Epidemiological and Cytological Studies on

Pyrenophora tritici-repentis

BY

MARYAM REZAEY

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
*****
COPYRIGHT PERMISSION

Epidemiological and Cytological Studies on *Pyrenophora tritici-repentis*

BY

MARYAM REZAEY

A Thesis
Submitted to the Faculty of Graduate Studies
of the University of Manitoba
in Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE

Acknowledgments

I am sincerely grateful to my supervisor **Dr. Lakhdar Lamari** who provided me this research opportunity, and guided me throughout the entire course of this research as well as writing this thesis, and also for his continuous patience during the period of thesis writing.

I would like to express my deep appreciation to my research committee members for their advices. I am also very grateful to **Dr. Fouad Daayf** for his continuous guidance, and also his invaluable help with the final correction and editing of my thesis. I am also grateful and greatly admire the dedication of **Dr. Murray Ballance** for precisely reading my thesis and providing valuable and detailed comments which helped a lot toward more improvement of my thesis. I also acknowledge **Dr. Brent Maccallum** for his considerable constructive comments.

I would also like to appreciate **Richard Smith**, **Ralph Kowatsch**, and **Ardelle Grieger** who have helped me and taught me all the lab techniques I needed to conduct this research. Thanks to all staff of the general office who find delight in assisting and supporting students. I would also like to give thanks to staff of greenhouse for all their care towards my plants.

My heartfelt gratitude goes to my husband, **Ahad Baghery,** for all his love and support. Without his encouragement and understanding, it would have been impossible for me to finish this work.

The financial support of the **Natural Sciences and Engineering Research Council** of Canada is gratefully acknowledged. I must also thank **Dr. Peter McVetty** for his financial support.

TABLE OF CONTENTS

LIST OF TABLES

Table ..... Page

LIST OF FIGURES

Figures **Page**

GENERAL ABSTRACT

Rezaey, Maryam. M.Sc., The University of Manitoba. May, 2009. Epidemiological and Cytological Studies on *Pyrenophora tritici-repentis.*, Major Advisor; L. Lamari.

The epidemiological value of Ptr toxins of *Pyrenophora tritici-repentis* was studied on seven wheat cultivars. Starting with a pathogen population consisting of eight races, four consecutive generations were constructed. Population structure at the second and fourth generations was determined by PCR. Ptr ToxA-producing isolates were either the most frequent or their frequencies were increasing. Ptr ToxB-producing isolates survived on all cultivars. The infection process of Ptr ToxA- and/or Ptr ToxB-producing isolates was also studied using a fluorescence microscopy technique. No significant difference was observed between Ptr ToxA- and/or Ptr ToxB-producing isolates in terms of germination percentage, number of germ tubes, appressoria, penetrated epidermal cells per spore, and also the percentage of mycelial area coverage of mesophyll layer in their incompatible interaction. Ptr ToxA-producing isolates had significantly larger mycelium percentage in mesophyll layer than Ptr ToxB-producing isolates in their compatible interaction from 48 to 72 hours post inoculation.

FOREWORD

This thesis is written in manuscript format. Preceding the two manuscripts is a general introduction and a review of the literature. Each manuscript includes an abstract, introduction, materials & methods, results, and discussion.

1.0 GENERAL INTRODUCTION

Tan spot is caused by the fungus *Pyrenophora tritici-repentis*. It is an economically important leaf spotting disease of wheat in the major wheat-growing regions throughout the world (Hosford, 1982). Tan spot may cause yield losses ranging from 3% to 50% (Rees *et al.*, 1982). Since the 1970s, economical importance, incidence, and severity of tan spot have increased in Canada because of changes in cultural practices (Rees, 1982; Bockus, 1998).

Tan necrosis, and chlorosis are two typical symptoms of tan spot (Lamari and Bernier, 1989b). Specific interactions between the wheat genotypes and individual isolates of the pathogen lead to differential development of both symptoms (Lamari and Bernier, 1989b, 1991). Isolates of *P. tritici-repentis* have been categorized into eight physiologic races based on their ability to induce tan necrosis and/or chlorosis on wheat differentials Glenlea, 6B365 and 6B662 (Lamari *et al.*, 2003).

P. tritici-repentis produces three independent host selective toxins (Ptr toxins). Ptr ToxA induces necrosis in sensitive wheat genotypes (Lamari and Bernier, 1989c; Ballance *et al.*, 1989; Tomas *et al.*, 1990; Tuori *et al.*, 1995; Meinhardt *et al.,* 1998). The encoding gene for Ptr ToxA is a single copy gene termed *ToxA* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1998), which is present only in the genomes of Ptr ToxA-producing isolates, and it is absent from isolates producing no Ptr ToxA (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Ptr ToxB causes chlorosis in sensitive wheat genotypes, and its encoding gene is present in multiple copies in Ptr ToxB-producing isolates (Martinez *et al.*, 2001; Strelkov, 2002). The only Canadian race 5, isolate 92-171R5, was found to carries a less active form of *ToxB* gene (Strelkov *et al.*, 2002). *ToxB* homologs are present

in single copies, and mutated forms in races 3 and 4, which do not have Ptr ToxB activity (Strelkov, 2002). Putative Ptr ToxC induces chlorosis in wheat genotypes different from those sensitive to Ptr ToxB (Strelkov and Lamari, 2003).

In western Canada the predominance of isolates producing Ptr ToxA is consistent with cultivar sensitivity to Ptr ToxA in this region (Strelkov and Lamari, 2003; Lamari *et al.* 2005a). Since isolate(s) with fully functional *ToxB* of race 5 were reported on durum wheat only in North Dakota (Ali and Francl, 1999) and not from other regions of western Canada or the USA, Strelkov and Lamari (2003) hypothesized that "a functional wild-type *ToxB* was not present (or was present at an extremely low frequency) in the pathogen in the native grasses when it moved to its wheat host".

The main objective of the present investigation is to provide an explanation for the absence of fully active Ptr ToxB-producing races of *P. tritici-repentis* in western Canadian populations, despite the presence of sensitive cultivars to this toxin for more than 80 years. We hypothesize that Ptr ToxB confers less selective advantage for competition to the pathogen in comparison to what Ptr ToxA provides to its producing isolates.

In the first manuscript, competition among the Ptr ToxA-and/or Ptr ToxB-producing isolates of *P. tritici-repentis* was studied on several Canadian cultivars to determine whether Ptr ToxB-producing isolates would have been selected by susceptible hosts if they were part of the pathogen population. The second manuscript compares, using fluorescence microscopy, the Ptr ToxA-and/or Ptr ToxB-producing isolates of *P. tritici-repentis* in establishing their relationship with wheat plants in both necrotic and chlorotic lesions.

2.0 LITERATURE REVIEW

2.1 The Pathogen

The causal organism for tan spot of wheat is the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoemaker (synonym *Helmintosporium tritici-repentis* Died.). Conidia are cylindrical with 4-7 septa and ascospores range from oval to spherical with 3 septa (Shoemaker, 1962). Ascospores in pseudothecia overwinter on the stubble (Conner, 1937; Hosford, 1971). Conidia are the secondary inoculum (Hosford, 1972). The pathogen has been reported on many grass species from different parts of the world (Hosford, 1971; Krupinsky, 1992a). It is also found to be the main pathogen of grasses in a native prairie in Western Canada (Morrall and Howard, 1975). The fungus was recovered from barley by Ali and Francl (2001). The most economically important host of *P. tritici-repentis* includes *Triticum aestivum* (Conners 1937).

2.2 Tan Spot Diseases

Tan spot is a destructive, stubble-born, polycyclic disease of wheat throughout the world (Hosford, 1982; Morrall and Howard, 1975; Rees and Platz, 1979). In Canada, it was first identified in 1939 (Conners, 1937). The first modern tan spot epidemic took place in 1974, and was reported as an important leaf spot disease on the Canadian Prairies (Tekauz, 1976). Changes in cultural practices (Rees, 1982) including shifts from conventional tillage to soil conservation tillage practices to reduce erosion (Bailey, 1996; Sutton and Vyn, 1990; Fernandez *et al.*, 1998), no or short crop rotations, introduction and continuous cultivation of susceptible wheat cultivars (Rees and Platz 1992, Rees *et al.*, 1982; Bockus and Claasen, 1992), a trend away from stubble burning (Rees and

Platz, 1983) may have contributed to the increased incidence and severity of tan spot since the 1970s. In Canada, most widely grown wheat genotypes in the 1970s and 1980s, such as 'Neepawa' and 'Katepwa', were susceptible to tan spot (Lamari *et al.* 1995, 1998; Lamari *et al*, 2005a). Since tan spot susceptibility was always present in the cultivated wheat in Canada, and has not changed through the 20th century, the actual cause of the 1970's epidemic may be changes in cultural practices (Rees and Platz, 1992).

Tan spot may cause significant yield losses, ranging from 3% to 50% in major wheat growing areas (Rees *et al.*, 1982; Rees and Platz, 1983). The amount of yield loss depends on cultivar susceptibility (Lamari and Bernier, 1989a, b; Sykes and Bernier, 1991), environmental conditions such as moisture, light, and temperature (Khan, 1971; Hosford *et al.*, 1987; Pfender *et al.*, 1988), and virulence of the pathogen population (Krupinsky, 1987; Lamari and Bernier, 1989b; Rees and Platz, 1983).

2.2.1 Epidemiology

P. tritici-repentis survives as pseudothecia (sexual fruiting bodies) on infested crop residue, infected seed, and overwintering grasses from one cropping season to the next (Hosford, 1971; Rees and Platz, 1980a; Rees *et al.*, 1982; Krupinsky, 1992a; Schilder and Bergstorm, 1995). Ascospores are considered as the primary source of inoculum in spring (Hosford, 1971; Reez and Platz, 1983; Sutton and Vyn, 1990). The conidia are repeatedly produced during the growing season on infected plants (Shoemaker, 1962; Hosford, 1972; Schilder and Bergstorm, 1992), and their dispersal is by wind (Platt and Morrall, 1980; Wright and Sutton and Vyn, 1990). In Canada and the northern Great Plains of the United States, tan spot severity has been correlated with top soil-preserved infested wheat residues, which gives rise to the pathogen inoculum on wheat, and alternative hosts from

season to season (Rees and Platz, 1980a, 1980b; Sutton and Vyn, 1990; Ali and Francl, 2001). Ali and Francl (2001) studied the reaction of barley cultivars to races of *P. tritici-repentis*, and found them to be highly resistant to the pathogen. They suggested that the role of barley as a secondary host in tan spot epidemiology is insignificant compared to wheat, and could not promote variation in the pathogen population.

2.2.2 Temperature and Wetness Duration

Temperature and duration of moisture are the two main factors in infection and disease development of wheat-*P. tritici-repentis* pathosystem (Hosford *et al.*, 1987; Lamari and Bernier, 1994). Maturation of pseudothecia and liberation of ascospores on crop residue were found to be dependent on cool and dry conditions (Hosford, 1971; Morrall and Howard, 1975; Summerell and Burgess, 1989). Hosford (1987) reported that when the post inoculation, relative-wetness period and/or temperature are increased, spore germination, number of germ tubes per spore, length of germ tubes, appressoria and papilla formation and colonized host cells also increased. Ronis and Semaskiene (2006) found that a sufficient amount of rain and an optimum temperature (21°C) resulted in a severe epidemic. At temperatures above the optimum, susceptible and resistant cultivars inoculated with the pathogen were found to react similarly, with smaller lesions on susceptible cultivars at 10°C (Lamari and Bernier, 1994). A humidity period of 24 h is sufficient to separate susceptible and resistant wheat genotypes (Gilchrist *et al.*, 1984). Although resistance breakdown in leaf-wetness durations over 30 h was reported by Hosford and Busch (1973), no association was found between resistance and the length of the wetness period by Lamari and Bernier (1989b), who observed no resistance breakdown even after 72 h of continuous leaf wetness.

2.2.3 Control of Tan Spot of Wheat

Control measures for tan spot are considered as follows: tillage practices (Pfender and Wootke, 1987), crop rotation with non-host crops (Martens *et al.*, 1988; Rees and Platz, 1980b), burning or incorporation of crop residues (Pfender *et al.*, 1988; Reez and Platz, 1980b) to reduce the level of inoculum, and planting resistant cultivars (Lamari, 1988; Lamari and Bernier, 1989a; Rees and Platz., 1980b). Seed treatment and foliar spray of the crop with fungicides (Hosford, 1982; Rees and Platz, 1980b; Tekauz *et al.*, 1982) are also being used. Since the use of chemicals is costly, and environmental concerns oppose stubble burning, these methods are not sustainable (Tekauz *et al.*, 1982). Cultivation of resistant cultivars is a more economically and environmentally acceptable method of controlling tan spot (Green and Campbell, 1979). Using a lesion type-based system, some resistant lines have been identified in diploid, tetraploid, and hexaploid wheat genotypes (Lamari and Bernier, 1989a; Sykes and Bernier, 1991; Singh *et al.*, 2006a).

2.3 Symptomology

Tan necrosis and extensive chlorosis are two typical symptoms, which are produced by *P. tritici-repentis* on susceptible hosts (Lamari and Bernier, 1989a, 1989b). Necrosis and chlorosis symptoms of tan spot are developed differentially as a result of specific interactions between the wheat genotypes and individual isolates of the pathogen (Lamari and Bernier, 1989b, 1991). On susceptible wheat lines, necrosis symptoms are oval-shaped necrotic leaf spots, with a brown to black spot at the center (Lamari and Bernier, 1989b). When plants are severely infected, necrotic lesions enlarge, coalesce and cover the entire leaf, resulting in leaf death (Hosford, 1971). *P. tritici-repentis* can also cause

kernel discoloration during the grain filling period (Schilder and Bergestrom, 1994; Fernandez *et al.*, 1994), which reduces its market acceptability and seedling emergence (Fernandez *et al.*, 1997).

Lamari and Bernier (1989a) showed that individual isolates of *P. tritici-repentis* independently can induce either necrosis or chlorosis in specific wheat lines or cultivars, which provided a base for a disease symptom-based system. Based on this system isolates were differentiated into four pathotypes based on their ability to induce tan necrosis and/or chlorosis on wheat differential lines: pathotype 1 (nec+chl+), pathotype 2 (nec+chl–), pathotype 3 (nec–chl+), and pathotype 4 (nec–chl– or avirulent) (Lamari and Bernier, 1989b). When new chlorosis-inducing isolates were identified, from Algeria, which induced chlorosis on wheat genotypes known to be resistant to isolates of pathotype 3 (nec- chl+), a race classification system was adopted (Lamari *et al.*, 1995). Isolates of *P. tritici-repentis* now are categorized into eight physiologic races based on their ability to induce tan necrosis and/or chlorosis on an expanded wheat differential set (Lamari *et al.*, 2003).

From the eight known races of *P. tritici-repentis,* races 2, 3, and 5 show symptoms on only one of the three differential lines (Lamari and Bernier, 1989b; Lamari *et al.*, 1995, Lamari *et al.,* 2003). Races 1, 6, and 7 show virulence on two differential lines (Lamari *et al.*, 1995; Strelkov *et al.*, 2002; Lamari *et al.*, 2003). Race 8 is able to attack all three differential lines (Lamari *et al.*, 2003). Race 4 is an avirulent race, with only one reported isolate (90-2), which shows symptoms on neither differential lines nor any tested plants so far (Lamari *et al.*, 1995). Table (2.1) shows the sensitivity of wheat differential lines/cultivars to toxins of *P. tritici-repentis.* In general the gene-for-gene model,

described by Flor (1955, 1971), recognition between the resistance gene in the host and the corresponding avirulence gene in the pathogen will result in a resistance reaction of the host plant to the invading pathogen. In the wheat-*P. tritici-repentis* interaction, which is a toxin-based one-to-one model suggested by Lamari *et al.* (2003), recognition between the host and the pathogen, i.e., matching a putative receptor from the host with its corresponding toxin from the pathogen, results in compatibility, and the failure of this recognition results into incompatibility (resistance reaction).

Table 2.1. Sensitivity of differential lines or cultivars of wheat to host-specific toxins of races of *P. tritici-repentis.* (Source: Strelkov and Lamari, 2003).

	Races							
Differential line or cultivar	4	2	1	8	7	5	6	3
'Glenlea'		Ptr ToxA						
6B662				Ptr ToxB				
6B365			Ptr ToxC				Ptr ToxC	
Number of toxins produced*	0	1	2	3	2	1	2	1

Note: Shaded areas under each race indicate the toxin produced for the respective cultivars or differential lines. 'Glenlea' is sensitive to Ptr ToxA; lines 6B662 and 6B365 are sensitive to, respectively, Ptr ToxB and putative toxin or Pathogenicity factor Ptr ToxC.
*The eight races follow a 1:3:3:1 distribution for the production of 0, 1, 2 and 3 toxins, respectively: race 4 (0 toxin) : races 2, 3, 5 (1 toxin) : races 1, 6, 7 (2 toxins) : race 8 (3 toxins).
This Figure is reproduced by the permission from the Canadian Phytopathological Society.

2.4 Toxin Involvement in Tan Spot of Wheat

Hosford *et al.* (1987) observed differences between the length of colonizing hyphae and length of the lesions, and proposed that there were toxic substances produced by the colonizing hyphae which spread ahead of the hyphae, causing further development of the lesions. More evidences was collected by Lamari and Bernier (1989c) when they found

that all the necrosis-inducing isolates were able to produce toxins *in vitro*, and that the isolates which were not able to induce necrosis did not produce toxins *in vitro*. The corresponding toxin reproduced the necrosis symptom in all the tested sensitive wheat lines/cultivars, but all the resistant host plants were insensitive to the necrosis toxin. Necrosis and chlorosis symptoms of tan spot were later proved to be related to production of toxin(s) in different studies (Lamari *et al*, 1995; Orolaza *et al.*, 1995).

To date, there are three characterized toxic compounds (Ptr toxins) produced by *P. tritici-repentis* isolates (Ballance *et al.*, 1989; Orolaza *et al.*, 1995; Tuori *et al.*, 1995; Zhang *et al.*, 1997; Strelkov *et al.*, 1999; Effertz *et al.*, 2002). Ptr toxins are known to be host-selective toxins (HSTs) as they independently induce necrosis and chlorosis symptoms on sensitive wheat lines/cultivars, but not on the insensitive ones. HSTs enable the producing fungus to colonize the host tissue by changing the properties of cellular membranes in susceptible plants (Scheffer, 1976). Ptr toxins are considered as virulence factors by Friesen *et al.* (2004) or pathogenicity factors (Lamari and Bernier, 1989a, 1989b, 1989c; Ciuffetti *et al.*, 1997) in development of tan spot disease on susceptible wheat genotypes.

Toxins from *P. tritici-repentis*

Ptr ToxA

Ptr ToxA induces necrosis in susceptible wheat genotypes (Lamari and Bernier, 1989a). Ptr ToxA is a ribosomally synthesized, heat stable polypeptide, with a molecular-weight of 13.2 kDa (Ballance *et al.*, 1989; Tomas *et al.*, 1990; Tuori *et al.*, 1995). The encoding gene for Ptr ToxA is a single copy gene termed *ToxA* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Only Ptr ToxA-producing races carry *ToxA* in their genome

(Lamari *et al.*, 1995; Lamari *et al.*, 2003), and it is fully missing in non-Ptr ToxA-producing isolates (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Friesen *et al.* (2006) suggested that *P. tritici-repentis* acquired the *ToxA* gene via horizontal transfer of DNA from *Stagonospora nodurum* (Berk.), another pathogen of wheat. Although producing a HST compound is not sufficient to make a non-pathogen become a pathogen in some cases (Walton, 1996), Ciuffetti *et al.* (1997) transferred the *ToxA* gene into isolates with no Ptr ToxA activity and found those isolates capable of toxin production as well as inducing necrotic symptoms on susceptible wheat genotypes.

Manning and Ciuffetti (2005) found that the site of action for Ptr ToxA is present in both toxin-sensitive and -insensitive wheat plants. But in sensitive cultivars, Ptr ToxA is associated with a corresponding receptor or is internalized, but not in insensitive cultivars. They also found that Ptr ToxA is able to cross the plant cell plasma membrane to the inside of the cell in the absence of its producing fungus (Manning and Ciuffetti, 2005). This is supported by the cytological studies, which indicates intercellular growth of the tan spot fungus, with no evidence for mesophyll cells penetration (Lamari and Bernier, 1989b; Larez *et al.*, 1986; Loughman and Deverall, 1986). A requirement of active metabolism for the activity of Ptr ToxA was suggested by Kwon *et al.* (1998). Ptr ToxA is produced by races 1 and 2 (Ballance *et al.*, 1989; Tomas *et al.*, 1990; Tuori *et al.*, 1995; Zhang *et al.*, 1997), as well as races 7 and 8 (Lamari *et al.*, 2003).

Ptr ToxB

Ptr ToxB induces chlorosis in sensitive wheat plants. Ptr ToxB is a small, heat stable polar, non-ionic polypeptide of 6.6 kDa in mass (Orolaza *et al.*, 1995; 1998; Strelkov *et al.*, 1998b and 1999) with no homology with Ptr ToxA (Strelkov *et al.*, 1999).

Ptr ToxB causes chlorosis using a mechanism involving chlorophyll photo-oxidation to inhibit photosynthesis (Strelkov *et al.*, 1998a). *ToxB* in multiple copies is the encoding gene for Ptr ToxB (Martinez *et al.*, 2001; Strelkov *et al.*, 2002; Strelkov *et al.*, 2006). *ToxB* homologs are totally absent in Ptr ToxB-non-producing isolates from races 1 and 2 (Strelkov *et al.*, 2002; Lamari *et al.*, 2003; Strelkov *et al.*, 2006), but are present in single copies in isolates from races 3 and 4, isolates which do not have Ptr ToxB activity (Strelkov *et al.*, 2002; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). The *ToxB* homolog in race 3 isolates is different from the fully active *ToxB* form race 5 in the promoter and signal peptide coding regions sequence (Strelkov, 2002; Strelkov and Lamari, 2003). In race 4, the *ToxB* homolog has 86% similarity with the wild-type *ToxB* (Strelkov *et al.*, 2006), the differences are in the promoter and signal peptide coding region, but also extended multiple sites in the coding region for the protein (Strelkov, 2002; Martinez *et al.*, 2004). The Canadian isolate 92-171R5 of race 5 carries only 2 copies of *ToxB* gene, resulting into smaller chlorosis symptoms (Strelkov *et al.*, 2002). Ptr ToxB-producing races 5, 6, 7, and 8 of *P. tritici-repentis* carry multiple copies of *ToxB* (Strelkov and Lamari, 2003; Lamari *et al.*, 2003).

Ptr ToxC

Ptr ToxC is a partially characterized chlorosis-inducing toxin, which causes extensive chlorosis in wheat genotypes different from those on which Ptr ToxB can induce chlorosis (Lamari and Bernier, 1991). Ptr ToxC is a heat stable polar, non-ionic low-molecular-mass molecule, but it is not a protein (Effertz *et al.*, 2002). Ptr ToxC is assumed to be produced by races 1, 3, 6, and 8 (Strelkov *et al.*, 2002; Lamari *et al.*,

2003). So far, no information is available on Ptr ToxC, regarding the gene (s) involved and the way this non-proteinous compound is synthesized.

Ptr ToxD

The involvement of toxins other than ToxA, ToxB and ToxC was suggested by Gamba and Lamari (1998), based on the observation of a necrosis symptom induced by Ptr ToxB-producing isolates from races 3 and 5 on tetraploid wheat lines, Coulter and 4B160. There are also claims on a new proteinaceous Ptr toxin named Ptr ToxD. This Ptr toxin induces necrosis (Andrie *et al.*, 2007) and chlorosis (Ali *et al.*, 2002; Meinhardt *et al.*, 2003). Ptr ToxD is produced by isolates of race 9 (Manning *et al.*, 2002; Ciuffetti *et al.*, 2003). These may turn out to be false toxins with no involvement in the infection process. The claims of Ptr ToxD have not received any confirmation to date.

2.5 Resistance Genes to Tan Spot

Some lines with resistance to tan spot were identified in diploid species, hexaploid, and tetraploid wheat genotypes (Lamari and Bernier, 1989a; Sykes and Bernier, 1991; Singh *et al.*, 2006a). Resistance to tan spot is assigned to independent recessive loci (Lee and Gough, 1984; Lamari and Bernier, 1989c, 1991; Gamba and Lamari, 1998; Singh and Hughes, 2005). Resistance to tan necrosis inducing isolates is controlled by a single recessive gene, *tsn1*, located on the long arm of chromosome 5B in both durum and common wheat (Faris *et al.*, 1996). Two other recessive resistance genes, named *tsn3* and *tsn-syn1*, to tan necrosis were located on chromosome 3D (Tadesse *et al.,* 2006).

Resistance to chlorosis inducing isolates of races 1, 3 and 5 are provided by distinct genes (Lamari and Bernier, 1991; Orolaza *et al.*, 1995; Singh and Hughes, 2006). Resistance to isolates of races 1 and 3 is controlled by a single major quantitative trait

loci (QLT), named *QTsc.dnsu-1A* located on the short arm of chromosome 1A (Faris *et al.*, 1997; Effertz *et al.*, 2001). Friesen and Faris (2004) mapped another major QTL toxin insensitivity, called *tsc2* on the short arm of chromosome 2B, which confers resistance to race 5 isolates. They also found some other QTLs with minor effects on the short arm of 2A, the long arm of 4A, and on the long arm of chromosome 2B. Recently, a single recessive gene *tsn2* which controls resistance to necrosis induced by race 3 in tetraploid wheat was located on the long arm of chromosome 3B (Singh *et al.*, 2006b).

2.6 Wheat-*P. tritici-repentis* Infection Process

P. tritici-repentis like other foliar pathogens uses topographic features to produce appressoria to be attached at specific sites on the leaf surface (Larez *et al.*, 1986; Loughman and Deveral, 1986; Hoch *et al.*, 1987). Following the adhesion of the appressoria to the leaf surface (cuticle), an infection peg grows from the appressorium, and penetrates the cell walls of epidermal layers (Larez *et al.*, 1986; Loughman and Deverall, 1986; Lamari and Bernier, 1989b). As appressoria of *P. tritici-repentis* lack melanin to use mechanical pressure, probably it uses cell-wall degrading enzymes for penetration (Dickman *et al.*, 1989; Bucheli *et al.*, 1990). Subsequently, a vesicle is formed in the penetrated epidermal cell, and secondary hyphae are formed from the vesicle and colonize the mesophyll cell intercellularly (Larez *et al.*, 1986; Loughman and Deverall, 1986; Lamari and Bernier, 1989b). No mesophyll cell penetration has been previously observed (Lamari and Bernier 1998b; Dushinsky *et al.*, 1998).

No significant differences were observed between resistant and susceptible cultivars in terms of initial stages of infection, such as spore germination, appressoria formation,

penetration of epidermal cell walls, formation of intracellular vesicles, and intracellular hyphal growth in epidermal cells (Loughman and Deverall, 1986).

An avirulent isolate from race 4 of *P. tritici-repentis* was also able to infect both susceptible and resistant wheat genotypes. It germinated, formed appressoria, penetrated and colonized the host epidermal cells, but it was blocked shortly after its primary colonization of the mesophyll layer (Larez *et al.*, 1986; Lamari and Bernier, 1989b).

2.6.1 Defense Mechanisms of Wheat to Tan Spot

Formation of papillae occurs at the penetration site on the inner side of plant cell walls as a common mechanism of resistance of plant cells in both resistant and susceptible host plants to fungal attack (Aist 1976; Aist and Israel, 1977). However, higher frequency of papillae formation in infected resistant cultivars with *P. tritici-repentis* was not found to be associated with a lower frequency of penetration (Larez *et al.*, 1986; Loughman and Deverall, 1986). Resistance to *P. tritici-repentis* is not expressed until hyphae are established in the intercellular spaces of the mesophyll layer (Lamari and Bernier, 1989b). Following the intercellular growth of hyphae, mesophyll cells below the infection site become dark brown which corresponded to the dark brown-to-black spots observed on the resistant plant (Lamari and Bernier, 1989b).

The fleck condition or necrosis caused by *P. tritici-repentis* is perhaps a hypersensitive response according to Lee and Gough (1984). Lignin is believed to play a role in restricting *P. tritici-repentis* growth in resistant cultivars (Dushnicky *et al.*, 1998).

2.7 Worldwide Population Structure of *P. tritici-repentis*

The structure of *P. tritici-repentis* population varies in different parts of the world. Races 1 to 3 were identified in North America with the predominance of races 1 and 2 in

this region (Lamari and Bernier, 1989b; Lamari *et al.*, 1998). In the Middle East, race 1 was found to be predominant (Lamari *et al.,* 2005b). Isolates of races 1 and 2 were mainly collected from hexaploid wheat (Lamari *et al.,* 2005b). Race 4 isolates has been collected from North America (Lamari and Bernier, 1989b; Ali and Francl, 2003). No isolate of race 4 was reported from the Middle East (Lamari *et al.,* 2005b). Race 5 isolates were first reported on durum wheat from North Africa (Lamari *et al.*, 1995), and North America (Lamari *et al.*, 1998; Strelkov *et al.,* 2002; Ali and Francl, 2003), on tetraploid wheat in the Middle East (Lamari *et al.*, 2005b), and Europe (Tadesse *et al.,* 2006). To date, isolate 92-171R5 is the only reported Canadian isolate of race 5 from hexaploid wheat, and it is less aggressive than race 5 isolates reported from North Africa on susceptible lines (Lamari *et al.*, 1998; Strelkov *et al.,* 2002). Race 6 isolates have been reported from North Africa on tetraploid wheat hosts (Lamari *et al.*, 1995). Races 7 and 8 isolates were identified in the Middle East on tetraploid wheat (Strelkov *et al.* 2002; Lamari *et al.* 2003; Lamari *et al.*, 2005b).

