Comparison of *ToxA* and *ToxB* Genes of *Pyrenophora tritici-repentis* Isolates from Different Geographical Regions of the World

By

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A Thesis submitted to the Faculty of Graduate Studies of the

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ABSTRACT

Pyrenophora tritici-repentis (Died.) Drechs. is the causal agent of the foliar disease of wheat, tan spot. The disease is found worldwide in all wheat growing regions. The symptoms of the disease, necrotic lesions and chlorosis are the products of three independent host-selective toxins (HSTs): Ptr ToxA, Ptr ToxB, and Ptr ToxC. The genes encoding Ptr ToxA (necrosis toxin) and Ptr ToxB (chlorosis toxin), both protein toxins, have been characterized. Previous studies with the genes for Ptr ToxA and Ptr ToxB had involved a limited number of isolates. The *ToxA* gene is a single copy gene and the *ToxB* gene occurs as multiple copies in all the isolates tested that contain either of these genes. To study and understand the global diversity of isolates, 135 isolates of all eight currently characterized races, collected from around the world, were analyzed using PCR amplification analysis, Southern blot analysis, and sequencing of the toxin genes. PCR amplification results showed that the respective toxin genes were present in isolates that possessed the toxin phenotype. Southern blot analysis of isolates showed the number of copies *ToxA* and *ToxB* and their distribution in isolates studied. Sequencing of *ToxA* and *ToxB* in selected isolates revealed that gene sequence of each toxin were highly similar for all the isolates analyzed, regardless of race or geographical origin.

INTRODUCTION

Pyrenophora tritici-repentis is a stubble-borne pathogen and the causal agent of the foliar disease of wheat known as tan spot. The disease can decrease wheat yield by decreasing grain filling due to a loss of leaf photosynthetic capacity. Yield losses have been reported to be between 5 to 15% (Hosford and Busch, 1973; Rees, 1982) and in severe cases, up to 50% (Rees et al., 1982). The disease has been described worldwide in wheat-growing regions (Hosford, 1982).

Symptoms of the disease were first described as necrosis accompanied by chlorosis in some cases (Nisikado, 1928; Conners, 1939; Valder and Shaw, 1953; Hosford, 1971). More recently, the symptoms were described as two independent symptoms: necrosis and chlorosis (Lamari and Bernier, 1989a). Based on the symptoms produced on a wheat differential set, collected isolates of *P. tritici-repentis* were grouped into pathotypes (Lamari and Bernier, 1989b). However, a race system was proposed once new isolates were discovered that were able to induce symptoms in cultivars which were once thought to be resistant (Lamari et al., 1995a). There are currently eight described races (Lamari and Bernier, 1989b; Lamari et al., 1991; Lamari et al., 1995a; Strelkov, 2002; Strelkov et al., 2002; Lamari et al., 2003; Strelkov et al., 2003). *P. tritici-repentis* is found worldwide, with different races found differentially distributed throughout the world (Lamari et al., 1995a; Lamari et al., 1998; Ali et al., 1999; Strelkov et al., 2002; Ali and Francl, 2003; Andrie et al., 2003; Lamari et al., 2005b; Engle et al., 2006; Tadesse et al., 2006).

The necrosis and chlorosis symptoms were found to be caused by host-specific toxins, two of which have been extensively studied. Ptr ToxA is a host-specifc toxin

(HST) responsible for necrosis and was found to be a heat stable protein with a molecular weight of 13.2 kDa, which was able to cause necrosis symptoms at relatively low concentrations (Ballance et al., 1989; Tomas et al., 1990; Meinhardt et al., 1995; Tuori et al, 1995; Zhang et al., 1997). Ptr ToxB is one of the chlorosis toxins; proteinaceous in nature with a molecular weight of 6.6 kDa, heat stable and high activity at low concentrations (Orolaza et al., 1995; Strelkov, 1998; Strelkov et al., 1999). A third toxin, Ptr ToxC, also induces chlorosis and has been partially characterized and found to be a low molecular weight metabolite (Effertz et al., 2002).

The two independent genes encoding Ptr ToxA and Ptr ToxB have been characterized and sequenced independantly. The *ToxA* gene has been sequenced from race 2 isolates from Canada and the United States; the sequences were found to be identical and the gene present as a single copy gene in the genome (Ballance et al., 1996; Ciuffetti et al., 1997; Ballance and Lamari, 1998). The genomic sequence of the *ToxB* gene has been sequenced from two different race 5 isolates. The *ToxB* gene was found to be a multicopy gene with sequence conservation among copies and between the two isolates (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006). The sequence of a mRNA has also been obtained from a race 6 isolate (Strelkov, 2002; Strelkov et al., 2006), and a *ToxB* homolog from a Canadian race 3 isolate (Strelkov, 2002). As with *ToxA*, sequence conservation was observed for *ToxB* gene homologs but again this was from a limited number of isolates (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006).

To better understand the *ToxA* and *ToxB* genes and their potential variability worldwide, the objectives were to: 1) to analyze isolates from all eight races collected

from around the world and to compare the relatedness of *ToxA* and of *ToxB* in the isolates that contain the genes, and 2) to investigate the potential of a genotypic method based on PCR amplification to classify isolates into specific races.

LITERATURE REVIEW

The History of Tan Spot

Tan spot of wheat is caused by the ascomycetous fungus *Pyrenphora tritici-repentis* (Died.) Drechs (anamorph: *Drechslera tritici-repentis* (Died.) Shoem., synonym *Helminthosporium tritici-repentis*). Although an important disease of wheat worldwide (Hosford, 1982), the fungus was first described in Germany on the wild grass species *Triticum repens* (Quack grass, also known as *Elymus canadensis*) and identified as *Helminthosporium tritici-repentis* (Diedicke, 1902; 1904). Drechsler (1923) first described the perfect state on *Triticum repens*. A diverse genus (Shoemaker, 1959, 1962), *Helminthosporium* was separated into three different genera: *Drechslera, Bipolaris, and Exserohilum* (Alcorn, 1988). From that point on, the accepted name for the asexual state for *Pyrenophora tritici-repentis* was *Drechslera tritici-repentis*. Molecular evidence also supported the separation of the three genera (Zhang and Berbee, 2001).

Pyrenophora and *Drechslera* were associated with brown spot on grass species in Canada and the United Sates (Drechsler, 1923; Conners, 1934; Hosford, 1971; Krupinsky, 1982a, 1982b), though the disease levels were lower relative to those on wheat (Krupinsky, 1992). *D. tritici-repentis* reportedly has the widest host range of all *Drechslera* species (Shoemaker, 1962).

A previously undescribed disease of wheat was reported in Japan by Nisikado (1928) and in India by Mitra (1931, 1934). The symptoms were brown spots and chlorosis of the wheat leaves. Based on the conidia, the causal agent was identified as *H. tritici-vulgaris* in Japan (Nisikado, 1928) and as *H. tritici-repentis* in India (Mitra, 1931,

1934). The two different namings of the same pathogen would cause confusion when new wheat diseases were reported in Canada as *H. tritici-repentis* as the causal agent (Conners, 1937, 1939) and in the United States when *Pyrenophora tritici-vulgaris* was identified as the causal agent (Barrus, 1942; Johnson, 1942). Valder and Shaw (1953) described 'yellow spot of wheat' in Australia and identified the causal agent as *Helminthosporium tritici-repentis.* Confusion was added when *Pyrenophora trichostoma* was reported to be the causal agent of a new leaf spot in North Dakota (Hosford, 1971). Based on the description of the propagules, it was thought that *P. trichostoma* was a sub-race of *P. tritici-repentis* (Hosford, 1971, 1972). *Pyrenophora trichostoma* and *Pyrenphora tritici-repentis* were found to be the same pathogen (Hosford, 1982).

The name 'tan spot' (or yellow spot, as it is referred to in Australia, Rees and Platz, 1979) was derived from the tan coloured lesions that appear on the leaf. Tan spot disease symptoms were described as brown spots that enlarge and coalesce, sometimes surrounded by yellow halos (Nisikado, 1928; Conners, 1939; Valder and Shaw, 1953; Hosford, 1971). Lamari and Bernier (1989a) found that the symptoms of necrosis and chlorosis were independent from one another and caused by different toxins. A susceptible cultivar shows one or a combination of these symptoms of the disease (Lamari and Bernier, 1989a).

Isolates were differentiated into pathotypes based on the symptoms produced on susceptible wheat cultivars (Lamari and Bernier, 1989b). A race system for isolate differentiation was adopted when isolates with different pathogenicity were identified (Lamari et al., 1995a). The race system is still symptom based but takes into account all of the symptoms produced by an isolate on an expanded wheat differential set to

designate a specific race. The number of recognized races is currently eight, with the potential for identifying new races based on the assessment of symptoms of isolates on individual wheat cultivars. The pathotypes and races will be described in greater detail later on.

The symptoms of tan spot were found to be caused by host-selective toxins (HSTs) produced by *P. tritici-repentis* (Tomas and Bockus, 1987; Lamari, 1988; Ballance et al., 1989; Orolaza et al., 1995). There are currently two fully characterized HSTs. Ptr ToxA and Ptr ToxB induce necrosis and chlorosis, respectively, on the appropriate sensitive wheat cultivars. The protein structure and the genes of Ptr ToxA and Ptr ToxB have been characterized. There is another partially characterized chlorosis toxin, Ptr ToxC (Effertz et al., 2002) and another toxin, Ptr ToxD which had been proposed but not published in a peer-reviewed journal (Manning et al., 2002b; Ciuffetti et al., 2003). The nature of the toxins and the genes that encode them will be discussed in later sections.

The Impact of Tan Spot

Wheat is the largest crop in Canada and a major export commodity. Any disease, such as tan spot, which has the potential to decrease wheat yield, is important. Tan spot is primarily a disease of wheat leaves but *P. tritici-repentis* can also infect wheat kernels, causing pink/red discolouration. This disease is called red smudge (Francl and Jordahl, 1992; Fernandez et al., 1997). There is a significant amount of damage caused by red smudge. The infected grain is discoloured, shriveled and has a low germination rate (Francl and Jordahl, 1992; Schilder and Bergstrom, 1992a; Schilder and Bergstrom, 1995; Fernandez et al., 1996; Fernandez et al., 1997). The commercial value of the infected grain is decreased due to the symptoms (Fernandez et al., 1997). In Canada, all

registered durum wheat cultivars are susceptible to red smudge (Fernandez et al., 1997). Though not the focus of the research, red smudge results in decreased income and is economically important.

On wheat leaves, the necrosis and/or chlorosis symptoms of the disease decrease the active photosynthetic area of the leaf, resulting in incomplete grain filling and smaller sized grain. Yield losses range from 5 to 15%, but in an extreme case, Rees et al. (1982) reported that yield losses in Australia were between 40 to 50%. The yield losses were attributed to the small size of the grain and the method of harvesting (Rees et al., 1982). *Pyrenophora triciti-repentis* is a stubble-borne disease that became more important in the 1970's due to changes in agricultural practices which leave infected stubble on the soil surface (Hosford, 1971; Rees and Platz, 1979; Bailey, 1996). Hence, control of the pathogen is important. The different control techniques used will be discussed in later sections.

Tan spot of wheat occurs worldwide and affects all wheat growing areas of the world (Hosford, 1982). There were recent reports that tan spot was emerging for the first time from the Pacific Northwest (Peever and Murray, 2002), the Czech Republic (Sarova et al., 2003), Bulgaria (Todorova, 2006), and with increasing prevalence in Nepal (Sharma et al., 2003). As the disease has a worldwide distribution, all areas of cultivated wheat are likely affected.

Classification of Isolates

Pathotype

A pathotype system was developed to group isolates into different pathotypes based on their ability to produce different symptoms. The system is symptom-based and

Lamari and Bernier (1989a) classified isolates into one of four pathotypes based on the necrosis and chlorosis symptoms (and combinations) produced on a differential set of wheat cultivars. Isolates able to produce both necrosis and chlorosis were pathotype 1 (nec+chl+). Pathotype 2 isolates produced necrosis only (nec+chl-) while pathotype 3 isolates produced chlorosis only (nec-chl+). Pathotype 4 isolates were avirulent and produced no symptoms (nec-chl-) (Lamari and Bernier, 1989b, Lamari et al., 1991). The maximum number of pathotypes is four and the system worked well to classify isolates until isolates from Algeria were added to the tan spot story and an expanded classification system was required.

Races

A collection of isolates from Algeria caused the shift from the pathotype system to the race designation system. Thirty-nine isolates collected from Algeria were inoculated onto the wheat differential set to determine pathotype. These isolates induced chlorosis (similar to pathotype 3 isolates), but on the wheat line 6B662 which was resistant to chlorosis by isolates of pathotypes 1 and 3. Furthermore, these Algerian isolates were unable to produce chlorosis on wheat line 6B365 though race 3 pathotype isolates induced chlorosis on this line (Lamari et al., 1995a). Based on the chlorosis symptoms alone, these new isolates would have been classified as pathotype 3 isolates. To accommodate these isolates and their different interaction from pathotype 3 isolates, it was proposed that a race system be adopted. The race of a fungal isolate would be based on differentiation by individual cultivars. Isolates of pathotypes 1 to 4 became part of races 1 to 4, respectively, and the new isolates were placed in race 5. The race system could accommodate new races as they were discovered.

Isolates of race 6 were also collected from Algeria. Isolate AlgH-2 was able to produce chlorosis symptoms on both cultivar 6B365 (used to differentiate race 3 isolates) and cultivar 6B662 (used to differentiate race 5 isolates) after inoculation (Strelkov, 2002; Strelkov et al., 2002; Strelkov et al., 2003). The Algerian isolate, Alg H-2, was proposed as race 6, due to its combined pathogenicity of races 3 and 5 which, was previously undescribed (Strelkov, 2002; Strelkov et al., 2002; Strelkov et al., 2003).

Races 7 and 8 isolates were discovered from the Fertile Crescent and the Caucasus regions. Collected in 2001, these isolates were able to produce necrosis symptoms on cultivar Glenlea and chlorosis symptoms on cultivar 6B662, combining the pathogenicity of race 2 (necrosis only) and race 5 (chlorosis on 6B662 only); this race was proposed as race 7 (Lamari et al., 2003). Other isolates were able to cause necrosis on Glenlea and chlorosis symptoms on both 6B365 and 6B662, combining the pathogenicity of race 2 (necrosis only) with race 3 (chlorosis on 6B365) and race 5 (chlorosis on 6B662); these isolates were proposed as race 8 isolates (Lamari et al., 2003). These eight races are the currently characterized races, which is the maximum based on three effective differential lines. The formula 2^3 where three is the number of virulence factors (toxins) with three matching loci in the plant, and two is the number of outcomes (resistant or susceptible, toxin-producing or non-toxin-producing) (Lamari et al., 2003). A race 9 was proposed but never fully characterized (Ciuffetti et al., 2003).

The race of an isolate is determined by taking into account the symptoms, which are toxin-based, on the effective wheat differentials (Glenlea, 6B365, and 6B662). The three effective differential lines plus six other secondary cultivars make up the wheat differential set. Table 1 summarizes the nine wheat cultivars/lines along with their race-

specific reactions, adapted from Strelkov et al. (2002). Each differential cultivar/line interacts with the isolate (Table 1)

Table 1: Reaction of the nine wheat differential set cultivars in response to infection by isolates from eight races of *Pyrenophora tritici-repentis*. Cultivars Glenlea, 6B365, and 6B662 (in bold) are the effective differential lines; others serve as secondary checks. Reactions were either resistant (R) or susceptible. Where a susceptible reaction occurs, the observed symptoms are reported as either necrotic (N) or chlorotic (C).

		Race							
Cultivar	**Ploidy**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Glenlea	**6n**	**N**	**N**	**R**	**R**	**R**	**R**	**N**	**N**
6B365	**6n**	**C**	**R**	**C**	**R**	**R**	**C**	**R**	**C**
6B662	**6n**	**R**	**R**	**R**	**R**	**C**	**C**	**C**	**C**
Erik	6n	R	R	R	R	R	R	R	R
Katepwa	6n	N	N	R	R	C	C	C	C
Salamouni	6n	R	R	R	R	R	R	R	R
UM-1	6n	R	R	R	R	R	R	R	R
4B1149	4n	R	R	R	R	R	R	R	R
Coulter	4n	N	N	N	R	N	N	N	N

and when all the symptoms produced are taken into account, a race can be designated. The addition of cultivars/lines of tetraploid and hexaplod designation could identify more races (Lamari et al., 1995; Lamari et al., 2005b). The number of races currently characterized is eight, although with the addition of more effective differentials cultivars, new races can be identified should they exist.

Distribution of Races Worldwide

Tan spot has been reported all over the world (Hosford, 1982) and where characterized, it appears that races are differentially distributed worldwide and the

populations are dynamic. Race 1 isolates were collected from all over the world: in Canada (Lamari and Bernier, 1989b; Lamari et al., 1998), the United States (Ali and Francl, 2003; Engle et al., 2006), Uruguay (Lamari, personal communication), North Africa (Lamari et al., 1995a), Germany (Tadesse et al., 2006), and from bread wheat from Azerbaijan, Syria, Kyrghyzstan, Uzbekistan, and Kazakhstan (Lamari et al., 2005b). When race 1 isolates are collected, they appear to be the predominant race (Lamari et al., 1998; Ali and Francl, 2003; Lamari et al., 2005b; Engle et al., 2006). Race 2 isolates have a similar distribution to race 1 isolates. Both are collected from bread (hexaploid) wheat rather than on tetraploid wheat. Race 2 isolates have been collected from Canada, the United States, Azerbaijan, Syria, Kyrghyzstan, Uzbekistan, and Kazakhstan (Lamari et al., 2005b). The countries of Azerbaijan, Syria, Kyrghyzstan, Uzebekistan and Kazakhstan make up the countries of the historic Silk Road (Lamari et al., 2005b) and are considered the wheat center of origin (Harlan, 1987; Lamari et al., 2005b).

Race 3 isolates have been collected in Canada and the United States (Lamari and Bernier, 1989b; Lamari et al., 1998; Engle et al., 2006). They have also been collected from Azerbaijan and Syria on tetraploid wheat (Lamari et al., 2005b). Race 4 isolates have been collected in Canada and the United States on bread wheat and non-cereal grasses (Lamari and Bernier, 1989b; Ali and Francl, 2003). No race 4 isolates have been collected from the Fertile Crescent (Lamari et al., 2005b).

Race 5 isolates were first collected from durum wheat in Eastern Algeria (Lamari et al., 1995a) and have been collected from durum wheat in North America (Lamari et al, 1998; Ali et al., 1999; Strelkov, 2002; Strelkov et al., 2002; Ali and Francl, 2003). In the Fertile Crescent, race 5 isolates were collected exclusively from tetraploid wheat (Lamari

et al., 2005b). A Canadian race 5 isolate was collected from hexaploid wheat for the first time. This isolate was shown to have low virulence compared to Algerian race 5 isolates (Lamari et al., 1998; Strelkov, 2002; Strelkov et al., 2002). Race 5 isolates have also been collected in Germany (Tadesse et al., 2006). Race 6 isolates were collected only in North Africa (Lamari et al., 1995a). Race 7 and 8 isolates have been collected exclusively from tetraploid wheat in Azerbaijan and Syria (Lamari et al., 2003; Lamari et al., 2005b). Fig. 1 shows the worldwide distribution of races that have been characterized to 2007.

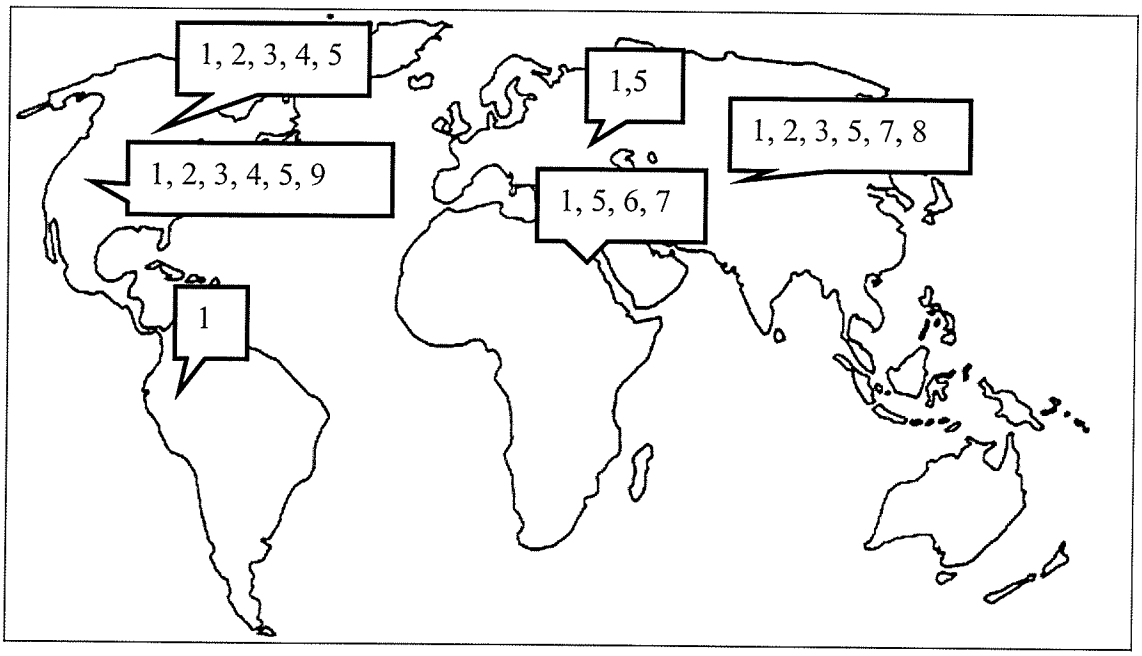


Fig. 1: The worldwide distribution of races of *P. tritici-repentis*. The races were indicated where isolates have been characterized. Although the disease is found worldwide, races may not be characterized as of 2007.

The populations of races of *P. tritici-repentis* are dynamic and can vary from year to year and by location. Lamari and Bernier (1989b) collected isolates of races 1, 2, 3 and 4 in Canada, with race 1 being predominant. In 1990, the two prevalent races were races 1 and 2, though race 1 was at a much higher proportion (Lamari et al., 1998). By

1994, the proportion of race 1 isolates collected was almost equal to the number of race 2 isolates collected, with race 3 isolates also collected. Ali and Francl (2003) studied the distribution of races in the Northern and Southern Great Plains of the United States. In the Northern Great Plains, the predominant race collected was race 1, though a small proportion of race 2 and 4 isolates were collected. In the Southern Great Plains, race 2 was the predominant race on bread wheat, race 5 isolates were collected from durum wheat, and race 4 isolates were collected from non-cereal grasses.

In the wheat center of diversity (Harlan, 1987), isolates of races are differentially distributed, with some countries having more race diversity than others. In Azerbaijan, isolates of races 1, 2, 3, 5, 7, and 8 were collected, with race 1 predominant. From wheat fields in Syria, isolates of races 1, 2, 3, 5, 7, and 8 were collected and race 3 made up 58% of the isolates found. In Kyrghyzstan and Uzbekistan, only race 1 isolates were collected. From Kazakhstan, isolates of races 1 and 2 were collected, with race 1 predominant (Lamari et al., 2005b). Isolates of race 1 and 2 were predominantly collected from hexaploid wheat hosts whereas all isolates of races 3, 5, 6, 7, and 8 were collected from tetraploid wheat hosts. Isolates of races 4 and 6 were not identified in any of these countries. Tan spot is a worldwide disease and the different races are distributed worldwide, with variations dependant on time and location.

Tan Spot Disease and Management

Life Cycle and Infection Process

P. tritici-repentis Life Cycle

In considering the life cycle of the pathogen, the infection of wheat leaves will be explored. The life cycle of *H. tritici-repentis* was fully described on wheat (Conners,

1939, 1937). The sexual fruiting bodies, the pseudothecia, are stubble-borne and mature on the over-wintered stubble in the spring (Conners, 1939; Hosford, 1971). Once the ascospores are mature, they are discharged towards light (Friesen et al., 2003), usually in May in Western Canada and very close to the ground. Ascospores are desseminated by strong winds to infect wheat and other host leaves (Morrall and Howard, 1975). The ascospores germinate, colonize leaf tissue, and conidiophores are produced on lesions.

The conidia, the secondary inoculum (Hosford, 1972), are wind-dispersed in June and July (Morrall and Howard, 1975; Wright and Sutton, 1990; Schilder and Bergstrom, 1992b) and can colonize new plants or re-colonize healthy tissue of the source wheat or other hosts. The conidiophores infect more tissue and produce more conidia; this cycle occurs repeatedly throughout the growing season (Morrall and Howard, 1975). A exponential relationship exists between epidemic development and conidia production. Thus small amount of infected stubble can lead to severe disease (Rees et al., 1982). As the weather becomes colder, pseudothecia form on wheat or other grass species and overwinter on stubble (Conners, 1939; Morrall and Howard, 1975; Hosford, 1982). Figure 2 is a representation of the life cycle of *P. tritici-repentis*.

Infection Process

During the initial stages of infection, there were no major differences between susceptible and resistant cultivars (Larez et al., 1986). Germination of conidia and germ tube formation on leaf tissue was not correlated to susceptibility or resistance of the host to the pathogen. During the initial stages of infection, both susceptible and resistant cultivars suffered intercellular damage, though papilla formation occurred at a higher rate

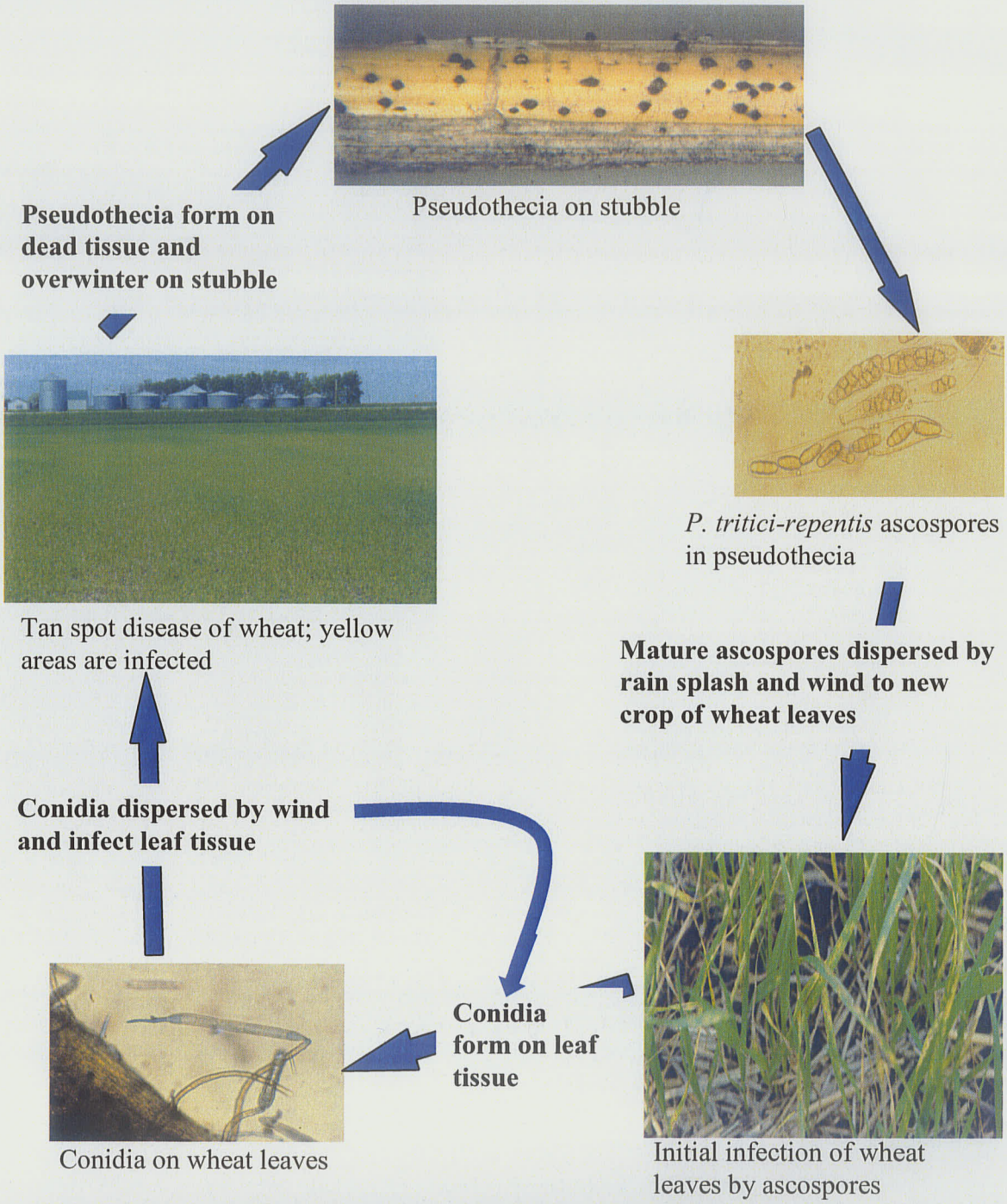


Fig. 2: The life cycle of *P. tritici-repentis* on wheat leaves. Photos provided by Dr. L. Lamari, University of Manitoba.

in resistant cultivars (Dushnicky et al. 1996; Dushnicky et al., 1998a) and in oat, which is resistant to *P. tritici-repentis* (Larez et al., 1986)

Dushnicky et al. (1998b) found cultivar-dependant differences in the infection process which became apparent after 96 h while studying a race 2 isolate and its interaction with resistant and susceptible cultivars. Colonization of susceptible leaf tissue led to the disruption of mesophyll and epidermal cells, resembling typical tan spot lesions. The lesions spread in a longitudinal manner and were somewhat limited from spreading laterally by sclerenchyma tissue of the midveins (Dushnicky et al., 1998b). Intact chloroplasts were undetectable in disrupted mesophyll (Dushnicky et al., 1998b). In resistant cultivars after the same time period, the mesophyll cells and chloroplasts were relatively intact. The mesophyll cells surrounding the infection site and intercellular hyphae appeared thickened, with no spaces between cells (Dushnicky et al., 1998b).

Lignin may play a role in cultivar resistance to infection. Lignin (or a lignin-like material) was stained in the thickened cell walls of the infection site and surrounding areas in resistant cultivars only. A lignin-like material was also deposited around the *P. tritici-repentis* hyphae growing intercellularily, presumably arresting further infection. The areas of lignin deposits corresponded to the necrotic lesions that are typically associated with a hypersensitive response (Dushnicky et al., 1989b). Initially, the infection process appeared to be the same between resistant and susceptible cultivars. Intercellular changes occurred between the two cultivars as time progressed; these changes were reflected outwardly as symptoms of the disease (Lamari and Bernier, 1989a; Dushnicky et al., 1998b). The *P. tritici-repentis* life cycle is influenced by many factors, making up the disease pyramid.

Factors Affecting Tan Spot Disease

The Host: Wheat

Tan spot disease will occur if the host is susceptible to the pathogen. Susceptibility to *P. tritici-repentis* is governed by independent host genes that are dominant over resistance (Lamari and Bernier, 1989c; Lamari and Bernier, 1991; Gamba and Lamari, 1998; Singh and Hughes, 2003). Sykes and Bernier (1991) found that in a hexaploid wheat cultivar, resistance to *P. tritici-repentis* was governed by two independent, recessive loci while in a tetraploid wheat cultivar studied, two complementary genes were involved in resistance. In a diploid cultivar studied, resistance was controlled by one recessive gene. Susceptibility to *P. tritici-repentis* is dominant for both the necrosis and chlorosis reactions which have been identified to date.

Historically in Canada, the different wheat cultivars and lines were unknowingly bred for susceptibility to *P. tritici-repentis.* Marquis, a cultivar grown in Canada from 1909, had good grain quality and resistant to stem rusts. Marquis was also susceptible to the necrosis-inducing *P. tritici-repentis* (Lamari et al., 2005a). Marquis was widely used for many years as breeding material. The beginnings of tan spot epidemics could be traced back to Neepawa, a cultivar derived from Marquis and grown from 1969. Neepawa was susceptible to both necrosis and chlorosis inducing isolates of *P. tritici-repentis* and was a parent to other necrosis-susceptible wheat cultivars (Lamari et al., 2005a). Susceptibility to Ptr ToxB chlorosis-inducing isolates was derived from the wheat cultivar, Thatcher a major cultivar in Canada from 1939 to 1968. As Neepawa was a backcross derivative of Thatcher, it was found to be sensitive to ToxB-chlorosis (Lamari et al., 2005a). In Canada, since 1994, a few wheat cultivars grown are resistant

to tan spot (Lamari et al., 2005a). The genes governing resistance/ susceptibility to tan spot will be discussed in a later section.

Environmental Factors

Temperature and relative leaf wetness are two important factors for propagule maturation and disease development. Cool temperatures were found to be necessary for maturation of pseudothecia and ascospores on stubble (Hosford, 1971; Morrall and Howard, 1975). Recovery of ascospores was greater after pseudothecia were exposed to cool, dry conditions rather than warm, wet conditions (Summerell and Burgess, 1989b). At both high and low temperatures, tan spot development was affected. Under greenhouse conditions, susceptible cultivars reacted similar to resistant cultivars to fungal inoculation at temperatures of 27°C or higher and resistant cultivars remained resistant (Lamari and Bernier, 1994). At 10°C, smaller lesions were observed on susceptible cultivars after pathogen inoculation, though the lesions were of the susceptible type (Lamari and Bernier, 1994). In the field, decreased disease severity was observed during hot summers (Conners, 1954; Shoemaker, 1954).

