tube. The excess water was discarded and the cultures were incubated under light for 18 h to induce conidiophore development (Lamari and Bernier, 1989c). The cultures then were transferred to an incubator and maintained in darkness at 15°C to induce formation of conidia. After 24 h, 10 mL of sd-H_2O was added to each culture and the conidial suspension was collected by gently scraping the colony surface with a sterilized wire loop. The collected conidial suspension was quantified with a Fuchs Rosenthal Counting Chamber (Hausser Scientific, Blue Bell, PA) and adjusted to 4000 conidia/mL.

2.2.2 Plant material and inoculation

Four hexaploid wheat genotypes (‘Katepwa’, ‘Glenlea’, ‘Salamouni’, 6B662) were included in the bioassay. ‘Katepwa’ (sensitive to Ptr ToxA and Ptr ToxB) was inoculated with all different treatments (combinations of isolates). ‘Glenlea’ (sensitive to Ptr ToxA), 6B662 (sensitive to Ptr ToxB) and ‘Salamouni’ (insensitive to both Ptr ToxA and Ptr ToxB) were included as controls. Seedlings were grown in Metro Mix 220 potting mixture in 10-cm diameter plastic pots at a density of 10 seedlings per pot (W.R. Grace and Co., Cambridge, MA). The seedlings were maintained in growth cabinets at 20/18°C (day/night) with a 16 h-photoperiod (180 µmol photons/m^2/s) until inoculation at the 2-3

leaf stage. The seedlings were inoculated with a suspension of 4000 conidia/mL supplemented with 1% Tween 20 [polyoxyethylene sorbitol monolaurate] per 1 L using a sprayer connected to an air line (Lamari and Bernier 1989a). When applying inoculum mixtures, conidia of each isolate were mixed in a 1:1 ratio (or 1:1:1 ratio when three isolates were co-inoculated). Negative controls were treated with ds-H2O and the same concentration of Tween 20. Immediately following inoculation, the seedlings were placed in darkness in a misting chamber (relative humidity ≥ 95%) for 24 h. The plants were then transferred to a growth cabinet kept at 20/18 °C (day/night) and a 16 h photoperiod (180 µmol photons/m^2/s) at 60% relative humidity. The second leaf of 1-2 plants selected randomly was harvested by cutting the central portion of each leaf (at least 2 cm from the tip and base) from each of a minimum three pots and subjected to analyses as described below. Leaf samples were collected at 0, 12, 24, 48, and 120 h post-inoculation (hpi). The experiment consisted of two biological replications, each consisting of six technical replicates. The results from the two biological replications are presented. To confirm infection by *P. tritici-repentis*, conidia were collected from the infected leaves and cultured as described by Lamari et al. (1995).

2.2.3 Measurement of the percent diseased leaf area

Groups of 3-4 leaves collected at 0, 12, 24, 48, and 120 hpi were mounted on blue-background cardboard and scanned with an Epson Perfection V19 scanner (Epson, Suwa, Nagano Prefecture, Japan). The resulting images were analyzed with the disease quantification software, ASSESS 2.0 (American Phytopathological Society, St. Paul, MN, USA). For consistency, only the central portion of the scanned leaves was analyzed. The total leaf and lesion areas were measured and the percent leaf area diseased (PLAD) was

calculated by the equation: (leaf area diseased/total leaf area) X 100. Each experiment was replicated and repeated as above. Means and standard deviations were calculated, and one-way and two-way ANOVA were used to detect whether time and/or treatment had an impact on PLAD measurement. Fisher's least significant difference (LSD) pairwise comparison was used to compare the treatments at each time point with RStudio (v. 3.4.3; RStudio Inc, Boston, MA). A simple two-tailed t-test was used to compare the difference in PLAD between wheat genotypes inoculated or co-inoculated with isolates of *P. tritici-repentis* at different time-points.

2.2.4 DNA extraction

DNA samples were extracted from wheat leaves and fungal tissue using a similar procedure. After collection, leaf samples were frozen at -80˚C and lyophilized. Once dried, the samples were homogenized with a TissueLyserII (QIAGEN, Hilden, Germany). DNA was isolated from 40 mg of leaf tissue with a Wizard® Genomic DNA Extraction Kit (Promega Corp, Madison, WI) following the manufacturer's instructions. DNA was further purified by two extractions with phenol:chloroform: isoamyl alcohol (25:24:1, v/v) followed by one extraction with chloroform:isoamyl alcohol (24:1, v/v). To isolate DNA of *P. tritici-repentis*, the fungus was grown under continuous agitation at room temperature in liquid Fries medium (Dhingra & Sinclair, 1986) containing 1.5% yeast extract. After three weeks, the mycelial mats were collected and lyophilized. Forty milligrams of each lyophilized isolate was used for DNA extraction as described above. DNA quantity and quality were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.2.5 Preparation of TaqMan probe and primers

All primers and probes used in quantitative PCR (q-PCR) analysis were designed with Primer Express Software (Applied Biosystems, Foster City, CA). Primers and probes specific to *ToxB* and *toxb* were designed to distinguish isolate 90-2 (race 4) from isolate Alg3-24 (race 5), respectively. The forward 90-2-F (5'-GGTCTATTCTGGAGCTCTTTTTGC-3') and reverse primers 90-2-R (5'-TTCCTTTTATCGAAAGTTTAGCATTG-3') were developed based on the unique sequence found of *toxb* in race 4 (R. Aboukhaddour, University of Alberta, unpublished data). Since the original sequence data were preliminary, these primers were used to amplify and re-sequence the amplicon obtained from race 4 isolate 90-2. The reaction conditions consisted of 1 min at 93°C, followed by 25 cycles of 1 min at 93°C, 1 min at 55°C, 3 min at 70°C, and a final extension of 5 min at 70°C (R. Aboukhaddour, unpublished data). The PCR products were visualized on a 1% agarose gel and purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The samples were sequenced on a 3730 DNA Analyzer (Thermo Fisher Scientific, Waltham, MA) at the Department of Biological Sciences, University of Alberta. The sequence obtained (5'-TATTCTGGAGCTCTTTTTGTTCACGTGCCACAGCTTGCTAACGCAAAAGTC AACGTTCTAAACAATGCTAAACTTTCGATAAAAGGAACTCTAACTCTAATCCTA TTTCTCTACCAATGACGA-3') was then used to design race 4-specific forward primer R4-F (5'-TTGTTCACGTGCCACAGCTT-3') and reverse primer R4-R (5'-TTCCTTTTATCGAAAGTTTAGCATTG-3') for q-PCR analysis, which amplified a 69 bp product. These primers were used in conjunction with the TaqMan minor groove-binding (MGB) probe R4-P (5'-CTAACGCAAAAGTCAACG-3').

The primers and probe for quantifying *ToxB* were based on publicly available sequence information (GenBank Accession no. AY243460.2). The forward R5-F (5'-CCTGCTGCCCTTGTTTCG-3') and reverse primers R5-R (5'-ACCGCTTCGTTGATGTTTAAGATAT-3') amplified a 59 bp product; the sequence of the MGB probe R5-P was 5'-CCAACTGCGTCGCC-3'. Similarly, the *ToxA*-specific primers and probe also were developed based on publicly available sequence information (GenBank Accession no. HM234160.1). The forward primer R2-F (5'-GGGAAGCTGCATGTCAATCA-3') and reverse primer R2-R (5'-TGGCCGATGTTGTTGACAGA-3') amplified a 60 bp product, which was used with the MGB probe R2-P (5'- AATCAACCCTAGTCGTCC-3') in the q-PCR analysis.

An additional primer and probe set was designed based on a partial chitin synthase gene sequence (*chitin synthase 4*; GenBank Accession no. XM_001937866.1). The forward primer chi-F, 5'- TGCCGCCTCCAAGACTTC-3' and reverse primer chi-R, 5'-AATCTGCTGCTTTCTCTTCTTCGA-3' amplified a 58 bp product, and were used in conjunction with the TaqMan MGB probe chi-P, 5'- TTGGGCCCGGTTGA-3'. The specificity of all primers was confirmed by conventional PCR assays with DNA extracted from fungal mycelial mat of the isolates used in this experiment.

2.2.6 Quantitative-PCR analysis

Total DNA from each sample was analyzed in 96-well reaction plates using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Reaction mixtures consisted of 5 µL TaqMan Universal Master Mix (Applied Biosystems), 0.1 µL each of 50 µM forward and reverse primers, 0.03 µL fluorogenic probe (100 µM), 4 µL (60 ng) template DNA sample, and 0.7 µL diethyl pyrocarbonate (DEPC)-treated water.

Reactions conditions included 2 min at 50°C, 10 min at 95°C, and 40 cycles of 10 s at 95°C and 30 s at 60°C.