2.8 Tan Spot and Host Susceptibility in Western Canada

Tan spot susceptibility for both the necrosis and chlorosis reactions is controlled by independent dominant genes (Gamba and Lamari, 1998).

In Canada, sensitivity to Ptr ToxA seems to have been transmitted from the first major cultivar, Red Fife, into Canadian cultivars, such as Marquis in the late 1800s, and propagated for a century into Canadian cultivars through the breeding programs (Lamari *et al.*, 2005a). Sensitivity to Ptr ToxB was also transferred from another major cultivar, Thatcher, in 1934 into many cultivars, such as Neepawa through backcross programs

(Lamari *et al.*, 2005a). Percentage of the occupied area by Ptr ToxA- and Ptr ToxB-sensitive Canadian wheat crops for over 60 years are presented in Figure (2.1).

Ptr ToxA sensitivity of cultivars is consistent with the predominance of Ptr ToxA-producing isolates in western Canada (Strelkov and Lamari, 2003), but the absence of Ptr ToxB-producing isolates remains unexplained with more than 80 years of large scale cultivation of Ptr ToxB sensitive wheat genotypes, such as Neepawa and Thatcher (Strelkov and Lamari, 2003; Lamari *et al.*, 2005a). Most Canadian cultivars are insensitive to Ptr ToxC (Strelkov and Lamari, 2003). Isolates of race 3 are also nearly absent from hexaploid wheat, but present in durum wheat, at less than one percent. Ptr ToxC does not appear to confer a selective advantage to the pathogen, and does not cause damage to wheat production in Western Canada (Strelkov and Lamari, 2003), as most cultivars are insensitive to it.

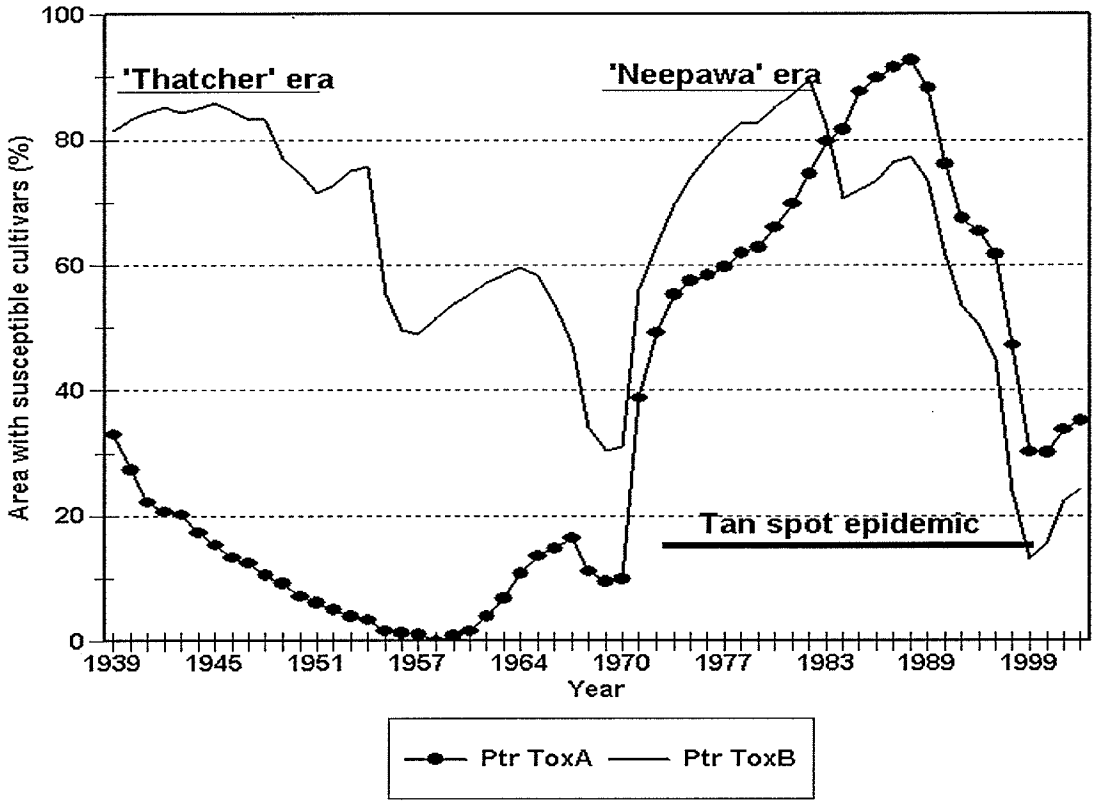


Figure 2.1. Sensitivity to Ptr ToxA and Ptr ToxB in the wheat crop produced in western Canada from 1939 to 2003. For each year, values were computed as the ratio of cumulative area occupied by toxin-sensitive cultivars to the total Canadian Western Red Spring (CWRS) wheat area. Only cultivars grown on at least 5% of the CWRS wheat area in western Canada were considered. Wheat cultivar production data compiled from annual surveys of western Canada done by Searle Grain (1939-1940), Line Elevator Farm Service (1941-1992), Federal Grain Ltd. (1966-1971), Prairie Wheat Pools (1971-1992), Secan (1993), and the Canadian Wheat Board (1998-2003) (Source: Lamari, *et al.*, 2005a).
This Figure is reproduced by the permission from the American Phytopathological Society.

3.0 SIMULATING PATHOGEN POPULATION SHIFTS OF *PYRENOPHORA TRITICI-REPENTIS* ON DIFFERENT CANADIAN WHEAT CULTIVARS

3.1 Abstract

The fungal pathogen *Pyrenophora tritici-repentis* causes tan spot, an important leaf spotting disease of wheat worldwide. The epidemiological value of Ptr ToxA and Ptr ToxB, the host-selective toxins of *P. tritici-repentis*, was studied on seven Canadian wheat cultivars. Six susceptible and one resistant cultivar (five hexaploid and two tetraploid) were inoculated with a pathogen population consisting of a mixture of isolates from the eight known races of the pathogen (one isolate per race). Subsequent pathogen generations were constructed by mixing 100 single-spore isolates obtained from individual lesions from infected plants. These populations were re-inoculated to produce the next generation. In this study, four consecutive generations were constructed, and the population structure at the second and fourth generations was assessed by PCR. Ptr ToxA-producing isolates were predominant on all of the hexaploid and one tetraploid (4B160) wheat cultivars, followed by isolates that produce both Ptr ToxA and Ptr ToxB. Ptr ToxB-producing isolates, which are not part of the Canadian pathogen population, survived on all the major wheat cultivars grown in western Canada over the past century which were included in this study. This suggests that Ptr ToxB-producing isolates would have been a matter of concern for wheat production if they were part of the pathogen population. Ptr ToxA and/or Ptr ToxB appear to confer a competitive advantage to the producing isolates, as determined by population shifts over four generations.

3.2 Introduction

Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs., (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.), is a foliar disease of wheat (*Triticum aestivum* L., *T. durum*) worldwide (Hosford, 1982). Since the 1970s, the disease has become a problem and caused yield losses in all major wheat growing areas (Tekauz, 1976; Rees *et al.*, 1982). Changes in cultural practices, cultivation of susceptible cultivars and changes in pathogen virulence may have contributed in the increased incidence of the disease (Rees and Platz, 1992).

To date, isolates of *P. tritici-repentis* have been categorized into eight races based on their virulence patterns on three effective wheat differential genotypes (Lamari *et al.*, 2003). Necrosis (nec+) and chlorosis (chl+), the two qualitative types of tan spot symptoms, have been found to be the results of specific host pathogen interactions (Lamari and Bernier, 1989a, 1989b, and 1991).

Necrosis on susceptible wheat genotypes is produced by Ptr ToxA (Lamari and Bernier, 1989a). Ptr ToxA is produced by races 1 and 2 (Ballance *et al.*, 1989), which were the prevalent races in western Canada over the past 20 years (Lamari and Bernier, 1989b; Lamari *et al.*, 1998), as well as by races 7 and 8 (Lamari *et al.*, 2003). Ptr ToxA is a proteinous toxin encoded by *ToxA*, a single copy gene (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997).

Chlorosis is caused by Ptr ToxB (Orolaza *et al.*, 1995; Strelkov *et al.*, 1998b). Ptr ToxB is produced by races 5, 6, 7, and 8 (Strelkov *et al.*, 2002; Lamari *et al.*, 2003; Strelkov and Lamari, 2003). Except for a single low virulence isolate of race 5, these races are nearly absent in Western Canada (Lamari *et al.*, 1998, Strelkov and Lamari

2003; Lamari *et al.*, 2005a). Ptr ToxB is also a protein, encoded by *ToxB,* which occurs in multiple copies (Ballance *et al.*, 1989; Strelkov *et al.*, 1999; Martinez *et al.*, 2001). *ToxB* homologs are totally absent in isolates of races 1 and 2 (Strelkov *et al.*, 2002). Canadian isolate 92-171R5 is the only isolate of race 5 which possesses a less active form of *ToxB* gene (Strelkov *et al.*, 2002). A single copy of mutated forms of *ToxB* is present in races 3 and 4 which do not have Ptr ToxB activity (Strelkov *et al.*, 2002; Strelkov and Lamari, 2003; Ciuffetti *et al.*, 2003). *ToxB* homologs in races 3 and 4 have mutation in their promoter and coding regions, respectively (Strelkov *et al.*, 2002, Strelkov and Lamari 2003).

Ptr ToxC, a nonionic polar low molecular weight peptide, is another chlorosis toxin (Effertz *et al.*, 2002), which induces chlorosis on wheat genotypes different from those that differentiate Ptr ToxB-producing races (Lamari and Bernier, 1991; Gamba and Lamari, 1998; Gamba *et al.*, 1998). Ptr ToxC is produced by races 1, 3, 6, and 8 of *P. tritici-repentis* (Strelkov *et al.*, 2002; Lamari *et al.*, 2003).

In the host, three dominant and independently inherited genes control the sensitivity to Ptr ToxA, Ptr ToxB, and Ptr ToxC, one gene for each toxin (Gamba *et al.*, 1998).

Except for the reported race 5 isolate from North America (Ali and Francl, 1999), fully active Ptr ToxB-producing races (races 5, 6, 7, and 8) are not present in the pathogen population in Western Canada, but have been reported from places other than North America, such as North Africa (Lamari *et al.*, 1995), Azerbaijan (Lamari *et al.*, 2003; Lamari *et al.* 2005b), and the Middle east (Lamari *et al.*, 2003).

Since sensitivity to both Ptr ToxB and Ptr ToxA, has always been present in wheat cultivars grown in western Canada (Lamari *et al.* 2005a), the isolates carrying a *ToxB* locus are expected to be present in the pathogen population.

The objectives of this research were: i) to determine whether Ptr ToxB-producing isolates, if present in the pathogen population, would have been selected by toxin-sensitive wheat hosts and ii) to compare the competitive advantages provided to the pathogen by Ptr ToxA and Ptr ToxB.

3.3 Materials and Methods

Plant materials

To assess competition among the races of *P. tritici-repentis*, wheat cultivars historically grown over large areas of western Canada were selected for the study. These cultivars are known to have different sensitivities to the Ptr toxins and represent all the known combinations of sensitivity to the Ptr toxins (Lamari *et al.* 2005a). Six susceptible and one resistant wheat cultivars (five hexaploid (*T. aestivum* L.), and two tetraploid (*T. turgidum* L. var. *durum*) were used in this study (Table 3.1).

Seeds of each line or cultivar were planted in 12-cm-diameter clay pots, containing soil:sand:peat moss mixture (2:1:1, v:v:v). Ten such pots were prepared for each cultivar. Five to six evenly paced seeds were planted per pot. Seedlings were maintained in a growth room set at 22/18°C (day/night) with a 16-h photoperiod at a light intensity of ≈180 $\mu Em^{-2}s^{-1}$. Plants were watered daily, and were fertilized with 10-20-0-14 (N-P-K-Ca) fertilizer weekly.

Table 3.1. Reaction of wheat lines/cultivars to known races of *Pyrenophora tritici-repentis.*

Cultivar	Ploidy[&]	Selection reason	*Ptr* toxin sensitivity	Susceptibility to the races	Cultivar release year
Red Fife	6x	The first major wheat cultivar grown in western Canada	A	1,2,7,8	1840's
Thatcher	6x	Predominant for 30 years, and occupied up to 50% of the acreage in past years	B	5,6,7,8	1935
Neepawa	6x	Predominant for 10 years, and occupied up to 60% of the acreage in past years	A+B	1,2,5,6,7,8	1969
AC Domain	6x	A modern cultivar	A+B+C	1,2,3,5,6,7,8	1993
4B160	4x	Tetraploid	Unknown	1, 3, 5, 6, 7, 8	Landrace
Coulter	4x	Tetraploid	A	1, 2, 3, 5, 6, 7, 8	1977
Selkirk	6x	Historic, resistant	No sensitivity	No susceptibility	1953

[&] Ploidy level: 6x = hexaploid, 4x = tetraploid

Pathogen isolates

In the first experiment, nine single-spore isolates representing the eight known races of *P. tritici-repentis* were selected for this study to provide all the combinations of the three Ptr toxins (Table 3.2). Isolate Alg3-24 was included as a wild-type for race 5 in addition to the only Canadian isolate (92-171R5) of race 5.

The experiment was repeated with Red Fife, Thatcher, Neepawa, and Coulter as hosts, using isolates different from the ones which were used in the first experiment for each race (Table 3.3). For the races with only one known isolate, the same isolate was used as in the first experiment. Prior to conducting the study, individual isolates were tested for their virulence pattern on lines from differential sets with known reactions to races 1-8 (Appendix 1), as well as on the wheat lines/cultivars used in this study (Appendix 2 and Appendix 3).

Table 3.2. *P. tritici-repentis* isolates used for the first experiment of population shift studies.

Race	Isolates(s)	Produced Ptr toxin	Place of origin
1	ASC1	A+C	Manitoba
2	86-124	A	Manitoba
3	D308	C	Manitoba
4	90-2	-	Manitoba
5	ALG3-24, 92-171R5	B	Algeria, Saskatchewan
6	ALGH2	B+C	Algeria
7	ICARDA35-5	A+B	Azerbaijan
8	ICARDA73-1	A+B+C	Azerbaijan

Table 3.3. *P. tritici-repentis* isolates used for the second experiment of population shift studies.

Race	Isolate(s)	Produced Ptr toxin	Place of origin
1	NA9-6	A+C	North Eastern Algeria
2	SK16-4	A	Saskatchewan
3	94-25, 331-9	C	Manitoba
4	90-2	-	Manitoba
5	NA4-4, ALG5-X1-1	B	North Eastern Algeria
6	ALGH2	B+C	Algeria
7	ICARDA36-3, ICARDA 36-4	A+B	Azerbaijan
8	ICARDA31-1, ICARDA31-2	A+B+C	Azerbaijan

Preparation of inoculum

Conidia were produced from single-spore cultures on V-8 potato-dextrose agar medium (V8-PDA) (150 mL V8 juice, 3 g $CaCo_3$, 10 g Difco PDA, 10 g Difco agar, and 850 mL distilled water) as described by Lamari and Bernier (1989a). To construct the initial population, conidial spore suspensions of 3000 spores per mL was produced for each isolate (Table 3.2). Equal volumes of the prepared spore suspension from each isolate were mixed to make a single inoculum population. This suspension was considered as the initial inoculum or generation zero (G0). The resultant suspension was inoculated onto each line/cultivar.

Inoculation with the initial inoculum (G0)

Seedlings were inoculated at the 2-3 leaf stage with a 3000 spores per mL suspension of the initial inoculum (G0). Ten drops of Tween 20 (polyoxyethylene sorbitol monolaurate) per L were added before inoculation to reduce surface tension. The spore suspension was agitated during inoculation to keep the spores suspended. A de Vilbiss sprayer, connected to an air line operated at a pressure of approximately 67 kPa, was used to spray the conidial suspension on the seedlings till run-off. Inoculated plants were incubated in a misting chamber. Continuous leaf wetness was provided by two ultrasonic humidifiers operated by an electronic leaf wetness control device for 24 h at 22/18°C (day/night) with a 16-h photoperiod. All plants were then incubated in a growth room set at 22:18°C (day:night) and 16-h photoperiod at a light intensity of $\approx$180 $\mu Em^{-2}s^{-1}$.

Recovering the pathogen to construct generations 1, 2, 3 and 4

Infected leaves from each cultivar/line were harvested 7-10 days post inoculation, and air-dried at room temperature for at least 48 h. Twenty infected leaves were then randomly selected and cut into 3-4 cm-pieces. Fifteen to twenty of such pieces were placed on moist filter paper in a Petri plate. Lesions were stimulated to sporulate by subjecting them to 18 h of continuous light at room temperature followed by 24 h of dark at 15°C. At least three such plates were prepared and used for recovering the resultant pathogen population on each cultivar at each generation.

To sample the pathogen population for each cultivar/line used in the experiment, 180-200 single spores were randomly picked from the cultivar at each generation and at a rate of one single-spore per lesion. Single spores obtained from each lesion were

transferred to 6 cm-in-diameter Petri plates containing 15 mL of V8-PDA. Cultures were incubated at 20°C until the colonies reached 3 cm in diameter. The pathogen cultures were labeled and kept separately in a refrigerator (4-5 °C) until used.

Inoculum preparation of the first generation

Subcultures from the single-spore plates were prepared in a 10 cm-in-diameter Petri plates containing 30 mL of V8-PDA. A 3000 spores per mL suspension was prepared from each plate for each single-spore as described before. At each generation, inoculum of at least 60 single-spore cultures from each cultivar were mixed in an equal proportion, and inoculated on the cultivar/ line from which they originated.

Inoculation of the first pathogen population

The first generation (G1) of the pathogen was inoculated on a new set of 12-day-old seedlings (two-leaf stage) of each cultivar/line to form the second generation (G2). The whole procedure (incubation of the infected plants, recovering the formed generation on the plants) was repeated for at least four consecutive generations on each cultivar/line.

Determining the structure of the resultant pathogen populations

To detect changes in the frequency of isolates producing Ptr ToxA and/or Ptr ToxB in the resultant populations, the structure of the pathogen population was characterized at the second and the fourth generations on each line/cultivar. Polymerase chain reaction (PCR) technique was performed on samples of 30-45 single spore cultures per population using primers for Ptr toxins (ToxA and ToxB primers).

Fungal Cultures and DNA Extraction

In this study, DNA was extracted from fresh fungal mycelia. One subculture was prepared for each of 30-45 single-spores, which were randomly selected at each generation. Subcultures were grown on V8-PDA in 10 cm-in-diameter Petri plates, and

incubated at 20°C. Colonies of about 4-5 cm were used for DNA extraction. Genomic DNA was extracted from mycelium of isolates using Véronique's method (Véronique *et al.,* 2001). The DNA pellet was air-dried, dissolved in 100 µL of TE buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA), and stored at 4 °C until used.

PCR Amplification Conditions

Amplification reactions were performed in a 50 µL volume consisting of 5 µL of 10X *Taq* polymerase reaction buffer (Gibco BRL), 1.5 µL of 50 mM $MgCl_2$ (Gibco BRL), 0.5 µL of 25 mM stock each of dNTP, 1.0 µL of *Taq* polymerase 5 units/µL (Gibco BRL), 1.0 µl of 20 pM Ptr toxin primers, 3 µL of 100 ng/µL of template DNA of each single-spore, and 37 µL of sterile HPLC-grade water. Amplification was carried out in a thermal cycler PTC-100 (MJ Researcher) programmed for 3 minute at 95°C, followed by 40 cycles of 45 seconds of 93°C, 30 seconds at 56°C, and 1 minutes at 68°C.

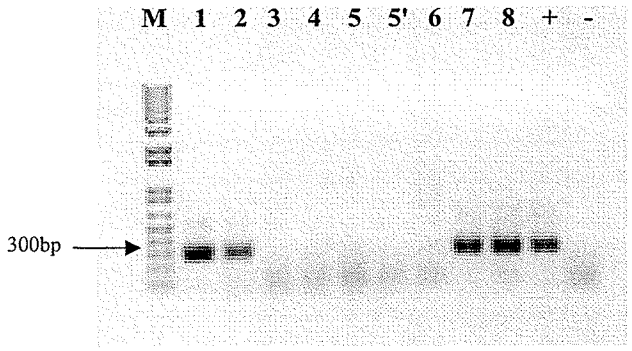
The amplified DNA products were electrophoresed in 1% agarose gel with 1× TAE running buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA pH= 8.0), and visualized with ethidium bromide staining. Digital images of the gels were captured, using an Alpha Image system PC from BIORAD.

Primers for PCR

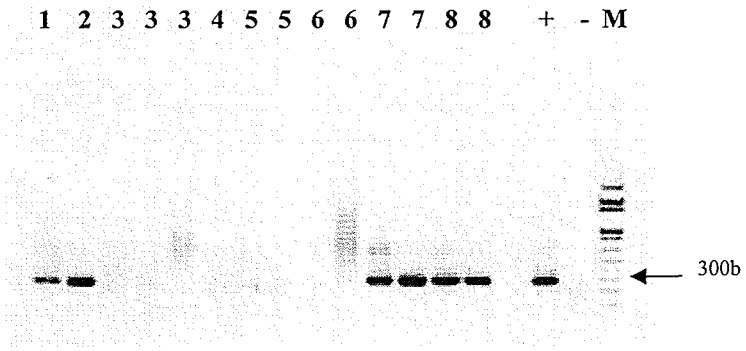
Primers ToxA1 (GTC ATG CGT TCT ATC CTC G) and ToxA2 (CCT ATA GCA CCA GGT CGT CC) amplify a 290 bp target region of races 1, 2, 7 and 8 (Fig. 3.1(a) and 3.1(a')). Primers ToxB2 (TAA CAA CGT CCT CCA CTT TGC) and ToxB3 (ATC AAC GAA GCG GTT ATT GC) amplify a product of 169 bp from races 3, 4, 5, 6, 7 and 8 (Fig. 3.1(b) and 3.1(b')). Primers R3RTPCR-1 (CTG TCT ACG GCT CTG CTG TCT G) and ToxB2 amplify a 402 bp product from race 3 (Fig. 3.1(c)). The primers were

constructed by Invitrogen Life Technologies based on sequences we provided. Location direction and of *ToxA* and *ToxB* primers are described in Fig. 3.2.

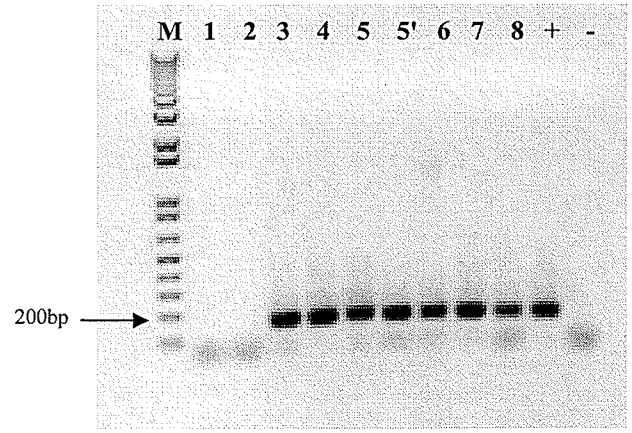
The primers were tested with all races to ensure their efficient amplification of the specific regions they were designed to amplify. The primers correctly differentiated, Ptr ToxA-producing isolates, Ptr ToxB-producing isolates, Ptr ToxA- and Ptr ToxB-producing isolates, as well as isolates of race 3. Sample gel pictures of this detection on populations are shown in Figures 3.2 and 3.3.



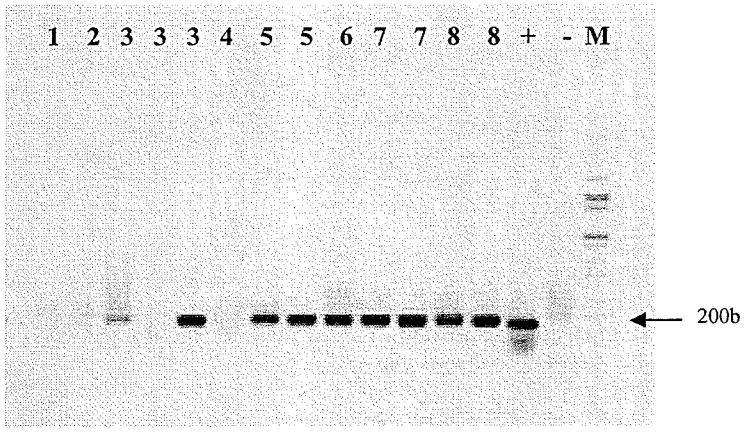
3.1a



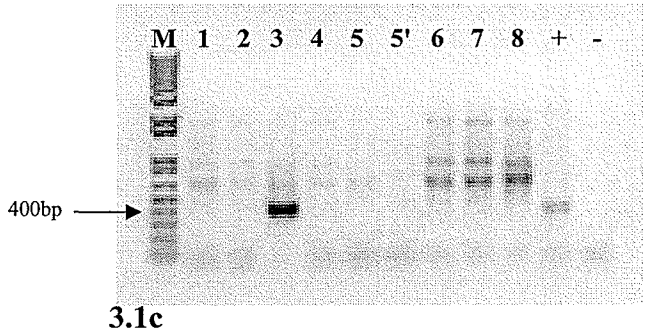
3.1a'



3.1b

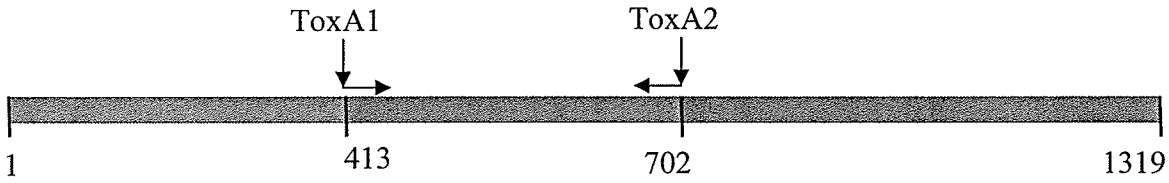


3.1b'

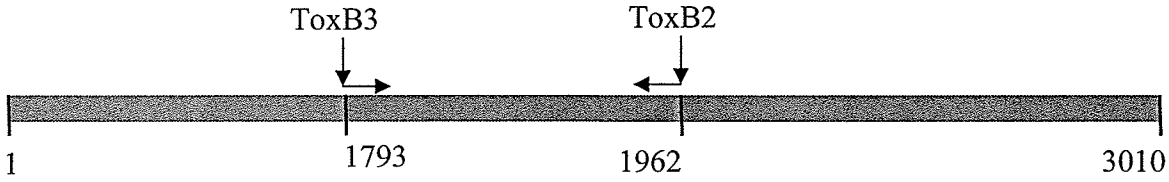


3.1c

Figure 3.1. Race identification of *P. tritici-repentis*, using primers for the encoding region of Ptr toxins. **(3.1a** and **3.1a')** Primers ToxA1 and ToxA2 amplify a 290 bp target region of races 1, 2, 7 and 8. **(3.1b** and **3.1b')** Primers ToxB2 and ToxB3 amplify a product of 169 bp from races 3, 4, 5, 6, 7 and 8. **(3.1c)** Primers R3RTPCR-1 and ToxB2 amplify a 402 bp product from race 3. **M**=1Kbp marker, (+) =Positive control, (-) =Negative control. Races: 1 (ASC1, NA9-6), 2 (86-124, SK16-4), 3 (D308, 331-9, 94-25), 4 (90-2), 5 (Alg3-24, NA4-4, and ALGX-1-1), 5' (92-17R5), 6 (AlgH2), 7 (ICARDA35-5, ICARDA 36-3, ICARDA 36-4), and 8 (ICARDA 73-1, ICARDA 31-1, ICARDA31-2).

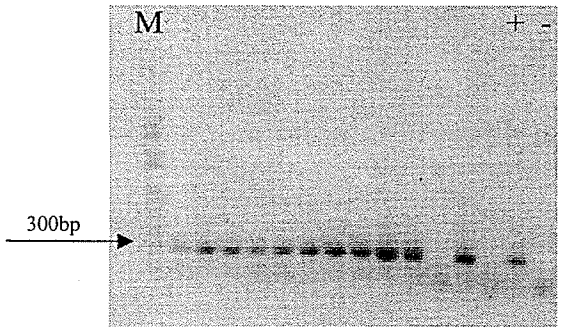


a.