Relative leaf wetness is an important factor for conidial germination and infection. A six-hour leaf wetness period with temperatures at 20°C and 30°C (and low wind) resulted in leaf spotting in susceptible cultivars. Major leaf spotting was observed between 6 to 30 h periods of leaf wetness (Hosford and Busch, 1973). A leaf wetness period of over 30 h has been reported to cause leaf spotting in resistant cultivars (Hosford and Busch, 1973). The size and number of lesions on leaves was related to the amount of moisture available. There appeared to be a positive correlation between wet weather and the development of tan spot epidemics. Tan spot infection appeared to be the most severe

when wet weather was reported (Conners, 1954; Hosford, 1971; Ronis and Semaskiene, 2006). Appropriate environmental conditions including temperature and humidity are needed for infection, but human influence also affects disease severity.

Human Influence on Tan Spot Development

As *P. tritici-repentis* is a stubble-borne pathogen, changes to agricultural practices that affect stubble will affect pathogen survival and inoculum levels. In the past, stubble management practices such as stubble-burning was considered to be the most effective form of control of tan spot as it yielded the greatest reduction in inoculum (Rees and Platz, 1979). Cultivation of stubble also reduced inoculum but was dependant on the depth of incorpo-ration which allowed for breakdown of residue by soil-borne bacteria (Rees and Platz, 1979; Summerell and Burgess, 1989a, 1989b). For environmental and soil conservation purposes, stubble burning and tillage have become less common practices for stubble management.

The retention of stubble at the surface of the soil decreases soil erosion but will affect the amount of inoculum at the soil surface. Conventional tillage is the practice of removing crop residues from the surface of the soil. Tillage with a mould-board plow buried the stubble under the soil, decreasing the number of pseudothecia and the resultant disease (Sutton and Vyn, 1990; Bockus 1992). Zero or no-till (leaving stub-ble on the soil) significantly increased disease in subsequent wheat crops, providing a so-urce of inoculum for the next growing season (Sutton and Vyn, 1990; Bockus, 1992). The retention of stubble not only provides an inoculum source for the next season, but Sutton and Vyn (1990) observed that the temperature at stubble level was found to be 1-3°C cooler in zero-tilled plots compared to conventional and minimum tilled plots. This can

positively affect pseudothecia development. Tan spot disease development is dependant not only on the host and the pathogen but on environmental factors. Good management can reduce the risk of disease occurring.

Control of Tan Spot

Crop rotation is an agricultural practice that can decrease occurrence of tan spot by reducing the level of inoculum (De Wolf et al., 1998). Rees and Platz (1979) wrote that crop rotation with a three-year period between wheat would decrease the disease incidence. Sutton and Vyn (1990) found that a two-year crop rotation with wheat following barley resulted in far less disease as compared to wheat-wheat planting. It was suggested that resistant crops such as soybean and alfafa be used between wheat plantings (Hosford, 1971). Crop rotation results in a reduced level of inoculum to initiate disease development when a susceptible wheat crop is planted.

Breeding for resistance is one of the best investments as a means for control of the pathogen (De Wolf et al., 1998; Gamba et al., 1998). Although the majority of the wheat cultivars grown in Canada are resistant to tan spot through breeding (Lamari et al. 2005a), this is not the case elsewhere in the world. In Australia, wheat cultivars were found to be susceptible to tan spot (Rees et al., 1988) but some cultivars were found to be partially resistant (Rees and Platz, 1989; Wilson and Loughman, 1998). Backcrossing with these moderately resistant cultivars are proving successful (Rees and Platz, 1989; Wilson and Loughman, 1998). As more races are discovered, new sources of resistance must be found. For resistance breeding, *Aegilops tauschii,* contributor of the D genome in synthetic wheat, may contain resistance genes (Zhang and Jin, 1998; Singh et al., 2006). The interaction between *P. tritici-repentis* and wheat is complex, with many

factors determining the success of infection. To better control tan spot, more information regarding the host-pathogen interaction must be gleaned.

Biochemical and Molecular Aspects of Host-Pathogen Interaction

Interaction Between Host and Pathogen

Gene-for-Gene Model

A general model was developed by Flor (1971) to describe the resistance reaction in a host plant. The gene-for-gene model stated that for every host resistance gene, there is a corresponding avirulence gene in the pathogen. The recognition of the avirulence gene (product) and a host gene (product) results in resistance and the reaction is unique and epistatic. The model was developed the model while Flor (1971) studied the flax-rust interaction. Figure 3a summarizes the reactions between the different genes and the outcomes of said interactions.

Toxin Model

In the case of wheat-*P. tritici-repentis*, a toxin model was proposed as an explanation of the interactions between a host and a toxin-producing pathogen (Gamba and Lamari, 1998; Strelkov and Lamari, 2003). The toxin model is the reverse of the gene-for-gene model for resistance and compatibility is the unique interaction. A successful interaction (from the pathogen side) occurs when the pathogen toxin interacts with a product of the host sensitivity gene, a receptor, though the exact protein product has not been determined (Gamba and Lamari, 1998). Once the interaction occurs, a signaling cascade may be activated and disease is manifested (Anderson et al., 1999). Absence of a sensitivity gene results in the absence of the receptor or binding target, the

signaling cascade does not occur and there is no disease (Anderson et al., 1999). Figure 3b is a representation of the different interactions and the outcomes.

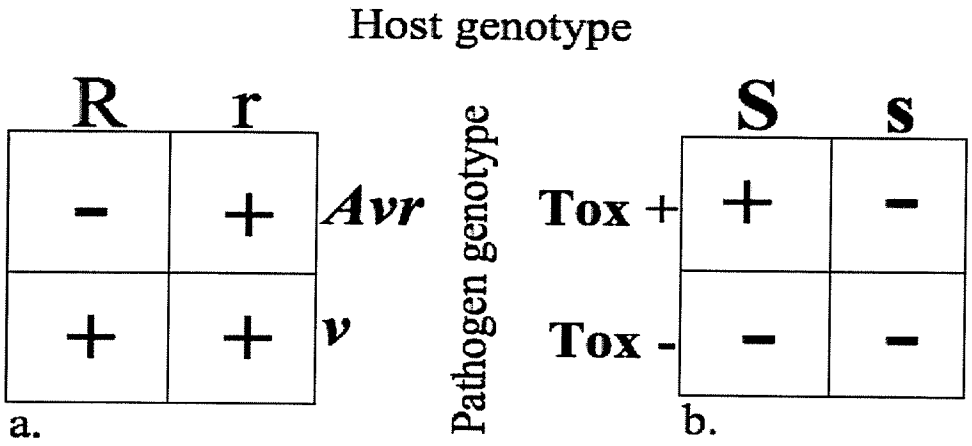


Fig. 3: The two host-pathogen interaction models with the host and pathogen genotypes indicated. **a.** Flor's gene-for-gene model. R is the dominant resistance gene and 'r' the recessive gene for susceptibility. *Avr* is the dominant avirulence gene in the pathogen, v is the recessive virulence gene. Resistance is the unique interaction and no disease occurs (-), whereas the other three interaction types lead to disease (+); **b.** the toxin model for interaction between a toxin-producing isolate and a sensitive and insensitive wheat hosts. The interaction between a Tox + isolate and a sensitive host (S), the dominant trait, results in disease (+). No disease (-) occurs in any other interaction, making disease the unique interaction.

Wheat Genes for Resistance to Tan Spot

The location of a resistance gene for reaction to race 2 isolates was mapped in wheat. The gene, named *tsn1*, was found on the long arm of chromosome of 5B in both durum and common wheat and was most likely derived from a common durum ancestor (Faris et al., 1996). Singh et al. (2006) found that the majority of wheat cultivars possessing the B genome were susceptible to tan spot. The importance of the gene on chromosome 5B was shown with deletion and substitution experiments (Anderson et al., 1999). In a synthetic wheat line, two resistance genes, named *tsn3* and *tsn-syn1*, were found to be located on chromosome 3D. The dominant gene (*Tsn-syn2*) was also located on the same chromosome (Tadesse et al., 2006).

Research studies on the gene in wheat for resistance to isolates expressing *ToxB* showed that a quantitative trait loci (QTL), named *QTsc.dnsu-1A* was found on chromosome 1AS (Effertz et al., 2001). A second QTL, designated *tsc2*, was found to be located on the short arm of chromosome 2B. The presence of *tsc2* gene is enough for susceptibility but they reported that other minor factors affected susceptibility/ resistance (Friesen and Faris, 2004). Other genes for resistance were located in the wheat genome. Two QTLs for a race non-specific resistance were located on chromosomes 1BS and 3BL in a Brazilian wheat cultivar (Faris and Friesen, 2005).

Host-Selective Toxins

Host-selective toxins (HST) are toxins produced by a microorganism and are selectively toxic to susceptible hosts but have little or no effect on nonsusceptible hosts (Scheffer and Briggs, 1981; Scheffer, 1983). Features that are characteristic of HSTs are: 1. The HST can reproduce the symptoms of the disease on sensitive plant genotypes and cultivars that are resistant to the pathogen should not be affected by the toxin, even at high levels (Graniti, 1991). Avirulent isolates are those that do not produce the toxin (Graniti, 1991). 2. The gene for host sensitivity to the toxin should be the same gene as host susceptibility. The reaction of the host is dependent on recognition of the toxin (Scheffer, 1983). 3. The pathogenicity of a pathogen and its virulence may be dependent on the ability to produce a HST (Graniti, 1991). 'Sensitivity' and 'insensitivity' refer to the reaction of the host wheat cultivars to the toxins produced by *P. tritici-repentis*. A cultivar is sensitive if symptoms manifest (Lamari and Bernier, 1989c).

The two independent symptoms of tan spot, necrosis and chlorosis (Lamari and Bernier, 1989a) were found to be due to the interaction between the host and HSTs

produced by *P. tritici-repentis* (Lamari, 1988; Lamari et al., 1995, Orolaza et al., 1995). Toxin involvement in disease has been reported in another related *Pyrenophora* species, *Pyrenophora teres* (Smedegard-Petersen, 1977). Furthermore, species of *Helminthosporium* were also shown to produce toxins (Pope et al., 1983). The toxins of *P. tritici-repentis* have been characterized (Tomas and Bockus, 1987; Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Meinhardt et al., 1998; Zhang et al., 1997; Orolaza et al., 1995; Strelkov, 1998; Strelkov et al., 1999; Effertz et al., 2002).

Necrosis Toxin: Ptr ToxA

Ptr ToxA Protein

The necrosis toxin was purified and characterized by several groups and identified by several names, but when it was apparent there was only one toxin responsible, the researchers gathered and jointly decided to standardize the nomenclature. The necrosis toxin is referred to as Ptr ToxA or ToxA (Ciuffetti et al., 1998). Other host-selective toxins characterized from *P. tritici-repentis* were renamed using a similar nomenclature.

Purification and characterization of Ptr ToxA was carried out by several groups. The toxin was a monomeric protein with a final size of 13.2 kDa, confirming the estimate of a large toxic compound (Lamari and Bernier, 1989c). The toxin only caused necrosis on sensitive wheat cultivars (Tomas and Bockus, 1987; Lamari and Bernier, 1989c). Ptr ToxA was also found to be relatively heat stable (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995; Meinhardt et al., 1998).

Two features of Ptr ToxA were found from the partial amino acid sequence obtained by Zhang et al. (1997). From the 101 amino acid sequence, the secondary structure of Ptr necrosis toxin was predicted to be mostly a β-sheet with little α-helix

structure. An important amino acid motif, arginyl-glycyl-aspartyl (RGD), was also revealed (Zhang et al., 1997).

Protein sequence analysis revealed different potential sites for phosphorylation and myristolyation. Utilizing site-directed mutagenesis, changes to individual amino acids were made and the results showed that a vitronectin-like motif and two sites for casein kinase 2 (CK2) phosphorylation were essential for toxin function (Ciuffetti et al., 1997; Tuori et al, 2000). The position of the amino acid threonine gave rise to the possibility of multiple phosphorylation sites by phosphokinase C and casein kinase II, especially important if the toxin is taken up into the cell (Zhang et al., 1997).

Ptr ToxA Gene: *ToxA*

The gene for Ptr ToxA has been characterized and sequenced independently by two groups with similar results. Ballance et al. (1996) isolated a cDNA clone, pPtrNEC, and Ciuffetti et al. (1997) also cloned the genomic Ptr ToxA gene, which they termed *ToxA*. The gene will be referred to as *ToxA* to be consistent with the current nomenclature (Ciuffetti et al., 1998). *ToxA* was found to have an open reading frame (ORF) of 534 nucleotides with two introns. A 55 nucleotide-long intron was found in the non-translated leader sequence and a second intron (50 nucleotides in length) was found in the C-terminus domain of the ORF (Ciuffetti et al. 1997). Expression of the *ToxA* gene in pPtrNEC and infiltration of products from bacterial cell lysates produced the same host-specific necrotic symptoms on sensitive wheat cultivars and no symptoms on insensitive wheat cultivars, comparable to pure toxin infiltration and the ToxA-phenotype (Ballance et al., 1996). The sequences of PtrNEC and *ToxA* have been submitted to GenBank (accession number U79662 and AF004369, respectively).

The information from *ToxA* revealed more of the protein nature of the toxin. *ToxA* was found to encode a 177 to 178 amino acid pre-pro-protein with a molecular weight of 19.7 kDa, 16 to 22 amino acids of which made up the signal peptide of the pre-pro-protein (Ballance et al., 1996, Ciuffetti et al., 1997). The remaining 156 to 163 amino acids make up the pro-protein, and included in the pro-protein is a 38 to 44 amino acid sequence called the pro-peptide, essential in proper folding of the protein (Tuori et al., 2000). The pro-peptide is cleaved before secretion to produce the mature 13.2 kDa protein (Tuori et al., 2000; Manning and Ciuffetti, 2005).

The *ToxA* gene was found as a single copy in isolates that possess the ToxA-phenotype and was absent in isolates that did not possess the phenotype (Ballance et al., 1996; Ciuffetti et al., 1997). Broader analysis of the *ToxA* gene in isolates of races 1, 2, 7, and 8 have shown *ToxA* to be present as a single copy in all cases (Lamari et al., 2003). Probing for the *ToxA* gene showed that the *ToxA* was located on a 3.0 Mb chromosome in four isolates tested, and it was suggested that it was a B-like supernumerary chromosome (Lichter et al., 2002). *ToxA* was found to be completely lacking in non-pathogenic isolates, which coincides with previous results (Ballance et al., 1996, Ciuffetti et al., 1997). Recently, a *ToxA* gene homolog was found in *Stagonospora nodorum,* another pathogen of wheat. The *Stagonospora* gene had a 99.7 % homology with *P. tritici-repentis ToxA* (Friesen et al., 2006).

Ptr ToxA Production and Mode of Action

Ptr ToxA was recognized by Ptr ToxA antibodies in intercellular washing fluid (IWF) collected from inoculated wheat leaves. This showed that Ptr ToxA was produced *in planta* and only in necrosis-inducing isolates (Lamari et al., 1995b). Kwon et al.

(1996) showed that infiltration with Ptr ToxA caused electrolyte leakage from cells of sensitive wheat cultivars and that wheat lines which developed necrosis showed electrolyte leakage. Treatment of sensitive wheat cultivars with inorganic calcium blockers and inhibitors of protein phosphatase showed reduced amounts electrolyte leakage when treated with the necrosis toxin, indicating the importance of calcium influxes and protein phosphorylation cascade in Ptr ToxA action (Rasmussen et al., 2004).

The arginyl-glycyl-aspartyl (RGD) motif may play a role in Ptr ToxA mode of action. The motif in mammelian proteins has been shown to interact with integrin proteins, a class of plasma membrane proteins. Mutations in the RGD motif of Ptr ToxA to arginyl-alanyl-aspartyl (RAD) and to arginyl-glycyl-glutamyl (RGE) resulted in absence of necrosis on sensitive cultivars and no electrolyte leakage (Kwon et al., 1996; Meinhardt et al., 2002). Tuori et al. (2000) demonstrated that a mutation of a threonine just N-terminal to the ToxA RGD motif and heterologous expression of Ptr ToxA with the RGD substituted by alanyl-glycyl-alanyl (AGA) decreased toxic activity. Manning et al. (2002a) found that the amino acid residues surrounding the RGD motif were similar to vitronectin, a mammelian integrin binding protein. It was suggested that ToxA may interact with an integrin-like receptor, which is different from mammelian integrin proteins (Manning et al., 2002a). Manning and Ciuffetti (2005) hypothesize that due to the absence of plasma membrane rupturing during toxin entrance, the internalization of the toxin into the cell may be due to receptor-mediated endocytosis.

The toxin did not play a role in initial infection (Dushnicky et al., 1996, 1998a) but plays a role later in infection when the symptoms are manifested, approximately 72 h

post infection (Lamari and Bernier, 1989b). Green fluorescent protein (GFP)-tagged ToxA was found compartmentalized in the mesophyll (chloroplasts and cytoplasm) and persisted there for 4 h until eventual cell death (Manning and Ciuffetti, 2005). The importance of the aggregations of Ptr ToxA in chloroplasts was not fully explained, but shows the importance of chloroplasts in disease development (Deshpandie, 1993).

Chlorosis Toxin: Ptr ToxB

Ptr ToxB Protein

A chlorosis toxin was found to be causing the chlorosis symptom produced by races 5, 6, 7, and 8 of *P. tritici-repentis* on wheat differential line 6B662. The toxin will be referred to as Ptr ToxB or ToxB to follow the nomenclature (Ciuffetti et al., 1998). Host susceptibility to the pathogen and host toxin sensitivity were identical. The toxin was found in culture filtrates, spore germination fluid, and IWF post-pathogen inoculation (Orolaza et al., 1995). The size of the protein was determined to be 6.612 kDa (Strelkov, 1998; Strelkov et al., 1999), confirming Orolaza et al. (1995) initial size estimates. Ptr ToxB production was independent of necrosis toxin production (Lamari and Bernier, 1989c). Ptr ToxB was characterized as a small, heat stable, hydrophilic protein (Orolaza et al., 1995; Strelkov, 1998; Strelkov et al., 1999). The activity of Ptr ToxB was quite high, with weak chlorosis detectable after 72 h even at a concentration as low as 14 nM (Strelkov, 1998, Strelkov et al., 1999).

The amino acid sequence of Ptr ToxB was determined by two groups (Martinez et al., 2001; Strelkov, 2002, Strelkov et al., 2006). Partial sequencing of the N-terminus revealed no homology with Ptr ToxA (Strelkov, 1998; Strelkov et al., 1999). Ptr ToxB was made up of 64 amino acid residues with no tryptophan residues detected. Before

proteolytic processing, the protein is 87 amino acids long with a calculated mass of 8.9 kDa (Martinez et al., 2001; Strelkov, 2002; Strelkov et al., 2006). The first 23 amino acids were found to be a signal peptide, which is characteristic of secreted proteins (Martinez et al., 2001; Strelkov et al., 2002). After removal of the signal peptide, the mature toxin protein is 64 amino acids long with a mass of 6.6 kDa, consistent with previous studies (Strelkov, 1998; Strelkov et al., 1999). There were two positions in the N-terminus where amino acid differences were reported. At position 2 and 18, Strelkov (1998, 1999) reported an unknown amino acid and serine, respectively while Martinez et al. (2001) reported cysteine residues at these positions, and these were subsequently confirmed by gene sequencing.

Ptr ToxB Gene: *ToxB*

The gene encoding Ptr ToxB, *ToxB*, has been sequenced and characterized. The *ToxB* gene consists of a 261 bp open reading frame (ORF) with no introns present (Martinez et al., 2001; Strelkov, 2002; Strelkov et al., 2006). In isolate Alg 3-24, a 151 bp non-translated leader sequence with a 52 nucleotide intron preceded the ORF (Strelkov, 2002; Strelkov et al., 2006). The ORF encoded an 87 amino acid protein with a calculated mass of 8.9 kDa, the size of Ptr ToxB prior to proteolytic processing (Martinez et al., 2001; Strelkov, 2002; Strelkov et al., 2006).

ToxB was found in multiple copies in ToxB-phenotype isolates by Southern blot analysis. An Algerian race 5 isolate, Alg 3-24, was shown to have between 8-10 copies of *ToxB* (Strelkov, 2002; Strelkov et al., 2006), and at least nine copies of *ToxB* were present in a North Dakota race 5 isolate (Martinez et al., 2004). Multiple copies of *ToxB* were also reported in isolates of races 5, 6, 7, and 8 (Strelkov et al., 2003; Lamari et al.,

2003). The *ToxB* probe did not hybridize to any fragments from genomic DNA of isolates of races 1 and 2 (Strelkov, 2002; Lamari et al., 2003; Strelkov et al., 2006).

Sequencing of the individual *ToxB* loci revealed that the ORFs from different isolates were identical. Three *ToxB* loci of Alg 3-24 were sequenced and the ORFs were identical to each other and to six of the estimated nine *ToxB* loci in DW-7 (Martinez et al., 2004; Strelkov et al., 2006). Although multiple copies of the *ToxB* loci exist, the ORFs are identical.

Sequencing of genomic clones containing the *ToxB* gene region revealed that sequence similarity was not limited to the ORF. Sequencing upstream of the ORF showed that a 197 bp repeated insertion was present in three of six DW7 *ToxB* loci and in two of three Alg 3-24 *ToxB* loci. In two of six loci of DW7 and one of three loci of Alg3-24, the same insert was found downstream of the ORF (Martinez et al., 2004; Strelkov, 2002; Strelkov et al., 2006). Upstream of the 197 bp insert was a 286 bp retrotransposon-like sequence in five of six *ToxB* of DW7. The retrotransposon was similar in sequence to a *gypsy*-like retrotransposon *Maggy* from *Magnaporthe grisea* (Martinez et al., 2004). A 288 bp retrotransposon-like sequence was also present upstream of the 197 bp insert in one of three *ToxB* loci of Alg 3-24. The retrotransposon was most similar to a *gypsy*-like *Afu2* retrotransposon from *Aspergillus fumigatus* (Strelkov et al., 2006). Further upstream was a truncated transposon (36 bp inverted repeats flanked by 6 bps of direct repeats) present in three of six DW7 *ToxB* loci and in two of three Alg 3-24 *ToxB* loci (Martinez et al., 2004; Strelkov, 2002; Strelkov et al., 2006). Sequencing downstream of the DW7 *ToxB* ORFs revealed that four of six loci had coding sequence similar to known

protein geranylgeranylpyrophosphate synthetase, a separate reverse transcriptase found in retrotransposons, and sequence similar to plant retrotransposons (Martinez et al., 2004).

In the case of isolates of races 3 and 4, the genotype and the phenotype did not match up. Based on phenotype, isolates of races 3 and 4 do not produce ToxB-type chlorosis on sensitive wheat cultivars. In isolates of races 3 and 4, a single hybridizing fragment was detected with the *ToxB* probe (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006); the *ToxB* homolog in a race 4 isolate was named *toxb* by Martinez et al. (2004). Comparison of the *ToxB* ORF and the ORF of *toxb* showed 86% similarity; the *toxb* ORF was also identical to a *ToxB* homolog found in Canadian race 4 isolates (Strelkov et al., 2006). In studies of the chromosomes, a single fragment hybridized to the *ToxB* probe in a race 4 isolate (Lichter et al., 2002). There were differences in the promoter and enhancer region of *toxb* (Martinez et al., 2004) and regions flanking *toxb* (Martinez et al., 2004; Strelkov et al., 2006). The protein encoded for by *toxb* (Ptr toxb) is 81% similar to Ptr ToxB and one amino acid shorter.

Orolaza et al. (1995) first remarked on the difference in the extent of chlorosis symptoms between a Canadian race 5 isolate, 92-171R5 and an Algerian race 5 isolate, Alg 3-24. Both induced chlorosis on sensitive wheat cultivars but the extent of chlorosis produced by 92-171R5 was significantly less (Strelkov, 2002; Strelkov et al., 2002; Strelkov et al., 2003). Infiltration of crude culture filtrates into sensitive wheat leaves produced symptoms only when concentrated. However, production and purification of Ptr ToxB from 92-171R5 was problematic due to low level of the toxin in culture filtrates (Strelkov, 2002; Strelkov et al., 2002). Genetic comparison between the two isolates showed only two copies of *ToxB* present in the genome of 92-171R5 and between 8-10

copies in Alg 3-24 (Strelkov, 2002; Strelkov et al., 2006). The difference in virulence between the two isolates could be attributed to the difference in copy number of *ToxB*.

Ptr ToxB Mode of Action

The mode of action of Ptr ToxB was found to be light dependant. Ptr ToxB degrades the chlorophyll in the cell but does not inhibit its synthesis and the role of photochemical bleaching was studied (Strelkov, 1998, Strelkov et al., 1998). Etiolated seedlings of sensitive cultivars infiltrated with toxin showed a decrease in chlorophyll content after light exposure. Seedlings infiltrated with ToxB followed by incubation in the dark for set amount of time and reintroduction to light delayed the onset of chlorosis by the amount of time seedlings were in the dark. Photochemical bleaching may be part of ToxB mode of action as treatment with different active oxygen (AO) scavengers prevented chlorosis (Strelkov, 1998).

Partially Characterized Toxins

Ptr ToxC

A third toxic compound (named Ptr ToxC) which produced chlorosis symptoms, was partially purified from the culture filtrate of a race 1 isolate (Effertz et al., 2002). When infiltrated into the leaves of the wheat cultivar 6B365 (used to differentiate ToxB chlorosis; Lamari and Bernier, 1989a), Ptr ToxC induced race 3-type chlorosis (effertz et al., 2002). Ptr ToxC appeared stable over a wide range of pH (from 2 to 10), was non-ionic, polar and was also heat stable. It was small in size, as dialysis with tubing with a cut off of 3,500 Da showed activity both inside and outside of the tubing (Effertz et al., 2002). At this time, there is no information available on how Ptr ToxC is synthesized or how many genes may be involved.

Ptr ToxD

Preliminary evidence for the existence of a fourth HST by *P. tritici-repentis* has been presented but never published in a peer-reviewed journal. Proposed as Ptr ToxD, this toxin was determined to exist in race 9 isolates (Manning et al., 2002b; Ciuffetti et al., 2003). The race 9 isolate was reported to have produced the same symptoms as races 2 and 8 isolates on the wheat differential set, but the genes for *ToxA* and *ToxB* were absent (Andrie et al., 2007a). Ptr ToxD was shown to be a protein in nature. Meinhardt et al. (2003) also discussed the possibility of a Ptr ToxD but have yet to publish results in a peer-reviewed journal.

Genetic Variation Among Isolates of *P. tritici-repentis*

The variation among isolates of *P. tritici-repentis* was studied in isolates in different parts of the world and there was no correlation found between colony morphology, region of collection or with race. Santos et al. (2002) studied the genetic variation among twelve different Brazilian *P. tritici-repentis* isolates by random amplified polymorphic DNA markers (RAPD). There was no correlation between polymorphisms detected with the primers, region of collection or morphology. Singh et al. (2006) tried to correlate the race of an isolate to its geographical origin. Using 30 RAPD primers and 11 isolates, high levels of polymorphisms were detected but no correlation could be found between the polymorphisms and race or geographical origin. Using isolates of the six races available at the time of study, Aung (2001) conducted a RAPD study and also found that polymorphisms could not be distinguish pathogenic and non-pathogenic races of *P. tritici-repentis*. Subsequent work by Santos et al. (2002) and

Singh et al. (2006) found similar results. Based on RAPD analysis, polymorphisms were detected but could not be correlated with race or geographical origin in the two studies.

At the chromosomal level, little variation in the karyotypes of pathogenic and nonpathogenic isolates of *P. tritici-repentis* was detected (Lichter et al., 2002). AFLP analysis of isolates from around the world showed diversity was reasonably high, with some isolates carrying unique alleles (Friesen et al., 2005). The isolates studied appeared as though they had been drawn from one large, stable interbreeding population. They found that there was no link between AFLP haplotype and pathogenic races (Friesen et al., 2005), which agrees with the karyotype analysis (Lichter et al, 2002). It appears that molecular techniques cannot differentiate pathogenic from nonpathogenic races nor by geographical origin. The genomes of different isolates of *P. tritici-repentis* were studied but the toxin genes themselves have not been studied from a large cross section of isolates of different races.

MATERIALS AND METHODS

Culture of Isolates

Isolates

In total, 135 isolates were chosen to represent the eight races of *Pyrenophora tritici-repentis*. The breakdown was as follows:

Race 1: 37 isolates
Race 2: 31 isolates
Race 3: 9 isolates
Race 4: 1 isolate
Race 5: 31 isolates
Race 6: 5 isolates
Race 7: 9 isolates
Race 8: 12 isolates

The complete list of isolates chosen based on race is listed in Appendix I; isolates are organized based on race and location of collection. Some isolates were obtained by Dr. Lamari from ICARDA (International Center for Agricultural Research in the Dry Areas) based in Aleppo, Syria.

Single-Spore Cultures and Mycelial Mat Initiation

Inoculum and mycelial mats were started from single spore cultures selected from Dr. L. Lamari's *P. tritici-repentis* collection. The single-spore cultures were started from the original spore cultures that had been stored in 25% glycerol at -70°C. Subculturing from the glycerol stocks insured that material was the correct isolate. Small aliquots were taken from the glycerol stocks using a sterile loop and streaked on V8-PDA (150 mL V8 juice, 10 g potato dextrose agar, 3g calcium carbonate, 10 g agar to 1 L with distilled water) plates (Lamari and Bernier, 1989c). The plates were incubated in the dark at 17°C for seven days until there was sufficient mycelial growth.

Mycelial plugs with a diameter of 1 cm were removed with a flamed cork borer and placed face down (mycelia in direct contact with the media) onto the center of fresh V8-PDA plates (Fig. 4a,b). Plates were incubated at 17°C for seven days, until the diameter of the mats are approximately 7 cm (Fig. 4c).

To induce sporulation, four small plugs of mycelia were removed from each plate and placed (mycelia side-up) on a fresh V8-PDA plate (Fig. 4d). Plates were left under light for 16 h followed by incubation at 12°C in the dark for 24 h. After the light-dark period, the plugs appeared green around the edges (Fig. 5a), indicating the presence of conidia. Inspection under a dissecting microscope showed that the banana-shaped conidia were present (Fig. 5b and c). One plug was cut in half and the edges were rubbed onto a fresh V8-PDA plate, distributing the conidia across the plate and facilitating the selection of single conidia. Using a dissecting microscope, a small, square section of agar with a single conidiospore was removed and placed onto the center of a fresh V8-PDA plate, conidiospore side up (Fig. 5d). The isolates were grown at 17°C in the dark for five days and became the source material. All isolates were prepared in the same manner. The purpose of this process was to select a single conidiospore as the source material to represent the isolate and thereby eliminating the possibility of contamination in the original glycerol stock.

Spore Production and Harvesting for Inoculum

When the mycelial mats derived from a single spore grew to a diameter of five centimeters, mycelial plugs were taken, placed mycelia side down in the center of a fresh V8-PDA plates and incubated at 17°C for five days until the mats were 5 cm in diameter. Sterile water was poured to flood each plate. A flamed, rounded test tube was used to

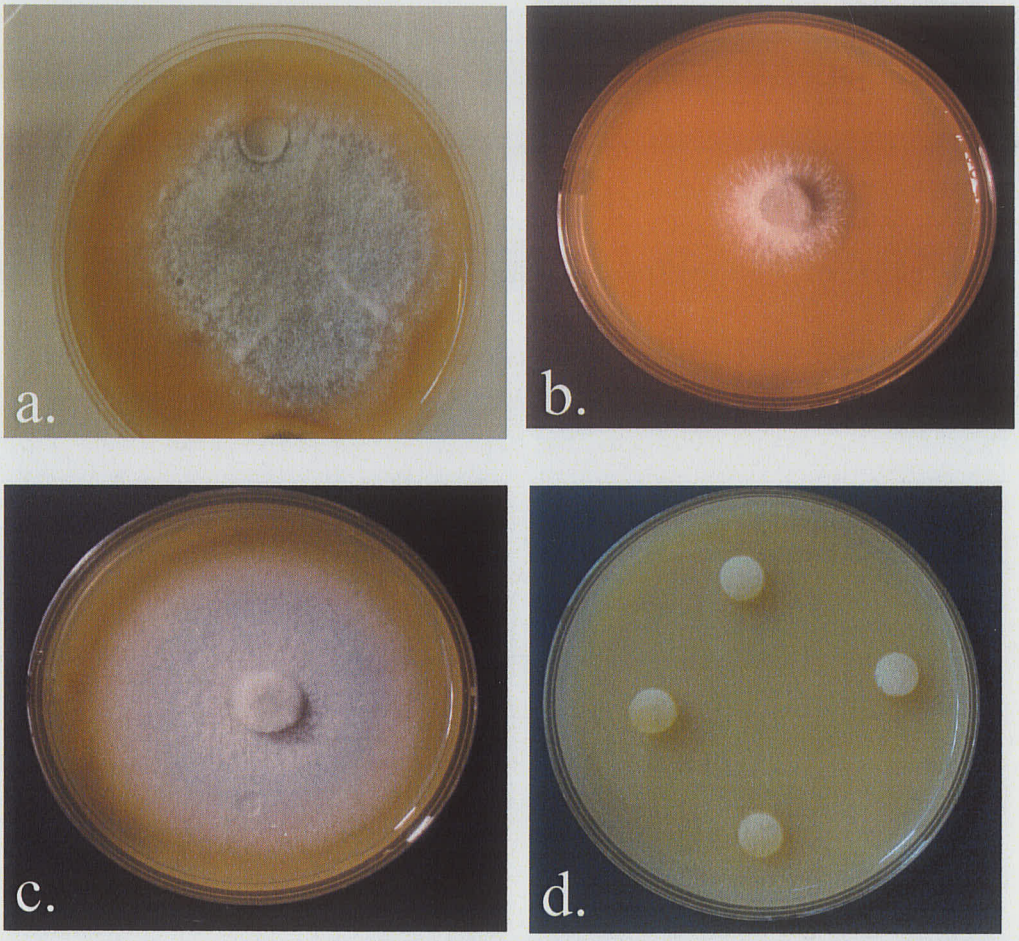


Fig. 4: Culturing of *P. tritici-repentis* spores from glycerol stocks. **a.** Plug removed from five-day old mycelial mat initiated from glycerol stocks; **b.** Mycelial growth from plug on V8-PDA media; **c.** Mycelial mat at a diameter of 7 cm; **d.** Four plugs removed from mycelial mat to induce sporulation.

