GENETICS OF VIRULENCE AND TOXIN PRODUCTION IN *Pyrenophora tritici-repentis*, THE CAUSAL AGENT OF TAN SPOT OF WHEAT.

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GENETICS OF VIRULENCE AND TOXIN PRODUCTION IN *Pyrenophora tritici-repentis*, THE CAUSAL AGENT OF TAN SPOT OF WHEAT

BY

HAMZA HABIL OTONDO

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FOREWARD

The materials, methods and results in this thesis are presented in the form of three manuscripts intended for publication. The style as well as the preparation of tables and figures comply with the requirements of Canadian Journal of Plant Pathology. A general discussion and bibliography are included following the manuscripts.

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GENERAL ABSTRACT

Resistance to *Pyrenophora tritici-repentis*(Died.) Drechs., the causal agent of tan spot of wheat, is expressed by the formation of small, dark-brown restricted lesions on host tissue, and susceptibility as tan necrotic lesions or extensive chlorosis. Isolates of *P. tritici-repentis* are currently grouped into four pathotypes based on their ability to induce in appropriate differential cultivars, tan necrosis, extensive chlorosis or a resistant reaction on either or both cultivars of a differential pair. Isolates that induce tan necrosis produce, *in-vitro* and *in-vivo,* a host-selective toxin referred to as Ptr-necrosis toxin. There is presently no information on whether genetic changes associated with either asexual or sexual reproduction of the fungus could give rise to new patterns of virulence. The objectives of this study were: to determine whether the asexual processes of anastomosis and heterokaryon formation could lead to variation in virulence; to study the inheritance of virulence and toxin production; and to determine the role of Ptr-necrosis toxin in the expression of virulence by isolates of *P. tritici-repentis.*

To assess heterokaryon formation, an iprodione resistant mutant derived from isolate 86-124, was paired with a hygromycin B resistant mutant derived from isolate D308. The pairings were observed for the incidence of hybridization which was indicated by the production of dense aerial hyphae where the hyphae met; this is known as the meld. Conidia were isolated from the meld and grown on a medium amended by adding both iprodione and hygromycin B at levels inhibitory to the wild types. Eleven percent of the conidia isolated from the meld grew on the doubly

amended medium indicating they were produced following anastomosis and the formation of heterokaryons. Subcultures were classified into two types based on mycelial growth, and on the ability of heterokaryons to sporulate on unamended medium: the first showed restricted growth and did not sporulate, the second, normal mycelial growth but reduced sporulation. When the heterokaryon stability of the subcultures was assessed on amended media, it was found that mycelial plugs taken from all sections of subcultures with restricted growth grew on doubly amended medium, but were non-sporulating. Mycelial plugs that were taken 1 cm from the edge of the colony of subcultures with normal growth and reduced sporulation grew only on V8PDA amended with hygromycin B, indicating that the heterokaryon had dissociated. Conidia from subcultures with normal mycelial growth were tested for virulence on differential cultivars, and were found to express only extensive chlorosis, confirming that the heterokaryon had dissociated.

Specific isolate virulence/avirulence phenotypes were used as genetic markers to study the inheritance of virulence and toxin production. Ascospores from individual pseudothecia or complements of eight ascospores from single asci were analysed for segregation of virulence on differential wheat cultivars. Sixteen percent of pseudothecia contained ascospores that segregated in a ratio of 1:1 (parental: recombinants). Five complements of eight ascospores from individual asci segregated in a ratio of 1:1:1:1 for the four disease phenotypes and in a ratio of 1:1 (Ptr-necrosis toxin producer : non-producer) suggesting the involvement of two genes, one controlling the ability to induce tan necrosis, the other extensive chlorosis. The factor

for Ptr-necrosis toxin was associated with the locus for tan necrosis.

Ptr- necrosis toxin was purified from culture filtrates of isolate 86-124 and used at several concentrations to test for its effect on the colonization of wheat leaves by selected isolates of *P. tritici-repentis*. Untreated leaves, the leaves that were inoculated only with spores of the avirulent isolate and those that were exposed only to the toxin at less than 0.1μg/mL, remained symptomless. When Ptr-necrosis toxin was present at 0.1 μg/mL, the avirulent isolate invaded and colonized intercellular spaces of the mesophyll in the susceptible cultivar Glenlea but not the resistant cultivar Salamouni, and induced symptoms as rapidly as the virulent isolate. Leaves of the resistant cultivar Salamouni were not colonized by either the toxin-producing isolate 86-124 or the avirulent isolate P8A6 3 supplemented with Ptr-necrosis toxin. This suggests that resistance to tan necrosis is based on insensitivity to the toxin produced by necrosis inducing isolates. These observations support the hypothesis that Ptr-necrosis toxin is a primary factor in the expression of virulence in the necrosis inducing pathotypes.

The occurrence of anastomosis followed by heterokaryon formation, and the reconstitution of the four pathotypes by sexual recombination, indicates the potential for *P. tritici-repentis* to evolve additional virulence that could overcome resistance genes in the previously resistant cultivars of wheat.

2. GENERAL INTRODUCTION

Tan spot of wheat caused by *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph : *Drechslera tritici-repentis* (Died.) Shoemaker 1962) has become an important disease world wide (Duff 1954; Valder and Shaw 1952; Misra and Singh 1972). The pathogen infects many grass species (Krupinsky 1982, 1987) and has the widest host range of any *Pyrenophora* species (Shoemaker 1962). The infection causes reduced test weight and a pink discolouration of the kernels, resulting in reduced grade. Under favourable conditions, epidemics of tan spot cause 12.9 to 49% losses in grain yield of spring wheat (Hosford and Busch 1974; Rees *et al.* 1981). Increased disease severity has been associated with change from conventional tillage to practices that retain stubble on the soil surface (Pfender *et al.* 1988).