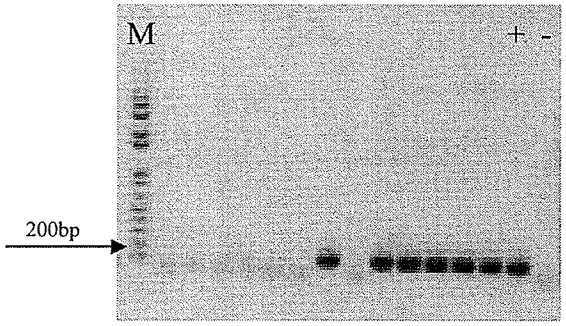


b.

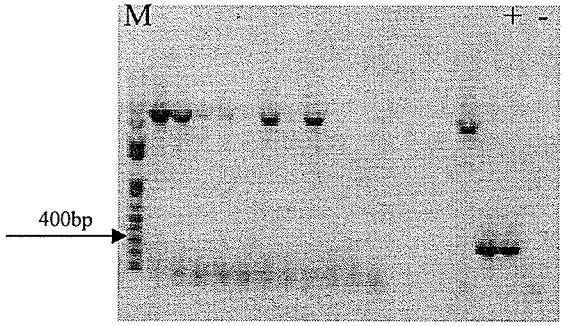
Figure 3.2. Location and direction of *ToxA* and *ToxB* primers used for PCR.. **a.** *ToxA:* GenBank accession number: AF004369. **b.** *ToxB:* GenBank accession number: AY242115



3.2a



3.2b



3.2c

Figure 3.3. Determining population structure at the second generation of *P. tritici-repentis* on Thatcher by PCR using Ptr toxin primers. **3.2a)** PCR amplification products of Ptr ToxA-producing isolates using *ToxA* primer set. **3.2b)** PCR amplification products of Ptr ToxB-producing isolates using *ToxB* primer set. **3.2c)** PCR amplification products of Ptr ToxC-producing isolates using race 3 primer set. **M**=1Kbp marker, (+) =Positive control, (-) =Negative control.

3.4 Results

First experiment

The structure of the recovered populations of *P. tritici-repentis* from the wheat lines/cultivars for the second and the fourth generations based on PCR are presented in Fig. 3.4 and Fig. 3.5, respectively, and their trend on each cultivar is presented in Fig. 3.6. Except for the race 3 isolate, which was eliminated on Red Fife (insensitive to Ptr ToxC) from the second generation and on Selkirk (resistant) at the fourth generation, all other isolates survived on all lines/cultivars.

Ptr ToxA-producing isolates

Except on Coulter, Ptr ToxA-producing isolates increased from generation zero to four on all the susceptible and resistant cultivars. They were predominant on all the hexaploid cultivars as well as on the tetraploid wheat 4B160 (insensitive to Ptr ToxA) at the second and fourth generations (Fig. 3.4). The highest frequency of Ptr ToxA-producing isolates was on Red Fife (sensitive to Ptr ToxA) at the second generation and on 4B160 at the fourth generation. Their lowest frequency was on Coulter (sensitive to Ptr ToxA) at both generations. Ptr ToxA-producing isolates were, as a group, at all times the most frequently recovered isolates.

Ptr ToxB-producing isolates

Ptr ToxB-producing isolates survived at all generations on all the susceptible and resistant lines/cultivars. Their frequency decreased from generation zero to the second generation, and slightly increased from the second to the fourth generation on Neepawa (Ptr ToxA- and Ptr ToxB-sensitive) and 4B160 (Ptr ToxA- and Ptr ToxB-insensitive). They also had higher frequency on the susceptible than on the resistant cultivars. On Red

Fife (Ptr ToxB-insensitive), Ptr ToxB-producing races were less frequent compared to their frequency on other lines and cultivars.

Ptr ToxA- and ToxB-producing isolates

Ptr ToxA- and Ptr ToxB-producing isolates (races 7 and 8) survived on all lines/cultivars, except on 4B160 (Ptr ToxA- and Ptr ToxB-insensitive) where they eliminated at the last generation. After decreasing from generation zero to the second generation, there was an increase in their frequency from the second to the fourth generation on hexaploid, but not on tetraploid cultivars. They were always the second predominant group on all wheat lines/cultivars. Races 7 and 8 were most frequently recovered from Red Fife (sensitive to Ptr ToxA only) at second generation, and from AC Domain (sensitive to all three toxins) and Thatcher (sensitive to Ptr ToxB only) at the fourth generation. At fourth generation, on AC Domain, Ptr ToxA- and ToxB-producing races were present at frequencies similar to those of Ptr ToxA-producing races. Races 7 and 8 (Ptr ToxA- and ToxB-producing isolates) had a similar frequency as the Ptr ToxB-producing races on Selkirk (resistant), at the second generations, and on Coulter at the fourth generation.

Ptr ToxC-producing isolate

The race 3 isolate used in this study decreased from generation zero to the second generation, except on 4B160 and Coulter both insensitive to Ptr ToxC. This Ptr ToxC-producing isolate was the first predominant group on Coulter at both the second and fourth generation, and formed the second predominant group on 4B160 at the second generation. It disappeared from the pathogen population at the second generation on Red Fife, and was not recovered at the fourth generation either. Race 3 was the least frequent

on all hexaploid cultivars at both the second and fourth generations. Except on AC Domain (sensitive to Ptr ToxC) and Coulter (insensitive to Ptr ToxC), there was a decrease from the second to fourth generation for this Ptr ToxC-producing isolate. Race 3 was eliminated from the pathogen population on Neepawa (insensitive to Ptr ToxC) and Selkirk (resistant) at the fourth generation.

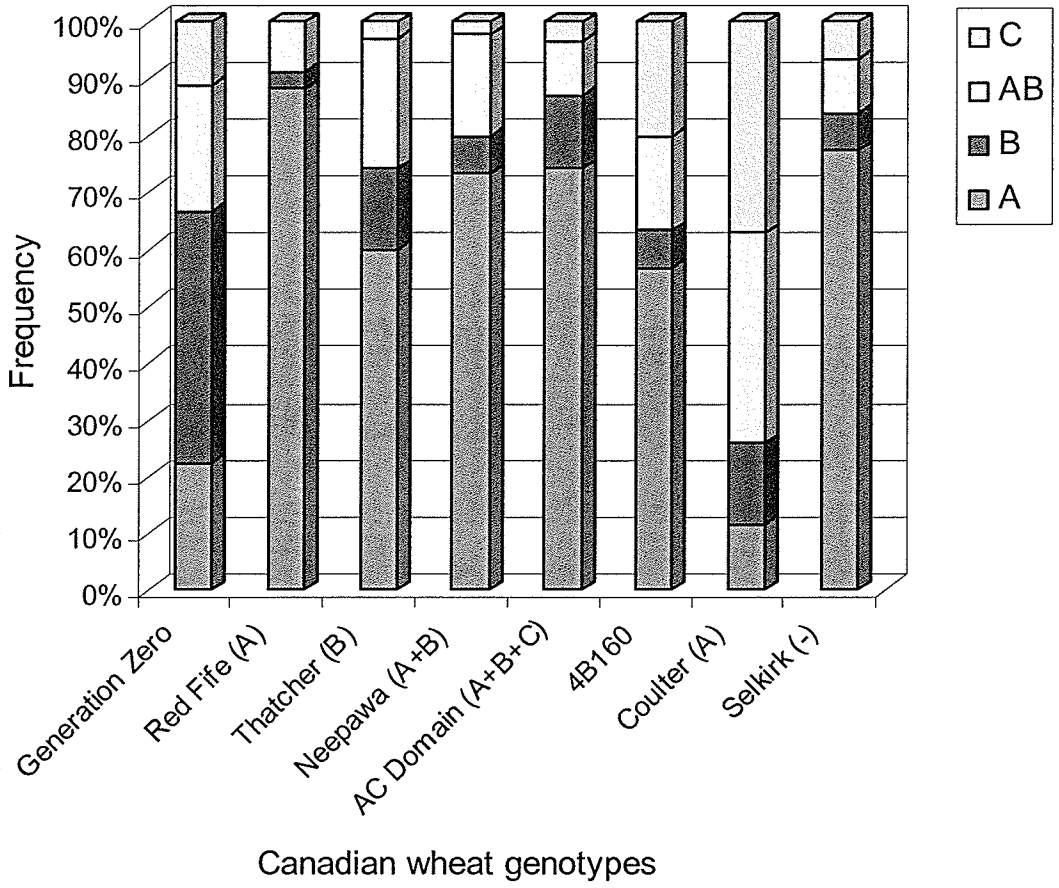


Figure 3.4. Population structure of *P. tritici-repentis* at the second generation on different Canadian cultivars for the first experiment. A=Ptr ToxA-producing isolates= ASC1 (Race 1), 86-124 (Race 2). B =Ptr ToxB-producing isolates= ALG3-24 (Race 5), 92-171R5 (Race 5), ALGH2 (Race 6). AB =Ptr ToxA and ToxB producing isolates= ICARDA 35-5 (Race 7), ICARDA 73-1 (Race 8). C = Ptr ToxC-producing isolate = D308 (Race 3)

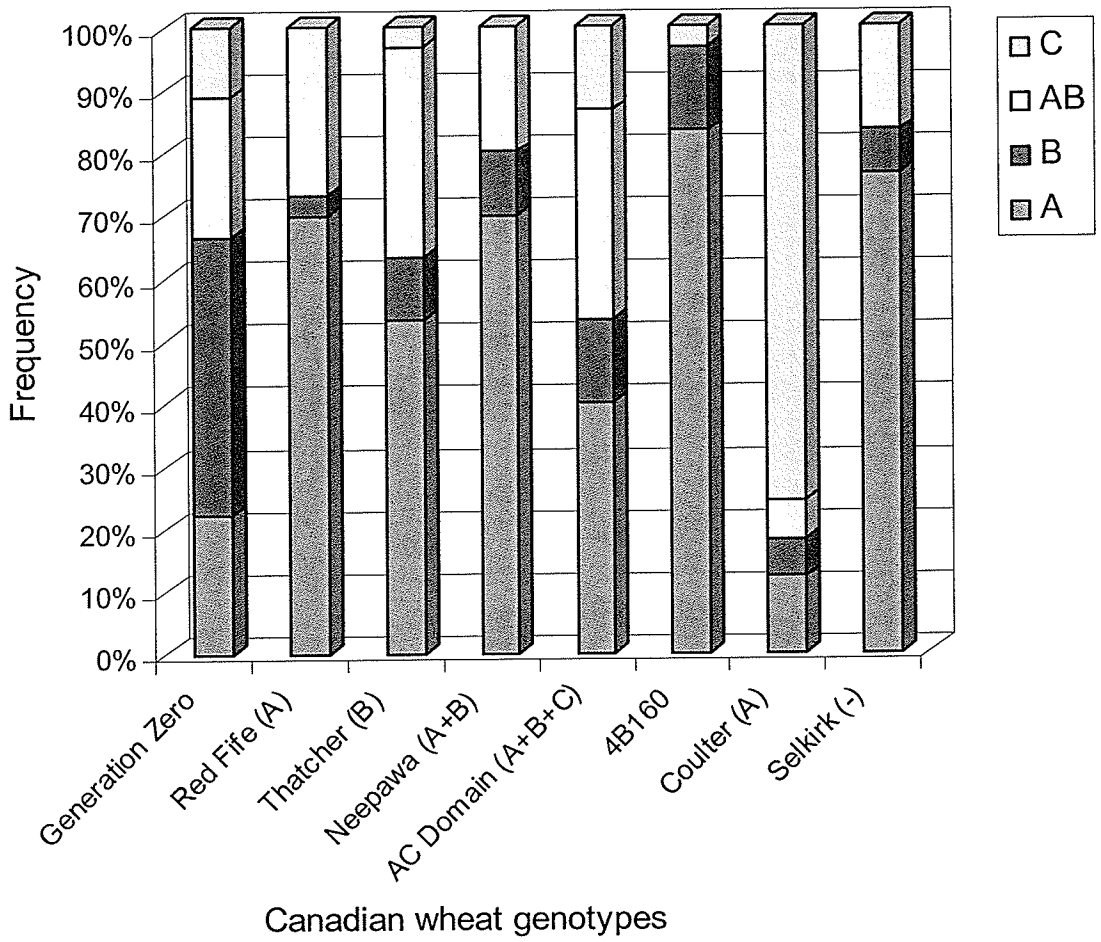


Figure 3.5. Population structure of *P. tritici-repentis* at the fourth generation on different Canadian cultivars for the first experiment. A=Ptr ToxA-producing isolates= ASC1 (Race 1), 86-124 (Race 2). B =Ptr ToxB-producing isolates= ALG3-24 (Race 5), 92-171R5 (Race 5), ALGH2 (Race 6). AB =Ptr ToxA and ToxB producing isolates= ICARDA 35-5 (Race 7), ICARDA 73-1 (Race 8). C = Ptr ToxC-producing isolate = D308 (Race 3).

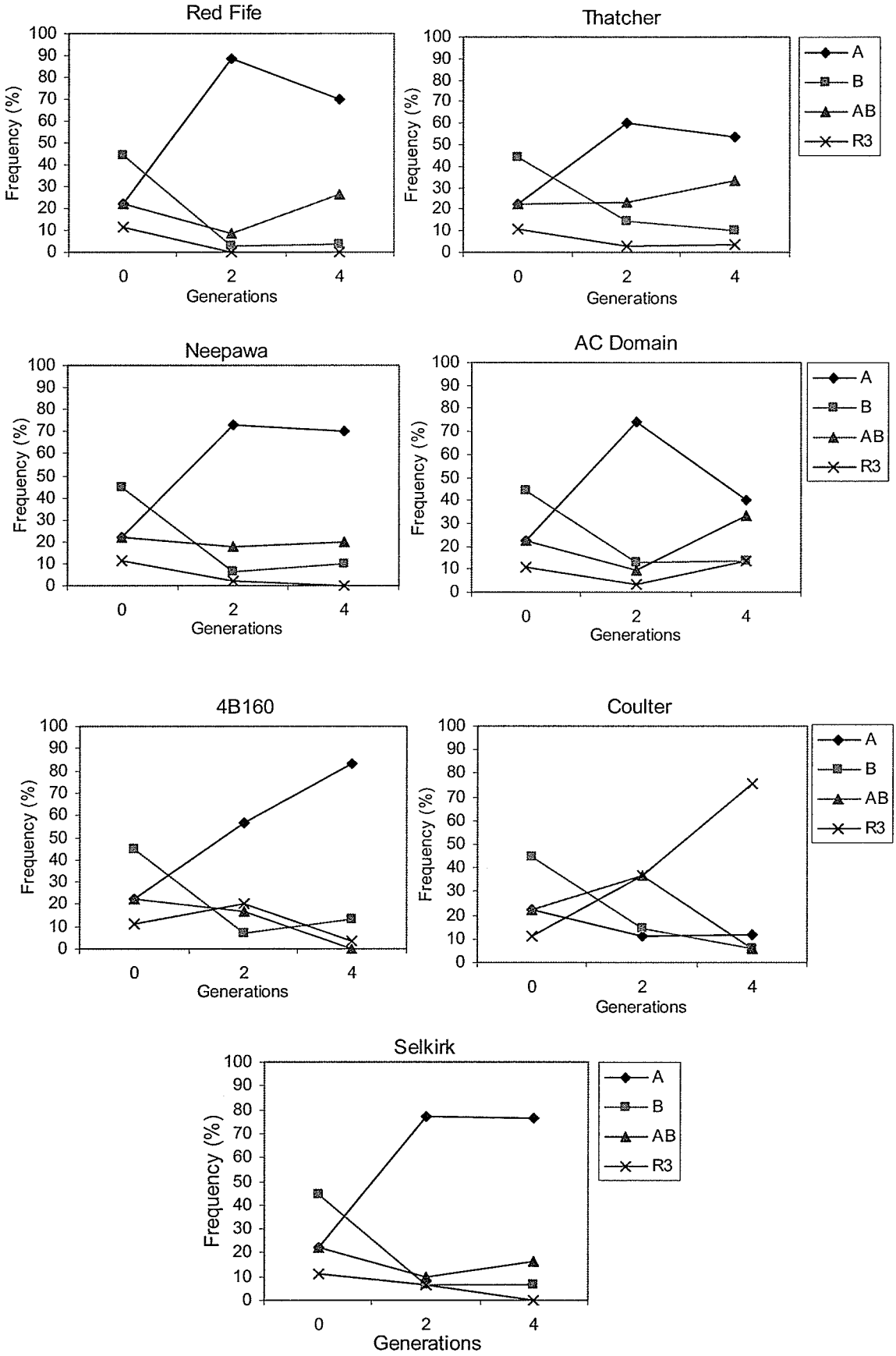


Figure 3.6. Population shifts of *P. tritici-repentis* on different Canadian cultivars for the first experiment.

Second Experiment

Population structures of *P. tritici-repentis* for the second and fourth generations are presented in Fig. 3.7 and Fig. 3.8, respectively, and their trend through the generation on each cultivar is shown in Fig. 3.9.

Ptr ToxA-producing isolates

Ptr ToxA-producing isolates survived on all the tested wheat cultivars. Their frequency increased from generation zero to the fourth generation, except on Thatcher (insensitive to Ptr ToxA). At the second generation, they were the third dominant members on all the cultivars except on Coulter (sensitive to Ptr ToxA) where they were the second dominant group. At the last generation, Ptr ToxA-producing isolates were the most predominant on Coulter (sensitive to Ptr ToxA) and the least frequent on Thatcher (insensitive to Ptr ToxA). At the fourth generation, Ptr ToxA-producing isolates had a frequency almost similar to the predominant isolates on Red Fife (sensitive to Ptr ToxA only), and were the second dominant group on Neepawa (Ptr ToxA- and Ptr ToxB-sensitive).

Ptr ToxB-producing isolates

Ptr ToxB-producing isolates survived on all the tested cultivars. Their frequency increased from generations zero to four on all the cultivars, except on Coulter (insensitive to Ptr ToxB). At the second generation, Ptr ToxB-producing isolates were the most predominant members on all cultivars, except on Thatcher (sensitive to Ptr ToxB) where they were the second dominant group. At the fourth generation, they were still the most predominant on all cultivars, except Coulter (insensitive to Ptr ToxB), where they were the second dominant isolates in the recovered population.

Ptr ToxA- and ToxB-producing isolates

Frequency of Ptr ToxA- and ToxB-producing isolates decreased from generation zero to four on all the cultivars, except on Thatcher (sensitive to Ptr ToxB only). At the second generation, they were the first predominant group on Thatcher (sensitive to Ptr ToxB only). On Red Fife (sensitive to Ptr ToxA only) and Neepawa (Ptr ToxA- and Ptr ToxB-sensitive) they were always the second dominant group of isolates. Their least frequency was on Coulter (sensitive to Ptr ToxA, insensitive to Ptr ToxB).

Ptr ToxC-producing isolates

Ptr ToxC-producing isolates decreased from generations zero to four, and were eliminated on Red Fife (insensitive to Ptr ToxC) and Thatcher (insensitive to Ptr ToxC). They were the least frequent group of isolates on Neepawa (insensitive to Ptr ToxC) and Coulter (insensitive to Ptr ToxC), and finally were eliminated at the last generation on Coulter.

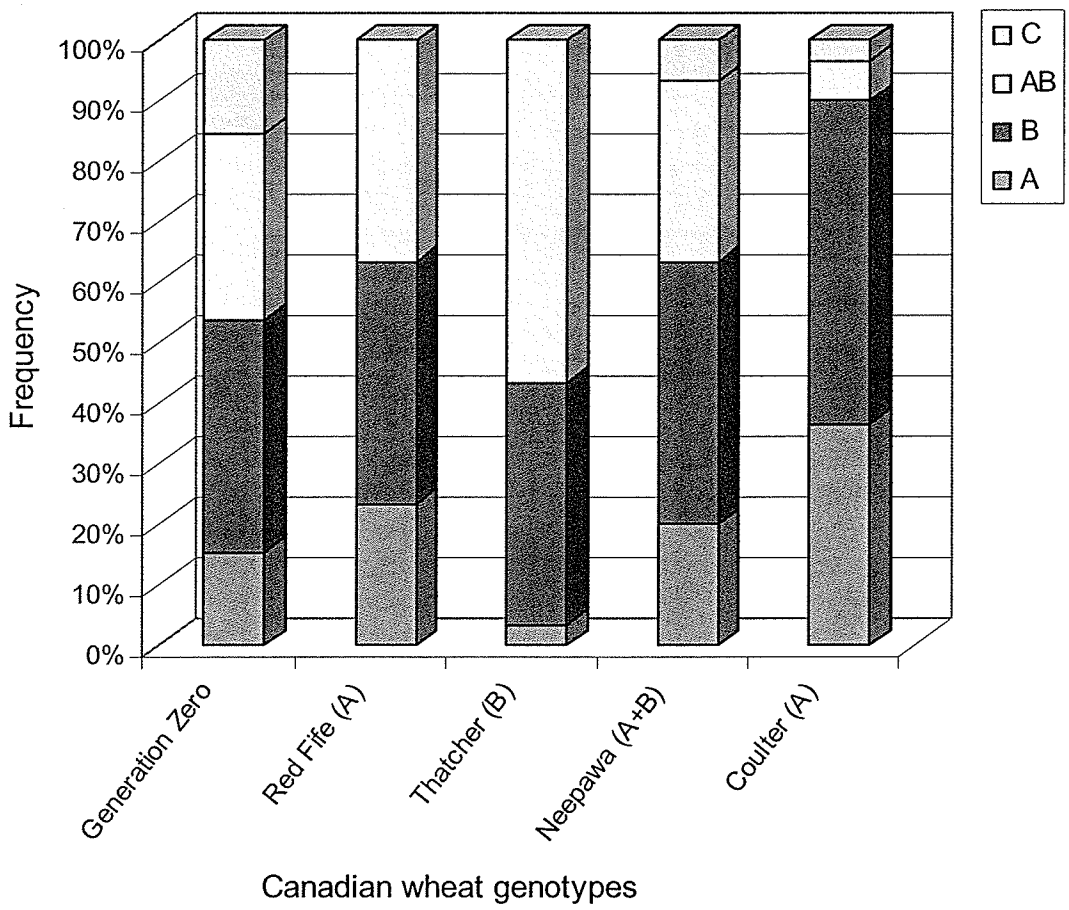


Figure 3.7. Population structure of *P. tritici-repentis* at the second generation on different Canadian cultivars for the second experiment. A=Ptr ToxA-producing isolates=NA9-6 (Race 1), SK16-4 (Race 2). B=Ptr ToxB-producing isolates=NA4-4 (Race 5), ALG5-X1-1 (Race 5), ALGH2 (Race 6), 90-2 (Race 4). AB=Ptr ToxA- and ToxB-producing isolates= ICARDA 36-3 (Race 7), ICARDA 36-4 (Race 7), ICARDA31-1 (Race 8), ICARDA 31-2 (Race 8). C=Ptr ToxC-producing isolates=331-9 (Race 3), 94-25 (Race 3).

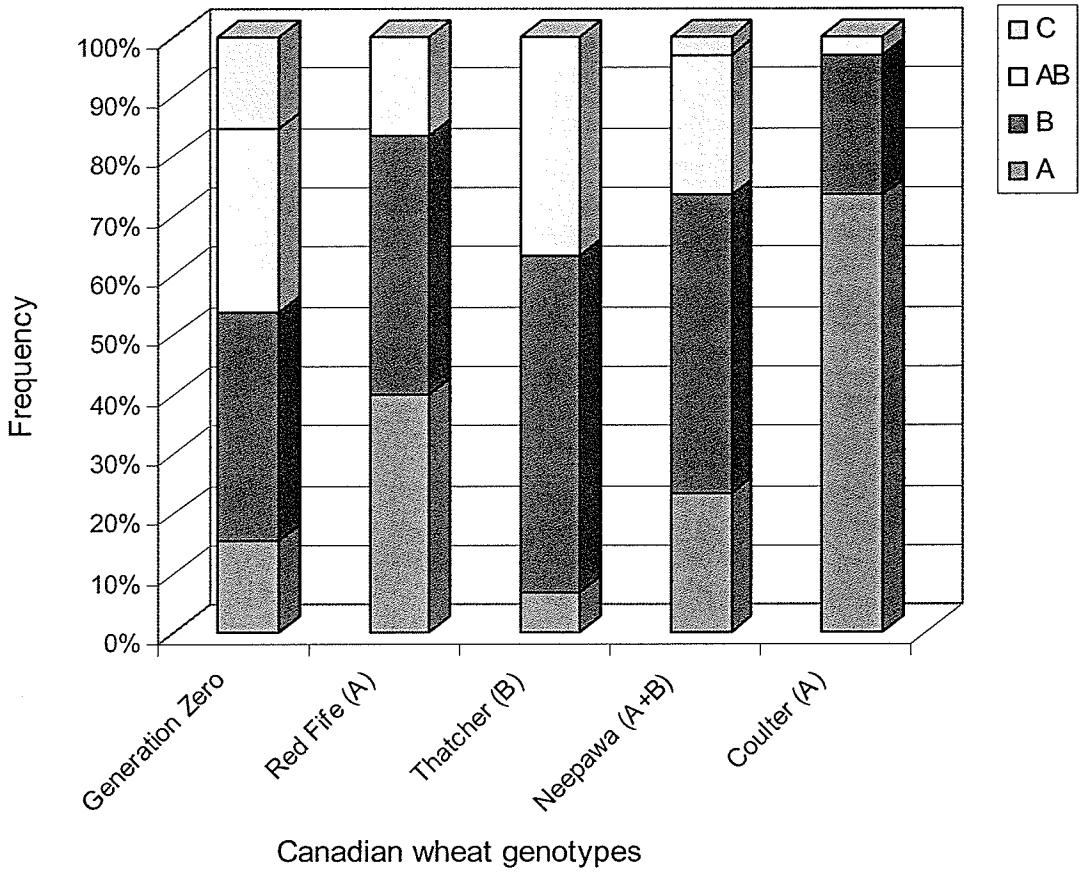


Figure 3.8. Population structure of *P. tritici-repentis* at the fourth generation on different Canadian cultivars for the second experiment. A=Ptr ToxA-producing isolates=NA9-6 (Race 1), SK16-4 (Race 2). B=Ptr ToxB-producing isolates=NA4-4 (Race 5), ALG5-X1-1 (Race 5), ALGH2 (Race 6), 90-2 (Race 4). AB=Ptr ToxA- and ToxB-producing isolates= ICARDA 36-3 (Race 7), ICARDA 36-4 (Race 7), ICARDA31-1 (Race 8), ICARDA 31-2 (Race 8). C=Ptr ToxC-producing isolates=331-9 (Race 3), 94-25 (Race 3).

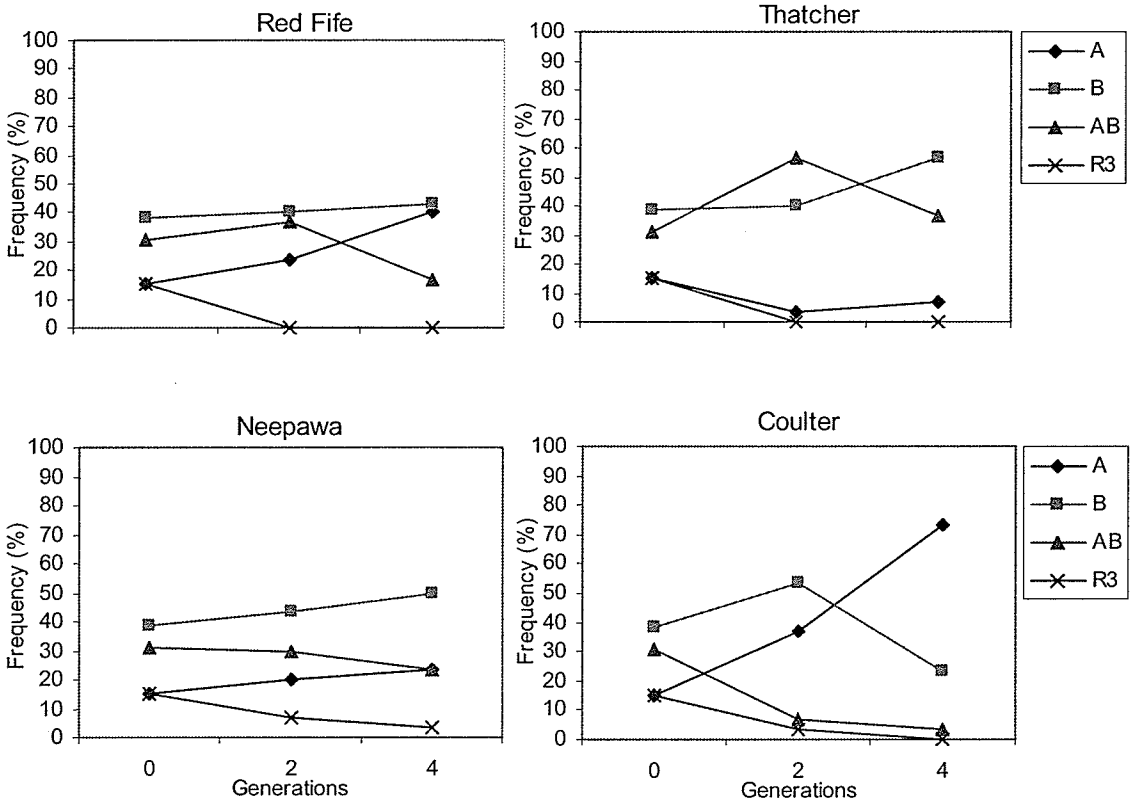


Figure 3.9. Population shifts of *P. tritici-repentis* on different Canadian cultivars for the second experiment.