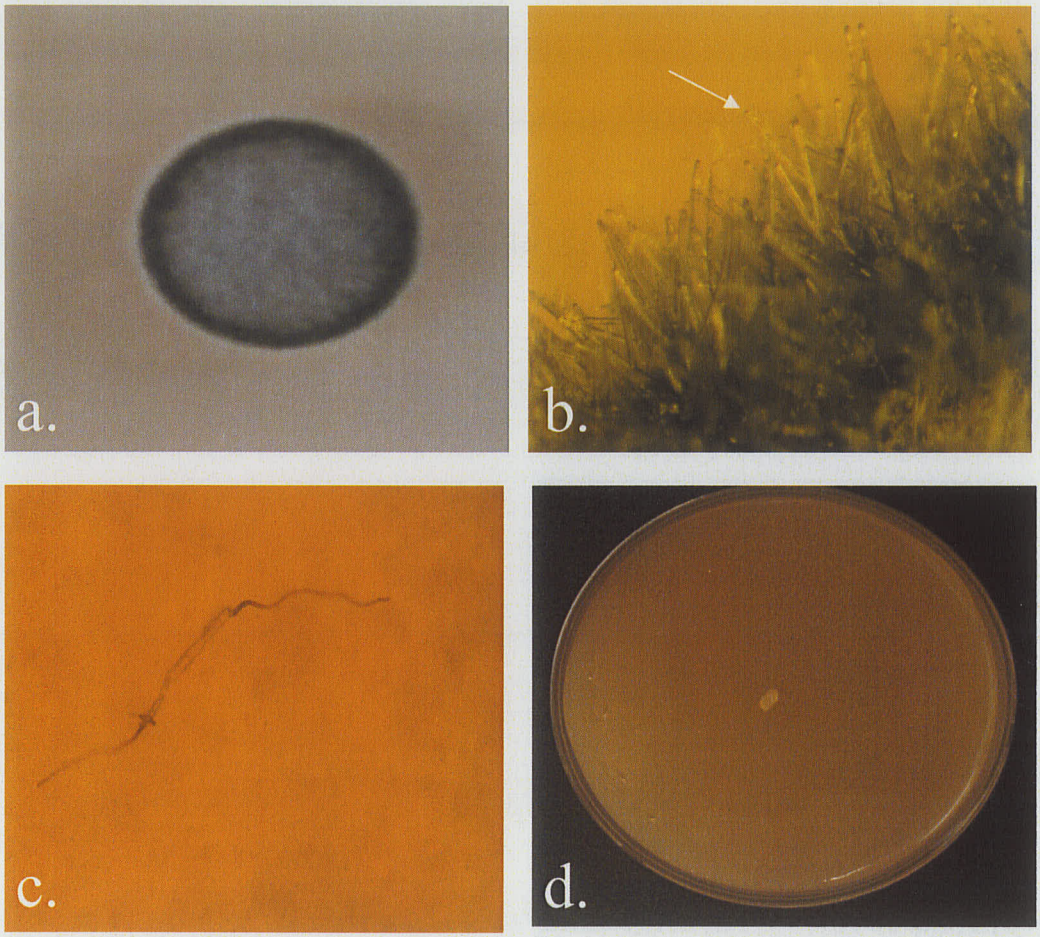


Fig. 5: Conidia of *P. tritici-repentis* for single spore cultures. **a.** Plug of mycelia with conidia in outer green area after 17 h light and 24 h dark treatment; **b.** Magnified view of conidia on a plug; **c.** Banana-shaped germinating conidium on V8-PDA media; **d.** Small section of agar with a single conidium on the center of a V8-PDA plate.

gently flatten mycelia. The water was completely poured off and plates were put under cool white fluorescent light for 16 h at room temperature. This was followed by a 24 h incubation in the dark at 12°C. If conidia formation occurred, the mycelia appeared green and velvety. Plates were flooded with sterile water and rubbed with a sterile loop to dislodge the conidia (Lamari & Bernier 1989b). Water (and conidia) was poured off and a second rinse was done. The spore suspension for each isolate was collected and agitated in a Waring blender to disrupt spore clumping and polyoxyethylene sorbitan monolaurate (Tween 20, Fisher Scientific) was added.

The concentration of spores in the spore suspension was determined by using a counting chamber (Hausser Scientific, Horsham, PA, USA). The spore suspension was adjusted to 3000 spores/ml by addition of distilled water. Throughout all stages of conidia harvesting, conidia were kept on ice until needed to prevent germination of the conidia.

Symptom Assessment on Wheat Differential Set

The spore suspension was sprayed onto the wheat leaves using a DeVilbis sprayer at a pressure of 15 psi. The DeVilbis sprayer was cleaned by running water, bleach and then water again through the gun prior to addition of each spore suspension. The leaves of test plants were sprayed until runoff. Inoculated plants were kept in a misting chamber where two ultrasonic humidifiers provided continuous leaf wetness in the misting chamber. Inoculated plants remained in the humidity chamber for 24 h then moved to a growth room (16/8 h light/dark cycle at 19°C) and were watered daily. Symptoms of tan spot were observed 72 hours after inoculation. The reactions from the differential set differentiated an isolate into one of eight races (Lamari and Bernier, 1989b).

Mycelial Mat Production and Harvesting

Mycelial mats were the source of genomic DNA for extractions. Spores from single spore cultures were inoculated at a concentration of 2500 spores into 100 ml of liquid Fries Media (30 mM ammonium tartrate, 20 mM ammonium nitrate, 2 mM magnesium sulfate septahydrate, 10 mM potassium phosphate monobasic, 15 mM potassium phosphate dibasic, 90 mM sucrose, 1 g yeast extract, 2 ml of trace element solution containing 0.4 mM lithium chloride, 0.8 mM copper chloride, 0.3 mM molybdic acid, 0.4 mM manganese chloride tetrahydrate, 0.4 mM cobalt chloride tetrahydrate). The flasks were incubated at 17°C for two weeks in the dark without shaking. After the first three days of incubation, the flasks were gently agitated to dislodge germinating conidia from flask bottoms and to encourage growth at the air-media interface.

When mats reached a thickness of approximately 0.5 cm, they were harvested. To separate mycelia mats from the liquid media, the contents of the flask were poured through a ceramic funnel with Whatman #4 filter, 11 cm in diameter (Whatman, International, Maidstone, England) with vacuum applied. The mycelial mats were scraped from the filters and placed in sterile sample bags (Fisher), frozen completely and lyophilized for 48 hours. Mats were stored at –20°C.

Growth of Wheat Differential Lines

Ten different wheat cultivars/lines were used for symptom assessment. Seven seeds of each cultivar were planted in five inch clay pots containing a 2:1:1 mixture of soil, sand and peat, respectively. Each pot was divided in half allowing for two cultivars per pot. Seeds were planted 1 cm deep in the soil. The cultivars were grown in a growth

chamber with a 16/8 h light/dark cycle at 19°C. Plants were watered daily until plants reached the 2-3 leaf stage.

Genomic DNA Extraction

Genomic DNA from isolates was extracted using the Promega Wizard Genomic DNA extraction kit (Promega Corp., Madison, Wisconsin). The procedure for genomic DNA extraction was adapted from the plant material extraction method included in the kit. Lyophilised mycelial mat material (0.3 to 0.4 grams) was weighed into 1.5 ml microcentrifuge tubes (Fisher). Although mycelial mats were lyophilized prior to extraction, they seemed to be hygroscopic; as a result, the tubes were immersed in liquid nitrogen to flash-freeze the material. Frozen mycelia were ground with sterile wooden toothpicks rather than the recommended plastic pestles. It was found that toothpicks were easier to manipulate and prevented an accumulation of unground material at the bottom of the tube.

Once the material was sufficiently ground, 500 µl of the Wizard Nuclei Lysis solution was added. The toothpicks were used to insure that all the material was sufficiently wetted with the solution. The tubes were vortexed for three seconds in quick bursts and incubated in a stationary water bath (65°C) for 30 min, allowing the nuclei to rupture. After 30 min, the tubes were removed from the water bath.

To digest the RNA present, an RNaseA solution was added (RNase A in TE [10 mM Tris, 1 mM EDTA] buffer). The amount added was modified from the directions of the kit; the kit recommended a total of 12 µg and an incubation period of 15 min. The total RNase added was doubled and the incubation conditions were 2 h at 37°C. This was

done because it was found that RNA remained in the sample when the original kit directions were followed.

After the 2 h incubation period, tubes were cooled to room temperature and 200 µl of the Wizard Protein Precipitation solution was added. Tubes were vortexed in 5 s intervals for a total of 20 s to precipitate proteins, and then centrifuged (16,000 rcf) at room temperature in a microcentrifuge for 10 min. The DNA, which remained soluble in the supernatant, was recovered by transferring this supernatant (approximately 500 µl) to 600 µl of cold isopropanol. Tubes were inverted to mix thoroughly and the precipitated DNA appeared in strings. DNA was allowed to precipitate for a minimum of 1 h at 4°C rather than the 10 min recommended in the kit. Tubes were centrifuged at 16,000 rcf at room temperature for 10 min to pellet precipitated DNA; the supernatant was removed and discarded. Pelleted DNA from different preparations ranged in color from grey to reddish-brown.

DNA was washed by adding 500 µl of 70% ethanol to tubes and inverting. Tubes were centrifuged at 16,000 rcf at room temperature for 5 min. Supernatants were discarded, removing some of the color from the original DNA pellets, rendering them yellow-white to grey. DNA was air dried in a 37°C dry incubator (Isotemp Incubator, Model 655D, Fisher Scientific). On average, drying time was 20 min.

The dried DNA appeared as a colored crust at the bottom of the microcentrifuge tubes. DNA was rehydrated with the addition of 500 µl of Wizard DNA Rehydration solution (TE buffer) and incubated in a 65°C water bath for 1 h with periodic mixing to redissolve the DNA. After 1 h, the solutions appeared opaque with no particulate matter.

Samples were cooled to room temperature for 5 min, before being subjected to phenol-chloroform extractions.

Phenol-chloroform extractions and DNA recovery

Phenol-chloroform extractions were done to remove proteins, phenolic compounds and other contaminants. Equal volumes of phenol (Tris-buffered Phenol, pH 6.5, Fisher Scientific) and chloroform-isoamyl alcohol (24:1) were added directly to the DNA solution; in this case, 250 µl of phenol and 250 µl of chloroform. Tubes were inverted vigorously for 5 min. To separate the phases, samples were centrifuged at 18,300 rcf for 10 min. After the centrifugation, the aqueous (upper) phase was carefully removed with a micropipette and put into a clean tube. The phenol-chloroform extractions were repeated twice more for a total of three extractions. Phenol-chloroform extractions were followed by two chloroform-isoamyl alcohol extractions, each 500 µl, in order to remove any traces of phenol left in the DNA sample. After the extractions, the aqueous phase was pipetted into new 1.5 ml microcentrifuge tubes. Two volumes of 95% ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2 were added. The tubes were inverted to mix and the DNA was allowed to precipitate overnight at 4°C.

The DNA samples were recovered by centrifugation at 16,000 rcf for 10 min. The supernatant was removed and the pellets were washed with 300 µl of 70% ethanol. The tubes were centrifuged again at 16,000 rcf at room temperature for 5 min. Supernatant was removed, and at this point, the DNA pellet appeared as a small white pellet at the bottom of the tube. To evaporate any remaining ethanol, tubes were dried at 37°C for approximately 30 min. Once the DNA was completely dry, it was redissolved in 50 µl of TE buffer. The samples were incubated at 65°C (with occasional tapping) to

speed up dissolution and the DNA solution appeared clear. Tubes were centrifuged for 5 s to insure that contents were at the bottom of each tube.

DNA quantification

To quantify DNA, a 2 µl aliquot from each sample was added to 800 µl of distilled water. Using a quartz cuvette, the diluted DNA was analysed using an UltraSpec 2100 PW (Biochrom) spectrophotometer. The light path was 1 cm and absorbance readings were taken at wavelengths of 230, 260 and 280 nanometers. The 260/230 ratio provided an indication of contamination from phenolic compounds, carbohydrates and peptides (Manchester, 1996). The A_{260} value was used to calculate the quantity of DNA in the sample and the 260/280 ratio provided an indication of protein contamination in the sample (Sambrook et al, 1989). The desired absorbance ratio value for 260/230 was two or greater while the desired ratio for the 260/280 value was greater than 1.8 (Sambrook et al., 1989). The DNA concentration was calculated by multiplying the A_{260} value by 50 µg/ml and then by the dilution factor, in this case 400 (Sambrook et al., 1989). DNA aliqouts with a concentration of 100 ng/µl in water were prepared and stored at 4°C; undiluted stock DNA in TE buffer was stored at -20°C.

Electrophoresis

Agarose gel electrophoresis was carried out in 0.8% agarose prepared in 1x TAE (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA). Ethidium bromide was added directly to the agarose gel at a concentration of 0.5 µg/ml. Gels were run at a constant voltage. The time and voltage of run was dependant on the size of the gel; for example, a mini-gel with a width of 7 cm was run at 100 volts while a gel 24.5 cm in width was run

at 120 volts. The separated products were examined on a UV light box. Gels were photographed and recorded using a Alpha Imager (Bio-Rad).

Molecular Techniques

PCR

Polymerase Chain Reaction (PCR) is the amplification of a product from DNA/RNA. Two oligonucleotide primers are designed to anneal to target DNA. The denaturation step (around 95°C) separates the two DNA strands. At the appropriate annealing temperature, the binding of primer to complementary DNA is specific. A thermally stable DNA polymerase from *Thermus aquaticus* (*Taq*) elongates the strands by adding nucleotides (Saiki et al., 1988).

Primer Construction

The *ToxA* primers were constructed using the sequence of a genomic DNA *ToxA* gene clone GenBank (accession number AF004369) rather than the mRNA clone since all previous *ToxA* sequences were identical. Two pairs of nested primers were constructed using the website http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi. The internal primer set was TxA1 and TxA2 and the expected size of the product was 290 bp. The product started 3 bp upstream from the ATG translational start codon and ended 292 bp upstream from the stop codon (Fig. 6). The external primer set, TxAG1 and TxAG2, amplified a product that contained sequence from 274 bp upstream of the translational start codon to146 bp downstream from the TAG translational stop codon (Fig. 6). Based on the sequence, the expected amplified product size was 998 bp.

There were several genomic sequences for *ToxB* available in GenBank and comparison has shown that they are identical (Strelkov, 2002; Martinez et al., 2004;

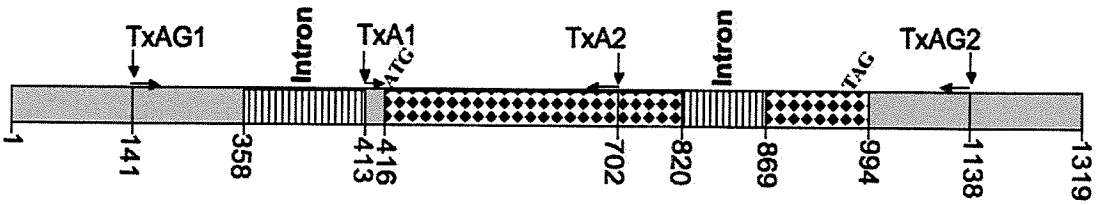


Fig. 6: Location and direction of *ToxA* primers used for PCR analysis. Primers were constructed from a genomic *ToxA* sequence (accession number AF004369). Coding region ( ) and introns ( ).

Strelkov et al., 2006). The reference sequence chosen (accession number AY424115) was a genomic DNA clone from a race 5 Algerian isolate, Alg 3-24. The sequence was imputed into a primer design site and three sets of nested primers were designed. TxBG1 and TxB7 was the external primer set and the product ran from 858 bp upstream of the ATG translational start codon to 93 downstream of the TAG translational stop codon (Fig. 7). Based on the reference sequence, the expected size product was 1224 bp. The product of the middle primer set, R5P-F and TxB7, ran from 400 bp upstream of the ATG translational start codon to TxB7 and the expected product size is 757 bp (Fig. 7). The internal primer set, TxB1 and TxB2 is expected to amplify a 245 bp product within the coding sequence. The product starts 17 bp downstream of the ATG translational start codon to the 'T' of the TAG translational stop codon (Fig. 7). Table 2 summarizes the primer information.

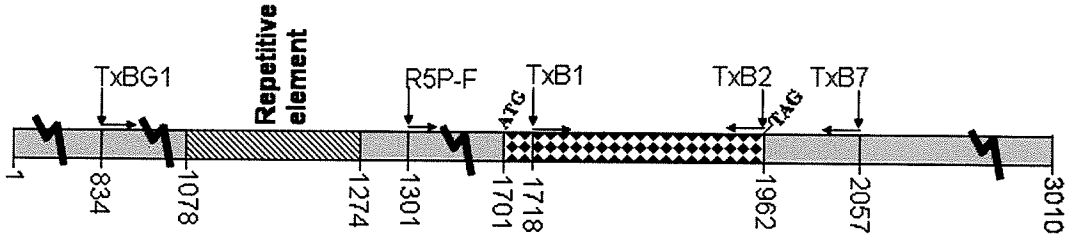


Fig. 7: Location and direction of *ToxB* primers used for PCR analysis. Primers were constructed from the genomic *ToxB* sequence of a race 5 isolate Alg 3-24 (accession number AY242115). Coding region ( ) and repetitive element ( ).

Table 2: The nested *ToxA* and *ToxB* primers used for PCR amplification analysis. *ToxA* primers were designed from a genomic *ToxA* sequence of a race 2 isolates (accession number AF004369) and *ToxB* primers were designed from a race 5 isolate (accession number AY242115). The sequence of the primers, the gene amplified, annealing sites, and the melting temperatures (Tm) based on 50mM of Na^+ are given.

Primer Name	Gene Amplified	Location in Toxin Sequence (bp)	Primer Sequence	Tm at 50 mM Na^+ (¼C)
TxAG1	*ToxA*	141	GGCA TCAT TGCA TGGA CATT	58
TxAG2	*ToxA*	1138	TCGA TCCG ACTC CTCT CCT	60
TxA1	*ToxA*	413	GTCA TGCG TTCT ATCC TCG	57
TxA2	*ToxA*	702	CCTA TAGC ACCA GGTC GTCC	60
TxBG1	*ToxB*	834	GGCG TGGT CTAG ACTG CCCTA	64
R5P-F	*ToxB*	1301	CGGT CTAC CGCA CATA GAGC	60
TxB1	*ToxB*	1718	GACT ACCA TGCT ACTT GCTG TG	58
TxB2	*ToxB*	1962	AACA ACGT CCTC CACT TTGC	60
TxB7	*ToxB*	2057	AGGA AAAC TGTG CTAT GCCA GA	60

PCR Product Amplification and Analysis

The template for PCR amplification was the genomic DNA extracted from the fungal mycelial mats. All of the components for the PCR reaction mixture (except DNA) were mixed in one tube to make a 'master mix' (Saiki et al. 1988). Master mix and samples were kept on ice during preparation prior to placing PCR reaction mixtures into the thermocycler.

The master mix components, volumes, and final concentrations were:

10x PCR Buffer	5.00 µl	1x
25 mM dNTPs	0.40 µl	0.2 mM
50 mM $MgCl_2$	1.25 µl	1.25 mM
Primer forward (20 pmoles/µl)	0.50 µl	0.2 nM
Primer reverse (20 pmoles/µl)	0.50 µl	0.2 nM
Taq polymerase (2.5 U/µl)	1.00 µl	0.05 U/µl
HPLC grade water	47.35 µl	
Sample DNA 100 ng/µl	1.00 µl	2 ng/µl
TOTAL VOLUME	50.00 µl	

Forty nine microliters of master mix were pipetted into 200 µl PCR tubes (Sarstedt) and 100 ng DNA was added. Tubes were vortexed briefly to mix components and centrifuged briefly (approximately 7 s) in a Micromax centrifuge prior to be being placed in a MJ Research PTC-100 thermocycler. Initial denaturation was carried out for 3 min at 95°C, all subsequent denaturation steps were for 45 s (step 1). The step 2 was annealing at 55°C for 30 s and elongation step was at 68°C for 1 min (step 3). Steps 1 to 3 were repeated 40 times. There was a final elongation step at 72°C for 10 min after which the tubes were held at 4°C. If necessary, reactions mixtures were stored at –20°C until analysed.

To analyse PCR products, agarose gels were prepared as described previously. To prepare PCR samples for analysis, 2 µl of 6x sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol; Sambrook et al., 1989) was spotted onto a piece of parafilm and 12 µl of PCR product were added and mixed with buffer. The resulting mixture was loaded into an agarose gel sample well. In addition to PCR products, 12 µl of a 1 Kb ladder (Invitrogen) were loaded along side the samples as a molecular size reference. Gels were run and the results visualized and recorded as described earlier in the thesis (page 44).

Southern Blot Analysis

Southern blot analysis was developed by E.M. Southern as a method to search for specific DNA with a small piece of RNA (as he originally used) or DNA for sequence similarities (Southern, 1975). The probe, a fragment of DNA used for searching for homologous DNA has radioactive isotope incorporated into the sequence (Feinberg and Vogelstein, 1983) with a Klenow enzyme (Klenow and Henningsen, 1970), allowing for

detection afterward. The hybridization solutions and temperature of hybridization allow for specific binding to occur (Southern, 1975).

Restriction Enzyme Digests

For analysis of the *ToxA* gene, genomic DNA from selected fungal isolates was digested with the restriction enzyme *Xho*I (Invitrogen) (Ballance et al. 1996). *Xho*I recognizes the sequence CTCGAG and cuts after the first 'C'. Based on the sequence of *ToxA* in Genbank (accession number AF004369), no internal *Xho*I sites are present. The components of digestion mixtures and the final concentrations were as follows:

XhoI (10 Units/µl, Invitrogen)	1.0 µl	0.2 U/µl
10x REACT buffer (Invitrogen)	5.0 µl	1x
Genomic DNA	10 µg (X µl)	0.2 µg/µl
HPLC grade water	44-X µl	
TOTAL VOLUME	50.0 µl	

Reactions were incubated at 37°C for 48 h to digest DNA to completion. Extra *Xho*I (10 units each) was added at 18 and 36 h.

The restriction enzymes *Xho*I and *Hind*III were used in combination or digestion of genomic DNA for ToxB analysis. *Hind*III recognizes the sequence AAGCTT and cuts after the first 'A'. For the *ToxB* sequence (accession number AY242115), the Sequence Manipulation Suite showed that *Xho*I nor HindIII cut within the *ToxB* coding region. The reaction mixtures with volumes and final concentrations were as follows:

*Xho*I (10 Units/µl, Invitrogen)	1.0 µl	0.2 U/µl
*Hind*III (10 Units/µl, Invitrogen)	1.0 µl	0.2 U/µl
10x REACT Buffer (Invitrogen)	5.0 µl	1x
Genomic DNA	10 µg (X µl)	0.2 µg/µl
HPLC grade water	43-X µl	
TOTAL VOLUME	50.0 µl	

Reactions were incubated at 37°C for 48 h until digested to completion. At 18 and 36 h, 10 units of both *XhoI* and *HindIII* were added.

After 48 h, the digestion products were fractionated on an 0.8% agarose gel as described earlier in the thesis (page 44). Gels for Southern blots were typically run overnight (approximately 18 h) at 25 volts to separate the DNA fragments. The next day, the gel was quickly (to minimize UV exposure) photographed with an Alpha Innotech gel-doc system. The migration distance of each ladder band was measured on the gels with the wells as the start point and recorded on the photograph of the gel.

A blot of each gel was prepared using a Hybond N^+ Nylon Membrane (Amersham Biosciences) and vacuum blotting apparatus (Pharmacia Biotech) according to the VacuGene XL transfer procedure 3. Each membrane was wet according to manufacture's instructions in 2xSSC (0.6 M sodium chloride, 60 mM sodium citrate) or 2xSSPE (0.6 M sodium chloride, 40 mM sodium phosphate monobasic, 4 mM EDTA), depending on the base for the prehybridization and hybridization solutions. After the gel was laid out on the rubber mask, air bubbles were removed with a glass pipette.

The depurination step was done to break the DNA into smaller fragments with a 0.2 M hydrochloric acid (HCl) solution as directed in the VacuGene XL protocol. Vacuum was maintained at 52.5 mBar for 30 min or until the bromophenol blue tracking dye band turned completely yellow. The HCl solution was refilled as needed.

To renature the DNA, the gel was covered with a 1 M NaOH solution while maintaining the vacuum at 52.5 mBar. Denaturation took 90 min; the NaOH solution was replenished as necessary, making sure not to let the gel dry. When 90 min had elapsed, the bromophenol blue band had returned to its original blue color and the xylene cyanol band was green in color.

A sharp pencil was used to pierce the gel and directly mark well location onto the membrane and a handheld UV lamp was used to mark off the ladder. The membrane was washed with 2x SSC solution for 10 s and UV crosslinked in a Stratagene UV Crosslinker 1800 (Stratagene) to fix the DNA to the membrane. The membrane was wrapped in Saran Wrap to prevent drying out, labeled with masking tape, and stored at -20°C until needed. Each membrane was assigned a letter for archiving purposes.

Probe DNA Preparation for Hybridization

ToxA probe DNA was prepared by PCR amplification with TxA1 and TxA2 of the plasmid pPtrNEC reported by Ballance et al. (1996). The PCR product was purified and the DNA recovered from an agarose gel using a modified freeze squeeze method (Thuring et al., 1975). The *ToxB* probe was made by PCR amplification of the *ToxB* genomic DNA of the race 5 isolate Alg 3-24 with internal primers TxB1 and TxB2. The PCR product was purified and recovered in the same way as the *ToxA* probe.

Labeling of Probes for Hybridization

Probe DNA was labeled with radioisotopes using the Megaprime labeling kit (Amersham Biosciences) by the addition of the following components with final concentrations:

Probe DNA	1.0 µl (50 ng)	1.0ng/µl
Sterile distilled water	27.0 µl	
Labeling Buffer	10.0 µl	0.2x
Primer mixture	5.0 µl	0.1x
^{32}P dCTP	5.0 µl (5 µCi)	0.5 µCi/µl
Klenow enzyme (1 U/µl)	2.0 µl	0.04 U/µl
TOTAL VOLUME	50.0 µl	

The water-DNA solution was denatured by heating at 95°C for 5 min in an Analog Heatblock (VWR) and then cooled on ice for 5 min. Components from the Megaprime

Labeling Kit were added after the cooling. The probe was labeled by the incorporation of ^{32}P (in the form of deoxycytidine 5' triphosphate, Perkin Elmer) from random hexanucleotide primers and Klenow enzyme. The reaction mixture tube was put into a lead pig to contain radiation and incubated at 37°C for 2 h for elongation to take place. All sample manipulation in which radioactivity was involved was carried out behind an acrylic shield.

Unincorporated nucleotides were removed by using Illustra MicroSpin S-400 HR Columns (Amersham Biosciences) following the manufacturer's instructions. The prepacked columns were filled Sephacryl equilibrated in 1x TE buffer. The columns were vortexed at high speed to resuspend the separation matrix. The plastic tip at the end of the column was snapped off (cap remained on) and the column was placed in a microcentrifuge tube. Tube and column were centrifuged for 30 s at 3,000 rpm to remove the TE buffer. The column was placed in a new microcentrifuge tube, the column cap was removed and the entire contents of labeled solution were carefully applied to the column. The column was centrifuged for 2 min at 3,000 rpm and the probe was recovered at the bottom of the microcentrifuge tube. The radioactivity of the probe was counted in counts per second (cps) and recorded; typically, the counts were over 4,000 cps. The probe solution was denatured by heating, as described above, and cooled on ice for five minutes.

Prehybridization and Hybridization

To prepare the membrane for hybridization, a prehybridization step was done to block non-specific sites on the membrane. The SSC prehybridization and hybridization solutions contained the following:

6x SSC
5x Denhardt's Solution (0.5 g Ficoll Type 400, Pharmacia Biotech, 0.5 g polyvinylpyrrolidone type 10, 0.5 g bovine serum albumin, Fraction V, Sigma)
0.5% SDS
20 µg/ml salmon sperm DNA
100 ml TOTAL VOLUME

Formamide was used in the prehybridization and hybridization solutions when high backgrounds were obtained. The formamide prehybridization and hybridization solutions were as follows:

6x SSPE
5x Denhardt's solution
0.5% SDS
20% Formamide
100 µg/ml salmon sperm DNA
100 ml TOTAL VOLUME

The salmon sperm DNA was denatured in boiling water for 5 min and then chilled for 5 min on ice prior to addition. SDS was added last. The solution was warmed to 65°C or 50°C (the hybridization temperatures of SSC and SSPE, respectively) prior to the addition of denatured salmon sperm DNA. Once the salmon sperm DNA was added, the solution was kept warm at the hybridizing temperature until needed.

The membranes were removed from -20°C, the Saran Wrap was gently removed and the membranes were wet in a solution of 2x SSC or SSPE. The membranes were rolled with the DNA side facing inward and gently inserted into glass hybridization tubes (VWR, Robbins Scientific) with a pair of long forceps. Tubes were rolled to unroll the membranes, affixing the membranes to the glass walls of the hybridization tubes. Twenty five milliliters of heated prehybridization solution was added to each tube and put into a Robbins Scientific Model 2000 Micro Hybridization Incubator. The prehybridization (approximately 6 h) and hybridization were conducted at the same

temperature; 65°C and 50°C were the hybridization temperatures when SSC and SSPE were used, respectively.

Twenty-five milliliters of warm hybridization solution were added to each tube (after discarding the prehybridization solution) and 25 µl of labeled probe were added to each hybridization tube. Tubes were placed in the hybridization oven at the appropriate hybridization temperature overnight, approximately 18 h.

The next morning, the hybridization solution was removed and a series of wash solutions with decreasing salt concentration with a constant amount of SDS and increasing temperature were employed to remove unbound probe and probe non-specifically bound (Southern, 1975). SSC was used in the wash solutions if SSC was used in the hybridization solutions. SSPE was used in the same manner in the wash solutions.

Twenty-five milliliters of the first wash solution (5x SSC or SSPE, 0.1% SDS) were added directly to each hybridization tube. The membranes were washed for 10 min in the hybridization oven at hybridization temperature. The counts on each membrane were monitored with a Geiger counter (Ludlum 44-9) and counts were recorded.

After the first wash, membranes were transferred to large plastic containers for subsequent washes and incubated in a shaking water bath. The amount of SSC or SSPE in the wash solutions was decreased to 2x at an incubation temperature of 62°C for 30 min. The 1x SSC or SSPE wash was incubated at 65°C for 30 min. SDS was kept constant at 0.1%. For the 1x SSC or SSPE wash, the temperature was increased to 65°C. The counts were measured and recorded after each wash.

If the counts were lower than 12 cps after the 1x SSC or SSPE wash, the washes were stopped. If the counts were still high, additional 30 min washes were carried with 0.1x SSC (SSPE) and 0.05x SSC (SSPE) and 0.1% SDS with each successive wash the temperature was increased by 2°C.

Visualization of Bands

To visualize the results, autoradiography was used. The membranes were gently blotted on paper towels to remove surface solution and wrapped in Saran Wrap, with the DNA side laying facedown on the Saran Wrap; insuring that the smooth side of the Saran Wrap was in contact with the X-ray film. The membrane was placed in a metal cassette, covered with an x-ray film and the cassette was closed and wrapped in a light-tight bag. Depending on the final count on the membrane, the x-ray was exposed for 10 to 14 days at -70°C.

Analysis of Copy Number

The presence or absence of a toxin gene was assessed by determining if hybridizing bands were present. The intensity of bands present on film in genomic Southern blots was used to estimate copy number against a known control sample. Controls and test samples were digested, blotted and probed at the same time. Band intensity was instrumentally estimated using the ASSESS program (American Phytopathological Society) and the Alpha Innotech Imager system. Copy number information was used in the Karyograph program (L. Lamari, personal communication) to plot out the pattern of hybridizing fragments against the migration distance on a linear scale.

Sequencing of Toxin Genes

The toxin genes were sequenced by Macrogen USA (Rockville, MD) based on the Sanger method (Sanger et al., 1977). The sequencing was conducted using Big Dye terminator cycling conditions. The products were purified using ethanol precipitation and run using ABI Automatic Sequencer 3730XL.