The standard curve method (Real-time PCR Handbook, 2014, Thermo Fisher Scientific) was used to calculate the amount of DNA for each of the four genes of interest (*ToxA*, *ToxB*, *toxb*, and *chitin synthase 4*) in a StepOnePlus Real-Time PCR System (Applied Biosystem). A standard curve of *P. tritici-repentis* DNA was created via serial dilutions starting at 1.25 ng (1.25 ng, 0.625 ng, 0.313 ng, 0.156 ng, 0.078 ng, 0.039 ng, 0.019 ng, 0.0098 ng). The quantity of the amplified fungal DNA was measured according to each corresponding standard curve based on its threshold value (C_t), and presented in ng per gram of inoculated and lyophilized host tissue (Cao et al., 2014). The amount of DNA per gram of leaf tissue was estimated at 0, 12, 24, 48, and 120 hpi for each treatment. Two-way analysis of variance (ANOVA) was used to determine whether time and/or treatment had an impact on the amount of DNA. Fisher's Least Significant Difference (LSD) pairwise comparison was used to compare the treatments at each time point with RStudio v. 3.4.3 (RStudio Inc, Boston, MA). Statistical analysis was applied on the means of two biological replicates and results shown are the average of the total 12 technical replicates.

2.2.7 Microscopy analysis

Fungal growth and colonization of host tissues were examined by fluorescence microscopy following staining with fluorescein isothiocyanate wheat germ agglutinin (WGA-FITC) (Ayliffe et al., 2011; Manning & Ciuffetti, 2015). Briefly, at least three leaf segments harvested as described above were placed in 15 mL Falcon tubes (Froggabio, North York, ON) containing 1 M KOH and 0.1% Tween 20 (Anachemia Canada, Inc.;

Lachine, QC/ Thermo Fisher Scientific; Hampton, NH). The leaf segments were autoclaved at 105˚C, 1 min (maximum pressure 0.186 MPa), washed in 1-2 mL of 50 mM Tris·HCL pH 7.5 (Thermo Fisher Scientific), and immersed in 10 mL 50 mM Tris·HCL pH 7.5 for 20 min. The solution was discarded and the leaf tissue transferred to small tubes containing 500 µL of 20 µg/mL WGA-FITC (Sigma-Aldrich). The samples were stained overnight in darkness at room temperature, and then rinsed gently with 50 mM Tris·HCL pH 7.5. The stained leaf segments were placed carefully on glass microscope slides (one segment per slide) and examined with a Zeiss Axioimager M1 microscope (Carl Zeiss AG, Oberkochen, Germany) using a AF488 fluorescence channel (excitation wavelength 493 nm; emission wavelength 517 nm). Images were captured with an Axiocam 506 mono camera (Carl Zeiss AG). Each sample unit consisted of three replicates for each of the two runs of the experiment.

2.3 Results

2.3.1 Symptom development

Tan spot symptom development on inoculated wheat leaves was assessed visually and by calculating the PLAD after image analysis with ASSESS 2.0. Both chlorosis and necrosis symptoms were observed on 'Katepwa' (sensitive to Ptr ToxA and Ptr ToxB) following inoculation with *P. tritici-repentis* isolate Ptf3 (Fig. 2-1). However, although Ptf3 represents the isolate 86-124 (Ptr ToxA^{+}) transformed to produce Ptr ToxB as well as Ptr ToxA, most of the lesions it caused on 'Katepwa' were necrotic (Fig. 2-1). The average PLAD at 120 hpi was 64.09% (Fig. 2-2). When the same cultivar was inoculated with 86-124, lesions also were necrotic (Fig. 2-1) with an average PLAD of 74.29% (Fig. 2-2). In contrast, inoculation with isolate Alg3-24 (Ptr ToxB^{+}) induced chlorotic lesions

on ‘Katepwa’ (Fig. 2-1), and the PLAD (52.41%) was significantly lower (P<0.05) than that observed with 86-124 (Fig. 2-2). As expected, inoculation with the avirulent race 4 isolate 90-2 (no HSTs produced) resulted in almost no lesion development on ‘Katepwa’ leaves (Fig. 2-1), with an average PLAD of <1%. This was almost identical to the reaction of the highly resistant ‘Salamouni’ to inoculation with this same isolate. This cultivar, which is insensitive to Ptr ToxA and Ptr ToxB, also was resistant to the isolates Ptf3, 86-124 and Alg3-24 (Fig. 2-1 and Fig. 2-2). ‘Salamouni’ developed only small dark-brown flecks, typical of a resistance response, following inoculation with Ptf3 and 86-124, and only a few small chlorotic flecks in response to Alg3-24. The wheat line 6B662, which is sensitive to Ptr ToxB but not Ptr ToxA, developed extensive chlorosis following inoculation with Alg3-24 (Ptr ToxB+), but much more restricted chlorosis symptoms following inoculation with Ptf3 (mainly around a central necrotic fleck in each lesion). The reaction of 6B662 to isolate Ptf3 resembled that to 86-124, with leaf yellowing even more limited and confined to a narrow halo around the necrotic flecks (Fig. 2-1).

Co-inoculation of ‘Katepwa’ with isolates 86-124 and Alg3-24 resulted in a PLAD (51.62%) that was not significantly different from that observed when this cultivar was inoculated with Alg3-24 by itself, but which was lower than the PLAD observed in response to inoculation with 86-124 on its own (Fig. 2-2). Similarly, when ‘Katepwa’ was co-inoculated with 86-124 and the avirulent isolate 90-2, the PLAD (30.82%) was significantly lower than when it was inoculated with 86-124 alone. Co-inoculation of Alg3-24 with the avirulent 90-2 resulted in a significantly lower PLAD (5.38%) than when ‘Katepwa’ was inoculated only with the former. The combined inoculation with three isolates (86-124 + Alg3-24 + 90-2) resulted in a PLAD (37.31%) that was similar to

that obtained following co-inoculation with 86-124 + 90-2 or with 86-124 + Alg3-24 (Fig. 2-2).

Symptom development on 'Katepwa' also was analyzed over a time-course at 0, 12, 24, 48, and 120 hpi (Fig. 2-3). The presence of developing lesions first became visible at 48 h, with the appearance of weakly chlorotic, localized spots in response to inoculation with isolates Ptf3, 86-124 and Alg3-24 (Fig. 2-3). By 120 hpi, the foliar lesions had expanded significantly and were coalescing. In response to inoculation with the Ptr ToxA-producing isolates 86-124 and Ptf3, these lesions were mainly necrotic and surrounded by restricted chlorotic haloes (Fig. 2-3). In contrast, the lesions induced by Alg3-24 (Ptr ToxB+) were predominantly chlorotic, with this chlorosis occasionally extending out from small necrotic centers. Although Ptf3 had been transformed to produce Ptr ToxB as well as Ptr ToxA (Kim et al., 2014), it induced symptoms that qualitatively most closely resembled those caused by 86-124 (Fig. 2-3). Symptoms caused by co-inoculation with 86-124 and Alg3-24 were similar those caused by either isolate alone up to 48 hpi, but at 120 hpi more chlorosis was evident following co-inoculation with Alg3-24 that following inoculation with 86-124 on its own. The severity of symptoms, as expressed by PLAD, also was similar in response to all isolates up to 48 hpi. At 120 hpi, however, PLAD was greatest in response to inoculation with 86-124 (67.79%) followed by Ptf3 (57.60%) and Alg3-24 (29.42%) (Fig. 2-4). Co-inoculation with 86-124 and Alg3-24 resulted in a PLAD (35.92%) that was not significantly different than that obtained with Alg3-24 alone. No distinct symptoms were visible in response to inoculation with the avirulent isolate 90-2 at any time (Fig. 2-3) and PLAD remained at trace levels (Fig. 2-4).

2.3.2 Gene quantification

The abundance of the *ToxA*, *toxb* and *ToxB* genes was monitored to estimate selectively the growth and proliferation of isolates representing races 2 (Ptr ToxA^{+}), 4 (avirulent) and 5 (Ptr ToxB^{+}), respectively, of *P. tritici-repentis* in 'Katepwa' leaf tissue. In inoculations or co-inoculations that included Ptr ToxA-producing isolates (Ptf3 and 86-124), the amount of *ToxA* DNA generally increased as infection progressed by time (Fig. 2-5). When either Ptf3 or 86-124 was inoculated on its own, there was a small increase in the quantity of *ToxA* detected from 0 to 12 hpi, followed by a partial decline at 24 hpi, and then a continued and significant increase until the final time-point at 120 hpi. This pattern changed when 86-124 was co-inoculated with Ptr ToxA-non-producing isolate(s) (Alg3-24 and/or 90-2). In these treatments, the quantity of *ToxA* remained fairly low and constant up to 48 h, increasing significantly only at 120 hpi. Nonetheless, at that time-point, the quantity of this gene in the co-inoculation treatments was less than half that observed when Ptf3 or 86-124 was inoculated alone. The *ToxA*-specific primers and probe did not amplify any target when 'Katepwa' was inoculated only with Alg3-24, as would be expected for an isolate that does not carry the *ToxA* gene (not shown).