3.5 Discussion

Isolates producing Ptr ToxA were found to be the most frequently recovered members of the population on both Ptr ToxA-sensitive and -insensitive wheat cultivars tested in the first experiment, except on Coulter. The frequencies of Ptr ToxA-producing isolates also increased on both the susceptible and resistant wheat cultivars from generation zero to four in both experiments, except on Thatcher (insensitive to Ptr ToxA). As Ptr ToxA-producing isolates finally became predominant at the fourth generation on susceptible cultivars Coulter and Red Fife in the first and second experiment, (respectively) they would be expected to predominate on other susceptible cultivars such as Neepawa, if the experiment went to further generations. Ptr ToxA-producing isolates used in the second experiment, were increasing but were not always the predominant members of the recovered populations. This suggests that there is a variation among isolates of certain races, (e.g. 1 and 2) in terms of their competitive ability.

Ptr ToxB-producing isolates showed a different behavior on the same cultivars in both experiments. In the first experiment, frequency of Ptr ToxB-producing isolates decreased from generation zero to four regardless of the susceptibility of the host genotypes, but in the second experiment, an increasing trend was observed for Ptr ToxB-producing isolates, even on Red Fife (insensitive to Ptr ToxB). ToxB-producing isolates not only were not eliminated from the pathogen population on all cultivars, but also were always the most recovered isolates on Red Fife (insensitive to Ptr ToxB) and Neepawa (sensitive to Ptr ToxB and Ptr ToxA). Widespread cultivation of susceptible host cultivars has been shown to favor virulent races of rust in Canada (Green, 1971; Johnson and Newton, 1941). By analogy, our results strongly support the idea that Ptr ToxB-

producing isolates were absent through so many years of cultivation of Ptr ToxB-sensitive wheat cultivars, or may have been less competitive as in the first experiment. Otherwise, they would have been selected by near a century of cultivation of susceptible genotypes in Western Canada.

As competition studies on races of *Puccinia striiformis* (Brown and Sharp, 1970) and *Puccinia graminis tritici* (Osoro and Green, 1976) have shown, races with the most virulence genes predominated in pathogen populations. Therefore, it was expected that having more than one Ptr toxin would give more selective advantage to the isolates of *Pyrenophora tritici-repentis* as well. However, the results of this study did not confirm it. Ptr ToxA- and ToxB-producing isolates were increasing from generations zero to four on all the tested susceptible cultivars, and were always the second predominant group of isolates except on the tetraploid cultivars used in the first experiment. In most of the recovered populations from the second experiment, Ptr ToxA- and ToxB-producing isolates kept their rank as the second predominant group after Ptr ToxB-producing isolates.

The decreasing trend and ultimately elimination of Ptr ToxC-producing isolates of race 3 on resistant cultivars, Red Fife, Thatcher and Neepawa (insensitive to Ptr ToxC) would be predictable in both experiments. Frequency of these isolates increased from generation zero to four on AC Domain (sensitive to Ptr ToxC) and Coulter (susceptible to race 3) in experiment one. However, a decrease leading to the disappearance of race 3 occurred on Coulter (susceptible to all race) in the second experiment, which represents a high level of variation among the isolates in race 3 in terms of their competitive ability in the pathogen population.

The results suggest that the structure of the formed generations is not necessarily consistent with the theory of race-specific interactions between isolates of the pathogen and sensitive lines/cultivars. Furthermore, the results reveal that different isolates from the same race do not behave similarly on the same cultivar. This is the case for race 3 isolates, D308 (producing putative Ptr ToxC) was the most predominant member in the pathogen population on Coulter (sensitive to Ptr ToxC) in the first experiment, but in the second experiment, isolates 331-9 and 94-25 (race 3, producing putative Ptr ToxC) became the least frequent in the second generation, and were even eliminated at the fourth generation from the pathogen population on Coulter.

Also, survival of isolates on cultivars resistant to them, survival of Ptr ToxC- and Ptr ToxA-producing isolates on Thatcher (insensitive to Ptr ToxA and Ptr ToxC), and survival of Ptr ToxB-producing races on Red Fife (insensitive to Ptr ToxB) are examples which indicate that cultivar sensitivity to Ptr toxins is not the only determinant factor for the selection or elimination of an isolate from the populations of *P. tritici-repentis*. This suggests that, besides the type of the known Ptr toxin(s) produced by the isolates of the pathogen, there may be other factors which contribute to the interaction of a specific isolate with its host. For example, differences among the individual isolates of one race in terms of their ability to infect and colonize wheat plants, and sporulation in terms of timing and quantity could be some of the factors which may be important for the success of an isolate to compete with other isolates present on a specific wheat line/cultivar. Therefore, regarding a population of different isolates, an isolate-specific interaction between *P. tritici-repentis* and wheat lines/cultivars is more probable than an interaction, based totally on host-specific toxins.

The ability of Ptr ToxB-producing races to compete and survive in the presence of Ptr ToxA-producing races in the *P. tritici-repentis* populations is a subject of concern. The large areas cultivated with Ptr ToxB-sensitive wheat lines/cultivars, along with the presence of the mutated encoding gene for Ptr ToxB in the pathogen population in western Canada (Strelkov *et al.*, 2003), could provide two essential components of the disease triangle. This might lead to major outbreaks if the environmental conditions favor. Hence, breeders may want to consider this fact in their breeding programs, and be alert not to introduce unintentionally the sensitivity to Ptr ToxB to cultivars in the future, as it happened before for Ptr ToxA sensitivity (Lamari *et al.*, 2005a). Providing resistance not only to necrosis-inducing isolates of *P. tritici-repentis*, but also to the chlorosis-inducing isolates of *P. tritici-repentis* should be included in their breeding programs.

4.0 CYTOLOGICAL STUDIES ON NECROTIC AND CHLOROTIC LESIONS INDUCED BY *PYRENOPHORA TRITICI-REPENTIS* ON WHEAT

4.1 Abstract

Pyrenophora tritici-repentis causes necrosis and chlorosis on susceptible wheat hosts. Ptr ToxA and Ptr ToxB are responsible for inducing necrosis and chlorosis respectively, in sensitive wheat lines/cultivars. Using a fluorescence microscopic technique, the pre-colonization and colonization events of necrosis- and/or chlorosis-inducing isolates of the pathogen were studied on four wheat lines/cultivars, three Ptr toxin-sensitive and one resistant. There was no significant difference between Ptr ToxA- and/or Ptr ToxB-producing isolates in terms of germination percentage, number of germ tubes and appressoria per conidia, as well as the penetrated epidermal cells per conidia. There was also no significant difference between isolates in their incompatible interaction with wheat lines/cultivars in terms of percentage of mycelial area coverage (mycelium %) in the mesophyll layer. But, there was a significant difference between Ptr ToxA- and Ptr ToxB-producing isolates in their compatible interaction with wheat lines/cultivars after 24 hours and thereafter till 72 hours post inoculation, necrosis-producing isolates had a higher percentage of mycelial area coverage than chlorosis-inducing isolates.

4.2 Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker) causes tan spot, a destructive disease of wheat worldwide (Hosford, 1982). Tan spot may cause yield losses up to 50% (Rees *et al.*, 1982). Changes in cultural practices have increased incidence of the disease (Rees and Platz, 1992). Tan necrosis and extensive chlorosis are two types of symptoms produced by the pathogen on susceptible wheat genotypes (Lamari and Bernier, 1989a, 1989b and 1991).

In susceptible wheat cultivars, necrosis and chlorosis symptoms are induced by two proteinaceous host specific toxins. Ptr ToxA induces necrosis (Lamari and Bernier, 1989a). Ptr ToxA is encoded by a single copy gene, named *ToxA*, (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Ptr ToxB induces chlorosis (Strelkov *et al.*, 1998b). Production of Ptr ToxB is controlled by *ToxB* gene, which is present in multiple copies (Martinez *et al.*, 2001; Strelkov *et al.*, 2002; Strelkov *et al.*, 2006). Sensitivity to Ptr ToxA and Ptr ToxB is controlled by two independent dominant genes in the host, one gene for each toxin (Gamba *et al.* 1998).

So far, studies on the infection process of *P. tritici-repentis* on its host plants included only the induced necrotic lesions by Ptr ToxA-producing isolates (Dushinsky *et al.* 1996; Larez *et al.*, 1986; Loghman and Deverall, 1986), except one histological study conducted by Lamari and Bernier (1998b) on chlorosis lesions induced by putative Ptr ToxC on wheat line 6B365 (chl+) with isolate ASC1 (chl+, nec+). To investigate differences in the infection process among Ptr ToxA and/or Ptr ToxB-producing isolates of *P. tritici-repentis*, we studied both the chlorotic and necrotic symptoms using

fluorescence microscopy. The main objective of this study was to compare the colonization process of Ptr ToxA and/or Ptr ToxB producing-isolates in necrotic and chlorotic lesions in wheat host plants.

4.3 Materials and Methods

Plant materials

Three susceptible and one resistant hexaploid wheat cultivars (*Triticum aestivum L.*) were selected for this study (Table 4.1). Wheat seeds were planted in 10-cm-diameter clay pots, containing a mixture of a soil:sand:peat moss (2:1:1, v:v:v). Ten pots containing five to six evenly spaced seeds were prepared for each line/cultivar. Seedlings were incubated in a growth room set at 22/18 °C (day/night) with a 16-h photoperiod at a light intensity of ≈180 $\mu E.m^{-2}.s^{-1}$. Plants were watered once a day, and fertilized using a 10-20-0-14 (N-P-K-Ca) fertilizer weekly.

Table 4.1. Wheat lines/cultivars and the type of lesion induced on them by Ptr toxins.

Cultivar	Lesion type	Ptr toxin sensitivity	Race Susceptibility
Glenlea	Necrotic	(A+)*	1, 2, 7, 8
Thatcher	Chlorotic	(B+)*	5, 6, 7, 8
Neepawa	Necrotic and Chlorotic	(A+) and (B+)	1, 2, 5, 6, 7, 8
Salamouni	Resistant	No sensitivity	No susceptibility

*(A+)= Sensitive to Ptr ToxA, (B+)= Sensitive to Ptr ToxB

Pathogen isolates

One isolate from each of races 2 (Ptr ToxA+), 5 (Ptr ToxB+), 7 (Ptr ToxA+ and Ptr ToxB+), and 4 (avirulent) were used in this study. The types of the induced lesions by each isolate on the wheat plants used in this study are presented in Table 4.2.

Table 4.2. *Pyrenophora tritici-repentis* isolates and the type of symptom(s) induced on wheat lines/cultivars.

			Host Cultivar			
Race	**Isolate**	**Ptr Toxin(s) produced**	**Glenlea**	**Thatcher**	**Neepawa**	**Salamouni**
2	86-124	A	necrosis	resistant	necrosis	resistant
5	ALG3-24	B	resistant	chlorosis	chlorosis	resistant
7	ICARDA 35-5	A+B	necrosis	chlorosis	necrosis	resistant
4	90-2	No Ptr toxin	resistant	resistant	resistant	resistant

Experimental design

In order to study the effect of Ptr toxins on pre-colonization stages from 3 to 24 hours post inoculation. (h.p.i.), a factorial experiment with the basic design of a randomized complete block design (RCB) was conducted. Cultivars in four levels (Glenlea, Thatcher, Neepawa, and Salamouni) were used as blocks. The three factors were: time in four levels of 3, 6, 12, and 24 h.p.i., host-pathogen interactions in two levels (compatible, incompatible), and isolate groups in four levels (necrosis-inducing, chlorosis-inducing, necrosis-chlorosis-inducing, and avirulent).

Logarithms of germination percentage along with the square root of the other three traits were used for analysis of variance (ANOVA) to normalize the distribution of the variance.

Inoculum production and inoculation

Single spores of each isolate were grown in 10 cm-in-diameter Petri plates containing 30 mL of V8-potato-dextrose agar (150 mL V8-juce, Difco PDA (10g), $CaCo_3$ (3g), and distilled water (850 mL). Single-spore cultures were incubated at 20°C until the colonies reached 5-6 cm in diameter. Plugs from single-spore plates were placed in 10 cm-in-diameter Petri plates containing 30 mL of V8-PDA. Subcultures were then placed in the dark at 15 °C until colonies had grown 4-5 cm in diameter. Inoculum was harvested

from the subcultures using the procedure described by Lamari and Bernier (1989a). For each isolate, a spore suspension (3000 spores/mL) was prepared. Plants were sprayed at 2-3 leaf stages with individual isolates using a sprayer connected to an air line, operated at a pressure of approximately 67 kPa. The spore suspension was constantly agitated during inoculation to ensure spore distribution uniformity. Prior to inoculation, ten drops of Tween 20 (polyoxyethylene sorbitol monolaurate) per L were added to reduce surface tension.

Inoculated plants were incubated in a misting chamber set at 22/18 °C (day/night) with a 16-h photoperiod for 24 h. Continuous leaf wetness was provided by two ultrasonic humidifiers. Infected plants were then placed in a growth room set at 22:18 °C (day/night) and 16-h photoperiod at a light intensity of ≈180 $\mu E.m^{-2}s^{-1}$ for 5 days. For each treatment, 2 pots of plants were inoculated with distilled water and considered as a control.

Sampling and leaf tissue clearing

Samples were taken for microscopic observations from 3 hours to 3 days after inoculation for each treatment. Two infected leaves per pot were sampled for each treatment at each sampling time. Pieces of about 1.5 cm were taken from the middle of the infected leaves. Samples of infected and healthy control plant leaf tissues were harvested from seedlings of Glenlea, Thatcher, Neepawa, and Salamouni at 3, 6, 12, and 24 h.p.i. for pre-colonization, and at 12, 24, 48, and 72 h.p.i. for evaluation of colonization of the mesophyll layer.

To examine the specimens using fluorescence microscopy, spores and intercellular hyphae were stained using a KOH-aniline blue fluorescence technique developed by

Hood and Shew (1996). Tissue segments were placed in 20 mL of 1M KOH for 24 hours at room temperature, and then autoclaved at 121 °C for 15 minutes. Autoclaved specimens were stored at 4 °C until used. Samples were placed at room temperature for at least 30 minutes before observations. Each specimen was rinsed three times in deionized water and mounted in a staining solution consisting of 0.05% aniline blue (CI #42755 Fisher Scientific) in 0.067M K_2HPO_4 at pH 9.0. This method resulted in a high degree of contrast between the host tissue and the fungal structures.

Samples were examined using a Zeiss microscope fitted with an ultraviolet excitation filter (G365) used in combination with a dichromatic beam splitter (FT395) and barrier filter (LP420) transmitting above 420nm. Digital photos were taken by a Nikon Coolpix 8800 camera.

Mycelium measurements

To compare the amount of colonizing hyphae in both the chlorotic and necrotic lesions, the total length and average thickness of mycelia were measured using the "Digital Ruler" feature of the image analysis software Assess 2.0 (APS Press, St. Paul, MN, USA). Mycelium area of each isolate was calculated in the pictures taken from the samples over the time of observations (5-15 pictures at each sampling time). Calibration was applied on each picture in micron scale, and the total tissue area was computed as the product of the picture's length x width in microns. The percentage of mycelial coverage of the picture for each sample was calculated as follows: % area covered by mycelium = mycelium area / picture area × 100.

Microscopic observations

Pre-colonization events of spore germination, and appressorium formation were measured in terms of germination percentage, number of germ tubes and appressoria per spore. Penetration of epidermal cell wall, vesicle formation, colonization of epidermal cells, as well as colonization of mesophyll layer from Ptr ToxA- and/or Ptr ToxB-producing isolates were observed for chlorotic, necrotic and lesions on resistant cultivars.

4.4 Results

Initial stages of infection

Pathogen isolates were all able to germinate, form germ tubes and appressoria by 3 h.p.i. (Fig. 4.9: A and B). Penetration and colonization of epidermal cells on all the lines/cultivars occurred by 6 h.p.i. irrespective of isolates and/or cultivars (Fig. 4.10: A1, A2, B1, B2, C, and D).

ANOVA (Tables 4.3 to 4.6) showed that sampling time, h.p.i., had a significant effect on germination percentage, germ tube per spore, appressorium per spore, and penetration per spore of the necrosis- and/or chlorosis-inducing isolates as well as the avirulent isolate used in this study. These differences were significant at 1% error level for the germination of germ tubes, appressorium, and number of penetrations per conidium and at 5% for conidium germination. By 6 h.p.i. spore germination of 90% occurred (Fig. 4.1), and there was no significant difference among times post inoculation from 6 h to 24 h.p.i. for all the used isolates (Table 4.7). The lowest percentage of spore germination was at 3 h.p.i. as expected. The number of germ tubes per spore was not significantly different from 6-24 h.p.i., but there was a significant difference between 3 h and 6-24 h.p.i. (Table 4.2, Fig 4.2). The number of appressoria per spore, as well as penetrations per spore at 3 h.p.i. was significantly different from those seen at 6-24 h.p.i. There was no significant difference between 6 and 12, as well as 12 and 24 h.p.i. terms of the number of appressoria per spore, but there was a significant difference between 6 and 24 h.p.i. (Table 4.7). The difference between 6 and 12 h.p.i. in terms of penetration per spore was significant, while there was no difference between 12 and 24 h.p.i. The initial

Table 4.3. Analysis of variance for germination percentage of different isolates of *P. tritici-repentis* on various wheat lines/cultivars.

S.o.V.	df	SS	MS	F value	Pr>F
Cultivar (C)	3	0.017	0.006	0.76 ^{ns}	0.5246
Reaction (R)	1	0.048	0.048	6.42 *	0.0150
Time (T)	3	0.090	0.030	4.02 *	0.0131
Isolate groups (I)	3	0.013	0.004	0.60 ^{ns}	0.6203
T×R	3	0.042	0.014	1.85 ^{ns}	0.1521
I×R	2	0.005	0.003	0.35 ^{ns}	0.7039
T×I	9	0.047	0.005	0.69 ^{ns}	0.7137
Error	43	0.322	0.007	-	-
Total	67	0.584	-	-	-

ns and *: non-significant, significant at error level of 5%, respectively.

Table 4.4. Analysis of variance for germ tube per spore of different isolates of *P. tritici-repentis* on various wheat lines/cultivars.

S.o.V.	df	SS	MS	F value	Pr>F
Cultivar (C)	3	0.120	0.040	3.45 *	0.0245
Reaction (R)	1	0.033	0.033	2.86 ^{ns}	0.0982
Time (T)	3	0.265	0.088	7.61 **	0.0003
Isolate groups(I)	3	0.082	0.027	2.35 ^{ns}	0.0853
T×R	3	0.029	0.010	0.83 ^{ns}	0.4871
I×R	2	0.027	0.013	1.15 ^{ns}	0.3268
T×I	9	0.014	0.002	0.13 ^{ns}	0.9985
Error	43	0.499	0.012	-	-
Total	67	1.068	-	-	-

ns, *, and **: non-significant, significant at error levels of 5% and 1%, respectively.

Table 4.5. Analysis of variance for appressorium per spore of different isolates of *P. tritici-repentis* on various wheat lines/cultivars.

S.o.V.	df	SS	MS	F value	Pr>F
Cultivar (C)	3	0.072	0.024	0.64 ^{ns}	0.9512
Reaction (R)	1	0.004	0.004	0.10 ^{ns}	0.7563
Time (T)	3	1.901	0.634	17.00 **	<0.0001
Isolate groups (I)	3	0.036	0.012	0.32 ^{ns}	0.8079
T×R	3	0.204	0.608	1.82 ^{ns}	0.1577
I×R	2	0.067	0.034	0.91 ^{ns}	0.4120
T×I	9	0.135	0.015	0.40 ^{ns}	0.9262
Error	43	1.603	0.037	-	-
Total	67	4.022		-	-

ns and **: non-significant, significant at error level of 1%, respectively.

stages of the infection process were similar on both the resistant and susceptible wheat lines/cultivars for Ptr ToxA- and/or Ptr ToxB-producing isolates of *P. tritici-repentis* used in this study (Tables 4.4 to 4.6); except for germination percentage where the type of reaction (resistant/susceptible) had a significant effect (Table 4.3). The type of cultivar had no significant effect on any stages of infection, except for germ tube per spore where it was significant at 5% (Table 4.4). There were no significant differences between Ptr ToxA- and/or Ptr ToxB-producing in terms of germination percentage, number of germ tubes and appressoria per spore (Tables 4.3 to 4.5). There were 12-20 germ tubes (Fig. 4.2) and 3-17 appressoria (Fig. 4.3) per every 10 spores. From all interactions between different factors, only time×reaction interaction was significant for penetration per spore (Table 4.6). Coefficients of variation (CV) were 4.5, 8.9, 20.7, and 18.1 percent for germination percentage, germ tube per spore, appressorium per spore, and penetration per spore, respectively.

Penetration and colonization of epidermal cell

All the tested pathogen isolates were able to penetrate the epidermal cells in both compatible and incompatible interactions (reaction). There was no significant difference among the necrosis- and/or chlorosis-inducing isolates in terms of the number of penetrated epidermal cells per spore (Table 4.6). Even the avirulent isolate 90-2 was able to penetrate the epidermal cells of all the inoculated line/cultivars. Direct penetration of intact epidermal cells was started for some conidia by 6 h.p.i., and more spores were involved in penetration of epidermal cells by 12 h.p.i. (Fig. 4.9: C, D, and E) A bulbous appressorium which is formed at the point of contact of germ tubes with the surface of plants adheres to the cuticle where it has a flat surface, and penetrating hypha

(penetration peg) is produced (Fig. 4.9: C and D). The penetration peg from the flat surface of appressorium grows through the cuticle and epidermal cell walls (Fig. 4.9: E). Following the entrance of penetrating hyphae to epidermal cells, a spherical vesicle is formed (Fig. 4.9: F and 4.2: A2). One to two branches of intracellular hyphae extends out from the sides of vesicle, which grows and colonizes the entire epidermal cell (Fig. 4.10: B1). There were 2-18 penetrated epidermal cells per every 10 spores (Fig. 4.4). There was a fluorescing halo at the penetration site on the outer wall of some epidermal cells (Fig. 4.9: G). Papillae were sometimes formed within the epidermal cells, although it was not successful in preventing the fungal entry (Fig. 4.9: H).

Table 4.6. Analysis of variance for penetration per spore of different isolates of *P. tritici-repentis* on various wheat lines/cultivars.

S.o.V.	df	SS	MS	F value	Pr>F
Cultivar (C)	3	0.050	0.017	0.71 ^{ns}	0.5515
Reaction (R)	1	0.003	0.003	0.14 ^{ns}	0.7132
Time (T)	3	3.088	1.029	43.64 **	<0.0001
Isolate groups (I)	3	0.006	0.002	0.09 ^{ns}	0.9654
T×R	3	0.292	0.097	4.12 *	0.0118
I×R	2	0.111	0.056	2.36 ^{ns}	0.1064
T×I	9	0.157	0.017	0.74 ^{ns}	0.6725
Error	43	1.014	0.024	-	-
Total	**67**	**4.722**	**-**	**-**	**-**

ns, *, and **: non-significant, significant at error levels of 5% and 1%, respectively.

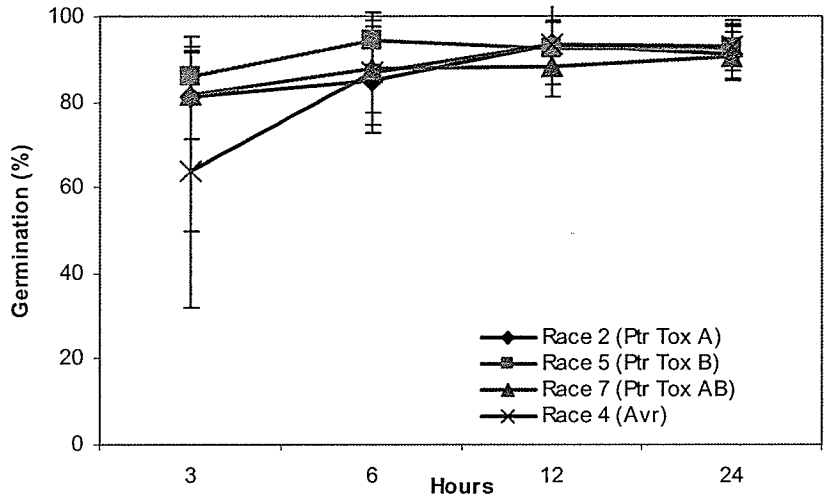


Figure 4.1. Germination percentage of *P. tritici-repentis* spores of isolates 86-124 (race 2), ALG3-24 (race 5), ICARDA 35-5 (race 7) and 90-2 (race 4) on different wheat cultivars from 3-24 h.p.i.

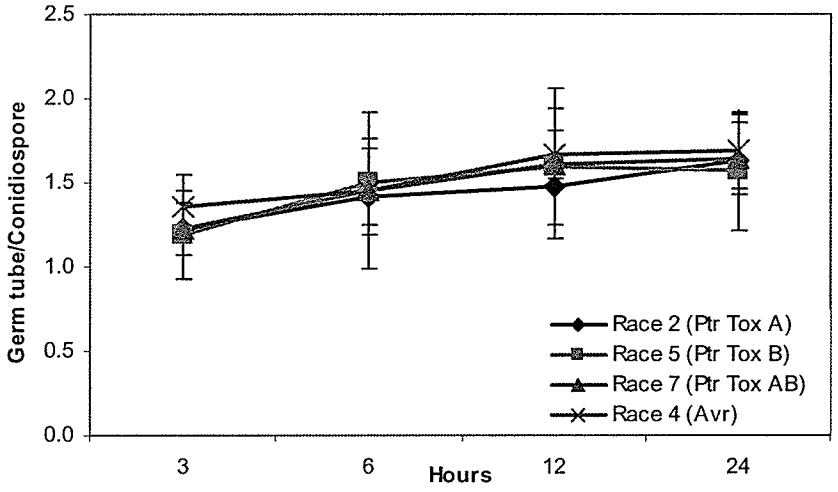


Figure 4.2. Average number of germ tube(s) /spore of *P. tritici-repentis* isolates 86-124 (race 2), ALG3-24 (race 5), ICARDA 35-5 (race 7) and 90-2 (race 4) on different wheat cultivars from 3-24 h.p.i.

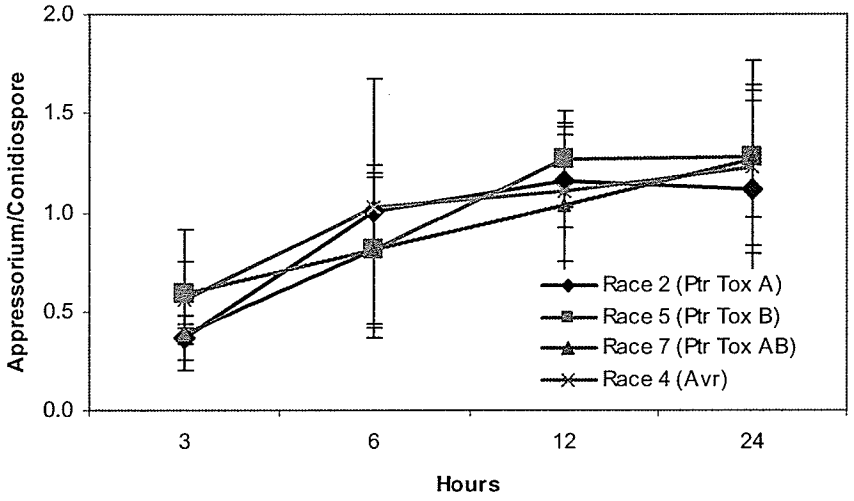


Figure 4.3. Average number of appressorium/spore of *P. tritici-repentis* isolates 86-124 (race 2), ALG3-24 (race 5), ICARDA 35-5 (race 7) and 90-2 (race 4) on different wheat cultivars from 3-24 h.p.i.

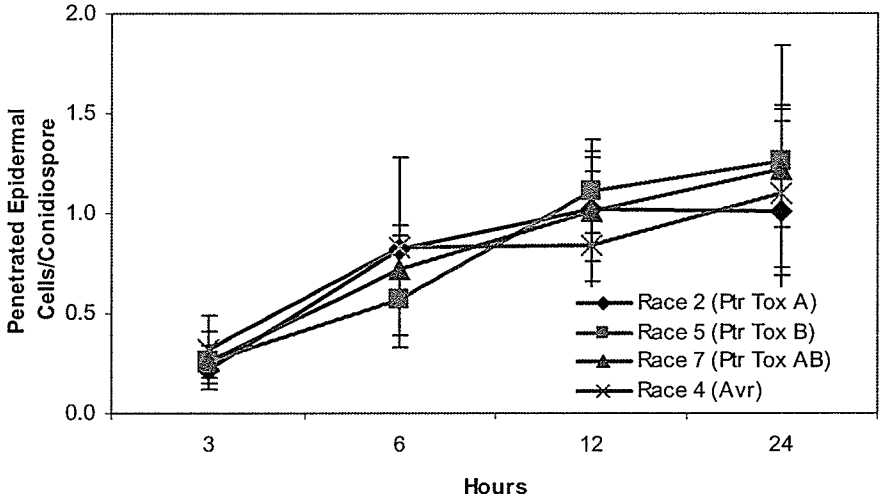


Figure 4.4. Average number of penetrated epidermal cell(s) per spore of *P. tritici-repentis* isolates 86-124 (race 2), ALG3-24 (race 5), ICARDA 35-5 (race 7) and 90-2 (race 4) on different wheat cultivars from 3-24 h.p.i.