Isolates of *P. tritici-repentis* are currently grouped into four pathotypes based on the ability of isolates to induce in appropriate differential cultivars, tan necrosis or extensive chlorosis or a resistant reaction in either or both cultivars of a differential pair. Resistance to tan spot, expressed as small, dark-brown lesions, has been identified at all ploidy levels of wheat (Lamari and Bernier 1989a) while susceptibility is expressed as tan necrotic lesions or extensive chlorosis, or both. Resistance to tan necrosis and insensitivity to the Ptr-necrosis toxin produced specifically by the isolates that induce tan necrosis is recessive, while resistance to extensive chlorosis varies from dominant to partially dominant (Lamari and Bernier 1991).

Several methods have been tried in attempts to control tan spot of wheat. Burying infected crop residue was considered because the fungus survives poorly on buried stubble (Pfender and Wootke 1987), however, this practice leads to soil erosion (Raymond *et al.* 1985). Control of tan spot using foliar fungicides is feasible (Tekauz *et al.* 1983; Watkins *et al.* 1982) but the cost may be prohibitive. The incorporation of resistance into susceptible wheat cultivars through breeding is now possible. In order to determine how effective incorporation of resistance would be, we require a better understanding of the host-pathogen interaction, durability of resistance and the degree of variability of virulence patterns. Presently, there is no information on whether asexual or sexual reproduction of the fungus can give rise to increased levels or new patterns of virulence, and this study sought to provide such information.

Hybridization and recombination have been demonstrated in *P. tritici-repentis*. Mutants resistant to either the fungicide iprodione or the antibiotic hygromycin B were generated via ultraviolet light mutagenesis and used in sexual crosses (McCallum 1991; McCallum *et al.* 1994). Crosses were made in all possible combinations between derived subcultures of isolate 86-124 which were resistant to either iprodione or hygromycin B, and derived subcultures of isolate D308 which were resistant to either iprodione or hygromycin B. Hybrid progeny were obtained only from crosses between subcultures of isolate 86-124 resistant to iprodione and of 86-124 resistant to hygromycin B. Hybridization did not occur for all pairings, thus further work is required to understand hybridization and recombination in *P. tritici-repentis*.

The objectives of the present study were: i) to assess the occurrence of

anastomosis and heterokaryon formation as asexual means of variation in *P .tritici-repentis;* (ii) to determine the inheritance of virulence and toxin production; and (iii) to determine the role of the Ptr-necrosis toxin in virulence of isolates of *P. tritici-repentis.*

3. REVIEW OF LITERATURE

3.1. Taxonomy of *Pyrenophora tritici-repentis.*

P. tritici-repentis, the causal agent of tan spot in wheat, is an ascomycete of the class Loculoascomycetes, order Pleosporales, family Pleosporaceae (Luttrel 1973). The conidia are cylindrical, divided into 5-7 multinucleate cells (Shoemaker 1962; Wehmeyer 1954) and measure 95-165 x 14-18 μm. Pseudothecia are black and produce abundant eight-spored asci which vary in size from 200-700μm (Pfender *et al.* 1988). Asci are bitunicate, cylindrical and narrow at the base. Ascospores are hyaline at maturity, have three transverse septa and one longitudinal septum and measure 47-65 x 20-26 μm.

3:2 Host range of *Pyrenophora tritici-repentis.*

P. tritici-repentis is reported to have a broad host range among grass species (Morrall and Howard 1975). At least 26 species of grasses are infected by this fungus (Krupinsky 1982, 1987) and in moist weather it can cause yield losses of up to 49% (Rees *et al.* 1982). The wide host range of this fungus is in contrast to that of other members within the genus e.g. *P. teres* Drechs., which only infects barley. The ability of *P. tritici-repentis* to colonize such a large number of grasses, most of which are perennial and grow in wheat producing areas, facilitates overwintering and provides inoculum to initiate tan spot epidemics in farm fields (Krupinsky 1982, 1987).

3:3 **Epidemiology**

Several factors contribute to the production and spread of inoculum, and the resulting disease severity in wheat. *P. tritici-repentis* can survive saprophytically on infested residues of wheat and grasses between cropping seasons (Adee and Pfender 1989; Hosford 1971), and in infested seed (Schilder and Bergstrom 1990). Ascocarps that are formed on infested stubble release ascospores as primary inoculum in spring. Early season infection is largely initiated from inoculum formed on nearby stubble since ascospores are not readily wind dispersed (Wright and Sutton 1990; Krupinsky 1992). Following primary infection, conidia are produced on lesioned tissue and these are wind disseminated and cause secondary infection cycles in the field.

3:4 **Sporulation in culture**

The conditions required for conidial production by *P. tritici-repentis* have been studied to some degree. Mycelial growth in culture is optimal at 20-25^{0}C and the fungus is a diurnal sporulator, requiring light at wavelengths less than 350ηm to form conidiophores (Odvody *et al.* 1982). Production of conidia occurs over a temperature range of 10-25 ^{0}C (Platt *et al.* 1977). High relative humidity (80-100 %) is essential for conidia formation and promotes the formation of pseudothecia and ascospores (Platt and Morrall 1980). Maximum pseudothecial formation is attained at -0.2Mpa and ceases under -2.4 Mpa (Pfender *et al.* 1988). Ascospores develop most quickly at 15^{0}C (Summerell and Burgess 1988b).

3:5 Conidial liberation

Release of conidia has been shown to be dependent on wind speed and relative humidity (RH) (Platt and Morrall 1980). Wind speeds of 3.3 ms^{-1} result in the liberation of 100% of spores at all RH values. Even at a relatively low wind speed, 60% spore liberation was obtained at 35% RH. Liberation was greater with changing than with constant RH.

