GENETIC STUDIES ON THE RESISTANCE TO TAN SPOT OF WHEAT AND GENETIC SIMILARITY AMONG ISOLATES OF *PYRENOPHORA TRITICI-REPENTIS*

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ABSTRACT

Tan spot of wheat, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs., has become a major component of the leaf spot disease complex in the prairie region in recent years. On susceptible host cultivars *P. tritici-repentis* induces two distinct symptoms, tan necrosis and extensive chlorosis. To date, five races of *P. tritici-repentis* have been identified based on their reaction on a set of wheat differential cultivars. The major objective of this study was to investigate the genetics of resistance to tan spot in several diverse hexaploid wheat cultivars to races 1, 2, and 3, and to the culture filtrate of *P. tritici-repentis*, race 2. The F_1 and F_2 generations and $F_{2:3}$ and $F_{7:8}$ families of crosses between the seven selected genotypes were tested with each race in growth room experiments. Two independently inherited genes controlled resistance to tan spot of wheat. A single dominant gene controlled resistance to chlorosis induced by races 1 and 3, while a single recessive gene controlled resistance to necrosis induced by races 1 and 2. The same recessive gene controlled resistance to necrosis induced by spore-inoculation or culture filtrate infiltration of *P. tritici-repentis*, race 2. This indicated that infiltration with the culture filtrate could replace spore-inoculation when testing for resistance to the necrosis component of tan spot. No segregation was observed in crosses among the resistant cultivars for both the necrosis and the chlorosis components, indicating that the resistant cultivars carried the same resistance genes.

The second objective of this study was to assess the genetic similarity among different isolates of *P. tritici-repentis* and to determine if this similarity is related to race classification or geographic origin of the isolates. Thirty-eight isolates including other

Pyrenophora species were studied with 30 RAPD primers. Cluster analysis and AMOVA indicated that high genetic variability existed among the isolates of *P. tritici-repentis.* but this variability was independent of race classification or geographic origin of the isolates.

The last objective of this study was to identify molecular markers linked to the resistance gene controlling the necrosis component of tan spot. No RAPD marker linked to gene controlling resistance to the necrosis component was identified.

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1. INTRODUCTION

Tan spot, a foliar disease of wheat, is caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoem. (syn. *Helminthosporium tritici-repentis* Died.) (Shoemaker 1962; Krupinsky 1987; De Wolf et al. 1998). Tan spot has been reported to occur throughout the major wheat growing regions of the world (Hosford 1982; Krupinsky 1982; Weise 1987; Ciuffetti and Tuori 1999). The pathogen infects many grass species and has the widest host range of any *Pyrenophora* species (Shoemaker 1962; Krupinsky 1982, 1987; De Wolf et al. 1998). Hence, the pathogen is able to overwinter on a number of hosts and is also capable of surviving saprophytically on infected wheat stubble and crop residues (Morrall and Howard 1975; Krupinsky 1992).

Increases in the incidence and severity of tan spot have been attributed to changes in cultural practices, including shift from conventional tillage and stubble burning to conservation tillage systems, shorter rotations, continuous wheat cultivation and growing of susceptible wheat cultivars (Bockus 1998; De Wolf et al. 1998). Tan spot, on average, causes a 3-15% yield loss which under conditions favorable to the disease can be as high as 50% (Hosford 1982; Rees and Platz 1983, 1992; Shabeer 1986)

A number of management practices are useful in controlling tan spot. These include the use of non-host plants in the crop rotations, destruction and avoidance of infested straw, stubble and volunteer plants by either burning or burying. However, stubble burning and tillage increase the risk of soil erosion and can contribute to pollution

of the environment. The application of fungicides is also effective in controlling tan spot, but when grain prices are low their use is not cost-effective (Raymond et al. 1985). Therefore, resistant cultivars are the most effective and economical means of controlling tan spot of wheat.

The tan spot syndrome consists of two phenotypically distinct symptoms, tan necrosis and extensive chlorosis. Resistance is expressed as small, dark brown lesions that do not increase in size with time and leaf wetness periods, while susceptibility is expressed as small dark brown spots surrounded by tan necrosis and/or extensive chlorosis that often cover the entire leaf (Lamari and Bernier 1989b). Resistance to tan spot of wheat has been reported to be either quantitatively (Nagle et al. 1982; Shabeer 1986; Elias et al. 1989; Faris et al. 1997) or qualitatively (Lamari and Bernier 1989b; Sykes and Bernier 1991; Duguid 1995; Faris et al. 1996; Gamba et al. 1998) inherited.

Isolates of *P. tritici-repentis* are classified into five races based on their ability to induce necrosis and/or extensive chlorosis on a set of differential cultivars. Race 1 induces both necrosis and chlorosis while race 2 induces necrosis only. Races 3 and 5 induce extensive chlorosis only; however, they differ in virulence. Race 4 is avirulent and fails to induce any disease symptoms (Lamari et al. 1995a). In culture, the tan spot fungus produces host specific toxins, designated as Ptr ToxA, Ptr ToxC, and Ptr ToxB, produced by races 1 and 2, 1 and 3, and 5 respectively (Ciuffetti and Tuori 1999).

The five races of tan spot fungus cannot be differentiated morphologically and the isolates have to be tested on differential cultivars to determine their race classification. DNA amplification fingerprinting could be used for race classification and estimation of pathogenic variability (Shi et al. 1996; Achenbach et al. 1996). However, to date, there

has been no reported use of such techniques to measure the extent of genetic variability among and within the different races of *P. tritici-repentis*. An understanding of such variability is important in breeding for resistance to tan spot. Therefore, to provide basic information for such breeding, these studies were undertaken with the following objectives:

1) to determine the genetic control of resistance to races 1, 2 and 3 of *P. tritici-repentis* and to establish the allelic relationships among the resistance gene(s) present in the different sources of resistance studied;
2) to determine the genetic control of resistance to the culture filtrate of the *P. tritici-repentis*, race 2 and to verify if culture filtrate infiltration can replace spore inoculation when testing for resistance;
3) to study the genetic similarity among isolates of *P. tritici-repentis*; and
4) to identify molecular markers useful for marker-assisted selection for tan spot resistance.

2. LITERATURE REVIEW

2.1 Taxonomy and Host Range of *Pyrenophora tritici-repentis*

Pyrenophora tritici-repentis is grouped in the Kingdom Fungi; Division Eumycota; Subdivision Ascomycotina; Class Loculoascomycetes; Order Pleosporales and Family Pleosporaceae (Ainsworth et al. 1973). This facultative pathogen is considered to be a necrotroph as it causes extensive tissue damage to the host in its parasitic phase, but can also survive on dead or dying host plant tissue in its non-parasitic phase.

Of all the *Pyrenophora* species, *P. tritici-repentis* is believed to have the widest host range on grasses and is capable of infecting at least 26 species (Shoemaker 1962; Morrall and Howard 1975). The ability of this fungus to colonize such a large number of grasses, most of which are perennial and grow in wheat producing areas, facilitates over-wintering and provides initial inoculum for tan spot epidemics (Krupinsky 1982, 1987, 1992). *P. tritici-repentis* is nonpathogenic on oat and barley, and weakly pathogenic on rye (Weise 1987; Maraite et al. 1992).

2.2 Epidemiology

The life cycle of *P. tritici-repentis* involves both a sexual and an asexual stage. The sexual stage is characterized by the production of ascospores, while the asexual stage is characterized by the production of conidia (Schilder and Bergstrom 1992b). The sexual spores, ascospores, are produced in asci within a pseudothecium. The

pseudothecia of *P. tritici-repentis* range from 200 to 700 μm in diameter and are spherical in shape and black in color. Asci are bitunicate, cylindrical and narrow at the base (Pfender et al. 1988). During the asexual stage, 5 to 7-celled conidia that are cylindrical, rounded at the apex, and measuring 95 to 165 μm in length and 14 to 18 μm in thickness are produced (Shoemaker 1962). Tan spot epidemics are characterized by a simple interest phase initiated by ascospores, followed late in the crop season by a short compound interest phase resulting from infection by conidia (Wright and Sutton 1990).

P. tritici-repentis survives between crops by growing saprophytically on infected wheat stubble, crop residues, native grasses and in infested seeds (Adee and Pfender 1989; Schilder and Bergstrom 1990). Pseudothecia provide the mechanism by which the fungus survives between wheat crops (Hosford 1972). Moist conditions with moderate temperatures (15-18°C) are required for maturation of pseudothecia and for optimum production of ascospores (Pfender et al. 1988; Wright and Sutton 1990).

Ascospores are the primary source of inoculum of *P. tritici-repentis* and can infect and produce lesions on young wheat seedlings (Howard and Morrall 1975; Weise 1987; Adee and Pfender 1989). Dispersal of the ascospores generally occurs under the damp conditions of night. The wind-dispersed ascospores are disseminated only short distances (Morrall and Howard 1975; Raymond et al. 1985) causing localized epidemics (Rees 1982; Schilder and Bergstrom 1992b).

Following primary infection of a susceptible plant leaf, lesions develop producing conidiophores and conidia, which serve as the secondary source of inoculum. Production of conidia is promoted by periods of rainfall and high humidity at night (Weise 1987). *P. tritici-repentis* requires a diurnal (light/dark) cycle to induce sporulation. Conidiophores

are produced during the light period while dark periods are necessary for conidial production (Francl and Jordahl 1997: De Wolf et al. 1998). This induction can occur over a temperature range of 10 to 31°C. but the optimum temperature is 21°C (Platt et al. 1977).

Under periods of rainfall and high humidity. multiple cycles of conidial production and release can occur. leading to rapid development of an epidemic in both space and time (Morrall and Howard 1975: Schilder and Bergstrom 1992b). The major factor affecting conidial dispersal is wind. Wind speeds as low as 2 ms^{-1} were sufficient to cause 100% conidial dispersal (Morrall and Howard 1975: Platt and Morrall 1980). However. Francl and Jordahl (1997) reported that wind speed had no detectable effect on conidial dispersal and that liberation of conidia was affected little by wind duration. They also observed that only 30 to 50% of conidia produced were liberated from leaves. suggesting that the liberation rate is lower than previously thought. Under optimal conditions the tan spot fungus can initiate a secondary disease cycle through conidiospores every eight days (Riaz et al. 1991).

P. tritici-repentis can also be seedborne. Infected seeds have a distinct pink discoloration and the fungus appeared to occur mainly as thick-walled resting mycelium within the pericarp. especially at the embryo and brush ends of the seed (Schilder and Bergstrom 1992a: Rees and Platz 1992). Seed-to-seedling transmission is almost 100% (Schilder and Bergstrom 1992a). Seedlings derived from infected seeds were shorter and lighter in color than their uninfected counterparts. The diseased seedlings develop small brown spots on the coleoptile as well as large black pseudothecia on the seed, but the importance of seed-to-seedling transmission in the development of tan spot epidemics is

not known (Schilder and Bergstrom 1992a; Fernandez et al. 1998). Fernandez et al. (1996) reported that there was transmission of *P. tritici-repentis* to the coleoptile but not to the true leaves.

2.3 Symptoms and Quantification

Infection and disease symptoms occur at all stages of growth of the wheat crop. Symptoms vary depending upon the race of the pathogen, host genotype and environmental conditions. Wheat seed infected by *P. tritici-repentis* have pink-red discoloration (red smudge), dark smudge/specks, black point, low germination and is shriveled (Valder 1954; Fernandez et al. 1998). Plants infected by *P. tritici-repentis* are shorter and weigh less than healthy plants and severe tan spot may reduce tiller number, grain number and grain size (Rees and Platz 1992). Symptoms begin as small dark brown to black spots on the leaf surface. On resistant cultivars no lesion expansion occurs, while on susceptible cultivars extensive necrotic and/or chlorotic lesions are observed (Lamari and Bernier 1989a; Orolaza et al. 1995).

Several rating systems have been used to describe the host reaction to *P. tritici-repentis*. These include percent infection (Nagle et al. 1982), lesion size and percent infection (Luz and Hosford 1980), an index combining lesion size, percent leaf area infected and leaf location (Raymond et al. 1985), and lesion type (Hosford 1971; Gilchrist et al. 1984). A 1-5 scale based on lesion type was developed by Lamari and Bernier (1989a). While previous researchers associated quantitative variables with the scales, Lamari and Bernier (1989a) used a qualitative measure only. On their scale the newest fully expanded leaf at the time of inoculation is rated numerically as follows:

1 = small, dark brown to black spots without any surrounding chlorosis or tan necrosis;
2 = small, dark brown to black spots with very little chlorosis or tan necrosis;
3 = small, dark brown to black spots completely surrounded by distinct chlorotic or tan necrotic ring, lesions are not coalescing;
4 = small, dark brown to black spots completely surrounded with tan necrotic or chlorotic zones, some of the lesions coalescing; and
5 = the dark brown or black centers may or may not be distinguishable, most lesions consist of coalescing chlorotic or tan necrotic zones.

2.4 Factors Affecting Disease Development

2.4.1 Association with Wheat Residues

Tan spot severity has been correlated with levels of infected wheat residue in the field (Ciuffetti and Tuori 1999). Historically, farmers have burnt residues and, as a consequence, achieved good control of tan spot. Experiments on tan spot and stubble management during the 1970's showed that burning resulted in less disease than in tilled or stubble retained treatments. Similarly disc ploughing resulted in less tan spot than stubble retained, sweep ploughing or zero tillage treatments (Rees and Platz 1992). However, the practices of leaving the soil surface bare results in soil erosion and moisture loss. Conservation farming practices have gained momentum in the last two decades and so has the severity of tan spot.

2.4.2 Spore Movement

There appears to be only limited movement of the primary inoculum (ascospores) with the majority of ascospores not travelling far from their source. In contrast, dissemination of conidia results in a rapid increase in the epidemic within a crop and in the spread of the disease to neighboring fields or areas. Both conidia and ascospores are

disseminated by wind (Wright and Sutton 1990). Schilder and Bergstrom (1992b) observed that the maximum number of conidia occurred within 3 m of the inoculum source, but conidia were recovered as far as 100 m from the source. Francl (1997) and Schilder and Bergstrom (1992b) indicated that long distance dispersal of viable conidiospores is possible.

2.4.3 Plant Growth Stage

Wheat leaf age has significant effects on the appearance and severity of disease symptoms. Older leaves were more susceptible to infection than younger leaves (Cox and Hosford 1987). However, Lamari and Bernier (1989a) reported that high level resistance is stable under different growth stages. While Lamari and Bernier (1989a) did observe a differential response in disease reaction at the 2-leaf and the 4 to 6-leaf stage in 12 of 116 accessions evaluated, they considered this to be the result of disease escape. Hosford et al. (1990) reported that as long as plants are actively growing vegetatively, susceptibility, when measured as lesion length, does not appear to vary greatly with growth stage. However, flag leaf susceptibility did increase at the dough stage. Fernandez et al. (1994) and Riede et al. (1996) have reported that adult plant reaction did not always agree with seedling reaction.

2.4.4 Temperature

The optimal temperature for expression of disease symptoms is 18 to 20°C (Luz and Bergstrom 1986) and 15 to 18°C for maturation of pseudothecia and ascospores (Wright and Sutton 1990). Conidia are produced at temperatures between 10 to 25°C with the optimum being 21°C. Increasing temperature increased disease development in

both resistant and susceptible cultivars (Luz and Bergstrom 1986; Hosford et al. 1987). However, Lamari et al. (1992) reported that high temperatures (>27°C) induced resistant reactions in moderately susceptible and susceptible cultivars. This resistance may result from loss of toxin activity as insensitivity to the Ptr ToxA was observed to occur at 27 to 30°C (Lamari et al. 1992).

2.4.5 Wet Period

High moisture *per se* is favorable to saprophytic growth of *P. tritici-repentis*. Prolonged wet periods of 48 h or longer have been reported to result in resistant cultivars developing tan spot disease (Hosford 1982; Weise 1987). Hosford et al. (1987) reported that increasing the post-inoculation wet period increased disease infection and development, although disease development in resistant cultivars was always less than in susceptible cultivars. However, Lamari and Bernier (1989a) found resistance to be independent of the length of the leaf wetness period.

2.5 Histology of Infection Process

Several studies have described the nature and timing of infection and colonization of resistant and susceptible cultivars of wheat by *P. tritici-repentis* (Larez et al. 1986; Loughman and Deverall 1986; Lamari and Bernier 1989c; Dushnicky 1993; Dushnicky et al. 1996, 1998). Larez et al. (1986) studied the period between 3 and 72 h after inoculation and found that most infection occurred between 6 and 24 h. Conidia germinated to produce germ tubes, which formed appressoria above epidermal cells or stomatal complexes. Infection tubes developed from the appressoria, and penetrated the epidermal cell by both mechanical puncture and enzymatic hydrolysis. Delayed infection

or failure to penetrate the epidermal cell wall was associated with the formation of papillae and occasionally haloes at the infection site in both susceptible and resistant hosts. If penetration did not occur below an appressorium, the germ tube grew further along the leaf surface to produce a secondary appressorium. Successful penetration of epidermal cells occurred with the formation of an intracellular vesicle, which produced one or more secondary intracellular hyphae (Larez et al. 1986; Lamari and Bernier 1989c; Dushnicky et al. 1996, 1998).

Lamari and Bernier (1989c) stated that differences between compatible and incompatible reactions were evident at 72 h after inoculation and that the specific host reaction was expressed only after the pathogen had grown intercellularly within the mesophyll. In compatible reactions the mesophyll cells surrounding the invading hyphae or within close proximity to the intercellular hyphae became disrupted. In susceptible plants the spread of fungal hyphae was limited by the larger mid-veins. However, in a resistant plant, the secondary hyphae were restricted to the infected epidermal cell and/or to a localized area of the mesophyll at the infection site. Mesophyll cell walls became thickened and intercellular spaces surrounding the infection site were filled with material, restricting the growth of the fungus. Histochemical staining of the thickened regions indicated the presence of lignin or lignin-like material. Lignification is the major resistance mechanism in the resistant host (Dushnicky 1993).

2.6 Pathogen Variability

The pathogenic variability of isolates of *P. tritici-repentis* has been studied (Misra and Singh 1972; Luz and Hosford 1980; Gilchrist et al. 1989; Schilder and Bergstrom

1990; Krupinsky 1987, 1992). In these studies quantitative measures such as disease severity, lesion number, lesion size and percentage leaf area infected were used to differentiate isolates of *P. tritici-repentis*.

Misra and Singh (1972) pioneered the study of variation in the pathogen population and observed variability based on lesion size. Similar differences in virulence, based on lesion size, were observed by Gilchrist et al. (1984) in eight isolates collected from the wheat cultivar Morocco in Mexico. Luz and Hosford (1980) screened 40 isolates collected from the USA and Canada and observed significant variation for percent leaf area infected and number of lesions produced per cm^2. Using means separation, they grouped the isolates into 12 races.

Schilder and Bergstrom (1990) tested 17 isolates of *P. tritici-repentis* on 12 wheat genotypes and found moderate physiological variation based on necrotic leaf area, whereas Krupinsky (1987) studied 27 isolates obtained from *Bromus inermis* and found them to be similar in virulence and morphologically identical. In a later study, however, Krupinsky (1992) found that 61 isolates from grass species and 26 isolates from barley differed for virulence on wheat genotypes, indicating some degree of specialization among the isolates from the two sources.

Quantitative measurements fail to precisely identify pathogen races as they imply a continuum of reaction types (Lamari 1988). Hence, Lamari and Bernier (1989c) reported that isolates of *P. tritici-repentis* could be classified into pathotypes based on their ability to induce tan necrosis and/or extensive chlorosis. Initially three pathotypes were identified which were referred to as pathotype 1 which induced both necrosis and extensive chlorosis, pathotype 2 which induced necrosis only and pathotype 3 which

induced extensive chlorosis only. Subsequently, Lamari et al. (1991) identified pathotype 4, which is avirulent and fails to induce any symptoms.

The symptom-based pathotype classification is limited to four broad categories. The ability of the pathogen to generate new virulence, as evidenced by the physiological variation reported in many parts of the world, suggests that new virulence types may exist within the current pathotypes. Lamari et al. (1995a) identified Algerian isolates, which induced chlorosis but reacted differently on the differential cultivars. This led to the classification of isolates based on their virulence on a set of differential cultivars. Currently isolates of *P. tritici-repentis* are classified into five races (Lamari et al. 1995a).

Most studies have detected variation in virulence on susceptible cultivars but not on resistant sources, indicating limited variability for genetic virulence (De Wolf et al. 1998). The native prairie grasses, acting as hosts for overwintering and as a primary source of fungal inoculum, could play an important role as a source of genetic variation for the fungus and as a reservoir of fungal populations genetically different from those prevalent on wheat (Krupinsky 1987; Ali and Lamari 1997; De Wolf et al. 1998).

2.7 Role of Toxins in Tan Spot

P. tritici-repentis produces several toxins that mimic the tan necrosis and extensive chlorosis symptoms in susceptible wheat cultivars (Tomas and Bockus 1987; Lamari and Bernier 1989b; Brown and Hunger 1993; Zhang et al. 1997; Anderson et al. 1999; Ciuffetti and Tuori 1999). Tomas and Bockus (1987) were the first to report the occurrence of toxic compound(s) that induced tan spot symptoms when infiltrated into the host plants. They concluded that the different reactions obtained from infiltration of

resistant and susceptible plants suggested that culture filtrates could be used to screen for resistance. They also suggested that disease resistance might be due, at least in part, to insensitivity to the toxic compounds.

2.7.1 Necrosis Inducing Toxins

Lamari and Bernier (1989b) reported that crude and dialyzed culture filtrates from isolates of pathotype 1 and 2 contained a heat labile (121°C for 20 min) toxin(s), which induced necrotic symptoms on cultivars susceptible to necrosis inducing pathotypes of *P. tritici-repentis*. Isolates from pathotypes 3 and 4 did not contain the toxin(s). Lamari and Bernier (1989b) reported that the toxin(s) of *P. tritici-repentis* is cultivar-specific, has a high molecular weight and appears to be a pathogenicity factor.

The toxic compound(s) from necrosis inducing *P. tritici-repentis* culture filtrates have been purified and characterized (Ballance et al. 1989; Thomas et al. 1990; Tuori et al. 1995; Zhang et al. 1997). Ballance et al. (1989) reported that the toxin was a heat labile protein of M_r 13,900 (designated as the Ptr necrosis toxin) with an average minimum active concentration of 0.2 nM. Zhang et al. (1997) found that the toxin was a heat labile protein of M_r 13,215 with an isoelectric point at pH 10. Thomas et al. (1990) described the toxin as a heat-stable protein of M_r 14,700 (designated as Ptr toxin) with an average minimum active concentration of 90 nM. Tuori et al. (1995) reported a 13.2 kDa heat labile protein (designated as ToxA), which induced visible necrosis in sensitive cultivars at an average minimum concentration of 60 nM. Tuori et al. (1995) also observed other less abundant necrosis inducing components in culture filtrate that were chromatographically and immunologically distinct from ToxA. Comparison of these proteins showed only minor differences in amino acid content. Differences in molecular

weight, specific activity and heat lability suggested that multiple toxins might be produced by *P. tritici-repentis* (Tuori et al. 1995; Meinhardt et al. 1997). At the Third International Tan Spot Workshop it was decided to refer to the necrosis-inducing toxin produced by race 1 and 2 as Ptr ToxA (Lamari and Gilbert 1998).

P. tritici-repentis produces a second class of necrosis-inducing phytotoxins that are composed of spirocyclic lactams named triticones (Kenfield et al. 1988). These phytotoxins consist of six structurally similar triticones (A, B, C, D, E and F). Triticones have been reported to be host non-selective in their ability to induce necrosis (Kenfield et al. 1988; Hallock et al. 1993).

The role of the necrotic toxin(s) in the infection process is to cause further fungal growth and movement into the intercellular spaces of the mesophyll after penetration and colonization of the epidermal cells have been accomplished (Lamari and Bernier 1989b). The observation that both virulent and avirulent isolates of *P. tritici-repentis* are capable of penetration and colonization, but only the toxin producing isolates are able to complete the infection process, supports the above hypothesis.

Lamari and Bernier (1994) reported that cultivars that are sensitive to the necrosis inducing toxin become insensitive at 27°C. However, if plants infiltrated with toxin at 27°C were transferred to 22°C, the plants will again become toxin-sensitive. Lamari and Bernier (1994) concluded that this was consistent with protein behavior and suggested that the breakdown of the compatible interaction (sensitivity/susceptibility) may be caused by a failure of the toxin to interact with its receptor. Anderson et al. (1999) proposed that insensitivity was not conferred by a gene product *per se*, but rather by the absence of a gene for sensitivity to the toxin.

Anderson et al. (1999) hypothesized that the necrosis inducing toxin interacts with the product of the gene conferring sensitivity in the host. Kwon et al. (1998) examined the role of wheat metabolism in the host-pathogen interaction and indicated that active transcription, active translation, and functional host H-ATPase was required for toxin activity. The absence of the sensitivity gene would result in the absence of a receptor or binding site for the toxin(s), leading to a disruption of the signaling cascade required for toxin(s) activity and ultimately to insensitivity of the host (Anderson et al. 1999)

A single recessive nuclear gene controls insensitivity to the Ptr-necrosis toxin (Lamari and Bernier 1989b; Lamari and Bernier 1991; Duguid 1995; Faris et al. 1996; Anderson et al. 1999). Faris et al. (1996) and Stock et al. (1996) reported that the gene for resistance to the necrosis inducing race 2 and insensitivity to the Ptr-necrosis toxin was located on chromosome arm 5BL, and proposed the symbol *tsn1* to designate this gene.

Genetic studies indicate that the same gene controls resistance to necrosis induced by spore inoculation and culture filtrate infiltration however, differential responses by host plants to spore inoculation and culture filtrate have been observed. Lamari and Bernier (1989b) found that wheat cultivar Columbus was a mixture of two lines. These lines were morphologically identical and susceptible to isolate ASC1 but demonstrated opposite reactions to the Ptr necrosis toxin. Riede et al. (1996) reported differential responses to toxin and conidial inoculation in two synthetic lines PF844005 and PF844008. Similarly, Zhang and Jin (1998) reported differential responses in wild relatives of wheat when tested with fungal spore inoculation and culture filtrate infiltration. Although the toxin is a major factor in the host-parasite interaction,

additional factors operate in the system. Further research will be necessary to determine other genes responsible for resistance (Riede et al. 1996).

2.7.2 Chlorosis Inducing Toxins

Genetic data indicates that chlorosis induced by race 3 in line 6B-365 follows the toxin model proposed by Ellingboe (1981) (Lamari and Bernier 1991). The chlorotic symptom is suppressed at temperatures above 27°C in a manner which parallels the suppression of the tan necrotic symptom (Lamari and Bernier 1994).

Several attempts to isolate toxin(s) from chlorosis inducing isolates and culture media were unsuccessful (Lamari and Bernier 1989b). However, Brown and Hunger (1993) isolated low molecular weight (800 and 1800 Da) toxins, which induced the characteristic chlorosis associated with tan spot. Meinhardt et al. (1997) and Effertz (1998) identified a *P. tritici-repentis* isolate that produced a chlorosis toxin in culture. This toxin induced chlorosis in the wheat line 6B-365, had a small molecular weight (<2000 Da), was very polar, non-ionic and not extractable by butanol. Although this toxin was isolated from a race 1 isolate, the toxin mimics the pathogenicity of race 3. This toxin has not been structurally characterized. The relationship of the low molecular weight chlorosis inducing toxin reported by Brown and Hunger (1993) to that reported by Meinhardt et al. (1997) and Effertz (1998) is unknown.

Orolaza et al. (1995) reported that a host-specific toxin was involved in development of the chlorosis phenotype of tan spot. The chlorosis toxin appeared to be a pathogenicity factor and was designated as Ptr-chlorosis toxin. This toxin was isolated from race 5, a new race found in Algeria, and exhibited the same host genotype specificity as the fungus on hexaploid wheats. Preliminary characterization suggested

that the Ptr-chlorosis toxin was much smaller than the Ptr-necrosis toxin molecule and was water soluble, heat labile and stable to exposure to organics (Orolaza et al. 1995). Susceptibility to the tan spot fungus and sensitivity to the toxin were under the same genetic control (Orolaza et al. 1995; Gamba et al. 1998). At the Third International Tan Spot Workshop, it was agreed to designate the chlorosis inducing toxin produced by races 1 and 3 as Ptr ToxC and that produced by race 5 as Ptr ToxB (Lamari and Gilbert 1998).