Penetration per spore was significantly different at 3 and 6, and 12 h.p.i. There was not a significant difference between 12 and 24 h, (Table 4.7).

Table 4.7. Comparison of pre-colonization events means for different times post inoculation using Duncan method and 5% error level.

	Time post inoculation (hours)			
Pre-colonization event	3	6	12	24
Germination (%)	78.2	88.5	91.9	91.8
Germ tube per spore	1.25	1.45	1.59	1.63
Appressorium per spore	0.47	0.91	1.14	1.22
Penetration per spore	0.27	0.74	1.00	1.15

Extended lines indicate lack of significant difference.

Colonization of the mesophyll layer

General observations

Following the infection of epidermal cells, colonizing hyphae grew intercellularly (no mesophyll cell penetration) in the mesophyll layer in both necrotic (Fig. 4.11 and 4.15) and chlorotic lesions (Fig. 4.13 and 4.15) as well as in the lesions in the resistant cultivars, where the advance of the invading hyphae were halted and remained localized (Fig. 4.12, 4.14, 4.16 and 4.17).

The results of ANOVA (Table 4.8) shows that time post inoculation (T) and the type of cultivar (C) had a significant effect on the percentage of the area of colonizing hyphae at 1 % level. Similarly, reaction (R) and pathogen isolates (I) had significant effect on mycelium area percentage at 1% error level. R×I as well as R×T×I interactions were significant at 5% error level while R×T and I×T interactions were not significant. Coefficient of variation (CV) for area percentage was 66.3%.

ANOVA on thickness of colonizing hyphae (Table 4.9) indicates that cultivar, reaction type, and isolate groups had significant effect at 1 % level while time had no

significant effect. All interactions between factors were significant at 1 % except for R×T interaction. The coefficient of variation (CV) for mycelium thickness was 22.7

Table 4.8. Analysis of variance for mycelium area (%) of *P. tritici-repentis* isolates on various wheat lines/cultivars.

S.o.V.	df	SS	MS	F value	Pr>F
Cultivar (C)	3	171.56	57.19	17.42 **	<0.0001
Reaction (R)	1	36.25	36.25	11.04 **	0.0011
Time (T)	3	242.08	80.69	24.58 **	<0.0001
Isolate groups(I)	3	49.84	16.61	5.06 **	0.0022
T×R	3	19.77	6.59	2.01 ns	0.1147
I×R	2	22.24	11.12	3.39 *	0.0360
T×I	9	38.57	4.29	1.31 ns	0.2370
T×I×R	6	45.68	7.61	2.32 *	0.0352
Error	177	581.11	3.28	-	-
Total	207	625.98	-	-	-

ns, *, **: non-significant, significant at error levels of 5%, 1% respectively.

Table 4.9. Analysis of variance for mycelium thickness (μ) of *P. tritici-repentis* isolates on various wheat lines/cultivars.

S.o.V.	df	SS	MS	F value	Pr>F
Cultivar (C)	3	8.19	2.73	6.40 **	0.0004
Reaction (R)	1	17.00	17.00	39.85 **	<0.0001
Time (T)	3	0.93	0.31	0.73 ns	0.5385
Isolate (I)	3	24.32	8.11	19.00 **	<0.0001
T×R	3	1.84	0.61	1.43 ns	0.2343
I×R	2	8.62	4.31	10.11 **	<0.0001
T×I	9	26.47	2.94	6.90 **	<0.0001
T×I×R	6	9.18	1.53	3.59 **	0.0022
Error	177	75.49	0.43	-	-
Total	207	172.03	-	-	-

ns and **: non-significant and significant at error level of 1%, respectively.

Ptr ToxA-producing isolates (Necrosis-inducing isolate)

Compatible interaction

Branches of intercellular hyphae of Ptr ToxA-producing isolate (86-124) on the susceptible plants Glenlea and Neepawa growing in all directions were observed at the zone of infection. Individual mycelia also spread out of the infection zone in diverse directions (Fig. 4.11: A to H). No sign of fluorescing plant cells was observed.

Percentage of mycelial area coverage (mycelium %) was about 2.8 percent at 12 h.p.i. (Fig. 4.5a), which did not change at 24 h.p.i. From 24-72 h.p.i., there was a dramatic increase in the mycelium percentage in necrotic lesions, and reached to about 8.2 percent (Fig. 4.5a). Colonizing intercellular hyphae with a thickness of 2.6 μ at 12 h grew thicker to about 3.4 μ by 72 h.p.i. (Fig. 4.5b).

Incompatible interaction

Over the time of observation, the mycelium area percentage on Thatcher and Salamouni expanded from 0.9 to 3.4 percent from 12-72 h.p.i. (Fig. 4.5a). Penetrated epidermal cells were fluorescing (Fig. 4.12: B and D). Mesophyll cells near the colonizing hyphae were also fluorescing (Fig. 4.12: F and H). The intercellular hyphae had a thickness of 2.1 μ at 12 h.p.i., which increased to 2.5 μ by 48 h.p.i., and remained almost the same up to 72 h.p.i. (2.7 μ) (Fig. 4.5b). Mycelial networks were formed in the mesophyll (Fig. 4.12: A, C, E, and G).

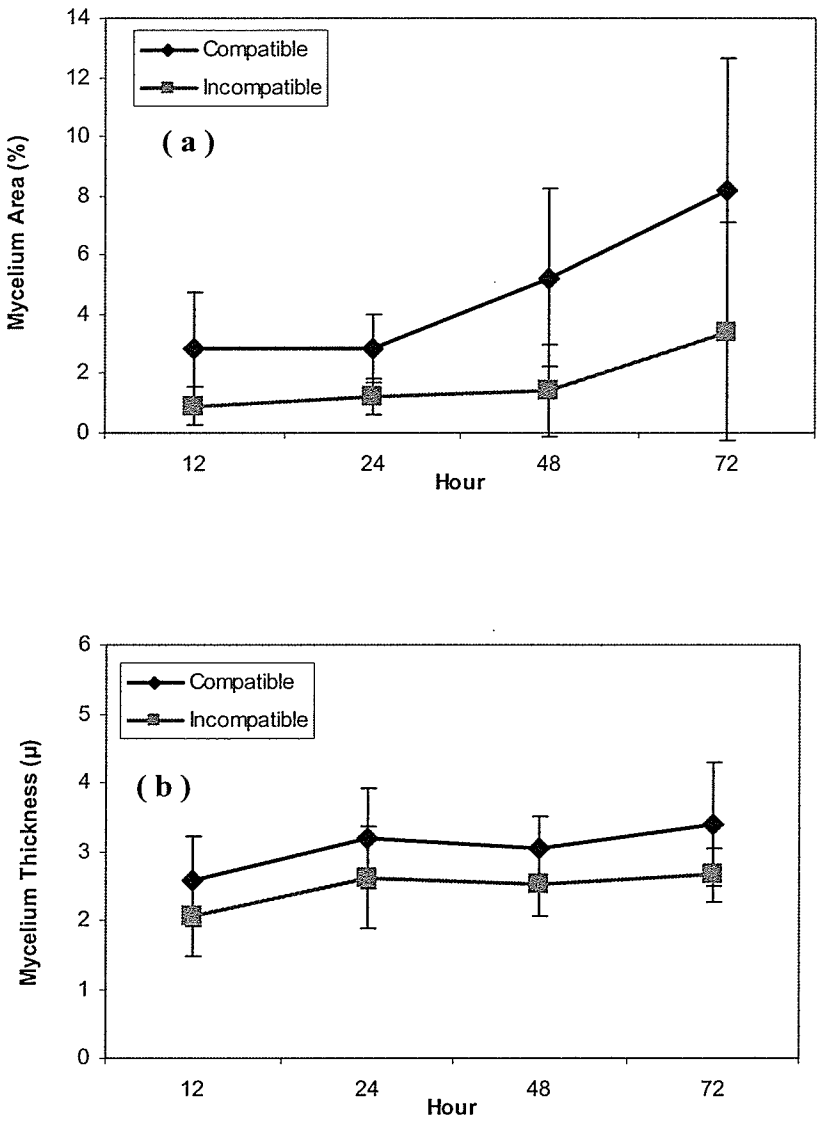


Figure 4.5. Mycelium area (%) (a), and mycelium thickness (µ) (b) of necrosis-inducing isolate (86-124) of *P. tritici-repentis* on susceptible wheat cultivars Glenlea, and Neepawa, and on resistant cultivars Thatcher and Salamouni, from 12-72 h.p.i.

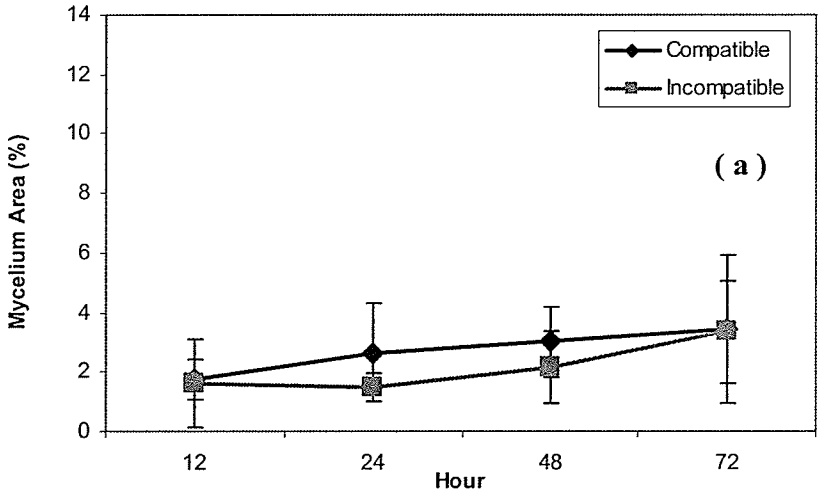
Ptr ToxB-producing isolates (Chlorosis-inducing isolate)

Compatible interaction

Mycelia of the Ptr ToxB-producing isolates in chlorotic lesions on susceptible cultivars Thatcher and Neepawa grew intercellularly in the mesophyll layer. By 12 h.p.i., the area of colonizing hyphae was about 1.8 percent, and increased slightly up to 48 h.p.i. (Fig. 4.6a, and Fig. 4.13: A to F). By 48 h.p.i., there was a higher slope of growth, which led to a 3.0 percent of mycelial area coverage (Fig. 4.6a). By 12 h.p.i., the thickness of invading hyphae was about 4.4 μ, which decreased to a minimum of 2.0 μ by 72 h.p.i. (Fig. 4.6b). Usually a network of mycelia is formed in the mesophyll (Fig. 4.13: G and H). No sign of fluorescing mesophyll cells was observed.

Incompatible interaction

In the incompatible interactions between Ptr ToxB-producing isolates and resistant cultivars Glenlea and Salamouni, mycelial area coverage was about 1.6 percent by 12 h.p.i., and reached a maximum of 3.4 percent by 72 h.p.i. (Fig. 4.6a). Thickness of colonizing hyphae was 2.7 μ by 12 h.p.i., and increased to 2.6 μ by 72 h.p.i. (Fig. 4.6b). Mesophyll cells and penetrated epidermal cells were fluorescing at 48 and 72 h.p.i. (Fig. 4.14: D and F). There were only branches of individual intercellular hyphae in the mesophyll by 12 to 24 h.p.i. (Fig. 4.14: B1 and B2). Mycelial networking was observed from 48 to 72 h.p.i. in the mesophyll (Fig. 4.14: C and E).



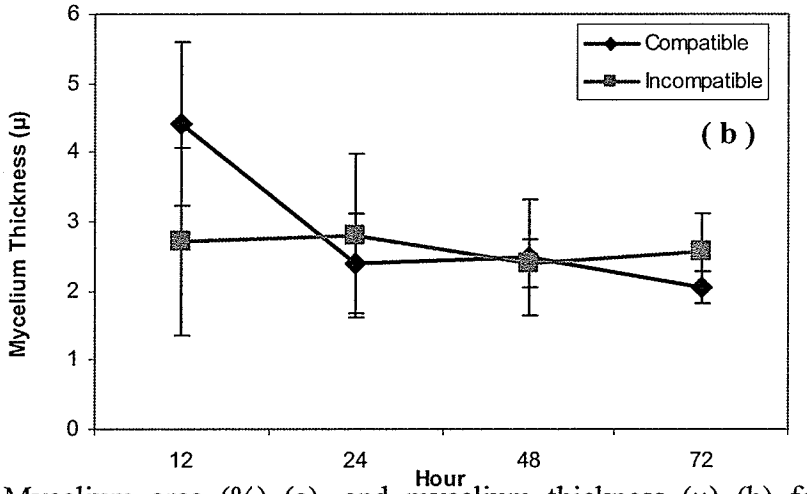


Figure 4.6. Mycelium area (%) (a), and mycelium thickness (µ) (b) for chlorosis-inducing isolate (ALG3-24) of *P. tritici-repentis* on susceptible wheat cultivars Thatcher and Neepawa, and on resistant wheat cultivars Glenlea and Salamouni, from 12-72 h.p.i.

Ptr ToxA- and ToxB- producing isolate (Necrotic-chlorotic-inducing isolate)

Compatible interaction

Area coverage by colonizing hyphae of isolate ICARDA 35-5, which produces both Ptr ToxA and Ptr ToxB, on the susceptible plants Neepawa, Thatcher and Glenlea was about 1.6 percent by 12 h.p.i., and increased to a maximum of 6.8 percent by 72 h.p.i. (Fig. 4.7a). There were only branches of hyphae from 12 to 24 h.p.i. (Fig. 4.15: A1, A2, B, and C). A growing mycelial network was observed by 48 h.p.i. and thereafter till 72 h.p.i. (Fig. 4.15: D, E, F, and G). The thickness of the invading hyphae on susceptible plants by 12 h.p.i. was about 3.2 μ and increased to 4.4 μ by 72 h.p.i. (Fig. 4.7b).

Incompatible interaction

In the incompatible interaction of the Ptr ToxA- and ToxB-producing isolate of *P. tritici-repentis* with the resistant cultivar Salamouni, mycelial area coverage was 0.8 percent at 12 h.p.i., which had a slight rate of increase up to 72 h.p.i. where it reached to 1.2 percent (Fig. 4.7a). By 12 h.p.i., the colonizing hyphae was about 3.5 μ thick, which increased in diameter to 3.1 μ by 12 h.p.i. and remained the same size up to 72 h.p.i. (Fig. 4.7b). Fluorescing mesophyll cells were observed as early as 12 h.p.i. (Fig. 4.16: A1). Penetrated epidermal cells were also fluorescing (Fig. 4.16: B2). There were just branches of individual hyphae near the fluorescing mesophyll cells from 12 to 72 h.p.i. (Fig. 4.16: A2, B1, C1, and D1). Papillae were observed near the penetration site (Fig. 4.16: C2 and D2).

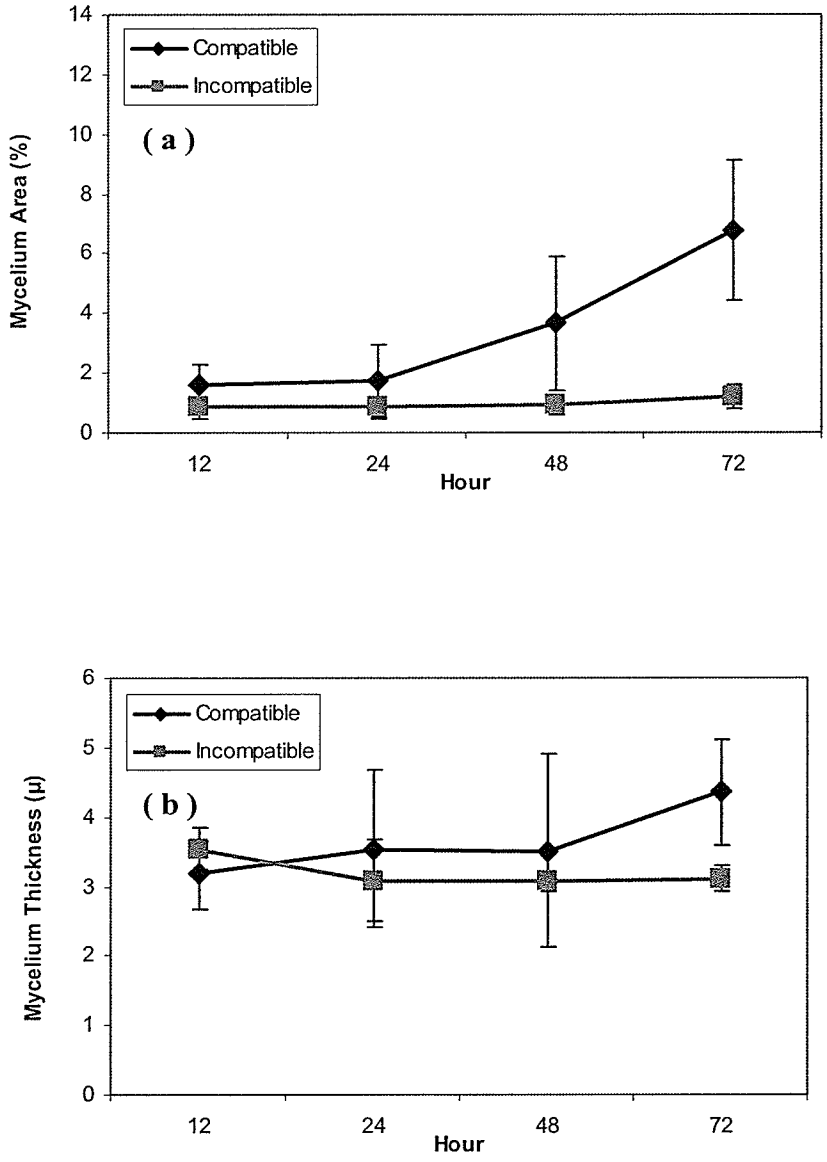


Figure 4.7. Mycelium area (%) (a), and mycelium thickness (µ) (b) for necrosis- and chlorosis-inducing isolate (ICARDA 35-5) of *P. tritici-repentis* on susceptible wheat cultivars Neepawa, Thatcher and Glenlea and on resistant wheat cultivar Salamouni, from 12-72 h.p.i.

Isolate producing no Ptr Toxins (Avirulent isolate)

On Neepawa and Salamouni the avirulent isolate 90-2 colonized the mesophyll layer intercellularly (Fig. 4.17: A and B). The mycelial area coverage was about 0.4 percent by 12 h.p.i., and advanced more to 1.7 percent by 72 h.p.i. (Fig. 4.8a). There were only 1-2 branches of hyphae at the colonized spot with a thickness of 2.3 μ by 12 h.p.i. They grew thicker to about 3.0 μ by 48 h.p.i., and did not change till 72 h.p.i. (Fig. 4.8b). There were some fluorescing cells near the colonizing hyphae at the infection site (Fig. 4.17: H). The hyphal network described above for compatible interactions was not observed. Only individual branches of hyphae were seen spreading out from the infected epidermal cells through the mesophyll layer, and not far from the infection site from 12 to 72 h.p.i. (Fig. 4.17: C and D).

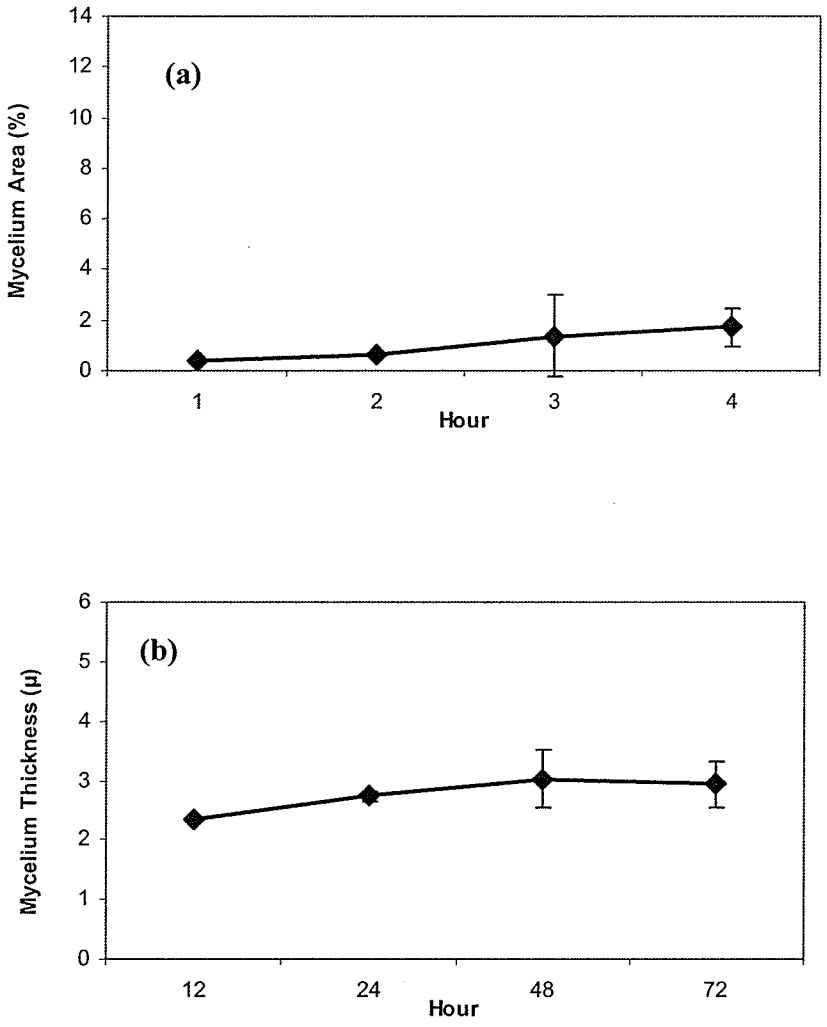


Figure 4.8. Mycelium area (%) (a), and mycelium thickness (µ) (b) for avirulent isolate (90-2) of *P. tritici-repentis* on wheat cultivars Salamouni and Neepawa, from 12-72 h.p.i.

Mean comparison

Mean comparisons for percentage of mycelial area coverage (mycelium %) showed, that there were no significant differences in the mycelium are percentage among the Ptr ToxA- and/or Ptr ToxB-producing isolates and the avirulent isolate on their incompatible interaction with corresponding wheat cultivars. Also there were no a significant differences between Ptr ToxA- and/or Ptr ToxB-producing isolates on their compatible interaction with wheat lines/cultivars at 12, and 24 h.p.i. However, there was a significant difference between Ptr ToxA-producing isolates and Ptr ToxB-producing isolates on their compatible interaction with wheat lines/cultivars at 48 h and 72 h.p.i. in their mycelium percentage (Table 4.10), where Ptr ToxA-producing isolates had higher percentage of the colonizing hyphae than Ptr ToxB-producing isolates. By 48 h.p.i., Ptr ToxA- and ToxB-producing isolates were not significantly different from both Ptr ToxA-producing isolates, and Ptr ToxB-producing isolates. There was not a significant difference between Ptr ToxA- and ToxB-producing isolates and Ptr ToxA-producing isolates, but the difference was significant with Ptr ToxB-producing isolates at 72 h.p.i., where Ptr ToxA- and ToxB-producing isolates had a higher percentage area of the colonizing hyphae.

Mean comparison results for mycelium thickness (μ) (Table 4.11) showed that for the compatible interaction, there was always significant difference between the three type of isolate other than at 24 h.p.i., where Ptr ToxA producing isolates and Ptr ToxA- and ToxB-producing isolates were similar. For all the sampling times, Ptr ToxA- and ToxB-producing isolates had the largest thickness followed by Ptr ToxA-producing isolates and Ptr ToxB-producing isolates. Only at 12 h.p.i., Ptr ToxB-producing isolates had the

largest thickness, followed by Ptr ToxA- and ToxB-producing isolates, and Ptr ToxA-producing isolates.

For the incompatible interactions, there were no significant differences between Ptr ToxA-producing isolates and Ptr ToxB-producing isolates, except at 12 h.p.i., where Ptr ToxB-producing isolates had larger mycelium thickness than Ptr ToxA-producing isolates. Ptr ToxA- and ToxB-producing isolates always showed a significant difference with both Ptr ToxA-producing isolates and Ptr ToxB-producing isolates, except for 24 h.p.i., and always had the largest mycelium thickness.

Table 4.10. Mean comparison for mycelium area (%) among necrosis- and/or chlorosis-inducing isolates of *P. tritici-repentis* on susceptible wheat lines/cultivars using Duncan method and 5% error level.

	Necrotic- and/or chlorotic-inducing isolates/Mycelium Area (%)		
Time	Necrosis-inducing isolate (race 2)	Necrosis-chlorosis-inducing isolate (race 7)	Chlorosis-inducing isolate (race 5)
12h	2.81	1.58	1.76
24h	2.84	1.71	2.64
48h	5.23	3.66	3.02
72h	8.16	6.77	3.43

Extended lines under the mycelium area (%) area indicate lack of significant difference.

Table 4.11. Mean comparison for mycelium thickness (μ) among necrosis- and/or chlorosis-inducing isolates of *P. tritici-repentis* on susceptible and resistant wheat lines/ cultivars, using Duncan method and 5% error level.

Interaction	Time post inoculation (hours)	Necrosis- and/or chlorosis-inducing isolates / Mycelium thickness(μ)			
Compatible	12	B	AB	A	
		4.4	3.2	2.6	
	24	AB	A	B	
		3.5	3.2	2.4	
	48	AB	A	B	
		3.5	3.0	2.5	
	72	AB	A	B	
		4.4	3.4	2.0	
Incompatible	12	AB	B	Avr.	A
		3.6	2.7	2.3	2.1
	24	AB	B	Avr.	A
		3.1	2.8	2.8	2.6
	48	AB	Avr	A	B
		3.1	3.1	2.5	2.4
	72	AB	Avr	A	B
		3.1	2.9	2.7	2.6

Extended lines under isolate group names indicate lack of significant difference between them. A, B, AB, and Avr. represent necrosis-, chlorosis-, necrosis & chlorosis-producing, and avirulent isolates respectively.