3:6 The effect of leaf wetness duration and temperature on infection.

By correlating the performance of wheat genotypes in the field with their post-inoculation wet period requirement in the green house, Hosford and Busch (1974) showed that moderately resistant genotypes required wet periods of 18-24 h and susceptible cultivars wet periods of 6-12 h to develop similar amounts of disease as measured by the number of lesions per cm^2. In a similar study, Hosford *et al.* (1987) showed that an increase in temperature and/or post-inoculation leaf wetness increased disease severity in both a susceptible cultivar and, to a lesser extent also in a resistant cultivar. The range of lesion size depended on the cultivar, with smaller lesions occurring in a resistant cultivar and larger lesions in a susceptible cultivar. Luz and Bergstrom (1986) also showed that wheat cultivars differ in the amount of disease they develop in response to changes in post-inoculation temperature. Their data indicates that temperature optima for disease development is between 18-28^{0}C, with the precise range being dependent on the cultivar. On the cultivars Max and BR8, the number of lesions per cm^2 remained relatively high over a wide temperature range (18-24^{0}C) whereas on the cultivar BH1146, the range is narrow (25-28^{0}C) but the temperature is higher.

The reported breakdown of resistance with increased temperature and/or post-inoculation wetness period is contrary to the findings of Lamari and Bernier (1989a), who found no significant differences in the percent leaf area infected for wetness durations ranging from 24-72 h at 22^{0}C. The reason resistance was found to break down with prolonged wetness can be explained by the way Hosford *et al.* (1987) characterized resistance or susceptibility on the basis of lesions per cm^2 or percent leaf area infected. A large number of lesions is not necessarily synonymous with susceptibility, which implies genetic action (or lack of it) and results in a "typical" phenotype. Lamari and Bernier (1989a, b) showed that the tan spot syndrome consists of two phenotypes, tan necrosis and chlorosis, and that resistance based on lesion type did not change following post-inoculation wetness periods. The increase in amounts of disease with prolonged wetness as reported by Hosford *et al.* (1987) could be due to a larger number of infections/unit area as a result of multiple infections/conidium arising from branching and/or germination of several "cells" per conidium.

3:7 **Infection process**

Studies that have been conducted on the infection process of *P. tritici-repentis* in the susceptible and resistant wheats reported no differences in % spore germination, the number of appressoria produced, or the successful penetration of epidermal cells between such wheats within 12 h post-inoculation at 18-22^{0}C and 90-100% RH (Larez *et al.* 1986; Loughman and Deverall 1986; Lamari and Bernier 1989b). However, differences were observed between the susceptible and resistant wheats after 48 h post-

inoculation when hyphae became established in the intercellular space of the mesophyll tissue showing a susceptible reaction, but not in resistant tissue. Whether differences occur between the infection processes of virulent and avirulent isolates have not been investigated.

3:8 **The effect of leaf position and plant growth stage on disease development.**

Wheat leaf position, or age, has been shown to affect significantly the appearance and severity of disease symptoms. Both Raymond *et al.* (1985) and Riaz *et al.* (1991) noted that despite the fact that leaves were inoculated at the same time, lesions on lower (older) leaves coalesced earlier than did the lesions on upper (younger) leaves, indicating that older leaves are more susceptible to infection than younger leaves. Raymond *et al.* (1985) also confirmed that the growth stage at which infection occurs can influence yield loss. Studies by Rees and Platz (1983) showed that a 13% yield loss occurred at the seedling stage following infection, compared to a 35% loss at the jointing stage following infection. Shabeer and Bockus (1988) reported that plants are most susceptible physiologically to infections occurring before the boot or flowering stages, and that about half of the total yield loss due to such infections results from multiple infections by secondary inoculum. Losses are a result of a significant reduction in kernel weight and the number of grains per spike, and not of a reduced number of spikes per plant.

3:9 CONTROL OF TAN SPOT OF WHEAT.

3:9:1 Cultural methods for control.

Several cultural methods have been tried in attempts to control tan spot in wheat. Control practices consisting of rotations with non-host species have been attempted to control tan spot in Australia (Rees and Platz 1980, 1981). However, since there is evidence that *P. tritici-repentis* infects several species of grasses which may provide inoculum in the absence of wheat stubble (Krupinsky 1982, 1987), rotations may not be always effective. Fungicides have been used to effectively control tan spot in the field, but such practices are cost prohibitive (Tekauz *et al.* 1983).

Applications of nitrogen as either ammonium sulphate or calcium nitrate in the field are reported to reduce disease severity (Huber *et al.* 1987), and both the rate and form of nitrogen influenced the severity of tan spot in winter wheats. Thus Huber *et al.* (1987) thought that management of nitrogen might provide a cultural control for this disease. In a separate study, Bockus and Davis (1993) also found that fertilizer applications significantly reduced disease severity in inoculated treatments. However, the severe physiological necrosis and chlorosis from nitrogen deficiency in nonfertilized, noninoculated checks were indistinguishable from the symptoms of the disease. These symptoms were alleviated with both nitrogen forms in noninoculated treatments. The apparent reduction of disease by nitrogen fertilizers in inoculated treatments was due to alleviating physiological necrosis and chlorosis, and therefore both ammonium sulphate and calcium nitrate reduced disease by delaying leaf

senescence but not by directly reducing the disease.

3:9:2 **Biological control**

Biological control involves both the manipulation of the environment to the disadvantage of the harmful organisms, and the use of other organisms to reduce the levels of the harmful agent on the host. Manipulation of the environment could involve changes in water potential, temperature, competition for nutrients and release of toxic substances into the environment (Campbell 1989).