2.8 Control Measures

Control of tan spot is possible by chemical, cultural, genetic resistance and biological measures (Ciuffetti and Tuori 1999). Destroying and/or removing infested straw, stubble and volunteer wheat from the field by either burying the residue through tillage or burning it will control primary inoculum levels of *P. tritici-repentis* (Adee and Pfender 1989; Rees and Platz 1992).

Summerell and Burgess (1989) reported that *P. tritici-repentis* could survive on wheat chaff and stubble for at least two years under cool and dry conditions, which inhibit stubble decomposition. Therefore, for successful control of tan spot by crop rotation, wheat should be not grown more frequently than once every three years and should be replaced in the rotation by nonhost crops (Summerell and Burgess 1989). Application of nitrogen fertilizer has also been reported to reduce disease severity of tan spot (Huber et al. 1987).

Chemical treatments involve application of fungicides. Protectant fungicides such as manocozeb (Dithane M-45, Manzate 200) can effectively control tan spot of wheat, but their use may not be economically or environmentally safe. The systemic fungicide

Tilt® (propiconazole) is also used in the prairie region (Duguid 1995; Schilder and Bergstrom 1992a; Bockus 1998). Systemic seed dressing fungicides such as tridimenol have been used to control disease early in the season (Duguid 1995; De Wolf et al. 1998).

Another means of controlling tan spot involves use of biocontrol agents. These biocontrol agents are antagonistic microorganisms, which could displace *P. tritici-repentis* from the stubble and wheat residues (Bockus 1998). *Limonomyces roseipellis* (Pfender et al. 1991) and *Cochliobolus sativus* (Luz and Bergstrom 1987) have been shown to antagonize *P. tritici-repentis* and have effectively reduced *P. tritici-repentis* populations. However, both *L. roseipellis* and *C. sativus* are pathogenic to wheat.

Seed-applied bioprotectants may be a useful tactic for enhancing wheat health, specifically for managing seedborne inoculum of *P. tritici-repentis* (Luz 1992; Luz et al. 1998). The bioprotectants *Paenibacillus macerans* (Embrapa Trigo), *Pseudomonas putida* (Embrapa Trigo), *Trichoderma harzianum* (BioWorks T-22) and *T. virens* (BioWorks G-41) increased seedling emergence and grain yield of wheat significantly in field tests. Grain yield was equivalent or superior to that when seed treatment with iprodione and thiram was used (Luz et al. 1998).

The most effective, economical and environmentally friendly means of controlling tan spot disease involves incorporation of genetic resistance into commercially grown wheat cultivars. As colonization and growth of the pathogen on senesced tissue is independent of the reaction of the living host to infection, use of resistant cultivars is unlikely to cause a reduction in disease carry over (Summerell and Burgess 1988), however it will reduce wheat yield losses due to tan spot. Effective

resistance has been identified and is being used in different breeding programs to develop tan spot resistant cultivars (Lamari and Bernier 1989a; Gilchrist 1992; Riede et al. 1996).

Lamari and Bernier (1989a) found high levels of resistance to tan spot in all ploidy levels of wheat. Resistance was also found in related wild species especially *T. dicoccoides* (AABB), *T. dicoccum* (AABB), *T. persicum* (CCUU), *T. timopheevii* (AAGG), and *T. zukhovskii* (AAAAGG). Similarly, Alam and Gustafson (1988) observed resistant accessions in *Aegilops speltoides* (SS), *Ae. triaristata* (UUMM), *Ae. cylindrica* (CCDD), and *Ae. ovata* (UUMM). In a recent study by Zhang and Jin (1998), high levels of resistance were observed in accessions of *T. monococum* (AA), *Ae. tauschii* (DD) and *Ae. speltoides* (SS).

Rees and Platz (1990) found no immune reaction to tan spot in more than 1,400 bread wheat accessions. However, resistance occurs at different levels in many spring and winter types especially in Brazilian spring wheats. Gilchrist (1992) reported resistance to tan spot in Chinese, Brazilian, and Mexican lines and the progeny from one interspecific cross with *Agropyrum curvifolium*. High levels of resistance in bread wheat and synthetic wheat have been observed by other researchers (Evans et al. 1992; Riede et al. 1996).

2.9 Inheritance of Resistance

Prior to the identification of the two separate components of tan spot of wheat and the development of the qualitative rating scale by Lamari and Bernier (1989a), few genetic studies of the inheritance of resistance to tan spot had been conducted and those studies had used quantitative parameters for disease assessment. Nagle et al. (1982)

assessed disease severity as percentage leaf area infected in studies of the inheritance of resistance in six hexaploid and five tetraploid sources. They concluded that the inheritance of resistance was complex since the data obtained did not fit simple Mendelian segregation ratios.

Other reports of quantitative control of resistance were made by Shabeer (1986) who studied resistant cultivar Red Chief and Rees (1987) who found resistance to be incomplete and controlled by four or more recessive genes in eight sources of resistance. Elias et al. (1989) reported that resistance in durum wheat was polygenic. Faris et al. (1997) observed that resistance to the chlorosis component of tan spot was controlled quantitatively and that a large portion of the variation was controlled by quantitative trait loci (QTL) on chromosome 1AS in the cross of resistant synthetic hexaploid W-7984 with susceptible Opata 85.

Lee and Gough (1984) reported resistance to be controlled by a single recessive gene in the Chilean cultivar Carifen 12 when crossed with the susceptible cultivar TAM W-101. After Lamari and Bernier (1989a) developed the 1 to 5 qualitative rating scale and identified races of *P. tritici-repentis* most genetic studies have used isolate ASC1 which belongs to race 1 (Lamari and Bernier 1989b: Sykes and Bernier 1991; Duguid 1995. Gamba and Lamari 1998). Sykes and Bernier (1991) studied two accessions of diploid wheat with isolate ASC1 and reported that a single recessive gene controlled resistance. Lamari and Bernier (1989b) studied one tetraploid accession (4B242) and also reported that resistance was controlled by a single recessive gene. Sykes and Bernier (1991) studied crosses involving resistant cultivars Salamouni, Erik and Carifen 12 with the susceptible cultivar Columbus. They reported that two nuclear recessive genes

controlled resistance to the isolate ASC1. However, Lamari and Bernier (1989b) had previously reported that resistance in cultivars Erik and Salamouni was due to a single gene. Monogenic control was also reported by Duguid (1995) who studied resistance to race 1 (isolate ASC1) in four cultivars Erik, ST6, 6B367, and 6B1043. Resistance to the tan necrosis component was controlled by a single recessive gene while a single dominant gene controlled resistance to the chlorosis component. Lack of segregation in crosses involving the resistant sources indicated that they shared at least one resistance gene. Gamba and Lamari (1998) found resistance to race 1 in the tetraploid wheat cross 4B160 x Coulter to be controlled by two independently inherited genes.

Lamari and Bernier (1991) studied reciprocal crosses in all combination between Glenlea (susceptible-necrosis), 6B-365 (susceptible-chlorosis) and Salamouni (resistant-both necrosis and chlorosis). They studied resistance to necrosis induced by isolate Ptr 86-124 (race 2) and resistance to chlorosis induced by isolate Ptr D308 (race 3). The F_2 generation and $F_{2:3}$ family segregation ratios were consistent with the action of two independent genes, one recessive gene controlling resistance to tan necrosis and the other dominant gene controlling resistance to extensive chlorosis.

Duguid (1995), using races 2 (isolate Ptr 86-124) and 3 (isolate Ptr D308) obtained results similar to those of Lamari and Bernier (1989b, 1991). In four of nine crosses studied, resistance to necrosis in the F_2 generation was found to be under two-gene control, but, the $F_{2:3}$ family segregation ratio indicated single gene control. Similar findings were observed in two crosses, ST15 x BH1146 and ST15 x Katepwa, which segregated for resistance to the chlorosis component. Stock (1996) studied the F_1 and F_2 generations and $F_{2:3}$ families in a cross between Chinese Spring and Kenya Farmer and

identified a single nuclear recessive gene governing resistance to necrosis induced by isolate Ptr 86-124 (race 2).

Although quantitative inheritance of resistance to tan spot has been reported (Nagle et al. 1982; Shabeer 1986; Elias et al. 1989; Faris et al. 1997), most recent studies have indicated qualitative genetic control (Lamari and Bernier 1989b, 1991; Sykes and Bernier 1991; Duguid 1995; Gamba et al. 1998; Anderson et al. 1999). Currently, it is believed that four independent genes control resistance to tan spot in tetraploid wheat, one for resistance to chlorosis induced by race 1 and the remaining three for resistance to necrosis induced by races 1 and 2, 3 and 5, respectively (Gamba and Lamari 1998). Gamba et al. (1998) found that three independent genes controlled resistance to tan spot in hexaploid wheat, one gene for resistance to necrosis caused by race 2 and two genes for resistance to chlorosis, one for chlorosis induced by races 1 and 3 and the other for race 5. The relationship between the resistance genes in tetraploid and hexaploid wheat is not known.

2.10 Molecular Markers

Molecular markers are of interest to plant breeders as a source of genetic information and for use in indirect selection of traits linked with the markers (Kelly 1995). Prior to DNA-based markers, morphological markers, protein and isozyme markers were used (Bonde at al. 1993; Mikas et al. 1993), but the number of traits that could be tagged was small because of the limited number of such markers (Mikas et al. 1993).

Disease screening based on the phenotype of an individual is less effective if the effect of the environment is high and/or if resistance is polygenic. Conventional disease screening is laborious, time consuming, expensive and may involve complicated procedures (Wechter et al. 1995). Marker-based selection would be more efficient provided there was tight linkage between the marker and the trait of interest and that selection for the marker was more convenient (faster, cheaper, reproducible, expressed earlier). Markers for disease resistance offer the additional advantage of permitting selection in absence of the pathogen and facilitate gene pyramiding (Kelly 1995; Naqvi and Chattoo 1996). Successful marker assisted selection depends on identification of markers that are user friendly and are tightly linked to the trait of interest.

2.11 Molecular Markers Relevant to this Study

2.11.1 RAPD Analysis

The discovery of heat-stable DNA polymerase, in conjunction with the automated temperature cycler led to the development of polymerase chain reaction (PCR) in 1985 by K.B. Mullis. The PCR involves a series of cycles each comprising of three steps: a) heat denaturation of the double stranded DNA, b) primer annealing to complementary sites to the DNA template, and c) synthesis of a new DNA strand by extension from the primers with a DNA polymerase using nucleoside triphosphates (Mullis and Faloona 1987).

The random amplified polymorphic DNA (RAPD) technique (Williams et al. 1990; Welsh and McClelland 1990) reveals DNA variation through the selective amplification of a specific segment within the total genomic DNA by the polymerase chain reaction (PCR). The RAPD assay is based on the use of synthetic 10-mer

oligonucleotides as primers in a DNA amplification reaction (Williams et al. 1990). The primer, when mixed with genomic DNA and thermostable polymerase, and subject to temperature cycling similar to PCR, will prime the amplification of several fragments. Amplification products can then be separated by electrophoresis on agarose or polyacrylamide gels and visualized by staining with ethidium bromide or silver.

The RAPD assay is highly sensitive to nucleotide differences between the primer and the template (Williams et al. 1990) assaying single nucleotide differences. The RAPD assay functions as a convenient, effective and relatively inexpensive method of identifying molecular markers. For RAPD analysis only small amounts of genomic DNA (15-25 ng) of moderate quality are required (Williams et al. 1990). A major advantage of RAPD assay is that it can easily be applied to a large number of samples and can be automated (Lu et al. 1996).

However, RAPD analysis has several disadvantages that restrict its value as a selection tool in marker-assisted selection. Since RAPD is a dominant marker, it cannot differentiate between heterozygous and homozygous genotypes and, thus, its reliability is reduced in early generation selection. Difficulties in ensuring repeatability and reliability of RAPD marker can create problems. RAPD analysis is highly sensitive to the reaction conditions, in particular the template-primer ratio, the thermocycling conditions and Mg^{2+} concentration (Penner et al. 1993; Morrell et al. 1995).

2.11.2 SCAR Analysis

Utility of a desired RAPD marker can be increased by sequencing its termini and designing longer primers for specific amplification of markers (Paran and Michelmore 1993; Morrell et al. 1995; Naqvi and Chattoo 1996). These SCAR primers are then used

in a normal PCR with high annealing temperature. DNA sequence differences are manifested by the presence or absence of a single unique band (Staub et al. 1996).

SCAR markers are more reproducible than RAPD markers and can be developed into a plus/minus array where electrophoresis is not needed (Staub et al. 1996). The advantages of SCAR over RAPD markers are that as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into codominant markers (Paran and Michelmore 1993; Naqvi and Chattoo 1996; Staub et al. 1996).

2.12 Bulked Segregant Analysis

Michelmore et al. (1991) developed the bulked segregant analysis, which involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool or bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other genes. Two bulked DNA samples (i.e. one DNA bulk from homozygous resistant and one from homozygous susceptible plants for a particular disease) are analyzed to identify markers that distinguish them. Polymorphic markers are potentially linked to the locus controlling the trait of interest. Each polymorphic marker is then studied over the whole population to establish linkage and to determine the genetic distance between the trait and the marker.

The number of individuals used to constitute the bulks depends upon the frequency with which unlinked loci might be detected as polymorphic between the bulk samples. This, in turn, depends upon the type of marker and the type of population used. Bulks can be based on as few as 3-4 individuals, however, most bulks involve 7-10

individuals. With a smaller bulk size, the frequency of false positive increases and with a larger bulk size, polymorphisms observed between bulks decrease (Michelmore et al. 1991).

2.13 Molecular Markers for Tan Spot of Wheat

2.13.1 RFLP Markers

Faris et al. (1996) identified RFLP markers linked to gene *tsn1* for resistance to the necrosis component of tan spot in the cross of resistant synthetic hexaploid W-7976 with the susceptible cultivar Kulm. They found fragments detected by probes *Xbcd 1030* and *Xwg 583* flanking the *tsn1* locus at distances of 5.7 cM and 16.5 cM, respectively. Through aneuploid analysis they determined that *tsn1* was located on the long arm of chromosome 5B.

In a later study, Faris et al. (1997) used RFLP to analyze the cross of the resistant synthetic hexaploid W-7984 with susceptible Opata 85. They observed that resistance to the chlorosis component was controlled quantitatively, but that a large portion of the variation observed was accounted for by quantitative trait loci (QTL) on chromosome 1AS. Further RFLP analysis indicated a gene with a minor effect on 4AL and an interaction between the 1AS gene and a gene on 2DL. Together these loci explained 49% of the variation for resistance to chlorosis in this population.

2.13.2 RAPD Markers

Stock (1996) identified two RAPD markers loosely linked to the tan spot resistance gene *tsn1* using temperature sweep gel electrophoresis. Linkage analysis indicated that the polymorphic fragments amplified by the primers UBC 195 and UBC

102 were located 23.4 ± 5.7 cM and 27 ± 6.9 cM from *tsn1*, respectively. UBC 195 and UBC 102 were tightly linked (4.2 ± 2.5 cM).

2.14 Genetic Similarity among Fungal Isolates

The analysis of genetic diversity and relatedness between or within different individuals, populations and species is central to many disciplines of biological science. The classical strategies of assessing genetic variability such as comparative anatomy, morphology, embryology, and physiology are being increasingly replaced by more accurate and economic molecular techniques.

Mycologists and plant pathologists have adopted various molecular biology techniques to settle taxonomic disputes, identify cultures, fingerprint fungal isolates, analyze genetic variability, trace pathogen spread and identify ploidy levels in fungi and other plant pathogens (Bonde et al. 1993). In recent years the utilization of molecular markers for the detection of species and races of various diseases has become very popular.

Most of the current tests are based on exploiting sequence polymorphism within internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA). Alternative strategies for developing species or race specific markers have employed unique sequences of mitochondrial DNA or cloned restriction fragments of genomic DNA (Schilling et al. 1996). Caetano-Anolles et al. (1991) reported that DNA amplification fingerprinting (DAF), using one oligonucleotide primer of arbitrary sequence, produced a characteristic spectrum of short DNA products of varying complexity that could detect differences between organisms. Genetic markers generated by RAPD and other PCR

based techniques have proven to be useful for studying genetic variability and phylogenetic relationships within and between a wide range of organisms (Achenbach et al. 1996; Shi et al. 1996).

Several molecular techniques have been used to study the bio-diversity of fungi. These techniques include protein and DNA-based methodologies. At the protein level immuno-chemistry, amino-acid sequence data, allozyme and isozyme analyses have been used. At the DNA level, various assays such as DNA-DNA hybridization, restriction enzyme analysis, DNA sequencing and PCR based techniques like RAPD and AFLP are available.

Beck (1999) developed a diagnostic PCR assay based on an internal transcribed spacer (ITS) for the detection of *P. tritici-repentis* in wheat. These primers are very specific to *P. tritici-repentis* but fail to differentiate the different races of *P. tritici-repentis*. A preliminary study by Zinno et al. (1998) with a limited number of isolates of *P. tritici-repentis* indicated the presence of polymorphism between isolates, but no correlation could be established between these differences and geographic origin, pathogenicity and toxin production. A few studies to assess the genetic variability of the closely related species *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* (Crous et al. 1995; Peltonen et al. 1996; Campbell et al. 1999) have been conducted, but no comprehensive study to assess the genetic composition of isolates of *P. tritici-repentis* in relation to their race classification and their geographical origin has been reported. The extent of variability among and within the races of *P. tritici-repentis* is yet to be assessed.

The analysis of molecular variance (AMOVA) developed by Excoffier et al. (1992) is designed to analyze molecular marker data. The AMOVA analysis estimates

and partitions marker variance into hierarchical source levels, e.g. among populations and within populations. It uses the genetic distance matrix generated from the molecular data as the input data and performs variance partitioning analogous to the ANOVA analysis.

Use of AMOVA has increased the utility of molecular markers for assessment of genetic relationship among and between species. The AMOVA analysis has been used for RFLP data (Rosewich et al. 1999), RAPD data (Nebauer et al. 2000) and AFLP data (Shim and Jorgensen 2000). When used with RAPD data, however, the AMOVA results must be interpreted with caution due to the dominant nature of RAPD, which can cause overestimation of the similarity among or between populations.

3. GENETICS OF RESISTANCE TO TAN SPOT OF WHEAT

3.1 Introduction

Tan spot of wheat, caused by the fungus *Pyrenophora tritici-repentis*, is a destructive disease worldwide that can lead to serious losses both in grain quantity and quality of wheat (Hosford 1982; Weise 1987; Ciuffetti and Tuori 1999). The tan spot disease syndrome consists of two phenotypically distinct and independent symptoms, tan necrosis and extensive chlorosis (Lamari and Bernier 1989a). Within these components a range of reaction types has been described by Lamari and Bernier (1989a). Resistance is expressed as small, dark brown lesions that do not increase in size while susceptibility is expressed as dark brown spots surrounded by tan necrosis and/or extensive chlorosis that may involve the entire leaf. Necrotic lesions are well defined and tan in color while chlorotic lesions are less well defined and exhibit gradual yellow discoloration (Lamari and Bernier 1989a).

Initially, isolates of *P. tritici-repentis* were grouped into four pathotypes (P) based on their ability to induce, on a differential set of wheat cultivars, tan necrosis and extensive chlorosis (P1: n+c+), tan necrosis only (P2: n+c-), extensive chlorosis only (P3: n-c+) or no necrotic or chlorotic symptoms (P4: n-c-) (Lamari and Bernier 1989c, 1991). More recently, however, isolates of *P. tritici-repentis* have been classified into five races based on their virulence on a set of differential wheat cultivars (Lamari et al. 1995a).

Resistance to tan spot of wheat has been reported to be inherited either quantitatively (Nagle et al. 1982; Shabeer 1986; Elias et al. 1989; Faris et al. 1997) or

qualitatively (Lamari and Bernier 1989b, 1991; Duguid 1995; Faris et al. 1996; Gamba et al. 1998; Gamba and Lamari 1998; Anderson et al. 1999). Information from genetic studies helps in deciding the breeding strategies to be adopted and the resistant sources to be utilized in developing resistant cultivars. Hence, the objectives of this study were:

1) to study the genetic control of resistance to *P. tritici-repentis* races 1, 2 and 3;
2) to determine the allelic relationship among the resistance gene(s) possessed by the different sources of resistance;
3) to study the relationship between the gene(s) controlling resistance to necrosis caused by races 1 and 2; and
4) to determine the relationship between the gene(s) controlling resistance to chlorosis induced by races 1 and 3.

3.2 Materials and Methods

3.2.1 Population Development

Based on their reaction to different races of *P. tritici-repentis*, seven cultivars were selected for this study. Their growth habit, country of origin, pedigrees and response to *P. tritici-repentis* races 1, 2 and 3 are given in Table 3.1. The seven selected cultivars Erik, Hadden, Red Chief, 86ISWN 2137, 6B-365, Glenlea and Kenyon were crossed in all possible combinations, without reciprocals, to produce the cross populations used in this study (Appendix I). The cross Kenyon x 6B-365 was not studied. Each F_1 plant grown to produce F_2 seed was harvested separately. The individual

Table 3.1 Cultivars selected for the study of the inheritance of resistance to tan spot of wheat

Cultivar	Growth habit	Origin	Reaction to *P. tritici-repentis*			Pedigree
			Race 1	Race 2	Race 3	
Erik	Spring	USA	R-Nec[a]/R-Chl[b]	R-Nec	R-Chl	Kitt/2/Waldron/Era
86ISWN 2137	Spring	CIMMYT	R-Nec/R-Chl	R-Nec	R-Chl	Not Known
Glenlea	Spring	Canada	S-Nec[c]/R-Chl	S-Nec	R-Chl	Pembina2/Bage/2/CB100
Kenyon	Spring	Canada	S-Nec/Mod. R-Chl	S-Nec	Mod. R-Chl	Neepawa*5/Buck Manantial
6B-365	Winter	Lebanon	R-Nec/S-Chl[d]	R-Nec	S-Chl	Not Known
Red Chief	Winter	USA	R-Nec/R-Chl	R-Nec	R-Chl	Red Clawson/Red Arcadian
Hadden	Winter	USA	R-Nec/R-Chl	R-Nec	R-Chl	Trumbull*2/Red Wonder/2/ *T. timopheevi*/Steinwedel/3/ Asosan/Hardired/4/Chancellor/ 5/Coker/47-27/6/Coastal

[a] Resistant to the necrosis component of tan spot of wheat.

[b] Resistant to the chlorosis component of tan spot of wheat.

[c] Susceptible to the necrosis component of tan sot of wheat.

[d] Susceptible to the chlorosis component of tan spot of wheat.

F_2 populations of each cross were tested for segregation for disease reaction to eliminate non-hybrid populations. $F_{2:3}$ families. $F_{3:4}$ families and $F_{7:8}$ families were produced by single seed descent in the greenhouse and/or the field from random F_2 plants.

Crosses of the resistant cultivars Erik. 86ISWN 2137. Hadden and Red Chief with each of the two susceptible cultivars Glenlea and Kenyon were studied to determine the inheritance of resistance to necrosis induced by race 2. The allelism studies involved the six possible crosses among the four resistant cultivars Red Chief. 86ISWN 2137. Erik and Hadden. and the cross between susceptible cultivars Kenyon and Glenlea. In both the inheritance and allelism studies. the F_1 and F_2 generations and $F_{2:3}$ families were tested with race 2. $F_{3:4}$ families were also tested with race 2 to provide additional data for the inheritance study.

To determine the inheritance of resistance to the chlorosis component caused by race 3. crosses of resistant cultivars Hadden. 86ISWN 2137. Glenlea. Red Chief and Erik with the susceptible line 6B-365 were studied. All possible crosses excluding reciprocals among the five resistant cultivars were studied to determine the allelism of the genes controlling resistance to the chlorosis component. The F_1 and F_2 generations and $F_{2:3}$ families were tested with race 3 in both the inheritance and allelism studies.

The following five crosses were studied to determine the inheritance of resistance to the necrosis and the chlorosis components of tan spot caused by race 1:

1) Erik x Glenlea - segregating for resistance to the necrosis component;
2) Hadden x Glenlea - segregating for resistance to the necrosis component;
3) 86ISWN 2137 x 6B-365 - segregating for resistance to the chlorosis component;
4) Erik x 6B-365 - segregating for resistance to the chlorosis component; and

5) Glenlea x 6B-365 - segregating for resistance to both the necrosis and the chlorosis components of tan spot.

To establish the relationship between the genetic control of resistance to the necrosis component of tan spot caused by races 1 and 2. $F_{7:8}$ lines of the cross Erik x Glenlea were tested with both races. $F_{2:3}$ families of the cross Erik x 6B-365 were tested with races 1 and 3 to determine the relationship between gene(s) controlling resistance to the chlorosis component caused by both races.

The family size tested in the allelism studies was 12 plants. Assuming a single gene control for resistance, this family size gave a 95% probability of being able to distinguish between a segregating family and a non-segregating family (Hanson 1959). At least 40 families per cross were tested in the allelism study. A family size of 16-18 plants was used in the inheritance studies. This gave a 99% probability of being able to distinguish between a segregating and a non-segregating family if resistance was controlled by a single gene. The cross Glenlea x 6B-365 segregated for both the necrosis and the chlorosis components of tan spot when tested with race 1. It was assumed that resistance to tan spot in this cross was controlled by two genes and at least 36 plants per family were screened to ensure a 90% probability of being able to distinguish between a segregating and a non-segregating family (Hanson 1959). At least 60 families per cross were tested in the inheritance study.

To establish the relationship between the gene(s) controlling resistance to the necrosis component caused by races 1 and 2, and the chlorosis component caused by races 1 and 3, at least 16 plants per family were screened with each race. In this study, the same 80 families per cross were tested with each race.

3.2.2 Inoculum Production

A culture of isolate Ptr-200 (race 1), provided by Dr. G.R. Hughes, University of Saskatchewan, and two cultures of isolates Ptr 92-164 (race 2) and Ptr 94-8-2 (race 3), provided by Dr L. Lamari, University of Manitoba, were used. Single-spore cultures were produced for each of the three isolates and stored at 4°C in a refrigerator for inoculum production.

Inoculum was produced using a modification of the method of Lamari and Bernier (1989a). Mycelial plugs of 0.5 cm diameter from the stock cultures were transferred to 10 cm petri plates containing V8P agar [150 ml V8-juice, 10 g PDA, 10 g agar, 3 g $CaCO_3$ and 850 ml distilled water (Dhingra and Sinclair 1985)]. The cultures were incubated in the dark at 20-22°C for six days. The plates were then flooded with sterile distilled water, the mycelium flattened with the base of a sterile test tube and the excess water poured off. To induce conidiophore production the plates were incubated under continuous light at room temperature for two days followed by one day in the dark in an incubator at 15-16°C to induce conidia production.

The plates were flooded with distilled water and the conidia were suspended in the distilled water by gently brushing the mycelium with a camel-hair brush to dislodge the conidia from the conidiophores. In order to reduce surface tension 5-7 drops of Tween 20 (polyxyethlene sorbitan monolaurate) were added per liter of spore suspension. Spore concentration was measured with a haemocytometer and adjusted to 3000 conidia per milliliter by the addition of distilled water.

3.2.3 Disease Screening

Seed of the populations to be screened, along with appropriate checks, was pre-germinated and seeds at a similar stage of germination were planted in 6-inch diameter pots containing Redi-Earth (W.R Grace & Co. of Canada Ltd., Ajax, Canada) to maximize uniform emergence. In the allelism studies, 12 seeds per pot were planted, while nine seeds per pot were planted in the remaining studies. The pots were arranged at random when inoculation was done. Using a hand sprayer, plants at the two-leaf stage were sprayed until runoff with the conidial suspension of the appropriate isolate. Following inoculation, the seedlings were incubated for 24 h in continuous leaf wetness in a mist chamber located in a growth room at 22/17°C (day/night) with a 16 h photoperiod and then returned to benches in the same growth room.

Eight to ten days after spore-inoculation, the seedlings were rated for disease reaction. The 1-5 lesion type rating scale developed by Lamari and Bernier (1989a) was used to rate resistance to the necrosis component. Seedlings with ratings of 1-2 were considered resistant, while those with ratings of 3-5 were considered susceptible. For the chlorosis component, plants were rated as resistant or susceptible depending on the presence or absence of chlorosis. Since the chlorosis component is less well defined and is continuous in expression, rating of the segregating populations was based on the extent of chlorosis developed by the resistant and susceptible checks in each test.