Figure 4.9. Spore germination, appressorium formation, epidermal cell penetration and host reaction at the penetration site of *Pyrenophora tritici-repentis* on leaf surface. A) Germinated spore of isolate ICARDA 35-5 on resistant cultivar Salamouni 3 hours post inoculation (h.p.i.) (×300). B) Germinated spore of isolate ALG3-24 on susceptible cultivar Thatcher 6 h.p.i. (×300). C) Germinated spore with appressoria and penetration peg of isolate 86-124 on susceptible cultivar Glenlea 12 h.p.i. (×300). D) Germinated spore with two appressoria and penetration peg of isolate ALG3-24 on susceptible cultivar Thatcher 12 h.p.i. (×300). E) Germinated spore of isolate 86-124 penetrated the epidermal cell of susceptible cultivar Glenlea with penetration peg 12 h.p.i. (×300). F) Penetration of epidermal cell, vesicle formation, and colonization of epidermal cell by isolate ALG3-24 on susceptible cultivar Thatcher 24 h.p.i. (×300). G) Fluorescing halo on epidermal cells at the penetration site of isolate ALG3-24 on susceptible cultivar Thatcher 24 h.p.i. (×200). H) Fluorescing halo on epidermal cells at the penetration site of isolate 90-2 on cultivar Neepawa 24 h.p.i. (×300). Abbreviations: Ap = appressorium, E = epidermal cell, FH = fluorescing halo, G = germ tube, H = intracellular hyphae, IE = infected epidermal cell, Pp = penetration peg, S = spore, St = stomate, V = vesicle

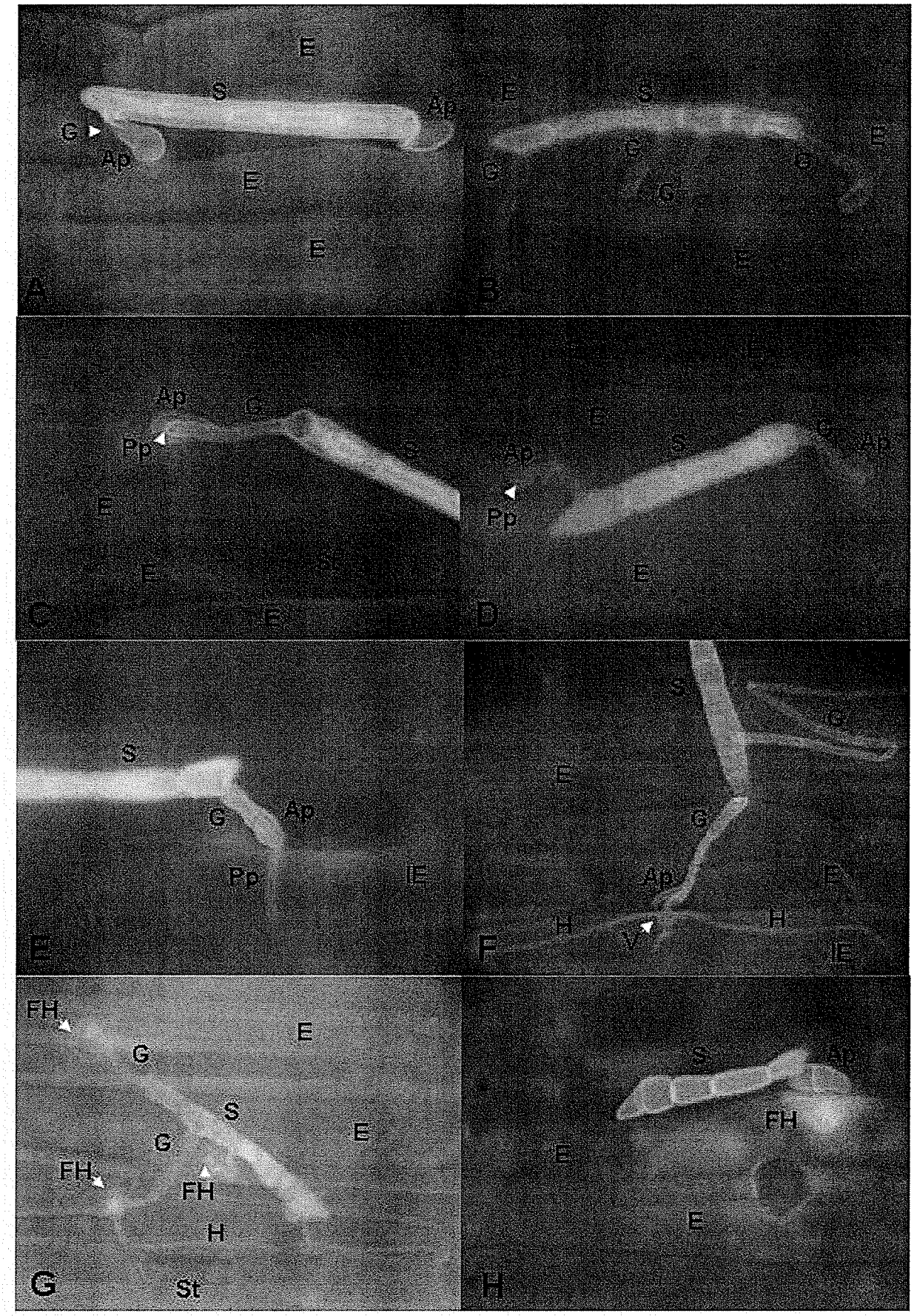

E
S
Ap
G
Ap
E
E
A
E
S
G
E
G
G
G
E
B
Ap
G
Pp
S
E
E
S
C
E
E
S
G
Ap
Ap
Pp
E
D
S
G
S
E
G
Ap
Pp
E
E
F
H
V
H
E
S
G
Ap
FH
E
G
E
S
G
E
FH
FH
H
G
St
S
Ap
FH
E
E
H

Figure 4.10. Colonization of epidermal cells, and initial colonization of mesophyll layer by isolates of *P. tritici-repentis*. A1) Germinated spore of isolate ALG3-24 penetrating the epidermal cell of susceptible cultivar Thatcher 24 h.p.i. (×200). A2) Germinated spore of isolate ALG3-24 colonizing the epidermal cells of susceptible cultivar Thatcher 24 h.p.i. (×200). B1) Germinated spore of isolate ICARDA 35-5 penetrating and colonizing the epidermal cell of susceptible cultivar Glenlea 12 h.p.i. (×200). B2) Epidermal cell and mesophyll layer colonization of susceptible cultivar Glenlea by isolate ICARDA 35-5 12 h.p.i. (×200). C) Germinated spore of avirulent isolate 90-2 penetrated and colonized the epidermal cell of cultivar Neepawa 24 h.p.i. (×200). D) Epidermal cell of resistant plant Salamouni Colonized by isolate 86-124 is fluorescing 12 h.p.i. (×500).**Abbreviations:** Ap = appressorium, E = epidermal cell, FE = fluorescing epidermal cell, G = germ tube, H = intracellular hyphae, IE= infected epidermal cell, IH = intercellular hyphae, MC = mesophyll cell, Pp = penetration peg, S = spore, St = stomate, V = vesicle.

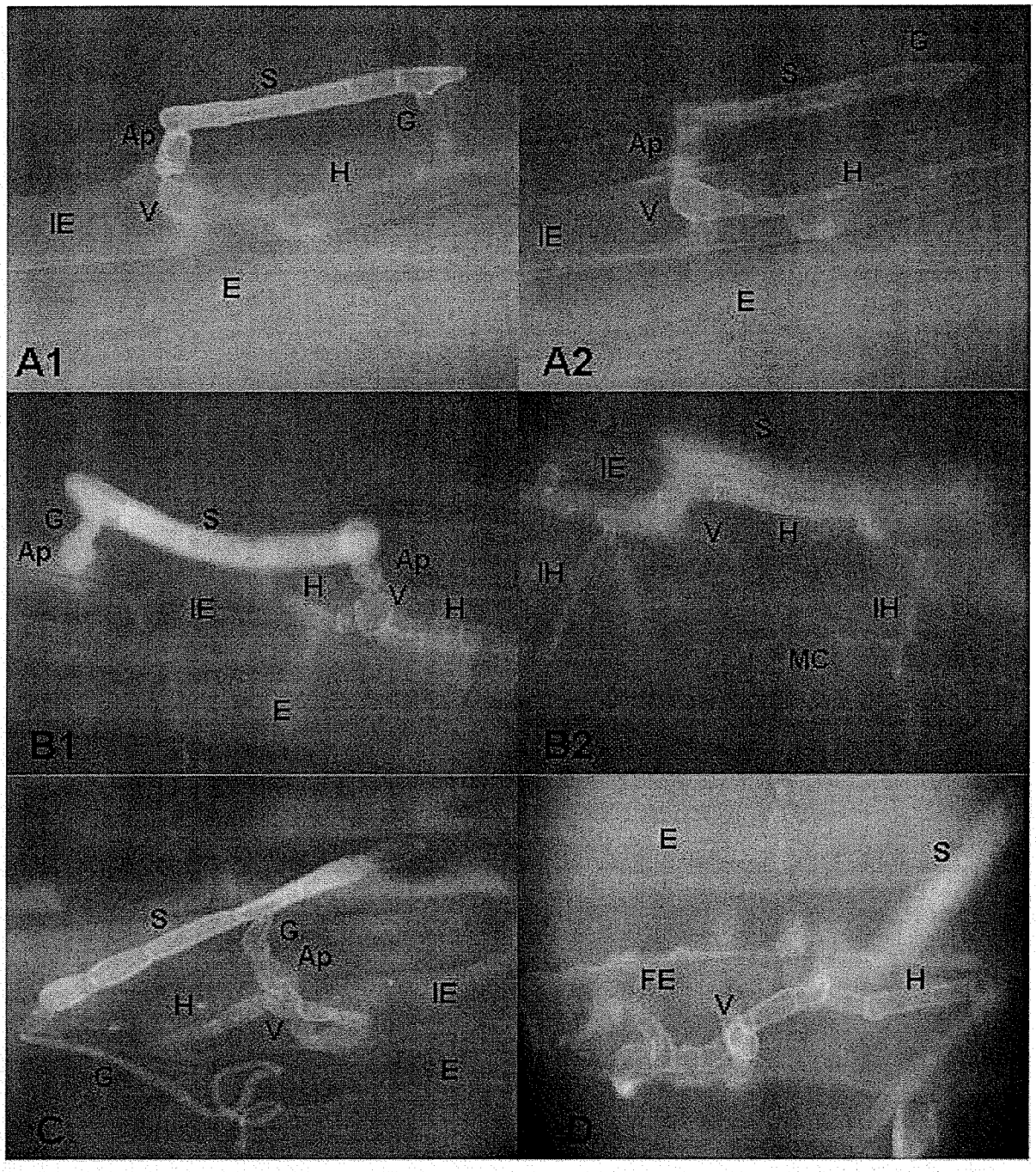

S
G
Ap
H
IE
V
E
A1
G
S
Ap
H
V
IE
E
A2
G
S
Ap
H
V
IE
B1
S
IE
V
H
IH
IH
MC
B2
S
G
Ap
IE
H
V
E
C
E
S
FE
V
H
D

Figure 4.11. Colonization of mesophyll layer of susceptible cultivars by necrosis-inducing isolate 86-124 of *P. tritici-repentis*. A) Mesophyll colonization of cultivar Neepawa by intercellular hyphae 12 h.p.i. (×300). B) Intercellular colonizing hayphae in mesophyll layer of cultivar Glenlea 12 h.p.i. (×300). C) Colonizing hyphae in mesophyll layer of cultivar Neepawa 24 h.p.i. (×300). D) Hyphal network in mesophyll layer of cultivar Glenlea 24 h.p.i. (×300). E) Intercellular hyphae colonizing the mesophyll layer of cultivar Neepawa 48 h.p.i. (×300). F) Colonization of cultivar Glenlea by intercellular hayphae 48 h.p.i. (×300). G) Hyphal network in covered the mesophyll layer of cultivar Neepawa by 72 h.p.i. (×200). H) Network of intercellular hyphae in mesophyll layer of cultivar Glenlea 72 h.p.i. (×100). Abbreviations: E = epidermal cell, HN = hyphal network, IH = intercellular hyphae, C = mesophyll cell, S = spore, St =stomate, T = trichome.

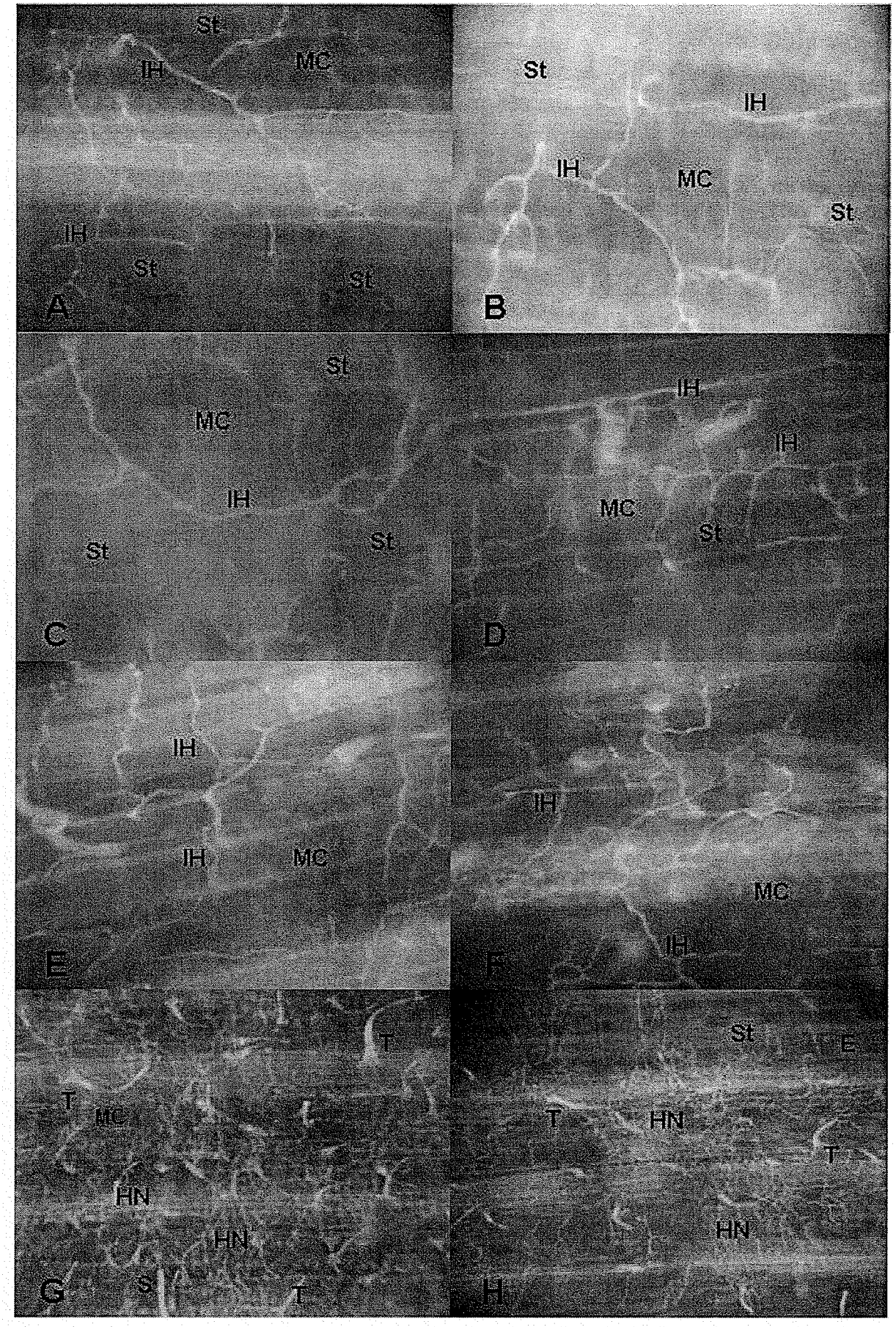

St
IH
MC
IH
St
St
A
St
IH
IH
MC
St
B
St
MC
St
St
C
IH
IH
MC
St
D
IH
IH
MC
E
IH
MC
IH
F
T
T
MC
HN
HN
S
T
G
St
E
T
HN
T
HN
H

Figure 4.12. Epidermal cell and mesophyll layer colonization of resistant cultivars to necrosis-inducing isolate 86-124 of *P. tritici-repentis*. A) Colonizing hyphae in mesophyll layer of cultivar Thatcher by 12 h.p.i. (×300). B) Infected fluorescing epidermal cell of cultivar Salamouni 12 h.p.i. (×300). C) Intercellular hyphal network in mesophyll layer of cultivar Thatcher 24 h.p.i. (×300). D) Fluorescing infected epidermal cells of cultivar Salamouni 24 h.p.i. (×300). E) Colonizing intercellular hyphae in mesophyll layer of cultivar Thatcher 48 h.p.i. (×300). F) Fluorescing mesophyll cells near and far from the intercellular hyphae in cultivar Salamouni 48 h.p.i. (×300). G) Intercellular hyphae in mesophyll layer of cultivar Thatcher by 72 h.p.i. (×300). H) Fluorescing mesophyll cells under the infected epidermal cell of cultivar Salamouni 72 h.p.i. (×300). Abbreviations: Ap = appressorium, E = epidermal cell, FE = fluorescing epidermal cell, FMC = fluorescing mesophyll cell, G = germ tube, H = intracellular hyphae, HN = hyphal network, IH = intercellular hyphae, MC = mesophyll cell, S = spore, St =stomate, T = trichome.

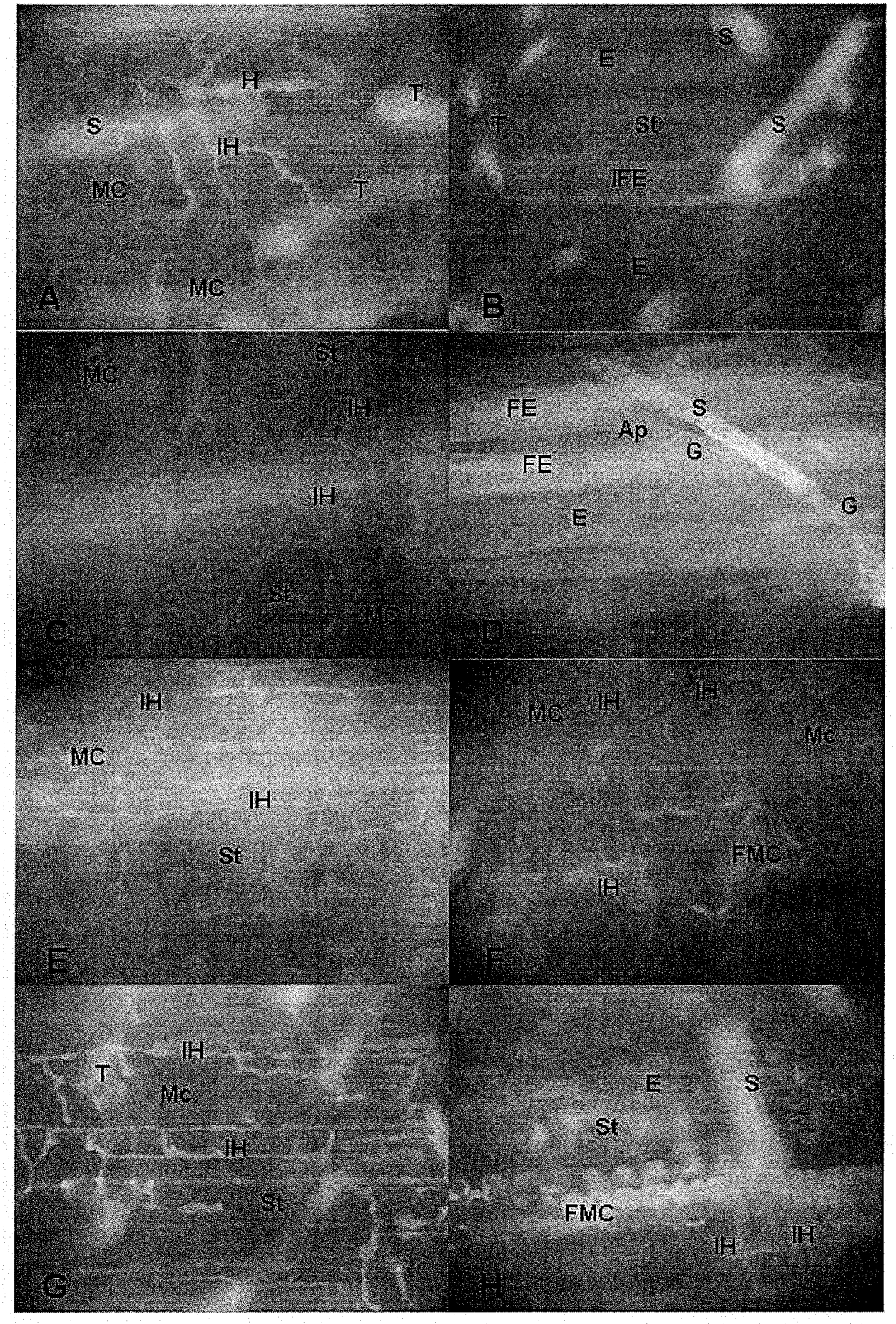

H
T
S
IH
MC
T
MC
A
S
E
T
St
S
IFE
E
B
St
MC
IH
IH
St
MC
C
FE
S
Ap
G
FE
E
G
D
IH
MC
IH
IH
MC
Mc
IH
St
FMC
IH
E
F
IH
T
Mc
IH
St
G
E
S
St
FMC
IH
IH
H

Figure 4.13. Colonization of mesophyll layer of susceptible cultivars by chlorosis-inducing isolate ALG3-24 of *P. tritici-repentis*. A) Intercellular colonizing hayphae in mesophyll layer of cultivar Thatcher by 12 h.p.i. (×300). B) Intercellular colonizing hayphae in mesophyll layer of cultivar Neepawa 12 h.p.i. (×300). C) Intercellular colonizing hayphae in mesophyll layer of cultivar Thatcher 24 h.p.i. (×300). D) Intercellular colonizing hayphae in mesophyll layer of cultivar Neepawa 24 h.p.i. (×300). E) Intercellular colonizing hayphae in mesophyll layer of cultivar Thatcher 48 h.p.i. (×300). F) Hyphal network in mesophyll layer of cultivar Neepawa 48 h.p.i. (×100). G) Intercellular colonizing hyphae in mesophyll layer of cultivar Thatcher by 72 h.p.i. (×300). H) Hyphal network in mesophyll layer of cultivar Neepawa 72 h.p.i. (×100). Abbreviations: E = epidermal cell, HN = hyphal network, IH = intercellular hyphae, MC = mesophyll cell, S = spore, St = stomate, T = trichome

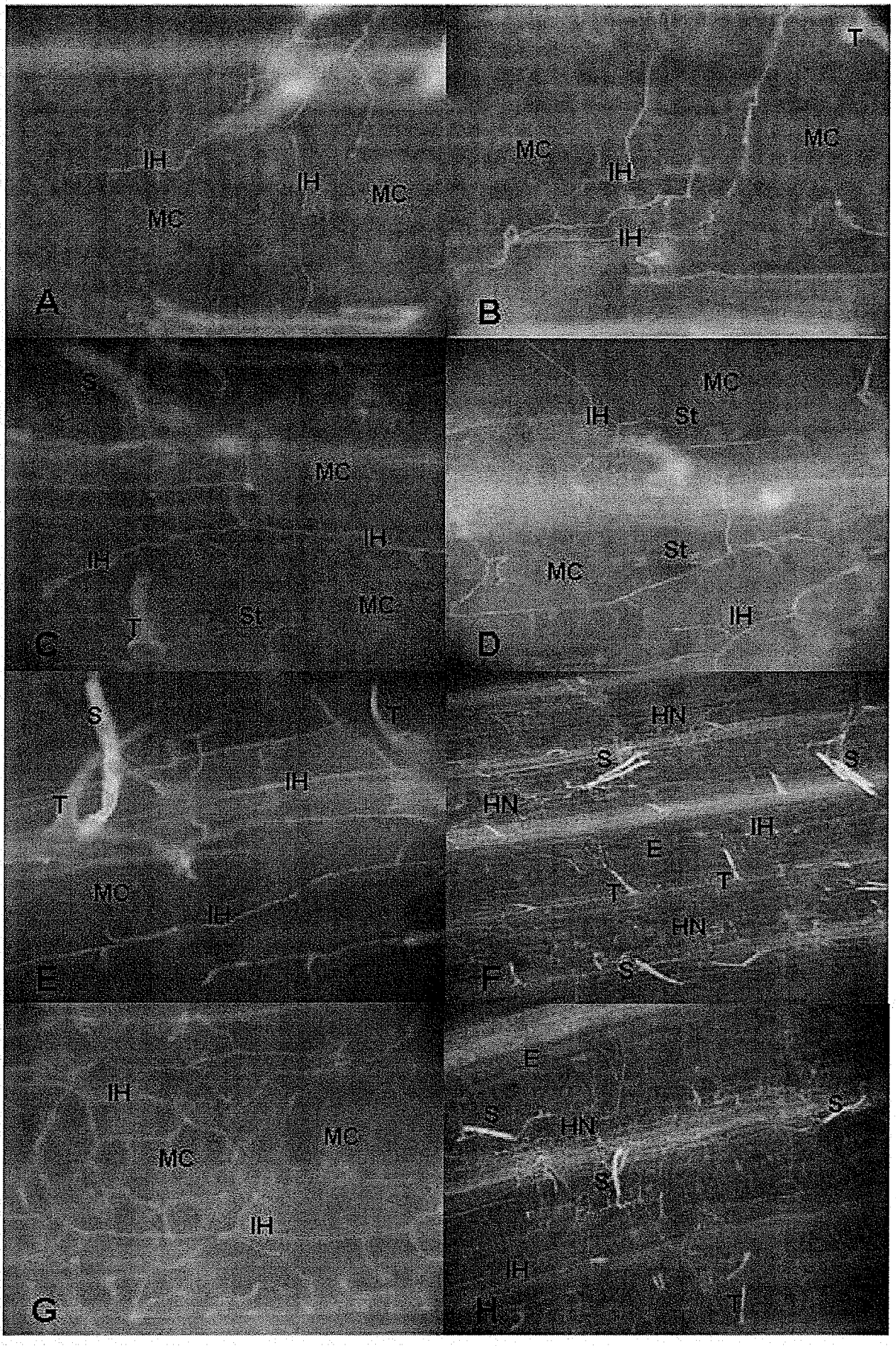

IH
IH
MC
MC
A
T
MC
MC
IH
IH
B
S
MC
IH
IH
MC
T
St
C
MC
IH
St
St
MC
IH
D
S
T
IH
T
MC
IH
E
HN
S
S
HN
IH
E
T
T
HN
S
F
E
S
S
HN
S
IH
T
H
IH
MC
MC
IH
G

Figure 4.14. Mesophyll layer colonization and reaction of resistant cultivars to chlorosis-inducing isolate ALG3-24 of *P. tritici-repentis*. A1, A2) Colonizing intercellular hyphae in mesophyll of cultivar Glenlea 12 h.p.i. (×300). B1, B2) Branches of colonizing intercellular hyphae in mesophyll of cultivar Glenlea 24 h.p.i. (×300). C) Branches of colonizing intercellular hyphae in mesophyll of cultivar Glenlea 48 h.p.i. (×300). D) Fluorescing infected mesophyll cells of cultivar Salamouni 48 h.p.i. (×100). E) Network of colonizing intercellular hyphae in mesophyll layer of cultivar Glenlea by 72 h.p.i. (×300). F) Fluorescing infected epidermal cells of cultivar Salamouni 72 h.p.i. (×100). Abbreviations: E = epidermal cell, FE = fluorescing epidermal cell, FMC = fluorescing mesophyll cell, IGC = infected guard cell, IH = intercellular hyphae, MC = mesophyll cell, NE = narrow epidermal cell, S = spore, St = stomate, T = trichome.

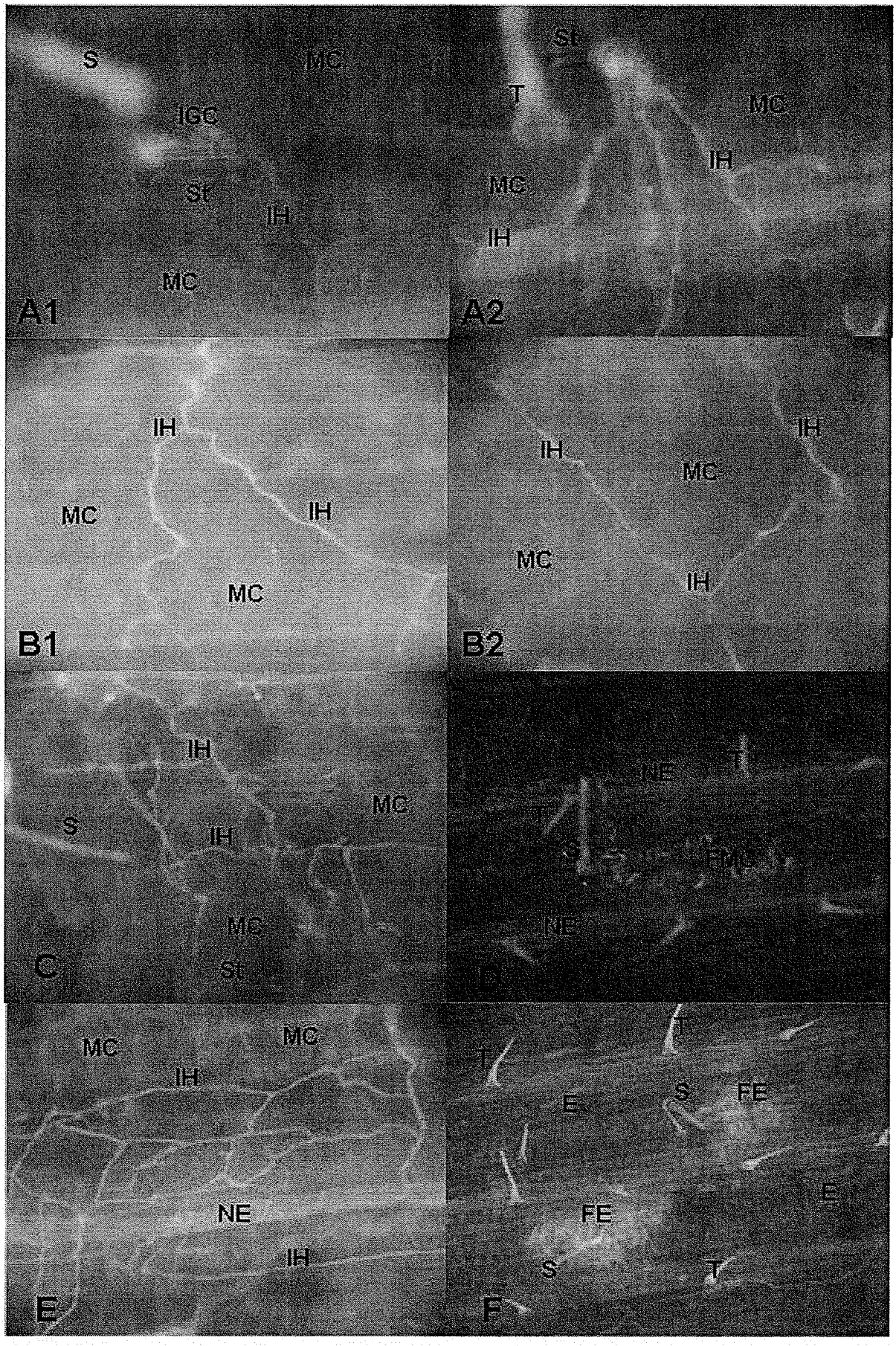

S
MC
IGC
St
IH
MC
A1
St
T
MC
IH
MC
IH
A2
IH
MC
IH
MC
B1
IH
IH
MC
IH
MC
B2
IH
MC
S
IH
MC
St
C
T
NE
T
S
IH
NE
T
MC
MC
IH
NE
IH
E
T
T
S
FE
E
FE
S
T
E
F

Figure 4.15. Colonization of mesophyll layer of susceptible cultivars by necrosis-chlorosis-inducing isolate ICARDA35-5 of *P. tritici-repentis*. A1, A2) Branches of intercellular hyphae in mesophyll layer of cultivar Neepawa 24 h.p.i. (×300). B) Geminated spore infected the epidermal cell and branches of intercellular hyphae is colonizing mesophyll layer of Colonization of cultivar Glenlea 24 h.p.i. (×300). C) Branches of intercellular hyphae in mesophyll layer of cultivar Thatcher 24 h.p.i. (×300). D) Intercellular hyphal network in mesophyll layer of cultivar Glenlea 48 h.p.i. (×300). E) Spores on the epidermal layer and the hyphal network in mesophyll layer around the spores for cultivar Thatcher 48 h.p.i. (×100). F) Colonizing intercellular hyphae in mesophyll layer of cultivar Glenlea 72 h.p.i. (×300). G) Spots of hyphal network in mesophyll layer of cultivar Thatcher by 72 h.p.i. (×100). Abbreviations: E = epidermal cell, H = intracellular hyphae, HN = hyphal network, IH = intercellular hyphae, MC = mesophyll cell, NE = narrow epidermal cell, S = spore, St = stomate, T = trichome.