In q-PCR analysis to quantify the amount of *ToxB*, its abundance was found to be greatest following inoculation with the transformed isolate Ptf3, at all time-points examined (Fig. 2-6). As was observed with *ToxA*, there was a small increase in the quantity of *ToxB* from 0 to 12 hpi, followed by a partial decline at 24 hpi, and then a continued and significant increase until 120 hpi. The amount of *ToxB* measured after inoculation with Ptf3 was significantly greater than all other treatments, including the race 5 (Ptr ToxB^{+}) isolate Alg3-24. Nonetheless, the only other significant increase in *ToxB* over the time-course was observed with Alg3-24, where the abundance of the gene

increased significantly at 120 hpi. When Alg3-24 was co-inoculated with the Ptr ToxB-non-producing isolates 86-124 or 90-2, there was no significant increase in *ToxB* at any time-point, not even at 120 hpi. As expected, *ToxB* could not be quantified in leaves inoculated with 86-124 (*ToxB*$^{-}$) alone (not shown).

Measurements of *toxb* showed that it was never as abundant in host tissue as *ToxA* or *ToxB* (it was an order of magnitude lower, when peak amounts were compared) (Fig. 2-7). The amount of *toxb* measured over the time-course also was quite distinct from *ToxA* and *ToxB*. When isolate 90-2 was inoculated on its own, the abundance of *toxb* increased rapidly between 0 and 24 hpi, peaked at 24 and 48 hpi, and then declined back to almost baseline levels at 120 hpi. Co-inoculation of 90-2 with Alg3-24 or 86-124 + Alg3-24 significantly reduced this initial increase. In the case of co-inoculation with 90-2 + Alg3-24, there were still significant increases detected at 12 and 24 hpi, but the amount of *toxb* was about a third of that measured in the 90-2 treatment. In the 86-124 + Alg3-24 + 90-2 treatment, the only significant increase in *toxb* was observed at 12 hpi. There were no significant increases found when 90-2 was co-inoculated only with 86-124.

Trends in the proliferation and abundance of *P. tritici-repentis* DNA across all isolates were monitored by measurements of *chitin synthase 4* (*CHS4*), which is conserved between races of the fungus. The abundance of this gene was greatest at 120 hpi in all treatments except inoculation with 90-2 on its own, in which no significant increase was detected at any time-point (Fig. 2-8). In general, co-inoculation of 86-124 with Alg3-24 and/or 90-2 resulted in a decrease in total *CHS4* at 120 hpi relative to 86-124 alone. Similarly, co-inoculation of Alg3-24 with 90-2 decreased the abundance of *CHS4* at this time-point, relative to Alg3-24 on its own. In contrast, co-inoculation of

Alg3-24 with both 90-2 and 86-124 did not have such a negative influence on the abundance of this gene (Fig. 2-8).

2.3.3 Microscopy analysis

Fungal mycelium was stained *in planta* with a WGA-FITC fluorescent conjugate, which binds to chitin in the fungal cell walls. As expected, no fungal development or hyphae were observed in leaf tissue sampled at 0 hpi (not shown). By 12 and 24 hpi, however, the conidia had germinated, with germ tubes visible and limited colonization of leaf tissue; at these time-points, no differences were evident between any of the treatments (Fig. 2-9). At 48 hpi, limited colonization of the host tissues was observed following inoculation with isolates Ptf3, 86-124 or Alg3-24, with some hyphae spreading intercellularly in the mesophyll. In contrast, growth by 90-2 still was confined mainly to the leaf surface at 48 hpi, in the form of numerous long germ tubes emerging from the conidia. By 120 hpi, colonization of the host tissue was extensive for Ptf3 and 86-124, with proliferation of intercellular hyphae evident in the mesophyll. Growth of Alg3-24, however, remained much more limited, and generally was restricted to the vicinity of the penetration point. There was little if any ingress of 90-2 into inoculated tissues, although what appeared to be intracellular vesicles were observed occasionally (Fig. 2-9).

In general, the infection process when leaf tissues were co-inoculated with isolate mixtures was variable, with fungal development resembling one or the other of the isolates included in the inoculation, depending on the particular conidium examined (Fig. 2-10). This likely reflected the origin of the specific conidia, since individual conidia in the mixture represented 86-124, Alg3-24 or 90-2, and the WGA-FITC staining method cannot distinguish between different isolates of *P. tritici-repentis*. No novel colonization

patterns or obvious interactions between isolates were observed in the co-inoculated treatments.

2.4 Discussion

Within-host competition between fungal pathogens is common in many pathosystems (Koskella et al., 2006; Gold et al., 2009; Seemuller et al., 2010). A typical case of within-host competition, the selective sweep, occurs when a more competitive pathogen out-competes and gradually takes the place of another less virulent pathogen when the two occur on the same host (Nowak & May, 1994; Zhan & McDonald, 2013). Studies of within-host competition were conducted on *Puccinia graminis titici* (Osoro & Green, 1976) and *Puccinia striiformis* (Brown & Sharp, 1970), where the results showed that the isolate that possessed more virulence genes predominated over races with fewer virulence genes. In the tan spot pathosystem, the question of competitive ability was examined by Rezaey (2009), who inoculated wheat genotypes with a mixture of isolates representing the eight races of *P. tritici-repentis*, and then conducted conventional end-point PCR analysis with *ToxA* and *ToxB*-specific primers on a set of recovered isolates. While Rezaey (2009) was able to recover $ToxA^{+}$ and $ToxB^{+}$ isolates across all generations from susceptible and resistant cultivars, the ability to recover an isolate may not provide a full indication of its competitive ability, especially under growth room conditions, since this measure does not reflect total biomass or relative abundance. In the present study, microscopy, q-PCR analysis and evaluations of symptom development and severity were used together to compare the *in planta* proliferation of isolates representing different races of *P. tritici-repentis*.

When isolates were inoculated on their own, the symptoms they induced were consistent with what was expected for each race-cultivar interaction, reflecting the interaction of the HSTs produced and the matching sensitivity genes in the host (Lamari and Strelkov, 2010; Faris et al., 2013). Qualitatively, however, the symptoms caused by Ptf3 (transformed to produce Ptr ToxB; Kim et al., 2014) on the Ptr ToxB-sensitive hosts 'Katepwa' and 6B662 most closely resembled the non-transformed isolate 86-124 (Ptr ToxA^{+}, Ptr ToxB^{-}), from which Ptf3 was generated. While Ptf3 still carried *ToxB*, as was evident from the q-PCR analysis, it nonetheless caused much weaker chlorosis than the wild-type Ptr ToxB-producing isolate Alg3-24, both in this study and when it was first described (Kim et al., 2014). Originally, despite the weaker chlorosis-inducing activity of Ptf3, it was found to produce the Ptr ToxB protein (Kim et al., 2014). Since the level of *ToxB* expression or Ptr ToxB protein product were not measured in the current study, however, it is not possible to rule out that reduced transcription and/or translation contributed to the weak chlorosis-inducing activity. On 'Katepwa', which is sensitive to both Ptr ToxA and Ptr ToxB, it is also possible that the reaction to the former may have masked the reaction to the latter.

More generally, PLAD was greatest in response to inoculation with 86-124 and Ptf3. This would suggest that Ptr ToxA-producing isolates, and 86-124 in particular, were more aggressive than the Ptr ToxB-producing Alg3-24. Necrosis symptoms caused by Ptr ToxA generally develop more quickly (14-48 h) (Pandelova et al., 2009, 2012) than those caused by Ptr ToxB (48-72 h) (Strelkov et al., 1999), and it is likely that more rapid death of host tissues would facilitate colonization by this necrotrophic fungus. Indeed, the microscopy analysis indicated greater proliferation of 86-124 and Ptf3 than Alg3-24 in host tissues, at least at 120 hpi. Although fungal sporulation *in planta* was not assessed in

this study, it also is reasonable to hypothesize that quicker and more rapid colonization would facilitate greater sporulation. In many necrotrophic pathogens, the uptake of nutrients and initiation of the production of the next generation largely depend on the destruction of host tissues (Anderson & May, 1979, 1991; May & Anderson, 1979, 1983). Nonetheless, greater virulence can be associated with lower transmission efficiency in some parasites (Levin & Pimentel, 1981), and so it may be important to compare sporulation rates in future studies.