3.3 Results

In all tests, Erik, Red Chief, Hadden and 86ISWN 2137 were resistant to both the necrosis and the chlorosis components. However, these resistant sources differed in the

level of resistance expressed. Erik and Red Chief were rated 1 and 86ISWN 2137 was rated 2, whereas Hadden was more variable, showing a 1-2 rating for the necrosis component. Glenlea and Kenyon were susceptible to the necrosis component, but were resistant and partially resistant to the chlorosis component, respectively. Line 6B-365 was susceptible to the chlorosis component but resistant to the necrosis component.

3.3.1 Genetics of Resistance to the Necrosis Component Caused by Race 2

F_1 plants of all eight resistant x susceptible crosses studied were susceptible indicating that resistance to the necrosis component is recessive (Table 3.2). However, a range of susceptible reaction types (ratings of 3-5) for F_1 plants in all crosses was observed. This range was more pronounced in some crosses than in others. F_1 plants of crosses involving Hadden as the resistant parent were less susceptible than F_1 plants of the other crosses.

The F_2 generation of all eight crosses segregated in a 1 resistant: 3 susceptible ratio indicating single gene control and confirming that resistance to the necrosis component of tan spot is recessive (Table 3.2). $F_{2:3}$ families segregated in 1 homozygous resistant: 2 segregating: 1 homozygous susceptible ratio supporting the hypothesis of monogenic control of resistance (Table 3.3). This hypothesis was further confirmed by $F_{3:4}$ family segregation which fit a 3 homozygous resistant: 2 segregating: 3 homozygous susceptible ratio for all eight crosses studied (Table 3.4).

All F_1 and F_2 plants of the cross between the susceptible cultivars Kenyon and Glenlea were susceptible, indicating that both parents share the same susceptibility gene(s) for the necrosis component of tan spot caused by race 2 (Table 3.2). The $F_{2:3}$

families tested were also susceptible confirming that there was no allelic difference for the susceptibility gene(s) possessed by those cultivars (Table 3.3).

Table 3.2. F_1 plant disease reaction to isolate Ptr 92-164 (race 2) and chi-square tests of F_2 segregation ratios of crosses used to study the inheritance and allelic relationships among the gene(s) controlling resistance to necrosis caused by Ptr 92-164 (race 2)

Cross	F_1 plants		F_2 plants		Ratio tested	χ^2 value	Probability value[a]
	R	S	R	S			
Resistant x Susceptible							
2137 x Kenyon	0	9	102	255	1:3	2.428	0.12
Erik x Kenyon	0	9	90	264	1:3	0.034	0.85
Red Chief x Kenyon	0	9	72	254	1:3	1.476	0.22
Hadden x Kenyon	0	8	95	305	1:3	0.333	0.56
2137 x Glenlea	0	9	80	274	1:3	1.089	0.30
Erik x Glenlea	0	9	96	256	1:3	0.969	0.33
Red Chief x Glenlea	0	9	112	320	1:3	0.197	0.65
Hadden x Glenlea	0	8	92	248	1:3	0.768	0.38
Susceptible x Susceptible							
Kenyon x Glenlea	0	9	0	340	0:1	-	-
Resistant x Resistant							
Erik x 2137	9	0	315	7	15:1	9.130	0.00
Erik x Hadden	8	0	338	5	15:1	13.444	0.00
Erik x Red Chief	8	0	311	9	15:1	6.453	0.01
2137 x Hadden	9	0	312	8	15:1	7.680	0.00
2137 x Red Chief	8	0	336	3	15:1	16.653	0.00
Hadden x Red Chief	8	0	308	13	15:1	2.652	0.10

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Table 3.3. Chi-square tests for $F_{2:3}$ family segregation ratios of crosses used to study the inheritance and allelic relationships among the gene(s) controlling resistance to necrosis caused by Ptr 92-164 (race 2)

Cross	$F_{2:3}$ families			Ratio tested	χ^2 value	Probability value[b]
	Resistant	Seg[a]	Susceptible			
			Resistant x Susceptible			
2137 x Kenyon	27	56	37	1:2:1	2.200	0.33
Erik x Kenyon	28	60	32	1:2:1	0.267	0.88
Red Chief x Kenyon	23	61	36	1:2:1	2.849	0.24
Hadden x Kenyon	26	61	33	1:2:1	0.850	0.65
2137 x Glenlea	28	54	38	1:2:1	2.866	0.24
Erik x Glenlea	31	55	34	1:2:1	0.983	0.61
Red Chief x Glenlea	25	64	31	1:2:1	1.133	0.57
Hadden x Glenlea	28	63	29	1:2:1	0.317	0.85
			Susceptible x Susceptible			
Kenyon x Glenlea	0	-	40	0:1	-	-
			Resistant x Resistant			
Erik x 2137	40	-	0	1:0	-	-
Erik x Hadden	40	-	0	1:0	-	-
Erik x Red Chief	40	-	0	1:0	-	-
2137 x Hadden	40	-	0	1:0	-	-
2137 x Red Chief	40	-	0	1:0	-	-

[a] Segregating for resistance.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Table 3.4. Chi-square tests for $F_{3:4}$ family segregation ratios of crosses used to study the inheritance of resistance to necrosis caused by Ptr 92-164 (race 2)

Cross	$F_{3:4}$ families			Ratio tested	χ^2 value	Probability value[b]
	Resistant	Seg[a]	Susceptible			
2137 x Kenyon	33	24	23	3:2:3	2.734	0.25
Erik x Kenyon	29	23	28	3:2:3	0.617	0.73
Red Chief x Kenyon	27	24	29	3:2:3	1.133	0.57
Hadden x Kenyon	26	23	31	3:2:3	1.017	0.60
2137 x Glenlea	24	22	34	3:2:3	1.950	0.38
Erik x Glenlea	25	23	32	3:2:3	1.402	0.50
Red Chief x Glenlea	29	25	26	3:2:3	1.817	0.40
Hadden x Glenlea	34	33	23	3:2:3	2.617	0.27

[a] Segregating for resistance.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

F_1 plants of the six possible crosses among the four parents resistant to the necrosis component of tan spot gave a resistant reaction to race 2. However, while most of the plants of the F_2 generations of these crosses were resistant, a few plants which showed a moderately susceptible (rating 3) reaction were observed. All crosses between the resistant parents, with the exception of Hadden x Red Chief, failed to fit a 15 resistant: 1 susceptible ratio suggesting that they carry the same gene (Table 3.2). None of the $F_{2:3}$ families of the crosses studied were susceptible indicating the presence of the same resistant gene among the resistant parents (Table 3.3). The F_2 generation segregation ratio in the cross Hadden x Red Chief indicated that Hadden and Red Chief possessed different resistance genes. This hypothesis could not be verified as $F_{2:3}$ families of the cross Hadden x Red Chief were not tested due to unavailability of seed.

3.3.2 Genetics of Resistance to the Chlorosis Component Caused by Race 3

F_1 plants of five resistant x susceptible crosses studied for inheritance of resistance to the chlorosis component caused by race 3 were resistant indicating that resistance was dominant (Table 3.5). The F_2 segregation data for each cross fit a 3 resistant: 1 susceptible ratio expected for single gene control of resistance and confirmed that resistance to the chlorosis component of tan spot was dominant (Table 3.5).

Monogenic control of resistance to the chlorosis component in four of the five crosses was confirmed by $F_{2:3}$ family segregation which fitted a 1 homozygous resistant: 2 segregating: 1 homozygous susceptible ratio (Table 3.6).

All plants tested in both the F_1 and F_2 generations of the ten crosses studied to determine allelism of the gene(s) controlling resistance to the chlorosis component of tan spot were resistant (Table 3.5). This indicated that the resistant cultivars Red Chief, 86ISWN 2137, Erik, Glenlea, and Hadden share the same resistance gene. To confirm this hypothesis, $F_{2:3}$ families of all crosses except Hadden x Red Chief were tested. All $F_{2:3}$ families were resistant thus supporting the hypothesis of no allelic differences among the resistance genes possessed by the five resistant parents in this study (Table 3.6).

Due to lack of seed, $F_{2:3}$ families of the cross Hadden x 6B-365 were not tested. However, the allelism study indicated that all the resistant sources possess the same resistance gene. Hence, based on those results and the F_2 segregation data, it can be inferred that resistance to the chlorosis component caused by race 3 in this cross is also monogenically controlled (Table 3.6).

Table 3.5. F_1 plant disease reaction to isolate Ptr 94-8-2 (race 3) and chi-square tests of F_2 segregation ratios of crosses used to study the inheritance and allelic relationships among the gene(s) controlling resistance to chlorosis caused by Ptr 94-8-2 (race 3)

Cross	F_1 plants		F_2 plants		Ratio tested	χ^2 value	Probability value[a]
	R	S	R	S			
			Resistant x Susceptible				
2137 x 6B-365	9	0	328	104	3:1	0.198	0.66
Erik x 6B-365	8	0	205	72	3:1	0.146	0.70
Red Chief x 6B-365	8	0	235	85	3:1	0.417	0.52
Hadden x 6B-365	7	0	216	64	3:1	0.685	0.41
Glenlea x 6B-365	9	0	236	93	3:1	1.873	0.17
			Resistant x Resistant				
Erik x Glenlea	9	0	265	0	1:0	-	-
Erik x 2137	8	0	340	0	1:0	-	-
Erik x Hadden	8	0	260	0	1:0	-	-
Erik x Red Chief	7	0	325	0	1:0	-	-
Hadden x 2137	8	0	275	0	1:0	-	-
Hadden x Red Chief	9	0	285	0	1:0	-	-
Hadden x Glenlea	8	0	255	0	1:0	-	-
Red Chief x 2137	9	0	265	0	1:0	-	-
Red Chief x Glenlea	8	0	250	0	1:0	-	-
2137 x Glenlea	7	0	295	0	1:0	-	-

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Table 3.6. Chi-square tests for $F_{2:3}$ family segregation ratios of crosses used to study the inheritance and allelic relationships among gene(s) controlling resistance to chlorosis caused by Ptr 94-8-2 (race 3)

Cross	$F_{2:3}$ families			Ratio tested	χ^2 value	Probability value[b]
	Resistant	Seg[a]	Susceptible			
			Resistant x Susceptible			
2137 x 6B-365	16	32	12	1:2:1	0.800	0.67
Erik x 6B-365	24	43	13	1:2:1	3.475	0.18
Red Chief x 6B-365	25	41	14	1:2:1	3.075	0.21
Glenlea x 6B-365	26	38	16	1:2:1	2.700	0.26
			Resistant x Resistant			
Erik x Glenlea	80	-	0	1:0	-	-
Erik x 2137	80	-	0	1:0	-	-
Erik x Hadden	80	-	0	1:0	-	-
Erik x Red Chief	60	-	0	1:0	-	-
Hadden x 2137	80	-	0	1:0	-	-
Hadden x Glenlea	80	-	0	1:0	-	-
Red Chief x 2137	80	-	0	1:0	-	-
Red Chief x Glenlea	80	-	0	1:0	-	-
2137 x Glenlea	80	-	0	1:0	-	-

[a] Segregating for resistance.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

3.3.3 Genetic Control of Resistance to the Necrosis and the Chlorosis Components of Tan Spot Caused by Race 1

F_1 plants of the crosses Erik x Glenlea and Hadden x Glenlea were susceptible to the necrosis component indicating that resistance to the necrosis component of tan spot was recessive (Table 3.7). Segregation of the F_2 generation fit a 1 resistant: 3 susceptible and $F_{2:3}$ families of these crosses fit a 1 homozygous resistant: 2 segregating: 1 homozygous susceptible ratio indicating that a single recessive gene controlled resistance to the necrosis component (Tables 3.7. 3.8). These results agreed with the previous result of single gene control for resistance to the necrosis component caused by race 2 (Section 3.3.1).

Table 3.7. F_1 plant disease reaction to isolate Ptr 200 (race 1) and chi-square tests of F_2 segregation ratios of crosses used to study the inheritance of resistance to necrosis and chlorosis caused by Ptr 200 (race 1)

Cross	F_1 plants		F_2 plants		Ratio tested	χ^2 value	Probability value[a]
	R	S	R	S			
Erik x Glenlea	0	9	94	266	1:3	0.237	0.63
Hadden x Glenlea	0	9	95	257	1:3	0.742	0.39
Glenlea x 6B-365	0	8	54	252	3:13	0.244	0.88
2137 x 6B-365	9	0	234	86	3:1	0.600	0.44
Erik x 6B-365	8	0	255	105	3:1	3.333	0.19

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Table 3.8. Chi-square tests for $F_{2:3}$ family segregation ratios of crosses used to study the inheritance of resistance to necrosis and chlorosis caused by Ptr 200 (race 1)

Cross	$F_{2:3}$ families			Ratio tested	χ^2 value	Probability value[b]
	Resistant	Seg[a]	Susceptible			
Erik x Glenlea	26	53	21	1:2:1	0.860	0.65
Hadden x Glenlea	26	36	18	1:2:1	2.400	0.30
Glenlea x 6B-365	03	33	24	1:8:7	0.643	0.73
2137 x 6B-365	19	28	13	1:2:1	1.467	0.48
Erik x 6B-365	24	41	15	1:2:1	2.075	0.35

[a] Segregating for resistance.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

The F_1 plants of the crosses 86ISWN 2137 x 6B-365 and Erik x 6B-365, when tested with race 1, were resistant to the chlorosis component indicating that resistance was dominant (Table 3.7). The F_2 generation segregation data fit a 3 resistant: 1 susceptible ratio in both crosses (Table 3.7). $F_{2:3}$ family segregation data fit a 1 homozygous resistant: 2 segregating: 1 homozygous susceptible ratio confirming single dominant gene control of resistance to the chlorosis component induced by race 1 (Table 3.8). A single dominant gene also controlled resistance to the chlorosis component caused by race 3 (Section 3.3.2).

F_1 plants of the cross Glenlea x 6B-365 showed only necrotic symptoms indicating that resistance to the chlorosis component and to the necrosis component caused by race 1 is dominant and recessive, respectively (Table 3.7). Since rating individual plants for resistance to both necrosis and chlorosis was difficult and error prone, plants were rated for either the presence or absence of disease. On this basis, the

F_2 generation segregated in a 3:13 (absence: presence) ratio. indicating that two genes controlled resistance to tan spot caused by race 1 (Table 3.7).

The cross Glenlea x 6B-365 segregated for resistance to both the necrosis and the chlorosis components. Earlier studies (Sections 3.3.1. 3.3.2) showed that resistance to the necrosis component was controlled by a single recessive gene and resistance to the chlorosis component was controlled by a single dominant gene. If these two genes are independent then plants in the F_2 generation would have a phenotypic composition of 9 A_B_: 3 A_BB: 3 aaB_: 1 aabb. Plants with the A_B_ and A_BB phenotypes would be susceptible to the necrosis component. but resistant to the chlorosis component. Plants with genotype aabb would be susceptible to the chlorosis component. but resistant to the necrosis component. Plants with phenotype aaB_ would be resistant to both the necrosis and the chlorosis components. Therefore. the expected ratio of plants resistant to both the necrosis and the chlorosis components and plants susceptible to either or both the necrosis and the chlorosis components should fit a 3 (3 aaB_): 13 (9 A_B_ + 3 A_BB + 1 aabb) ratio.

With two independent genes controlling resistance. the genotypic ratios of the F_2 plants would be 1 AABB: 2 AABb: 1 AAbb: 2 AaBB: 4 AaBb: 2 Aabb: 1 aaBB: 2 aaBb: 1 aabb. Plants with genotype aaBB would produce families resistant to both the necrosis and the chlorosis components. Plants with the AaBB. AaBb and aaBb genotypes would produce families segregating for the necrosis and/or the chlorosis components. Plants with genotypes AABB, AABb, Aabb, Aabb and aabb would produce families susceptible for the necrosis and/or the chlorosis components. Therefore, the expected segregation ratio for the $F_{2:3}$ families is 1 homozygous resistant (1 aaBB): 8 segregating (2 AaBB + 4

AaBb + 2 aaBb): 7 homozygous susceptible (1 AABB + 2 AABb + 1 Aabb + 2 Aabb + 1 aabb) families.

$F_{2:3}$ family segregation ratio fit a 1 homozygous resistant (necrosis and chlorosis): 8 segregating (necrosis and/or chlorosis): 7 homozygous susceptible (necrosis and/or chlorosis). This confirmed that two independent genes. a recessive gene for the necrosis and a dominant gene for the chlorosis component. controlled resistance to tan spot caused by race 1 (Table 3.8).

3.3.4 Relationship between Gene(s) Controlling Resistance to Necrosis Induced by Races 1 and 2

$F_{7:8}$ lines of the cross Erik x Glenlea were tested with isolates Ptr 200 (race 1) and Ptr 92-164 (race 2) to study the relationship between gene(s) controlling resistance to necrosis caused by races 1 and 2. Resistance to necrosis caused by races 1 or 2 of *P. tritici-repentis* in the cross Erik x Glenlea was found to be controlled by a single recessive gene (Sections 3.3.1. 3.3.3).

The expected segregation for single gene control in $F_{7:8}$ lines is 63 homozygous resistant: 2 segregating: 63 homozygous susceptible lines. Segregation of $F_{7:8}$ lines of the cross Erik x Glenlea did fit this ratio when tested with race 1 and with race 2 (Table 3.9). These genes controlling resistance to the necrosis induced by races 1 and 2 were not independent (Table 3.9).

It was observed that 40 lines were resistant to both isolates Ptr 92-164 (race 2) and Ptr 200 (race 1) and 37 lines were susceptible to both isolates. Two lines segregated for resistance to both isolates and one line was susceptible to isolate Ptr 92-164 but segregated for resistance to isolate Ptr 200. All but one line gave similar disease

reactions to isolates Ptr 200 (race 1) and Ptr 92-164 (race 2). This indicates that the same gene controls resistance to necrosis caused by races 1 and 2.

Table 3.9. Chi-square tests for $F_{7:8}$ line segregation ratios of the cross Erik x Glenlea for resistance to the necrosis component induced by isolates Ptr 200 (race 1) and Ptr 92-164 (race 2) and test of independence of resistance gene(s)

Isolate	$F_{7:8}$ lines			Ratio tested	χ^2 value	Probability value[b]
	Resistant	Seg[a]	Susceptible			
Ptr 92-164	40	2	38	63:2:63	0.508	0.78
Ptr 200	40	3	37	63:2:63	2.603	0.27

		Ptr 92-164					
		R_2	Seg_2	S_2			
Ptr 200	R_1	40	0	0	R.T.[c]	276.625	0.00
	Seg_1	0	2	1			
	S_1	0	0	37			

[a] Line has individuals expressing both resistant and susceptible reaction.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

[c] R.T. = 3969 R_1R_2: 126 R_1Seg_2: 3969 R_1S_2: 126 Seg_1R_2: 4 Seg_1Seg_2: 126 Seg_1S_2: 3969 S_1R_2: 126 S_1Seg_2: 3969S_1S_2, where R_1 and R_2 = resistant to race 1 and race 2, respectively. Seg_1 and Seg_2 = segregating to race 1 and race 2, respectively, and S_1 and S_2 = susceptible to race 1 and race 2, respectively

3.3.5 Relationship between Gene(s) Controlling Resistance to Chlorosis Induced by Races 1 and 3

In order to study the relationship between gene(s) controlling resistance to chlorosis induced by races 1 and 3, $F_{2:3}$ families of the cross Erik x 6B-365 were tested with isolates Ptr 200 (race 1) and Ptr 94-8-2 (race 3). Monogenic inheritance of resistance to the chlorosis component caused by races 1 and 3 of *P. tritici-repentis* was observed in the cross Erik x 6B-365 (Sections 3.3.2, 3.3.3).

For single gene control the expected segregation in $F_{2:3}$ families would be 1 homozygous resistant: 2 segregating: 1 homozygous susceptible. $F_{2:3}$ families of the cross Erik x Glenlea segregated in this expected ratio when tested with race 1 and with race 3 (Table 3.10). A test of independence indicated that the genes controlling resistance to chlorosis induced by races 1 and 3 were not independent (Table 3.10).

Table 3.10. Chi-square tests for $F_{2:3}$ family segregation ratios of the cross Erik x 6B-365 for resistance to the chlorosis component induced by isolates Ptr 200 (race 1) and Ptr 94-8-2 (race 3) and test of independence of resistance gene(s)

		$F_{2:3}$ families			Ratio	χ^2 value	Probability
Isolate		Resistant	Seg[a]	Susceptible	tested		value[b]
Ptr 94-8-2		24	43	13	1:2:1	3.475	0.16
Ptr 200		24	41	15	1:2:1	1.675	0.43
		Ptr 94-8-2					
		R_2	Seg_2	S_2			
Ptr 200	R_1	24	0	0	R.T.[c]	153.450	0.00
	Seg_1	0	41	0			
	S_1	0	2	13			

[a] Family has individuals expressing both resistant and susceptible reaction

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

[c] R.T. = 1 R_1R_2: 2 R_1Seg_2: 1 R_1S_2: 2 Seg_1R_2: 4 Seg_1Seg_2: 2 Seg_1S_2: 1 S_1R_2: 2 S_1Seg_2: 1 S_1S_2, where R_1 and R_2 = resistant to race 1 and race 3, respectively, Seg_1 and Seg_2 = segregating to race 1 and race 3, respectively, and S_1 and S_2 = susceptible to race 1 and race 3, respectively

All 24 families resistant to Ptr 200 were also resistant to isolate Ptr 94-8-2 and 13 families were susceptible to both isolates. However, there were differences in the number of segregating and susceptible families for the two races. Forty-one families segregated when tested with either isolate. However, two families, segregated for

resistance to race 3. but were susceptible to race 1. All resistant families. whose disease phenotype is clearer and thus could be identified accurately. gave similar responses to both isolates Ptr 200 (race 1) and Ptr 92-164 (race 3). This suggests strongly that resistance to chlorosis caused by race 1 or 3 is controlled by the same gene.

3.4 Discussion

Throughout this study it was observed that genetic control of resistance to tan spot of wheat was qualitatively inherited. This agrees with the findings of other researchers (Lamari and Bernier 1989b. 1991: Sykes and Bernier 1991: Duguid 1995: Gamba et al. 1998: Anderson et al. 1999). Other studies have reported resistance to tan spot to be quantitatively inherited (Nagle et al. 1982: Shabeer 1986: Elias et al. 1989: Faris et al. 1997). However. comparison among these studies cannot be made due to lack of standardization of factors such as inoculation method. environmental conditions. rating scales and isolate used.

The development of the necrosis-chlorosis model for tan spot of wheat has been of great value to studies of the genetic control of resistance. Differentiation between the necrotic and the chlorotic components of tan spot has led to reliable and accurate assessment of resistance and susceptibility. The identification of isolates capable of inducing necrosis only or chlorosis only symptoms has further aided these genetic studies.

In this study. a single recessive gene was found to control resistance to the necrosis component of tan spot. A similar finding has also been reported by others (Lamari and Bernier 1989b, 1991: Duguid 1995; Gamba et al. 1998). Duguid (1995)

observed that the F_2 segregation in cross Erik x Katepwa fit a 5 resistant: 11 susceptible ratio while in cross Erik x BH1146 the F_2 segregation ratio was 1 resistant: 3 susceptible. However, the $F_{2:3}$ family segregation for both the crosses indicated single gene control. In this study, crosses involving Erik as the resistant parent segregated as expected for single gene control, as was also reported by Lamari and Bernier (1989b). These differing results demonstrate the errors associated with basing segregation data on single plant ratings for tan spot. Misclassification or disease escape can create incorrect segregation data, which will result in a wrong interpretation of genetic control. Single plant data is useful to derive preliminary results but final conclusions should be made on the basis of family segregation data, which is less liable to such errors.

While studying the inheritance of resistance to the necrosis component, plants of crosses involving winter cultivars were not vernalized. If there was linkage between genes for vernalization and tan spot resistance then the segregation patterns observed in the crosses involving spring and winter resistant cultivars would be different. However no such results were observed, indicating that the resistance gene for the necrosis component was not linked to genes for vernalization. The allelism studies indicated that the same gene was present in both spring and winter resistant cultivars. It was also observed that the same recessive gene controls resistance to necrosis caused by race 1 and by race 2. Preliminary studies by Anderson et al. (1999) indicated that the same recessive gene controlled resistance to the necrosis component of tan spot in both durum and hexaploid wheat. This indicates that resistance to the necrosis component caused by races 1 and 2 in both durum and hexaploid wheat is controlled by a single gene.

Although a few moderately susceptible plants (rating 3) were observed in the F_2 generation of the resistant x resistant crosses, all crosses except Hadden x Red Chief failed to fit a 15 resistant: 1 susceptible ratio indicating that the resistant parents possessed the same resistant gene. The occurrence of moderately susceptible plants could be due to misclassification or high inoculum load causing resistant plants to be rated moderately susceptible, or there could be genetic reasons. Progeny testing of these moderately susceptible plants was not possible due to lack of space and time. Instead, $F_{2:3}$ families of the resistant x resistant crosses were tested. All the families tested were resistant confirming that the resistant parents possessed the same resistance gene.

To date, all allelism studies of genotypes resistant to the necrosis component of tan spot have failed to show any segregation, indicating that the resistance sources share the same resistance gene (Sykes and Bernier 1991; Duguid 1995; Gamba et al. 1998; Gamba and Lamari 1998; Anderson et al. 1999). Stock et al. (1996) and Anderson et al. (1999) reported that this gene (*tsn1*) is located on chromosome 5BL in both durum and hexaploid wheat.

Lamari and Bernier (1989a) found a high level of resistance in all ploidy levels of wheat. They also found resistance in related wild species, especially *T. dicoccoides* (AABB), *T. dicoccum* (AABB), *T. persicum* (CCUU), *T. timopheevii* (AAGG), and *T. zukhovskii* (AAAAGG). High levels of resistance in accessions of *T. monococum* (AA), *Aegilops tauschii* (DD), *Ae. speltoides* (SS), *Ae. triaristata* (UUMM), *Ae. ovata* (UUMM), and *Ae. cylindrica* (CCDD) have also been observed (Alam and Gustafson 1988; Cox et al. 1992; Zhang and Jin 1998). Most of these wild relatives of wheat do not share the 5BL chromosome on which the resistance gene *tsn1* is located. This indicates

that resistance genes may also be located on other chromosomes. The resistance observed in the wild relatives is likely to be different to what is presently observed in wheat and durum cultivars.

The narrow genetic base for resistance to tan spot and the occurrence of new races of *P. tritici-repentis* in recent years emphasizes the need to identify new resistance genes to develop durable and effective resistant varieties. Study of interspecific crosses between resistant wild relatives of wheat and durum and hexaploid wheat is likely to identify new genes for resistance to tan spot of wheat. These new resistance genes can be utilized to broaden the genetic base of resistance and thus reduce the possibility of existing resistance genes breaking down.

The occurrence of a few moderately susceptible F_2 plants in the allelism study could indicate the occurrence of a cluster of tightly linked genes, which may differ for tan spot resistance and would appear as a single gene in classical inheritance studies using normal population sizes. Studies with large populations and progeny testing of plants showing moderately resistant reaction are needed to examine the hypothesis of existence of a gene family. Molecular techniques could also be used to identify such a gene family.

Other researchers have reported that resistance genes are often clustered as a multiallelic series at a locus or as multiple linked loci (e.g. Ellis et al. 1997). One such example exists at the L and M gene loci for rust resistance in flax. The L locus is a single gene with multiple alleles and the M locus is a complex locus with an array of about 15 similar genes (Ellis et al. 1997). Thirteen alleles at the L gene locus in flax have been sequenced and their rust resistance specificity determined using various molecular techniques (Ellis et al. 1999). Islam and Shepherd (1991) have demonstrated the

complexity of the L and M loci in flax through genetic studies. Similarly in maize, the *rp*1 locus for resistance to maize rust is a complex locus whose structure has been verified by both genetic and molecular studies (Hulbert 1997).

The fact that only a single recessive resistant gene for necrosis component of tan spot has been identified need not necessarily be very alarming. Colonization and growth of fungus on senesced tissue is independent of the reaction of the living host to infection (Summerell and Burgess 1988). Hence, incorporation of this resistant gene into presently grown wheat cultivars is unlikely to bring a selection pressure on the fungus, but it would reduce yield losses due to tan spot (Duguid 1995). However, attempts to find new sources of resistance should be carried on extensively.