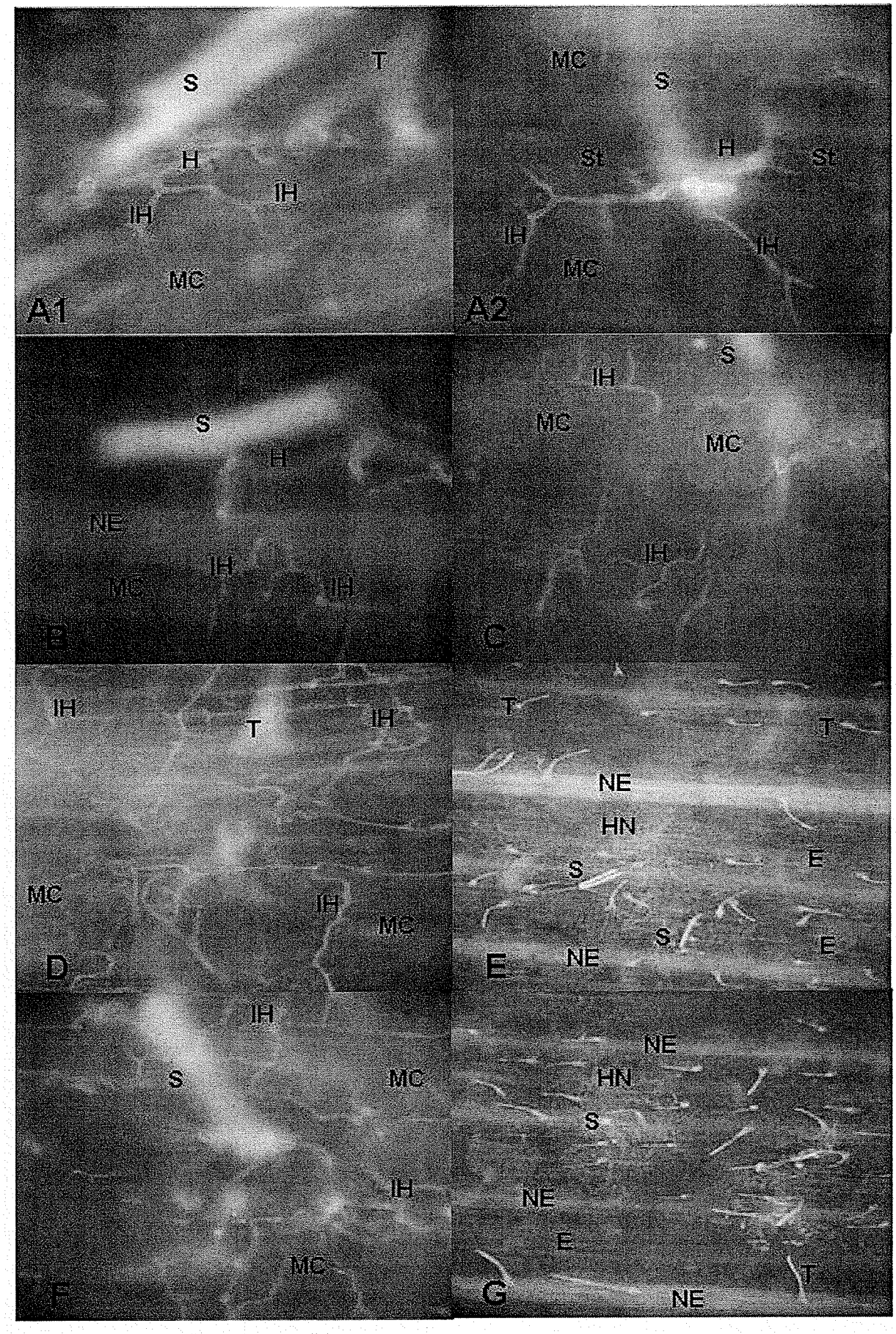

T
S
H
IH
IH
MC
A1
MC
S
St
H
St
IH
IH
MC
A2
S
H
NE
IH
MC
IH
B
S
IH
MC
MC
IH
C
IH
T
IH
MC
IH
MC
D
T
T
NE
HN
S
E
S
E
NE
E
IH
S
MC
IH
MC
F
NE
HN
S
NE
E
T
G
NE

Figure 4.16. Mesophyll layer colonization and reaction of resistant cultivar Salamouni to necrosis-chlorosis-inducing isolate ICARDA 35-5 of *P. tritici-repentis*. A1) Fluorescing mesophyll cells (×300), A2) branches of intercellular colonizing hyphae in mesophyll layer of cultivar Salamouni, and 12 h.p.i. (×500). B1) Fluorescing mesophyll cells, and intercellular colonizing hyphae in mesophyll layer of cultivar Salamouni 24 h.p.i. (×300). B2) Fluorescing epidermal cells of cultivar Salamouni 24 h.p.i. (×300). C1) Branches of colonizing intercellular hyphae, C2) fluorescing mesophyll cells and papilla formation of cultivar Salamouni 48 h.p.i. (×300). D1, D2) Fluorescing mesophyll cells near the intercellular colonizing hyphae, and formation of papilla in mesophyll cells of cultivar Salamouni 72 h.p.i. (×300). Abbreviations: E = epidermal cell, FE = fluorescing epidermal cell, FMC = fluorescing mesophyll cell, IH = intercellular hyphae, MC = mesophyll cell, Pa = papilla, St = stomate.

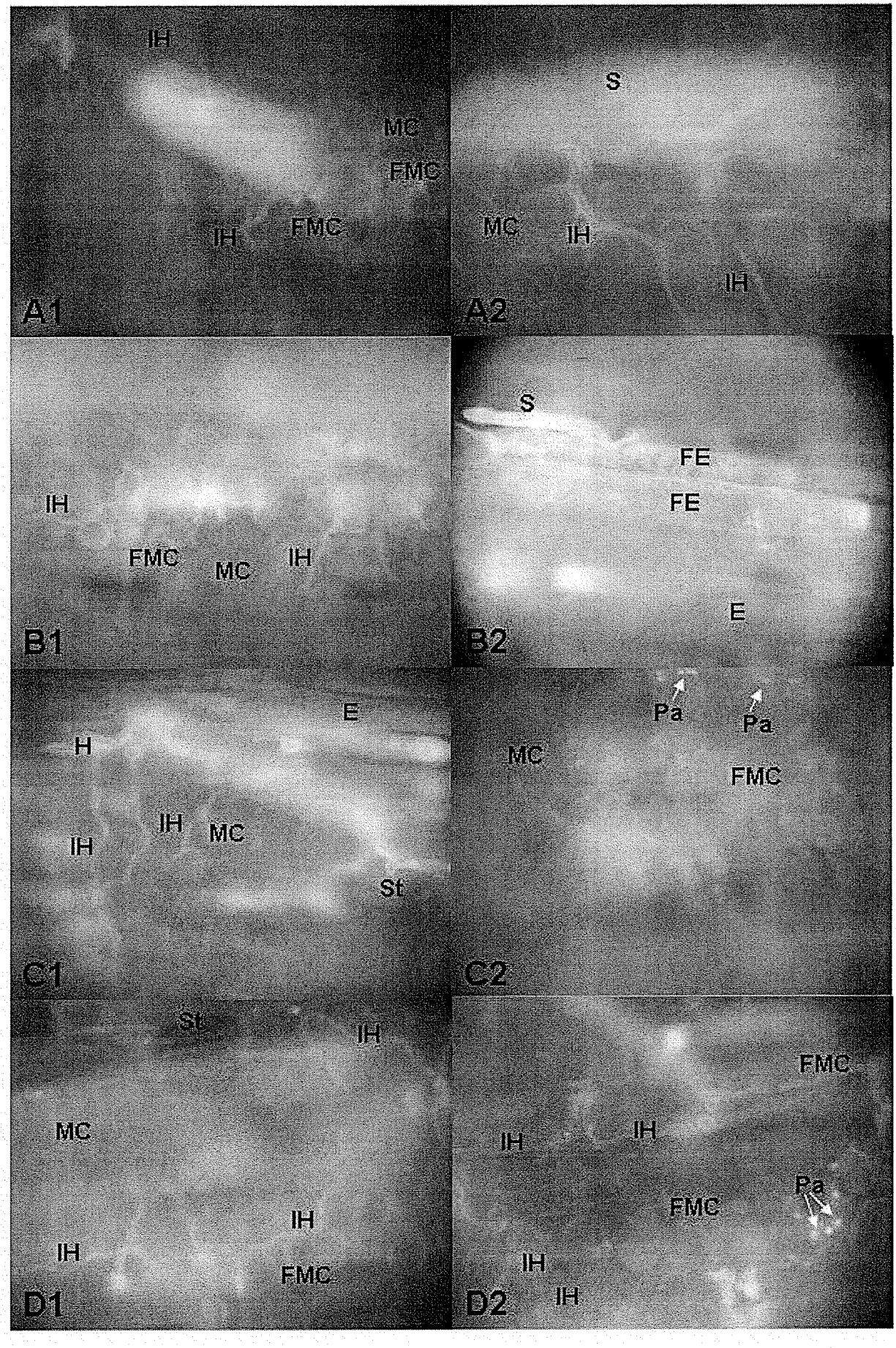
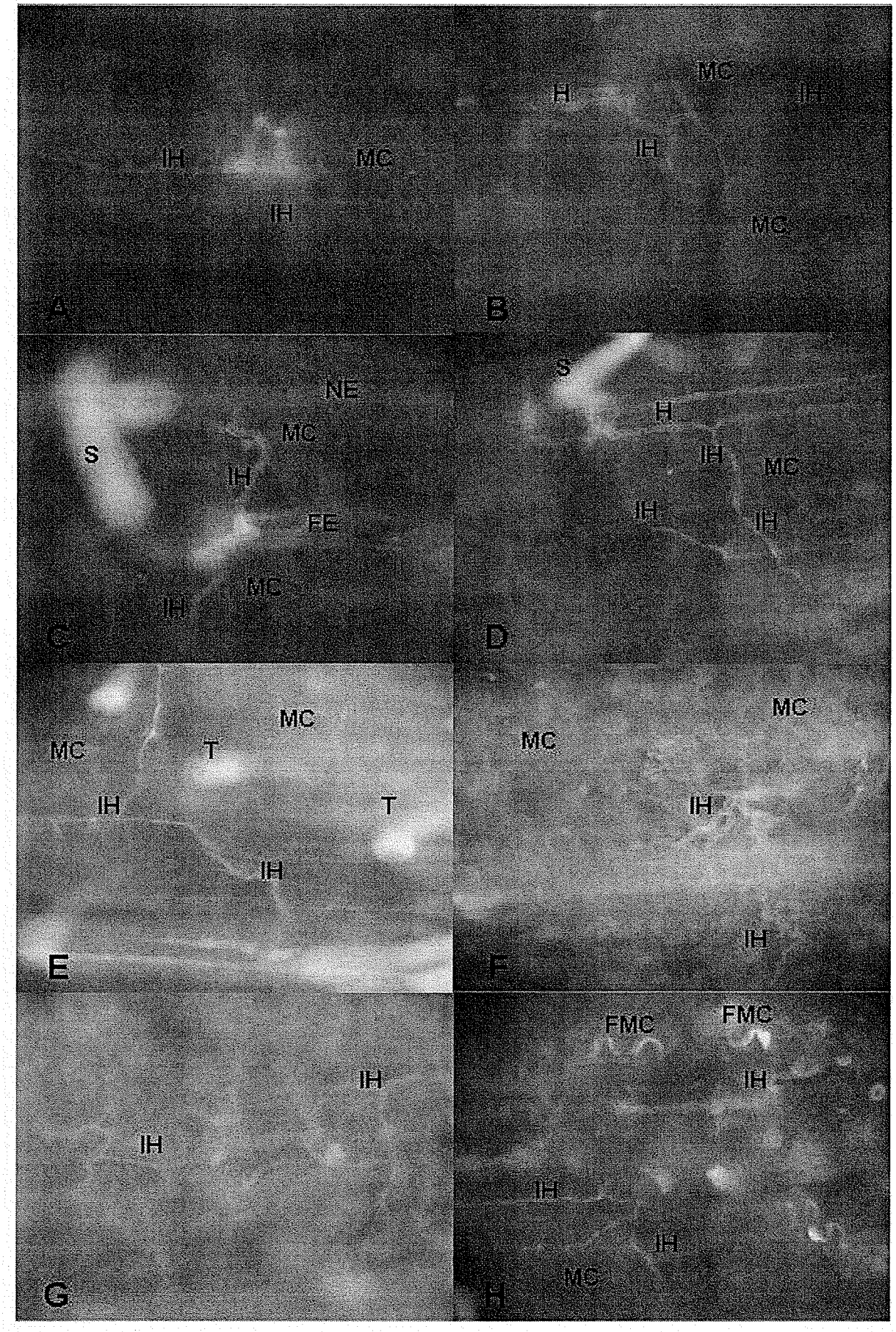

IH
MC
FMC
IH
FMC
A1
S
MC
IH
IH
A2
S
FE
FE
E
B2
IH
FMC
MC
IH
B1
E
H
IH
IH
MC
St
C1
Pa
Pa
MC
FMC
C2
St
IH
MC
IH
FMC
D1
FMC
IH
IH
Pa
FMC
IH
IH
D2

Figure 4.17. Mesophyll layer colonization and reaction of wheat cultivars to avirulent isolate 90-2 of *P. tritici-repentis*. A) Branches of intercellular colonizing hyphae in mesophyll layer of cultivar Neepawa 12 h.p.i. (×300). B) Branches of intercellular colonizing hyphae in mesophyll layer of cultivar Salamouni 12 h.p.i. (×300). C) Branches of intercellular colonizing hyphae in mesophyll layer of cultivar Neepawa 24 h.p.i. (×300). D) Branches of intercellular colonizing hyphae in mesophyll layer of cultivar Salamouni 24 h.p.i. (×300). E) Branches of intercellular colonizing hyphae in mesophyll layer of cultivar Neepawa 48 h.p.i. (×300). F) More branches of intercellular colonizing hyphae in mesophyll layer of cultivar Salamouni 48 h.p.i. (×300). G) A few branches of intercellular colonizing hyphae in mesophyll layer of cultivar Neepawa 72 h.p.i. (×300). H) Fluorescing mesophyll cells and branches of intercellular colonizing hyphae in mesophyll layer of cultivar Salamouni 72 h.p.i. (×300). Abbreviations: FMC = fluorescing mesophyll cell, IH = intercellular hyphae, MC = mesophyll cell, S = spore, T = trichome.


A
IH
MC
IH
B
MC
H
IH
IH
MC
C
NE
MC
S
IH
FE
MC
IH
D
S
H
IH
MC
IH
IH
E
MC
MC
T
IH
T
IH
F
MC
MC
IH
IH
G
IH
IH
H
FMC
FMC
IH
IH
IH
MC

4.5 Discussion

We investigated differences in pre-penetration, penetration, epidermal cell colonization and mesophyll colonization among the necrosis- and chlorosis-inducing isolates of *P. tritici-repentis* in compatible and incompatible situations. Based on the results of this study, all the Ptr ToxA- and/or Ptr ToxB-producing isolates as well as the avirulent isolate from race 4 completed all the stages from spore germination to penetration and colonization of the epidermal cells on both susceptible and resistant cultivars. Loughman and Deverall (1986) had also observed similar initial stages of infection of *P. tritici-repentis* on susceptible and resistant cultivars. There were no significant differences among the isolates regarding germination percentage, the number of germ tubes, and appressoria as well as the number of the penetrated epidermal cells per spore (Tables 4.3 to 4.6). Formation of vesicle and presence of intracellular colonizing hyphae within epidermal cells was also observed for all the Ptr ToxA- and/or Ptr ToxB-producing isolates as well as the avirulent isolate on both susceptible and resistant lines/cultivars. Our results were consistent with those of Larez *et al.* (1986) on the infection of epidermal cells of two susceptible and resistant spring wheats as well as one resistant oat by *P. tritici-repentis*. Dushnisky *et al.* (1996) had also observed a similar infection process of a race 2 isolate (necrosis-inducing) on susceptible and resistant cultivars.

Following penetration of epidermal cells, establishment of *P. tritici-repentis* occurred in the mesophyll layer in both the susceptible and resistant cultivars for necrosis- and/or chlorosis-inducing isolates as well as the avirulent isolate within 12 h.p.i. No penetration of mesophyll cell was observed in our study, and was not reported in

previous studies (Larez *et al.*, 1986; Lamari and Bernier, 1989b; Loughman and Deverall, 1986; Dushnisky *et al.* 1996).

Necrosis-inducing isolates were more successful in their compatible interactions with Glenlea (sensitive to Ptr ToxA) and Neepawa (sensitive to Ptr ToxA and ToxB) than were the chlorosis-inducing isolates in their compatible interactions with Thatcher (sensitive to Ptr ToxB) and Neepawa in terms of growth and expansion in the mesophyll layer. Ptr ToxA-producing isolates expanded their mycelial area coverage (mycelium %) from 2.6% at 12 h.p.i. to 8.7% at 72 h.p.i., whereas Ptr ToxB-producing isolates increased to just 3.2% of area, occupying less than half the area of the colonizing hyphae in necrotic lesions. Differences of mycelium percentage between the necrosis-inducing isolates and chlorosis-inducing isolates of *P. tritici-repentis* were significant at 48 to 72 h.p.i.

The results from the incompatible interaction of the necrosis-inducing isolates, and the chlorosis-inducing isolates with wheat lines cultivars showed seemingly similar growth patterns (no significant differences in mycelium %). The interesting fact was that chlorosis-inducing isolates showed the same rate of growth in the mesophyll layer both in their compatible and incompatible interactions with the corresponding wheat cultivars. In contrast, the necrosis-inducing isolate was much more successful on the susceptible wheat plants than on the resistant wheat cultivars, which was significant by 48 h.p.i. and thereafter till 72 h.p.i. However, Lamari *et al.* (1989b) observed the difference between compatible and incompatible interactions to be evident later, at 72 h.p.i. Ptr ToxA appears to be of benefit to *P. tritici-repentis* isolates by enabling them to colonize the

mesophyll tissue more successfully than Ptr ToxB-producing isolates by covering larger areas of mesophyll with more mycelia.

Since Ptr ToxA has been found to be advantageous to the producing isolates, there was an expectation that production of Ptr ToxB along with Ptr ToxA could add to the ability of the pathogen to attack the susceptible wheat cultivars such as Neepawa (sensitive to Ptr ToxA and Ptr ToxB), in a more aggressive way than the isolates producing a single Ptr toxin. However, our results did not confirm such a hypothesis. Growth rate of Ptr ToxA- and ToxB-producing isolates were not significantly different from the Ptr ToxA- and Ptr ToxB-producing isolates in their incompatible interactions with wheat cultivars. Ptr ToxA- and ToxB-producing isolates were as successful as Ptr ToxA- and Ptr ToxB-producing isolates in the compatible interactions till 48 h.p.i. Ptr ToxA- and ToxB-producing isolates were significantly more successful than Ptr ToxB-producing isolates at 48 h and thereafter till 72 h.p.i. Our results from the population shifts of *P. tritici-repentis* confirmed that production of more than one Ptr toxin does not necessarily make the producing isolates more competitive than the isolates producing only one toxin on the tested individual cultivars.

Regarding the thickness of the colonizing hyphae (Table 4.11), necrosis-inducing isolates in compatible interactions had significantly larger mycelium thickness than chlorosis-inducing isolates, except for 12 h.p.i., where chlorosis-inducing isolates had significantly lager mycelium thickness than necrosis-inducing isolates. Necrosis- and chlorosis-inducing isolates in both interactions had significant larger mycelium thickness than necrosis-inducing isolates at all sampling times, except for 24 h.p.i., where both were similar. In incompatible interactions, chlorosis-inducing isolates still had a larger

mycelium thickness than necrosis-inducing isolates at 12 h.p.i., but were similar in terms of mycelium thickness thereafter till 72 h.p.i. This may be an indication of higher growth rate of necrosis-inducing isolates than the chlorosis-inducing ones, which will result in higher percentage of mycelium in a given area. Since the invading hyphae grew intercellularly, the thickness of mycelia could be affected by factors such as space limitation or nutrition accessibility, or could be a morphological characteristic of the individual isolates. The increasing growth rate of the intercellular hyphae in the mesophyll suggests a favorable condition for the isolates. In other words, the increase in hyphal thickness could be an indication of a successful and healthy growth of the invading intercellular hyphae from a particular isolate. These intercellular hyphae are the origin of the conidiophores, which later, in a favorable condition, are stimulated to produce conidia. So, the thickness of the colonizing hyphae in the mesophyll layer could represent more stored nutrients for the time of spore production. The more lesions producing conidia, the greater chance for a particular isolate to show up in the population. This would partially explain why Ptr ToxA-producing isolates are more successful than Ptr ToxB- producing isolates in their ability to sporulate and become dominant in the population.

Based on the results of this study, Ptr ToxA appears to provide more competitive advantage to the producing isolates, which is consistent with the results of the competition studies and partially explain the finding that ToxA-producing isolates were the predominant group or their frequencies were increasing in the recovered pathogen population on both the susceptible and resistant lines/cultivars. (Chapter 3)

5.0 GENERAL DISCUSSION

5.1 Selection of Ptr ToxB-producing Isolates by the Sensitive Wheat Hosts

We compared the competitive ability of Ptr ToxA- and/or Ptr ToxB-producing isolates of *P. tritici-repentis* in a population consisting of all the eight known races, on seven historic and modern Canadian cultivars (Chapter 3). Their infection process was also individually observed on four of the same cultivars used in competition studies (Chapter 4).

The results of this study support the hypothesis, that Ptr ToxB-producing isolates were absent in the *P. tritici-repentis* population through a century of cultivation of Ptr ToxB-sensitive wheat cultivars in Western Canada. This conclusion is justified by the fact that Ptr ToxB-producing isolates were not eliminated on all cultivars tested, and were predominant in the second experiment on all cultivars tested, except Coulter. They also have the ability to reproduce on resistant hosts, which would have kept them in the population in the absence of compatible genotypes. Ptr ToxB-producing isolates had a higher frequency on Ptr ToxB-sensitive lines/cultivars than on the resistant ones. They were reaching higher frequencies on compatible genotypes from generation two to four, suggesting that Ptr ToxB-producing isolates were likely to be dominant on the Ptr ToxB-sensitive lines/cultivars in the first experiment, if we were able to run additional generations. This suggests that Ptr ToxB-sensitive wheat cultivars cultivation in western Canada from 1932 to 2003 (Lamari *et al.,* 2005a), would have selected the Ptr ToxB-producing isolates, if they were part of the pathogen population even at a low frequency. This makes the only report of wild-type isolates of race 5 from North Dakota (Ali and Francl, 1999) quite puzzling.

The selection of Ptr ToxB-producing isolates by susceptible wheat cultivars points out their potential destructive ability to wheat production, if homologues for the inactive *ToxB,* which is part of the gene pool of the pathogen in western Canada (Strelkov *et al.,* 2002, Martinez *et al.,* 2004), become fully active. Considering that *P. tritici-repentis* cause a polycyclic disease, the high spore quantity produced makes it likely for mutation to activate *ToxB* homologues. Furthermore, race-specific resistance (only to Ptr ToxA-producing races) in wheat cultivars may exert a selection pressure beneficial to isolates carrying *ToxB* and ToxB-homologues. This was suggested to be the cause of the rise of race 2 which was rare from 1984 to 1987 (Lamari and Bernier 1989c) and became the most prevalent race from 1991 to1994 (Lamari, L., 1998), and from 2000 to 2002 (Sighn, *et al.,* 2007) in western Canada. Kutcher *et al.,* (2007) also proposed selection pressure on *Leptosphaeria maculans* from race-specific resistance genes in canola as the probable cause for the change in virulence of the pathogen.

This information could be useful for breeders to include Ptr ToxB resistant cultivars in their breeding programs to provide a more appropriate and durable resistance to tan spot disease and also help to stabilize the pathogen population.

5.2 Competitive Advantages Provided to the Pathogen by Ptr ToxA and Ptr ToxB

The lack of significant differences in the initial stages of the infection process among Ptr ToxA- and/or Ptr ToxB-producing, as well as the avirulent isolates of *P. tritici-repentis* (chapter 4), confirmed that Ptr toxins were not involved in the pre-mesophyll colonization events.

We also investigated the differences, in the behavior, in the mesophyll layer, of Ptr ToxA- and/or Ptr ToxB-producing isolates used in the first competition experiment

(Chapter 4). First of all, establishment of the intercellular hyphae in the mesophyll of the corresponding susceptible or resistant wheat cultivars occurred for both the necrosis- and chlorosis-inducing isolates. The growth rate of the colonizing hyphae in the mesophyll layer was not significantly different among the Ptr ToxA- and Ptr ToxB-producing isolates when the wheat genotypes were Ptr toxin-insensitive (i.e. with respect to given toxins). This emphasizes the advantage provided to the fitness of the fungal isolates by the Ptr toxin(s), when there was a higher rate of intercellular mycelium growth on the Ptr toxin-sensitive lines/cultivars.

To find out which Ptr toxin is more advantageous to the virulence of *P. tritici-repentis* isolates, cytological studies were carried out on the Ptr toxin-sensitive cultivars (Chapter 4). Although we could not detect a significant difference among the Ptr ToxA- and Ptr ToxB-producing isolates up to 24 h.p.i., the survival of some isolates on resistant cultivars (incompatible genotypes) suggested that cultivar-sensitivity to Ptr toxins is not the only determinant factor leading to the selection of the pathogen. By 72 h.p.i., the Ptr ToxA-producing isolate had a higher growth rate than the tested Ptr ToxB-producing isolates. This suggested that the production of Ptr ToxA is more advantageous than the production of Ptr ToxB, a finding that was consistent with the predominance of Ptr ToxA-producing isolates in this thesis (Chapter 3) and in surveys in many parts of the world. Seemingly, Ptr ToxA provides more fitness ability to its producing isolates for survival and reproduction.

Producing more than one Ptr toxin did not give more selective advantage to the producing isolates on a given cultivar on a given cultivar. This was based on the finding that Ptr ToxA- and ToxB-producing isolates were the second, but not the first

predominant group of isolates in both competition experiments conducted in this study (Chapter 3). They were not more successful than isolates producing only one toxin (e.g. Ptr ToxA) in the colonization of the mesophyll tissue (Chapter 4). Although Ptr ToxA- and ToxB-producing isolates were more successful than Ptr ToxB-producing isolates, their performance may be due to their ability to produce Ptr ToxA. Apparently, the type of the produced toxin seems to be more important than the number of toxins. In wheat stem rust, carrying more virulence genes was found to be advantageous to the *Puccinia graminis tritici* races, as races carrying more virulence genes were predominant in the population shift studies conducted by Osoro and Green (1976). They concluded that races with more virulence genes are better competitors. They also suggested that genes for competitive ability were independent and that their distribution in the population was random (i.e., their association with virulence genes was by chance).

Most of the evidence supports the hypothesis that sensitivity to Ptr toxins is the determinant factor which gives a selective advantage to the corresponding Ptr toxin-producing isolates. Differences in the frequency of isolates from the same race on the same cultivar (i.e. race 5) suggests that the structure of the formed generations is not necessarily consistent with the hypothesis of a race-specific interaction between isolates of the pathogen and sensitive lines/cultivars. The effect of some characteristics of individual isolates, independent of toxins, is suspected. These characteristics could be related to the sporulation ability of individual isolates, in terms of timing and quantity, in the amount of toxin production as well as in the procurement and utilization of nutrients from the mesophyll. This may result in healthy mycelia, which will form more basal conidiophores and, consequently, lead to more sporulation. The presence of different

isolates may also exert different selection pressures. In *P. tritici-repentis*, aggressiveness of isolates may also be different from one isolate to another and be independent of Ptr toxins production. Aboukhaddour (2008) reported that karyotypes of isolates within the races of *P. tritici repentis* are variable among the races of the pathogen, which is probably an explanation for the phenotypic differences of the isolates from the same race. Regarding variation of virulence among the necrosis-inducing isolates of *P. tritici repentis,* Misra and Singh (1972), reported variations in lesion size among three necrosis-inducing single-spore isolates on 50 wheat cultivars. Krupinsky (1992b) grouped 88 isolates in 3 classes based on the lesion size and percentage of necrotic area. Graniti (1991) considered both the pathogenicity and virulence of a pathogen to be related to the production of host-specific toxins.