While more rapid and severe symptoms were associated with inoculation of 'Katepwa' with 86-124, it is notable that co-inoculation with other isolates significantly reduced PLAD. Co-inoculation of 86-124 with Alg3-24 resulted in a PLAD similar to that obtained by inoculation with Alg3-24 alone, while inoculation with the avirulent isolate 90-2 reduced PLAD even further. Similarly, co-inoculation of Alg3-24 with 90-2 resulted in a significant decline in PLAD relative to inoculation with Alg3-24 alone. These results suggest some competition or negative interaction from co-inoculation between isolates, especially when a more aggressive isolate is inoculated together with a less aggressive (or avirulent) isolate. This has been observed in other pathosystems. For instance, in chestnut blight, it was reported that the disease was reduced when the hypovirulent strain of *Cryphonectria parasitica* was co-inoculated with the wild-type strain on the same hosts (reviewed in Heiniger & Rigling, 1994). In the case of *P. tritici-repentis*, it is possible that isolates compete for limited nutrients or space on the host itself. There also have been suggestions that this fungus produces compounds, such as the anthroquinone catenarin, to inhibit the growth of competing microorganisms (Wakuliński et al., 2003), although in the microscopy analysis the growth of individual colonies of virulent isolates did not appear to differ when co-inoculated with less virulent

isolates. While resistance to *P. tritici-repentis* is regarded mainly as passive (Strelkov & Lamari, 2003), it also is possible that co-inoculation with the avirulent isolate 90-2 helped to induce defense responses in the host, thereby reducing the extent of disease development. Active defense against the tan spot pathogen has been observed in previous studies, with some evidence of papilla formation and/or lignification near the appressorium in wheat hosts (Larez et al., 1986; Dushnicky et al., 1998a, 1998b).

The q-PCR analysis provided a wider indication of fungal proliferation in the wheat leaves than could be obtained by microscopy, but yielded results that were generally consistent with the evaluation of symptom development and the microscopic examination. Interestingly, a small peak in the amount of *ToxA*, *ToxB* and *CHS4* DNA was detected at 12 h following inoculation with some isolates, followed by a decline at 24 hpi prior to continued increases at 48 and 120 hpi. This may have reflected the presence, at 12 hpi, of conidia on the leaf surface that had not successfully penetrated the host tissue. Conidia of *P. tritici-repentis* germinate soon after landing on the leaf surface (Larez et al., 1986; Dushnicky et al., 1996), and these could have contributed to the fungal DNA detected at earlier time-points. As time progressed, the contribution of non-germinated and/or non-penetrating conidia to total DNA would have declined, and the increases observed at 48 and 120 hpi would have more accurately reflected fungal colonization of host tissues. Most fungi produce adhesive compounds to help the spores attach to the leaf surface firmly at the early infection stages (Edwards & Allen, 1970; Nicholson, 1984; Hamer et al., 1988). These adhesive compounds, however, cannot be produced by conidia continuously without successful penetration into the host tissue. A study of the pre-penetration process of *Magnaporthe oryzae* indicated that a spore tip mucilage was found in conidia once they landed on the leaf surface (Wilson & Talbot, 2009). After

germination, however, the mucilage did not provide sufficient adhesion force. It is possible that in the current study, similar adhesive compounds were no longer produced between 12 to 24 hpi, resulting in the conidia of *P. tritici-repentis* falling off the leaf surface. The transient increase in the amount of *toxb* DNA observed with 90-2 also likely reflected this phenomenon; however, in the case of 90-2, no further increases in DNA were detected at later time-points, since little if any host colonization occurred with this isolate.

The q-PCR analysis also indicated greater proliferation of 86-124 and Ptf3 in the host than either Alg3-24 or 90-2. This was evident when comparing the quantities of *ToxB* following inoculation with Ptf3 vs. Alg3-24. The former was transfected with more than 12 copies of *ToxB* (Kim et al., 2014), while Alg3-24 is estimated to have 8-10 copies of the gene (Martinez et al., 2004; Strelkov et al., 2006). Nonetheless, there was ~6-fold more *ToxB* quantified in Ptf3 than in Alg3-24, suggesting much greater growth by Ptf3 in the inoculated leaves. Similarly, there was significantly more *CHS4* quantified following inoculation with 86-124 and Ptf3 than with Alg3-24. These findings are consistent with the microscopy analysis and again suggest greater colonization of host tissue by the Ptr ToxA-producing isolates vs. the Ptr ToxB-producer. The low levels of *toxb* and *CHS4* found following inoculation with 90-2 were not surprising, given the avirulent nature of this isolate. Co-inoculation of 86-124 with Alg3-24 and/or 90-2, or Alg3-24 with 90-2, was associated with declines in *ToxA*/*ToxB*/*CHS4* relative to inoculation of each of the more virulent isolates alone, further indicating negative interactions or competition between isolates.

The results of this study showed that Ptr ToxA-producing isolates of *P. tritici-repentis* colonized host tissue more extensively and produced more severe tan spot

symptoms than did the Ptr ToxB-producing isolate Alg3-24. This implies that in terms of damage caused on susceptible wheat, races of the fungus that can produce Ptr ToxA may represent a greater threat than those that produce only Ptr ToxB. As such, Ptr ToxA may have greater epidemiological significance than Ptr ToxB for *P. tritici-repentis*. Nonetheless, the observation that co-inoculation of races 2, 5 and/or 4 resulted in reductions in symptom severity and total fungal biomass (as reflected by the quantity of *ToxA*/*ToxB*/*CHS4*), indicates that avirulent (race 4) or less virulent races (race 5) may compete to some extent with the more virulent races (race 2) when these occur together. Moreover, even limited colonization by Alg3-24 resulted in fairly significant chlorosis symptoms on susceptible wheat. As such, while Ptr ToxA-producing isolates of the fungus may represent the greatest risk to wheat production in Canada, the possible occurrence and impact of Ptr ToxB-producing isolates should not be ignored.

2.5 References

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2.6 Figures

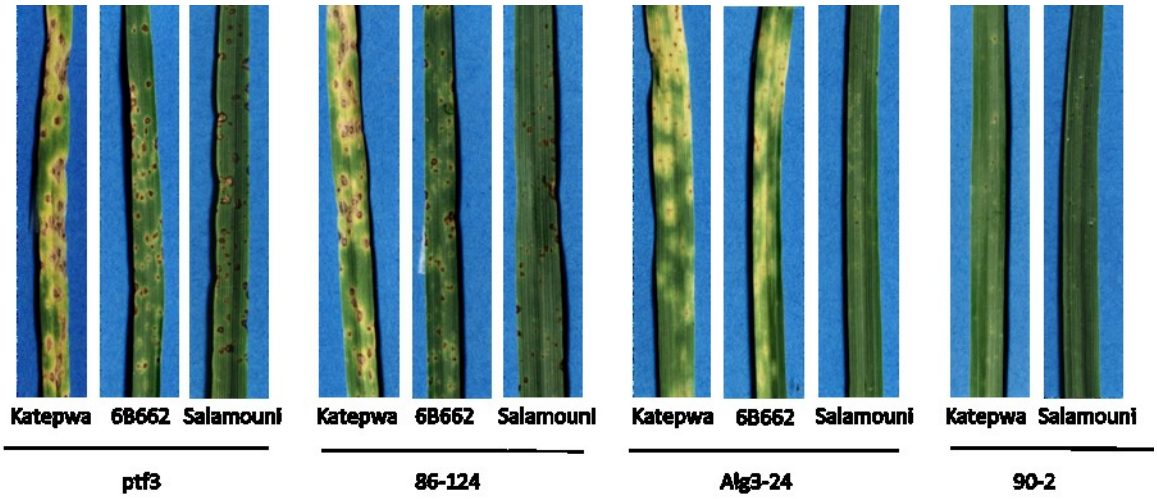

Figure 2-1. Reaction of wheat genotypes 120 hours post-inoculation with the *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA^{+}, Ptr ToxB^{+}), 86-124 (Ptr ToxA^{+}), Alg3-24 (Ptr ToxB^{+}), and 90-2 (avirulent). The wheat genotype 'Katepwa' is sensitive to both Ptr ToxA and Ptr ToxB, line 6B662 is sensitive to Ptr ToxB, and 'Salamouni' is insensitive to both Ptr ToxA and Ptr ToxB.