Monogenic dominant inheritance of resistance to the chlorosis component of tan spot caused by either race 1 or 3 was observed in this study. This finding agrees with those of Duguid (1995) and Lamari and Bernier (1991). However, Gamba et al. (1998) observed single recessive gene control for resistance to the chlorosis component caused by race 3. Lamari and Bernier (1991) found that the mode of inheritance of resistance to race 3 varied with the parental lines used. In some crosses they had observed resistance to be dominant while in others it was partially dominant indicating a background effect of the different resistant sources. This could explain the difference between the findings of this study and those of Gamba et al. (1998).

Expression of the chlorosis component of tan spot is highly influenced by environmental conditions and this may explain the differences observed in this study and in that of Gamba et al. (1998). In this study plants showing tip chlorosis were rated as resistant and only plants showing chlorosis symptoms comparable to the susceptible

check (Line 6B-365) were rated as susceptible. This method of rating may be different to that used by Gamba et al. (1998). The fact that Gamba et al. (1998) used isolate HY 331-9 (race 3) whereas isolate Ptr 94-8-2 (race 3) was used for disease induction in this study could also contribute to the difference. The two isolates may differ in virulence, resulting in different results. However, Duguid (1995), who also used isolate Ptr D308, obtained results similar to those of this study.

The absence of segregation for resistance to the chlorosis component in crosses between resistant parents indicates that the resistant parents share at least one gene for resistance. This agrees with the findings of Lamari and Bernier (1991), Duguid (1995) and Gamba et al. (1998). The same single dominant gene controls resistance to chlorosis induced by either race 1 or 3. However, when cross Erik x 6B-365 was tested with races 1 and 3, two $F_{2:3}$ families were susceptible to race 1 but segregated for resistance to race 3. This differential response of two families to races 1 and 3 is considered more likely to be due to disease escape or to errors in rating than to genetic reasons. Also, environmental conditions highly influence expression of the chlorosis component and this may have contributed to these differential responses.

Race 1 causes necrosis and chlorosis on both durum and hexaploid wheat while race 3 causes chlorosis in hexaploid wheat and necrosis in durum (Lamari and Bernier 1989c; Gamba and Lamari 1998). This suggests that besides carrying a virulence gene for induction of necrosis, race 1 also carries a virulence gene(s) for induction of chlorosis in durum wheat. Hence it can be hypothesized that race 1 possesses one virulence gene for necrosis induction and two virulence genes for chlorosis induction, one for hexaploid

wheat and another for durum wheat. Race 3 would posses two virulence genes. one for necrosis induction in durum and the other for chlorosis induction in hexaploid wheat.

The results from this study indicate that the gene for resistance to the chlorosis component caused by races 1 and 3 is the same in hexaploid wheat. Therefore. the virulence gene for chlorosis induction in races 1 and 3 may be identical. However. if races 1 and 3 do possess different virulence genes. this would provide an explanation for the differential response to the two families i.e. susceptible to race 1. but segregating for resistance to race 3.

Results from the study of the cross Glenlea x 6B-365 with isolate Ptr 200 (race 1) indicated that resistance to the necrosis and to the chlorosis components of the tan spot syndrome was controlled by independent genetic systems. This is the first report. using an isolate different to ASC1. that confirms the findings of Sykes and Bernier (1991) and Duguid (1995). The number of resistance gene(s) identified in a cross when disease is induced by race 1 depends on the components of tan spot for which the cross is segregating for resistance. In crosses Erik x Glenlea and Hadden x Glenlea which were segregating for resistance to necrosis. resistance was controlled by a single recessive gene. However. in crosses Erik x 6B-365 and 86ISWN 2137 x 6B-365 which were segregating for resistance to chlorosis. resistance was due to a single dominant gene.

Lamari and Bernier (1991) and Gamba et al. (1998) have hypothesized that due to the absence of an epistatic effect of compatibility which occurs in pathosystems following gene-for-gene model. wheat-*P. tritici-repentis* pathosystem follows the toxin model proposed by Ellingboe (1981). In the toxin model. compatibility between the host and the pathogen leads to susceptibility while in the gene-for-gene model. compatibility

leads to resistance. The toxin model applies to host-pathosystems wherein toxin(s) are the primary factor(s) of pathogenicity (Ellingboe 1981) and would apply to this pathosystem since different races of *P. tritici-repentis* produce different toxins, which have been demonstrated to be pathogenicity factor(s) (Lamari and Bernier 1991; Orolaza et al. 1995; Gamba et al. 1998; Anderson et al. 1999). Anderson et al. (1999) further supported that the wheat-*P. tritici-repentis* pathosystem follows the toxin model by indicating that resistance is not due to a gene product *per se* but rather to the absence of a gene for sensitivity, i.e. recognition of the toxin leads to susceptibility, a property of the toxin model. Hence to develop complete resistance to tan spot of wheat, all available resistance gene(s) must be incorporated into the present wheat varieties.

The simplest explanation of the results of this study is that resistance in hexaploid wheat to the necrosis component caused by races 1 and 2 is controlled by the same single recessive gene and that resistance to the chlorosis component caused by race 1 and 3 is controlled by the same single dominant gene. Lamari and Bernier (1991) hypothesized that the hexaploid wheat-*P. tritici-repentis* pathosystem follows the toxin model and for every resistance gene in the host plant there is a corresponding virulence gene in the pathogen. If this hypothesis is true, the same virulence gene for necrosis induction would be present in races 1 and 2 and the same virulence genes for chlorosis induction would be present in races 1 and 3. Hence combination of the virulences of races 2 and 3 would produce race 1. This agrees with the findings of Otonda (1995). Otonda (1995) crossed the necrosis-inducing race 2 (n+c-) with the chlorosis-inducing race 3 (n-c+) and obtained isolates belonging to all four races 1 (n+c+), 2 (n+c-), 3 (n-c+), and 4 (n-c-) of *P. tritici-repentis*.

In this study, it was observed that it was possible to predict the response to race 1 after a combined study with races 2 and 3 in the hexaploid wheat-*P. tritici-repentis* pathosystem. Based on study of F_2 populations, Duguid (1995) reported that the combined response to race 2 and 3 did not predict the response to race 1. However, Duguid (1995) was studying crosses that did not have monogenic resistance to both the components of tan spot and hence obtained results different to this study.

To date, three independent genes conferring resistance to tan spot have been identified in hexaploid wheat (Gamba et al. 1998). One gene for resistance to necrosis caused by races 1 and 2 and two genes for resistance to the chlorosis component, one for chlorosis caused by races 1 or 3 and the other for race 5 induced chlorosis. In durum wheat four independent genes for resistance to tan spot have been identified (Gamba and Lamari 1998). Three genes control resistance to necrosis induced by races 1 and 2, 3 and 5 and the fourth gene controls resistance to chlorosis induced by race 1. Preliminary studies by Anderson et al. (1999) reported that the gene for insensitivity to necrosis caused by toxin ToxA derived from a race 2 isolate is the same in both durum and hexaploid wheat. The relationship between the other genes has yet not been investigated. It would be interesting to study the relationship between these genes, as it would indicate possible reasons why races 3 and 5 of *P. tritici-repentis* induce necrosis in durum but chlorosis in hexaploid wheat. It could also lead to better understanding of the genetics of resistance in the *Triticum-P. tritici-repentis* pathosystem.

This study found that there are two independent genes controlling resistance to tan spot in hexaploid wheat. A single recessive gene controlled resistance to the necrosis

component caused by either race 1 or race 2 while resistance to the chlorosis component caused by race 1 or race 3 was controlled by a single dominant gene.

4. STUDIES WITH CULTURE FILTRATE OF *PYRENOPHORA TRITICI-REPENTIS*, RACE 2

4.1 Introduction

Tan spot, caused by the fungus *Pyrenophora tritici-repentis*, is an important foliar disease of wheat worldwide. Tan spot causes loss in both quality and quantity of wheat grain production. *P. tritici-repentis* produces several toxins that mimic the tan necrosis and extensive chlorosis symptoms in wheat cultivars (Tomas and Bockus 1987; Lamari and Bernier 1989b; Brown and Hunger 1993; Tuori et al. 1995; Anderson et al. 1999).

Tomas and Bockus (1987) were the first to report the occurrence of toxic compound(s) that induced tan spot symptoms when the crude culture filtrate was infiltrated into wheat leaves. They concluded that because of the distinct and different reactions obtained from culture filtrate infiltration of resistant and susceptible plants, culture filtrate could be used to screen for resistance. They also suggested that disease resistance might be due, at least in part, to insensitivity to the toxic compounds.

There are two known types of necrosis-inducing toxins. A host selective proteinaceous toxin produced by races 1 and 2, designated as Ptr ToxA (Lamari and Gilbert 1998), is the main factor causing the necrotic symptoms in susceptible wheat cultivars. *P. tritici-repentis* produces another class of necrosis-inducing phytotoxins composed of spirocyclic lactams named triticones. Triticones have been reported to be host nonselective in their ability to induce necrosis (De Wolf et al. 1998).

A host-specific toxin designated as Ptr ToxB (Orolaza et al. 1995; De Wolf et al 1998; Lamari and Gilbert 1998) causes the chlorosis induced by race 5. The chlorosis toxin appeared to be a pathogenicity factor and exhibited the same wheat genotype specificity as the fungus on hexaploid wheat. Meinhardt et al. (1997) and Effertz (1998) identified a second chlorosis toxin designated Ptr ToxC from race 1 isolate that produces chlorosis in the wheat line 6B-365. Structural characterization of Ptr ToxC has yet to be done.

Various studies (Lamari and Bernier 1989b; Duguid 1995; Gamba and Lamari 1998; Gamba et al. 1998) have indicated that resistance to the necrosis component of tan spot and to the Ptr necrosis toxin (ToxA) is controlled by the same single recessive gene. They also observed that toxin ToxA can be used as a surrogate for spore-inoculation with necrosis-inducing races 1 and 2. However, Riede et al. (1996) and Zhang and Jin (1998) reported differential responses to toxin infiltration and fungal spore inoculation. Tuori et al. (1995) and Meinhardt et al. (1998) reported that multiple toxins might be produced by necrosis inducing isolates of *P. tritici-repentis*. Therefore, to assess if spore inoculation can be replaced by toxin(s) infiltration, a culture filtrate, which supposedly contains all types of toxins, should be used to induce disease instead of a single toxin. Hence, this study was conducted to:

1) study the inheritance of resistance to necrosis caused by culture filtrate infiltration and spore inoculation with isolate Ptr 92-164 (race 2); and
2) establish the relationship between the host reaction to culture filtrate infiltration and spore inoculation for the necrosis component of tan spot.

4.2 Materials and Methods

4.2.1 Population Development

The F_1, F_2, BC_1F_1 and $F_{7:8}$ generations of the crosses of resistant cultivars Erik and 86ISWN 2137 with susceptible cultivars Glenlea and Kenyon, and resistant cultivar Red Chief with susceptible cultivar Kenyon were studied. The $F_{7:8}$ population of the cross Erik x Kenyon was not available. At least sixteen plants per $F_{7:8}$ line were inoculated with spore inoculum and an additional eight plants of each line were infiltrated with culture filtrate to establish if culture filtrate can replace spore inoculation when testing for resistance to the necrosis component of tan spot of wheat.

4.2.2 Culture Filtrate Production

Fungal inoculum was produced by the procedure described in Section 3.2.2. Culture filtrates were produced from isolate Ptr 92-164 (n+c-) following the procedures described by Orolaza et al. (1995). Initially, cultures were grown on V8P medium. Five 0.5 cm diameter plugs were cut from the agar culture and transferred to a 250-ml Erlenmeyer flask containing 100 ml of modified Fries medium plus 0.1% yeast extract (Appendix II). Each culture was incubated without shaking at 20°C in continuous light for 21 days and the culture filtrate harvested by sequential passage through Whatman number 2 filter paper (Whatman, Inc., Clifton, NJ). The culture filtrate was either used immediately or stored in a refrigerator at 4°C.

4.2.3 Disease Screening

Disease screening using spore inoculation was done following the procedures described in Section 3.2.3. Four days after spore inoculation, the newly emerged and

uninoculated third leaf was infiltrated with culture filtrate at a site marked by a permanent marker. The culture filtrate was infiltrated into wheat leaves by a lab-made device modelled after the Hagborg device used for infiltrating thin leaves (Fig. 4.1) (Hagborg 1970). Absence and presence of the necrosis at the site of infiltration indicated insensitivity and sensitivity to the culture filtrate, respectively (Fig. 4.2a, 4.2b).

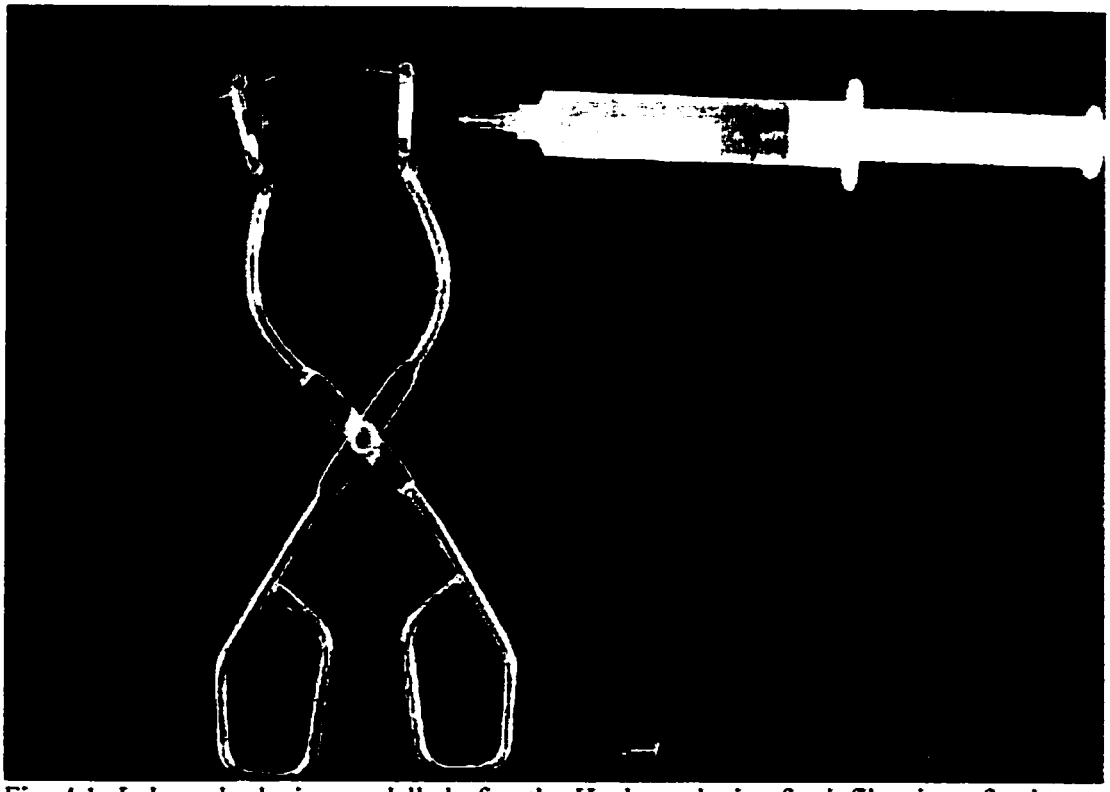


Fig. 4.1. Lab-made device modelled after the Hagborg device for infiltrating of culture filtrate into wheat leaves

Plant reactions for insensitivity/sensitivity to the culture filtrate infiltration and resistance/susceptibility to spore-inoculation were recorded simultaneously eight days after spore inoculation. Infiltration of check cultivars with the culture filtrate was done to check the concentration of toxin in the culture filtrate prior to the screening of the segregating populations. In preliminary studies 50%, 25%, 10% and 1% dilutions of the

culture filtrate with distilled water were tested. Based on these studies, a 25% dilution of the culture filtrate was used to differentiate clearly between the resistant and susceptible checks. The culture filtrate was further diluted if the resistant checks showed a sensitive reaction or collapse of leaf tissue.

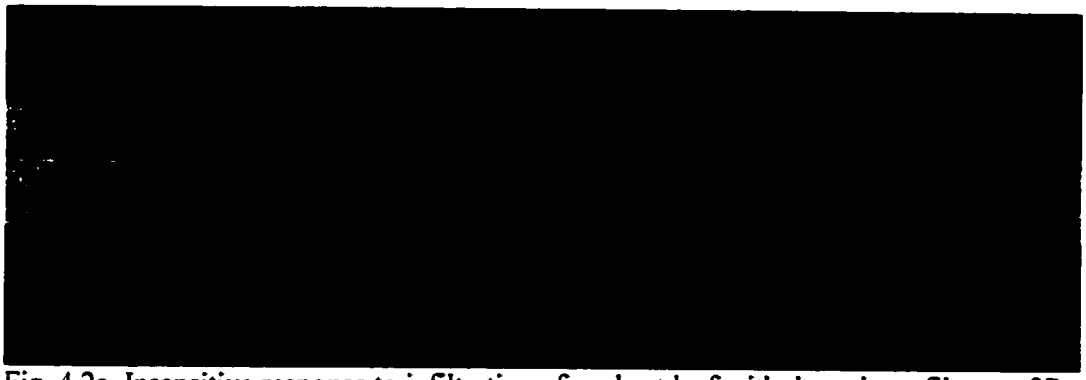


Fig. 4.2a. Insensitive response to infiltration of a wheat leaf with the culture filtrate of Ptr 92-164 (race 2). Infiltration was done between the marked lines

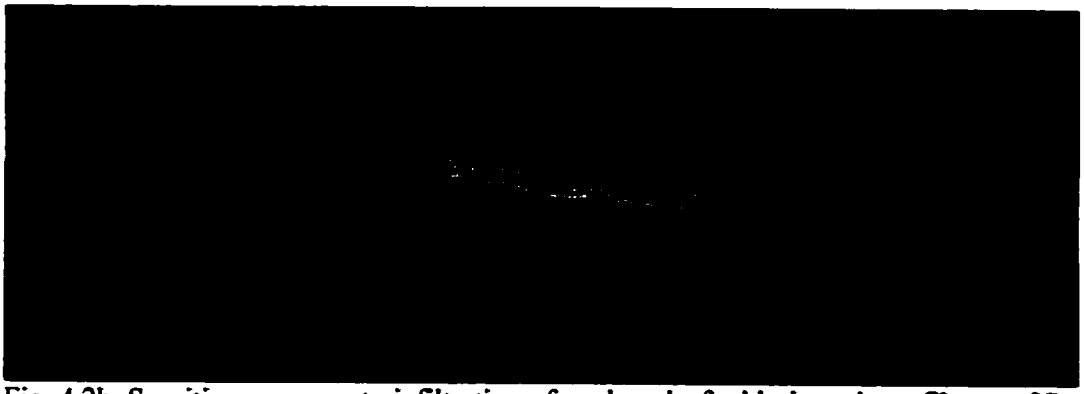


Fig. 4.2b. Sensitive response to infiltration of a wheat leaf with the culture filtrate of Ptr 92-164 (race 2). Infiltration was done between the marked lines

4.3 Results

In preliminary studies with seven cultivars, it was observed that there was a similar response to culture filtrate and spore inoculation. Plants resistant to spore-

inoculum were insensitive to the culture filtrate and plants susceptible to spore-inoculum were sensitive to the culture filtrate (Table 4.1). The presence of necrosis was caused by the culture filtrate infiltration and not by the process of infiltration or by the infiltration of modified Fries medium because infiltration with water or modified Fries medium failed to produce necrosis.

Table 4.1. Reaction of different wheat cultivars to infiltration with water, Fries medium and culture filtrate, and to fungal spore-inoculation with *P. tritici-repentis* isolate Ptr 92-64 (race 2)

Cultivars	Water	Fries medium	Culture filtrate	Spore inoculum
Erik	Insensitive	Insensitive	Insensitive	Resistant
86ISWN 2137	Insensitive	Insensitive	Insensitive	Resistant
Hadden	Insensitive	Insensitive	Insensitive	Resistant
Red Chief	Insensitive	Insensitive	Insensitive	Resistant
6B-365	Insensitive	Insensitive	Insensitive	Resistant
Kenyon	Insensitive	Insensitive	Sensitive	Susceptible
Glenlea	Insensitive	Insensitive	Sensitive	Susceptible

All F_1 plants (8-9 plants) of each cross tested were susceptible and sensitive indicating that resistance/insensitivity was recessive. F_2 segregation ratios gave a good fit to a 1 resistance: 3 susceptible ratio (Table 4.2). Segregation in the BC_1F_1 generation fitted a 1 resistance: 1 susceptible ratio (Table 4.3), confirming single gene control of resistance to the necrosis component when fungal spore-inoculation was used. Similarly, when the culture filtrate was used, the segregation data indicated that insensitivity to the culture filtrate was controlled by a single gene (Tables 4.2, 4.3).

Table 4.2. Genetics of insensitivity/resistance to culture filtrate infiltration and spore-inoculation of the necrosis inducing isolate Ptr 92-164 (race 2) in the F_2 generation of the selected crosses

Inoculation method	Disease reaction		Ratio tested	χ^2 value	Probability value[a]
	Resistant/ Insensitive	Susceptible/ Sensitive			
Erik x Glenlea					
Culture Filtrate	91	243	1:3	0.898	0.34
Spore Inoculation	93	241	1:3	1.441	0.23
Erik x Kenyon					
Culture Filtrate	94	268	1:3	0.181	0.67
Spore Inoculation	97	265	1:3	0.622	0.43
86ISWN 2137 x Glenlea					
Culture Filtrate	92	225	1:3	2.735	0.10
Spore Inoculation	91	226	1:3	2.323	0.13
86ISWN 2137 x Kenyon					
Culture Filtrate	72	193	1:3	0.665	0.41
Spore Inoculation	71	194	1:3	0.454	0.50
Red Chief x Kenyon					
Culture Filtrate	96	240	1:3	2.286	0.13
Spore Inoculation	97	239	1:3	2.683	0.10

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Table 4.3. Genetics of insensitivity/resistance to culture filtrate infiltration and spore-inoculation of the necrosis inducing isolate Ptr 92-164 (race 2) in the BC_1F_1 generation of the selected crosses

Inoculation method	Disease reaction		Ratio tested	χ^2 value	Probability value[a]
	Resistant/ Insensitive	Susceptible/ Sensitive			
(Erik x Glenlea) x Erik					
Culture Filtrate	39	35	1:1	0.216	0.64
Spore Inoculation	40	34	1:1	0.486	0.49
(Erik x Kenyon) x Erik					
Culture Filtrate	30	37	1:1	0.181	0.67
Spore Inoculation	32	35	1:1	0.622	0.43
(86ISWN 2137 x Glenlea) x 86ISWN 2137					
Culture Filtrate	42	38	1:1	0.200	0.65
Spore Inoculation	43	37	1:1	0.450	0.50
(86ISWN 2137 x Kenyon) x 86ISWN 2137					
Culture Filtrate	41	34	1:1	0.653	0.42
Spore Inoculation	40	35	1:1	0.333	0.56
(Red Chief x Kenyon) x Red Chief					
Culture Filtrate	7	5	1:1	0.333	0.56
Spore Inoculation	7	5	1:1	0.333	0.56

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Most plants screened in the F_2 and BC_1F_1 generations gave similar reactions to spore-inoculation and culture filtrate infiltration. However, a few plants in both generations showed a differential response. The test of independence for the gene(s) controlling resistance to spore inoculation and culture filtrate in both the F_2 and BC_1F_1 generations, i.e. goodness-of-fit to a 1:3:3:9 and a 1:1:1:1 ratio, respectively, indicated that the resistance gene(s) were not independently inherited (Tables 4.4, 4.5). Single gene control for resistance to both spore-inoculation and culture filtrate, and similar

reactions to spore-inoculation and culture filtrate infiltration by majority of plants tested indicated that the same gene controls resistance to both spore-inoculation and culture filtrate.

Table 4.4. Tests of independence of gene(s) controlling insensitivity/resistance to culture filtrate infiltration and spore-inoculation of the necrosis inducing isolate Ptr 92-164 (race 2) in the F_2 generation of the selected crosses

Inoculation method		Spore Inoculation		Ratio tested	χ^2 value	Probability value[a]
		Resistant	Susceptible			
		Erik x Glenlea				
Culture filtrate	Insensitive	89	2	1:3:3:9	349.81	0.00
	Sensitive	4	239			
		Erik x Kenyon				
Culture Filtrate	Insensitive	92	2	1:3:3:9	352.21	0.00
	Sensitive	5	263			
		86ISWN 2137 x Glenlea				
Culture Filtrate	Insensitive	90	2	1:3:3:9	373.31	0.00
	Sensitive	1	224			
		86ISWN 2137 x Kenyon				
Culture Filtrate	Insensitive	69	3	1:3:3:9	267.45	0.00
	Sensitive	2	191			
		Red Chief x Kenyon				
Culture Filtrate	Insensitive	92	4	1:3:3:9	359.89	0.00
	Sensitive	5	235			

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

Table 4.5. Tests of independence of gene(s) controlling insensitivity/resistance to culture filtrate infiltration and spore-inoculation of the necrosis inducing isolate Ptr 92-164 (race 2) in the BC_1F_1 generation of the selected crosses

Inoculation method		Spore Inoculation		Ratio tested	χ^2 value	Probability value[a]
		Resistant	Susceptible			
(Erik x Glenlea) x Erik						
Culture Filtrate	Insensitive	39	0	1:1:1:1	70.756	0.00
	Sensitive	1	34			
(Erik x Kenyon) x Erik						
Culture Filtrate	Insensitive	30	0	1:1:1:1	60.104	0.00
	Sensitive	2	35			
(86ISWN 2137 x Glenlea) x 86ISWN 2137						
Culture Filtrate	Insensitive	42	0	1:1:1:1	76.700	0.00
	Sensitive	1	37			
(86ISWN 2137 x Kenyon) x 86ISWN 2137						
Culture Filtrate	Insensitive	40	1	1:1:1:1	72.040	0.00
	Sensitive	0	34			
(Red Chief x Kenyon) x Red Chief						
Culture Filtrate	Insensitive	7	0	1:1:1:1	12.667	0.00
	Sensitive	0	5			

[a] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

To establish the relationship between spore inoculation and culture filtrate infiltration, and to confirm the findings of the F_2 and BC_1F_1 individual plant data, $F_{7:8}$ lines of all crosses except Erik x Kenyon were tested. The segregation pattern of the $F_{7:8}$ lines confirmed that resistance to necrosis induced by either culture filtrate infiltration or spore-inoculation was also controlled by a single gene (Table 4.6).

Table 4.6. Genetics of insensitivity/resistance to culture filtrate infiltration and spore-inoculation of the necrosis inducing isolate Ptr 92-164 (race 2) in $F_{7:8}$ lines of the selected crosses

Inoculation method	Disease reaction			Ratio tested	χ^2 value	Prob. value[b]
	Resistant/ Insensitive	Seg[a]/ Seg[c]	Susceptible/ Sensitive			
Erik x Glenlea						
Culture Filtrate	40	2	38	63:2:63	0.508	0.78
Spore Inoculation	40	2	38	63:2:63	0.508	0.78
86ISWN 2137 x Kenyon						
Culture Filtrate	37	2	41	63:2:63	0.653	0.72
Spore Inoculation	37	2	41	63:2:63	0.653	0.72
86ISWN 2137 x Glenlea						
Culture Filtrate	38	2	40	63:2:63	0.508	0.78
Spore Inoculation	38	3	39	63:2:63	2.502	0.29
Red Chief x Kenyon						
Culture Filtrate	38	1	41	63:2:63	0.165	0.92
Spore Inoculation	38	2	40	63:2:63	0.508	0.78

[a] Segregating for resistance.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

[c] Segregating for insensitivity.

In the two crosses 86ISWN 2137 x Kenyon and Erik x Glenlea, all $F_{7:8}$ lines gave similar responses to both culture filtrate infiltration and spore inoculation. However, in each of the crosses Red Chief x Kenyon and 86ISWN 2137 x Glenlea, one $F_{7:8}$ line showed a differential response (Table 4.7). These lines were sensitive to culture filtrate and segregated for resistance when spore-inoculated. A test of independence confirmed that the gene(s) controlling resistance to necrosis induced by spore-inoculation and culture filtrate infiltration were not independent (Table 4.7). Results from testing of $F_{7:8}$

lines also support the conclusion that resistance to both culture filtrate infiltration and spore inoculation is controlled by the same gene.