The saprophytic sexual stage of *P. tritici-repentis* is apparently a complex one. Mycelial growth and pseudothecial numbers have been shown to decline as the external water potentials decrease (Summerell and Burgess 1988a). The fungus grows maximally at -0.2Mpa and produces the maximum number of pseudothecia at this water potential. Mycelial growth and pseudothecial formation is reduced at -10.5 Mpa indicating that this fungus can not survive in water deficient areas.

The recovery of *P. tritici-repentis* from stubble, and the production of fertile pseudothecia on stubble residues that have been weathered over a number of years in various environments, have both been used as indicators of viability of the fungus (Summerell and Burgess 1989; Adee and Pfender 1989). Incorporation and burial of stubble is inimical to the survival of the fungus. The fungus is infrequently recovered from stubble after 26 weeks of burial, 52 weeks of incorporation and 104 weeks on stubble that is retained on the soil surface.

3:9:3 **Competition and pathogen survival.**

P. tritici-repentis survives well in association with both a number of other parasites as well as primary saprophytes that occur on straw that remains on the soil surface. Persistence of the pathogen in infested straw was correlated with the composition of the microbial communitity in various macroenvironments in no till fields (Zhang and Pfender 1992; Pfender *et al.* 1993a). The results showed that in soil-borne straw, this pathogen was displaced by a community composed of Actinomycetes and soil-borne fungi. At water potentials higher than -39 Mpa, *P. tritici-repentis* was initially displaced by *Pythium oligandrum* Drechs., at 10^{0}C and *Aspergillus terreus* Thom., and *Chaetomium globosum* Kunze:Fr., at 20^{0}C and 30^{0}C respectively, and finally *Penicillium* spp and *Fusarium solani* (Mart.)Sacc., (Summerell and Burgess 1989). No fungi other than *P. tritici-repentis* were recovered at -150Mpa. Prolonged periods of moisture occurred in those microenvironments in which the survival and pseudothecial production was poorest (Summerrel and Burgess 1989).

3:9:4 **Antagonism**

Several organisms have been reported to exhibit antagonism towards *P. tritici-repentis*. Such antagonism by *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur., was observed when the two organisms were inoculated simultaneously, or when *C. sativus* was inoculated up to six hours after *P. tritici-repentis* (Luz and Bergstrom 1987). The antagonism of *C. sativus* towards *P. tritici-repentis* is expressed as a suppression of conidial germination, appressorium formation, and germ

tube elongation on the leaf surface. Several straw-associated fungi, including *Limonomyces roseipellis* Stalpers & Loerakker, are capable of inhibiting both growth and pseudothecial production by *P. tritici-repentis* (Pfender 1988; Pfender *et al.* 1993a). These straw associated fungi reduce ascocarp and ascospore production, with the degree of suppression varying between 50-90 %, depending on the test conditions. The antagonism was thought to be due to mycoparasitism, because straw associated fungi are known to have chitinolytic ability.

Antibiotic producing bacterial strains inhibit the growth of *P. tritici-repentis* in culture and on wheat straw maintained under conditions of high humidity. Inhibition by *Pseudomonas fluorescens* (Trevisan) Migula, was expressed as a reduction in mycelial growth and is due to pyrrolnitrin, one of the antibiotics produced by *P. fluorescens,* strain Pf-5 (Pfender *et al. 1*993b).

Among a group of herbicides tested for their ability to inhibit pseudothecial formation in infested straw, the glyphosate containing herbicide Roundup was the most effective, if applied before infection (Sharma *et al.* 1989). However, it had no effect on mycelial growth rate. This report did not specify whether the inhibitory effect on pseudothecial formation was due specifically to glyphosate or to some non-herbicide component of the preparation.

3:9:5 **Host resistance to tan spot.**

Resistance to tan spot in wheat has been reported at all ploidy levels of wheat and is usually visually expressed by the formation of restricted small, dark-brown lesions (Gough 1982; Lamari and Bernier 1989a). And while the incorporation of

resistance into susceptible cultivars through breeding is now possible, we require a full understanding of the nature of the interaction between wheat and *P. tritici-repentis,* durability of resistance and the degree of variability of virulence patterns.

3:9:6 THE NATURE OF HOST-PARASITE INTERACTION.

Compatible and incompatible interactions

The interaction of the host and pathogen genotypes is influenced by the environment over the course of time, and the result is a "typical" phenotype or expression of disease symptoms. The phenotype of the interaction is used to characterize the host as being resistant (the pathogen may penetrate the cuticle and epidermal cells, but subsequent growth is restricted making the interaction incompatible) or susceptible (the pathogen penetrates and grows unimpeded making the interaction compatible). In the pathogen, the counterpart of resistance is avirulence and for susceptibility it is virulence. Virulence is the ability of a pathogen to infect and cause disease on different host genotypes while avirulence is the inability to infect and cause disease on different genotypes (Andrivon 1993).

Aggressiveness

The quantitative measure of disease expression by an isolate on a susceptible cultivar is known as aggressiveness (Andrivon 1993). Aggressiveness is a characteristic of the host-pathogen interaction as influenced by environmental conditions. The same pathogen strain can induce different amounts of disease on a series of susceptible hosts. Thus, aggressiveness depends on the partial resistance

features of the host on which this character is measured, and as it can be scored repeatedly under standard environmental conditions, this makes it a stable trait for a given host-pathogen pair.

The traits of aggressiveness which can be measured directly in the disease cycle are: (i) incubation and latent periods; (2) number of spores produced in a given area or in a fixed time period; (3) the lesion sizes and (4) the duration of the sporulation period. Short incubation and latent periods, long sporulation periods, large cumulative spore counts, and large areas of infected tissue can be indicative of an isolates high levels of aggressiveness on a specific cultivar if it is being compared to another isolate. Isolates of *P. tritici-repentis* have been found to differ in aggressiveness as measured by the percent leaf area infected (Krupinsky 1992b) but not on the basis of individual components of disease reaction.