DNA Preparation for *ToxA* and *ToxB* Sequencing

The templates for the sequencing of *ToxA* and *ToxB* genes were generated by PCR amplification of genomic DNA. For *ToxA*, the primers used both to amplify the target DNA and to sequence the product were TxAG1 and TxAG2. To amplify *ToxB*, the external primer set, TxBG1 and TxB7, was used. Primers TxBG1, TxB1, and TxB7 were used as the primers to sequence the product. In total, there were 16 isolates chosen for *ToxA* sequencing from races 1, 2, 7, and 8 and assigned identification numbers and 13 isolates of races 5 and 8 for *ToxB* sequencing with identification numbers.

PCR amplification was carried out following the method of Saiki et al. (1988) but FideliTaq DNA Polymerase (USB) was used instead of *Taq* polymerase. The high-fidelity polymerases have a proofreading ability, resulting in a low error frequency, which is necessary when searching for differences in sequence that can vary by a single base pair (Barnes, 1992). Different reaction mixture conditions as well as PCR programs were tested before the selected mix of all components was found. The total volume of the reaction mixture was 25 µl and the volume of components and final concentrations of the components were as follows:

10x PCR buffer (provided with FideliTaq)	2.50 µl	1x
25 mM dNTPS	0.20 µl	0.1 mM
25 mM $MgCl_2$ (provided with FideliTaq)	1.50 µl	0.75 mM
Forward Primer (20 pmoles/µl)	0.25 µl	0.2 nM
Reverse Primer (20 pmoles/µl)	0.25 µl	0.2 nM
FideliTaq (5 U/µl)	0.125 µl	0.013 U/µl
DNA (50 ng/µl)	1.00 µl	1 ng/µl
HPLC Grade Water (Fisher)	19.2 µl	

The master mix, which consisted of all components except the template DNA, was prepared on ice and mixed to homogeneity before 24 µl aliquots of the maters mix were added to 50 ng of DNA template in 200µl PCR tubes (Sarstedt). To generate enough DNA for sequencing, normally three 25 µl PCR reactions were run with each target DNA sample. Tubes were vortexed to mix all components and then pulsed in a Micromax microcentrifuge for five seconds, ensuring that all components were at the bottom of the tube.

The thermocycler used for all reactions was a MJ Research PTC-100 (MJ Research, Waltham, MA) with the heated lid. Initial denaturation was at 95°C for 3 min. Subsequent denaturations were at 95°C for 30 s. Annealing temperature was between 55° to 57°C, depending on the primer set with an annealing time of 30 s. Elongation was done at 68°C for 1 min followed by cycling forty times from the denaturation step to the elongation step. Once all 40 cycles were completed, there was a final elongation step at 68°C for 2 min. The temperature was then held at 4°C until the tubes were needed. The preferred method of storing the PCR products was freezing at -20°C until needed.

PCR products were separated by agarose gel electrophoresis as described previously. In this case, the entire mixture (25 µl) was mixed with sample loading buffer (4 µl) prior to loading on the gel. For a gel 14 cm in width, the voltage applied was 120 volts for two hours.

Once the gel finished running, it was photographed. If a single band was present, the gel was moved to the darkroom for excision of bands. Using a handheld UV lamp as a guide, the product was quickly cut with the razor blade, minimizing UV exposure. The DNA was stored at -20°C until agarose was completely frozen.

Freeze Squeeze Method

To extract DNA, a modified 'freeze squeeze' method was used (Thuring et al. 1975). A 5 X 5 cm square of Parafilm was cut and folded into a small packet. The edges were folded over and warmed to seal the edges, preventing the escape of liquid. The frozen DNA – agarose band was inserted into the Parafilm packet. Gentle pressure was applied to agarose band to squeeze out the liquid containing the DNA. Liquid was collected into fresh microcentrifuge tubes and kept on ice. Generally, the combined amount of liquid collected from three PCR replicates of each sample was close to 400 µl. The volume was brought up to 500 µl with TE buffer. DNA was purified and precipitated by extracting once with phenol-chloroform and once with chloroform and precipitating DNA as described previously. Recovery of DNA involved multiple centrifuge cycles as the amount of recovered DNA was small. The recovered and dried DNA was redissolved in 30 µl of HPLC-grade water. DNA was quantified and stored as described earlier (page 44).

The required DNA concentration was 50 ng/µl of DNA in 15 µl for each reaction. Primers were sent at a concentration of 5 pmoles in 10 µl of water, which could be used for five separate reactions. Sequencing was carried out at Macrogen USA using their primer extension sequencing option.

Editing of forward sequences was straightforward. In the case of reverse sequences, the edited sequences were reverse-complemented using the 'reverse complement' tool on the Sequence Manipulation Suite site (http://bioinformatics.org/sms/). Alignment of the forward and reverse sequences was done using NCBI's blast2seq function found at: (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Once the forward and reverse sequences were aligned, a more complete sequence was obtained. The complete sequences were used for comparison among isolates and for alignment with the existing toxin gene sequences in the NCBI database. *ToxA* sequences from the isolates were compared with both genomic clone sequence and the mRNA sequence in Genbank (accession number AF004369 and UPTU79662, respectively). *ToxB* sequences were compared for alignment to one of the copies of *ToxB* found in Alg 3-24, a race 5 isolate (accession number AY242115). All sequences were aligned using Genebee Multiple Alignment (http://www.genebee.msu.su/services/malign_reduced.html).

RESULTS

Symptom Assessment

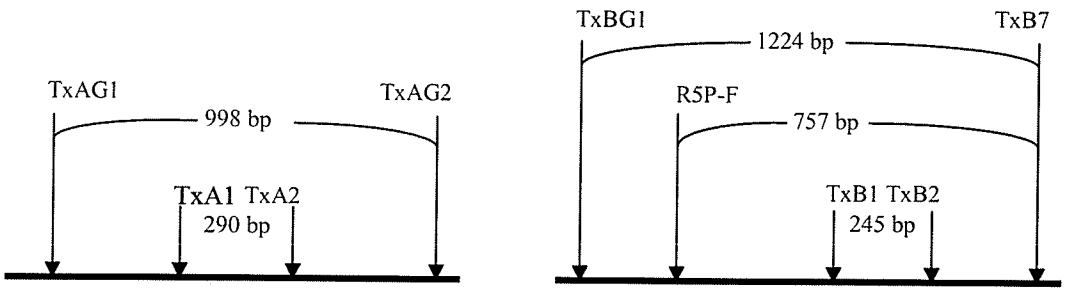
Inoculation of the differential set of wheat cultivars with each isolate was the primary method used to characterize isolates based on the symptoms generated; each isolate was classified into one of eight races. The cultivars of the differential set have different sensitivity to the toxins and produce symptoms accordingly. When the isolates used in this study were first collected, they were inoculated onto the wheat differential cultivars to classify each isolate into one of eight races. In several cases when other analyses gave results which were inconsistent with the original race designation of isolates, those isolates were re-analysed to confirm the race classification.

Although nine different cultivars were used to assess symptoms, the three effective wheat differentials were Glenlea, 6B365 and 6B662 (Lamari et al., 2003). Three symptom types were possible: necrosis, chlorosis or a resistant reaction (Fig. 8). Glenlea, the ToxA-sensitive cultivar, produced tan necrotic symptoms when inoculated with isolates of races 1, 2, 7, and 8 (Fig. 8a, Lamari & Bernier, 1989a; Lamari et al., 2003). All other isolates induced the resistant reaction, which appeared as small brown flecks on wheat leaves (Fig. 8c, Lamari & Bernier, 1989a). Cultivars 6B365 and 6B662 were used to differentiate chlorosis caused by Ptr ToxB and Ptr ToxC. Isolates of races 5, 6, 7, and 8 induced chlorosis on ToxB-sensitive cultivar 6B662; all other isolates induced a resistant reaction on this line. ToxC chlorosis was induced on ToxC -sensitive cultivar 6B365 by isolates or races 1, 3, 6, and 8 (Fig. 8b, Lamari & Bernier, 1989b; Lamari et al., 1995). All other isolates induced the resistant reaction. The other host lines/cultivars served as secondary checks.

Fig. 8: The symptoms of tan spot on wheat leaves. Based on the symptoms produced on the wheat differential set, isolates can be differentiated into one of eight races. **a.** Tan necrotic lesions; **b.** Chlorosis on leaves; **c.** Small brown flecks of the resistant reaction.

Identification of Toxin-like Sequences by PCR

The basis of PCR amplification identification of toxin-like sequences is that a product can be amplified if specific primers are able to find and anneal to their complementary sequences in the target DNA (Saiki et al. 1988). PCR analysis was carried out on 135 isolates of all eight races collected from different regions around the world. With PCR analysis, the presence of *ToxA* or *ToxB* genes in isolates could be detected by the presence of products consistent with the isolates' race. Products amplified with toxin primers in isolates that are not known to produce a toxin could indicate the presence of toxin-like sequences. The expected sizes of the products are based on the highly conserved characterized sequences (Ballance et al., 1996; Ciuffetti et al., 1997; Ballance and Lamari, 1998; Martinez et al., 2004; Strelkov et al., 2006). If the product was larger or smaller than expected, it would indicate a mutation in the gene region by insertions or deletions, respectively. Point mutations of nucleotides may not be detectable with electrophoresis product size analysis but if the mutation occurred under one of the priming sites and disrupts hybridization, no product would be amplified. This latter possibility was addressed by using nested primer sets. If there were no sequence changes in the isolates tested relative to the limited number of previously characterized sequences, the expected product size for each primer set studied would be as indicated below:



ToxA

Genomic DNA served as the template for PCR amplification with the internal *ToxA* primer set, TxA1 and TxA2. This primer set was used to verify the presence of the *ToxA* (or *ToxA*-like) coding sequence. All isolates were subjected to PCR amplification and only isolates that produced Ptr ToxA necrosis symptoms (races 1, 2, 7, and 8) on sensitive wheat cultivars amplified the expected size product (Tables 3, 4, 5, and 6); Figure 9 shows a limited number of isolates of races 1 and 7 and the amplified products. Although agarose gel electrophoresis cannot detect single nucleotide substitutions and deletions, it appeared that the *ToxA* coding region in the ToxA-producing isolates was conserved in size.

No PCR product was amplified from isolates of races 3, 4, 5, or 6 (Tables 7, 8, and 9) which, based on the phenotype reactions, do not express a ToxA-related necrosis and were not expected to have a *ToxA* gene. The lack of amplified product indicated the absence of *ToxA*-like sequences under the internal *ToxA* primer set in non-ToxA-producing isolates.

PCR amplification of genomic DNA with the external *ToxA* primer set, TxAG1 and TxAG2 could show modifications in sequence outside of the coding region in the form of deletions and insertions. The PCR amplification with TxA1 and TxA2 showed that the coding region was present and conserved in ToxA-phenotype isolates based on size and presence of PCR amplified product. Isolates of races 1, 2, 7, and 8 amplified product of the expected size (Tables 3, 4, 5, and 6). A limited number of PCR amplification products of ToxA-phenotype isolates are shown in Figure 10. The similarity in product size indicated no major insertions or deletions were present in the

Table 3: PCR amplification products results of race 1 isolates with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F & TxB7	TxBG1 & TxB7
Isolate	Race	Region of Collection	Expected Size (bp)	290 bp	998 bp	245 bp	757 bp	1224 bp
ICARDA 1-5	1	Kazakhstan		E	E	NP	NP	NP
ICARDA 3-1	1	Kazakhstan		E	E	NP	NP	NP
ICARDA 7-1	1	Kyrgyzstan		E	E	NP	NP	NP
ICARDA 8-4	1	Kyrgyzstan		E	E	NP	NP	NP
ICARDA 14-1	1	Kyrgyzstan		E	E	NP	NP	NP
ICARDA 15-1	1	Kyrgyzstan		E	E	NP	NP	NP
ICARDA 18-4	1	Azerbaijan		E	E	NP	NP	NP
ICARDA 21-4	1	Azerbaijan		E	E	NP	NP	NP
ICARDA 22-17	1	Azerbaijan		E	E	NP	NP	NP
ICARDA 29-3	1	Azerbaijan		E	E	NP	NP	NP
ICARDA 32-2	1	Azerbaijan		E	E	NP	NP	NP
ICARDA 33-1	1	Azerbaijan		E	E	NP	NP	NP
ICARDA 67-3	1	Uzbekistan		E	E	NP	NP	NP
ICARDA 67-4	1	Uzbekistan		E	E	NP	NP	NP
ICARDA 67-5	1	Uzbekistan		E	E	NP	NP	NP
ICARDA 68-2	1	Uzbekistan		E	E	NP	NP	NP
NA 3-4	1	North Eastern Algeria		E	E	E (VF)	NP	NP
NA 5-3	1	North Eastern Algeria		E	E	E (VF)	NP	1000 (VF)
NA 8-3	1	North Eastern Algeria		E	E	E (VF)	NP	NP
NA 9-4	1	North Eastern Algeria		E	E	E (VF)	NP	NP
UY 127-2	1	Uruguay		E	E	NP	NP	NP
PDY-7	1	North Dakota		E	E	NP	NP	NP
99-49-2	1	Manitoba		E	E	NP	NP	NP
98-MD2	1	Morden, MB		E	E	NP	NP	NP
98-MD4	1	Morden, MB		E	E	NP	NP	NP
98-MD7	1	Morden, MB		E	E	NP	NP	NP
98-MD8	1	Morden, MB		E	E	E (VF)	NP	1000 (VF)
98-MD10	1	Morden, MB		E	E	E (VF)	NP	NP
SC 10-1	1	Swift Current, SK		E	E	E (VF)	NP	NP
SC 12-3	1	Swift Current, SK		E	E	E (VF)	NP	1000 (VF)
SC 24-3	1	Swift Current, SK		E	E	NP	NP	NP
SC 25-3	1	Swift Current, SK		E	E	E (VF)	NP	NP
SC 29-3	1	Swift Current, SK		E	E	E (VF)	NP	NP
SC 36-3	1	Swift Current, SK		E	E	E (VF)	NP	NP
SK 104-1	1	Saskatchewan		E	E	E (VF)	NP	1000 (VF)
SK 105-2	1	Saskatchewan		E	E	NP	NP	NP

Table 4: PCR amplification products results of race 2 isolates with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F & TxB7	TxBG1 & TxB7
Isolate	**Race**	**Region of Collection**	**Expected Size (bp)**	**290 bp**	**998 bp**	**245 bp**	**757 bp**	**1224 bp**
ICARDA 33-4	2	Azerbaijan		E	E	NP	NP	NP
ICARDA 34-7	2	Azerbaijan		E	E	NP	NP	NP
ICARDA 34-10	2	Azerbaijan		E	E	NP	NP	NP
ICARDA 35-37	2	Azerbaijan		E	E	NP	NP	NP
ICARDA 58-3	2	Kazakhstan		E	E	NP	NP	NP
ICARDA 62-6	2	Kazakhstan		E	E	NP	NP	NP
ICARDA 63-8	2	Kazakhstan		E	E	NP	NP	NP
ICARDA 63-9	2	Kazakhstan		E	E	NP	NP	NP
32JA	2	Alberta		E	E	E (VF)	NP	NP
2004-38-1	2	Cypress Hills, AB		E	E	E (VF)	NP	E (VF)
2004-38-3	2	Cypress Hills, AB		E	E	NP	NP	NP
2004-53-1	2	Cypress Hills, AB		E	E	E (VF)	NP	NP
2004-54-1	2	Cypress Hills, AB		E	E	E (VF)	NP	NP
SK 103-1	2	Saskatchewan		E	E	E (VF)	NP	E (VF)
SK 109-1	2	Saskatchewan		E	E	NP	NP	NP
86-124	2	Portage La Prairie, MB		E	E	NP	NP	NP
90-68	2	Manitoba		E	E	NP	NP	NP
91-18	2	Manitoba		E	E	NP	NP	NP
94-94	2	Manitoba		E	E	NP	NP	NP
94-70	2	Manitoba		E	E	NP	NP	NP
94-106	2	Manitoba		E	E	NP	NP	E (VF)
SC 3-7	2	Swift Current, SK		E	E	NP	NP	NP
SC 5-2	2	Swift Current, SK		E	E	1000 (VF)	NP	E (VF)
SC 6-2	2	Swift Current, SK		E	E	NP	NP	E (VF)
SC 18-2	2	Swift Current, SK		E	E	1000 (VF)	NP	E (VF)
SC 19-2	2	Swift Current, SK		E	E	NP	NP	NP
SC 21-1	2	Swift Current, SK		E	E	1000 (VF)	NP	E (VF)
SC 23-2	2	Swift Current, SK		E	E	NP	NP	E (VF)
SC 27-1	2	Swift Current, SK		E	E	NP	NP	NP
SC 28-2	2	Swift Current, SK		E	E	NP	NP	E (VF)
SC 30-1	2	Swift Current, SK		E	E	NP	NP	E (VF)

Table 5: PCR amplification products results of race 7 isolates with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F & TxB7	TxBG1 & TxB7
Isolate	Race	Region of Collection	Expected Size (bp)	290 bp	998 bp	245 bp	757 bp	1224 bp
ICARDA 17-5	7	Azerbaijan		E	E	E	E	E
ICARDA 17-10	7	Azerbaijan		E	E	E	E	1000, E
ICARDA 35-5	7	Azerbaijan		E	E	E	E	1000
ICARDA 35-17	7	Azerbaijan		E	E	E	E	1000, E
ICARDA 35-21	7	Azerbaijan		E	E	E	E	1000
ICARDA 35-24	7	Azerbaijan		E	E	E	E	E
ICARDA 35-25	7	Azerbaijan		E	E	E	E	1000
ICARDA 36-3	7	Azerbaijan		E	E	E	E	E
ICARDA 36-4	7	Azerbaijan		E	E	E	E	1000

Table 6: PCR amplification products results of race 8 isolates with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F & TxB7	TxBG1 & TxB7
Isolate	Race	Region of Collection	Expected Size (bp)	290 bp	998 bp	245 bp	757 bp	1224 bp
ICARDA 17-11	8	Azerbaijan		E	E	E	E	1000, E
ICARDA 31-1	8	Azerbaijan		E	E	E	E	1000, E
ICARDA 31-2	8	Azerbaijan		E	E	E	E	1000, E
ICARDA 34-1	8	Azerbaijan		E	E	E	E	1000
ICARDA 34-2	8	Azerbaijan		E	E	E	E	1000
ICARDA 35-6	8	Azerbaijan		E	E	E	E	1000
ICARDA 35-13	8	Azerbaijan		E	E	E	E	E
ICARDA 35-16	8	Azerbaijan		E	E	E	E	E
ICARDA 35-18	8	Azerbaijan		E	E	E	E	E
ICARDA 35-19	8	Azerbaijan		E	E	E	E	E
ICARDA 35-33	8	Azerbaijan		E	E	E	E	E
ICARDA 42-14	8	Azerbaijan		E	E	E	E	1000

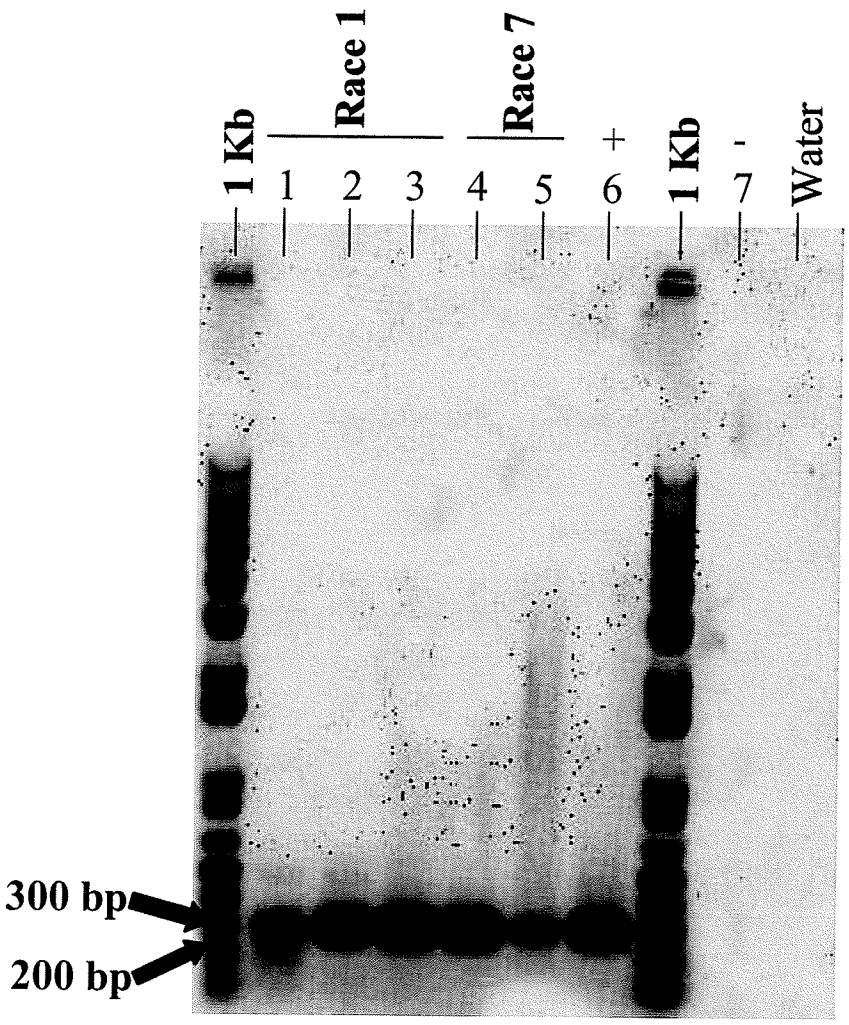


Fig. 9: PCR amplification products of ToxA-phenotype isolates using the *ToxA* internal primer set. TxA1 and TxA2 primers amplified the expected size product (290 bp) in isolates of races 1 and 7. The agarose gel was stained with ethidium bromide to visualize the PCR amplification products. For size estimation, a 1 Kb ladder was run alongside the samples. The water PCR reaction sample contained no DNA and was tested to insure that there were no contaminants in the primers or other reaction constituents. Isolates with the ICARDA designation were be abbreviated 'I'. 1) I 32-2; 2) I 33-1; 3) I 67-4; 4) I 35-24; 5) I 35-25; 6) 86-124 (race 2 positive control); 7) Alg 3-24 (race 5 negative control).

Table 7: PCR amplification products results of isolates of races 3 and 4 with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F & TxB7	TxBG1 & TxB7
Isolate	Race	Region of Collection	Expected Size (bp)	290 bp	998 bp	245 bp	757 bp	1224 bp
ICARDA 72-1	3	Syria		NP	NP	NP	1700	2100
ICARDA 72-2	3	Syria		NP	NP	E	1700	2100
ICARDA 72-3	3	Syria		NP	E (VF)	E	1700	2100
ICARDA 72-5	3	Syria		NP	NP	E	1700	2100
ICARDA 72-7	3	Syria		NP	NP	E	1700	2100
94-115	3	Manitoba		NP	NP	E	E	1000
331-9	3	Manitoba		NP	NP	E	NP	NP
SC 29-1	3	Swift Current, SK		NP	NP	NP	NP	NP
90-2	4	Manitoba		NP	NP	E (VF)	NP	NP

Table 8: PCR amplification products results of race 5 isolates with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F & TxB7	TxBG1 & TxB7
Isolate	Race	Region of Collection	Expected Size (bp)	290 bp	998 bp	245 bp	757 bp	1224 bp
ICARDA 17-1	5	Azerbaijan		NP	NP	E	E	1000
ICARDA 17-8	5	Azerbaijan		NP	NP	E	E	1000
ICARDA 34-3	5	Azerbaijan		NP	E (VF)	E	E	1000
ICARDA 34-6	5	Azerbaijan		NP	NP	E	E	1000
ICARDA 35-1	5	Azerbaijan		NP	NP	E	E	1000
ICARDA 35-20	5	Azerbaijan		NP	E (VF)	E	E	1000
ICARDA 35-57	5	Azerbaijan		NP	E (VF)	E	E	1000
ICARDA 36-1	5	Azerbaijan		NP	NP	E	E	1000
ICARDA 73-2	5	Syria		NP	NP	E	E	1000
ICARDA 73-3	5	Syria		NP	NP	E	E	1000
ICARDA 73-4	5	Syria		NP	NP	E	E	1000
NA 4-4	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-1	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-2	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-4	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-5	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-6	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-7	5	North Eastern Algeria		NP	NP	E	E	1000
NA 6-8	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-2	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-3	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-4	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-5	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-7	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-8	5	North Eastern Algeria		NP	NP	E	E	1000
NA 7-9	5	North Eastern Algeria		NP	NP	E	E	1000
Alg 3-24	5	Algeria		NP	NP	E	E	1000, E
Alg 3X-1	5	Algeria		NP	NP	E	E	1000, E
Alg 4-X1	5	Algeria		NP	NP	E	E	1000, E
Alg 4-X1-1	5	Algeria		NP	NP	E	E	E
Alg 5-X1-1	5	Algeria		NP	NP	E	E	E

Table 9: PCR amplification products results of race 6 isolates with *ToxA* and *ToxB* nested primers. 'E' indicated that the amplified product was the expected size; when product was larger or smaller than expected, the size of the product was indicated in bp; 'NP' signified the absence of amplified product; (VF) indicated that the product signal was very weak in the agarose gel.

				Primer Sets				
				ToxA		*ToxB*		
				TxA1 & TxA2	TxAG1 & TxAG2	TxB1 & TxB2	R5P-F &TxB7	TxBG1 & TxB7
Isolate	**Race**	**Region of Collection**	**Expected Size (bp)**	**290 bp**	**998 bp**	**245 bp**	**757 bp**	**1224 bp**
Alg H-1	6	Algeria		NP	NP	E	E	1000
Alg H-2	6	Algeria		NP	NP	E	E	E
Alg H-2A	6	Algeria		NP	NP	E	E	1000, E
Alg H-2G	6	Algeria		NP	NP	E	E	1000, E
Alg-H2 on 6B365	6	Algeria		NP	NP	E	E	E

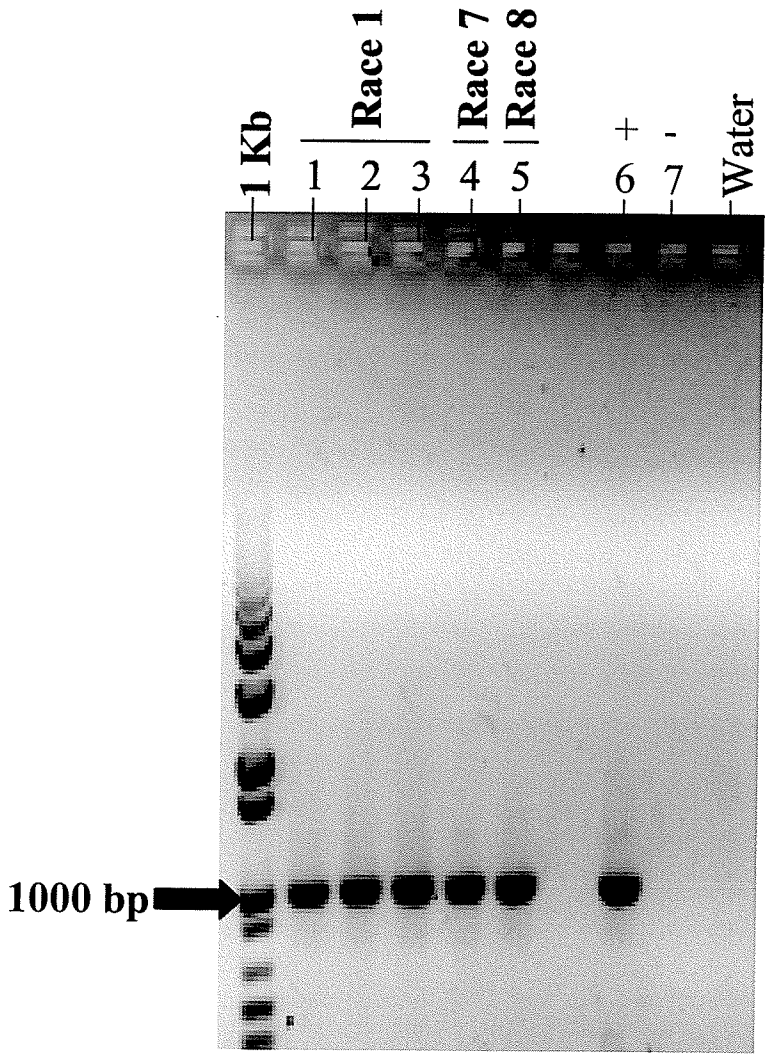


Fig. 10: PCR amplification products of ToxA-phenotype isolates using the *ToxA* external primer set. The isolates were of races 1, 2, 7, and 8.The expected size of the product amplified with TxAG1 and TxAG2 primers is approximately 1000 bp. The agarose gel was stained with ethidium bromide to visual products. No product was amplified in the negative control. The water PCR reaction sample contained no DNA and was tested to insure that there were no contaminants in the primers or other reaction constituents. For size estimation, a 1 Kb ladder was run alongside the samples. 'ICARDA' was abbreviated 'I'. 1) I 1-5; 2) 98-MD-7; 3) I 67-4; 4) I 17-5; 5) I 17-11; 6) 86-124 (race 2 positive control); 7) Alg 3-24 (race 5 negative control).

ToxA gene region between the two priming sites. Products were not amplified in isolates of races 3, 4, 5, and 6 (Tables 7, 8, and 9), confirming the absence of *ToxA*-like sequences for TxAG1 and TxAG2 annealing, which agree with previous results (Ballance et al., 1996; Ciuffetti et al., 1997).

One race 3 isolate (ICARDA 72-3) and three race 5 isolates (ICARDA 34-3, 35-20 and 35-57) weakly amplified a product with TxAG1 and TxAG2 (Tables 7 and 8; Fig. 11a). To confirm the findings, a second PCR amplification on recovered and purified PCR product DNA from an agarose gel showed that PCR amplification remained weak (Fig. 11b). Further analysis with Southern blots and sequencing was used to help elucidate these results to determine their validity (see page 93 for results).

The results obtained with both sets of *ToxA* primers confirm the presence of the *ToxA* gene in all ToxA phenotypes. The PCR product size with both *ToxA* primer sets was found to be very similar in all isolates, regardless of race or geographic origin. The absence of the *ToxA* (or *ToxA*-like) gene was confirmed in isolates of races 3, 4, 5, and 6 that do not induce the ToxA-sensitive response in sensitive wheat cultivars.

ToxB

Three sets of nested primers were used to assess the presence or absence of the *ToxB* gene region in all isolates. The internal primer set, TxB1 and TxB2, was used to detect the *ToxB* (or a *ToxB*-like) coding region in isolates. Isolates of races 5, 6, 7, and 8 amplified a product of the expected size of 245 bp, indicating the presence of a sequence homologous to the *ToxB* coding region. These races induce ToxB-chlorosis on Ptr ToxB-sensitive wheat cultivars. The product size indicated the absence of mutations in the form of insertions or deletions in the coding region (Tables 5, 6, 8, and 9; Fig. 12a)

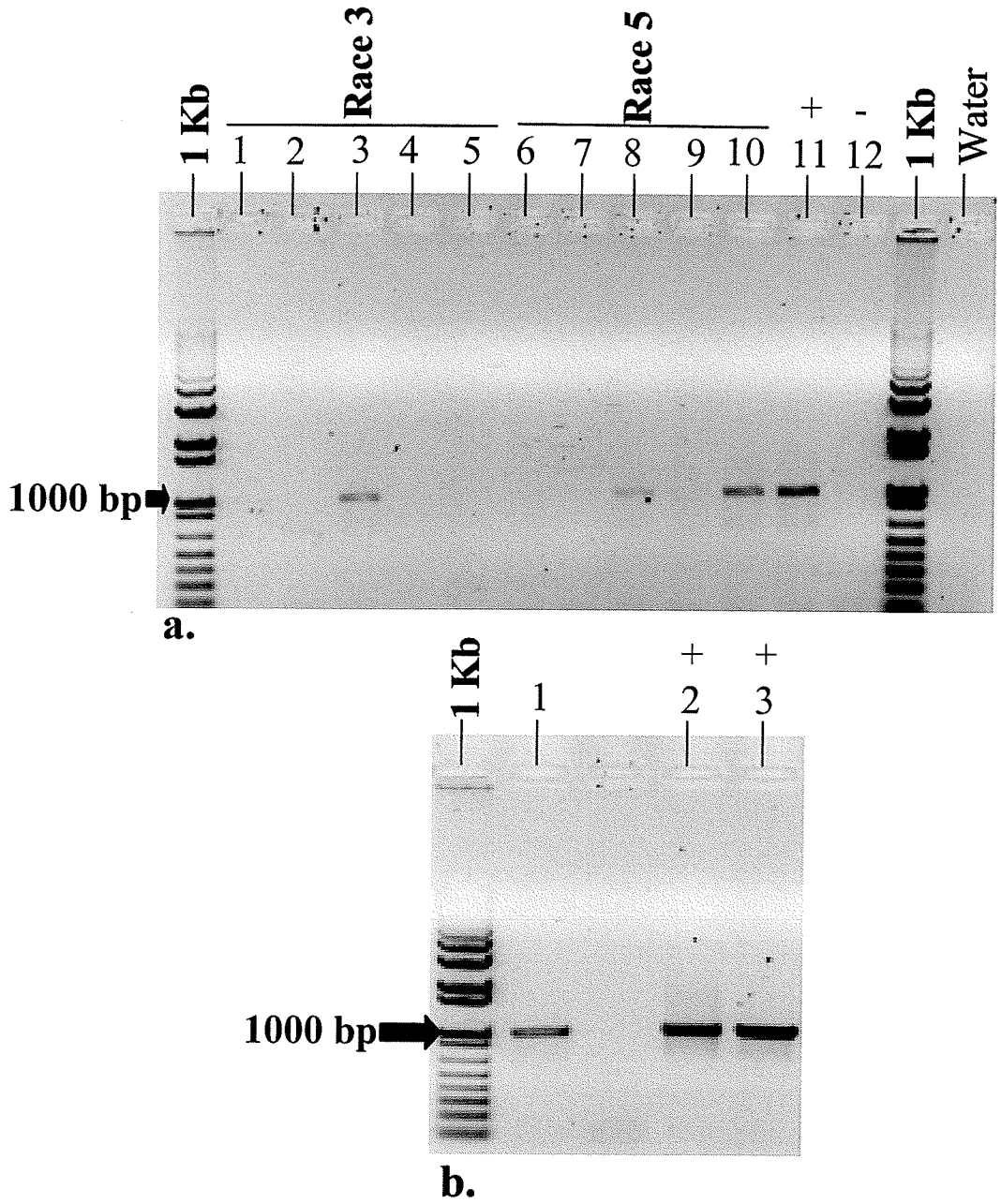


Fig. 11: PCR amplification products of TxAG1 and TxAG2 of non-ToxA-phenotype isolates. The agarose gel was stained with ethidium bromide to visualize the approximately 1000 bp product from isolates of races 3 and 5. ICARDA was abbreviated 'I'. **a.** Weakly amplified products of I 72-3, I 34-3, I 35-20, and I 35-57 of 1000 bp. 1) I 72-1; 2) I 72-2; 3) I 72-3; 4) I 72-5; 5) I 72-7; 6) I 17-1; 7) I 17-8; 8) I 34-3; 9) I 35-20; 10) I 35-57; 11) 86-124 (race 2 positive control); 12) Alg 3-24 (race 5 negative control). **b.** Reamplification of recovered PCR product with TxAG1 and TxAG2 from I 72-3 with TxAG1 and TxAG2. 1) I 72-3 (race 3); 2) I 41-4 (race 1 positive control); 3) 86-124 (race 2 positive control).