Table 4.7. Tests of independence of gene(s) controlling insensitivity/resistance to culture filtrate infiltration and spore-inoculation of the necrosis inducing isolate Ptr 92-164 (race 2) in $F_{7:8}$ lines of the selected crosses

Inoculation method		Spore Inoculation			Ratio tested	χ^2 value	Prob. value[b]
		Resistant (R)	Seg[a]	Susceptible (S)			
Erik x Glenlea							
Culture Filtrate	Insensitive (Ins)	40	0	0	R.T	281.9	0.00
	Seg[c]	0	2	0			
	Sensitive (Sen)	0	0	38			
86ISWN 2137 x Kenyon							
Culture Filtrate	Insensitive (Ins)	37	0	0	R.T.	282.2	0.00
	Seg[c]	0	2	0			
	Sensitive (Sen)	0	0	41			
86ISWN 2137 x Glenlea							
Culture Filtrate	Insensitive (Ins)	38	0	0	R.T.	276.7	0.00
	Seg[c]	0	2	0			
	Sensitive (Sen)	0	1	39			
Red Chief x Kenyon							
Culture Filtrate	Insensitive (Ins)	38	0	0	R.T.	129.9	0.00
	Seg[c]	0	1	0			
	Sensitive (Sen)	0	1	40			

[a] Segregating for resistance.

[b] Probability of obtaining deviations from the expected ratio by chance alone. A probability value greater than 0.05 indicates that segregation in the observed population does not differ significantly from the expected ratio.

[c] Segregating for insensitivity.

R.T. = The ratio tested was 3969 R.Ins: 126 R.Seg[c]: 3969 R.Sen: 126 Seg[a]Ins: 4 Seg[a]Seg[c]: 126 Seg[a]Sen: 3969 S.Ins. : 126 S.Seg[c] : 3969 S.Sen

4.4 Discussion

The development of a necrosis-chlorosis model and identification of isolates inducing either necrosis or chlorosis only has made genetic studies with spore-inoculation or toxin infiltration more accurate and consistent. Mendelian inheritance of resistance to culture filtrate infiltration and spore-inoculation was observed in the five crosses studied. The segregation pattern of F_2 and BC_1F_1 plants, and $F_{7:8}$ lines confirmed that the same recessive gene controls resistance to necrosis caused by either culture filtrate infiltration or spore-inoculation. These findings agree with previous reports (Lamari and Bernier 1989b; Duguid 1995; Gamba et al. 1998; Gamba and Lamari 1998).

Tests of independence of the segregating data in the F_2, BC_1F_1 and $F_{7:8}$ generations indicate that gene(s) controlling resistance to necrosis caused by spore-inoculation and culture filtrate infiltration are not independently inherited. Although the majority of F_2 and BC_1F_1 plants showed similar reactions to spore-inoculation and culture filtrate infiltration, a few plants in both generations that gave a differential response to spore-inoculation and culture filtrate were observed. This differential response could be either the result of errors associated with testing on a single plant basis or due to genetic reasons. Because of lack of space and time, progeny testing of the plants showing differential response could not be done. However, $F_{7:8}$ lines were tested to confirm the findings. All lines except two, gave similar reactions to spore-inoculation and culture filtrate infiltration and the segregation pattern of the $F_{7:8}$ lines confirmed that the same single gene controlled resistance to necrosis induced by spore-inoculation or culture filtrate. The two lines giving a differential response segregated for resistance to necrosis

induced by spore-inoculation and were sensitive to the culture filtrate. This difference is considered more likely due to disease escape or errors in rating than to genetic reasons.

Apart from the exceptions just discussed, an exact correspondence between spore resistant/susceptibility and toxin(s) insensitivity/sensitivity was obtained. As well, the same gene controlled resistance to necrosis induced by toxin(s) infiltration or spore inoculation. These findings indicate that a toxin(s) is the pathogenicity factor for induction of the necrosis component of tan spot. This was observed also by other researchers (Tomas and Bockus 1987; Lamari and Bernier 1989b; Duguid 1995; Gamba et al. 1998).

Approaches such as isolation of toxin from diseased plants, ability to reproduce typical disease symptoms when toxin is applied to healthy plants, similar host specificity shown by the pathogen and toxin, and correlation of virulence with ability to produce toxin determines whether or not toxin(s) is a pathogenicity factor (Yoder 1980). Lamari et al. (1995b) showed that the necrosis-producing toxin was present in the intercellular washing fluids of leaves infected with necrosis-inducing isolates. Lamari and Bernier (1989b) and Orolaza et al. (1995) reported that necrosis and chlorosis are produced when toxin(s) are infiltrated into susceptible cultivars. Genetic studies indicate that the same gene(s) controls resistance to necrosis and chlorosis induced by spore-inoculum and toxin infiltration (Duguid 1995; Gamba et al. 1998; Gamba and Lamari 1998). Finally, Ciuffetti et al. (1997) isolated a gene, which encodes for the necrosis-inducing toxin ToxA. They transformed non-toxin-producing isolates of *P. tritici-repentis* with this gene to produce a toxin-producing isolate that induced necrosis. This finding confirmed

that toxin ToxA is the primary determinant of pathogenicity for the necrosis component in the wheat-*P. tritici-repentis* pathosystem.

In several host-pathogen systems, toxin(s) may be pathogenicity or virulence factors. Here pathogenicity is considered to be the ability to cause disease, a qualitative term, whereas virulence refers to the extent or amount of disease produced, a quantitative term. When a toxin acts as a pathogenicity factor, its presence is necessary for disease development. However, when a toxin(s) is a virulence factor, its presence enhances disease development or is required for a portion of the disease development process. When a toxin(s) is a pathogenicity factor, resistance in host plants to both the pathogen and the toxin(s) is likely to be controlled by the same gene(s) whereas, different host resistance gene(s) may control resistance to the pathogen and the toxin(s) when the toxin(s) is a virulence factor.

Host-pathosystems where toxins have been shown to be pathogenicity factors include oat-*Helminthosporium victoriae*, maize-*H. carbonum* race 1, sorghum-*Pericona circinata*, and sugarcane-*H. sacchari* (Pringle and Scheffer 1964; Scheffer et al. 1967; Yoder 1980). Host-pathosystems where toxins act as virulence factors include maize-*H. maydis* race T and tobacco-*Pseudomonas tabaci* (Yoder 1980). The bacterium *Pseudomonas syringae* has a wide host range and produces a toxin, syringomycin, which can act as either a pathogenicity factor or a virulence factor depending upon the host plant. In maize and cowpea, the toxin acts as a pathogenicity factor, while in peach it is a virulence factor (Yoder 1980). Toxin(s) in the wheat-*P. tritici-repentis* pathosystem act as a pathogenicity factor.

Lamari and Bernier (1989b) reported a differential response of certain cultivars to culture filtrate and spores produced by race 1 (n+c+). These cultivars were susceptible to the chlorosis component but not to the necrosis component. However, if the two components of tan spot were analyzed separately, no differential response was observed. Therefore, when studying the relationship between reaction to the toxin infiltration and spore-inoculation, it is better to use isolates from races 2, 3 or 5, which induce only one symptom on susceptible wheat cultivars with both toxin infiltration and spore-inoculum.

In growth chamber studies, Riede et al. (1996) observed differential responses to ToxA infiltration and spore inoculation although both toxin and spores came from race 2 (n+c-). Cultivar Ponta Grossa 1 was insensitive to the toxin but highly susceptible to spore-inoculation, whereas synthetic lines PF844005 and PF844008 were resistant to spore-inoculation but sensitive to toxin infiltration. Similarly Zhang and Jin (1998), studying wild relatives of wheat, observed differential responses to culture filtrate infiltration and spore inoculation. This indicates that there may be factors other than the toxin(s), which may be responsible for disease development. It also indicates that host plants may have different factors, which respond differently to the toxin(s) and spore-inoculum.

Genetic studies in the wheat-*P. tritici-repentis* pathosystem have indicated that the same recessive gene controls resistance and insensitivity to necrosis induced by spore-inoculum and culture filtrate, respectively. These studies have all involved genotypes that showed a similar response to spore-inoculum and culture filtrate. Future studies should be done with accessions that show differential responses for they may give a better understanding of resistance to spore-inoculation and culture filtrate infiltration

for the necrosis component caused by races 1 and 2 and the chlorosis component induced by race 5.

In this study, it was observed that if the parents showed similar responses to culture filtrate and spore inoculation, then their cross progeny also showed the same relationship. This result is important from a plant breeder's perspective, as segregating populations could then be screened with the culture filtrate instead of using spore-inoculation. The process of culture filtrate infiltration may be difficult but the total time required and the expense involved are small compared to spore inoculation. The culture filtrate reaction is clear and easily differentiates between sensitive and insensitive plants provided the concentration of toxin(s) in the culture filtrate is optimized. Culture filtrate infiltration is less influenced by environmental conditions than spore-inoculation and can easily be combined with screening for resistance to other diseases.

Naitao et al. (1998) reported that *in vitro* selection of embryonic calli using *H. sativum* toxins was effective in selecting for resistance to spot blotch of wheat. Similarly Wicki et al. (1999) reported that a crude extract of *Septoria nodorum* could be used for *in vitro* screening for resistance to septoria nodorum blotch in wheat. Yu (1995) demonstrated that anther culture medium incorporated with toxin ToxA could be used for *in vitro* selection for tan spot resistance.

That culture filtrate containing necrosis inducing toxin can be used as a surrogate technique for fungal screening enhances the potential of tissue culture techniques which permit using the toxins to select for resistant cells and protoplasts. *In vitro* screening for tan spot resistance could be an important tool in a microspore-based doubled-haploid production program. However, only the toxins for necrosis induced by races 1 and 2, and

for chlorosis induced by race 5 have been isolated. Toxin(s) for chlorosis caused by races 1 and 3 have yet to be completely isolated and characterized. Therefore, all the toxins need to be identified and their interactions studied before *in vitro* screening for tan spot resistance could be recommended.

5. GENETIC SIMILARITY AMONG ISOLATES OF THE TAN SPOT FUNGUS

5.1 Introduction

The fungus *Pyrenophora tritici-repentis* (Died.) Dreches causes tan spot, a foliar disease of wheat. The tan spot disease syndrome consists of two distinct symptoms, tan necrosis and extensive chlorosis, for which host responses are independently inherited (Lamari and Bernier 1991). Initially isolates of *P. tritici-repentis* were grouped into four pathotypes based on their ability to induce tan necrosis and/or extensive chlorosis on a differential set of cultivars. The symptom-based pathotype classification is limited to four broad categories: pathotype 1 induces both necrosis and chlorosis; pathotype 2 induces necrosis only; pathotype 3 induces chlorosis only; and pathotype 4 fails to induce any symptoms.

The ability of the pathogen to generate new virulence, as evidenced by the physiologic variation reported in many parts of the world (Misra and Singh 1972; Luz and Hosford 1980; Krupinsky 1987, 1992), suggests that new virulence types may exist within the current pathotypes. Lamari et al. (1995a) identified Algerian isolates that induced chlorosis, similar to that induced by race 3, but reacted differently on a differential set of cultivars. This led to the classification of isolates based on their virulence on a set of differential host genotypes. Presently there are five races of *P. tritici-repentis* (Lamari et al. 1995a). In culture, all five races are morphologically similar and can be distinguished only by their reaction on the differential set.

Beck (1999) developed a diagnostic PCR assay based on an internal transcribed spacer (ITS) for the detection of *P. tritici-repentis* in wheat. These primers are very specific and do not amplify other common cereal pathogens or healthy wheat tissue DNA. However, this PCR assay failed to differentiate races of *P. tritici-repentis*.

Genetic information on the fungal population assists in disease resistance breeding and helps in understanding the sources of epidemics. Pathogen populations with large genetic variation are capable of rapidly evolving responses to changing environments. These pathogens cause major problem for durability of host resistance and develop resistant biotypes to fungicides (Peltonen et al. 1996). Frequent development of virulent races may result in the break down of host resistance genes. Analysis of genetic variation in populations of phytopathogenic fungi has been successfully done using molecular markers (Dusabenyagasani et al. 1999). Use of molecular markers to examine population dynamics of plant pathogens is providing levels of precision not previously available and is revolutionizing the analysis of population biology of plant pathogens (Dusabenyagasani et al. 1999).

Sexually reproducing plant pathogen populations have been reported to be more variable than asexually reproducing plant pathogens (Peever and Milgroom 1994). *P. tritici-repentis* reproduces both sexually and asexually with one cycle of sexual reproduction occurring on the wheat stubble between crops, and several cycles of asexual reproduction occurring during the growing season of the wheat crop. A preliminary study with limited number of isolates of *P. tritici-repentis* by Zinno et al. (1998) indicated polymorphisms among isolates, but no correlation could be established between RAPD polymorphisms and geographic origin, toxin production and pathogenicity of the

isolates. Extensive studies have been done assessing the genetic variability of the closely related species *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* (Crous et al. 1995; Peltonen et al. 1996; Campbell et al. 1999), but, to date, there has been no publication reporting on the genetic variability among and within the races of *P. tritici-repentis*.

The objectives of this study were to determine the genetic similarity and variability among different isolates of *P. tritici-repentis* using RAPD and to establish the relationship between genetic similarity of the different isolates, race classification and geographical origin.

5.2 Material and Methods

5.2.1 Isolates and Race Classification

Sixty-six isolates of *P. tritici-repentis* were screened on a differential set of wheat cultivars to determine their race classification. Most of these isolates were isolated from wheat leaves collected throughout Saskatchewan as a part of the Saskatchewan Wheat Disease Survey (Fernandez et al. 1999).

All isolates were screened twice on a differential set of host genotypes. In the first screening the differential set consisted of nine wheat cultivars, while in the second screening there were six differential cultivars (Table 5.1). Cultivars 6B-662, Red Chief and 86ISWN 2137 were excluded from the second screening as they gave resistant reactions to all the races tested, similar to cultivars Erik and Hadden. Only 15 isolates were tested at a time due to the space limitations of the mist chamber. Inoculum production and disease screening followed the procedures described in Sections 3.2.2 and 3.2.3, respectively.

Table 5.1. Cultivars included in the differential sets used for race classification of the isolates selected for the genetic similarity study

Wheat cultivar	Differential set for		Reaction to		
	1st Test	2nd Test	Race 1	Race 2	Race 3
Erik	Included	Included	Resistant	Resistant	Resistant
86ISWN 2137	Included	Excluded	Resistant	Resistant	Resistant
Glenlea	Included	Included	Susceptible	Susceptible	Resistant
Kenyon	Included	Included	Susceptible	Susceptible	Mod. Resistant
6B-365	Included	Included	Susceptible	Resistant	Susceptible
Red Chief	Included	Excluded	Resistant	Resistant	Resistant
Hadden	Included	Included	Resistant	Resistant	Resistant
6B-662	Included	Excluded	Resistant	Resistant	Resistant
Katepwa	Included	Included	Susceptible	Susceptible	Resistant

5.2.2 Isolates and DNA Extraction

To study genetic similarity and variation of tan spot fungus, 33 isolates of *P. tritici-repentis* and an "outlier" group consisting of one isolate each of *P. teres* f. sp. *teres*, *P. teres* f. sp. *maculata*, *P. graminea*, *Helminthosporium sativum* and an unknown isolate Canora Field 9 were selected (Appendix IV). Dr. A. Tekauz, Agriculture and Agri-Food Canada, Winnipeg, Manitoba provided cultures of all the outlier species except the unknown isolate (Canora Field 9). For DNA extraction, the fungal cultures were grown on V8P media in 100 mm-diameter petri dishes. After 7-8 days the mycelium was carefully scraped from the agar surface using a scalpel and placed in a 1.5 ml microfuge tube. The mycelium was then dried overnight in an oven at 37°C. DNA extraction followed a modification of the procedure of Cenis (1992).

Liquid nitrogen was added to the fungal mycelium in the microfuge tube. After

the mycelium was ground to powder. 300 µl of extraction buffer (200 mM Tris HCl. 25 mM EDTA. 250 mM NaCl. 0.5% w/v sodium dodecyl sulphate) was added. and tubes were placed in a freezer for 20 minutes at –20°C. The tubes were then centrifuged at 13.000 rpm for 10 minutes and the 300 µl supernatant transferred to another tube. 150 µl of 3 M sodium acetate added and the tube centrifuged again at 13.000 rpm for 15 minutes. After centrifugation. 450 µl of the supernatant was transferred to a fresh tube. an equal amount of isopropanol added and after standing for 15 minutes at room temperature. the tubes were centrifuged for 10 minutes and the supernatant discarded. The DNA pellet was washed with 70% cold ethanol. dried at room temperature and resuspended in 150 µl of sterile double distilled water. The DNA concentration was quantified by GeneQuant fluorimeter (Pharmacia LKB Biochrom Ltd.. Cambridge. UK) on the basis of UV absorption at 260 nm. The DNA concentration was diluted to 25 ng/µl and stored in freezer at –20°C until used.

5.2.3 Primer Screening and Amplification Conditions

The RAPD primers used in this study were obtained from Operon Technologies Inc.. Alameda. USA. and the Biotechnology Laboratory. University of British Columbia. Vancouver. Canada. PCR amplification was performed in a 40-well RoboCycler® Gradient 40 thermocycler (Stratagene®. La Jolla. USA). The reaction mixture contained 75 ng of fungal DNA template. 1 unit of Taq DNA polymerase. 50 mM KCl, 20 mM Tris-HCl (pH 8.4). 2 mM $MgCl_2$, 0.4 µM of primer and 200 µM of each of dTTP. dATP. dCTP and dGTP (Gibco BRL). The reaction mixture was brought to a volume of 25 µl by addition of sterile double distilled water and was overlaid by 25 µl of mineral oil before amplification.

The PCR amplification protocol included initial denaturation at 94°C for 6 minutes followed by 45 cycles of PCR involving a denaturation temperature of 92°C for 1 minute. an annealing temperature of 36°C for 1 minute and an extension temperature of 72°C for 1 minute. The final extension cycle was for 6 minutes at 72°C and then the PCR product was held at 6°C.

5.2.4 Gel Electrophoresis

After PCR amplification. 4 µl of gel loading buffer (6X) (Appendix III) was added to each tube containing the PCR reaction product. The PCR reaction products were separated through electrophoresis by running on a horizontal agarose gel [1.5 % (w/v) agarose cast in 1X TAE buffer (Appendix III) containing ethidium bromide (0.5 µg/ml)] at 80 to 100 V for two to three hours. All the 38 isolates studied. along with two 1 kb DNA ladders. which were used as a marker for comparison of molecular sizes of the amplified product were electrophoresed on the same gel. The gel was viewed under UV transilluminator (Cole-Parmer Instrument Co.. Chicago. USA) and photographed using the gel documentation system UVP ImageStore 7500 (DiaMed Lab Supplies. Mississauga. Canada).

5.2.5 Analysis of RAPD Data

The presence (1) or absence (0) of bands for each primer-isolate combination was scored. The data was then entered into a binary matrix and the SIMQUAL (Similarity of Qualitative Data) program of the NTSYS-pc 2.02i package (Numerical Taxonomy and Multivariate Analysis System) (Rohlf 1998) was used to generate a similarity matrix using the coefficients of Jaccard. Jaccard coefficients are calculated according to the

formula a/(a+b+c) where a is the number of bands shared by both isolates (X and Y). and b and c are the number of bands present only in X and Y. respectively. Bands absent in both isolates are excluded when calculating the Jaccard coefficients (Rohlf 1993). The similarity matrix was then used to find the tree matrix using the UPGMA (unweighted pair-group method of arithmetic averages) method of the SAHN (sequential. hierarchic. non-overlapping) program (Rohlf 1993).

Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was used as a second approach for studying the relationships among the isolates. AMOVA estimates and partitions the total genetic variation present in the fungal population into among and within-race variability. The binary matrix produced by scoring for presence (1) or absence (0) of bands for each primer-isolate combination was used as input data for the SIMINT (Similarity of Interval Data) program of the NTSYS-pc 2.02i package to generate genetic distances using the coefficient of squared euclidian distance (E_{ij}^2) (Rohlf 1998).

$$E_{ij}^2 = \sum_k (X_{ki} - X_{kj})^2$$

where E_{ij}^2 = coefficient of squared euclidian distance between isolates X_i and X_j

$\sum_k$ = summation of K bands

AMOVA was performed using the coefficient of squared euclidian distance as the input data and was limited to race 1 and race 2 isolates of *P. tritici-repentis*. Races 3 and 4 of *P. tritici-repentis* and the outliers were represented each by only one isolate and were excluded from the analysis.

5.3 Results

All isolates of *P. tritici-repentis* tested in this study belonged to race 1 or race 2 except isolates Ptr D308 and Ptr 90-2 which were known to belong to race 3 and race 4, respectively. For this study 33 isolates of *P. tritici-repentis* were selected of which 12 isolates were race 1, 19 were race 2, and 1 isolate each of race 3 and race 4 (Appendix IV).

Initially, 90 primers were screened on four random isolates of *P. tritici-repentis*. Most of the RAPD primers produced between five and 15 clear informative bands although a few gave no amplification at all. Thirty primers were selected on the basis of their ability to give clear amplification and the quantity of primer available. These 30 primers produced 244 scorable polymorphic bands. All the bands were polymorphic due to the inclusion of diverse outliers in this study. The amplification pattern observed using primer UBC 598 is shown in Fig. 5.1.

Based on Jaccard's coefficients of similarity, 32 isolates of *P. tritici-repentis* had similarity coefficients of over 59% but isolate Ptr D308 (race 3) was distinct in showing a similarity of only 40% to 52% to the other isolates (Appendix V). Isolates originating from the same location had similarity coefficients ranging from 69% (Outlook Fields 8 and 10) to 88% (Humbold Fields 8 and 9), whereas those from different locations ranged from similarities of 59% (Kelvington Field 1 and Ptr 200) to 93% (Battleford Field 1 and Canora Field 10). Similarity coefficients within races ranged from 59% (Kelvington Field 1 and Ptr 200) to 93% (Battleford Field 1 and Canora Field 10) for race 1 isolates and 75% (Davidson Field 6 and Outlook Field 10) to 86% (Saskatoon Fields 4 and 5) for race 2 isolates.

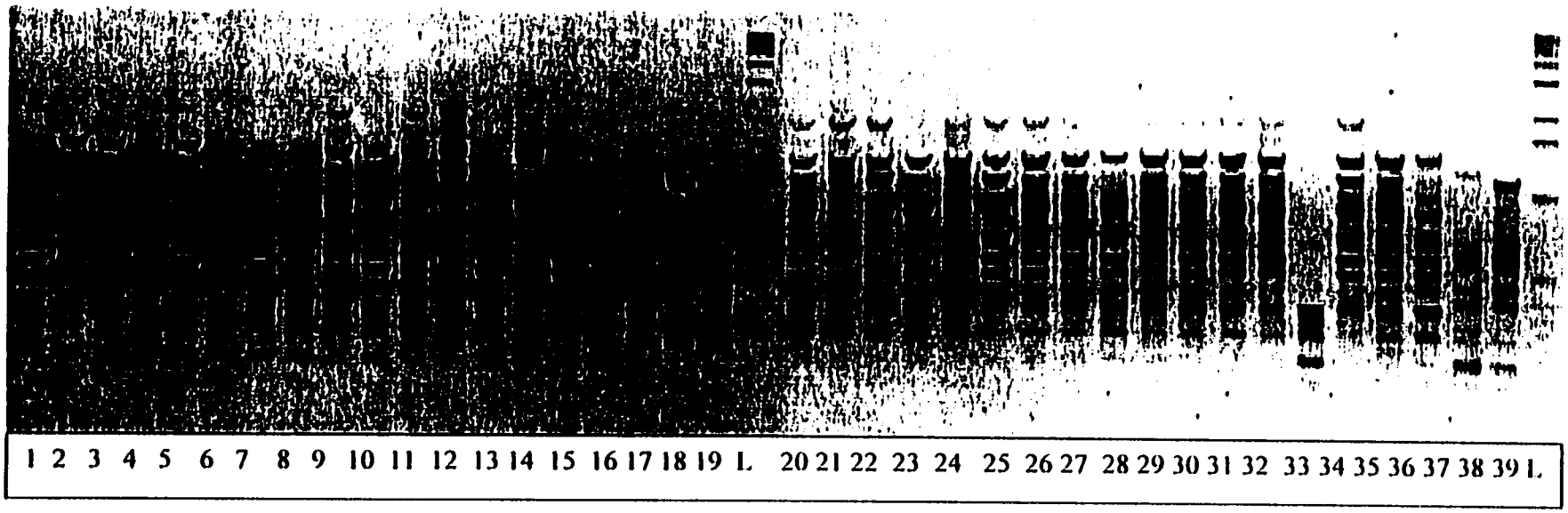


Fig. 5.1. Amplification profiles generated by the 38 isolates using primer UBC 598. Lane numbers 18, 19, 38, 39 and 33 are outliers while the remaining lanes are isolates of *P. tritici-repentis*. L represents 1 kb ladder used to compare the size of bands.

The outliers *P. teres* f. sp. *teres* and *P. teres* f. sp. *maculata* had a similarity coefficient of 41%, and similarity coefficients of 26% and 24%, respectively, with *P. graminea.* These three species had similarities of 13% to 28% with the isolates of *P. tritici-repentis*. The unknown isolate, Canora Field 9, and the *H. sativum* isolate showed similarities of 17% to 27% and 15% to 25% with the *P. tritici-repentis* isolates, respectively.

Based on UPGMA clustering analysis using Jaccard coefficients, the dendrogram produced showed that all the isolates had unique banding patterns and no two isolates were identical (Fig. 5.2). Clusters with greater than 80% similarity were observed, but these groupings did not reflect the race structure or geographical origin of the isolates. Even though there was high variability among the isolates of *P. tritici-repentis*, they clustered together in a single group compared to the outliers.

Primers which could differentiate the tan spot fungus from the four outlier species, were identified but none which could differentiate the different isolates of *P. tritici-repentis* in terms of race classification or geographical origin were found. This indicated that the primers studied were neutral for these characters. The outlier fungal species produced markedly different banding patterns compared to the *P. tritici-repentis* isolates and along with the unknown isolate, Canora Field 9, formed a separate cluster from that of the *P. tritici-repentis* isolates (Fig. 5.2).