The Gene-for-Gene hypothesis.

Many important advances in plant pathology have come from the genetic analysis of the host and the pathogen. For example, the detailed studies of the inheritance of virulence in flax rust pathogen *Melampsora lini* (Ehrenb.) Desmaz., and resistance in flax *Linum usitatissimum* L., led Flor (1942) to propose the gene-for-gene hypothesis of specificity in host-pathogen interactions. The gene-for-gene hypothesis states that for every resistance gene in the host there is a corresponding avirulence gene in the pathogen. Genetic analysis of *Venturia inaequalis* (Cooke) G. Wint., on apple (Bagga and Boone 1968; Boone and Keitt 1957) , *Bremia lactucae* Regel, on lettuce (Michelmore *et al.* 1984; Norwood and Crute 1983) and *Puccinia recondita*

f. sp. tritici (Roberge ex Desmaz.), on wheat (Kolmer and Dyck 1994) among others have supported the existence of the gene-for-gene relationships between fungi and their hosts.

The Wheat : *P. tritici-repentis* system

The host

Resistance to tan spot in wheat, as reported by Hosford (1971), Hosford and Busch (1974) and Raymond *et al.* (1985) was largely quantitative, and based on the number of lesions per cm^2 or percent disease severity. This did not provide a clear concept of resistance or susceptibility, which imply genetic interactions with "typical" phenotypes. The recognition of the interaction of the tan spot fungus with wheat genotypes as a syndrome consisting of two phenotypes, necrosis and chlorosis (Lamari and Bernier 1989a, b) led to studies on host-parasite interactions in this system. Susceptible wheat cultivars develop either tan necrosis or extensive chlorosis, while resistance to both tan necrosis and extensive chlorosis is expressed as the formation of small dark-brown lesions. Resistance to tan necrosis is controlled by one recessive gene while resistance to extensive chlorosis varies from partial to completely dominant (Lamari and Bernier 1991). However, preliminary results from an on-going study (Duguid, S.; personal comm.) and from other reports (Nagle *et a*l. 1982; Sykes and Bernier 1991) indicate that resistance to tan spot is controlled by more than one gene in some cultivars of wheat.

The pathogen

Variation among isolates of *P. tritici-repentis* has been reported for colony morphology and the amounts of pigments produced in culture, and for virulence and aggressiveness on wheat cultivars. While in culture most isolates form dark-gray colonies that produce black-green pigments, on subculture some strains give rise to variants that produce wine-red to purplish or reddish or bright orange pigments (Wehmeyer 1954; Hosford 1971; Hunger and Brown 1987). While such variants differ in morphology and growth rates, Wehmeyer (1954) also noted that the wine-red variants remained completely sterile and failed to form even the "sclerotial" primordia common to all other strains.

Misra and Singh (1972) reported that there were significant differences between isolates based on the severity of the reactions they induced on the host plants, and based on the percent leaf area infected and numbers of lesions formed per cm^2, Luz and Hosford (1980) distinguished 12 races amongst the 40 isolates they studied. During an attempt to verify the potential of smooth brome grass to serve as an alternative source of inoculum, Krupinsky (1987) found he could not differentiate between isolates from brome and wheat on the basis of reaction they induced on wheat leaves; the reaction was measured by the number of lesions per cm^2 and disease severity. Smooth brome grass serves as a host to *P. tritici-repentis,* and therefore inoculum can easily be transmitted between the two hosts.

Schilder and Bergstrom (1990a) showed that isolates they studied did not differ widely in disease reaction as measured by percent leaf area infected, but had a

moderate degree of physiological specialization based on the amount of disease induced on both winter and spring wheats. Winter wheats had moderate to high levels of resistance, while spring wheats were susceptible. Lamari *et al.* (1991) grouped their isolates of *P. tritici-repentis* into four pathotypes based on the ability of isolates to induce either tan necrosis, extensive chlorosis or a combination thereof on appropriate differential cultivars. Isolates of pathotype one induce both tan necrosis and extensive chlorosis (nec+chl+), pathotype two only tan necrosis (nec+chl-), pathotype three only extensive chlorosis (nec-chl+), while pathotype four, classified as avirulent, induces only small dark-brown lesions without either characteristic tan necrosis on Glenlea, or extensive chlorosis on line 6B365.

Ptr-necrosis toxin

Isolates that induce tan necrosis produce a cultivar specific Ptr-necrosis toxin in liquid culture (Tomas and Bockus 1987; Ballance *et al.* 1989, Lamari and Bernier, 1989c). When the toxin is infiltrated into susceptible host leaves, it produces tan-coloured necrotic lesions similar to those induced by the necrosis-inducing isolates (nec+chl- and nec+ chl+), and isolates that lack the ability to induce tan necrosis (nec-chl+ or nec-chl-) do not produce Ptr-necrosis toxin. Ptr-necrosis toxin was characterized and found to be a high molecular weight (Mw 13,900) protein (Ballance *et al.*1989), and Brown and Hunger (1993) isolated a low-molecular weight (Mw 800-1800) toxin which induces the characteristic chlorosis that is associated with tan spot disease, and also differentially inhibits elongation of wheat seedling coleoptiles. This Ptr-toxin does not induce necrosis. The relationship between these toxins, and their

roles in tan spot disease development, have not been investigated.