All isolates of races 3 and 4 amplified the expected size product (Table 7), demonstrating that a *ToxB*-like sequence was present but unexpressed as Ptr ToxB-chlorosis was not observed on the wheat differential set from any of these isolates, agreeing with previous results (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006). Under the conditions for amplification of *ToxB* product in isolates of races 5, 6, 7, and 8, a product was weakly amplified in the race 4 isolate (Fig. 12a, #1). In order to amplify a product from the race 4 isolate 90-2 with the TxB1 and TxB2 primer set, the annealing temperature was decreased to 50°C (Fig. 12b, #2). For further confirmation, a second internal primer set, TxB3 and TxB4, was used and amplified a *ToxB*-like product (Fig. 13).

The majority of isolates of races 1 and 2 were unable to amplify a product with the primer set TxB1 and TxB2, confirming the absence of a sequence homologous to the *ToxB* coding region. This was expected because phenotypically, these races do not induce ToxB-chlorosis on sensitive cultivars. In 24 isolates of races 1 and 2, the expected size product was amplified with TxB1 and TxB2. The products with TxB1 and TxB2 were strong when PCR cycle was 40 (Fig. 14a) but were weakly amplified or disappeared altogether when cycle number was 30 (Table 3 and 4; Fig. 14b). Table 3 and 4 reflect the results of 30 PCR cycles. Increasing the temperature to 57°C decreased the intensity of the band or eliminated it altogether. To test the validity of the results, Southern blot analysis will be used (see page 102 for results).

Outside of TxB1 and TxB2 was the middle primer set: R5P-F and TxB7. Forward primer R5P-F was designed from the sequence within the promoter region of the

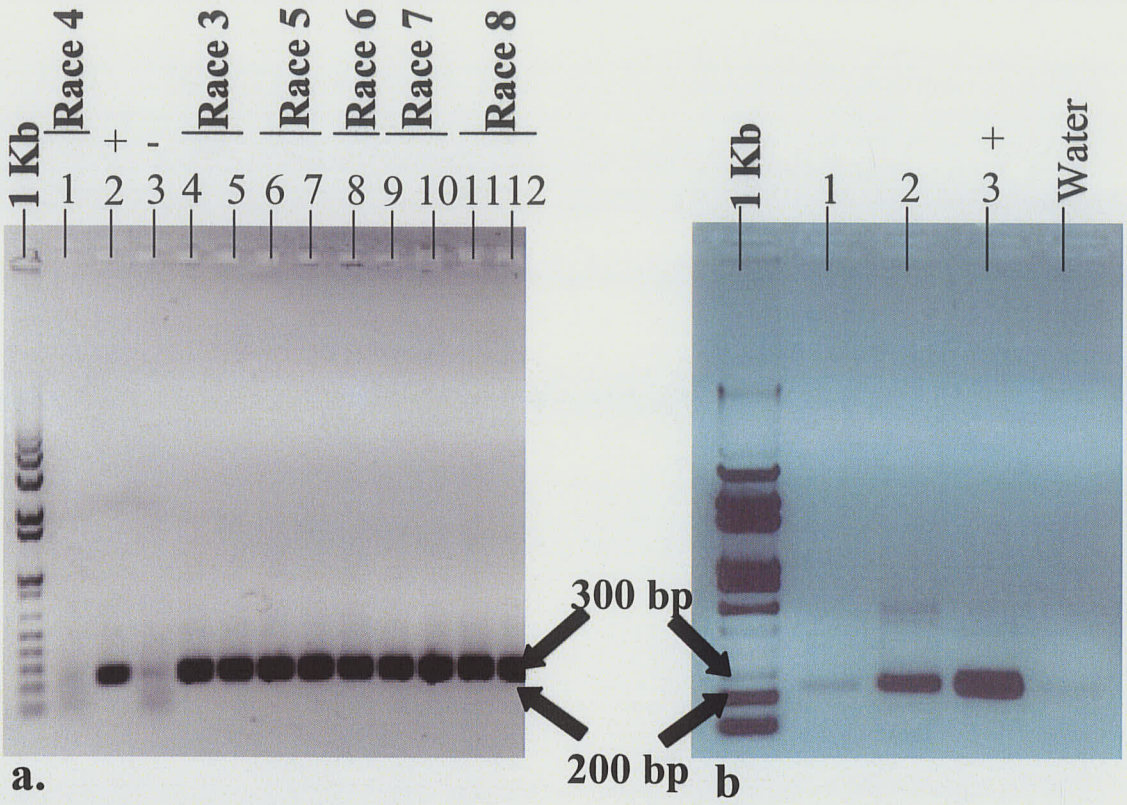


Fig. 12: TxB1 and TxB2 PCR amplification products of isolates of races 3, 4, 5, 6, 7, and 8. The agarose gel was stained with ethidium bromide to visualize bands of 245 bp and to estimate size of amplified bands, a 1 Kb ladder was run alongside the samples. ICARDA was abbreviated 'I'. **a.** PCR products of isolates of races 3, 4, 5, 6, 7, and 8 with an annealing temperature was 57°C. 1) 90-2; 2) Alg 3-24 (race 5 positive control); 3) I 8-4 (race 1 negative control); 4) I 72-1; 4) I 72-7; 5) I 35-1; 6) I 73-2; 7) Alg H-1; 8) I 17-5; 9) I 35-25; 10) I 17-11; 11) I 42-14. **b.** Results of PCR amplification when annealing temperature was decreased from 57°C to 50°C and two different 90-2 DNA samples were used. The water PCR reaction sample contained no DNA and was tested to insure that there were no contaminants in the primers or other reaction constituents. 1) 90-2 (race 4); 2) 90-2 (race 4); Alg 3-24 (race 5 positive control) with the internal *ToxB* primers TxB1 and TxB2.

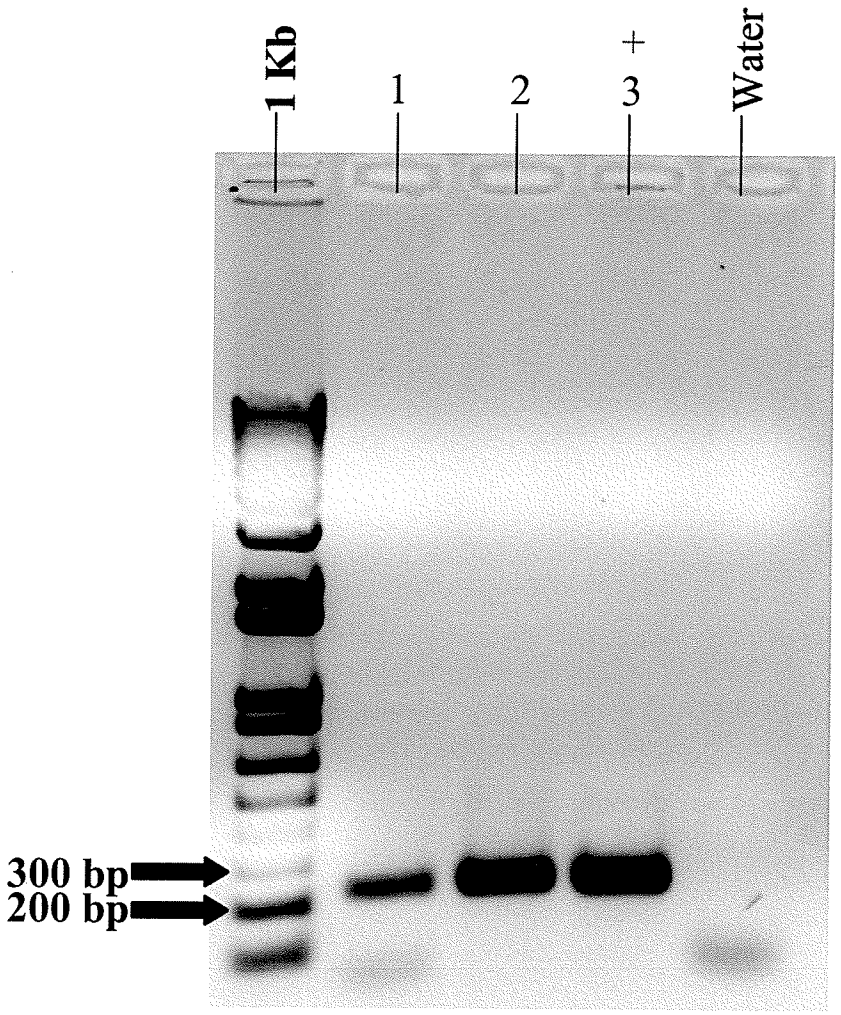


Fig. 13: TxB3 and TxB4 PCR amplification products of race 4 and 5 isolates. The expected size product was 244 bp. The agarose gel was stained with ethidium bromide to visualize the products and for size estimation, a 1 Kb ladder was run along side the samples. Two different 90-2 DNA samples were tested. The water PCR reaction sample contained no DNA and was tested to insure that there were no contaminants in the primers or other reaction constituents. 1) 90-2 (race 4); 2) 90-2 (race 4); 3) Alg 3-24 (race 5 positive control).

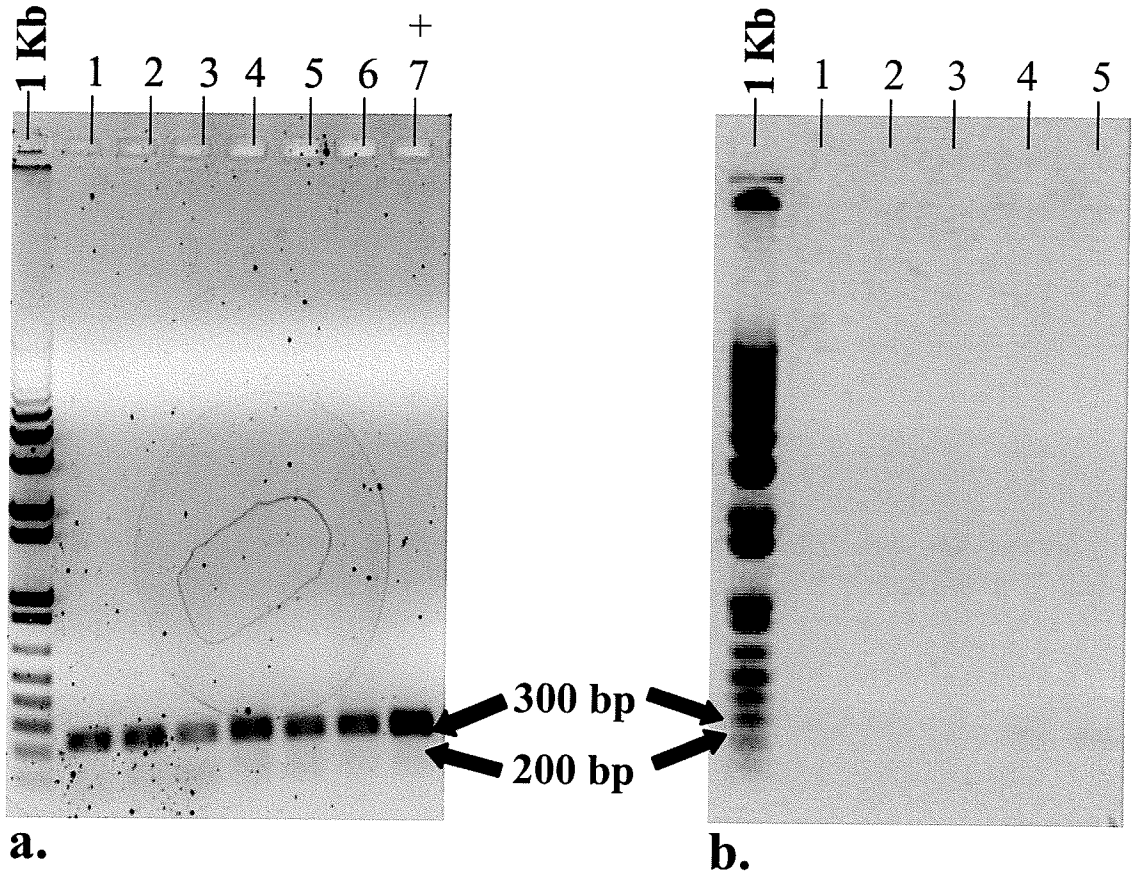


Fig. 14: TxB1 and TxB2 PCR amplification products of isolates of races 1 and 2. Agarose gel was stained with ethidium bromide and a 1 Kb ladder was run to visualize the expected size product of 245 bp. ICARDA was abbreviated 'I' **a.** PCR product when PCR cycle number was 40 and annealing temperature was 55°C. 1) 32JA; 2) I 33-4; 3) I 34-7; 4) 2004-38-1; 5) 2005-53-1; 6) NA 5-3; 7) Alg 3-24 (race 5 positive control for gel 'a'). **b.** PCR products of the same isolates when cycle number was decreased to 30 and annealing temperature was increased from 55°C to 57°C. ICARDA was abbreviated 'I'. 1) 32JA; 2) I 33-4; 3) I 34-7; 4) 2004-38-1; 5) 2005-53-1.

race 5 isolate Alg 3-24. The annealing site for primer R5P-F is found 205 bp upstream of the TATATAA promoter sequence. The annealing site of TxB7 is 93 bp downstream of the translational stop codon. The results from this primer set may elucidate differences outside of coding region in the form of insertions and deletions in different isolates, including those that do not induce ToxB-chlorosis but in which *ToxB*-like sequences have been found.

All tested isolates of races 5, 6, 7 and 8 amplified a product of the expected size of 757 bp (Table 5, 6, 8, and 9; Fig. 15). A product was expected because all of these isolates have been shown to induce ToxB-chlorosis on sensitive wheat cultivars. The similarity of product size suggested that the region between the primer annealing sites was conserved among isolates of different races.

The results of PCR amplification with R5P-F and TxB7 from the race 3 isolates were varied. The five Syrian isolates amplified a product of 1800 bp, much larger than the expected size of 757 bp (Table 7, Fig. 15). The larger product would suggest the presence of insertions in the sequence between the annealing sites of TxB1 and R5P-F. The presence of the insert may affect expression of *ToxB*. Isolate 94-115 amplified a product of the expected size while 331-9 and SC 29-1 were both unable to amplify a product, suggesting changes in the promoter region sequence (Table 7). The findings agreed with previous results that *ToxB*-like sequences occurred in race 3 isolates (Strelkov, 2002; Strelkov et al., 2006). No product was amplified in isolates of races 1 and 2 (Tables 3 and 4), consistent with the results with TxB1 and TxB2 because these races do not induce ToxB-chlorosis on the wheat differential set (Lamari and Bernier,

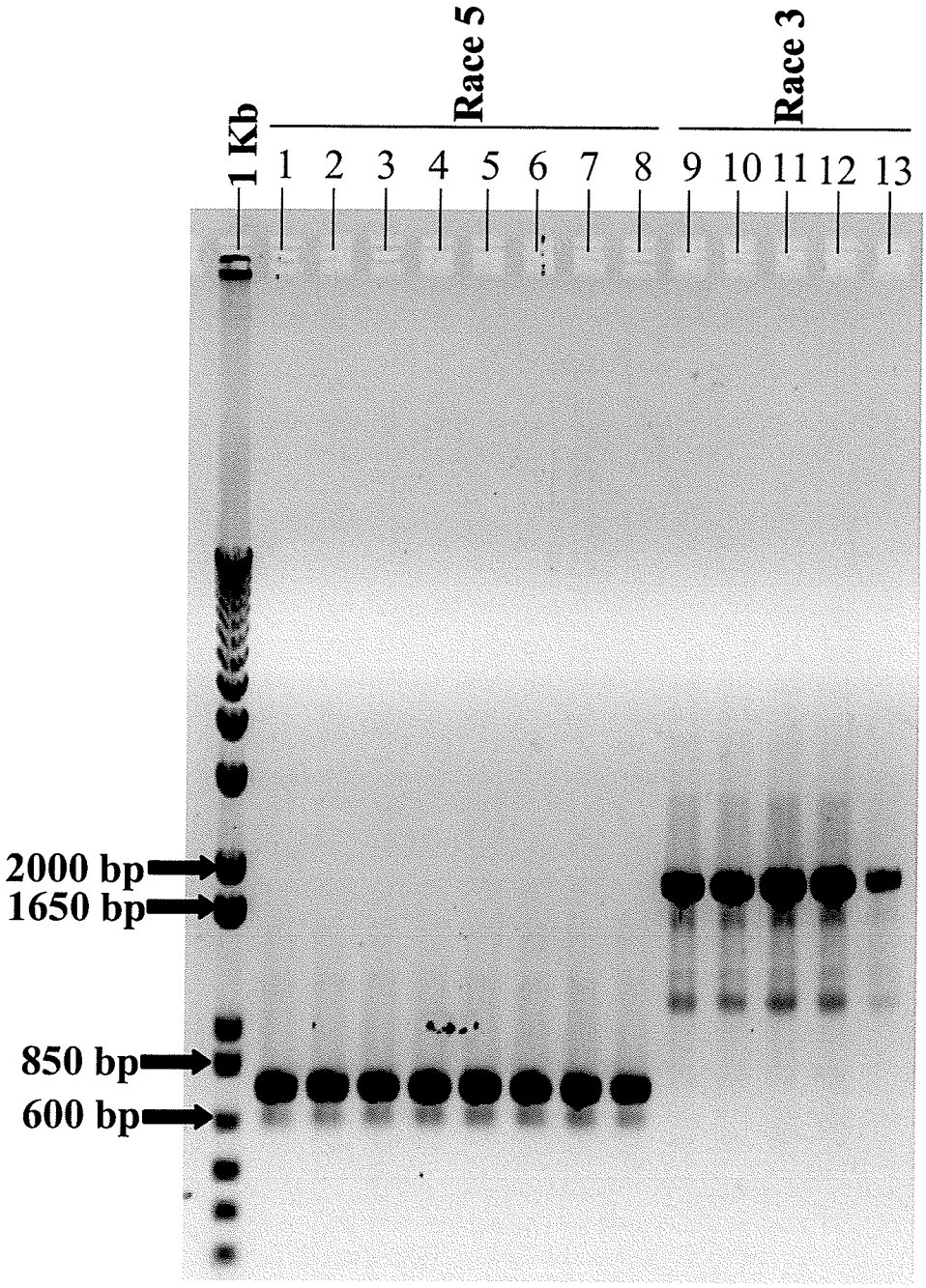


Fig. 15: PCR amplification products of race 3 and 5 isolates with RFP-F and TxB7. Based on a race 5 sequence, the expected product size was 757 bp. The agarose gel was stained with ethidium bromide and to visualize product size, a 1 Kb ladder was run alongside the samples. ICARDA was abbreviated 'I'. 1) I 17-1; 2) I 34-3; 3) I 35-20; 4) I 36-1; 5) I 73-3 6) NA 4-4; 7) Alg 3X-1; 8) Alg 3-24 (race 5 positive control); 9) I 72-1; 10) I 72-2; 11) I 72-3; 12) I 72-5; 13) I 72-7.

1989b). No product was amplified from 90-2 with primers R5P-F and TxB7 (Table 7), suggesting changes in sequences under the primer prevented the amplification of product.

The outer most primer set, TxBG1 and TxB7, was designed from the sequence of Alg 3-24, a race 5 isolate (accession number AY242115). This particular sequence contained 202 bp insert found in some *ToxB* sequences (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006) and the annealing location of TxBG1 is upstream of the insert. Based on the sequence from Alg 3-24, the expected size of the PCR product was 1224 bp with the insert. The size of the product will determine the presence of the 202 bp insert or other insertion or deletion changes between TxBG1 and R5P-F.

Isolates of races 5, 6, 7, and 8 amplified a product with TxBG1 and TxB7 (Tables 5, 6, 8, and 9, Fig. 16). This was expected as these races were classified based on their ability to induce ToxB chlorosis on the appropriate wheat cultivars. In 12 isolates of races 5, 6, 7, and 8, the amplified product was 1224 bp (the expected size) while in the remaining isolates the amplified product was approximately 1000 bp. The difference between them was approximately 200 bp, the size of the insert. Although the insert was previously described in race 5 isolates, it was found distributed amongst all ToxB phenotype isolates. There were isolates of races 5, 6, 7 and 8 that amplified both products simultaneously (Fig. 16, Tables 5, 6, 8, and 9).

The five race 3 Syrian isolates amplified a band of 2100 bp in size with TxBG1 and TxB7, much larger than expected (Table 7, Fig. 17). These results, taken with the PCR amplification results with R5P-F and TxB7, suggest that there are insertions present between the coding region and the annealing sites in these particular race 3 isolates. Isolate 94-115 amplified a product approximately 1000 bp, very much like ToxB-

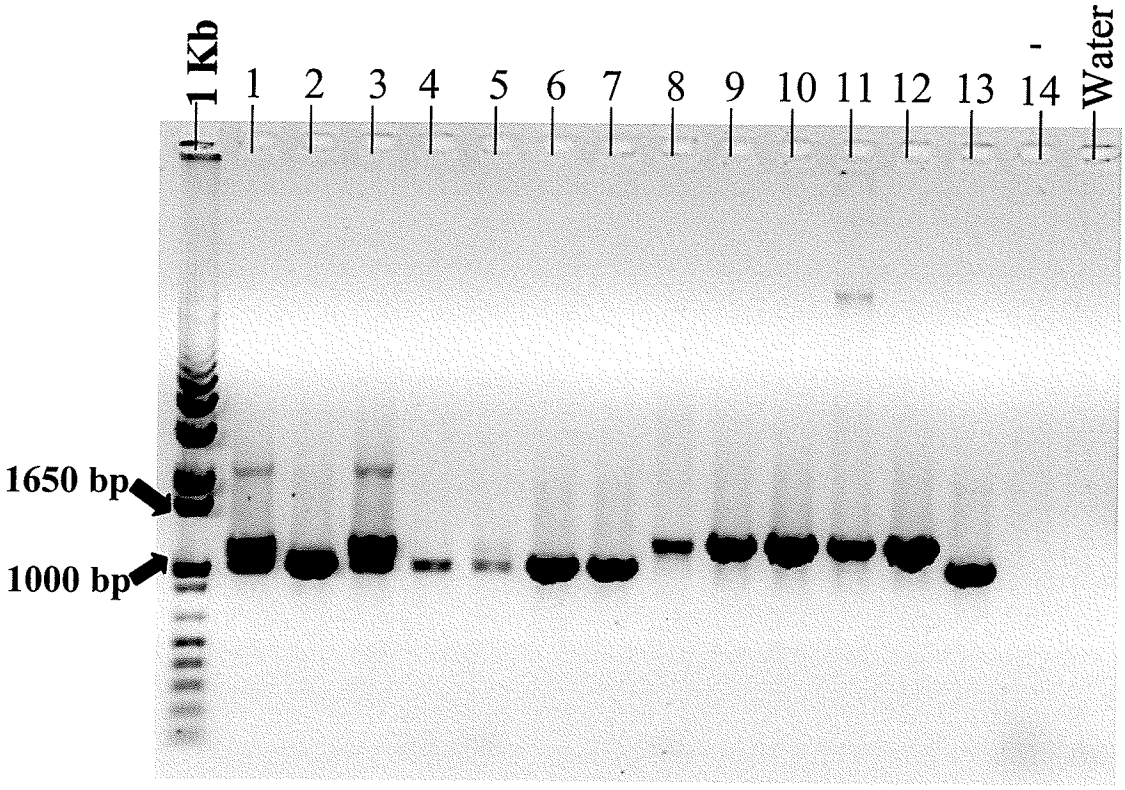


Fig. 16: PCR amplification products of race 8 isolates with TxBG1 and TxB7. The expected size of the product was 1224 bp, although some isolates were able to amplify a product that was smaller than expected (1000 bp) or both sizes of product. The agarose gel was stained with ethidium bromide to visualize the products. Only race 8 isolates were shown but the size variation also occurred in races 5, 6, and 7. The water PCR reaction sample contained no DNA and was tested to insure that there were no contaminants in the primers or other reaction constituents. ICARDA was abbreviated 'I'. 1) I 17-11; 2) I 31-1; 3) I 31-2; 4) I 32-2; 5) I 33-1; 6) I 34-1; 7) I 34-2; 8) I 35-6; 9) I 35-13; 10) I 35-16; 11) I 35-18; 12) I 35-19; 13) I 42-14; 14) 86-124 (race 2 negative control).

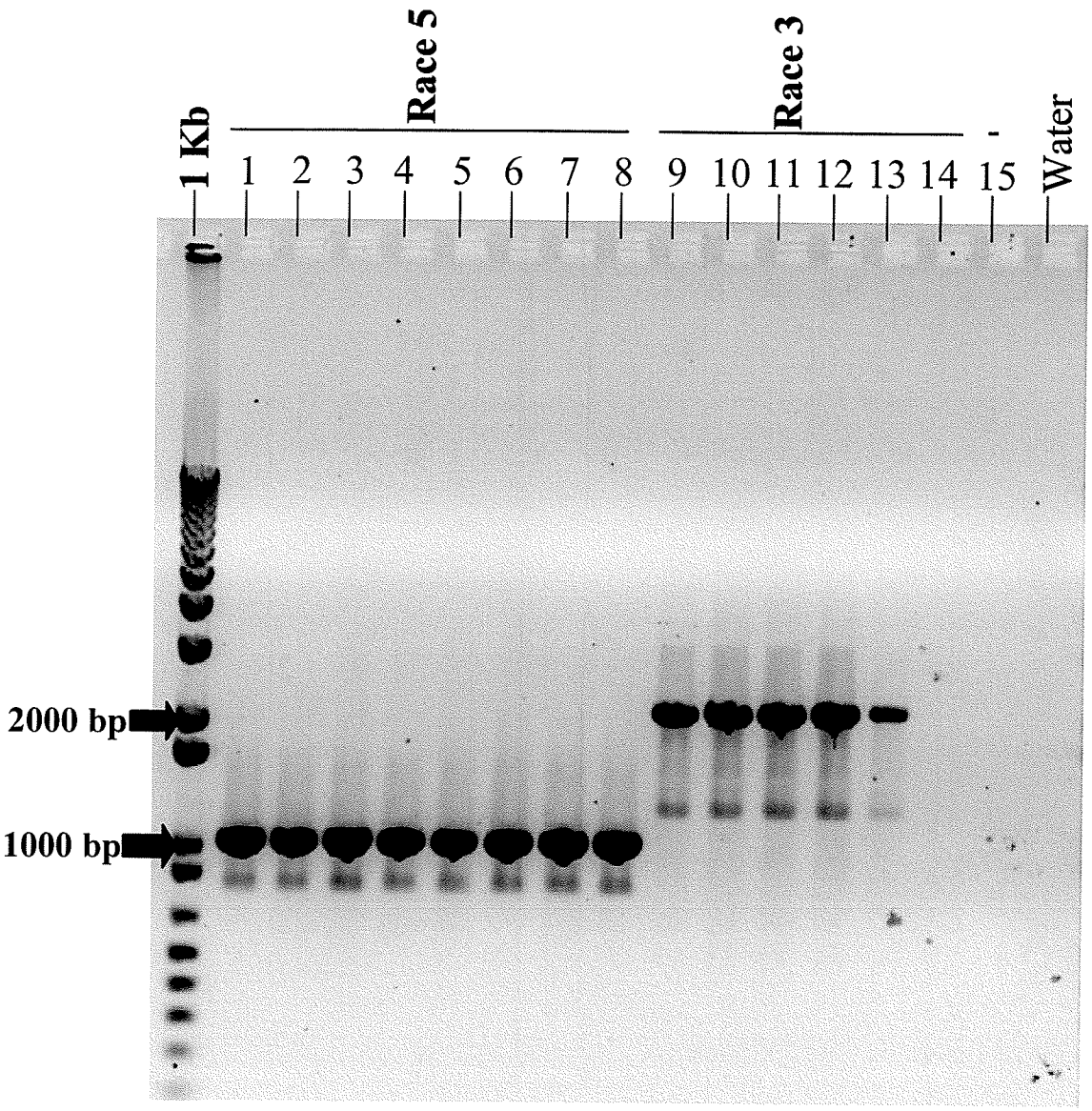


Fig. 17: The PCR products of race 3 ICARDA isolates and TxBG1 and TxB7. The expected size product of TxBG1 and TxB7 is 1216 bp but these particular race 5 isolates amplified a product of 1000 bp. The products amplified by the race 3 ICARDA isolates are approximately 2100 bp. The agarose gel was stained with ethidium bromide and a 1 Kb ladder was run along side the samples for size estimation. The water PCR reaction sample contained no DNA and was tested to insure that there were no contaminants in the primers or other reaction constituents. ICARDA was abbreviated 'I'. 1) I 17-1; 2) I 34-3; 3) I 35-1; 4) I 73-2; 5) NA 6-7; 6) NA 6-8; 7) NA 7-2; 8) NA 7-8; 9) I 72-1; 10) I 72-2; 11) I 72-3; 12) I 72-5; 13) I 72-7; 14) SC 29-1; 15) 86-124 (race 2 negative control).

phenotype isolates. This may indicate the presence of a sequence very similar to *ToxB*. No product was amplified from 331-9 and SC 29-1 (Table 7), indicating that sequences homologous to the primer sequences were absent in these isolates.

The race 4 isolate 90-2 amplified a product with TxB1 and TxB2 but no product was amplified with TxBG1 and TxB7. It appeared that the only homologus *ToxB* region present in 90-2 was the coding region. In isolates of races 1 and 2 studied, the majority of isolates did not amplify a product with TxBG1 and TxB7 (Tables 3 and 4). These isolates do not induce ToxB-related chlorosis on the differential set nor have they been shown to have *ToxB* homologs (Lamari & Bernier, 1989b; Strelkov, 2002). In ten isolates of races 1 and 2, a PCR product was weakly amplified with TxBG1 and TxB7 (Fig. 18). The validity of these results was unknown but may be elucidated with further analysis with Southern blots and sequencing (see page 102 for results).

There were four isolates with questionable PCR results. Isolates NA 7-8 and NA7-9 were originally classified in race 2. However, neither set of the *ToxA* primer sets were able to amplify a product while all three *ToxB* primers amplified a product. Two ICARDA isolates, 32-2 and 33-1, initially classified as race 8 isolates, were unable to amplify a product with any of the *ToxB* primers. Based on the symptoms after reinoculation on the wheat differential set, NA 7-8 and 7-9 were race 5 isolates and ICARDA 32-2 and 33-1 were race 1 isolates. Thus the PCR results and inoculation results were consistent for these isolates.