Based on the results of this study, production of Ptr ToxA provides more competitive advantage to the producing isolates. This conclusion is based on the fact that Ptr ToxA-producing isolates were either the predominant group (experiment 1), or their frequencies were increasing through the consecutive generations (experiment 2) (Chapter 3). These isolates had higher growth rate in mesophyll layers on both the susceptible and resistant wheat lines/cultivars (Chapter 4).

6.0 REFERENCE MATTER

6.1. Literature Cited

Aboukhaddour, R. (2008) *Pyrenophora tritici-repentis*: genome structural organization and genetic diversity. Ph. D. Thesis, The University of Manitoba, Winnipeg. 113pp.

Aist, J. R. (1976) Papillae and related wound plugs of plant cells. Ann. Rev. Phytopathol. **14:**145-163.

Aist, J. R., and Israel, H. W. (1977) Effects of heat shock inhibition of papilla formation on compatible host penetration by two obligate parasites. Physiol. Plant Pathol. **10:**13-20.

Ali, S, and Francl, L. J. (1999). Races of *Pyrenophora tritici-repentis* on durum in the northern Great Plains of the U.S. (Abstr.) Phytopathology 89:S2.

Ali, S., and Francl, L. J. (2001) Recovery of *Pyrenophora tritici-repentis* from barley and reaction of 12 cultivars to five races and two host-specific toxins. Plant Dis. **85:**580-584.

Ali, S., and Francl, L. J. (2003) Population race structure of *Pyrenophora tritici-repentis* prevalent on wheat and noncereal grasses in Great Plains. Plant Dis. **87:**418-422.

Ali, S., Ling, H., Meinhardt, S., and Francl, L. (2002) A new race of *Pyrenophora tritici-repentis* that produces a putative host-selective toxin. (Abstr.) Phytopathology 92 (suppl.):S3.

Andrie, R. M., Pandelova, I., and Ciuffetti, L. M. (2007) A combination of phenotypic and genotypic characterization strengthens *Pyrenophora tritici-repentis* race identification. Phytopathology **97:**694-701.

Bailey, K. L. (1996) Disease under conservation tillage system. Can. J. Plant Sci. **76:** 635-639.

Ballance, G. M., Lamari, L., and Bernier, C. C. (1989) Purification and characterization of a host selective toxin from *Pyrenophora tritici-repentis*. Physiol. Mol. Plant Pathol. **35:**203-213.

Ballance, G. M., Lamari, L., Kowatsch, R., and Bernier, C. C. (1996) Cloning, expression and occurrence of the gene encoding the Ptr necrosis toxin from *Pyrenophora tritici-repentis*. Mol. Plant Pathol.
URL: **http:**//www.bspp.org.uk./mppol/1996/1209ballance

Bockus, W. W. (1998) Control strategies for stubble-born pathogens of wheat. Can. J. Plant Pathol. **20:**371-375.

Bockus, W. W., and Claassen, M.M. (1992) Effects of crop rotation and residue management practices on severity of tan spot of winter wheat. Plant Dis. **76:** 633-636.

Brown, J,. F., and. Sharp, E. L. (1970). The relative survival ability of pathogenic types of *Pileeinis striiformis* in mixtures. Phytopathology **60:** 529-533.

Bucheli, P., Doares S. H., Albersheim P., and Darvill A. (1990) Host-pathogen interactions *XXXVI.* Partial purification and characterization of heat-labile molecules secreted by the rice blast pathogen that solubilize plant cell wall fragments that kill plant cells. Physiol. Mol. Plant Pathol. **36:**159–73.

Ciuffetti, L. M., Francl, L. J., Ballance, G. M., Bockus, W. W., Lamari, L., Meinhardt, S. W., and Rasmussen, J. B. (1998) Standardization of toxin nomenclature in the *Pyrenophora tritici-repentis*/wheat interaction. Can. J. Plant Pathol. **20:**421-424.

Ciuffetti, L. M., Manning, V. A., Martinez. J. P., Pandelova, I., and Andrie, R. M. (2003) Proteinaceous toxins of *Pyrenophora tritici-repentis* and investigation of the site-of-action of Ptr ToxA. Pages 96-102 in: Proceedings from the Fourth International Wheat Tan Spot and Spot Blotch workshop. J. B. Rasmussen, T. L. Friesen, and S. Ali, eds. North Dakota Extension Service, ND, USD.

Ciuffetti, L. M., Tuori, R. P., and Gaventa, J. M. (1997) A single gene encodes a selective toxin causal to the development of tan spot on wheat. Plant Cell. **9:**135-144.

Conner, L.L. (1937) P. 5. In: 17th Annu. Rep. Can. Plant Dis. Surv. Canadian Department of Agriculture. Division of Botany and Plant Pathology. Canada.

Dickman, M. B., Podila, G. K., and Kolattukudy, P. E. (1989) Insertion of cutinase gene into a wound pathogen enables it to infect intact host. Nature **342:**446–448.

Dushinsky, L. G., Ballance, G. M., Summer, M. J., and MacGregor, A. W. (1998) The role of lignification as a resistance mechanism in wheat to a toxin producing isolate of *Pyrenophora tritici-repentis*. Can. J. Plant Pathol. **20:**35-47.

Dushinsky, L. G., Ballance, G. M., Summer, M. J., and MacGregor, A. W. (1996) Penetration and infection of susceptible and resistant wheat cultivars by a necrosis toxin-producing isolate of *Pyrenophora tritici-repentis.* Can. J. Plant Pathol. **18:**392-402.

Effertz, R. J., Anderson, J. A., and Francl, L. J. (2001) Restriction fragment length polymorphism mapping of resistance to two races of *Pyrenophora tritici-repentis* in adult and seedling wheat. Phytopathology **91:**572-578.

Effertz, R. J., Meinhardt, S. W., Anderson, J. A., Jordahl, J. G., and Francl, L. J. (2002) Identification of a chlorosis-inducing toxin from *Pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat. Phytopathology **92:**527-533.

Faris, J. D., Anderson, J. A., Francl, L. J., and Jordahl, J. G. (1996) Chromosomal location of a gene conditioning insensitivity in wheat to a necrosis-inducing culture filtrate from *Pyrenophora tritici-repentis*. Phytopathology **86:**459-463.

Faris, J. D., Anderson, J. A., Francl, L. J., and Jordahl, J. G. (1997) RFLP mapping of resistance to chlorosis induction by *Pyrenophora tritici-repentis* in wheat. Theor. Appl. Genet. **94:**98-103.

Fernandez, M. R., McConkey, B. G., and Zentner, R. P. (1998) Tillage and summer fallow effects on leaf spot diseases of wheat in the semiarid Canadian Prairies. Can. J. Plant Pathol. **20:**376-379.

Fernandez, M.R., Clarke, J.M., and DePauw, R.M. (1994). Response of durum wheat kernels and leaves at different growth stages to *Pyrenophora tritici-repentis*. Plant Dis. **78:**597–600.

Fernandez, M.R., Clarke, J.M., DePauw, R.M., and Lefkovitch, L.P. (1997) Emergence and growth of durum wheat derived from red smudge-infected seed. Crop Sci. **37:**510–514.

Flor, H. H. (1955) Host–parasite interactions in flax rust – its genetics and other implications. Phytopathology **45:**680–685.

Flor, H. H. (1971) Current status of the gene-for-gene concept. Ann. Rev. Phytopathol. **9:**275-296.

Friesen T. L., and Faris J. D. (2004) Molecular mapping of resistance to *Pyrenophora tritici-repentis* race 5 and sensitivity to Ptr ToxB in wheat. Theor. Appl. Genet. **109:**464–471.

Friesen T. L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., Rasmussen, J.B., Solomon, P.S., McDonald, B.A. and Oliver, R.P. (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. Nature Genetics **38 (8):**953-956.

Gamba, F. M., and Lamari, L. (1998) Mendelian inheritance of resistance to tan spot (*Pyrenophora tritici-repentis*) in selected genotypes of durum wheat (*Triticum turgidum*). Can. J. Plant Pathol. **20:**408-414.

Gamba, F. M., Lamari, L., and Brûlé-Babel, A. (1998) Inheritance of race specific necrotic and chlorotic reactions induced by *Pyrenophora tritici-repentis* in hexaploid wheats. Can. J. Plant Pathol. **20:**401-407.

Gilchrist, S. L., Fuentes, S. F., and. de L. De la Isla de Baur, M. (1984) Determination of sources of resistance to *Helminthosporium tritici-repentis* under field and glasshouse conditions. Agrociencia. **56:**95-105.

Graniti, A. (1991) Phytotoxins and their involvement in plant diseases. Experimentia **47:**751-755

Green, G. J. (1971) Physiologic races of wheat stem rust in Canada from 1919 to 1969. Can. J. Bot. **49:**1575-1588.

Green, G. J. and Campbell, A.B. (1979) Wheat cultivars resistant to *Puccinia graminis tritici* in Western Canada: their development, performance, and economic value. Can. J. Plant Pathol. **1:**3-11.

Hoch, H., Staples, R. C., Whitehead, B., Comeau, J., and Wolf, E. D. (1987) Signaling for growth orientation and cell differentiation by surface topography in Uromyces. Science **235:**1659–1662.

Hood, M. E., and Shew, H. D. (1996) Application of KOH-aniline blue fluorescence in the study of plant-fungal interactions. Phytopathology **86:**704-708.

Hosford, Jr. R. M. (1971) A form of *Pyrenophora trichostoma* pathogenic to wheat and other grasses. Phytopathology **61:**28-32.

Hosford, Jr. R. M. (1972) Propagules of *Pyrenophora trichostoma*. Phytopathology **62:**627-629.

Hosford, Jr. R. M. (1982) Tan spot developing knowledge 1902-1981, virulent races and wheat differentials, methodology, rating systems, other leaf diseases, literature. P. 1-24. In: *Tan Spot of Wheat and Related Diseases* Workshop. (Hosford R. M., Jr.,ed.) North Dakota Agric. Exp. Station, Fargo.

Hosford, Jr. R. M., and Busch, R. H. (1973) Losses in wheat caused by *Pyrenophora trichostoma* and *Leptospheria avenaria* f. sp. *Triticea.* Phytopathology **64:**184-187.

Hosford, Jr. R. M., Larez, C. R., and Hammond, J. J. (1987) Interaction of wet period and temperature on *Pyrenophora tritici-repentis* infection and development in wheats of differing resistance. Phytopathology **77:**1021-1027.

Johnson, T. and Newton, M. (1941) The predominance of race 56 in relation to the stem rust resistance of Ceres wheat. Sci. Agric. **22:**152-156.

Khan, T. N. (1971) Effect of light on sporulation in *Drechslera tritici-repentis*. Br. Mycol. Soc. Trans. **56:**309-311.

Krupinsky, J. M. (1987) Pathogenicity on wheat of *Pyrenophora tritici-repentis* isolated from *Bromus inermis*. Phytopathology **77:**760-765.

Krupinsky, J. M. (1992a) Aggressiveness of isolates of *Pyrenophora tritici-repentis* obtained from wheat in the northern Great Plains. Plant Dis. 76:87-91.

Krupinsky, J. M. (1992b) Grass hosts of *Pyrenophora tritici-repentis*. Plant Dis. **76:**92-95.

Kutcher, H. R., Keri, M., McLaren, D. L., and Rimmer, S. R. (2007) Pathogenic variability of *Leptosphaeria maculans* in western Canada. Can. J. Plant Pathol. 29:388-393.

Kwon, C. Y., Rasmussen, J. B., and Meinhardt, S. W. (1998) Activity of Ptr ToxA of *Pyrenophora tritici-repentis* requires host metabolism. Physiol. Mol. Plant Pathol. **52:**201-212.

Lamari, L. (1988). Tan spot of wheat, caused by *Pyrenophora tritici-repentis*: Host and pathogen variability, host-pathogen interaction and involvement of toxin in disease. Ph. D. Thesis, The University of Manitoba, Winnipeg. 107pp.

Lamari, L., and Bernier, C. C. (1989a) Toxin of *Pyrenophora tritici-repentis*: Host-specificity, significance in disease, and inheritance of host reaction. Phytopathology **79:**740-744.

Lamari, L., and Bernier, C. C. (1989b) Virulence of isolates of *Pyrenophora tritici-repentis* on 11 wheat cultivars and cytology of the differential host reactions. Can. J. Plant Pathol. **11:**284-290

Lamari, L., and Bernier, C.C. (1989c). Evaluation of wheat lines and cultivars for reaction to tan spot (*Pyrenophora tritici-repentis*) based on lesion types. Can. J. Plant Pathol. **11:**49-56.

Lamari, L., and Bernier, C. C. (1991) Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. Phytopathology **81:**1092-1095.

Lamari, L., and Bernier, C. C. (1994) Temperature induced resistance to tan spot (*Pyrenophora tritici-repentis*) of wheat. Can. J. Plant Pathol. **16:**279-286.

Lamari, L., Gilbert, J., and Tekauz, A. (1998) Race differentiation in *Pyrenophora tritici-repentis* and survey of physiologic variation in western Canada. Can. J. Plant Pathol. **20:**396-400.

Lamari, L., McCallum, B. D., and DePauw, R. M. (2005a) Forensic pathology of Canadian bread wheat: The case of tan spot. Phytopathology **95:**144-152.

Lamari, L., Sayoud, R., Boulif, M., and Bernier, C. C. (1995) Identification of a new race of *Pyrenophora tritici-repentis*: Implications for the current pathotype classification system. Can. J. Plant Pathol. **17:**312-318.

Lamari, L., Strelkov, S. E., Yahyaoui, A., Amedov, M., Saidov, M., Djunusova, M., and Koichibayev, M. (2005b) Virulence of *Pyrenophora tritici-repentis* in the countries of the Silk Road Can. J. Plant Pathol. **27:**383–388.

Lamari, L., Strelkov, S. E., Yahyaoui, A., Orabi, J., and Smith, R. B. (2003) The identification of two new races of *Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. Phytopathology **93:**391-396.

Larez, C. R., Hosford, Jr. R. M., and Freeman, T. P. (1986) Infection of wheat and oats by *Pyrenophora tritici-repentis* and initial characterization of resistance. Phytopathology **76:**931-938.

Lee, T. S., and Gough, F. J. (1984) Inheritance of *Septoria* leaf blotch (*S. tritici*) and *Pyrenophora* tan spot (*P. tritici-repentis*) resistance in *Triticum aestivum cv.* Carifen 12. Plant Dis. **68:**848-851.

Loughman, R. and Deverall, B. J. (1986). Infection of resistant and susceptible cultivars of wheat by *Pyrenophora tritici-repentis*. Plant Pathol. **35:**443-450.

Manning, V. A., and Ciuffetti, L. M. (2005) Localization of Ptr ToxA produced by *Pyrenophora tritici-repentis* reveals protein import into wheat mesophyll cells. Plant Cell. **17:**3203-3212

Manning, V. M., Pandelova, I., and Ciuffetti, L. M. (2002) A race for a novel host-selective toxin. Phytopathology **92:**S51

Martens, J. W., Seaman, W. L., and Atkinson, T. G. (1988) Disease of Field crops in Canada. The Canadian Phytopathol. Society, Harrow, Ontario, Canada. 160 pp.

Martinez, J. P., Osch, N. W. and Ciuffetti, L. M. (2004) Characterization of the multiple copy host-selective toxin gene, *ToxB*, in pathogenic and nonpathogenic isolates of *Pyrenophora tritici-repentis* Mol. Plant-Microbe Interact. **17:**467-474.

Martinez, J. P., Ottum, S. A., Ali, S., Francl, L. J., and Ciuffetti, L. M. (2001) Characterization of the ToxB gene from *Pyrenophora tritici-repentis*. Mol. Plant-Microbe Interact. **14:**675-677.

Meinhardt, S., Ali, S., Ling, H., and Francl, L. (2003) A new race of *Pyrenophora tritici-repentis* that produces a putative host selective toxin. P. 117-121. In Proceedings of the Fourth International Wheat Tan Spot and Spot Blotch Workshop. (Rasmussen, J. B., Friesen, T. L., and Ali, S. eds) North Dakota Extension Service, ND, USA.

Meinhardt, S., Zhang, H. F., Effertz, R. J., and Francl, L. J. (1998) Characterization of additional peaks of necrosis activity from *Pyrenophora tritici-repentis.* Can. J. Plant Pathol. **20:** 436-437.

Misra, A. P., and Singh, R. A. (1972) Pathogenic differences among three isolates of *Helminthosporium tritici-repentis* and the performance of wheat varieties against them. Indian Phytopathol. 25:350-353.

Morrall, R. A. A., and Howard, R. J. (1975) The epidemiology of leaf spot disease in a native prairie. II. Airborne spore populations of *Pyrenophora tritici-repentis*. Can. J. Bot. **53:**2345–2353.

Orolaza, N. P., Lamari, L., and Ballance, G. M. (1995) Evidence of a host-specific chlorosis toxin from *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat. Phytopathology **85:**1282-1287.

Osoro, M. O., and Green, G. J. (1976) Stabilizing selection in *Puccinina graminis tritici* in Canada. Can. J. Bot. **54:**2204-2214.

Pfender, W. F., and Wootke, S. L. (1987) Production of pseudothecia and ascospores by *Pyrenophora tritici-repentis* in response to micronutrient concentrations. Phytopathology **77:**1213-1216.

Pfender, W. F., Pacey, C. A., and Zhang, W. (1988) Saprophytic growth and pseudothecia production by *Pyrenophora tritici-repentis* in plant tissue held at controlled water potentials. Phytopathology **78:**1205-1210.

Platt, H. W., and Morrall, R. A. A. (1980) Effects of wind speed and humidity on conidium liberation of *Pyrenophora tritici-repentis* Can. J. Plant Pathol. **2:**58-64.

Rees, R. G. (1982). Yellow spot, an important problem in the North-Eastern wheat areas of Australia. P. 68-70. In: Tan spot of wheat and related diseases workshop. (Hosford, Jr., R. M. de.), North Dakota State University, Fargo.

Rees, R. G., and Platz, G. J. (1979) The occurrence and control of yellow spot of wheat in northeastern Australia. Aust. J. Experimental Agric. Anim. Husb. **19:**369-372.

Rees, R. G., and Platz, G. J. (1980a). The epidemiology of yellow spot of wheat in Southern Queensland. Aust. J. Agric. Res. **31:**259-267.

Rees, R. G., and Platz, G. J. (1980b) The epidemiology of yellow spot of wheat in north-eastern Australia. Aust. J. Exp. Agric. Animal Husbandry. **19:**369-372.

Rees, R. G., and Platz, G. J. (1983) Effect of yellow spot on wheat: Comparison of epidemics at different stages of crop development. Aust. J. Agric. Res. **34:**39-46.

Rees, R. G., and Platz, G. J. (1992) Tan spot and its control: some Australian experiences. P. 1-9 In: Advances in Tan Spot Research. (Francl,L. J., Krupinsky, J. M., and McMullen, M. P. eds), North. Dakota. Agric. Exp. Station Fargo.

Rees, R. G., Platz, G.J., and Myer, R.J. (1982) Yield losses in wheat from yellow spot. Comparison of estimates derived from single tillers and plots. Aust. J. Agric. Res. **38:**899-908.

Ronis, A. and Semaskien, R. (2006) Development of tan spot (*Pyrenophora tritici-repentis*) in winter wheat under field conditions. Agronomy Res. (special issue). **4:**133-134.

Scheffer, R. P. (1976) Host-specific toxins in relation to pathogenesis and disease resistance. P. 247-269 In: Encylopedia of Plant Pathology, new series. Vol. 4. Physiological Plant Pathology. (Heitefuss R. and Williams P. H., eds.) Springer-Verlag, Berlin.

Schilder, A. M. C., and Bergstrom, G. C. (1992) The dispersal of conidia and ascospores of *Pyrenophora tritici-repentis*. P. 96-99 In: Proceeding from the Second International Tan Spot Workshop. (Francl, L. J. Krupinsky, J. M., and McMullen, M. P., eds.) North Dakota Agric. Exp. Station, Fargo.

Schilder, A. M. C., and Bergstrom, G. C. (1994) Infection of wheat seed by *Pyrenophora tritici-repentis*. Can. J. Bot. **72:**510-519.

Schilder, A. M. C., and Bergstrom, G. C. (1995) Seed transmission of *Pyrenophora tritici-repentis*, causal fungus of tan spot of wheat. Eur. J. Plant Pathol. **101:**81-91.

Shoemaker, R. A. (1962) *Drechslera* ITO. Can. J. Bot. **40:**809-836.

Singh, P. K., and Hughes, G. R. (2005) Genetic control of resistance to tan necrosis induced by *Pyrenophora tritici-repentis*, races 1 and 2, in spring and winter wheat genotypes. Phytopathology **95:**172-177.

Singh, P. K., and Hughes, G. R. (2006) Inheritance of resistance to the chlorosis component of tans pot of wheat caused by *Pyrenophora tritici-repentis*, races 1 and 3. Euphytica. **152:**413–420.

Singh, P. K., Gonzalez-Hernandez, J. L., Mergoum, M., Ali, S., Adhikari, T. B., Kianian, S. F., Elias, E. M., and Hughes, G. R. (2006b) Identification and molecular mapping of a gene conferring resistance to *Pyrenophora tritici-repentis* race 3 in tetraploid wheat. Phytopathology **96:**885-889.

Singh, P. K., Mergoum, M., Ali, S. Adhikari, T. B. Elias, E. M and Hughes, G. R. (2006a) Identification of new sources of resistance to tan spot, *Stagonospora nodorum* blotch, and *Septoria tritici* blotch of wheat. Crop. Sci. **46:**2047–2053.

Singh, P. K., Mergoum, M., and Hughes, G. R. (2007) Variation in virulence to wheat in *Pyrenophora tritici repentis* population from Saskatchewan, Canada, from 2000 to 2002. Can. J. Plant Pathol. 29:166-171.

Strelkov, S. E. (2002). Occurrence and expression of ToxB and comparative virulence of chlorosis-inducing races of *Pyrenophora tritici-repentis*. PhD thesis, University of Manitoba, Winnipeg.

Strelkov, S. E., and Lamari, L. (2003) Host-parasite interactions in tan spot [*Pyrenophora tritici-repentis*] of wheat. Can. J. Plant Pathol. **25:**339-349.

Strelkov, S. E., Kowatsch, R. F., Ballance, G. M., and Lamari, L. (2006) Characterization of the *ToxB* gene from North Africa and Canadian isolates of *Pyrenophora tritici-repentis*. Physiol. Plant Pathol. **67:**167-170.

Strelkov, S. E., Lamari, L., and Ballance, G. M. (1998a) Induced chlorophyll degradation by a chlorosis toxin from *Pyrenophora tritici-repentis*. Can. J. Plant Pathol. **20:**428-435.

Strelkov, S. E., Lamari, L., and Ballance, G. M. (1999) Characterization of a host-specific protein toxin (Ptr ToxB) from *Pyrenophora tritici-repentis*. Mol. Plant-Microbe Interact. **12:**728-732.

Strelkov, S. E., Lamari, L., Ballance, G. M., and Orolaza, N. P. (1998b) Isolation and mode of action of Ptr chlorosis toxin from *Pyrenophora tritici-repentis*. Pages 137-138 in: Molecular Genetic of Host-Specific Toxins in Plant Disease: Proceedings of the Third Tottori International Symposium on Host-Specific Toxins. K. Kohmoto and O. C. Yoder, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Strelkov, S. E., Lamari, L., Sayoud, R., and Smith, R. B. (2002) Comparative virulence of chlorosis-inducing races of *Pyrenophora tritici-repentis*. Can. J. Plant Pathol. **24:**29-35.

Summerell, B, A, and Burgess, L.W. (1989) Factors influencing survival of *Pyrenophora tritici-repentis:* water potential and temperature. Mycol. Res. **93:**41-45.

Sutton, J. C., and Vyn, T. J. (1990) Crop sequences and tillage practices in relation to disease of winter wheat in Ontario. Can. J. Plant Pathol. **12:**358-368.

Sykes, E. E., and Bernier, C. C. (1991) Qualitative inheritance of tan spot resistance in hexaploid, tetraploid, and diploid wheat. Can. J. Plant Pathol. **13:**38-44.

Tadess, W., Hsm, S. L. K., and Zeller, F. J. (2006) Identification and monosomic analysis of tan spot resistance gene from synthetic wheat genotypes. Crop Sci. **46:**1212-1217.

Tekauz, A. (1976) Distribution, severity and relative importance of leaf spot disease of wheat in western Canada in 1974. Can. Plant Dis. Surv. **56:**36-40.

Tekauz, A., Platford, R. G., and English, N. C. (1982) Tan spot on wheat. Manitoba Agronomists Proceedings pp. 60-65.

Tomas, A., Feng, G. H., Reeck, G. R., Bockus, W. W., and Leach, J. E. (1990) Purification of a cultivar-specific toxin from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. Mol. Plant-Microbe Interact. **3:**221-224.

Tuori, R. P., Wolpert, T. J., and Ciuffetti, L. M. (1995) Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. Mol. Plant-Microbe Interact. **8:**41-48.

Véronique, E., Christian, S., Nadine, G., Ghislaine, R., and Claude, A. (2001) Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. FEMS Microbiol. Ecol. **36:**61-71.

Walton, D. J. (1996) Host-selective toxins: agents of compatibility. The plant Cell. **8:**1723-1733.

Watson, I. A. (1970) Changes in virulence and population shifts in plant pathogens. Annu. Rev. Phytopathol. **8:**209-230.

Wright, K. H., and Sutton, J. C. (1990) Inoculum of *Pyrenophora tritici-repentis* in relation to epidemics of tan spot of winter wheat in Ontario. Can. J. Plant Pathol. **12:**149-157.

Zhang, H., Francl, L. J., Jordahl, J. G., and Meinhardt, S. W. (1997) Structural and physical properties of a necrosis-inducing toxin from *Pyrenophora tritici-repentis*. Phytopathology **87:**154-160.

6.2. Appendices

Appendix 1. Induced reaction by individual isolates of the eight known races of *P. tritici-repentis* on a set of differential lines.

Differentials	(Races), Isolates							
	(1) ASC1, NA9-6	(2) 86-124, SK16-4	(3) D308 331-9, 94-25	(4) 90-2	(5) ALG3-24, 92-171R5, NA4-4, ALG5X1-1	(6) ALGH2	(7) ICARDA35-5, ICARDA 36-3, ICARDA 36-4	(8) ICARDA73-1 ICARDA31-1, ICARDA31-2
Glenlea	N+	N+	R	R	R	R	N+	N+
6B662	R	R	R	R	CHL+	CHL+	CHL+	CHL+
6B365	CHL++	R	CHL++	R	R	CHL++	R	CHL++

N+= Sensitive to Ptr ToxA, and shows necrosis
CHL+= Sensitive to Ptr ToxB, and shows chlorosis
CHL++= Sensitive to Ptr ToxC, and shows extensive chlorosis

Appendix 2. Induced reaction by individual isolates of *P. tritici-repentis* used at the first experiment on the lines/cultivars used in this study.

	ASC1 (1)	86-124 (2)	D308 (3)	90-2 (4)	ALG3-24 (5)	92-171R5 (5)	ALGH2 (6)	ICARDA 35-5 (7)	ICARDA 73-1 (8)
4B160	N	R	N	R	N	N	N	N	N
AC Domain	N+CHL	N	CHL	R	CHL	CHL	CHL	N+CHL	N+CHL
Coulter	N	N	N	R	N	N	N	N	N
Neepawa	N	N	R	R	CHL	CHL	CHL	N+CHL	CHL+N
Thatcher	R	R	R	R	CHL	CHL	CHL	CHL	CHL
Selkirk	R	R	R	R	R	R	R	R	R
Red Fife	N	N	R	R	R	R	R	N	R+N

N= Necrosis symptom
CHL= Chlorosis symptom
R = Resistant

Appendix 3. Induced reaction by individual isolates used at the second experiment on the lines/cultivars used in this study.

	NA9-6 (1)	SK16-4 (2)	331-9, 94-25 (3)	90-2 (4)	NA4-4, ALG5X1-1 (5)	ALGH2 (6)	ICARDA 36-3, ICARDA 36-4 (7)	ICARDA 31-1, ICARDA 31-2 (8)
Coulter	N	N	N	R	N	N	N	N
Neepawa	N	N	R	R	CHL	CHL	N+CHL	CHL+N
Thatcher	R	R	R	R	CHL	CHL	CHL	CHL
Red Fife	N	N	R	R	R	R	N	N

N= Necrosis symptom
CHL= Chlorosis symptom
R= Resistant