The analysis of molecular variation (AMOVA) showed that the major portion of genetic variation in the fungus (96.8%) was within races or among isolates (Table 5.2). There was very little variation between races 1 and 2 (3.2%). This was evident also from the cluster analysis dendrogram (Fig. 5.2). Hence the results from both the AMOVA and

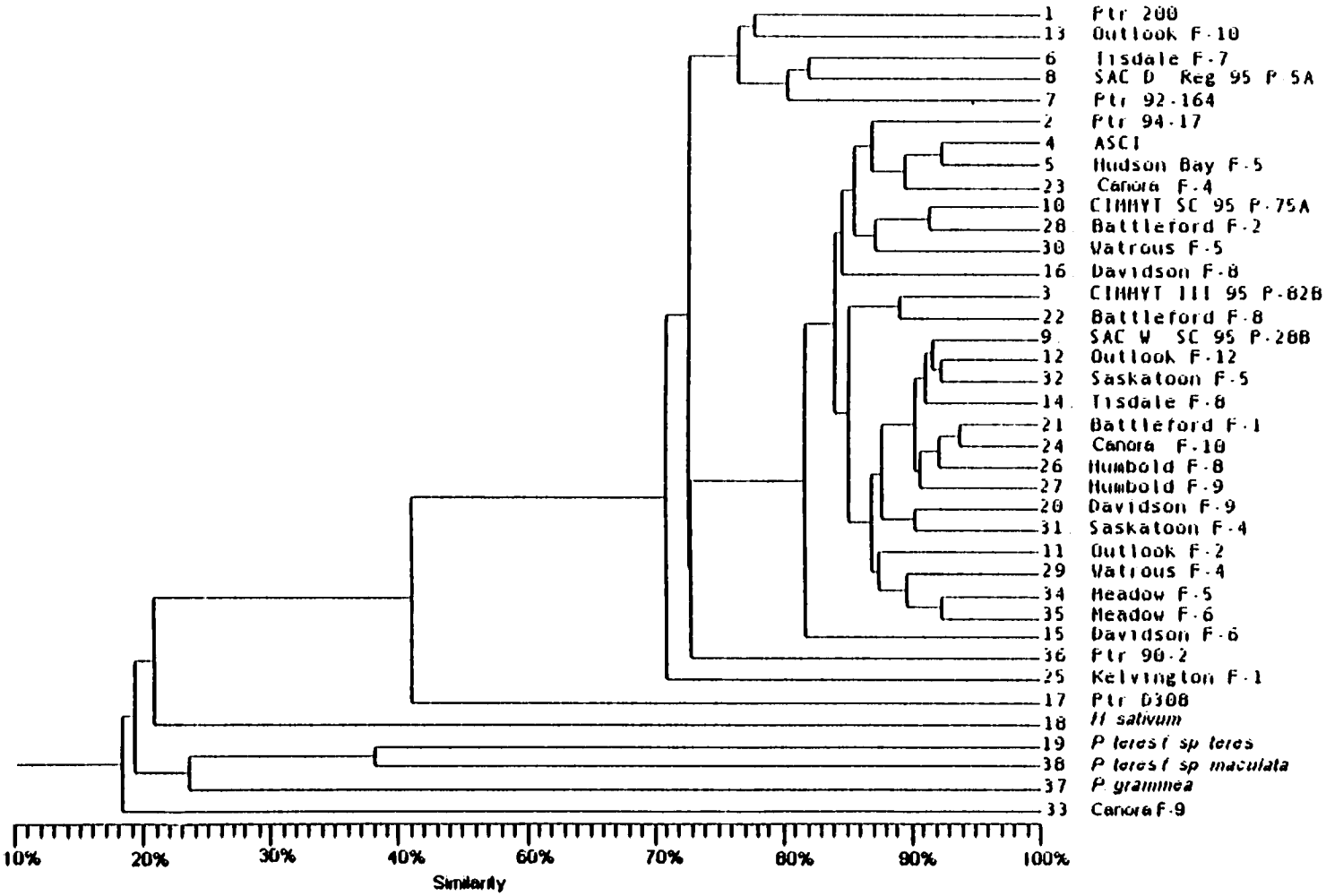


Fig. 5.2. Dendrogram based on RAPD polymorphism in 38 isolates. Cluster analysis was conducted using UPMGA method. Isolates 1-6 and 20-25 (race 1), 7-16, 26-32 and 34-35 (race 2), 17 (race 3) and 36 (race 4) belong to *P. tritici-repentis* while remaining 18, 19, 33, 37 and 38 are outliers

the cluster analysis indicated that the variability observed among the *P. tritici-repentis* isolates was independent of both the race structure and the geographic origin of the isolates.

Table 5.2. Analysis of molecular variance (AMOVA) of 31 isolates belonging to races 1 and 2 of *P. tritici-repentis*

Source of variation	d.f.	Sum of squares	Variance	Percentage
Among races	1	2.42	0.05415	3.22
Within races	29	47.18	1.62696	96.78
Total	30	49.61	1.68111	100

5.4 Discussion

The present race structure of tan spot fungus *P. tritici-repentis* is based on the virulence response to a set of wheat differentials. Race 5, initially identified in Algeria (Lamari et al. 1995a), has now been identified in North America (Lamari et al. 1998; Ali and Francl 1999). All five races of *P. tritici-repentis* are present in North America. However, knowledge of the extent of genetic variability among and within-races is lacking. This study was conducted to determine the extent of genetic similarity among isolates of *P. tritici-repentis* and to observe if a relationship between genetic similarity and race or geographic origin existed.

This study found that there was a high degree of genetic variability among isolates of *P. tritici-repentis*. Similar observations were made by Zinno et al. (1998) working with *P. tritici-repentis*, while others observed similar findings in other

Pyrenophora species (Crous et al. 1995: Peltonen et al. 1996: Ganeshan 1997). The AMOVA indicated that most of the variability occurred among the isolates or within races.

The results from the AMOVA and the cluster analysis indicated that the genetic similarity among the isolates of *P. tritici-repentis* studied was independent of race classification or geographical origin. A similar result was obtained by Zinno et al. (1998). who found that genetic polymorphism among isolates did not correlate with geographical origin. pathogenicity or ability to produce toxins. Such results are not surprising. since RAPD markers detect variation that is distributed throughout the genome while race classification is based on specific loci within the genome. The RAPD primers used in this study have fingerprinted regions or sequences of the fungal genome. which are different to those containing the genes for virulence (race classification).

In the prairie region the sexual stage of *P. tritici-repentis* occurs on the wheat stubble between crops while the asexual stage occurs during the crop growth. Occurrence of sexual recombination in nature could result in high genetic variability among the isolates of *P. tritici-repentis* and this variability could be independent of race or geographic origin. as observed in this study. Under favorable conditions conidiospores can travel 10 to 200 km (De Wolf et al. 1998). The tan spot fungus can also be seed-borne and hence long distance travel of fungal inoculum is possible. The occurrence of sexual reproduction and long distance dispersal of inoculum could result in occurrence of genetic variability independent of race structure or geographic origin.

The genetic polymorphism detected by RAPD analysis among the five fungal species *P. tritici-repentis*, *P. teres* f. sp. *teres*, *P. teres* f. sp. *maculata*, *P. graminea* and

H. sativum is an indication of the variability that exists among them. This agrees with the findings of other researchers (Peltonen et al. 1996; Ganeshan 1997). Although RAPD analysis failed to identify race-specific markers for *P. tritici-repentis*, markers which could differentiate the four other species were observed. More isolates of these species need to be tested to assess the specificity of these markers.

This study revealed that the tan spot fungus is highly variable and, therefore, increased efforts should be made to assess the variability present in the fungus through regular pathogenicity and virulence studies. In this study the majority of isolates were from races 1 and 2 hence, the true extent of variability present in the fungus could not be assessed. Races 1 and 2 induce similar disease reaction in both durum and hexaploid wheat however, races 3 and 5 cause chlorosis in hexaploid wheat, but necrosis in durum (Gamba and Lamari 1998). It is also observed that most of the isolates identified of races 3 and 5 come from durum host. It could be possible that host response could put a selection pressure on the genetic composition of *P. tritici-repentis*. Future studies should include isolates from all the fives races of *P. tritici-repentis* coming from different host plants to observe if host selection on tan spot fungus occurs or not.

The native prairie grasses act as host for overwintering and as a primary source of fungal inoculum. They could play an important role as a source of genetic variation in the fungal population and as a reservoir of fungal biotypes genetically different to those prevalent on wheat (Krupinsky 1987; Ali and Lamari 1997; De Wolf et al. 1998). Hence to develop a complete understanding of the population structure of *P. tritici-repentis*, isolates from native grasses should be included in future genetic and pathogenicity studies.

Results of the race classification of the isolates of *P. tritici-repentis* supported the previous findings that races 1 and 2 were the predominant races of this fungus in the prairie region (Lamari and Bernier 1989c; Lamari et al. 1998; Ali and Francl 1999). Since races 1 and 2 both cause necrosis on susceptible wheat cultivars, breeding programs should emphasize breeding for resistance to the necrosis component of tan spot.

6. IDENTIFICATION OF MOLECULAR MARKERS LINKED TO RESISTANCE TO THE NECROSIS COMPONENT OF TAN SPOT

6.1 Introduction

Tan spot, caused by the fungus *Pyrenophora tritici-repentis*, is an important foliar disease of wheat, causing significant losses in grain yield and quality. In recent years, it has become a major component of the leaf spot disease complex of western Canada (Fernandez et al. 1999). Tan necrosis and extensive chlorosis are two phenotypically distinct symptoms associated with this disease.

Three independent genes in hexaploid wheat are responsible for resistance to tan spot (Gamba et al. 1998). A single recessive gene controls resistance to necrosis induced by races 1 and 2, while two genes control resistance to chlorosis, one for chlorosis induced by races 1 and 3, and the other for chlorosis induced by race 5 (Lamari and Bernier 1991; Duguid 1995; Orolaza et al. 1995; Gamba et al. 1998).

The gene controlling resistance to the necrosis component of tan spot was designated as *tsn1* (Stock et al. 1996; Faris et al. 1996). Through aneuploid, substitution and deletion line analysis, *tsn1* has been located on the long arm of chromosome 5B (Faris et al. 1996; Stock et al. 1996; Anderson et al. 1999).

Faris et al. (1996) identified two RFLP probes, *Xbcd 1030* and *Xwg 583*, which flank the gene (*tsn1*) locus at distances of 5.7 cM and 16.5 cM, respectively, in a cross of resistant synthetic hexaploid W-7976 with susceptible cultivar Kulm. Using temperature

sweep gel electrophoresis. Stock (1996) identified two RAPD markers. UBC 109 and UBC 102. loosely linked to *tsn1*.

Identification and selection of plants for a trait of interest can be accelerated by the use of marker-assisted selection. Marker-assisted selection utilizes molecular markers linked to a major gene(s) for the trait of interest to select indirectly for the trait. A prerequisite for marker-assisted selection is that the molecular markers be closely linked to the gene(s) controlling the trait of interest. The objective of this study was to identify molecular markers linked to the gene controlling resistance to the necrosis component of tan spot.

6.2 Materials and Methods

6.2.1 Population Development and Disease Screening

Three populations. $F_{6:7}$ lines of the crosses. 86ISWN 2137 x Glenlea. 86ISWN 2137 x Kenyon. and Red Chief x Kenyon. were studied to identify RAPD markers linked to the gene controlling resistance to necrosis. Genetic studies indicated that a single recessive gene controlled resistance to necrosis in these populations (Section 3.3.1). These populations were developed from random F_2 plants using single seed descent both in the greenhouse and the field. Plants from which DNA was extracted were grown to maturity and progeny tested for disease reaction to necrosis inducing isolate Ptr 92-164 (race 2) to identify the phenotypes used to construct the resistant and susceptible bulks. A minimum of 16 plants per line was tested. This gave a 99% probability of accurately identifying segregating and non-segregating lines to resistance to necrosis (Hanson

1959). Inoculum production, disease screening and disease rating was done as described in Sections 3.2.3 and 3.2.4.

6.2.2 DNA Extraction

DNA extraction followed the method developed by Procunier et al. (1991). From each F_7 plant, one tiller was harvested at the 3-4-leaf stage for DNA extraction. From this tiller approximately 0.2-0.3 g fresh tissue was cut into small pieces and placed in a 1.5-ml microfuge tube. The tissue was frozen with liquid nitrogen, ground to fine powder. 0.5 ml 2X CTAB extraction buffer (Appendix III) at 65°C added and the tube contents were mixed well. The tubes were then placed in a 65°C water bath for 10 min before adding 0.5 ml chloroform/isoamyl alcohol (24:1) to each tube. After thorough mixing, the tubes were centrifuged at 13,000 rpm for 10 min.

After centrifuging, the upper aqueous phase (about 450 µl) was decanted into a new tube and 0.1 volume (45 µl) of 10% CTAB (Appendix III) at 65°C was added followed by one volume of 24:1 chloroform/isoamyl alcohol. The content of each tube were then mixed well, centrifuged at 13,000 rpm for 10 min and the supernatant transferred to a new tube. Two volumes (900 µl) of cold (-20°C) 95% ethanol was added to each tube which was then placed on ice for 5 min to precipitate the DNA. The DNA was pelleted by centrifugation at 13,000 rpm for 10 min. The alcohol was poured off and 500 µl of cold (-20°C) 70% ethanol was added again to the tube followed by centrifuging for 5 min to desalt the DNA pellet. The alcohol was removed, the DNA pellet air-dried at room temperature for 4-6 hrs, resuspended in 100 µl of sterile double distilled water and kept at 4°C overnight for rehydration. Finally, the isolated DNA was

quantified on the basis of UV absorption at 260 nm using a GeneQuant fluorimeter (Pharmacia LKB Biochrom Ltd., Cambridge, UK), diluted to a final concentration of 25 ng/µl and stored at -20°C until use.

6.2.3 Bulked Segregant Analysis

Two attempts were made to identify RAPD markers linked to tan spot resistance using bulked segregant analysis. In the first attempt bulks were composed of $F_{6:7}$ lines from the crosses 86ISWN 2137 x Glenlea and 86ISWN 2137 x Kenyon. The resistant and susceptible bulks were made of seven lines of which five lines were from cross 86ISWN 2137 x Glenlea and two lines from 86ISWN x 2137 Kenyon. The two populations were used to create the bulks with the objective of identifying cross non-specific RAPD markers. In the second attempt, resistant and susceptible bulks were composed of ten $F_{6:7}$ lines each from the cross Red Chief x Kenyon.

6.2.4 Primers Screening and Amplification Conditions

The RAPD primers used in this study were obtained from Operon Technologies Inc., Alameda, USA, and the Biotechnology Laboratory, University of British Columbia, Vancouver, Canada. The PCR reaction mixture consisted of 1 unit of Taq DNA polymerase, 25 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.4 µM of primer, and 200 µM of each of dTTP, dATP, dCTP and dGTP (Gibco BRL). The reaction was brought to a volume of 25 µl by the addition of sterile double distilled water and overlaid with 25 µl of mineral oil.

The PCR reaction was run in a RoboCycler® Gradient 40 thermocycler (Stratagene®, La Jolla, USA). The PCR amplification protocol was: initial denaturation

at 94°C for 6 minutes followed by 45 cycles of PCR involving a denaturation at 92°C for 1 minute; an annealing at 36°C for 1 minute: and an extension at 72°C for 1 minute. The final extension cycle was for 6 minutes at 72°C. The PCR reaction product was maintained at 6°C until prepared for gel electrophoresis.

6.2.5 Gel Electrophoresis

After PCR amplification. 4 µl of gel loading buffer (6X) (Appendix III) was added to each tube containing the PCR reaction product. The PCR reaction products were separated through electrophoresis by running on a horizontal agarose gel [1.5 % (w/v) agarose cast in 1X TAE buffer (Appendix III) containing ethidium bromide (0.5 µg/ml)] at 80 to 100 V for two to three hours. The gel was viewed with a UV transilluminator (Cole-Parmer Instrument Co.. Chicago. USA) and photographed using the gel documentation system UVP ImageStore 7500 (DiaMed Lab Supplies. Mississauga. Canada).

6.2.6 PCR Primer Development and Amplification Conditions

The sequence of PCR primers developed from RFLP probe *Xbcd 183* were provided by Dr. J.D. Faris. Kansas State University. Manhattan. USA. The probe *Xbcd 183* is closely linked (1-3 cM) to the gene controlling resistance to the necrosis component of tan spot (Faris. personal communication). These PCR primers amplify a single strong band of 1.2 kb. which upon restriction digestion should give polymorphisms between resistant and susceptible genotypes (Faris, personal communication). The PCR primers were synthesized by GIBCO and have the following sequences:

BCD 183 Forward: 5'GGATATAACCAGTGTTACTTTCCAACCGG3'

BCD 183 Reverse: 5'CACGGCGCTGCACCTCATCTAATTG3'

The PCR reaction was run in a RoboCycler® Gradient 40 thermocycler (Stratagene®, La Jolla, USA). The PCR amplification protocol was: initial denaturation at 94°C for 3 minutes followed by 30 cycles of PCR involving denaturation at 94°C for 1 minute; annealing at 53°C for 1 minute; and extension at 72°C for 2 minutes. The final extension cycle was at 72°C for 7 minutes and then the PCR product was maintained at 6°C until used.

The PCR reaction mixture consisted of the following reagents: 1 unit of Taq DNA Polymerase, 200 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.2 µM of forward and backward PCR primers and 200 µM of each of dTTP, dATP, dCTP and dGTP (Gibco BRL). The reaction was brought to a volume of 50 µl by the addition of sterile double distilled water. The above PCR reaction mixture was overlaid with 25 µl of mineral oil.

Restriction digestion with various restriction enzymes was performed on the 1.2 kb band produced by amplification of wheat DNA by Xbcd 183 PCR primers. Using the buffer and conditions best suited for the particular restriction enzyme, digestion was performed with the following restriction enzymes:

1) Rsa I	6) Eco RV	11) Hae III	16) Hinf I
2) Mse I	7) Hind III	12) Apa I	17) Mbo I
3) Tha I	8) Xho I	13) Sau 3 AI	18) Tsp 5091
4) Alu I	9) Bgl II	14) Sma I	
5) Taq I	10) Eco RI	15) Msp I	

The restricted digested product was subjected to gel electrophoresis as described in Section 6.2.5.

6.2.7 Cloning and Sequencing of 1.2 kb Fragment

The 1.2 kb fragments amplified by the PCR primers BCD 183F and BCD 183R were cloned into the PCR II vector (plasmid) using the TA Cloning Kit (Invitrogen BV. San Diego. USA). This vector was designed for direct cloning of PCR products. It has a 3'-thymidine overhang that binds to adenine residues naturally added to the 5' end of PCR products by thermostable polymerase. It carries a *Lac Z* gene for blue/white colour selection of transformed bacteria. ampicillin and kanamycin resistance genes. The recombinant PCR II vector was transformed into *Escherichia coli* and plated on a selective medium containing 50 µg/ml X-gal and 50 µg/ml ampicillin. White colonies. containing the recombinant plasmids. were carefully picked off and cultured overnight in liquid broth media (Bacto Tryptone-10 g/L. yeast extract-5 g/L. NaCl-10 g/L and sterile double distilled water to bring the volume to 1 L) at 37°C on an orbital shaker. To observe that the desired fragment was cloned into the PCR II vector. various bacterial colonies were picked and the cloned fragments amplified with primers T_7 and M_{13}. which are designed specifically to amplify cloned fragments in PCR II vector.

The 1.2 kb fragments amplified by PCR primers BCD 183F and BCD 183R were sequenced to develop possible sequence characterized amplified region (SCAR) markers. Plasmid extraction was done using the Wizard™ Miniprep kit (Promega, Madison, USA). Plasmids with the correct size of inserts were sent to Plant Biotechnology Institute, Saskatoon. Canada for DNA sequencing. Both strands of the cloned fragments were sequenced using the Dyedeoxy Terminator cycle sequencing kit (Applied

Biosystems, Foster City, USA) on a 370 A sequencer (Applied Biosystems, Foster City, USA). The computer program DNAMAN was used to align the sequences.

6.2.8 Association between Amplified Fragments and Chromosomes 5A, 5B and 5D

The association between the different 1.2 kb fragments amplified by primers BCD 183F and BCD 183R and wheat chromosomes 5A, 5B and 5D was attempted. Using the PCR primers Xbcd 183, DNA from hexaploid wheat, durum wheat, *T. tauschii*, and cytogenetic stocks nullisomic 5A tetrasomic 5B (N5A/T5B), nullisomic 5A tetrasomic 5D (N5A/T5D), nullisomic 5B tetrasomic 5A (N5B/T5A), nullisomic 5B tetrasomic 5D (N5B/T5D), nullisomic 5D tetrasomic 5A (N5D/T5A) and nullisomic 5D tetrasomic 5B (N5D/T5B) lines was amplified and digested with Bam HI enzyme. Subsequently, gel electrophoresis as reported in Section 6.2.5 was performed to observe the different bands produced by digestion.

6.3 Results

6.3.1 RAPD Analysis

In the first attempt to identify RAPD markers linked to tan spot resistance, resistant and susceptible bulks comprised of $F_{6:7}$ lines from the crosses 86ISWN 2137 x Glenlea and 86ISWN 2137 x Kenyon were screened with 440 UBC primers (UBC # 1-140 and 500-800). One hundred and nineteen primers produced polymorphisms, but only 40 primers produced repeatable polymorphisms. Polymorphisms for both strong and faint bands were observed. However, the banding patterns were not consistent over the bulks. As well, primers UBC 608 and UBC 618, which were polymorphic on the bulks,

failed to produce polymorphisms when screened on the individual lines constituting the bulks. At this stage study with these populations was discontinued.

The second attempt to identify a RAPD marker linked to the gene controlling resistance to necrosis, was made using $F_{6:7}$ lines from the cross Red Chief x Kenyon. A total of 240 primers (Operon series I_{1-20}, H_{1-20}, K_{1-20}, L_{1-20}, M_{1-20}, N_{1-20}, O_{1-20}, and UBC # 600-700) were screened. Fourteen primers produced repeatable polymorphic amplification and of these, only five primers, Operon I_1, I_{18}, and N_1, and UBC 612 and 628, produced consistent repeatable polymorphisms on the bulks. These five primers were subsequently screened on the individual lines constituting the bulks but none showed any linkage with the gene controlling resistance to the necrosis component of tan spot. The RAPD markers UBC 109 and UBC 102, identified by Stock (1996) to be linked to the necrosis controlling resistance gene (*tsn1*), failed to produce polymorphisms between the resistant and susceptible bulks of the cross Red Chief x Kenyon.

6.3.2 PCR Primer Analysis

Analysis with the PCR primers used only resistant cultivars Erik (R1) and Red Chief (R2) and susceptible cultivars Glenlea (S1) and Kenyon (S2). After optimization of the amplification conditions, the PCR primers BCD 183F and BCD 183R amplified the DNA from both resistant and susceptible cultivars. One monomorphic 1.2 kb fragment was amplified in all cultivars however, a faint 2 kb fragment was also amplified in the susceptible cultivars (Fig. 6.1). Since the 1.2 kb fragment is located close to the gene controlling resistance to necrosis (Faris, personal communication), further studies concentrated on this fragment only.

To observe polymorphisms, the 1.2 kb fragment was digested with different

restriction enzymes, but while most digested the 1.2 kb band, none produced polymorphisms between the resistant and susceptible cultivars. Digestion of the 1.2 kb fragment with enzyme Bam HI produced one band of 1.2 kb and two other bands of 800 bp and 400 bp length. This indicated that the 1.2 kb fragment observed after DNA amplification was composed of more than one fragment.

Under the assumption that the 1.2 kb length fragment was from the region adjoining the gene controlling resistance to necrosis, this fragment was cloned. Prior to sequencing, plasmids inserted with the 1.2 kb fragment, were amplified with primers T_7 and M_{13} and digested with restriction enzyme Bam HI to confirm that the correct size fragment was being sequenced.

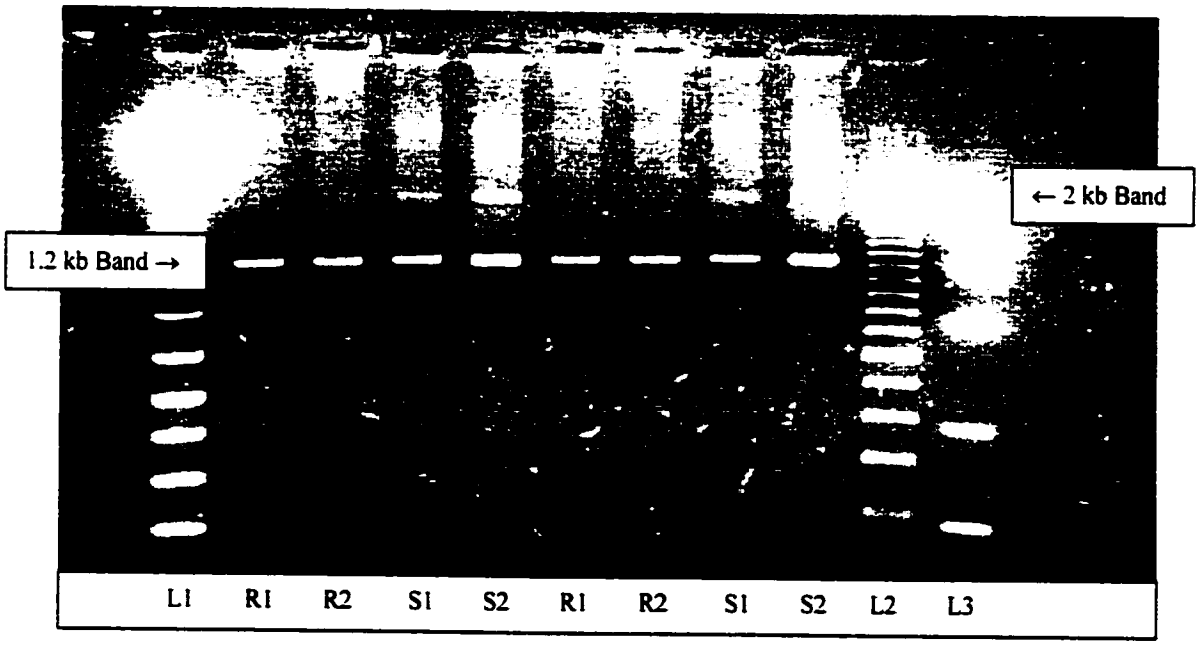


Fig. 6.1. Agarose gel electrophoresis of polymerase chain reaction products from resistant cultivars, Erik (R1) and Red Chief (R2), and susceptible cultivars, Glenlea (S1) and Kenyon (S2), using PCR primer XBCD 183. A 1.2 kb monomorphic band was observed in both resistant and susceptible cultivars while a 2 kb polymorphic band was observed only in susceptible cultivars.

Unexpectedly, amplification followed by restriction digestion of plasmids containing the 1.2 kb fragment insert from the resistant cultivars produced three types of 1.2 kb fragments, fragment F3 which remained undigested, fragment F2 which upon digestion produced bands of 800 bp and 400 bp in length, and fragment F1 which produced bands of 700 bp and 500 bp in length (Fig. 6.2). PCR of the plasmids with the 1.2 kb fragment insert from the susceptible cultivars amplified two types of fragments, the undigested fragment F3 of 1.2 kb length and the fragment F2 that upon digestion produced 800 bp and 400 bp bands (Fig. 6.2).

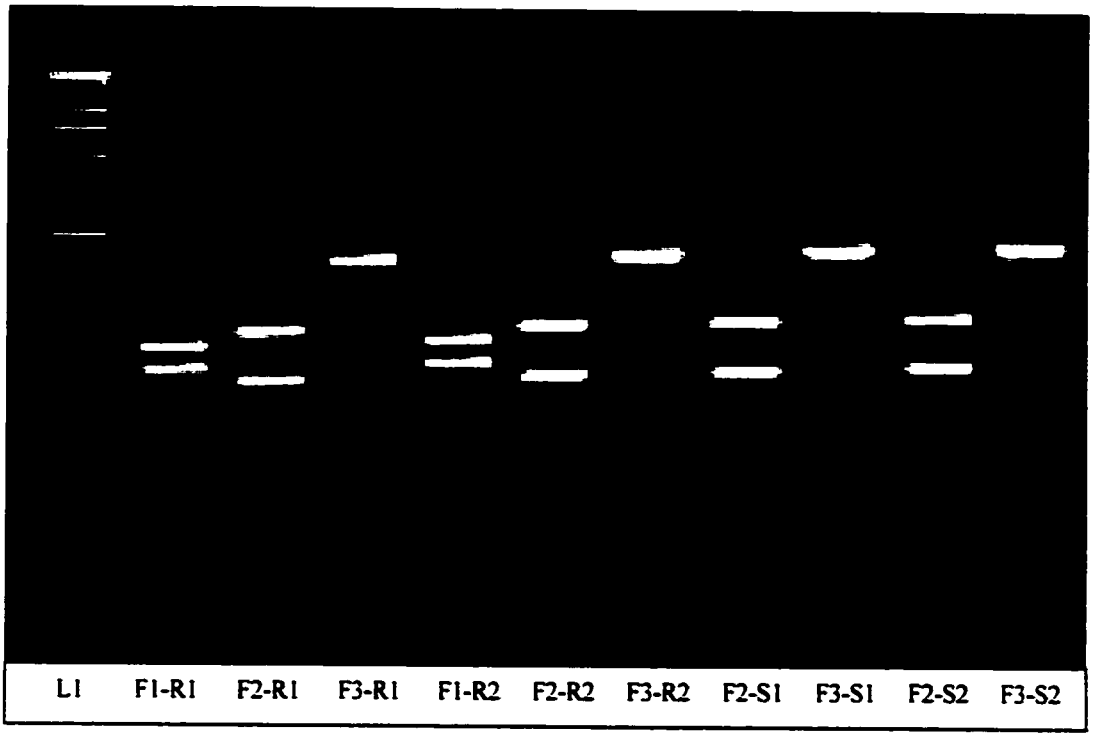
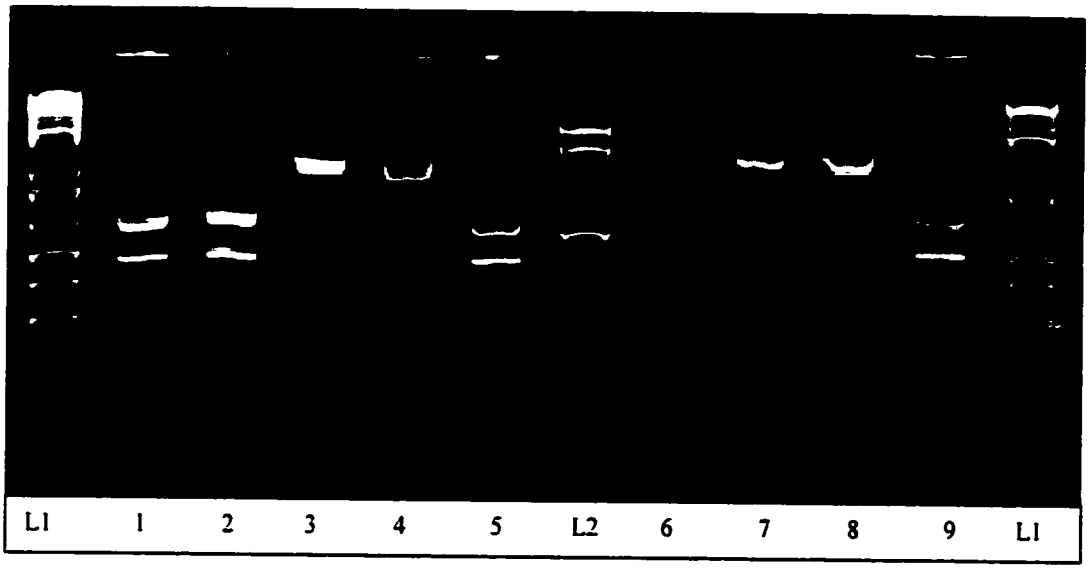


Fig. 6.2. Agarose gel electrophoresis of polymerase chain reaction products digested by restriction enzyme Bam HI from plasmids containing the different 1.2 kb inserted fragments. Three (F1-R1, F2-R1, F3-R1 and F1-R2, F2-R2, F3-R2) and two (F2-S1, F3-S1 and F2-S2, F3-S2) types of fragments were obtained from resistant and susceptible sources, respectively.