3:9:7 GENETICS OF FUNGAL PATHOGENS

Genetic studies of any organism require the use of characteristics which can be precisely defined and easily scored, and that provide information about an organism's genotype. The key to understanding variation in fungi lies in their reproductive systems. Investigations in other fungi indicate that variation is caused by, and distributed through, asexual and sexual processes (Burnett 1975; Fincham *et al.* 1979). However, there is presently no information on whether either asexual or sexual reproduction or both can give rise to increased levels or patterns of virulence in *P. tritici-repentis.*

3:9:7:1 Variation arising during asexual reproduction.

Mutations

During asexual reproduction, the origination of variants chiefly involves mutations, anastomoses followed by nuclear exchange to form heterokaryons, and parasexuality. The advancement of fungal genetics has been based on the use of mutations at specific loci to create genetic markers (Flor 1960; Griffiths and Carr 1961). As mutations occur spontaneously at a low frequency of approximately 10^{-6} in all cell populations of fungi (Fincham *et al.* 1979), mutagens such as X-rays, gamma rays, ultraviolet light and certain chemicals have been used to increase mutation rates in fungi. The rate and extent to which mutant genes can spread through, and between, populations is of importance in determining how fast a species can evolve, whether it will evolve eventually into two or more species and, for the plant pathologist, whether

new virulent forms could arise to overcome previously resistant genes.

The large size of most fungal populations means that mutant alleles arise continuously despite the rarity of mutation. The absence of a distinct germline in fungi ensures that mutations arising in virtually any tissue can be transmitted to subsequent generations either sexually or asexually. The dominant haploid phase that occurs in the life cycles of many fungi, means that new mutations can be expressed immediately if they are segregated into uninucleate spores or monokaryotic hyphae (Caten 1987). And although when they first arise new mutant alleles are so rare that they generally pass undetected, the high asexual reproductive rate of most fungi permits a rapid increase in number and frequency to those mutations that confer benefit under natural selection. Genetic markers created by mutagenesis have been used in several ascomycetous fungi including *Saccharomyces cerevisiae* (Meyen ex. E. Hans), *Aspergillus nidulans* (Eidam.) G. Wint., and *Neurospora crassa* Shear and B. O. Dodge, to develop genetic model systems (Fincham *et al.* 1979).

Heterokaryosis

Intraspecific hyphal fusion and nuclear migration occurs regularly between genetically identical (homogenic) hyphae of many fungi. And if such fusions and nuclear migrations occurs between thalli whose nuclei differ, heterokaryons, (i.e. mycelia containing a mixture of two or more types of nuclei) can be established. (Esser and Blaich 1973; Esser and Meinhardt 1984; Puhalla 1985; Burnett 1975). However, if hyphae are not genetically identical at certain loci (heterogenic), fusion may be prevented, terminated or limited in its effects, and since vegetative

incompatibility is based on genetic differences, the term heterogenic vegetative incompatibility is used to explain lack of fusion between hyphae. Heterogenic incompatibility can also affect certain sexual fusions (Esser and Blaich 1973; Burnett 1975; Fincham *et al.* 1979; Anagnostakis 1984). Vegetative incompatibility is divided into fusion incompatibility and post-fusion incompatibilty. In the former, cell fusion between the two strains does not occur whereas in the latter, fusion reactions cause "barrage" effects between colonies (Esser and Blaich 1973; Esser and Meinhardt 1984; Anagnostakis 1984). Hyphal death or repulsion occurs within the barrage zone in Basidiomycetes as well as *N. crassa*, while in most other Ascomycetes, aversion or development of reproductive structures occurs after a period of time within this region, depicting post-fusion incompatibility.

Fusion and post-fusion incompatibility have been widely studied in fungal species (Puhalla and Mayfield 1974; Pontecorvo and Sermonti 1954; Zambino and Harrington 1990). In each of the species studied, numerous vegetative incompatibility groups (VIG) have been recognized, each of which has two alleles at the controlling loci. An incompatibility reaction occurs when strains differing at one or more vegetative incompatibility (VI) loci are brought together. Incompatibility due to the interaction of alleles of a VI gene is termed allelic incompatibility. If a species has n loci at which allelic incompatibility occurs, then 2^n VIG are possible. Interaction between alleles at different VI loci is known as non-allelic incompatibility and has only been described in *Podospora anserina* (Ces.ex Rob.) Niessl, (Esser and Blaich 1973).

Studies of hyphal fusions and heterokaryon formation in some species of fungi indicate that once established, heterokaryons can be maintained in several ways. In *N. crassa* and *A. nidulans* (Pontecorvo *et al.* 1953), and *Penicillium chrysogenum* Thom., (Pontecorvo and Sermonti 1954), nuclei of paired strains migrate reciprocally, and then divide in the hyphae of each of the pair. Heterokaryons become established in multinucleate hyphal tips, and thus the heterokaryon can be maintained by further hyphal growth without recurrent anastomosis. In some other fungi, *Verticillium dahliae* Kleb., (Puhalla and Mayfield 1974), *Gibberella fujikuroi* (Sawada) Ito., (Puhalla and Spieth 1985) and *Leptographium wageneri* (Kendrick) M. J. Wingfield., (Zambino and Harrington 1990), recurrent anastomoses is required probably because the nuclei do not migrate from the cell in which anastomosis has occured, and the heterokaryon cannot be perpetuated without recurrent fusion.

The parasexual cycle

In the parasexual cycle, mycelium becomes heterokaryotic due to anastomosis or mutation. A portion of the haploid nuclei in the heterokaryon fuse to form diploid nuclei, and these divide mitotically and increase in number together with the remaining haploid nuclei (Kafer 1961; Buxton 1956; Hartley and Williams 1971). At mitosis, some of the diploid nuclei undergo recombination due to crossing over. Other diploid nuclei may enter spores which germinate and produce homokaryotic mycelia. The diploid nuclei in these mycelia may be haploidized, and from those which undergo mitotic recombination, haploid nuclei may result which differ in genetic constitution from those of the original heterokaryotic mycelium. The various

processes of diploidization, haploidization, aneuploidy and recombination may all be occurring simultaneously in the thallus, and not in any regular sequence or at any specific stage (Kafer 1961). There is much speculation about the evolutionary benefits of heterokaryosis as a means of exploiting hybrid vigour, storing genetic variation and adjusting somatically to changing environment (Buxton 1956; Caten and Jinks 1966; Caten 1987). The significance of heterokaryosis as a means of genetic variation will depend not only upon its occurrence, but also upon the degree of the diversity between the associated genomes.