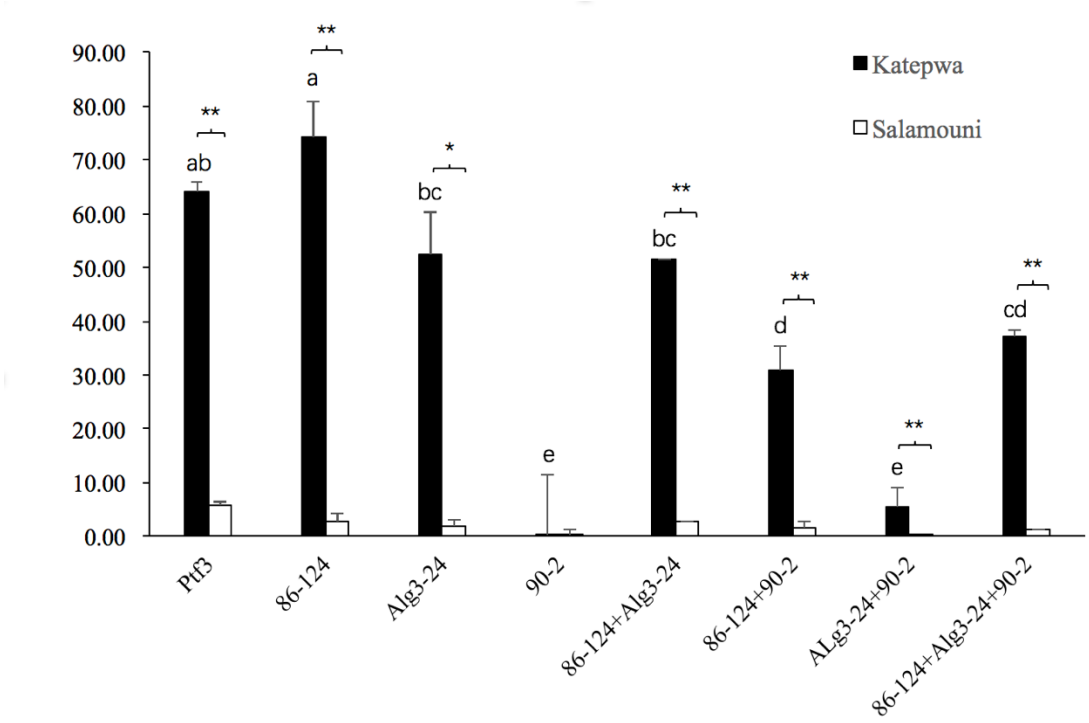

Figure 2-2. Percent leaf area diseased (PLAD) in the tan spot-susceptible wheat genotype 'Katepwa' and the resistant genotype 'Salamouni' 120 hours post-inoculation with isolates Ptf3 (Ptr ToxA$^+$, Ptr ToxB$^+$), 86-124 (Ptr ToxA$^+$), Alg3-24 (Ptr ToxB$^+$), and 90-2 (avirulent) of *Pyrenophora tritici-repentis*, alone or in combination. The means of two biological replicates are shown. Error bars represent the standard error of the mean. Different letters indicate significant differences across treatments based on Fisher's Least Significant Difference (LSD) (LSD = 18.04587). A simple two-tailed t-test was used to compare PLAD between the susceptible and resistant genotype in each treatment (*P < 0.05; **P < 0.01).

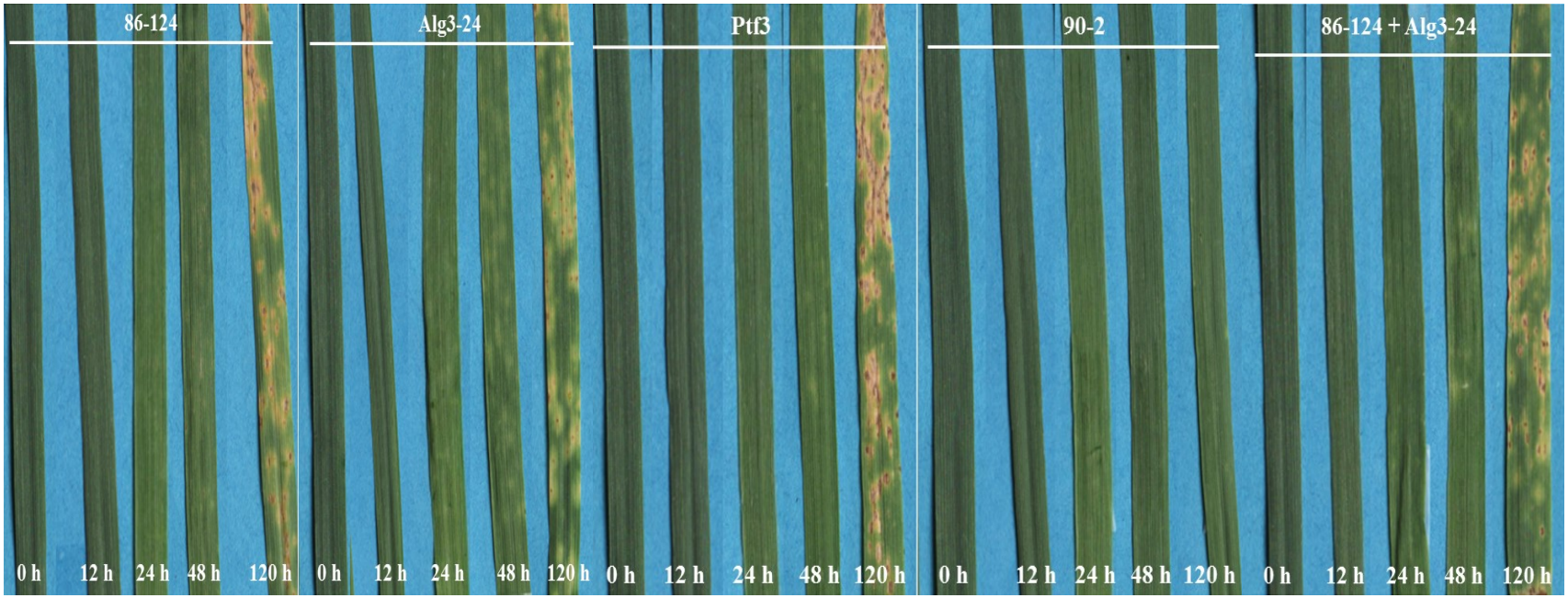

Figure 2-3. Leaves of the wheat genotype 'Katepwa' at different times following inoculation with *Pyrenophora tritici-repentis* isolates 86-124 (Ptr ToxA+), Alg3-24 (Ptr ToxB+), Ptf3 (Ptr ToxA+, Ptr ToxB+), and 90-2 (avirulent), or a combination of isolates 86-124 + Alg3-24.

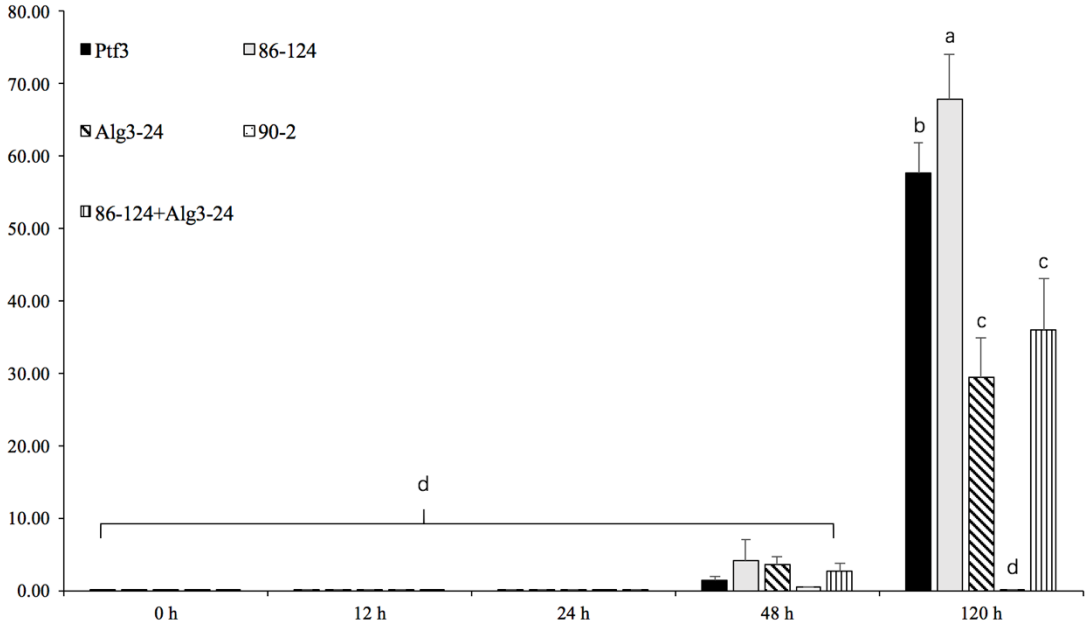

Figure 2-4. Percent leaf area diseased (PLAD) in the tan spot-susceptible wheat genotype 'Katepwa' at different times following inoculation with *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA$^+$, Ptr ToxB$^+$), 86-124 (Ptr ToxA$^+$), Alg3-24 (Ptr ToxB$^+$), and 90-2 (avirulent), or a combination of 86-124 + Alg3-24. Error bars indicate the standard error of the mean. Different letters indicate significant differences across treatments based on Fisher's Least Significant Difference (LSD) (LSD = 5.613083).