The PCR results showed that the *ToxA* gene was present in all isolates of races that show the ToxA-phenotype and absent in isolates of races that did not demonstrate the

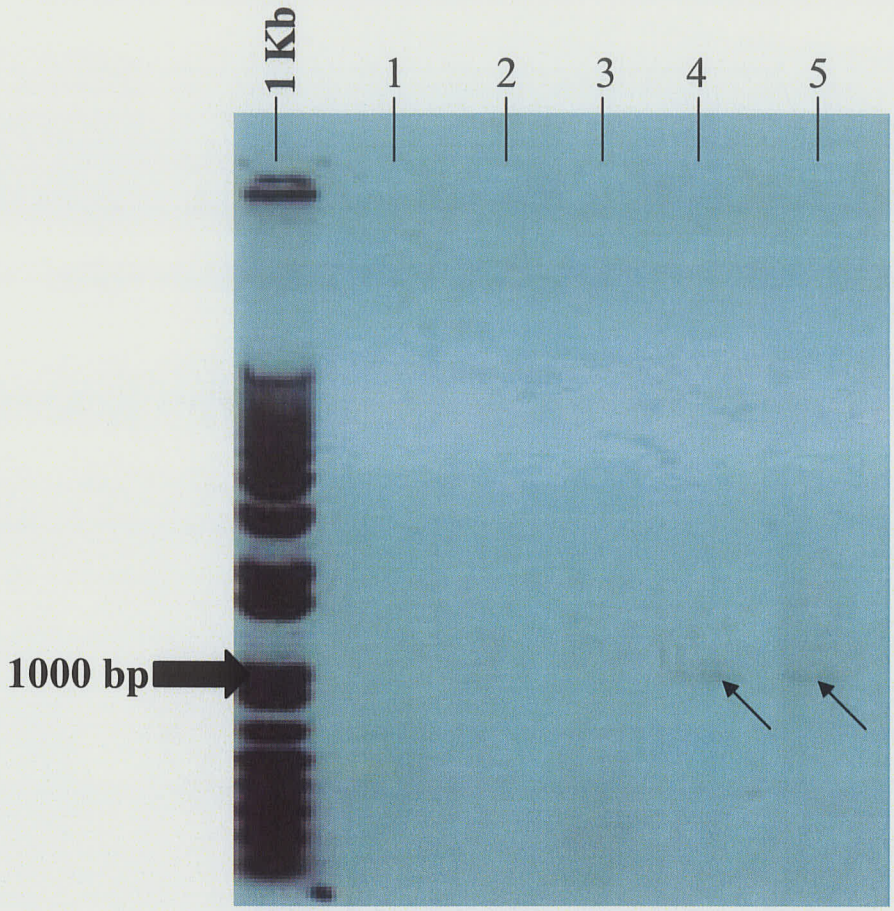


Fig. 18: The PCR products of race 1 isolates with TxBG1 and TxB7. Faint products approximately 1000 bp in size are visible. The agarose gel was stained with ethidium bromide to visualize bands and for size estimation, a 1 Kb ladder was run along side the samples. Although only race 1 isolates are shown, race 2 isolates also amplified the same faint product. ICARDA was abbreviated with 'I'. 1) I 1-5; 2) I 18-4; 3) 86-124; 4) NA 5-3; 5) 98-MD-8.

ToxA-phenotype on sensitive wheat cultivars. The lack of variation in product size demonstrated the absence of insertions and deletions. The race 3 and race 5 isolates proved to be exceptions with the external *ToxA* primer set but further analysis is needed to prove the validity of these results.

The *ToxB* primer sets amplified product in all isolates of races of the ToxB-phenotype. Product size variation was observed with primer set TxBG1 and TxB7. Based on the sequence used for constructing the primers which contained a 202 bp insert upstream of the coding region, the expected size product would be 1224 bp. Forms of *ToxB* lacking the insert have been found within the same isolate (Martinez et al., 2004; Strelkov et al., 2006). The 'smaller' sized product was amplified and indicated the absence of the 202 bp insert. In some isolates tested, both size products were amplified. *ToxB*-like sequences were detected by PCR in race 3 and race 4 isolates, although not shown to induce ToxB chlorosis. Isolates of races 1 and 2 were for the most part unable to amplify product with the *ToxB* primer sets, which confirmed the phenotype. There were a few isolates that weakly amplified *ToxB*-like products, but it remains to be seen if these were true products or anomalies, which will be addressed in the following sections.

PCR amplification was used as a screening tool to check for the presence or absence of the toxin genes and toxin-like genes, to detect mutations in the form of insertions and/or deletions and to verify the genotype to the phenotype found with inoculation of the wheat differential set. The PCR results seemed to confirm previous results that *ToxA* is conserved and is only found in isolates of races that show the ToxA-phenotype. The *ToxB* (or a *ToxB*-like) gene was amplified in isolates that showed the ToxB-phenotype (race 5, 6, 7, and 8) and in isolates with the ToxB-phenotype absent

(races 3 and 4), which is consistent with previous studies. Isolates of races 1 and 2 that amplified a product require further analysis. PCR analysis confirmed the phenotypes of isolates with genotypic evidence while further testing will be required on the isolates with PCR product amplification that did not match their respective phenotypes.

Southern Blot Analysis

The Southern blots were used here as a secondary confirmation method of the PCR results to verify the existence of *ToxA* and/or *ToxB* sequences in the genomes of selected isolates, and as a method to assess copy number of toxin genes in the isolates tested. Cleavage sites for *Xho*I and *Hind*III, the restriction enzymes chosen for digestion of genomic DNA, are not known to occur within the coding regions of either *ToxA* or *ToxB* based on genes sequenced to date. Therefore, each hybridizing band should represent a single copy as opposed to a split sequence.

For all gels, a control isolate, which had been treated in the same manner as the samples, was run and transferred with the samples. These control samples aided in assessing copy number. The control for *ToxA* probing was 86-124 (a race 2 isolate) and the *ToxB* control Alg 3-24, a race 5 isolate. Both of these isolates have been studied and characterized (Ballance et al. 1996, Strelkov, 2002, Strelkov et al. 2006). To estimate the size of the hybridizing fragment, a 1 Kb ladder was run along side the samples and transferred to the membrane. To assess copy number, the films were scanned and the density of each band was measured and compared to the control using the Alpha Innotech program. Although multiple copies have been shown to occur in Alg 3-24 (Strelkov, 2002; Strelkov et al., 2006), the hybridizing fragment with one *ToxB* copy was used as the baseline for density measurements.

ToxA

Previous studies have shown that *ToxA* is found as a single copy in the ToxA-producing isolates which have been examined (Ballance et al. 1996, Ciuffetti et al. 1997). However, these earlier studies examined a limited number of isolates. By evaluating more isolates from each of the ToxA-producing races, a more general understanding of the *ToxA* gene frequency in isolates was assured. DNA from 20 isolates of races 1, 2, 7, and 8 collected worldwide were probed with *ToxA*.

All of the 20 isolates tested hybridized a single band to the *ToxA* probe. The presence of a single band confirmed that *ToxA* was found as a single gene (Ballance et al., 1996; Ciuffetti et al., 1997). The size of the hybridizing fragment was the same in 14 of 20 isolates; the size was approximately 6500 bp (Fig. 19). Size variation was observed in 98-MD-7 (race 1), PDY-7 (race 1), ICARDA 17-10 (race 7), ICARDA 36-4 (race 7), ICARDA 35-33 (race 8), and ICARDA 42-14 (race 8). The North Dakota isolate, PDY-7, hybridized a band slightly larger than 6500 bp. The isolates of races 7 and 8 appeared to show the most variation in size of the hybridizing fragment (Fig. 19). For proper estimation of copy number, the DNA loads of each sample should be comparable. The hybridizing band of isolate NA 5-3 and SK 105-2 in particular appeared less intense than others (Fig. 19, #3 and 6). This was due to lower loading of sample initially as seen by ethidium bromide staining (Fig. 20, #3 and 6). All agarose gels which were blotted to membranes for Southern blot analysis are included for comparison of relative loading of relative loading of DNA.

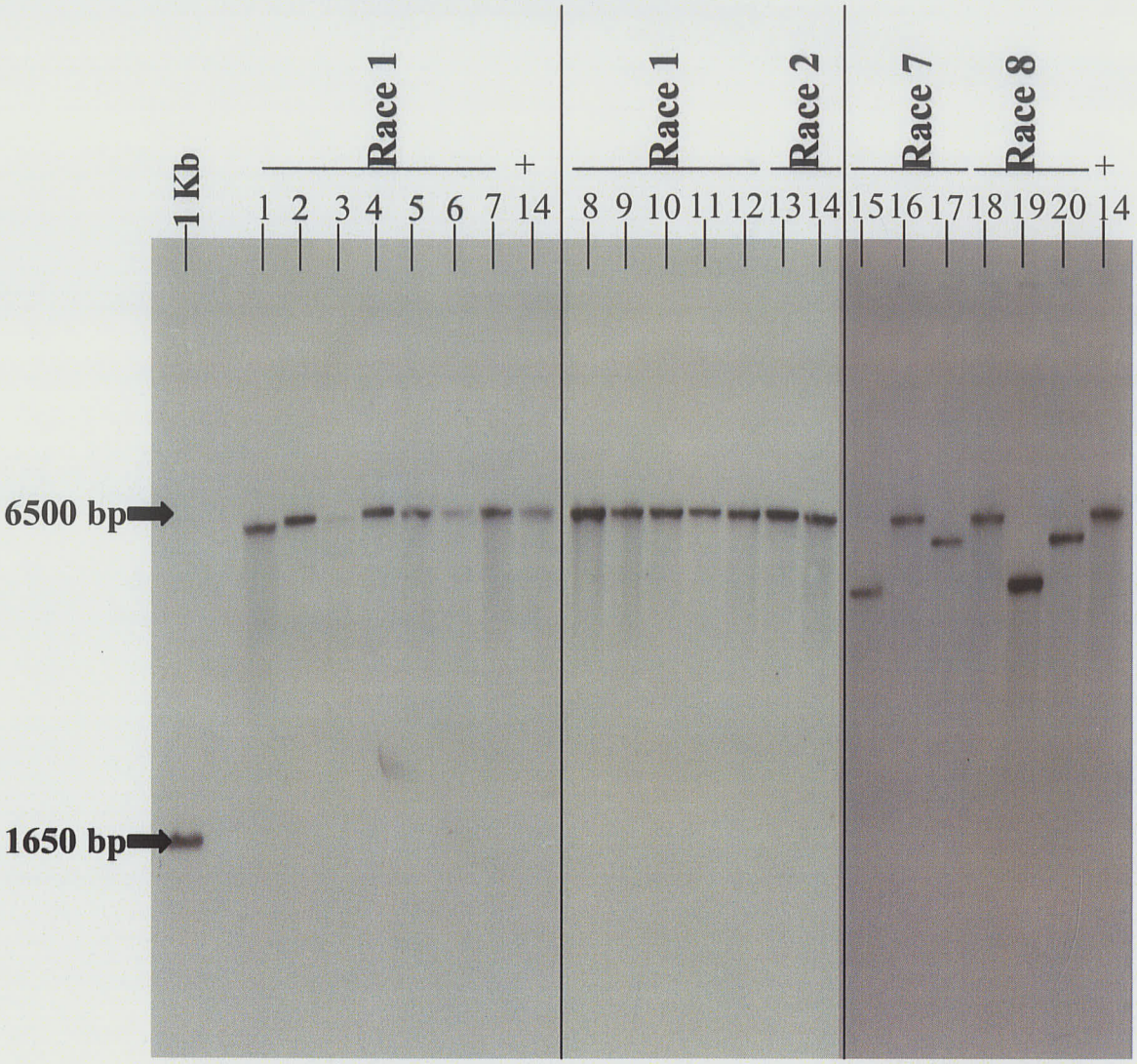


Fig. 19: Southern blots of ToxA-phenotype isolates probed with *ToxA* DNA. The DNA of 20 isolates of races 1, 2, 7, and 8 collected from different regions of the world was digested with *Xho*I, blotted and probed with *ToxA* DNA. The size of the hybridizing fragment was determined by comparison to the 1 Kb ladder. Isolate 86-124 was the control and is shown multiple times in the films. The image is a composite of three different films, which are separated by the vertical lines. The 1650 bp band from the 1 Kb ladder indicated. ICARDA was abbreviated 'I'. 1) 98-MD-7; 2) 99-49-2; 3) NA 5-3; 4) PDY-7; 5) SC 25-3; 6) SK 105-2; 7) UY 127-2; 13) 86-124 (race 2 control isolate); 8) I 1-5; 9) I 8-4; 10) I 18-4; 11) I 35-35; 12) I 67-4; 13) I 33-4; 14) 86-124; 15) I 17-10; 16) I 35-24; 17) I 36-4; 18) I 17-11; 19) I 35-33; 20) I 42-14; 13) 86-124 (race 2 control isolate).

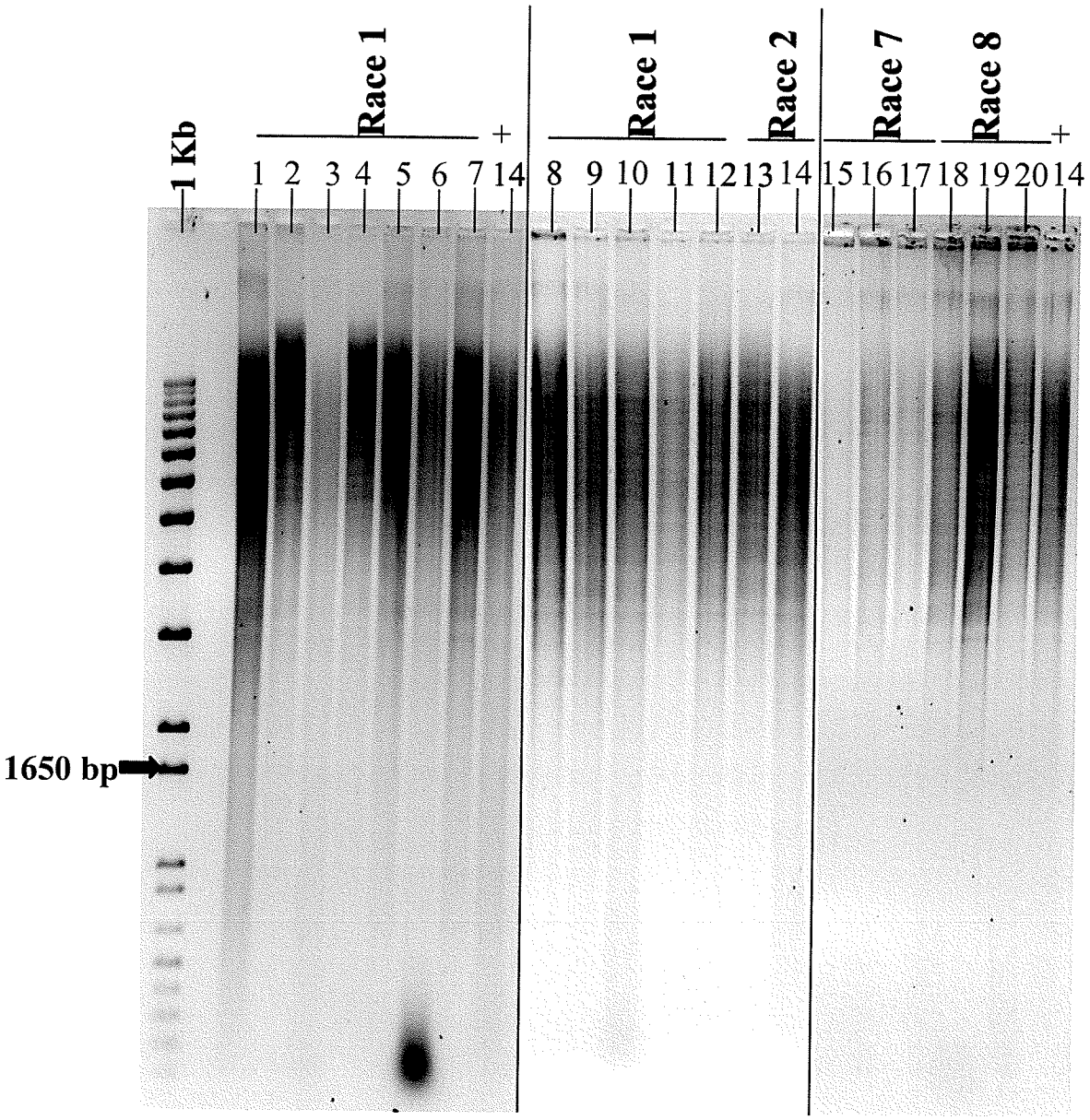


Fig. 20: *Xho*I-digested genomic DNA of isolates of races 1, 2, 7, and 8. The digested DNA was run on an agarose gel prior to blotting to a membrane. A 1 Kb ladder was run alongside the samples for size estimation purposes. The agarose gel was stained with ethidium bromide to visualize the relative loads of each sample. The image is of the three agarose gels which are separated by the vertical lines. ICARDA was abbreviated 'I'. 1) 98-MD-7; 2) 99-49-2; 3) NA 5-3; 4) PDY-7; 5) SC 25-3; 6) SK 105-2; 7) UY 127-2; 13) 86-124 (race 2 control isolate); 8) I 1-5; 9) I 8-4; 10) I 18-4; 11) I 35-35; 12) I 67-4; 13) I 33-4; 14) 86-124; 15) I 17-10; 16) I 35-24; 17) I 36-4; 18) I 17-11; 19) I 35-33; 20) I 42-14; 13) 86-124 (race 2 control isolate).

There appeared to be no correlation between hybridizing fragment size and region of collection of isolates. The Azerbaijan isolates showed the greatest amount of variation in the size of the hybridizing band (Fig. 21). Figure 21 is a computer rendered representation of all isolates, done with the Karyograph program (Lamari, personal communication) showed the size of the hybridizing fragment and region of collection. Table 10 is a list of all the ToxA-phenotype isolates probed with *ToxA*.

Table 10: The twenty isolates probed with *ToxA* for Southern blot analysis. These isolates were of races 1, 2, 7, and 8, the known ToxA phenotype isolates collected from different regions of the world.

Isolate	Race	Region of Collection
98-MD-7	1	Morden, Canada
99-49-2	1	Manitoba, Canada
NA 5-3	1	North Eastern Algeria
PDY-7	1	North Dakota, USA
SC 25-3	1	Swift Current, Canada
SK 105-2	1	Saskatchewan, Canada
UY 127-2	1	Uruguay, South America
ICARDA 1-5	1	Kazakhstan
ICARDA 8-4	1	Kyrgyzstan
ICARDA 18-4	1	Azerbaijan
ICARDA 35-35	1	Azerbaijan
ICARDA 67-4	1	Uzbekistan
ICARDA 33-4	2	Azerbaijan
86-124	2	Portage La Prairie, Canada
ICARDA 17-10	7	Azerbaijan
ICARDA 35-24	7	Azerbaijan
ICARDA 36-4	7	Azerbaijan
ICARDA 17-11	8	Azerbaijan
ICARDA 35-33	8	Azerbaijan
ICARDA 42-14	8	Azerbaijan

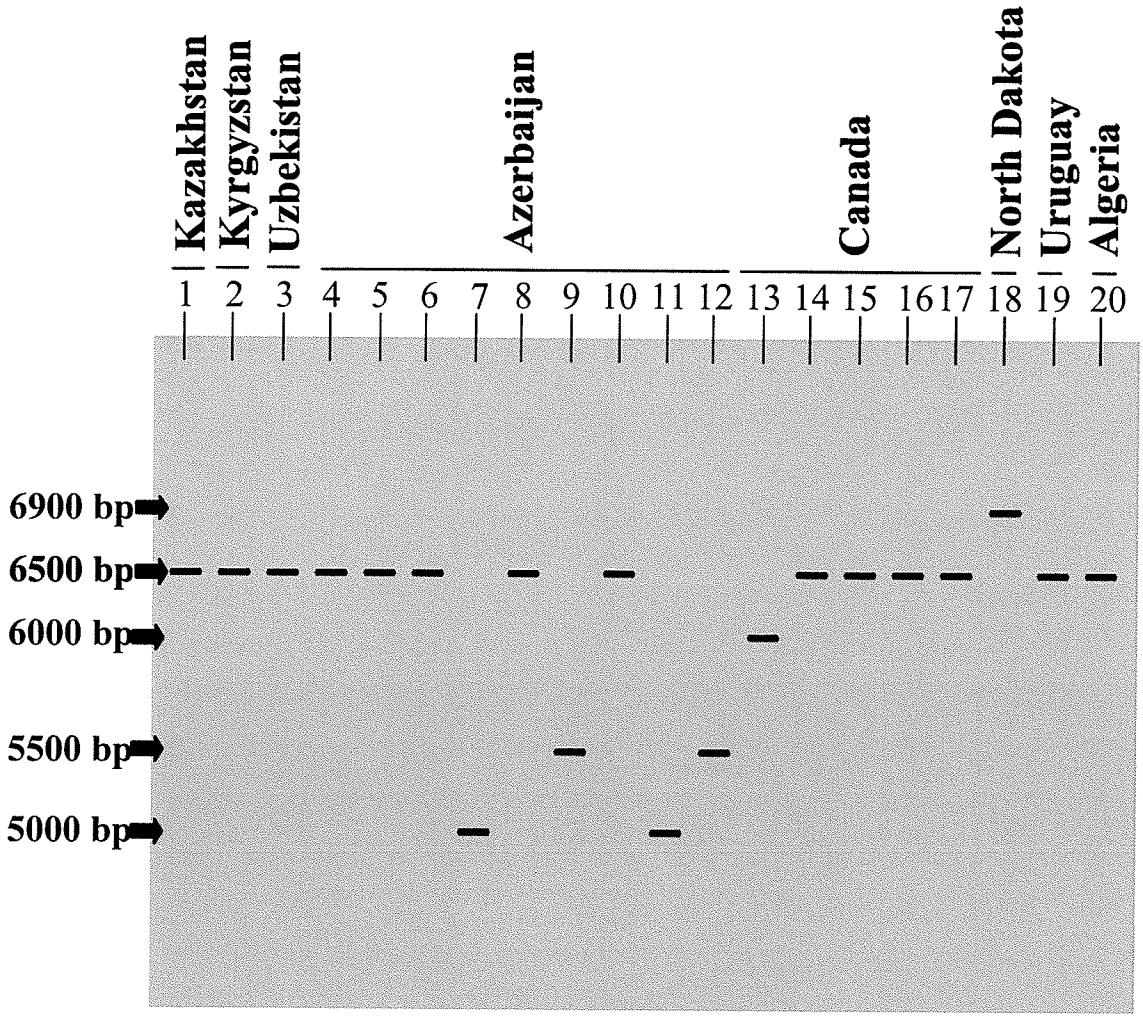


Fig. 21: The 'Karyograph' image of Southern blots of ToxA-phenotype isolates organized by region of collection. The isolates were of races 1, 2, 7, and 8, digested with restriction enzyme *Xho*I, blotted, and probed with *ToxA* DNA. The bands were generated using the Karyograph program and the size of each band is indicated. Isolates are grouped by region of collection. ICARDA was abbreviated 'I'. 1) I 1-5; 2) I 8-4; 3) I 67-4; 4) I 18-4; 5) I 33-4 6) I 35-35; 7) I 17-10; 8) I 35-24; 9) I 36-4; 10) I 17-11; 11) I 35-33; 12) I 42-14; 13) 98-MD-7; 14) 99-49-2; 15) SC 25-3; 16) SK 105-2; 17) 86-124; 18) PDY-7; 19) UY 127-2; 20) NA 5-3.

There were isolates of races 3 and 5 that amplified a product with the *ToxA* primers. To confirm if *ToxA*-like sequences were being detected, isolates of race 3 (5) and race 5 (7) were probed with *ToxA*. None of the isolates hybridized the *ToxA* probe, indicating the absence of a *ToxA*-like sequence in these isolates, agreeing with previous results of Ballance et al. (1996), Ciuffetti et al. (1997), and Lamari et al. (2003). Due to the absence of a *ToxA* hybridizing band, the PCR products amplified in isolates of races 3 (ICARDA 72-3) and 5 (ICARDA isolates 34-3, 35-20 and 35-57) may have been contaminants and unrelated to the *ToxA* gene.

Probing with *ToxA* DNA confirmed the presence of a *ToxA* sequence in ToxA-phenotype isolates. In the isolates tested, a single copy was found. The variation in band size could indicate the position of restriction sites for *Xho*I varied in the genomes of isolates tested. A *ToxA*-like sequence was absent in all non ToxA-phenotypes tested.

ToxB

The DNA used for the *ToxB* probe was the PCR amplified product of TxB1 and TxB2 of Alg 3-24, a race 5 isolate. This probe contained a portion of the coding sequence of the *ToxB* gene. It has been shown previously that *ToxB* exists as multiple copies in races 5, 6, 7 and 8 (Strelkov, 2002; Lamari et al. 2003; Martinez et al. 2004; Strelkov et al. 2006), with some race 5 isolates reportedly containing 8 to 10 copies (Strelkov, 2002; Martinez et al., 2004; Strelkov et al. 2006). Assessing *ToxB* copy number proved to be more difficult than with *ToxA* due to the common size of several hybridizing restriction fragments and because 8-10 copies are more difficult to assess than 1 copy. Alg 3-24 was the control isolate for which the estimated copy number is between 8 and 10 (Strelkov, 2002; Strelkov et al., 2006). In total, 25 isolates from races

5 (4), 6 (5), 7 (4) and 8 (12) were probed with the *ToxB* probe to assess copy number. Based on previous results, multiple copies of *ToxB* would be expected. Copy number was assessed as previously described.

Among the four race 5 isolates probed with *ToxB* DNA, the pattern of hybridizing bands varied from a single band to multiple bands and the intensities of each band were indicative of copy number. The arrows and numbers by each of the bands is an example of how copy number was determined for each isolate. The agarose gel of the restriction enzyme-digested genomic DNA was included to compare the relative amount of DNA loaded (Fig. 22a). Isolate Alg 3-24 hybridized five fragments with varying intensities: 2700, 1800, 1650, 900 and 875 bp (Fig. 22b, #1) for a total copy number of nine. Isolate Alg 4X1-1 hybridized the probe at six locations: 2700, 2500, 1800, 1650, 900, and 875 bp (Fig. 22b, #2) and the total copy number was ten. ICARDA 17-1 and NA 7-9 hybridized a single band at 1650 bp (Fig. 22b, #3 and 4) and the copy number was estimated to be eight.

Five Algerian race 6 isolates were probed with *ToxB* DNA. The agarose gel with the restriction enzyme-digested DNA was included for comparison of relative DNA loading (Fig. 23a). Isolate Alg H-1 was found to have one hybridizing band at 1650 bp with an estimated eight copies (Fig. 23b, #1), the same as two race 5 isolates, ICARDA 17-1 and NA 7-9. The other four isolates were found to have five hybridizing bands at 2700, 1800, 1650, 900, and 875 bp (Fig. 23b, #2 to 5), with an estimated *ToxB* copy number of nine. Although Alg H2-G appears more intense, the amount of DNA loaded initially appeared higher (Fig. 23a, #4). The hybridizing patterns of Alg H2, Alg H2-A, Alg H2-G, and Alg H2-G on 6B365 all resembled Alg 3-24 (Fig. 23b, #6).

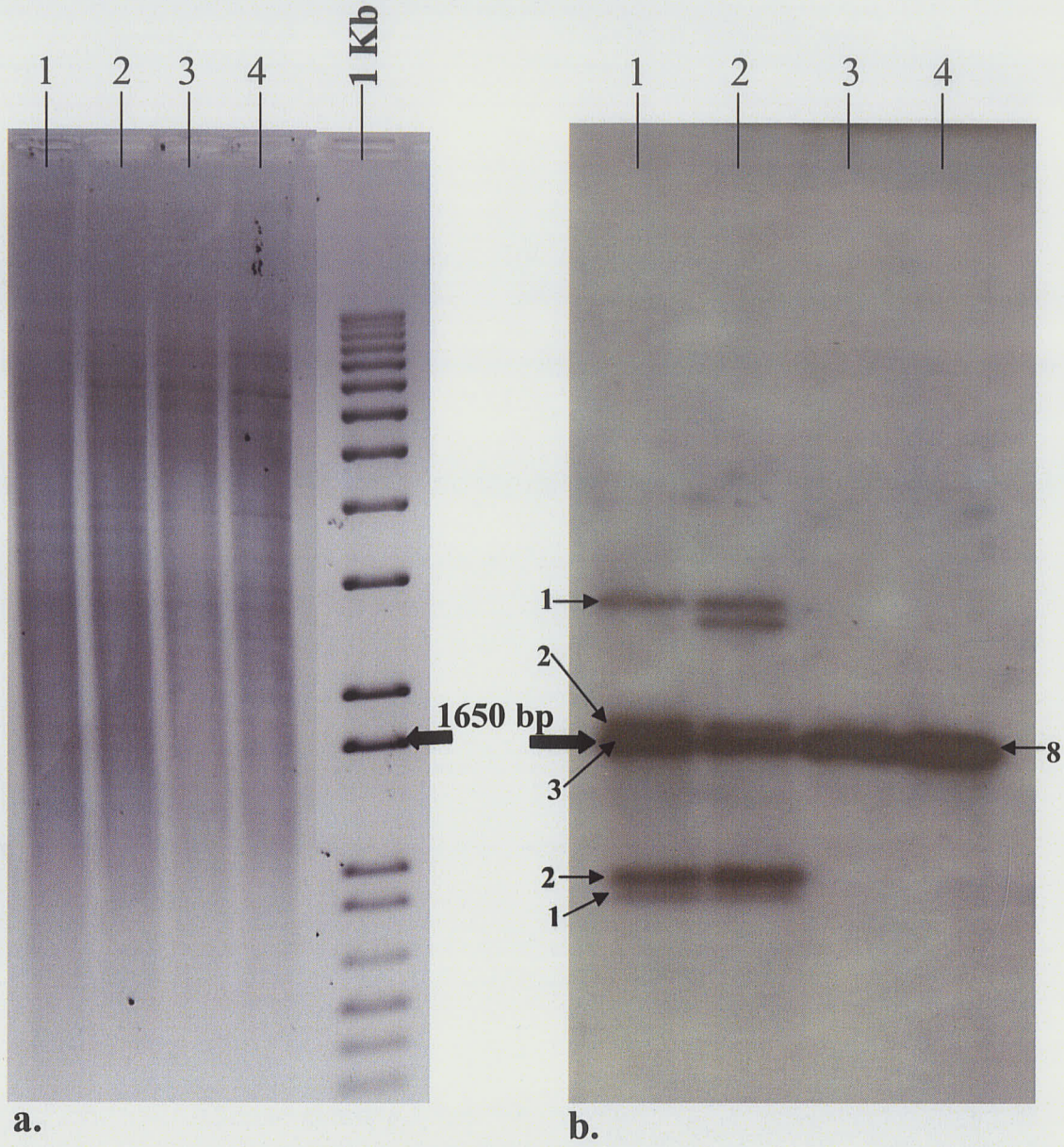


Fig. 22: The agarose gel and Southern blots of four race 5 isolates probed with *ToxB* DNA. **a.** Genomic DNA was digested with *Hind*III and *Xho*I, and run on a agarose gel. The 1650 bp band, common to all five isolates is indicated and was determined by the 1 Kb ladder run alongside the samples during agarose gel electrophoresis. The gel was stained with ethidium bromide for DNA visualization and comparison of relative loading intensities. ICARDA was abbreviated 'I'. 1) Alg 3-24; 2) Alg 4X1-1; 3) I 17-1; 4) NA 7-9. **b.** The X-ray film of race 5 isolates probed with *ToxB*. The arrows (→) and numbers by samples 1 and 4 indicate the estimated copy number of *ToxB* of the hybridizing fragment. ICARDA was abbreviated 'I'. 1) Alg 3-24; 2) Alg 4X1-1; 3) I 17-1; 4) NA 7-9.