3:9:7:2 **Variation arising during sexual reproduction.**

Mating types

Throughout most of the life of a fungus, fusion between genetically different strains is prevented or severely limited by the presence of heterogenic VI systems. However, when the sexual process occurs, these systems are largely or entirely suppressed, so out-crossing is possible (Esser and Blaich 1973). In many fungi the sexual process is initiated only as a result of an interaction between cells that differ genetically with respect to mating type, and probably also in other ways, thus promoting genetic exchange (Fincham *et al.* 1979; Burnett 1975; Anagnostakis 1984).

Heterothallism

Fungi in which the co-operation of genetically different strains is required for the initiation or completion of the sexual process are referred to as self-sterile or, since different strains need to be involved, as heterothallic (Pontecorvo *et al.* 1953;

Wheeler 1954; Olive 1958). The controlling mechanisms have been classed as either homogenic or heterogenic incompatibility (Burnett 1975; Esser and Blaich 1973; Esser and Kuenen 1967; Esser and Meinhardt 1984). In fungi that have two mating types based on alternate alleles at a single locus; homogenic incompatibility prevents mating between genetically similar progeny. Heterogenic incompatibility prevents mating between genetically dissimilar progeny.

Homothallism

A fungus in which every thallus is sexually self-fertile and can reproduce sexually without the aid of another thallus is referred to as homothallic. Homothallism is referred to as primary if there is no evidence for a heterothallic ancestor. If there is evidence that an earlier heterothallic state has been circumvented, then it is referred to as secondary homothallism or pseudocompatibility (Wheeler 1954; Olive 1958; Carlile 1987). Secondary homothallism can result from the inclusion of two nuclei of different mating type in a single spore, and the subsequent growth of a mycelium heterokaryotic for mating type. Secondary homothallism due to mutation for mating type in large-spored strains of *Sclerotinia trifoliorum* Eriks. is known (Uhm and Fuji 1983). In addition to *S. trifoliorum*, the existence of both heterothallic and homothallic strains of the same fungus has been reported in *Ceratocystis ulmi* (Buism.) Nannf. (Brasier and Gibbs 1975), *Cryphonectria parasitica* (Murrill) Barr (Anagnostakis 1984, 1988), and *Glomerella graminicola* Politis (Vaillancourt and Hanau 1991). With any homothallic fungus, when two strains are grown in mixed culture, the ascospores from any single ascus may derive their nuclei from a selfed

zygote of one strain or the other, or from a hybrid zygote (Pontecorvo *et al.* 1953).

Genetic analysis

For genetic analysis, the interest is primarily in crossed asci. Ascospore colour has been widely utilized to study the inheritance of spore colour, because progeny can be analysed directly by viewing the progeny with a microscope. Ascospore colour mutants have been utilized in the homothalic ascomycete *Sordaria fimicola* (Roberge ex Desmaz.) Ces. and De Not. (Olive 1956) as well as in the heterothallic plant pathogen *V. inaequalis* (Boone and Keitt 1957). Ascospore colour mutants have been used to directly identify hybrid perithecia and cleistothecia. Since ascospores of *S. fimicola* are produced in an ordered series, spore colour mutants make it possible to visually analyse the segregation of the spore colour-loci. Ascospore colour is an ideal marker for homothallic species because there is no need to germinate ascospores and form thalli to detect hybrid perithecia. Ascospores of *P. tritici-repentis* are hyaline and indistinguishable on the basis of colour. The most useful approach to genetic analysis is to germinate ascospores and score thalli for differences in colony morphology, pigments, virulence or toxin production.

The only *Pyrenophora* species that have been analysed genetically are *P. teres* and *P. graminea* Ito & Kurabayashi (McDonald 1963; Smedegard-Peterson 1977). Both species have bipolar heterothallic mating systems. *P. teres,* the causal agent of net-blotch of barley, occurs in two forms: the net form which produces a net-like pattern of necrotic lesions and the spot form which produces brown elliptical lesions. In crosses between the spot and net forms, four classes based on lesion type were

found in the progeny, the parental spot and net lesion inducing types, and two recombinant classes. The two recombinant classes were comprised of one class whose strains induced lesions intermediate between the spot type and the net type, while the strains of the second class induced fleck or non-pathogenic lesions (Smedegard-Peterson 1977). The two parental and two recombinants occurred in a 1: 1 : 1 : 1 ratio indicating that the ability to produce spot and net lesions is conditioned by two independent genes.