The F1 fragment was never observed after PCR amplification and subsequent digestion of genomic DNA of resistant wheat cultivars. The frequency of the bacterial colonies containing the recombinant plasmid with the F1 fragment insert was lower than those containing recombinant plasmids with the F2 or F3 fragment inserts, suggesting a low rate of amplification of the F1 fragment during PCR and therefore was not detected through gel electrophoresis. The amplification of three fragments (F1, F2 and F3) indicates that the PCR primers, BCD 183F and BCD 183R, possibly amplify the three copies of chromosome 5. Dr. Faris obtained similar results.

The association between the fragments, amplified by the PCR primers BCD 183F and BCD 183R, with chromosomes 5A, 5B and 5D was done by amplification of DNA from hexaploid wheat, durum wheat, *T. tauschii*, and the chromosome 5 aneuploid lines and subsequent digestion of the amplified fragments with the Bam HI enzyme. It was observed that the PCR primers Xbcd 183 amplified a 1.2 kb long fragment (presumably fragment F3), that was uncut by the Bam HI enzyme, from the 5D chromosome present in *T. tauschii*, hexaploid wheat (Glenlea, Erik, Kenyon, Red Chief and 86ISWN 2137), and aneuploid lines N5B/T5A, N5A/T5D and N5A/T5B (Fig. 6.3). Amplification from the 5A chromosome present in the durum wheat lines (DT 926, DT 925, DT 691, DT 522 and TM-23), hexaploid wheat (Erik, Red Chief, Kenyon, Glenlea and 86ISWN2137) and aneuploid lines N5B/T5A and N5D/T5B produced a 1.2 kb long fragment (presumably fragment F2) that was digested into 800 bp and 400 bp bands by Bam HI (Fig. 6.3).

Identification of the fragment amplified from the 5B chromosome was inconclusive. The banding pattern observed for N5A/T5D and N5A/T5B indicated that the fragment amplified from 5B chromosome was uncut. However, the 1.2 kb fragment



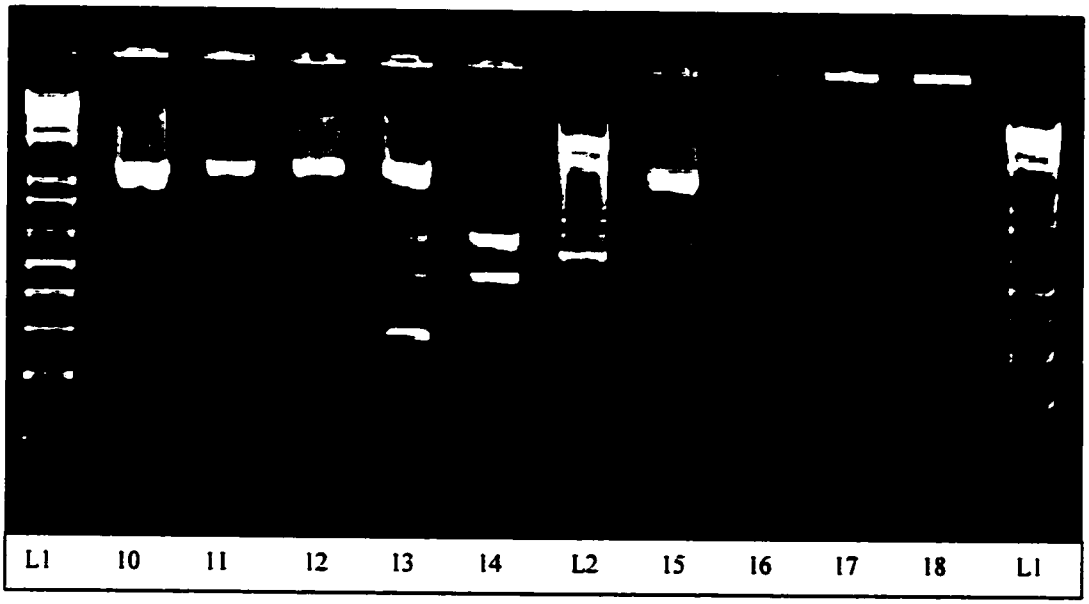


Fig. 6.3. Banding patterns observed after amplification by PCR primers Xbcd 183 and subsequent digestion with restriction enzyme Bam HI of genomic DNA of different genotypes studied to determine the association between the amplified fragments and chromosomes 5A, 5B, and 5D. The lanes and the banding patterns of the genotypes are as follows:

1= DT-926 (AABB), 2= TM-23 (AABB), 3= *T. tauschii* (DD), 4= Katepwa (AABBDD), 5= DT-925 (AABB), 6= N5B/T5D (AADD), 7= Glenlea (AABBDD), 8= N5B/T5A (AADD), 9= DT-691 (AABB), 10= N5A/T5D (BBDD), 11= N5A/T5B (BBDD), 12= Kenyon (AABBDD), 13= 2137 (AABBDD) 14=DT-522 (AABB), 15=Red Chief (AABDD), 16= Erik (AABBDD), 17= N5D/T5B (BBAA) 18=N5D/T5A (AABB) L1=1kb ladder L2= 100 bp ladder

Note: DNA amplification and digestion was not successful for entries in lanes 6, 16, and 18.

amplified from N5D/T5B upon digestion produced two bands of 800 bp and 400 bp in length. A faint 1.2 kb band was observed after digestion of fragments amplified from the durum lines and this could indicate that the fragment amplified from 5B chromosome was uncut (Fig. 6.3). None of the genotypes produced the F1 fragment that digested into bands of 700 bp and 500 bp in length.

The sequence data (Appendix VI) showed that there was a high degree of similarity between the three fragments (F1, F2 and F3) and between the fragments from the resistant and susceptible cultivars. Although attempted twice, sequencing of the F1 fragment of Red Chief (R2) was not accurate and therefore was not included in the multiple sequence alignment. Although the fragments F1 and F2 when digested with restriction enzyme Bam HI produced fragments of 700 bp and 500 bp and 800 bp and 400 bp, respectively, the sequencing data failed to show corresponding differences. The Bam HI cutting site was at exactly the same location on fragment F1 and fragment F2 (Appendix VI Page 141). Hence the occurrence of different banding patterns produced from the fragments F1 and F2 remains unexplained. Perhaps a sequencing error occurred.

Fragments F1 and F2 had exactly the same DNA sequence except for one or two base pair differences. However, the DNA sequence of fragment F3 showed greater differences when compared to the F1 and F2 fragments. The sequence data and the fragment association study indicated that the 1.2 kb uncut fragment F3 was amplified from the 5D chromosome and the 1.2 kb fragment F2 that was digested into 800 bp and 400 bp length bands was amplified from the 5A chromosome. The fragment amplified from the 5B chromosome cannot be determined conclusively.

The sequence data also revealed two sequences, which could possibly be exploited to develop tan spot markers. One is based on a single base pair difference (Appendix VI Page 141), the other on a 3-base pair difference (Appendix VI Page 140). These polymorphic sites are present in Erik and Kenyon but are absent in Red Chief and Glenlea and therefore, the markers developed may be cross-specific. The sequencing data further revealed that sufficient differences were present to develop markers that could map to chromosomes 5A and 5D of wheat. However, the lack of major differences among the cloned fragments and the inability to determine the sequence of the fragment amplified from the 5B chromosome where the gene for resistance to necrosis is located resulted in discontinuation of this study.

6.4 Discussion

To identify RAPD markers linked to the gene for resistance to the necrosis component of tan spot, three crosses, 86ISWN 2137 x Glenlea, 86ISWN 2137 x Kenyon and Red Chief x Kenyon, were studied. Altogether 580 primers were screened without success. In the first attempt the susceptible and resistant bulks were made from lines of two crosses, 86ISWN 2137 x Glenlea and 86ISWN 2137 x Kenyon. However, after screening 440 primers no success was observed. High polymorphism was observed among bulks, but was not repeatable. This could be due to incorrect composition of the bulks as lines from two crosses were involved in their formation. The parents Glenlea, Kenyon and 86ISWN 2137 involved in development of these crosses are spring wheat cultivars and may not be genetically diverse, which could have also contributed to the failure.

In the second attempt, the cross Red Chief x Kenyon was selected on the basis of the diverse genetic background of the parents. Two hundred and forty primers were screened without success. The level of polymorphism produced in the bulks in this cross was less than that observed in the first attempt. The low level of polymorphism could be due to the homeologous nature of wheat's three genomes and the poor resolving power of agarose gel electrophoresis. Stock (1996) observed that different fragments co-migrated as a single band on agarose gels resulting in failure to detect polymorphism.

The two markers, UBC 102 and UBC 195, identified by Stock (1996) to be linked to the gene controlling resistance to necrosis failed to produce polymorphism in this population. Stock (1996) obtained similar findings when he screened those primers on other populations, confirming that the markers were cross specific. He also observed that the markers were polymorphic when temperature sweep gel electrophoresis was used, but the polymorphism was absent with agarose gel electrophoresis. Since agarose gel electrophoresis was used in this study, this could have been another reason for failure to obtain polymorphisms.

Regardless of the detection of putative polymorphisms between the resistant and susceptible bulks, repeated amplification using the same PCR reaction mixture, primer and amplification conditions failed to produce consistent polymorphism between the bulks or among the lines constituting the bulks. Generally, strong bands tend to be reproducible while fainter bands tend to be inconsistent. However, in this study, both strong and faint bands were inconsistent in their reproducibility. Other researchers (Devos and Gale 1992; Stock 1996; Krasichynska 1997) have observed similar results demonstrating the low repeatability of RAPD.

Success in identifying RAPD markers linked to a trait of interest not only depends on the number of primers screened, but also on the genetic divergence among the parents used to produce the segregating population, the type of population studied and the type of molecular technique used (Devos and Gale 1992; Krasichynska 1997). Accurate phenotyping of the lines constituting the resistant and susceptible bulks is also very important. In this study, 16 plants of each line used in the bulks were progeny tested and the likelihood of error in phenotyping is considered low. PCR primers Xbcd 183 amplified the region adjoining the gene controlling resistance to the necrosis (Faris, personal communication). Sequence data from this region indicated that the region was highly conserved not only across resistant and susceptible sources but also across the three genomes of wheat. This could possibly be the reason for failure to identify RAPD markers linked to tan spot resistance.

The PCR primers BCD 183F and BCD 183R amplified two fragments from the genomic DNA of wheat cultivars, a 2 kb polymorphic fragment and a 1.2 kb monomorphic fragment. Since the 1.2 kb fragment was in the vicinity of the gene controlling necrosis, efforts were concentrated on sequencing this fragment with the objective to develop SCAR markers. However, due to lack of significant variation in the sequence of the fragments amplified from the resistant and susceptible cultivars, this objective was not achieved. No attempt was made to study the faint 2 kb polymorphic fragment as its location was unknown and it was difficult to obtain consistent polymorphisms for this faint fragment. However, future studies may work on this 2 kb polymorphic fragment to observe if this fragment is linked to tan spot resistance. The

differences observed in the sequence of the fragments amplified by PCR primers Xbcd 183 could be utilized in developing markers that could map to chromosomes 5A and 5D.

Even though RAPD analysis is user friendly and inexpensive, it does not appear to be the best choice when working with a polyploid species like wheat. The large genome of wheat with its homeologous chromosomes further adds to the limitations of RAPD analysis (Joshi and Nguyen 1993). A low level of polymorphism has been reported when using RAPD analysis on wheat and the repeatability of this polymorphism is variable across laboratories and across different crosses (Gale and Devos 1992; Joshi and Nguyen 1993). Gale and Devos (1992) further indicated that the sensitivity of the random amplification system does not make the use of RAPD markers for the construction of linkage maps in wheat worthwhile. More powerful techniques such as AFLP and microsatellites should be considered when attempting to identify markers linked to traits of interest in wheat.

7. GENERAL DISCUSSION

The tan spot disease syndrome consists of two phenotypically distinct symptoms. tan necrosis and extensive chlorosis. Prior to the development of the necrosis-chlorosis model of wheat-*P. tritici-repentis* pathosystem and the identification of races of *P. tritici-repentis*. most studies reported resistance to be quantitative (Nagle et al. 1982: Shabeer 1986: Elias et al. 1989). However. since the advent of the necrosis-chlorosis model. genetic control of resistance has generally been found to be qualitative (Lamari and Bernier 1989b: Lamari and Bernier 1991: Duguid 1995: Gamba et al. 1998).

Resistance to tan spot of wheat was observed to be qualitative throughout this study. All F_1 plant tested with isolates Ptr 200 (race 1) and Ptr 92-164 (race 2) were susceptible to necrosis and the segregation pattern observed in the F_2 generation and $F_{2:3}$ family ratio indicated that a single recessive gene controlled resistance to necrosis in both spring and winter wheat cultivars. The findings of this study are similar to those of other researchers (Lamari and Bernier 1989b. 1991: Duguid 1995: Gamba et al. 1998: Anderson et al. 1999).

All F_1 plants tested with isolates Ptr 200 (race 1) and Ptr 94-8-2 (race 3) were resistant to chlorosis and the F_2 generation and $F_{2:3}$ family ratio segregated in a pattern indicative of a single dominant gene controlling resistance to chlorosis. Monogenic control of resistance to chlorosis induced by races 1 and 3 has been previously reported (Lamari and Bernier 1991; Duguid 1995; Gamba et al. 1998).

In studies of resistance to the chlorosis component. Gamba et al. (1998) reported resistance to be recessive while Duguid (1995) reported resistance to be dominant. Lamari and Bernier (1991) observed resistance to the chlorosis component caused by race 3 to be dominant or partially dominant depending upon the cross studied. Several factors may have contributed to the difference in the findings of this study with that of Lamari and Bernier (1991) and Gamba et al. (1998). Chlorosis in this study was induced by isolates Ptr 94-8-2 (race 3) and Ptr 200 (race 1) which are different, and may differ in virulence, to the isolates used by Gamba et al. (1998) and Lamari and Bernier (1991). The chlorosis component is highly influenced by environmental conditions and a range of yellow discoloration may occur. For this reason, rating plants for resistance to chlorosis was based on the expression of chlorosis on the checks instead of 1-5 lesion type rating scale used by Lamari and Bernier (1991) and Gamba et al. (1998).

Two independent genes controlled resistance to the necrosis and chlorosis induced by race 1. A recessive gene controlled resistance to the necrosis component, while a dominant gene controlled resistance to the chlorosis component. Hence in order to achieve complete resistance to tan spot all available resistance gene(s) should be incorporated into wheat cultivars.

Allelism studies of the genes identified in resistant cultivars for resistance to the necrosis component revealed that they possessed the same resistance gene. Some F_2 plants showed a moderately susceptible reaction to necrosis, but none of the $F_{2:3}$ families tested were susceptible. The occurrence of these few moderately susceptible F_2 plants could be due to misclassification or receiving a high inoculum load when tested. Both would result in a resistant plant being rated as moderately susceptible and are considered

as errors in disease testing rather than to genetic reasons. In future studies large F_2 populations should be tested and any moderately susceptible plants progeny tested to confirm if their occurrence is due to errors associated with disease testing or to genetic reasons. Lack of segregation for resistance to chlorosis in the F_2 generation and $F_{2:3}$ families of the crosses among the resistant cultivars indicate that the resistant cultivars share the gene controlling resistance to the chlorosis component.

To date, three independent resistance genes have been identified in hexaploid wheat, one for resistance to necrosis caused by races 1 and 2 and two for resistance to chlorosis induced by races 1 and 3, and race 5 (Lamari and Bernier 1991, Duguid 1995 and Gamba et al. 1998). Four independently inherited resistance genes in durum have been reported, one for resistance to chlorosis caused by race 1 and three for resistance to necrosis caused by races 1 and 2, race 3 and race 5 (Gamba and Lamari 1998). A preliminary study by Anderson et al. (1999) indicated that the same gene in both durum and hexaploid wheat controlled insensitivity to necrosis caused by the toxin ToxA derived from a race 2 isolate. However no information is available for the relationship between the various genes controlling resistance in durum and hexaploid wheat.

The narrow genetic base for resistance to tan spot, especially for resistance to necrosis induced by races 1 and 2 in both durum and hexaploid wheat emphasizes the need to identify novel resistance genes to tan spot. Genetic studies have failed to identify new resistance gene(s) for the existing races of tan spot in tetraploid and hexaploid wheat, but several studies have identified new sources of resistance in wild relatives and synthetic wheat (Cox et al. 1992; Riede et al. 1996; Zhang and Jin 1998). However,

interspecific crosses between the resistant wild relatives and synthetic wheat and wheat should be studied to determine if these sources possess novel resistance genes.

Summerell and Burgess (1988) indicated that colonization and growth of *P. tritici-repentis* on senesced tissue is independent of the reaction of the living host to infection. Hence, incorporation of resistance into presently grown wheat cultivars is unlikely to bring selection pressure on the pathogen for increased virulence, but would reduce yield losses due to tan spot. Lamari et al. (1995a) first identified the chlorosis-inducing race 5 in Algeria, which was subsequently identified in North America (Lamari et al. 1998). In North America emphasis has been placed on breeding for resistance to the necrosis component and to date, no new necrosis inducing races have been identified, nor has the gene controlling resistance to necrosis shown any signs of breaking down. Hence although efforts should be made to broaden the genetic base of resistance to tan spot the narrow genetic base of resistance does not appear to be a cause for concern at present.

Durum and hexaploid wheat gave similar reactions to races 1 and 2 of *P. tritici-repentis*, but races 3 and 5 induce different disease symptoms (Gamba et al. 1998; Gamba and Lamari 1998). On durum wheat race 3 and race 5 induce necrosis, whereas on hexaploid wheat they cause chlorosis. The differential response requires further study to determine the genetic control involved. If new and different genes are identified, cultivars containing multiple genes for the different components of tan spot could be developed.

It was observed in this study that resistance/insensitivity to spore inoculation/ culture filtrate infiltration using necrosis inducing isolate Ptr 92-164 (race 2) was

controlled by the same recessive gene. The majority of F_2 and BC_1F_1 plants, and $F_{7:8}$ lines tested gave a similar reaction to spore inoculation and to infiltration with the culture filtrate. However, a few plants showed a differential response to spore inoculation and the culture filtrate. Due to lack of space and time progeny testing of these plants was not done. However, in future studies progeny test those plants showing the differential reaction should be done to establish whether the cause of their differential response is genetic or environmental.

The results from this study indicated that infiltration with the culture filtrate of race 2 isolates could replace spore inoculation when testing for resistance to the necrosis component of tan spot of wheat. Others have reported similar findings for necrosis caused by races 1 and 2 (Duguid 1995; Gamba et al. 1998). Replacement of spore inoculation with culture filtrate infiltration will speed and facilitate screening for resistance to tan spot of wheat.

Disease testing using culture filtrate infiltration is a cost-effective and less time consuming procedure, gives a clear disease reaction and can easily be combined with any other disease screening procedure. These reasons provide a strong case for culture filtrate infiltration to replace spore-inoculation. However, Riede et al. (1996) and Zhang and Jin (1998) observed differential responses by some genotypes to culture filtrate infiltration and spore-inoculation.

In this study it was observed that if infiltration with culture filtrate and spore inoculation gave similar reactions on the parents used in generating a segregating population, then the culture filtrate could be used instead of spore inoculation when screening that population. This information is important for plant breeders planning

screening tests. Culture filtrates of races 1 and 2, and of race 5 can be used to screen for resistance to necrosis (Duguid 1995; Gamba et al. 1998) and chlorosis (Orolaza et al. 1995; Gamba et al. 1998), respectively, in hexaploid wheat. However, the chlorosis symptom induced by race 1 and race 3 cannot be induced by their culture filtrates. Hence, it is important to know for which component of which race the screening is being done before a decision is made to replace spore inoculation with culture filtrate infiltration.

The culture filtrate of the necrosis inducing race 2 induced a similar disease reaction as the pathogen and resistance/insensitivity to necrosis induced by spore inoculation/culture filtrate infiltration was controlled by the same recessive gene. These results suggest strongly that toxin(s) in the wheat-*P. tritici-repentis* pathosystem act as the primary pathogenicity factor and agree with the hypothesis that the wheat-*P. tritici-repentis* pathosystem follows the toxin model proposed by Ellingboe (1981). In this toxin model, compatibility between the host and pathogen leads to susceptibility and toxins are involved in pathogenicity. Other studies have also indicated that the wheat-*P. tritici-repentis* pathosystem follows the toxin model (Lamari and Bernier 1991; Orolaza et al. 1995; Ciuffetti et al. 1997; Anderson et al. 1999).

It is hypothesized that race 1 isolates have three virulence genes, one gene induces necrosis in both durum and hexaploid wheat, and two genes induce chlorosis, one in hexaploid wheat and the other in durum wheat. Race 2 possesses one virulence gene which causes necrosis in both durum and hexaploid wheat. The virulence gene present in race 2 is the same as the necrosis inducing virulence gene of race 1. Race 3 possesses two virulence genes, one of which is the same as the chlorosis inducing virulence gene

possessed by race 1 and induces chlorosis in hexaploid wheat. The other virulence gene induces necrosis in durum wheat only (Lamari and Bernier 1991; Otonda 1995; Gamba and Lamari 1998). Race 4 is avirulent and thus possesses no virulence genes. Race 5 possesses two virulence genes, one induces chlorosis in hexaploid wheat and the other induces necrosis in durum wheat. The virulence genes possessed by race 5 have not been observed in any other races (Gamba and Lamari 1998). Since *P. tritici-repentis* can reproduce sexually, recombination between races possessing different virulence genes may result in the occurrence of new and more virulent races.

Recent disease surveys of durum wheat in western Canada resulted in the identification of race 5 (Lamari et al. 1998), which previously had been reported only in Algeria (Lamari et al. 1995a). Since native prairie grasses act as overwintering hosts and thus as a primary source of fungal inoculum, they could also play an important role as a source of new virulence for the fungus (Ali and Lamari 1997; Krupinsky 1987; De Wolf et al. 1998). Further studies on the pathogenicity, virulence and genetic structure of *P. tritici-repentis* should include isolates from different host plants including wild grasses. This will give the true population structure of *P. tritici-repentis* and would also indicate if the host plants put a selection pressure on the fungus.

Genetic similarity studies indicated that there was a high degree of genetic variability among the isolates. However, cluster analysis indicated that genetic similarity of isolates was independent of race structure or geographic origin of isolates. The high genetic variability indicates that sexual recombination is common for the tan spot fungus. Disease surveys in western Canada in the last decade have indicated that races 1 and 2 represented more than 90% of the fungal population and races 3 and 4, and more recently

race 5. accounted for the remaining 10% (Lamari et al. 1998). Race 1 still does not possess all the virulence genes available in the fungus so the potential exists for sexual combination of different races to produce new and more virulent races in the future. Constant monitoring of pathogen population through pathogenicity and virulence studies is important for identification of new races. which in turn would lead to identification of new resistance gene(s). Breeding programs to develop resistant cultivars can then use these new resistance genes.

In this study no RAPD marker linked to tan spot resistance was identified. This need not be of great concern when breeding for resistance to tan spot. The advantage of marker-assisted selection over conventional screening for tan spot resistance is limited. as conventional disease screening for tan spot is easy. effective and accurate. particularly for the necrosis component on which most wheat breeding programs place priority. The selection for insensitivity to toxin(s). which is highly correlated with resistance to spore inoculation. further reduces the advantage of marker assisted selection for tan spot. The large and polyploid nature of the wheat genome makes identification. development and use of molecular markers for marker-assisted selection difficult. Marker-assisted selection can be utilized to pyramid resistance genes. However. the limited number of available gene(s) for tan spot resistance has further reduced the potential of marker-assisted selection. However. as new resistance gene(s) are identified. use of molecular markers to assist pyramiding gene(s) can be desirable. Powerful molecular techniques like microsatellites and amplified fragment length polymorphism (AFLP) in future studies should be utilized to identify molecular markers linked to tan spot resistance.

8. CONCLUSIONS

1. Resistance to tan spot in the spring and winter wheat cultivars tested with races 1, 2, and 3 of *P. tritici-repentis* was qualitatively inherited. The same recessive gene controlled resistance to the necrosis component of tan spot caused by race 1 and race 2. Resistance to the chlorosis component caused by race 1 and race 3 was controlled by the same dominant gene. Resistance to the necrosis and the chlorosis components of tan spot are controlled by two independent genetic systems.

2. Allelism studies for both the necrosis and the chlorosis components of tan spot failed to identify new resistance genes.

3. The same recessive gene controlled resistance/insensitivity to necrosis caused by spore inoculation/culture filtrate infiltration of *P. tritici-repentis*, race 2. Infiltration with the culture filtrate of race 2 isolates can replace spore inoculation when testing for resistance to the necrosis component of tan spot of wheat.

4. High genetic variability was observed among isolates of *P. tritici-repentis*. AMOVA and cluster analysis revealed that the variability observed among *P. tritici-repentis* isolates was independent of both the race structure and the geographic origin of the isolates.

5. No RAPD marker linked to the resistance gene controlling the necrosis component of tan spot was identified.

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Appendix I

Appendix I. The list of different crosses studied for genetics of resistance to tan spot of wheat with races 1. 2 and 3 of *P. tritici-repentis*.