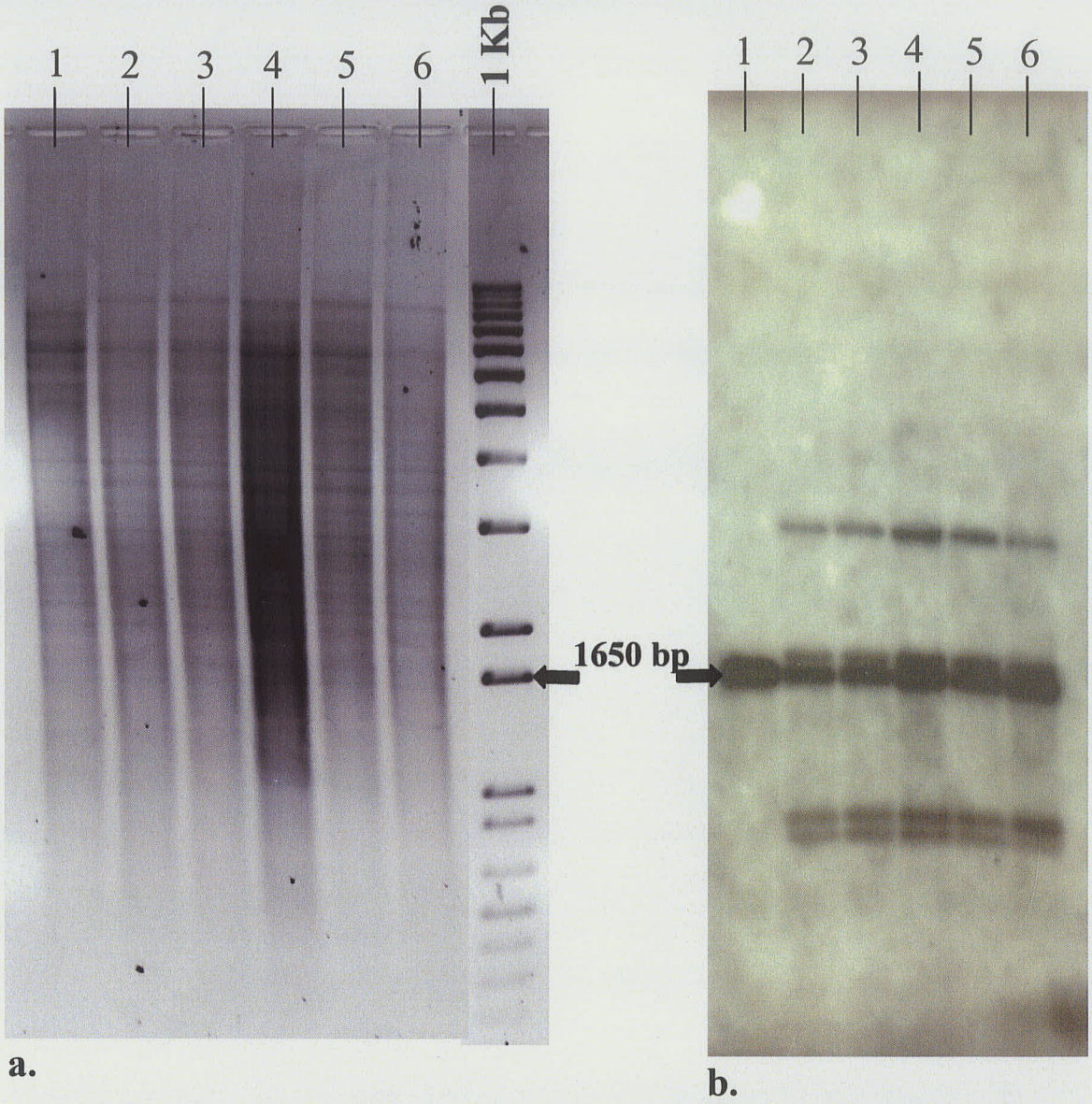


Fig. 23: The agarose gel and Southern blots of five race 6 isolates probed with *ToxB* DNA. **a.** The genomic DNA of isolates was digested with *Hind*III and *Xho*I and run on an agarose gel with a 1 Kb ladder alongside the samples for size estimation. The gel was stained with ethidium bromide to visualize relative DNA intensities. 1) Alg H-1; 2) Alg H-2; 3) Alg H2-A; 4) Alg H2-G; 5) Alg H2-G on 6B365; 6) Alg 3-24. **b.** The X-ray films after DNA was blotted, and probed with *ToxB* DNA. The race 5 control isolate, Alg 3-24, is also included. 1) Alg H-1; 2) Alg H-2; 3) Alg H2-A; 4) Alg H2-G; 5) Alg H2-G on 6B365; 6) Alg 3-24 (race 5 control isolate).

Among the five race 7 isolates tested, multiple hybridizing bands and multiple copies were found. The agarose gel was included for comparison of DNA loading (Fig. 24a). Isolates ICARDA 17-10 and 36-4 had three hybridizing bands at 2700, 1650, and 1275 bp with an estimated copy number of five (Fig. 24b, #1 and 3). Isolate 35-24 hybridized the *ToxB* probe at four locations (2700, 1900, 1800, and 1650 bp) with an estimated copy number of eight (Fig. 24b, #2). Isolate ICARDA 35-17 hybridized two bands at 2800 and 1650 bp with an estimated three copies of *ToxB* (Fig. 24b, #4). In four race 7 isolates tested, three different patterns of hybridization emerged and the copy number varied between isolates, although they were all collected from the same region.

In total, 12 race 8 isolates were probed and varying copy numbers. Isolate ICARDA 17-11 had three hybridizing fragments (2700, 1800, and 1650 bp) with an estimated copy number of six (Fig. 25, #1). Isolates ICARDA 31-1 and 31-2 had the same four hybridizing fragments (2700, 1900, 1800, and 1650 bp) and the copy number was eight (Fig. 25, #2 and 3), although it appeared that I 31-1 had more intense bands than I 31-2, the initial DNA loading of I 31-2 was less than I 31-2 (Fig. 26, #2 and 3). Five of the race 8 isolates probed had two hybridizing fragments at 2700 ad 1650 bp and the estimated copy number varied between two to five (Fig. 25, #7 to 11). Four isolates (ICARDA 34-1, 34-2, 35-6, 42-14) each hybridized a single fragment at 1650 bp (Fig. 25, #4 to 6, 12). Four copies were estimated in 34-1, 34-2 and 42-14 while the estimated copy number for 35-6 was two. The copy number of the isolates were variable and differences could be due to lower levels of DNA loading. Fig. 26 shows the agarose gels that were used to blotting of the membranes and subsequent probing with *ToxB*. Table 11 summarizes all the isolates that were probed with *ToxB* and the estimated copy number.

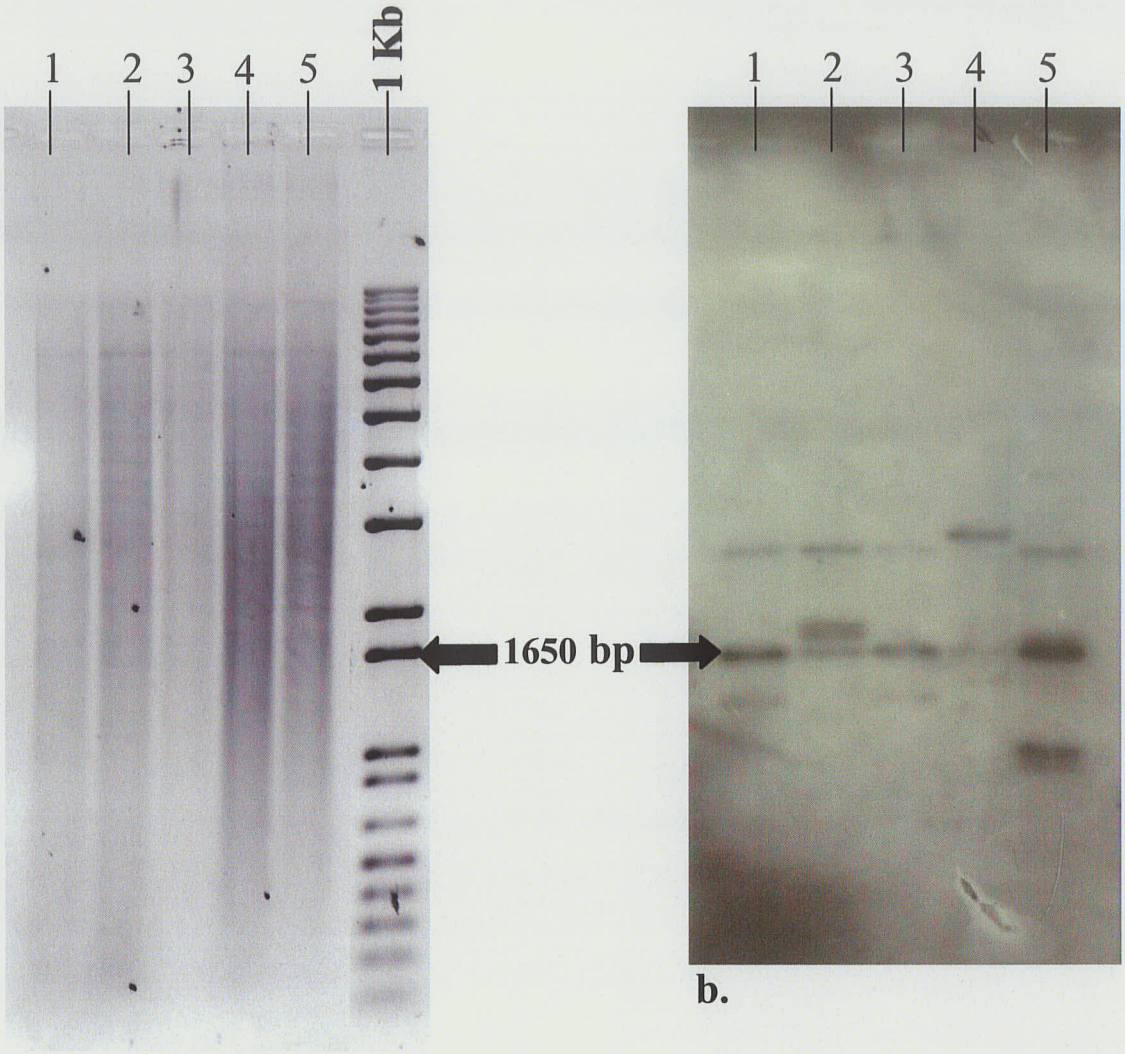


Fig. 24: The agarose gel and Southern blots of four race 7 isolates probed with *ToxB* DNA. **a.** Genomic DNA of the isolates was digested with *Hind*III and *Xho*I and run on an agarose gel. A 1 KB ladder was run alongside the samples for size estimation purposes. Gels were stained with ethidium bromide to visualize DNA and compare the relative amounts loaded. ICARDA was abbreviated 'I'. 1) I 17-10; 2) I 35-24; 3) I 36-4; 4) I 35-17; 5) Alg 3-24. **b.** The films after DNA was blotted and probed with *ToxB* DNA. The control isolate, Alg 3-24 (race 5) is also probed for comparison. ICARDA was abbreviated 'I'. 1) I 17-10; 2) I 35-24; 3) I 36-4; 4) I 35-17; 5) Alg 3-24 (race 5 control isolate).

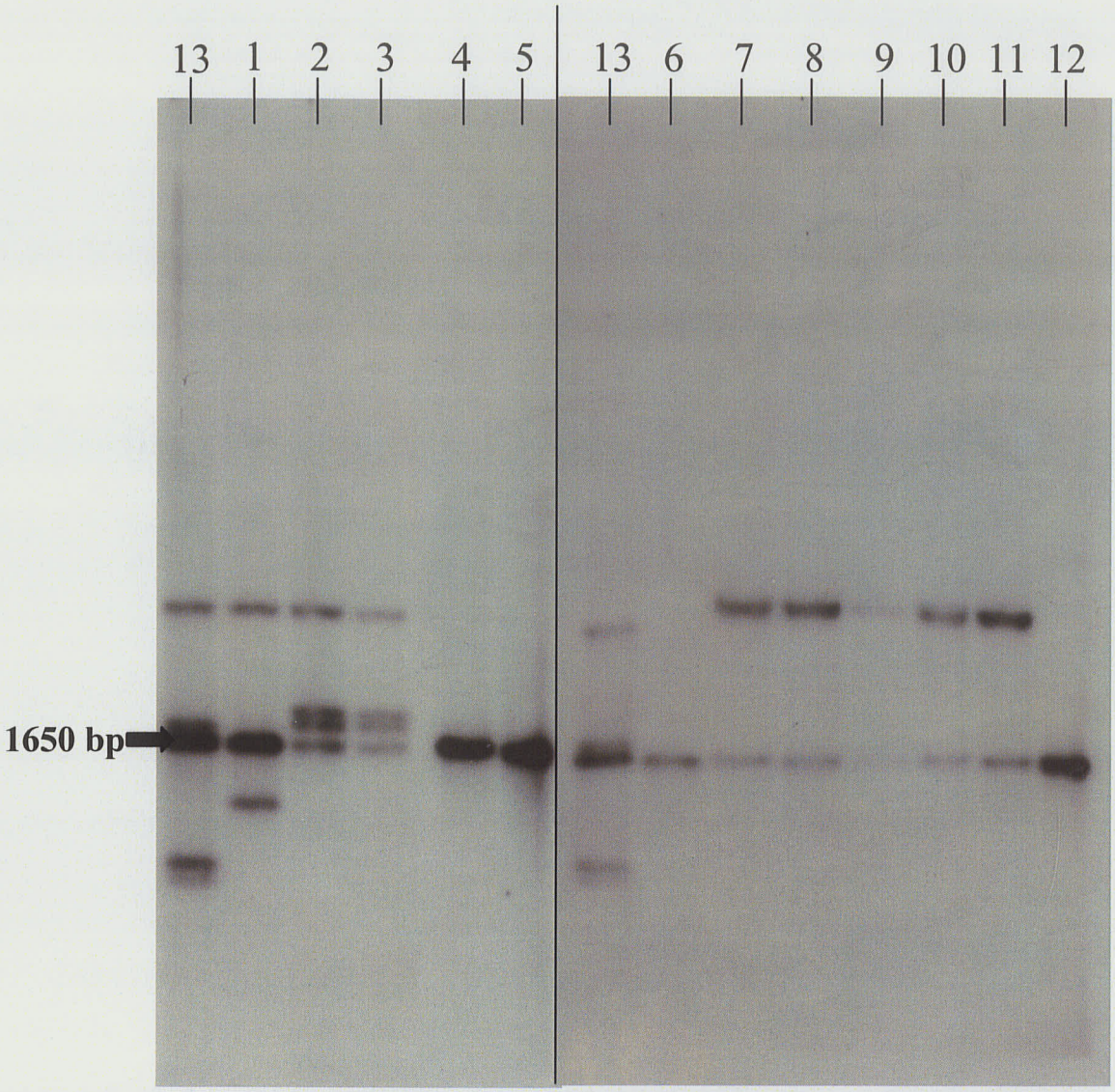


Fig. 25: Southern blots of 12 race 8 isolates probed with *ToxB* DNA. Genomic DNA was digested with *Hind*III and *Xho*I, blotted, and probed with *ToxB* DNA. Two films are shown here and are separated with a vertical line; the race 5 control, Alg 3-24, is shown on each film. The 1650 bp hybridizing band is indicated and was determined by running a 1 Kb ladder alongside the samples. For copy number estimation, Alg 3-24, the race 5 control isolate, was included. All race 8 isolates were collected from Azerbaijan. ICARDA was abbreviated 'I'. 1) I 17-11; 2) I 31-1; 3) I 31-2; 4) I 34-1; 5) I 34-2; 6) I 35-6; 7) I 35-13; 8) I 35-16; 9) I 35-18; 10) I 35-19; 11) I 35-33; 12) I 41-14; 13) Alg 3-24 (race 5 control isolate).

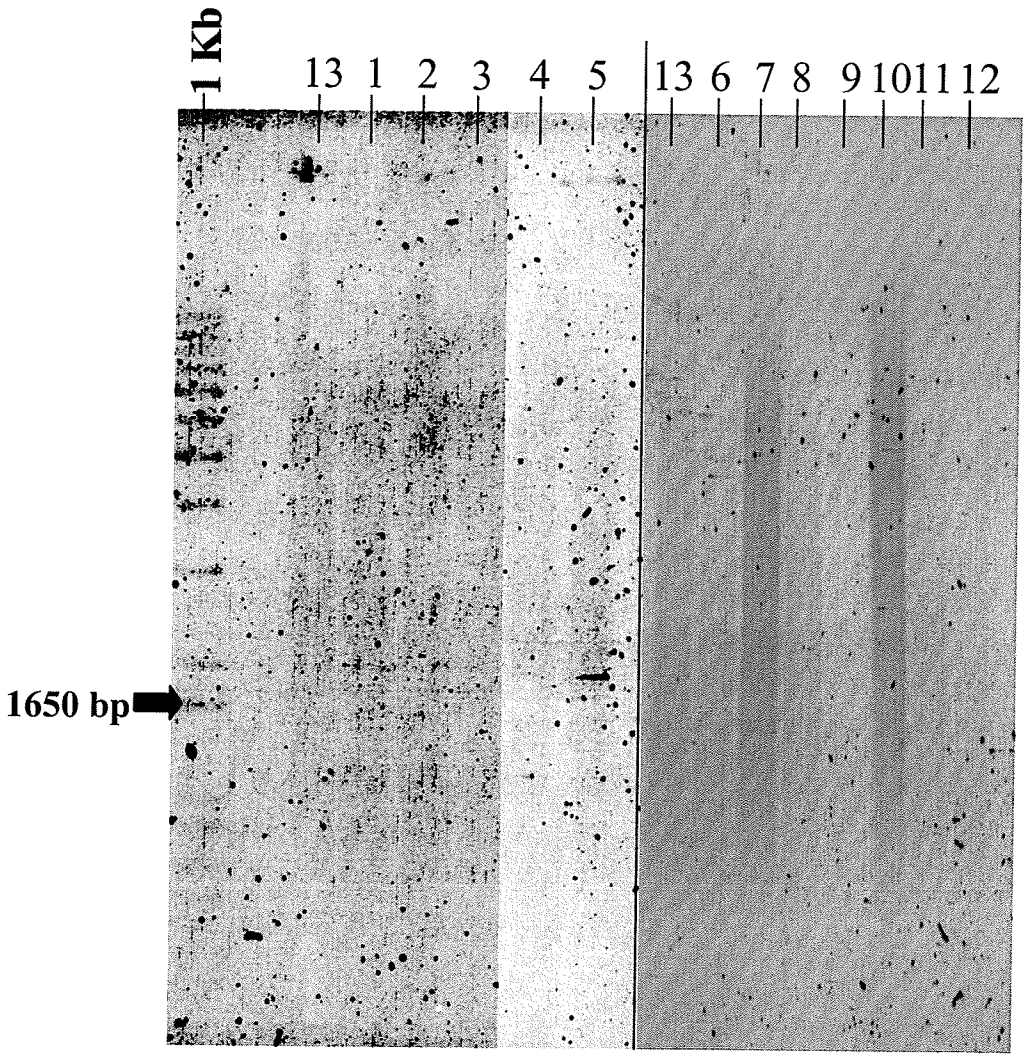


Fig. 26: The *Hind*III- and *Xho*I-digested genomic DNA of race 8 isolates run on agarose gels. Two gels were run and separated run by the vertical line. A 1 Kb ladder was run alongside the samples for size estimation purposes. The black spots are from the photographic paper and are not gel inclusions. The agarose gel shows five race 8 isolates with the race 5 positive control. ICARDA was abbreviated 'I'. 1) I 17-11; 2) I 31-1; 3) I 31-2; 4) I 34-1; 5) I 34-2; 6) I 35-6; 7) I 35-13; 8) I 35-16; 9) I 35-18; 10) I 35-19; 11) I 35-33; 12) I 41-14; 13) Alg 3-24 (race 5 control isolate).

Table 11: The twenty-five isolates probed with *ToxB* for Southern blot analysis. These isolates were of races 5, 6, 7, and 8, the known ToxB-phenotype isolates collected from different regions of the world. The region of collection, the total number of hybridizing fragments, and estimated copy number are included.

Isolate	Race	Region of Collection	Bands Present	Copy Number
Alg 3-24	5	Algeria	5	9
Alg 4x1-1	5	Algeria	6	10
ICARDA 17-1	5	Azerbaijan	1	8
NA 7-9	5	North Eastern Algeria	1	8
Alg H-1	6	Algeria	1	8
Alg H-2	6	Algeria	5	9
Alg H2-A	6	Algeria	5	9
Alg H2-G	6	Algeria	5	9
Alg H2-G on 6B365	6	Algeria	5	9
ICARDA 17-10	7	Azerbaijan	3	5
ICARDA 35-24	7	Azerbaijan	4	8
ICARDA 36-4	7	Azerbaijan	3	5
ICARDA 35-17	7	Azerbaijan	2	3
ICARDA 17-11	8	Azerbaijan	2	6
ICARDA 31-1	8	Azerbaijan	4	8
ICARDA 31-2	8	Azerbaijan	4	8
ICARDA 34-1	8	Azerbaijan	1	8
ICARDA 34-2	8	Azerbaijan	1	8
ICARDA 35-6	8	Azerbaijan	1	5
ICARDA 35-13	8	Azerbaijan	2	4
ICARDA 35-16	8	Azerbaijan	2	5
ICARDA 38-18	8	Azerbaijan	2	4
ICARDA 35-19	8	Azerbaijan	2	4
ICARDA 35-33	8	Azerbaijan	2	5
ICARDA 42-14	8	Azerbaijan	1	8

Figure 27 is the graphical representation of all isolates probed with *ToxB* assembled together for viewing of the similarities and differences that existed between isolates of different races.

Nine race 3 isolates and one race 4 isolate were probed with *ToxB* and were found to hybridize a single band. Previous studies have shown race 3 and 4 isolates were capable of hybridizing the *ToxB* probe which is indicative of the presence of a homologous *ToxB* sequence even though isolates do not produce *ToxB* as an expression phenotype (Strelkov, 2002; Martinez et al., 2004; Strelkov et al. 2006). Each isolate hybridized the *ToxB* probe once and at different size fragments. The ICARDA isolates all hybridized the probe to a 2000 bp fragment (Fig. 28, #1 to 5) while 331-9, 94-115, and SC 22-2 hybridized a 3000 bp fragment (Fig. 28, #6 to 8). Isolate SC 29-1, hybridized the probed to a fragment of 1300 bp (Fig. 28, #9). The race 4 isolate, 90-2 was shown previously to hybridize the *ToxB* probe (Fig. 28, #10). This time it did not, which can be attributed to lower levels of DNA initially loaded in the agarose gel (Fig. 29).

Previous studies have shown the absence of the *ToxB* gene in isolates of races 1 and 2 (Lamari et al. 2003; Strelkov, 2002; Martinez et al. 2004; Strelkov et al., 2006). However, PCR amplification performed on race 1 and 2 isolates showed a weakly amplified product in several isolates of the expected size. When these questionable isolates were examined by Southern blotting, no hybridizing fragments were detected. This is indicative that bands amplified by PCR were unrelated to *ToxB*.

Southern blot analysis with probes of *ToxA* and *ToxB* DNA confirmed the presence or absence of the toxin genes in isolates, confirming the phenotypes and PCR results. Although race 3 isolates have not been shown phenotypically to induce ToxB-

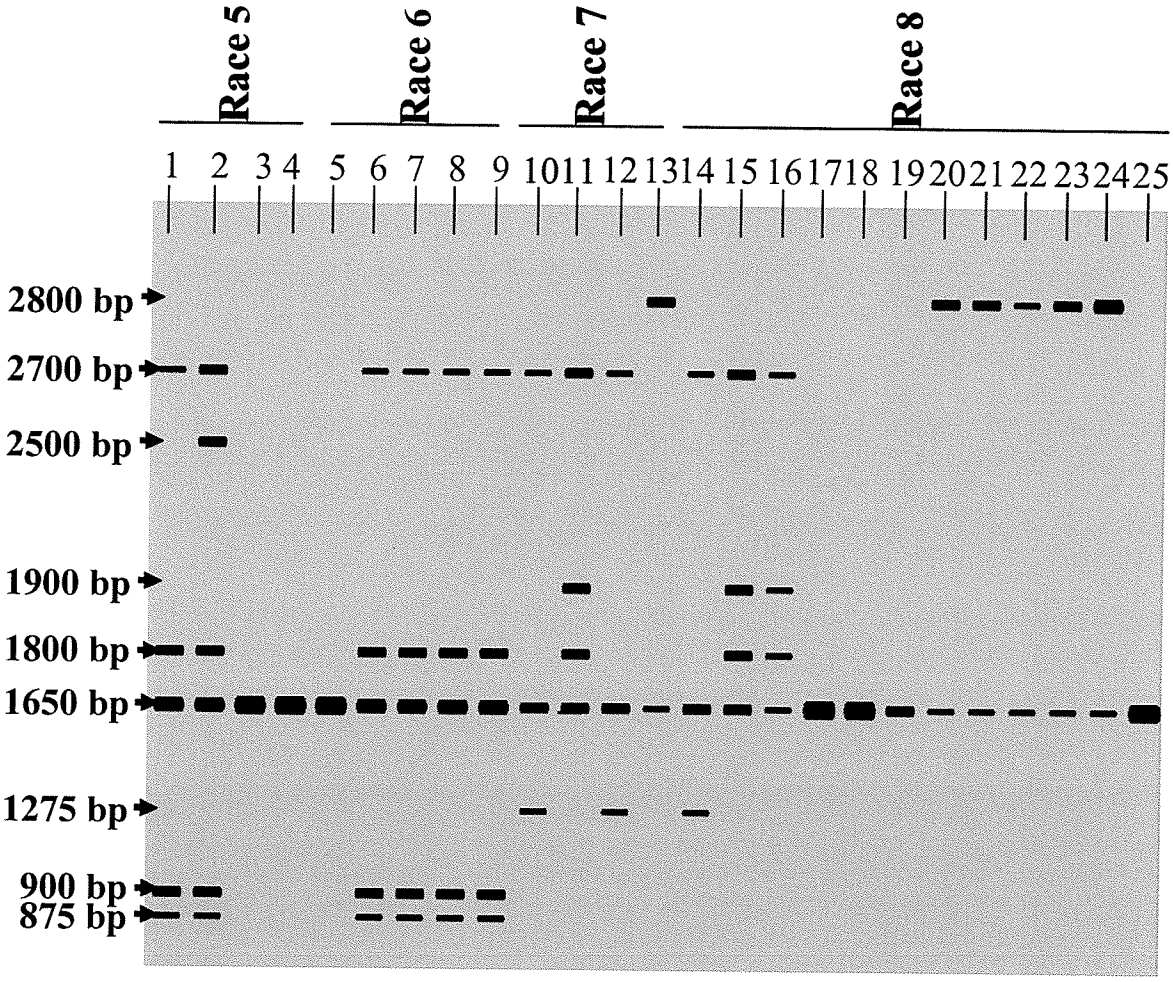


Fig. 27: The 'Karyograph' image of the Southern blot results of all 25 ToxB-phenotype isolates probed with *ToxB* DNA. The isolates were of races 5, 6, 7, and 8 and the hybridizing bands were represented graphically by the Karyograph program to demonstrate the variability in number and size of hybridizing fragments. The sizes of the hybridizing fragments are indicated and were determined from a 1 Kb ladder that was run alongside samples. ICARDA was abbreviated 'I'. 1) Alg 3-24; 2) Alg 4X1-1; 3) I 17-1; 4) NA 7-9; 5) Alg H-1; 6) Alg H-2; 7) Alg H-2A; 8) Alg H2-G; 9) Alg H-2G on 6B365; 10) I 17-10; 11) I 35-24; 12) I 36-4; 13) I 35-17; 14) I 17-11; 15) I 31-1; 16) I 31-2; 17) I 34-1; 18) I 34-2; 19) I 35-6; 20) I 35-13; 21) I 36-16; 22) I 35-18; 23) I 35-19; 24) I 35-33; 25) I 42-14.

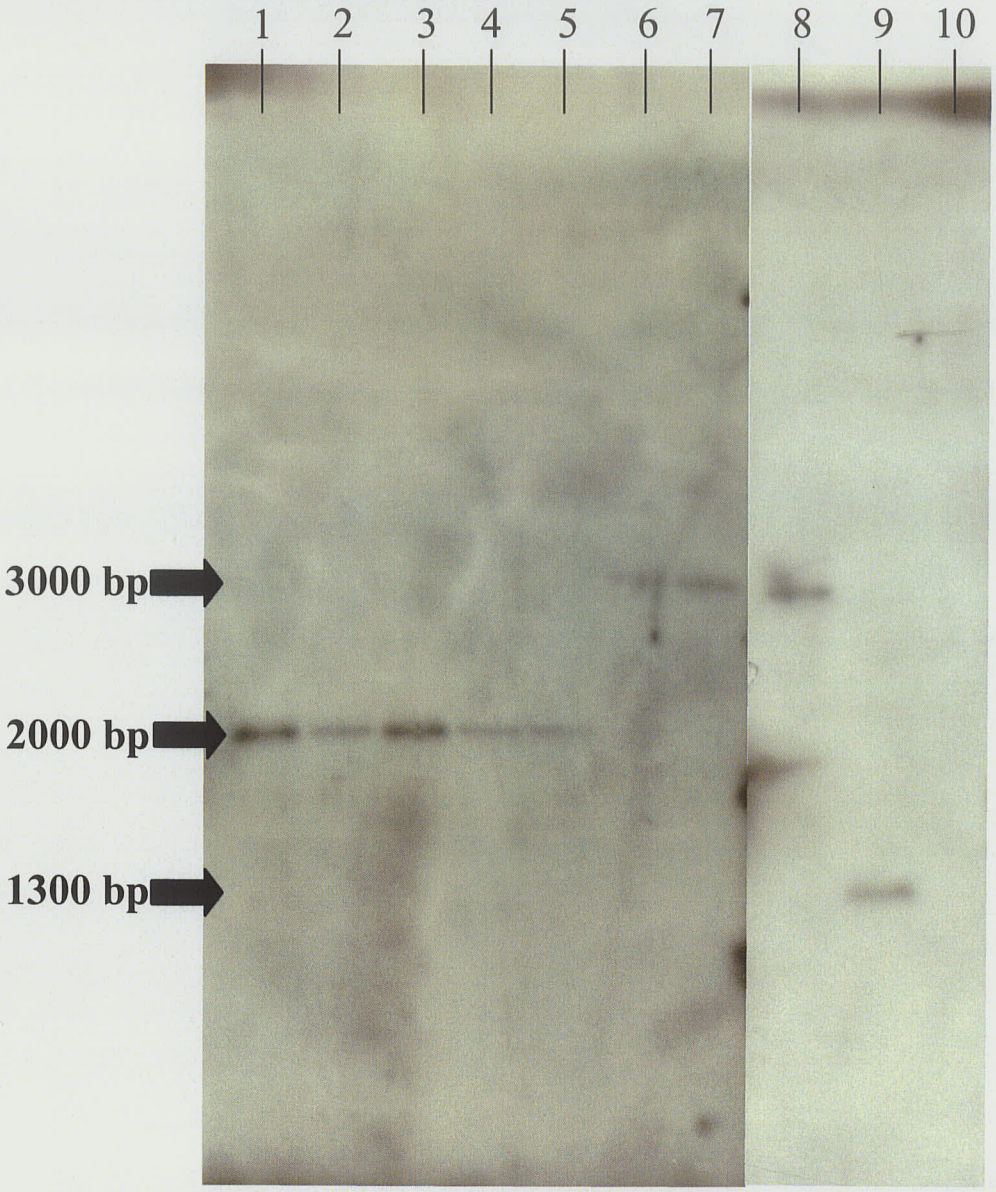


Fig. 28: Southern blots of race 3 and 4 isolates probed with *ToxB* DNA. The genomic DNA was digested with *Hind*III and *Xho*I, blotted, and probed with *ToxB* DNA. In total, there were nine race 3 isolates and one race 4 isolate that were probed and collected from different parts of the world. The sizes of the hybridizing fragments are indicated and the sizes were determined by running a 1 Kb ladder alongside the samples during agarose gel electrophoresis. ICARDA was abbreviated 'I'. 1) I 72-1; 2) I 72-2; 3) I 72-3; 4) I 72-5; 5) I 72-7; 6) 94-115; 7) 331-9; 8) SC 22-2; 9) SC 29-1; 10) 90-2.

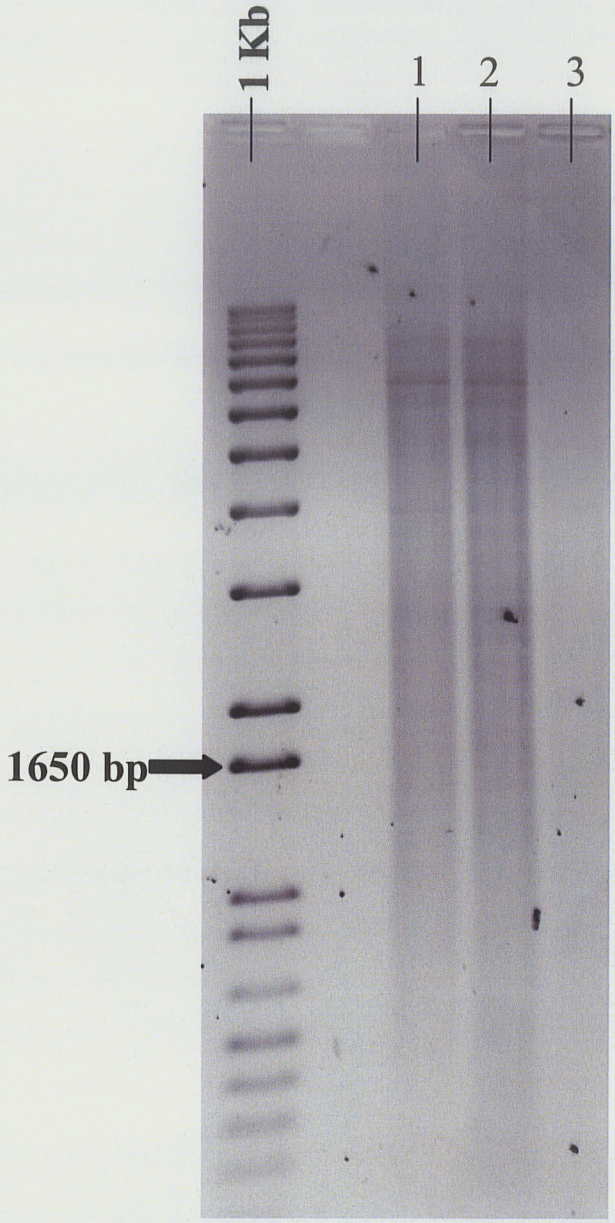


Fig. 29: *Hind*III- and *Xho*I-digested genomic DNA of isolates of races 3 and 4. Digested DNA was run on an agarose gel for separation prior to blotting and probing. A 1 Kb ladder was run alongside for size estimation. To visualize the DNA and compare the relative amounts of digested DNA loaded, the agarose gel was stained with ethidium bromide. From the intensity of the bands, 90-2 (#3) had little DNA loaded, resulting in no hybridization to the probe. 1) SC 22-2; 2) SC 29-1; 3) 90-2.

chlorosis, PCR products and hybridizing bands were detected with *ToxB* primers and probes, respectively. Based on the patterns of hybridizing bands with both probes, isolates could not be differentiated into races.