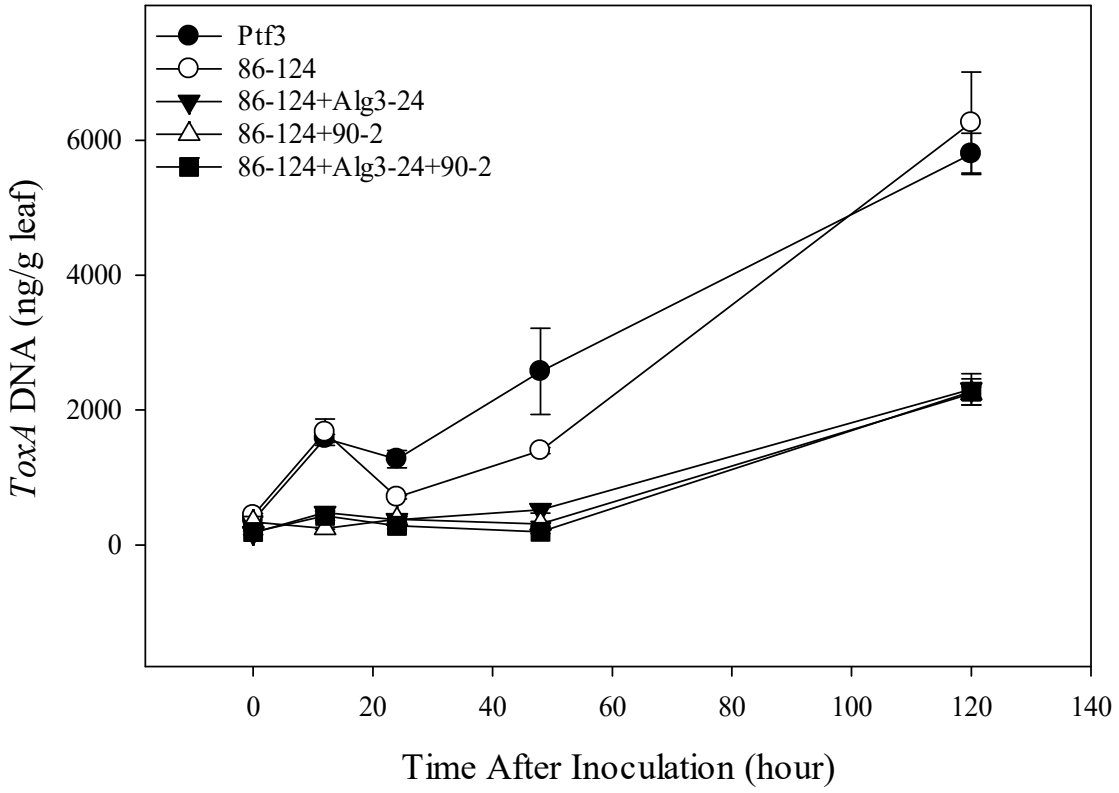

Figure 2-5. Quantification of the *ToxA* gene in 'Katepwa' wheat leaf tissue at various times following inoculation with *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA$^+$, Ptr ToxB$^+$) or 86-124 (Ptr ToxA$^+$), or combinations of 86-124 + Alg3-24, Alg3-24 + 90-2, and 86-124 + Alg3-24 + 90-2. Data points represent the mean of two biological replicates +/- standard error of the mean.

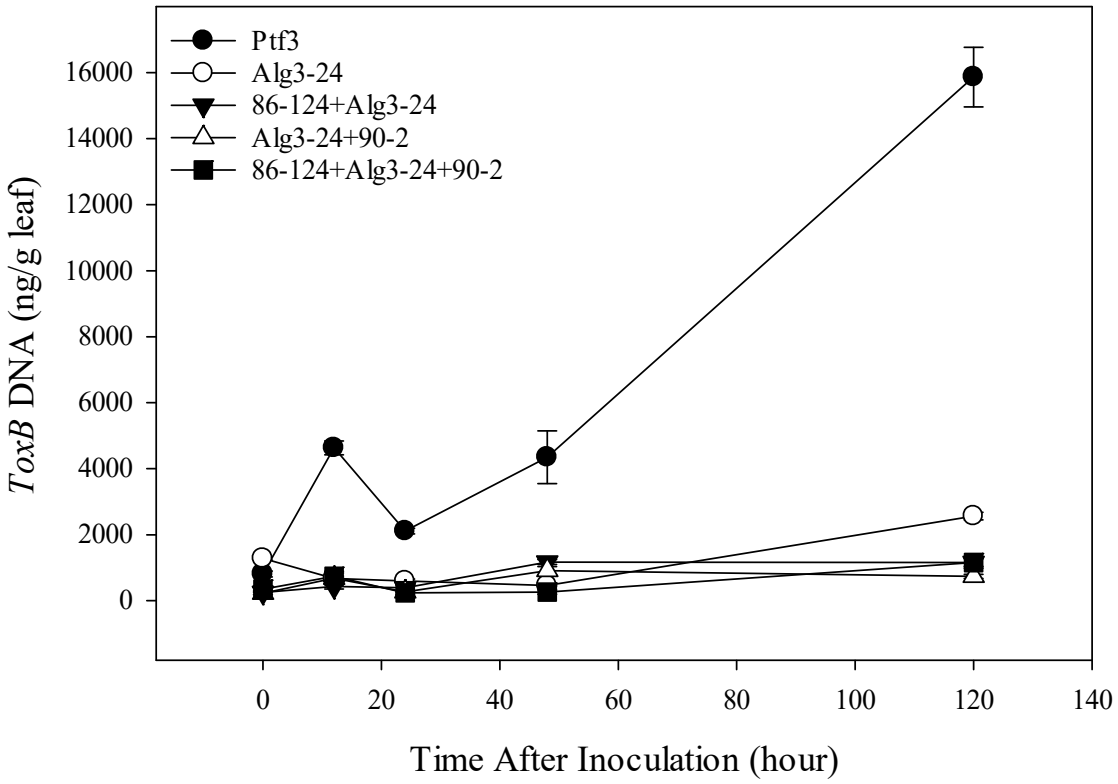

Figure 2-6. Quantification of the *ToxB* gene in 'Katepwa' wheat leaf tissue at various times following inoculation with *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA$^+$, Ptr ToxB$^+$) or Alg3-24 (Ptr ToxB$^+$), or combinations of 86-124 + Alg3-24, Alg3-24 + 90-2, and 86-124 + Alg3-24 + 90-2. Data points represent the mean of two biological replicates +/- standard error of the mean.

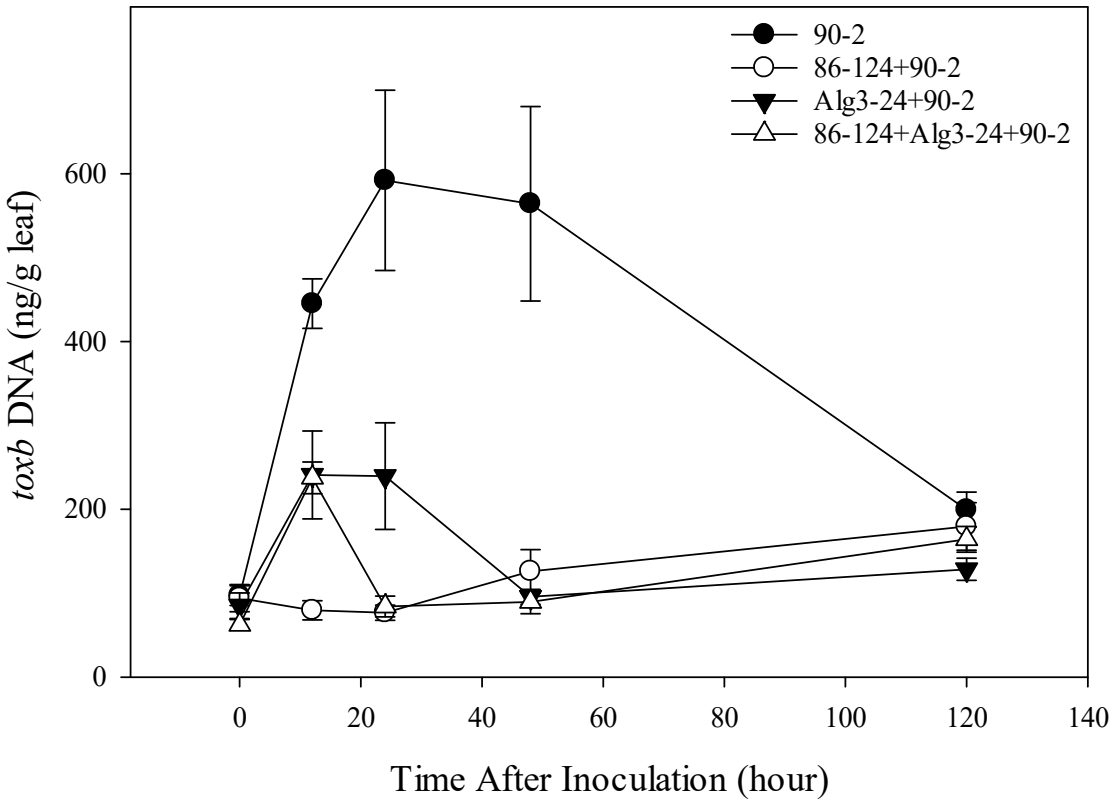

Figure 2-7. Quantification of the *toxb* gene in 'Katepwa' wheat leaf tissue at various times following inoculation with *Pyrenophora tritici-repentis* isolate 90-2 (avirulent), or combinations of 86-124 + Alg3-24, Alg3-24 + 90-2, and 86-124 + Alg3-24 + 90-2. Data points represent the mean of two biological replicates +/- standard error of the mean.