S.N.	Cross	Race 1	Race 2	Race 3
1	86ISWN 2137 x Kenyon	Not Studied	Studied	Not Studied
2	Erik x Kenyon	Not Studied	Studied	Not Studied
3	Red Chief x Kenyon	Not Studied	Studied	Not Studied
4	Hadden x Kenyon	Not Studied	Studied	Not Studied
5	86ISWN 2137 x Glenlea	Not Studied	Studied	Studied
6	Erik x Glenlea	Studied	Studied	Studied
7	Red Chief x Glenlea	Not Studied	Studied	Studied
8	Hadden x Glenlea	Studied	Studied	Studied
9	Kenyon x Glenlea	Not Studied	Studied	Not Studied
10	Erik x 86ISWN 2137	Not Studied	Studied	Studied
11	Erik x Hadden	Not Studied	Studied	Studied
12	Erik x Red Chief	Not Studied	Studied	Studied
13	86ISWN 2137 x Hadden	Not Studied	Studied	Studied
14	86ISWN 2137 x Red Chief	Not Studied	Studied	Studied
15	Hadden x Red Chief	Not Studied	Studied	Studied
16	86ISWN 2137 x 6B-365	Studied	Not Studied	Studied
17	Erik x 6B-365	Studied	Not Studied	Studied
18	Red Chief x 6B-365	Not Studied	Not Studied	Studied
19	Hadden x 6B-365	Not Studied	Not Studied	Studied
20	Glenlea x 6B-365	Studied	Not Studied	Studied

Appendix II

Appendix IIa. Composition of modified Fries medium for culture filtrate production

Ingredients	Amount
NH_4 Tartarate	5 g
NH_4NO_3	1 g
$MgSO_4.7H_20$	0.5 g
KH_2PO_4	0.13 g
K_2HPO_4	0.26 g
Sucrose	30 g
Yeast Extract	1 g
Trace Element Solution	2 ml
Water added to make the solution to one litre	

Appendix IIb. Composition of trace element solution for production of modified Fries medium

Ingredients	Amount
LiCl	167 mg
$CuSO_4.5H_2O$	174.4 mg
$Na_2MoO_4.2H_2O$	50.79 mg
$CoCl_2.4H_2O$	80 mg
$MnSO_4.H_2O$	61.49 mg
Water added to make the solution to one litre	

Appendix III

Buffers and solutions used in molecular studies

Solution/Buffer	Composition	Amount
Gel loading buffer (6X)	20% SDS	37.5 µl
	2% Bromophenol Blue	37.5 µl
	2% Xylene Cyanol	37.5 µl
	0.5 EDTA	150.0 µl
	30% Ficoll 400	625.0 µl
	dd H_2O	113.0 µl
Tris-Acetate buffer (50X)	Tris base	242.0 g
	Glacial acetic acid	57.1 ml
	0.5 EDTA	100.0 ml
2X CTAB buffer	2% CTAB (w/v)	20 g
	1% PVP (w/v)	10 g
	100 mM Tris	100 ml of 1 M Tris
	20 mM EDTA	40 ml of 0.5 M stock
	2.8 M NaCl	163.6 g
10% CTAB solution	10% CTAB (w/v)	100 g
	1.4 M NaCl	81.8 g
	dd. sterile water	818.2 g

Appendix IV

Different isolates of *P. tritici-repentis* and the outliers used in the genetic similarity study

S.N	Isolate/Species	Race
1	Ptr 200	1
2	Ptr 94-17	1
3	CIMMYT III 95 P-82B	1
4	ASCI	1
5	Hudson Bay Field 5	1
6	Tisdale F-7	1
7	Ptr 92-164	2
8	SAC D. Reg 95 P-5a	2
9	SAC W. SC 95 P-28B	2
10	CIMMYT SC 95 P-75A	2
11	Outlook Field 2	2
12	Outlook Field 12	2
13	Outlook Field 10	2
14	Tisdale Field 8	2
15	Davidson Field 6	2
16	Davidson Field 8	2
17	Ptr D308	3
18	*H. sativum*	-
19	*P. teres* f. sp. *teres*	-
20	Davidson Field 9	1
21	Battleford Field 1	1
22	Battleford Field 8	1
23	Canora Field 4	1
24	Canora Field 10	1
25	Kelvington Field 1	1
26	Humbold Field 8	2
27	Humbold Field 9	2
28	Battleford Field 2	2
29	Watrous Field 4	2
30	Watrous Field 5	2
31	Saskatoon Field 4	2
32	Saskatoon Field 5	2
33	Canora Field 9	Unknown
34	Meadow lake Field 5	2
35	Meadow lake Field 6	2
36	Ptr 90-2	4
37	*P. graminea*	-
38	*P. teres* f. sp. *maculata*	-

Appendix IV

Similarity matrix generated for 38 isolates using the coefficient of Jaccard

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
1	·																																					
2	66	·																																				
3	60	80	·																																			
4	72	86	88	·																																		
5	73	83	83	91	·																																	
6	77	67	65	68	70	·																																
7	74	64	64	67	69	78	·																															
8	70	67	69	71	72	79	77	·																														
9	68	77	84	85	80	69	67	72	·																													
10	72	82	81	85	85	73	70	78	80	·																												
11	70	80	86	87	83	66	68	73	83	81	·																											
12	65	79	85	87	82	64	63	68	91	80	82	·																										
13	75	71	73	74	72	69	74	73	70	77	77	69	·																									
14	68	79	85	87	82	65	65	69	90	80	85	89	69	·																								
15	71	76	80	82	81	66	68	71	79	82	78	78	74	80	·																							
16	70	78	79	85	85	69	69	71	84	81	80	82	70	79	79	·																						
17	51	44	43	44	44	50	52	50	42	46	43	42	47	40	47	46	·																					
18	21	22	22	22	21	25	22	24	23	23	20	22	21	22	21	24	15	·																				
19	20	22	23	21	22	25	27	24	22	22	24	22	23	24	21	21	21	22	18	·																		
20	66	80	86	85	83	69	64	66	88	77	82	88	69	84	76	82	43	21	21	·																		
21	67	83	90	90	85	66	63	69	90	81	87	91	71	90	78	82	43	22	21	88	·																	
22	65	74	87	80	74	62	60	65	80	73	79	78	69	81	77	74	41	22	22	83	83	·																
23	71	85	80	87	88	70	68	72	79	81	82	79	72	80	77	83	45	20	21	79	82	71	·															
24	65	84	86	87	84	64	64	68	86	78	84	89	70	87	75	82	44	20	21	86	93	79	85	·														
25	59	66	71	70	67	60	62	63	67	66	69	66	67	64	73	71	49	19	19	70	68	70	66	65	·													
26	66	82	88	88	85	68	65	69	87	80	82	89	70	90	78	79	44	22	21	87	91	81	81	90	64	·												
27	66	79	88	86	83	65	65	73	90	84	85	90	70	88	80	85	42	22	22	87	89	79	80	90	68	88	·											
28	75	83	81	86	85	72	72	76	80	90	83	76	79	78	78	83	45	22	20	79	81	74	85	80	68	78	80	·										
29	67	82	85	88	85	65	67	69	85	82	86	85	74	89	79	78	40	20	22	84	86	79	83	86	66	88	86	82	·									
30	69	79	85	86	82	67	68	71	84	86	84	83	74	85	82	81	43	21	22	80	85	78	81	83	69	85	87	84	85	·								
31	67	76	80	83	80	68	65	67	87	78	80	86	69	82	79	82	41	22	21	89	84	80	78	82	70	84	84	79	86	81	·							
32	67	80	84	86	82	67	63	66	90	78	83	91	70	89	77	82	39	21	21	89	89	81	80	88	64	89	87	77	89	82	86	·						
33	23	19	17	18	20	23	27	26	19	20	20	19	21	18	21	20	25	8	15	19	19	18	18	17	23	18	19	21	18	19	18	18	·					
34	68	83	86	89	83	70	68	70	87	85	85	85	72	88	82	83	43	22	20	86	86	81	81	82	70	86	85	85	89	86	86	86	18	·				
35	68	81	84	87	85	71	69	73	84	83	84	82	71	83	83	84	45	22	21	83	84	78	81	82	70	83	83	84	86	82	83	84	20	91	·			
36	63	69	66	69	71	62	64	66	68	74	69	67	66	68	70	68	44	20	21	64	67	65	71	68	61	70	67	71	69	72	69	68	18	71	71	·		
37	13	17	16	14	14	15	17	17	15	15	16	15	17	15	15	14	19	13	24	17	16	18	15	16	17	14	16	16	17	15	15	16	17	15	15	15	·	
38	23	23	23	21	21	28	27	24	22	22	22	21	23	21	20	24	16	25	41	22	21	24	23	21	22	20	22	24	21	22	22	21	7	23	23	21	26	·

Note: The name and race classification of 1-38 isolates given in Appendix III. For simplicity figures have been converted to percentage.

Appendix VI

Multiple sequence alignment of DNA sequence of fragments F1. F2. and F3 from cultivars Erik(R1). Red Chief(R2). Glenlea(S1). and Kenyon(S2)

ORIGIN

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F1-R1 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F2-R1 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F2-R2 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F2-S1 GA.....CCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   55
F2-S2 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F3-R1 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F3-R2 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F3-S1 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60
F3-S2 GATATAACCAGTGTTACTTTCCACCGGAGATACCCTCTTTTTGCCTCATCGTCGGAGGAT   60

F1-R1 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F2-R1 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F2-R2 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F2-S1 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   115
F2-S2 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F3-R1 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F3-R2 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F3-S1 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120
F3-S2 TGTACAGCCCATGTGTTCCATGGGATGGTTTATGCTGATCTCAACCAGAACCCACTTATT   120

F1-R1 GTGCCATTGGAGATTCTTCGTGGCCATTTGAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F2-R1 GTGCCATTGGAGATTCTTCGTGGCCATTTGAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F2-R2 GTGCCATTGGAGATTCTTCGTGGCCATTTGAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F2-S1 GTGCCATTGGAGATTCTTCGTGGCCATTTGAGTTCAGATAGAAGAGGTATATTGTCATAC   175
F2-S2 GTGCCATTGGAGATTCTTCGTGGCCATTTGAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F3-R1 GTGCCATTGGAGATTCTTCGTGGCCATTTaAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F3-R2 GTGCCATTGGAGATTCTTCGTGGCCATTTaAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F3-S1 GTGCCATTGGAGATTCTTCGTGGCCATTTaAGTTCAGATAGAAGAGGTATATTGTCATAC   180
F3-S2 GTGCCATTGGAGATTCTTCGTGGCCATTTaAGTTCAGATAGAAGAGGTATATTGTCATAC   180

F1-R1 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F2-R1 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F2-R2 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F2-S1 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   235
F2-S2 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F3-R1 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F3-R2 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F3-S1 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
F3-S2 ATCATGTGGTTTCTATAATTTGCATTATTTTACATTTTTTGTTGATTCTTATTTGCGTAG   240
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F1-R1 ACATTTCGATCAAAACAGATATATGGCAGTGGTTAAACTCTGTTTACATCGTTATGTTTT  300
F2-R1 ACATTTCGATCAAAACAGATATATGGCAGTGGTTAAACTCTGTTTACATCGTTATGTTTT  300
F2-R2 ACATTTCGATCAAAACAGATATATGtCAGTGGTTAAACTCTGTTTACATCGTTATGTTTT  300
F2-S1 ACATTTCGATCAAAACAGATATATGtCAGTGGTTAAACTCTGTTTACATCGTTATGTTTT  300
F2-S2 ACATTTCGATCAAAACAGATATATGGCAGTGGTTAAACTCTGTTTACATCGTTATGTTTT  295
F3-R1 ACATTTCGATCAAAgCAGATATATGtCAGTGGTcAAACTCTGTTTACATCGTTATGTTTT  300
F3-R2 ACATTTCGATCAAAgCAGATATATGtCAGTGGTcAAACTCTGTTTACATtGTgATGTTcT  300
F3-S1 ACATTTCGATCAAAgCAGATATATGtCAGTGGTcAAACTCTGTTTACATtGTgATGTTcT  300
F3-S2 ACATTTCGATCAAAgCAGATATATGtCAGTGGTcAAACTCTGTTTACATtGTgATGTTcT  300

F1-R1 TCACTGGAAGAATGCAAGTCATATACTTTGCTTTGAACCTTGCATTCTGGAGTCCTCGAA  360
F2-R1 TCACTGGAAGAATGCAAGTCATATACTTTGCTTTGAACCTTGCATTCTGGAGTCCTCGAA  360
F2-R2 TCACTGGAAGAATGCAAGTCATATACTTTGCTTTGAACCTTGCATTCTGGAGTCCTCGAA  360
F2-S1 TCACTGGAAGAATGCAAGTCATATACTTTGCTTTGAACCTTGCATTCTGGAGTCCTCGAA  355
F2-S2 TCACTGGAAGAATGCAAGTCATATACTTTGCTTTGAACCTTGCATTCTGGAGTCCTCGAA  360
F3-R1 TCACgGGAAGAATGCAAGTCATATACTTTGCaTTGAACCTTGCATTCTGcA.........A  352
F3-R2 TCACgGGAAGAATGCAAGTCATATACTTTGCaTTGAACCTTGCATTCTGcA.........A  352
F3-S1 TCACgGGAAGAATGCAAGTCATATACTTTGCaTTGAACCTTGCATTCTGcA.........A  352
F3-S2 TCACgGGAAGAATGCAAGTCATATACTTTGCaTTGAACCTTGCATTCTGcA.........A  352

F1-R1 TCCATATGACGTGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCCATGTTCT  420
F2-R1 TCCATATGACGTGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCCATGTTCT  420
F2-R2 TCCATATGAtGTGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCCATGTTCT  420
F2-S1 TCCATATGAtGTGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCCATGTTCT  415
F2-S2 TCCATATGACGTGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCCATGTTCT  420
F3-R1 TCCATATGAtGcGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCtATGTTCT  412
F3-R2 TCCATATGAtGcGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCtATGTTCT  412
F3-S1 TCCATATGAtGcGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCtATGTTCT  412
F3-S2 TCCATATGAtGcGAGTTTGAACTGAAGCATACATGTGCTTGTGCTGATATGCtATGTTCT  412

F1-R1 CCTATTTGATCACTTCTTACAAATTACAGTAAATTGCTTATAGCTGT.CCTGCTTTGTTG  479
F2-R1 CCTATTTGATCACTTCTTACAAATTACAGTAAATTGCTTATAGCTGTaCCTGCTTTGTTG  480
F2-R2 CCTATTTGATCAC...TTACAAATTACAGTAAATTGCTTATAGCTGTaCCTGCTTTGTTG  477
F2-S1 CCTATTTGATCAC...TTACAAATTACAGTAAATTGCTTATAGCTGTaCCTGCTTTGTTG  472
F2-S2 CCTATTTGATCACTTCTTACAAATTACAGTAAATTGCTTATAGCTGTaCCTGCTTTGTTG  480
F3-R1 CCTATTTGATCAC...TTACAAATTACAGTAAATTGCTTATAGCTtTtCCTGCTTTGTTG  469
F3-R2 CCTATTTGATCAC...TTACAAATTACAGTAAATTGCTTATAGCTtTtCCTGCTTTGTTG  469
F3-S1 CCTATTTGATCAC...TTACAAATTACAGTAAATTGCTTATAGCTtTtCCTGCTTTGTTG  469
F3-S2 CCTATTTGATCAC...TTACAAATTACAGTAAATTGCTTATAGCTtTtCCTGCTTTGTTG  469

F1-R1 NTGCTTGGA..TTTTTTTTGAGAACCAGGGACAGACTAGCGTAATATTGAGCCTGTAATG  537
F2-R1 gTGCTTGGA..TTTTTTTTGAGAACCAGGGACAGACTAGCGTAATATTGAGCCTGTAATG  538
F2-R2 gTGCTTGGA..TTTTTTTTGAGAACCAGGGACAGACTAGCGTAATATTGAGCCTGTAATG  535
F2-S1 gTGCTTGGA..TTTTTTTTGAGAACCAGGGACAGACTAGCGTAATATTGAGCCTGTAATG  530
F2-S2 gTGCTTGGA..TTTTTTTTGAGAACCAGGGACAGACTAGCGTAATATTGAGCCTGTAATG  538
F3-R1 gTGCTTGGAa.TTTTTTTTGAGAACCAGGGACAGgCTAGCGTAATATTGAGCCTGTAATG  528
F3-R2 gTGCTTGGAatTTTTTTTTGAGAACCAGGGACAGgCTAGCGTAATATTGAGCCTGTAATG  529
F3-S1 gTGCTTGGAatTTTTTTTTGAGAACCAGGGACAGgCTAGCGTAATATTGAGCCTGTAATG  529
F3-S2 gTGCTTGGAatTTTTTTTTGAGAACCAGGGACAGgCTAGCGTAATATTGAGCCTGTAATG  529
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F1-R1 TTGTAGCATGTATCTTGTTAAATTGAAATTATTCGCACGGAAGAAACTTAGTTTCAGTGT  597
F2-R1 TTGTAGCATGTATCTTGTTAAATTGAAATTATTCGCACGGAAGAAACTTAGTTTCAGTGT  598
F2-R2 TTGTAGCATGTATCTTGTTAAATTGAAATTATTCGCACGGAAGAAACTTAGTTTCAGTGT  595
F2-S1 TTGTAGCATGTATCTTGTTAAATTGAAATTATTCGCACaGAAGAAACTTAGTTTCAGTGT  590
F2-S2 TTGTAGCATGTATCTTGTTAAATTGAAATTATTCGCACGGAAGAAACTTAGTTTCAGTGT  598
F3-R1 TTGTAGCAcGTtTCTTGcTAAATcGAAATTATTCGtACGGAAGAAACTTAGTTTCAG..T  586
F3-R2 TTGTAGCAcGTtTCTTGcTAAATcGAAATTATTCGtACGGAAGAAACTTAGTTTCAG..T  587
F3-S1 TTGTAGCAcGTtTCTTGcTAAATcGAAATTATTCGtACGGAAGAAACTTAGTTTCAG..T  587
F3-S2 TTGTAGCAcGTtTCTTGcTAAATcGAAATTATTCGtACGGAAGAAACTTAGTTTCAG..T  587

F1-R1 CCATTGAACCAAGCACTCTAGGTATATATGGGCTCGCAAGGATGCATAGGCAAATTCAAG  657
F2-R1 CCATTGAACCAAGCACTCTAGGTATATATGGGCTCGCAAGGATGCATAGGCAAATTCAAG  658
F2-R2 CCATTGAACCAAGCACTCTAGGTATATATGGGCTCGCAAGGATGCATAGGCAAATTCAAG  655
F2-S1 CCATTGAACCAAGCACTCTAGGTATATATGGGCTCGCAAGGATGCATAGGCAAATTCAAG  650
F2-S2 CCATTGAACCAAGCACTCTAGGTATATATGGGCTCGCAAGGATGCATAGGCAAATTCAAG  658
F3-R1 tCATTGgACCAAGCACTCTAGGTATATATcGGCTCGCAAGGATGCATAGGCAAATTCAAG  646
F3-R2 CCATTGgACCAAGCACTCTAGGTATATATcGGCTCGCAAGGATGCATAGGCAAATTCAAG  647
F3-S1 CCATTGgACCAAGCACTCTAGGTATATATcGGCTCGCAAGGATGCATAGGCAAATTCAAG  647
F3-S2 CCATTGgACCAAGCACTCTAGGTATATATcGGCTCGCAAGGATGCATAGGCAAATTCAAG  647

                Bam HI
F1-R1 ATTTGGGCAGGATCCAACTTTGTACGCCCAGAAGGAAATATGTATTTATATTATATTTTG  717
F2-R1 ATTTGGGCAGGATCCAACTTTGTACGCCCAGAAGGAAATATGTATTTATATTATATTTTG  718
F2-R2 ATTTGGGCAGGATCCAACTTTGTACGCCCAGAAGGAAATATGTATTTATATTATATTTTG  715
F2-S1 ATTTGGGCAGGATCCAACTTTGTACGCCCAGAAGGAAATATGTATTTATATTATATTTTG  710
F2-S2 ATTTGGGCAGGATCCAACTTTGTACGCCCAGAAGGAAATATGTATTTATATTATATTTTG  718
F3-R1 ATTTGGGCAGGATC.AACTTTGTACGCgtAGAAGGAAATATGTATTTATgTTATATTTTG  705
F3-R2 ATTTGGGCAGGATC.AACTTTGTACGCgtAGAAGGAAATATGTATTTATgTTATATTTTG  706
F3-S1 ATTTGGGCAGGATC.AACTTTGTACGCgtAGAAGGAAATATGTATTTATgTTATATTTTG  706
F3-S2 ATTTGGGCAGGATC.AACTTTGTACGCgtAGAAGGAAATATGTATTTATgTTATATTTTG  706

F1-R1 CGTTGCTTGAA.GCTATATATTTTTGTTTATAACAATTATCTATGATATATTTTGC.TAA  775
F2-R1 CGTTGCTTGAA.GCTATATATTTTTGTTTATAACAATTATCTATGATATATTTTGC.TAA  776
F2-R2 tGTTGCTTGAA.GCTATATATTTTTGTTTATAACAATTATCTATGATATATTTTGC.TAA  773
F2-S1 tGTTGCTTGAA.GCTATATATTTTTGTTTATAACAATTATCTATGATATATTTTGC.TAA  768
F2-S2 CGTTGCTTGAA.GCTATATATTTTTGTTTATAACAATTATCTATGATATATTTTGC.TAA  776
F3-R1 CGTTGCT.GAAaGCTATATAgTTTTGTTTATAACAgTTATCTATGATATtTTTTGCaTAA  764
F3-R2 CGTTGCT.GAAaGCTATATAgTTTTGTTTATAACAgTTATCTATGATATtTTTTGCaTAA  765
F3-S1 CGTTGCT.GAAaGCTATATAgTTTTGTTTATAACAgTTATCTATGATATtTTTTGCaTAA  765
F3-S2 CGTTGCT.GAAaGCTATATAgTTTTGTTTATAACAgTTATCTATGATATtTTTTGCaTAA  765

F1-R1 CCAGTTAGCCACACAAGGTTATGCAAATA.......GCAAGTATCTGTAACTATATGTTT  828
F2-R1 CCAGTTAGCCACACAAGGTTATGCAAATA.......GCAAGTATCTGTAACTATATGTTT  829
F2-R2 CCAGTTAGCCACACAAGGTTATGCAAATA.......GCAAGTATCTGTAACTATATGTTT  826
F2-S1 CCAGTTAGCCACACAAGGTTATGCAAATA.......GCAAGTATCTGTAACTATATGTTT  821
F2-S2 CCAGTTAGCCACACAAGGTTATGCAAATA.......GCAAGTATCTGTAACTATATGTTT  829
F3-R1 CCAGTTAaCCACACAAGGTTATGCAAATAttgcaagGCAAGTATCTGTAACTATATGTTT  824
F3-R2 CCAGTTAaCCACACAAGGTTATGCAAATAttgcaagGCAAGTATCTGTAACTATATGTTT  825
F3-S1 CCAGTTAaCCACACAAGGTTATGCAAATAttgcaagGCAAGTATCTGTAACTATATGTTT  825
F3-S2 CCAGTTAaCCACACAAGGTTATGCAAATAttgcaagGCAAGTATCTGTAACTATATGTTT  825
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F1-R1 TGGTTTTGTTGCTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  888
F2-R1 TGGTTTTGTTGCTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  889
F2-R2 TGGTTTTGTTGCTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  886
F2-S1 TGGTTTTGTTGCTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  881
F2-S2 TGGTTTTGTTGCTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  889
F3-R1 TGGTTTTGTTGtTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  884
F3-R2 TGGTTTTGTTGtTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  885
F3-S1 TGGTTTTGTTGtTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  885
F3-S2 TGGTTTTGTTGtTTTGCAGGAGTTTTGGATTGCAAATTCCACCCAAGACAACCGTGGTTG  885

F1-R1 TTCACCGCCGGTGCCGACTCGATGATTAGGCTTTATTGCGACTGATGACGATGCTTTCAA  948
F2-R1 TTCACCGCCGGTGCCGACTCGATGATTAGGCTTTATTGCGACTGATGACGATGCTTTCAA  949
F2-R2 TTCACCGCCGGTGCCGACTCGATGATTAGGCTTTATTGCGACTGATGACGATGCTTTCAA  946
F2-S1 TTCACCGCCGGTGCCGACTCGATGATTAGGCTTTATTGCGACTGATGACGATGCTTTCAA  941
F2-S2 TTCACCGCCGGTGCCGACTCGATGATTAGGCTTTATTGCGACTGATGACGATGCTTTCAA  949
F3-R1 TTCACCGCCGGTGCCGACTCGATGATTAGGCTaTAcTGCGACTGATGACGATGCTTTCAA  944
F3-R2 TTCACCGCCGGTGCCGACTCGATGATTAGGCTaTAcTGCGACTGATGACGATGCTTTCAA  945
F3-S1 TTCACCGCCGGTGCCGACTCGATGATTAGGCTaTAcTGCGACTGATGACGATGCTTTCAA  945
F3-S2 TTCACCGCCGGTGCCGACTCGATGATTAGGCTaTAcTGCGACTGATGACGATGCTTTCAA  945

F1-R1 GGAGCATACATTTTAGGTGCCAATTTCTACGAGTAGCTTTTCATGATGATTATATTCTAC 1008
F2-R1 GGAGCATACATTTTAGGTGCCAATTTCTACGAGTAGCTTTTCATGATGATTATATTCTAC 1009
F2-R2 GGAGCATACATTTTAGGTGCCAATTTCTACGAGTAGCTTTTCATGATGATTATATTCTAC 1006
F2-S1 GGAGCATACATTTTAGGTGCCAATTTCTACGAGTAGCTTTTCATGATGATTATATTCTAC 1001
F2-S2 GGAGCATACATTTTAGGTGCCAATTTCTACGAGTAGCTTTTCATGATGATTATATTCTAC 1009
F3-R1 GGAGtATACgTTTTAGGTGgCAATcTCgACGgGTAGCTTTTCATGATGgTTATgTTCTgC 1004
F3-R2 GGAGtATACgTTTTAGGTGgCAATcTCgACGgGTAGCTTTTCATGATGgTTATgTTCTgC 1005
F3-S1 GGAGtATACgTTTTAGGTGgCAATcTCgACGgGTAGCTTTTCATGATGgTTATgTTCTgC 1005
F3-S2 GGAGtATACgTTTTAGGTGgCAATcTCgACGgGTAGCTTTTCATGATGgTTATgTTCTgC 1005

F1-R1 CGTTTCGCCAAGAGTTGCAGACCGGGGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTTTT 1068
F2-R1 CGTTTCGCCAAGAGTTGCAGACCGGGGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTTTc 1069
F2-R2 CGTTTCGCCAAGAGTTGCAGACCGGGGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTTTT 1066
F2-S1 CGTTTCGCCAAGAGTTGCAGACCGGGGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTTTT 1061
F2-S2 CGTTTCGCCAAGAGTTGCAGACCGGGGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTTTT 1069
F3-R1 CGTTTCGCCAAGAGTcGCAGACCGGaGCCAGGAAGGATTTCCTtTCGTGCTGCAGTTT.. 1062
F3-R2 CGTTTCGCCAAGAGTcGCAGACCGGaGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTT.. 1063
F3-S1 CGTTTCGCCAAGAGTcGCAGACCGGaGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTT.. 1063
F3-S2 CGTTTCGCCAAGAGTcGCAGACCGGaGCCAGGAAGGATTTCCTCTCGTGCTGCAGTTT.. 1063

F1-R1 GTGATCGCAGTTTTGTTTGGAGAGATTGTTGTATCTTTTTTCTTA..GTCATTATGTATA 1126
F2-R1 GTGATCGCAGTTTTGTTTGGAGAGATTGTTGTATCTTTTTTCTTA..GTCATTATGTATA 1127
F2-R2 GTGATCGCAGTTTTGTTTGGAGAGATTGTTGTATCTTTTTTCTTA..GTCATTATGTATA 1124
F2-S1 GTGATCGCAGTTTTGTTTGGAGAGATTGTTGTATCTTTTTTCTTA..GTCATTATGTATA 1119
F2-S2 GTGATCGCAGTTTTGTTTGGAGAGATTGTTGTATCTTTTTTCTTA..GTCATTATGTATA 1127
F3-R1 GcGATCGCAtTTTTGTTTGGgGAGATTGTTGTATCTTTTTTCTT gtca TtATTATGTATA 1122
F3-R2 GcGATCGCAtTTTTGTTTGGgGAGATTGTTGTATCTTTTTTCTT gtca TtATTATGTATA 1123
F3-S1 GcGATCGCAtTTTTGTTTGGgGAGATTGTTGTATCTTTTTTCTT gtca TtATTATGTATA 1123
F3-S2 GcGATCGCAtTTTTGTTTGGgGAGATTGTTGTATCTTTTTTCTT gtca TtATTATGTATA 1123
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F1-R1 ACAATTAGATGAGGTGCAGCGCCGT 1151
F2-R1 ACAATTAGATGAGGTGCAGCGCCGT 1152
F2-R2 ACAATTAGATGAGGTGCAGCGCCGT 1149
F2-S1 ACAATTAGATGAGGTGCAGCGCCGT 1144
F2-S2 ACAATTAGATGAGGTGCAGCGCCGT 1152
F3-R1 ACAATTAGATGAGGTGCAGCGCCGT 1147
F3-R2 ACAATTAGATGAGGTGCAGCGCCGT 1148
F3-S1 ACAATTAGATGAGGTGCAGCGCCG 1147
F3-S2 ACAATTAGATGAGGTGCAGCGCCGT 1148

Bold Letters: Possible sites for chromosome 5 specific and tan spot markers, and Bam HI enzymes cutting site.