Sequencing of Toxin Genes

PCR amplification did not detect any previously undescribed mutations in the form of deletions or insertions in the toxin genes. However, base pair mutations would be undetectable with agarose gel analysis. To detect single base-pair mutations within the genes, the *ToxA* and *ToxB* gene regions were sequenced from various selected isolates in the hopes of visualizing any differences that may exist in the sequences between isolates of different races from around the world. Sequencing was carried out commercially at Macrogen USA (Rockville, MD, USA) using the Sanger method (Sanger et al., 1977).

ToxA

The *ToxA* gene region was sequenced from 16 isolates using DNA amplified by PCR with primers TxAG1 and TxAG2. The selected isolates represented the four races that show the ToxA phenotype on sensitive wheat cultivars (races 1, 2, 7, and 8) collected from different areas of the world. The *ToxA* sequence has been evaluated in a small sampling of isolates studied (Ballance et al. 1996, Ciuffetti et al. 1997) and shown to be identical (Ballance and Lamari, 1998). Sequencing from a wider cross-section of isolates may present sequence differences among isolates from different races and geographical origin (Table 12).

The sequencing results obtained showed that the *ToxA* gene region for all 16 isolates tested were identical when compared to each other and to the ToxA genomic

clone in GenBank (accession number AF004369). The only differences found were the start and stop points of each sequence which was based on returned sequence quality.

Comparison of the *ToxA* sequences to the mRNA sequence in Genbank (accession number PTU79662) showed that sequences were identical except at a single nucleotide.

Table 12: The sixteen isolates sequenced for the ToxA gene. The gene was sequenced from isolates of races 1, 2, 7, and 8 from a PCR template with primers TxAG1 and TxAG2. The isolates were collected from different parts of the world and were identified by the identification numbers during sequencing and alignment.

Isolate	Race	Region of Collection	Identification Number
ICARDA 1-5	1	Kazakhstan	1
ICARDA 8-4	1	Kyrgyzstan	2
ICARDA 18-4	1	Azerbaijan	12
ICARDA 67-4	1	Uzbekistan	11
98-MD-7	1	Morden, Canada	3
SC 25-3	1	Swift Current, Canada	4
PDY-7	1	North Dakota, USA	5
UY 127-2	1	Uruguay	6
NA 5-3	1	North Eastern Algeria	7
SK 105-2	1	Saskatchewan, Canada	10
2004-38-3	2	Cypress Hills, Canada	15
86-124	2	Portage La Prairie, Canada	16
ICARDA 17-10	7	Azerbaijan	8
ICARDA 36-4	7	Azerbaijan	14
ICARDA 17-11	8	Azerbaijan	13
ICARDA 35-33	8	Azerbaijan	9

In the mRNA sequence, there is a 'T' at position 206 and at the same position in the genomic sequences (nucleotide 584 in AF004369, nucleotide 371 in isolates 1 to 4, 6 to 16 and nucleotide 335 in isolates 5), there is a 'C'. Original sequencing results were verified and found to be accurate. This difference had been reported in a previous study (Ballance and Lamari, 1998).

Despite this single nucleotide difference between the genomic clone and the mRNA sequence, the *ToxA* gene regions of the isolates from different races and

geographical origins studied here were identical. Unexpectedly, it appeared that the *ToxA* gene region was conserved within isolates of different races from different geographical regions worldwide.

ToxB

The *ToxB* gene region was sequenced using PCR amplified DNA from one race 5 isolate and twelve race 8 isolates using primers TxBG1 and TxB7. Currently, there are 11 complete *ToxB* sequences in GenBank from race 5 isolates. Based on sequence used for PCR primer design, the expected size product was 1224 bp or approximately 1000 bp, the difference being a 202 bp insert in the 5' untranslated region. There were isolates that were able to amply both products simultaneously.

Thirteen isolates were sent for sequencing with the TxBG1 and TxB7 as the primer set (Table 13). When sequencing results were obtained, the previously identified 261 bp open reading frame (Martinez et al., 2001, Strelkov, 2002) was identical in all isolates. Isolates whose PCR product with TxBG1 and TxB7 was approximately 1000 bp lacked the 202 bp section of sequence. Isolates whose PCR product was of the expected size contained the 202 bp portion of sequence. The sequence of the 202 bp element was conserved among all isolates possessing it. Within the 202 bp fragment, 197 bp were found to be repeated downstream of the coding region (Strelkov, 2002, Martinez et al., 2004; Strelkov et al., 2006). For the portion of the toxin region sequenced, there were no point mutations found and the presence or absence of the insert was the only difference among isolates.

Table 13: The twelve race 8 isolates and one race 5 isolate sequenced for the *ToxB* gene. The *ToxB* gene was sequenced from a PCR template using primers TxBG1 and TxB7. The identification numbers were used to identify isolates during sequencing and for the alignment of sequences.

Isolate	Race	Region of Collection	Identification Number
ICARDA 17-11	8	Azerbaijan	17
ICARDA 31-1	8	Azerbaijan	18
ICARDA 31-2	8	Azerbaijan	19
ICARDA 34-1	8	Azerbaijan	20
ICARDA 34-2	8	Azerbaijan	21
ICARDA 35-6	8	Azerbaijan	22
ICARDA 35-13	8	Azerbaijan	23
ICARDA 35-16	8	Azerbaijan	24
ICARDA 35-18	8	Azerbaijan	25
ICARDA 35-19	8	Azerbaijan	26
ICARDA 35-33	8	Azerbaijan	27
ICARDA 42-14	8	Azerbaijan	28
Alg 3-24	5	Algeria	29

In three race 8 isolates, ICARDA 17-11, 31-1 and 31-2 and in Alg 3-24, a race 5 isolate, two different sized bands were amplified and independently recovered and reamplified. The reamplified DNA was sent for sequencing. It was found that each type of sequence was represented by one of the recovered bands, ie. the expected size product as well as the shorter version of *ToxB*.

The limitation of sequencing with PCR product was that where multiple copies of *ToxB* exist (which has been shown with Southern blot analysis), all copies are amplified with PCR. If a single base pair difference did occur in one or two copies and a majority of the other copies had a different nucleotide, the dominant nucleotide would be reflected in the sequencing results, though multiple peaks may be present. To avoid this problem and to treat each *ToxB* copy separately, the amplified product would have to be cloned

and a sufficient number of clones sequenced to insure a reasonable confidence in the clone sequences.

Sequencing of *ToxA* and *ToxB* gene regions showed that both gene regions were highly conserved in isolates of different races collected from different geographical regions around the world. PCR amplification, Southern blot analysis, and sequencing methods showed that the genotypic evidence agreed with the phenotype expressed on sensitive wheat cultivars for the ToxA- and ToxB- phenotypes. Southern blot analysis estimated copy number and sequencing of the *ToxA* and *ToxB* gene regions showed the conservation of sequence of these genes.

DISCUSSION

The goal of the study was to find if differences existed in the *ToxA* and *ToxB* genes of *P. tritici-repentis* from isolates of different races collected from different areas of the world. The differences were assessed using three molecular techniques. PCR amplification analysis was used to assess if the phenotype observed on the wheat differential set was consistent with the genotype of the isolates, and to detect the presence of potential mutations in the genes in the form of insertions and/or deletions. Southern blot analysis was used to assess copy number of the toxin genes of different isolates and to confirm the phenotype of isolates as a secondary check for PCR results. Sequencing of the toxin genes was used to compare the toxin genes and pinpoint if even single nucleotide mutations occurred within the two genes among the isolates.

Similarities and Differences of Toxin Genes

ToxA

The *ToxA* genotype was confirmed by PCR and Southern blot analysis in isolates that showed the ToxA-phenotype, agreeing with previous results (Ballance et al., 1996, Ciuffetti et al., 1997). Isolates that did not induce necrosis on the wheat differentials were shown to lack *ToxA* sequences as verified by both PCR and the absence of hybridizing bands with the *ToxA* probe.

Sequencing showed the absence of any mutations in the *ToxA* gene of the 16 isolates from races 1, 2, 7, and 8. The gene coding region and surrounding regions were identical for all isolates among the four races studied. These isolates had been collected from different parts of the world and could have evolved differently. However, the similarity of *ToxA* genes agreed with previous results (Ballance et al., 1996; Ciuffetti et

al., 1997; Ballance and Lamari, 1998) where fewer isolates from fewer races and more limited collection region were studied. The *ToxA* gene was a single copy gene in all of these isolates, also agreeing with previous results (Ballance et al., 1996; Ciuffetti et al., 1997).

ToxB

PCR analysis confirmed the *ToxB* genotype in all ToxB-phenotype isolates of races 5, 6, 7, and 8. Each of the isolates produced the ToxB-related chlorosis symptoms on the wheat differentials. Furthermore, the *ToxB* gene was found in multiple copies in all isolates of races 5, 6, 7, and 8 collected from around the world. No correlation was found between copy number, race or geographical origin. These results expanded on previous results that showed that selected isolates of these races contained multiple copies of *ToxB* (Strelkov, 2002; Strelkov et al., 2002; Lamari et al., 2003; Martinez et al., 2004; Strelkov et al., 2006).

The sequences of 12 race 8 isolates from PCR products of the *ToxB* coding region and surrounding regions appeared to be highly similar to each other and to previously cloned sequences. The only difference observed between the isolates was the presence or absence of a 202 bp insert upstream of the coding region. Of the 202 bp, 197 bp of it make up a repeat found downstream of some but not all *ToxB* copies (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006). Downstream sequencing was not conducted because an external primer, with an annealing location downstream of coding regions, did not amplify a product after many trials. Therefore the prevalence of the downstream insert was not known. The importance of the 197 bp insert is not known although it may have some retrotransposon-like qualities (Martinez et al., 2004).

The PCR analysis confirmed the presence of a *ToxB*-like gene in isolates of races 3 and 4, which do not possess the ToxB-phenotype. Ptr ToxB symptoms are not produced by these isolates on the wheat differential set but toxin mRNA production has been observed in conidia and in ungerminated spores by RT-PCR of a race 4 isolate (Strelkov, 2002; Strelkov et al., 2006). Nine race 3 isolates probed with *ToxB* were collected from different continents of the world and a single *ToxB* homolog was hybridized in all isolates (Fig. 28), showing that *ToxB* homologs are found globally in race 3 isolates.

Previous sequencing efforts and comparison of the genomic clone of the Canadian race 3 isolate, D308, showed very strong homology to race 5 *ToxB* but differences exist between the two isolates. Sequence upstream of the *ToxB* gene coding region was non-homologous to race 5 *ToxB* and the race 3 *ToxB* lacked the first six nucleotides of the normal coding region (the ATG start codon and following codon). The remaining portion of the coding region sequence as well as 237 bp downstream of the TAA stop codon are identical to the sequence of the race 5 *ToxB* (Strelkov, 2002).

The Canadian race 4 isolate, 90-2, amplified a product with primer sets TxB1 and TxB2 and TxB3 and TxB4 but does not possess the ToxB-phenotype. A *ToxB* homolog, named *toxb*, has been has been sequenced in both 90-2 and an American race 4 isolate, SD-20; comparison of *toxb* to *ToxB* has shown 86% homology. There were 17 point mutations in *toxb* as well as three additional nucleotides in *toxb* not found in *ToxB*; these additional nucleotides resulted in amino acid substitutions (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006). *ToxB* PCR products were amplified in isolates that do not possess the ToxB-phenotype due to the presence of *ToxB* homolog(s).

In the *P. tritici-repentis*/ wheat pathosystem, there appears to be two toxin-related factors influencing the virulence of an isolate: toxin gene copy number and the structure of the toxin protein. Virulence, sensu Yoder (1980), is described as disease severity. The *ToxB* gene was found in multiple copies in isolates of races 5, 6, 7, and 8. Stelkov (2002) showed the implications of the multicopy nature of *ToxB* when Alg 3-24, an Algerian isolate, was compared to a less aggressive Canadian race 5 isolate, 92-171R5. Southern blot analysis showed Alg 3-24 had between 8-10 copies of *ToxB* compared to the two copies of *ToxB* in 92-171R5 (Strelkov, 2002; Strelkov et al., 2002). Expression of *ToxB* from 92-171R5 was lower in terms of total mRNA (Strelkov, 2002; Strelkov et al., 2002; Strelkov et al., 2006). Copy number appears to affect the virulence of an isolate, but different isolates have not been studied for the correlation between copy number and virulence.

Kim and Strelkov (2007) compared the expression and protein structure of *ToxB* and *toxb*. A *ToxB* homolog is found in race 4 isolates, which does not possess the ToxB- phenotype. Ptr ToxB expression of Alg 3-24 *ToxB* and 90-2 *toxb* were kept at the same level and the toxins were expressed under the same promoter so that gene copy number would not affect the results. The differences in virulence were attributed to the protein structure of Ptr ToxB of 90-2 (Kim and Strelkov, 2007). Although a *ToxB* homolog was detected with both PCR and Southern blot analysis, analysis of the actual sequence showed that differences between *ToxB* and *toxb* exist, and these changes affect the final translated protein and the virulence of the isolate.

Origin of Toxin Genes

ToxA

Friesen et al. (2006) purposed that *ToxA* originated in another wheat pathogen, *Stagonospora nodorum* due to the presence of a nearly identical *ToxA* gene. The coding region of the *Stagonospora nodorum ToxA* (abbreviated *SnToxA*) was found to be almost identical when to the coding region of the *P. tritici-repentis ToxA*. Collection and comparison of *SnToxA* globally resulted in 11 different forms of *SnToxA* being found (Friesen et al., 2006; Stukenbrock and McDonald, 2007). Due to the variation found within *SnToxA*, the conclusion drawn was that *ToxA* of *P. tritici-repentis* originated in *S. nodorum* and none of the *SnToxA* was shown to have complete homology to *ToxA*. The *ToxA* gene was proposed to have been transferred horizontally from *Stagonospora* to *Pyrenophora* via fungal anastomoses tubes (Friesen et al., 2006). The two genera are allied genera (Kodsueb et al., 2006)

The transfer of the toxin gene and selective pressures made *P. tritici-repentis* the pathogen it is today, according to Friesen et al. (2006). Once *ToxA* was successfully integrated into to the *P. tritici-repentis* genome, this allowed the pathogen to jump species from wild grasses where it was first reported (Diedicke, 1902) to wheat (Friesen et al., 2006). The pathogen was able to infect wheat because it carried the *ToxA* sensitivity gene, *Tsn1*. The new pathogen of wheat is presumed to have been distributed worldwide by infected seeds. According to the hypothesis of Friesen et al. (2006), tan spot emerged as a disease in the 1940's after reports in the United States and Australia of a new foliar disease of wheat. The disease was distributed all over the world by infected seed (Friesen et al., 2006).

The year that tan spot was first reported on wheat was not the 1940's as reported by Friesen et al. (2006). The first reports of tan spot were in Japan (Nisikado, 1928) and India (Mitra, 1929, 1931), and the disease was reported in Canada in the 1930's (Conners, 1939). Between the 1900's and 1928, the disease would have had to establish itself and be distributed all over the world. The fact that there was no variation observed indicates that the gene did arise quite recently in *P. tritici-repentis* (Friesen et al., 2006).

Friesen et al. (2006) hypothesized that *P. tritici-repentis* was distributed by infected seed. There is evidence that infected seed can transmit infection, but infection is transmitted ineffectively as seed germination rate is low and the seedlings have decreased vigour (Fernandez et al., 1996). Germination of infected seed can infect the coleoptile and to a lesser extent the true leaves, but not at epidemic proportions (Schilder and Bergstrom, 1995; Fernandez et al., 1996; Fernandez et al., 1997; Fernandez et al., 1998).

ToxB

Andrie et al. (2007b) recently hypothesized that *ToxB* originated from a common ancestor and has been passed down through vertical transfer. A *ToxB* homolog was found as multicopy genes in a related *Pyrenophora* species, *P. bromi*. ITS analysis showed *P. tritici-repentis* and *P. bromi* were the most related of the *Pyrenophora* species (Zhang and Berbee, 2001). The *ToxB* homolog was also found in another distantly related wheat pathogen, *Magnaporthe grisea* (Andrie et al., 2007b), which diverged from *P. tritici-repentis* approximately 100 million years ago. It is anticipated that *ToxB* homologs will be found in other species of the group Pleosporaceae (Andrie et al., 2007b).

If the both origin theories are correct, two separate genetic events occurred for some isolates to have both *ToxA* and *ToxB*. In the case of race 7 and 8 isolates, they could be considered the original condition having first inherited *ToxB* from the ancient ancestor and within the last 80 years inherited the *ToxA* gene which was integrated into the genome as it does not appear to be a supernumerary chromosome (Aboukhaddour, unpublished) and through reproduction isolation and genetic processes, both toxin genes were retained (Kohn, 2005). For all of its toxin advantages, isolates of races 7 and 8 are restricted to tetraploid wheats in the Fertile Crescent while races 1 and 2 are found on both hexaploid and tetraploid wheats in the same regions and worldwide (Lamari et al., 2005a). The Fertile Crescent is thought to be where both tetraploid and hexaploid wheats originated (Harlan, 1987).

It could be that the original isolates of *P. tritici-repentis* produced all three toxins (like race 8 isolates) which were then distributed all over the world through the exporting of wheat (Diamond, 1994; Germida and Siciliano, 2001) or the long distance transport of spores (Gregory, 1978). The arrival of the pathogen in countries could have allowed the pathogen to adapt to its surroundings and differentiation of races through gene loss and mutation. When the pathogen arose in Canada, the isolates of races 1 and 2 lost the *ToxB* gene (Strelkov and Lamari, 2003), as there are no *ToxB* homologs observed (Strelkov, 2002). The loss of *ToxB* would have to have occurred fairly recently as no homologs are detected in race 1 and 2 isolates but are still detectable in other related species (Andrie et al., 2007b). This loss is slightly inexplicable due to the fact wheat cultivars grown in Canada are susceptible to ToxB-type chlorosis. In Canada, ToxB-chlorosis has not been

observed on wheat (Lamari et al., 2005b). The race 3 and 4 isolates contained single *ToxB* homologs. The mutations of *ToxB* present in isolates of both races render it inactive.

The conservation of the toxin genes could involve the reproduction habits of *P. tritici-repentis*. Reproductively, *P. tritici-repentis* is the only homothallic fungus in the genus *Pyrenophora* (Turgeon, 1998). Mating does not require a second mating type and the fungus is essentially self-fertile (Alexopoulos et al., 1996). If there is no need for a separate partner, this could serve to keep the *ToxA* gene in ToxA phenotype isolates, as no homologs have been found in non-ToxA isolates (Ciuffetti et al., 1997).

Fungal toxin genes appear to be conserved in other toxin-producing plant pathogenic fungi. *Cochliobolus carbonum* is the causal agent of Northern leaf spot (Bailey et al., 2003). *C. carbonum* produces the HC toxin. *TOX2,* the HC toxin gene cluster, was found to be conserved among isolates worldwide (Cheng and Walton, 2000). Although the HC toxin is a secondary metabolite rather than a protein, the cluster of enzymes responsible for toxin production is found only in toxin-producing isolates (Panaccione et al., 1992). The *TOX2* locus contains at least two copies of the HST enzyme, both of which are identical (Panaccione et al., 1992). Like the *P. tritici-repentis* toxins, HC toxin is essential for infection to progress (Ciuffetti et al., 1997

Essential genes, such as the histone protein genes, were found to be different enough to distinguish between different mating types. Histone proteins are small proteins that interact with DNA, forming the nucleosomes, the smallest units of chromatin (Karp, 1999). These proteins are necessary for packaging the DNA and are extremely conserved between phylums (Karp, 1999). Steenkamp et al. (1999) compared the sequence of H3

histone gene from different mating populations of *Fusarium.* Although the coding region of the H3 genes were identical among the 60 strains used, the length of the introns were different enough to distinguish the different mating types, unlike *ToxA* and *ToxB* whose coding regions (and introns) are identical among different races.

Phenotype vs. Genotype

Currently, a phenotype-based method is used to differentiate isolates of *P. tritici-repentis* into one of eight races based on the symptoms produced on a set of wheat differentials. The advantage of the phenotypic method is that it is an overall evaluation of expressed genes of the isolate and relates to virulence but the method is not foolproof. The symptoms are evaluated by people and occasionally mistakes could be made. An isolate may be misclassified, isolates may be switched, and natural variation between isolates may affect the overall rating of an isolate. Using the phenotype method alone, the underlying genetics of the symptoms would be overlooked and new isolates could potentially be misclassified, as was the case in the United States (Andrie et al., 2007a). One of the objectives of the research was to determine if molecular techniques could be used to differentiate isolates into races. A genotypic method would rely on screening isolates based on the genes present in each isolate using PCR analysis with toxin-specific primer sets. Based on the presence or absence of amplified products, a race designation could be assigned.

Race designation based on amplified PCR products with the primers used in this research would be problematic. One difficulty is the absence of genetic markers for *ToxC.* Based solely on *ToxA* primers, isolates of races 1, 2, 7, and 8 would appear the same until *ToxB* primers were used. If no products are amplified, isolates of races 1 and

2 would appear the same. If products are amplified, isolates of races 7 and 8 would appear to be of the same race. The use of *ToxA* primers would separate isolates of races 1, 2, 7, and 8 from isolates of races 3, 4, 5, and 6 because these would not amplify a *ToxA* product. Using the *ToxB* primers that were used in the research, isolates of races 3, 4, 5, and 6 can not be differentiated from one another as isolates contain ToxB homologs, even if they do not possess the ToxB-phenotype (Strelkov, 2002; Martinez et al., 2004), and products were amplified. Once the isolates were divided into broader race grouping, the wheat differential line to distinguish the ToxC-phenotype are needed, but the total number of wheat cultivars/lines needed would be decreased.

Designing new primers specifically for the *ToxB* homologs could clarify the form of *ToxB* present in an isolate. From previous work (Strelkov, 2002; Martinez et al., 2004; Strelkov et al., 2006), *ToxB* and the *ToxB* homologs have been sequenced. Taking advantage of the characteristics of ToxB and the homologs, specific primers could be designed. With these primers, isolates of races 3 and 4 could be separated from one another and from races 5 and 6. However, without information on the genetics of ToxC, races 5 and 6 would appear the same genotypically.

The use of both methods at the outset of classification does not require extra material and the race of an isolate would be assessed on the wheat differentials and confirmed by PCR. The material for both methods starts from the same glycerol stock and subsequent isolated single spore. The inoculum would be prepared as described in Materials and Methods, and PCR analysis could be conducted directly on genetic material extracted from growing fungal mycelia (Edel et al., 1996).

To insure that ambiguous results do not arise from PCR analysis, the conditions must be stringent and fine-tuned. There were isolates that did not possess the phenotype but amplified PCR products. Southern blot analysis verified the absence of the genes. During PCR amplification, the primer annealing temperature should provide the highest stringency so that isolates that do not have *ToxA* or *ToxB* do not amplify a product. The annealing tenperature was increased for more specificity. The cycle number was decreased from 40 to 30 to prevent the amplification of potential contaminants. The conditions should be tested prior to large-scale amplification.

At this time, genotype alone cannot be used to differentiate isolates into races; phenotype is still the effective method for race differentiation. The genotypic method is complementary to the phenotypic method and can be used as a confirmation tool and a preliminary screening method. The phenotype alone can be used to differentiate isolates but may hinder the discovery of new races when symptoms are regarded separately from the underlying genetics (Andrie et al., 2007a); the genetics should not be ignored.

Experimental Considerations

At the end of the research, there is time for reflection of what could have been done and what could have been done differently. The portion of *ToxA* experimentation that could have been done differently would be the restriction enzymes used for Southern blot analysis. The results showed that when genomic DNA was digested with *Xho*I, one hybridizing fragment was detected. If by chance two copies of *ToxA* existed and when digested had the same size fragments which comigrated, two copies would appear as one. Another option would have been to use *Hind*III or *Sal*I. Ballance et al. (1996) showed that a single *ToxA* copy generated two hybridizing fragments because the restriction site

is within the coding region. If multiple copies of *ToxA* existed, at least two bands would hybridize.

If parts of *ToxB* experimentation could be redone, it would be a more in depth study into the correlation between copy number and virulence of *ToxB* and the sequencing of *ToxB*. Firstly, *ToxB* copy number of each isolate would have to be determined by Southern blot analysis, and then compared to amount of mRNA levels by Northern blot analysis. For a phenotypic approach, the isolates would be inoculated on to the appropriate wheat differential, cultivar 6B662 (sensitive to ToxB), and the extent of chlorosis could be compared, as was done by Strelkov (2002).

The determination of copy number was dependent on the number and the intensity of the hybridizing fragments during Southern blot analysis. Variability in copy number was obtained among the isolates. However, the copy number was variable because of the initial level of DNA loaded into the agarose prior to membrane blotting. If the Southern blot analysis could be redone, it should be done so that levels of DNA are consistent. When copy number is assessed, the variability would be due to the differences in copy number alone.

ToxB was sequenced from PCR products. The initial PCR amplification to obtain template DNA would have amplified all copies of *ToxB* in an isolate. To be certain that only single copies of *ToxB* are being sequenced, single clones of *ToxB* would need to be isolated prior to sequencing as was done by Martinez et al. (2004) and Strelkov et al. (2006). The process of recovering single clones for sequencing is a long process that involves insertion of amplified DNA in to a vector, bacterial transformation, and segregation to isolate single clones. The advantages of creating libraries would be

individual copies of *ToxB* that would be independently sequenced, ensuring that a single copy of the gene is being sequenced.

Implications of Research

Many factors are necessary for tan spot of wheat to occur. The factors, when taken together, form the disease pyramid. Of the points of the pyramid, neither the pathogen nor the weather conditions can be modified or controlled, but practices to reduce pathogen inoculum and resistance of wheat can be influenced.

Resistance to tan spot is a desired trait. Tan spot disease cannot occur without toxin production by isolates (Ciuffetti et al., 1997). By studying the toxin genes and understanding possible variations in the gene, more information for breeding could be provided. From the results, it appears that both *ToxA* and *ToxB* of *P. tritici-repentis* are identical from respective isolates, regardless of region of origin. The system is fairly stable, unlike the more dynamic rust system (Green, 1981; Webb and Fellers, 2006). Due to the stableness of the tan spot system and the successful integration of resistance genes, the introduction of an isolate from the Fertile Crescent would not be extremely detrimental to the health of Canadian wheat crops due to the fact that the toxin gene produced by the isolate is the same as the one produced by Canadian isolates.

General Conclusions

Based on the results, there appeared to be no differences of *ToxA* and *ToxB* among the isolates that carried the genes globally. The *ToxA* gene was a single copy gene, identical in sequence among isolates of ToxA-phenotype races collected from around the world. The *ToxB* gene was a multicopy gene found in ToxB-phenotype isolates from around the world. The copies of *ToxB* appeared to be identical, regardless

of race. From PCR amplification results with the specific primers used, isolates could not be differentiated into races based on genotype alone. The genotypic method was most affective when used in complement with the phenotypic method as a phenotype confirmation tool. The study provided more clues to the nature of *ToxA* and *ToxB* gene, two important pathogenic factors of the wheat pathogen *P. tritici-repentis*, with the hopes of providing information for plant breeding and the evolution of the pathogen.

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Appendix 1: The 135 isolates of *Pyrenophora tritici-repentis* from the collection of Dr. L. Lamari used in this study. The isolates are organized by race and region of collection. 'ICARDA' is the abbreviation for the International Center for Agricultural Research in the Dry Areas.

ISOLATE	RACE	REGION OF COLLECTION
ICARDA 1-5	1	Kazakhstan
ICARDA 3-11	1	Kazakhstan
ICARDA 7-1	1	Krygyzstan
ICARDA 8-4	1	Krygyzstan
ICARDA 14-1	1	Krygyzstan
ICARDA 15-1	1	Krygyzstan
ICARDA 18-4	1	Azerbaijan
ICARDA 21-4	1	Azerbaijan
ICARDA 22-17	1	Azerbaijan
ICARDA 29-3	1	Azerbaijan
ICARDA 32-2	1	Azerbaijan
ICARDA 33-1	1	Azerbaijan
ICARDA 35-35	1	Azerbaijan
ICARDA 67-3	1	Uzebekistan
ICARDA 67-4	1	Uzbekistan
ICARDA 67-5	1	Uzebekistan
ICARDA 68-2	1	Uzebekistan
NA 3-4	1	North Eastern Algeria
NA 5-3	1	North Eastern Algeria
NA 8-3	1	North Eastern Algeria
NA 9-4	1	North Eastern Algeria
PDY-7	1	North Dakota
99-49-2	1	Canada
98-MD2	1	Morden
98-MD4	1	Morden
98-MD7	1	Morden
98-MD8	1	Morden
98-MD10	1	Morden
SC 10-1	1	Swift Current
SC 12-3	1	Swift Current
SC 24-3	1	Swift Current
SC 25-3	1	Swift Current
SC 29-3	1	Swift Current
SC 36-3	1	Swift Current
SC 36-3	1	Swift Current

ISOLATE	RACE	REGION OF COLLECTION
SK 104-1	1	Saskatchewan
SK 105-2	1	Saskatchewan
UY 127-2	1	Uruguay
ICARDA 34-10	2	Azerbaijan
ICARDA 35-37	2	Azerbaijan
ICARDA 58-3	2	Kazakhstan
ICARDA 62-6	2	Kazakhstan
ICARDA 63-8	2	Kazakhstan
ICARDA 63-9	2	Kazakhstan
32JA	2	Canada
2004-38-1	2	Cypress Hills
2004-38-3	2	Cypress Hills
2004-53-1	2	Cypress Hills
2004-54-1	2	Cypress Hills
SK 103-1	2	Saskatchewan
SK 109-1	2	Saskatchewan
86-124	2	Canada
90-68	2	Canada
91-18	2	Canada
94-94	2	Canada
94-70	2	Canada
94-106	2	Canada
SC 3-7	2	Swift Current
SC 5-3	2	Swift Current
SC 6-2	2	Swift Current
SC 18-2	2	Swift Current
SC 19-2	2	Swift Current
SC 21-1	2	Swift Current
SC 23-2	2	Swift Current
SC 27-1	2	Swift Current
SC 28-2	2	Swift Current
SC 30-1	2	Swift Current

ISOLATE	RACE	REGION OF COLLECTION
ICARDA 72-1	3	Syria
ICARDA 72-2	3	Syria
ICARDA 72-3	3	Syria
ICARDA 72-5	3	Syria
ICARDA 72-7	3	Syria
94-115	3	Manitoba
331-9	3	Manitoba
SC 22-2	3	Swift Current
SC 29-1	3	Swift Current
90-2	4	Manitoba
ICARDA 17-1	5	Azerbaijan
ICARDA 17-8	5	Azerbaijan
ICARDA 34-3	5	Azerbaijan
ICARDA 34-6	5	Azerbaijan
ICARDA 35-1	5	Azerbaijan
ICARDA 35-20	5	Azerbaijan
ICARDA 35-57	5	Azerbaijan
ICARDA 36-1	5	Azerbaijan
ICARDA 73-2	5	Syria
ICARDA 73-3	5	Syria
ICARDA 73-4	5	Syria
NA 4-4	5	North Eastern Algeria
NA 6-1	5	North Eastern Algeria
NA 6-2	5	North Eastern Algeria
NA 6-4	5	North Eastern Algeria
NA 6-5	5	North Eastern Algeria
NA 6-6	5	North Eastern Algeria
NA 6-7	5	North Eastern Algeria
NA 6-8	5	North Eastern Algeria
NA 7-2	5	North Eastern Algeria
NA 7-3	5	North Eastern Algeria
NA 7-4	5	North Eastern Algeria
NA 7-5	5	North Eastern Algeria

NA 7-7	5	North Eastern Algeria

ISOLATE	RACE	REGION OF COLLECTION
NA 7-8	5	North Eastern Algeria
NA 7-9	5	North Eastern Algeria
Alg 3-24	5	Algeria
Alg 3X-1	5	Algeria
Alg 4-X1	5	Algeria
Alg 4-X1-1	5	Algeria
Alg 5-X1-1	5	Algeria
Alg H-1	6	Algeria
Alg H-2	6	Algeria
Alg H-2A	6	Algeria
Alg H-2G	6	Algeria
Alg -H2 on 6B365	6	Algeria
ICARDA 17-5	7	Azerbaijan
ICARDA 17-10	7	Azerbaijan
ICARDA 35-5	7	Azerbaijan
ICARDA 35-17	7	Azerbaijan
ICARDA 35-21	7	Azerbaijan
ICARDA 35-24	7	Azerbaijan
ICARDA 35-25	7	Azerbaijan
ICARDA 36-3	7	Azerbaijan
ICARDA 36-4	7	Azerbaijan
ICARDA 17-11	8	Azerbaijan
ICARDA 31-1	8	Azerbaijan
ICARDA 31-2	8	Azerbaijan
ICARDA 34-1	8	Azerbaijan
ICARDA 34-2	8	Azerbaijan
ICARDA 35-6	8	Azerbaijan
ICARDA 35-13	8	Azerbaijan
ICARDA 35-16	8	Azerbaijan
ICARDA 35-18	8	Azerbaijan
ICARDA 35-19	8	Azerbaijan
ICARDA 35-33	8	Azerbaijan
ICARDA 42-14	8	Azerbaijan