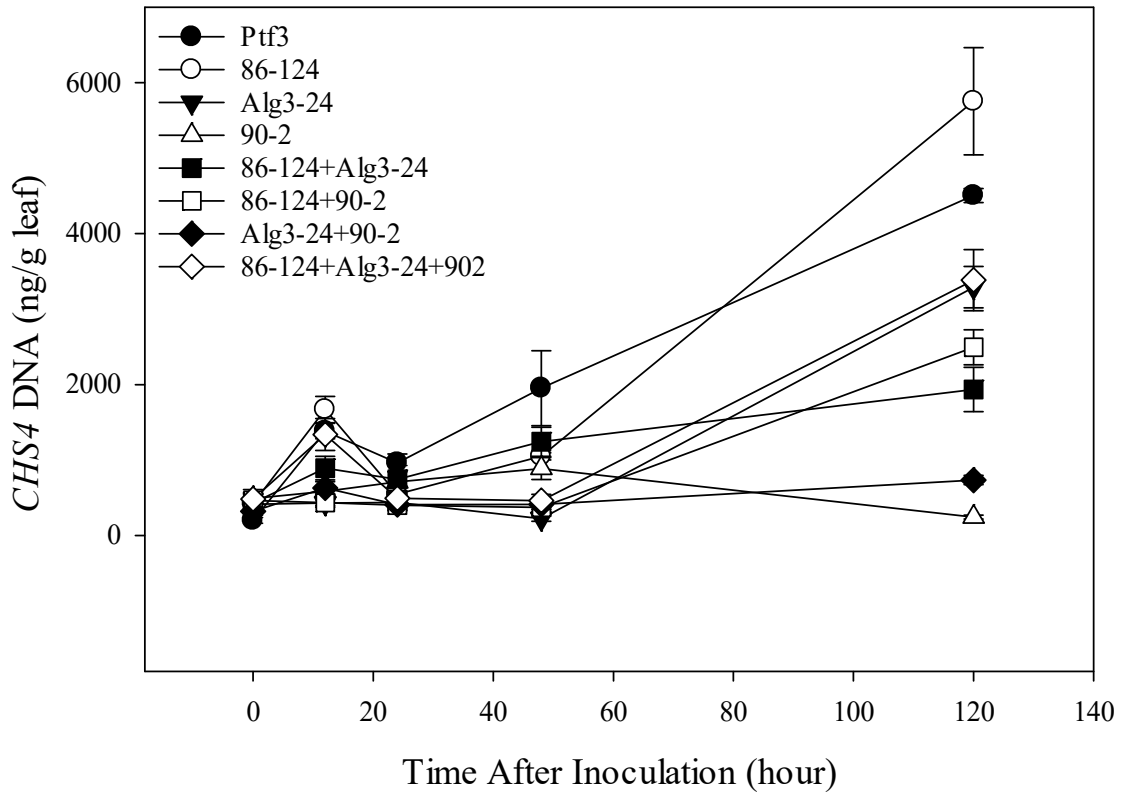

Figure 2-8. Quantification of *chitin synthase 4* in 'Katepwa' wheat leaf tissue at various times following inoculation with *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA$^+$, Ptr ToxB$^+$), 86-124 (Ptr ToxA$^+$), Alg3-24 (Ptr ToxB$^+$), 90-2 (avirulent) or combinations of 86-124 + Alg3-24, Alg3-24 + 90-2, and 86-124 + Alg3-24 + 90-2. Data points represent the mean of two biological replicates +/- standard error of the mean.

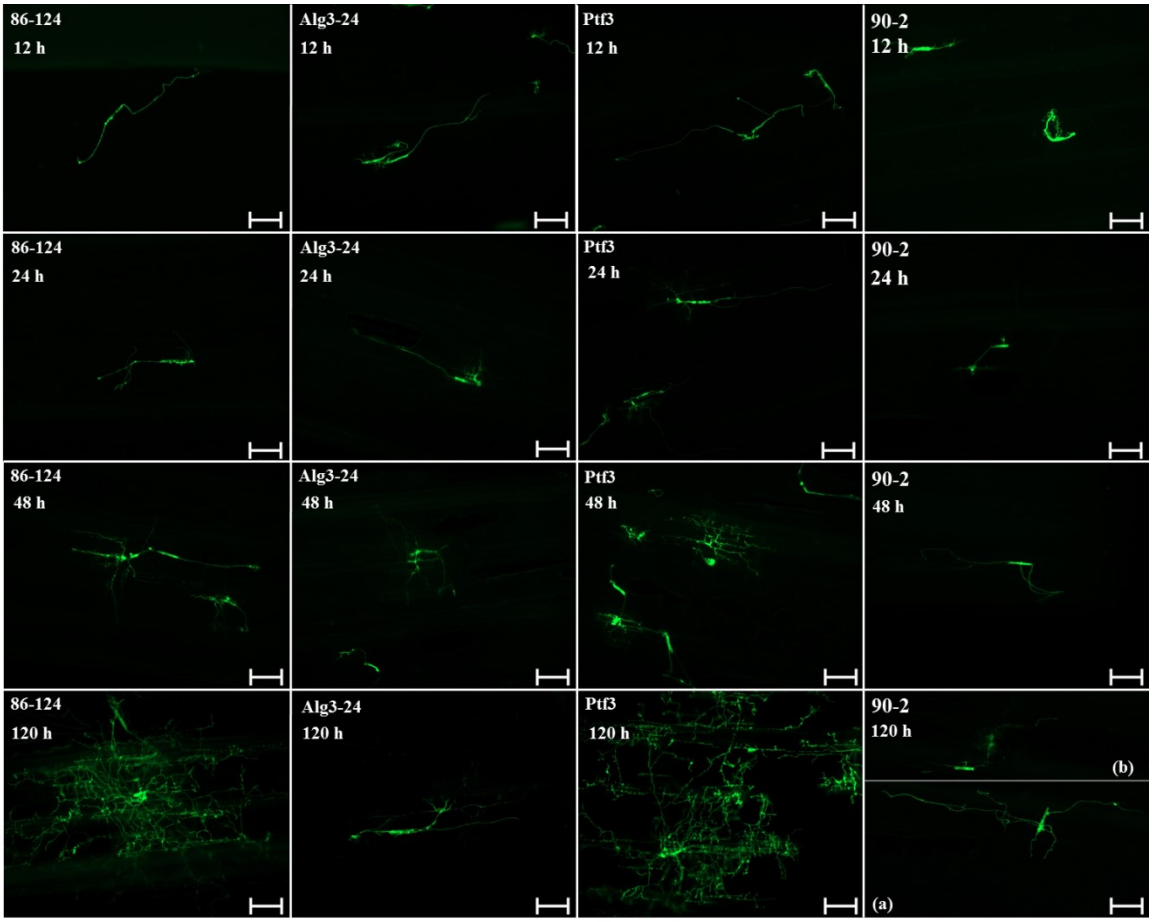

Figure 2-9. Colonization of tan spot-susceptible 'Katepwa' leaf tissue at various times following inoculation with *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA^{+}, Ptr ToxB^{+}), 86-124 (Ptr ToxA^{+}), Alg3-24 (Ptr ToxB^{+}), or 90-2 (avirulent). Inoculated leaf segments were stained fluorescein isothiocyanate wheat germ agglutinin (WGA-FITC) and analyzed by fluorescence microscopy. While some fungal penetration was observed with 90-2 at 120 h (a), in most cases conidia of this isolate produced long germ tubes but did not enter the host (b). Scale bars = 200 µm.

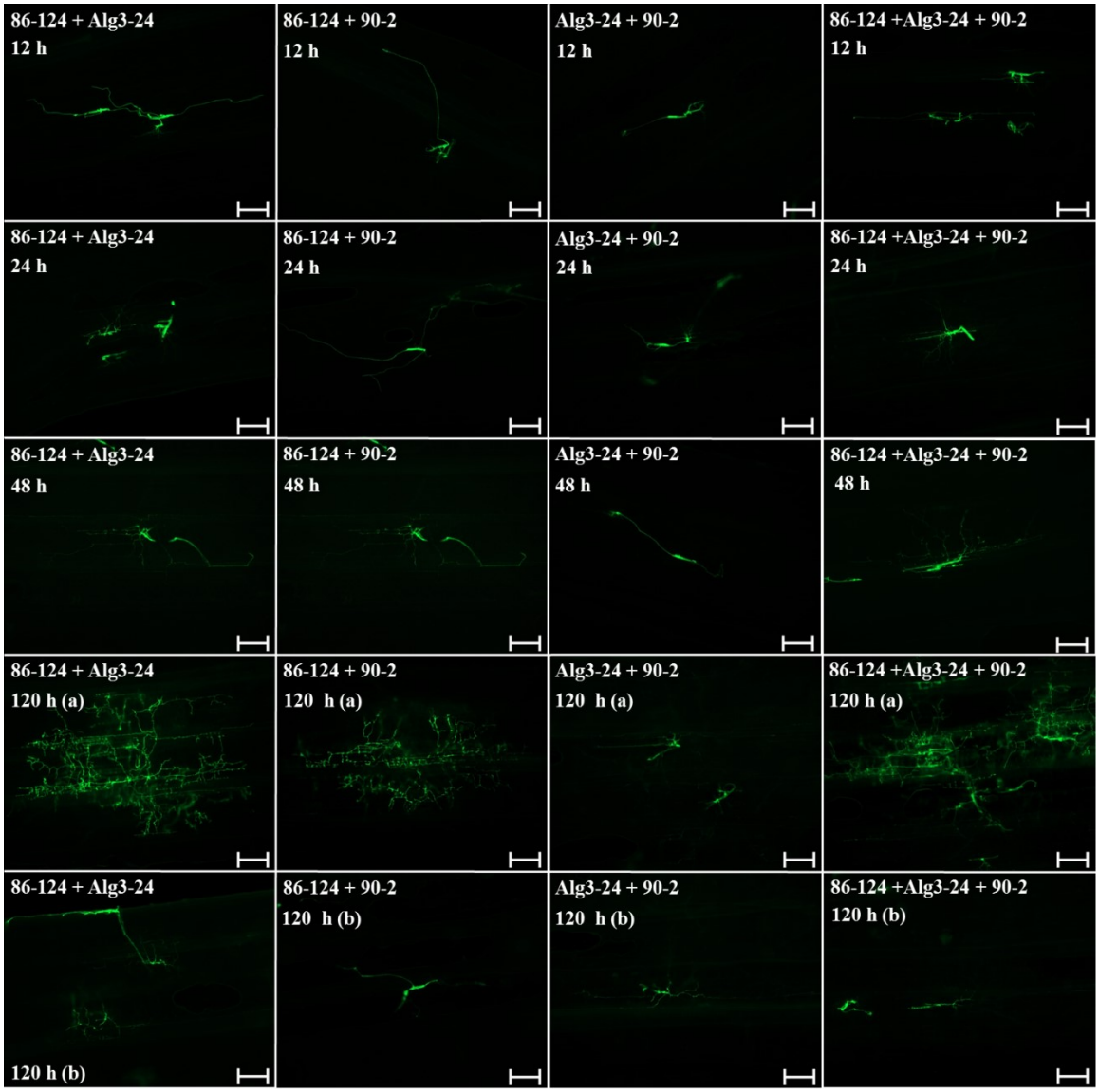

Figure 2-10. Colonization of tan spot-susceptible 'Katepwa' leaf tissue at various times following inoculation with various combinations of the *Pyrenophora tritici-repentis* isolates Ptf3 (Ptr ToxA$^+$, Ptr ToxB$^+$), 86-124 (Ptr ToxA$^+$), Alg3-24 (Ptr ToxB$^+$) and 90-2 (avirulent). Inoculated leaf segments were stained fluorescein isothiocyanate wheat germ agglutinin (WGA-FITC) and analyzed by fluorescence microscopy. Scale bars = 200 µm.

Chapter 3. Conclusions and Future Studies

3.1 General conclusions

In this thesis, the epidemiological significance of *ToxB* in *P. tritici-repentis* was explored to determine if the rarity of Ptr ToxB-producing isolates in Canada reflects the absence or rarity of wild-type *ToxB*, or if it suggests that the selective advantage provided by *ToxA* is greater than that provided by *ToxB*. This issue was examined via various experimental approaches, including evaluation of symptom development and percent leaf area diseased (PLAD), microscopy analysis to track conidial germination and the colonization of leaf tissue by isolates of *P. tritici-repentis*, and by using quantitative-PCR to measure the amount of *ToxA*, *ToxB*, *toxb* and *CHS4* at various times following inoculation with different isolates and combinations of isolates. As described in Chapter 2, the results of these experiments suggested that isolates producing Ptr ToxA (86-124, Ptf3) caused greater symptoms than the Ptr ToxB-producing isolate Alg3-24, and also proliferated to a greater extent in foliar wheat tissue. In general, therefore, it appeared that the $ToxA^{+}$ isolates were more competitive than the $ToxB^{+}$ isolate. An avirulent isolate 90-2 (classified as race 4 and possessing only an inactive homolog of *ToxB*, called *toxb*) was, as expected, the least competitive of all of the isolates examined. It appeared to grow very little if at all inside the host, consistent with the designation of race 4 as avirulent (Strelkov & Lamari, 2003).

At least from the stand-point of these results, it would appear that an important reason for the predominance of the Ptr ToxA-producing races 1 and 2 of *P. tritici-repentis* in western Canada is their ability to outcompete those races that do not produce this HST. There are many reports on the within-host competition between fungal pathogens

(Koskella et al., 2006; Gold *et al.*, 2009; Seemuller et al., 2010). In these studies, the more aggressive pathogen usually dominates the infection when co-inoculated with a less competitive one. This does not mean, however, that *P. tritici-repentis* isolates possessing the wild-type *ToxB* gene are not a threat to Canadian wheat production. Although proliferation of Alg3-24 within host tissues was limited, and PLAD was lower than what was observed after inoculation with 86-124 (Chapter 2), the severity of symptoms obtained following inoculation with this race 5 isolate, both in this thesis and in previous studies (Strelkov et al., 2002), was nonetheless significant. Indeed, PLAD just 120 hours after inoculation of the susceptible 'Katepwa' with Alg3-24 was 52.41%. This could reflect the high specific activity of Ptr ToxB (Strelkov et al., 1999), and potentially its movement or diffusion within the infected leaves. Regardless, even limited colonization of host tissue by a *ToxB*$^{+}$ isolate could result in severe chlorosis.

3.2 Future Studies

There are still unanswered questions and the potential for future studies to further explore the question of competitive ability in isolates of *P. tritici-repentis*. As noted in Chapter 2, sporulation was not measured or compared among isolates of the fungus. Ideally, sporulation levels could be measured following inoculation, to confirm whether or not the race 2 isolate (86-124) does in fact produce greater amounts of conidia than the race 5 isolate (Alg3-24) *in planta*. In culture, sporulation rates seem similar (unpublished data), but this may not be reflective of what is happening *in planta*. In order to conduct this work, the inoculated seedlings would have to be subjected to the conditions (day/light and temperature regimes) necessary to induce sporulation. With isolates of a

foreign origin, such as Alg3-24, proper biosafety precautions would also be required to prevent escape of the conidia.

Another potential study would be the co-inoculation of *ToxA* and *ToxB* knock-out isolates, to determine whether the lack of the HSTs would influence relative competitive advantage. For example, one could hypothesize that if 86-124 does not produce Ptr ToxA, necrosis would be significantly reduced. However, it would be important to determine if the reduction in symptoms would be matched by a reduction in colonization of the infected tissues by 86-124. This might also be a reasonable hypothesis, since host necrosis would be reduced, and *P. tritici-repentis* is a necrotrophic parasite. Silencing of *ToxB* in race 5 was conducted previously (Aboukhaddour et al., 2012), and while Ptr ToxB-production was reduced, it was not completely eliminated. A full knock-out strain might provide further insights into the contributions of this HST.

In the present study, no direct interactions were observed between hyphae of the different isolates in the co-inoculation experiments. Therefore, additional studies also could investigate the chemical ecology of this interaction. Chemically-mediated fungal interactions were studied previously in *Candida albicans*. This yeast was found to produce an acyclic sesquiterpene alcohol—farnesol in culture, which could inhibit the formation of hyphae and eventually regulate the population density of *C. albicans* in the adjacent area (Hornby et al., 2001; Ramage et al., 2002; Mosel et al., 2005). The release of inhibitory compounds by *P. tritici-repentis* isolates could look at both induced and constitutively produced chemicals; potentially, this could be carried out in culture first, which would be technically simpler, and if interesting results are obtained, attempts could be made to evaluate these types of interaction *in planta*.

Much effort has been devoted in recent decades to understanding host-pathogen interactions in tan spot of wheat. Given the importance of this disease, however, and the potential co-occurrence of multiple races of *P. tritici-repentis* in the field, additional attention should also be paid to interactions between isolates of the fungus, and how these could in turn affect disease development. Ultimately, a full understanding of the biology of this pathosystem will facilitate the management of tan spot of wheat.

3.3 References

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