4. Heterokaryon formation between iprodione and hygromycin B resistant mutants of *Pyrenophora tritici-repentis*.

ABSTRACT

Isolates of *P. tritici-repentis*, the causal agent of tan spot of wheat are currently grouped into four pathotypes based on their ability to induce tan necrosis, extensive chlorosis, or a combination of these or a resistant reaction on appropriate differential cultivars of wheat. The objective of this study was to assess the occurrence of anastomosis and heterokaryon formation as possible asexual means of making variation in the virulence of this fungus. A mutant of isolate 86-124 resistant to iprodione was paired with a mutant of isolate D308 resistant to hygromycin B. The pairings were observed for the incidence of heterokaryon formation as indicated by the presence of the meld. Conidia were isolated from the meld and grown on a medium amended with both iprodione and hygromycin B at levels inhibitory to the wild-type strains. Eleven percent of the conidia from the meld grew on the doubly amended medium indicating they were the result of anastomosis and heterokaryon formation. Subcultures were classified into two types based on mycelial growth and the ability of the heterokaryons to sporulate on unamended media: one with restricted growth and no sporulation, the other with normal growth but reduced sporulation. When the heterokaryon stability of the subcultures was assessed on amended media, it was found that mycelial plugs taken from all sections of subcultures with restricted growth grew on doubly amended medium, but were non-sporulating. Mycelial plugs that were taken 1 cm from the edge of the colony of subcultures with normal growth and

reduced sporulation grew on V8PDA amended with hygromycin B but not on V8PDA amended with iprodione, indicating that the heterokaryon had dissociated. Mycelial plugs that were taken 2 cm from the edge of the colony grew on the doubly amended medium, and mycelial plugs taken 3 cm from the edge of the colony were a mosaic that grew on media amended with either iprodione or hygromycin B, or both. Conidia from subcultures with normal mycelial growth and reduced sporulation were tested for virulence on differential cultivars that develop either tan necrosis or extensive chlorosis and were found to induce only extensive chlorosis also suggesting that the heterokaryon was not stable.

4.1. INTRODUCTION

Pyrenophora tritici-repentis (Died.) Drechsler, the cause of tan spot disease of wheat, has become an important disease world wide (Hosford 1971; Tekauz 1976; Rees and Platz 1981; Duff 1954). The pathogen has a broad host range among grass species (Morrall and Howard 1975; Krupinsky 1982, 1987) and causes yield losses of up to 49% in moist weather (Rees *et al.* 1982).

Isolates of *P. tritici-repentis* form either dark-gray or whitish-cream colonies on V8PDA medium and each type can form sectors that differ both morphologically and in the appearance of the pigments secreted into the medium; the latter range from dark-gray to orange or whitish cream (personal observations; Wehmeyer 1954; Hunger and Brown 1987). When axenic cultures from these sectors were made by subculturing hyphal tips and increased by growing colonies from single conidia, the dark-gray and whitish cream sectors produced abundant conidia whereas the white or albino sectors remained mycelial and did not sporulate.

Isolates of this fungus are currently grouped into four pathotypes based on their specific interactions with two differential wheat cultivars (Lamari *et al.* 1991). The interaction is characterized by the development of tan necrosis on cv. Glenlea and extensive chlorosis on line 6B365. Isolates of pathotype 1 induce both tan necrosis and extensive chlorosis (nec+chl+), pathotype 2 only tan necrosis (nec+chl-), pathotype 3 only extensive chlorosis (nec-chl+) and pathotype 4 (nec-chl-) lacks the ability to induce either symptom, and are classified as being avirulent.

Genetic analysis of this homothallic fungus was difficult until recently when mutants

resistant to either the fungicide iprodione or the antibiotic hygromycin B were created in isolates 86-124 (nec+chl-) and D308 (nec-chl+) via ultraviolet light mutagenesis and then used in sexual crosses (McCallum *et al.* 1994). The mutants were crossed in all possible combinations and the progeny analysed to determine the genetic basis of chemical resistance. Hybrid progenies were obtained in crosses involving mutants of isolate 86-124, but not of isolate D308. The results indicated that one locus controls iprodione resistance and a second locus controls hygromycin B resistance. Why hybridization did not occur between mutants of isolates 86-124 and D308 has not been investigated.

Genotypic variants brought about by mutations are distributed among progeny via asexual and sexual processes. The asexual process involves anastomosis and nuclear migration between vegetatively compatible strains to form heterokaryons (Tinline 1988; Heale 1988; Anagnostakis 1988). If hyphae are not genetically identical, hyphal fusion may be prevented, terminated, or limited in its effects by vegetative incompatibility. Vegetative incompatibility prevents mixing of protoplasm and nuclear migration (Leslie 1993). It is divided into fusion incompatibility and post-fusion incompatibility (Esser and Blaich 1973; Anagnostakis 1984; Caten 1986). In fusion incompatibility, cell fusion between the two incompatible strains does not occur and is usually expressed as aversion zones. In post-fusion incompatibility, fusion reactions cause barrage zones (an area between colonies where hyphae, after having attempted to anastomose, become curled, enlarged and degenerate). The barrage line eventually consists of paralyzed dead cells (Esser and

Meinhardt 1984).

The frequency of heterokaryon formation is usually increased a thousandfold by using either protoplast fusion or microinjection of donor protoplasmic fluids including nuclei into recipient strains. This technique assures successful crosses between strains in which crossing had failed previously, indicating that the hyphal wall is a major site of incompatibility (Heale 1988).

Studies on anastomosis and heterokaryon formation in some species of fungi indicate that heterokaryons once established can be maintained in several ways. In *N. crassa, A. nidulans* and *P. chrysogenum* (Pontecorvo *et al.* 1953), nuclei of both strains migrate and divide in the hyphae of paired strains. Heterokaryons become established in multinucleate hyphal tips, and are maintained by further hyphal growth without recurrent anastomosis. In other fungi like *V. dahliae* (Puhalla and Mayfield 1974) and *L. wageneri* (Zambino and Harrington 1990), recurrent anastomosis is required, probably because nuclei do not migrate from the cell in which anastomosis has occurred, and the heterokaryon can not be propagated without recurrent fusion. Heterokaryosis may contribute to fungal variation through the parasexual cycle (Pontecorvo 1956; Hartley and Williams 1971; Buxton 1956).

This study was undertaken to investigate the natural occurrence of hyphal fusion and heterokaryon formation between mutants of isolates 86-124 and D308. In order to assess the role of heterokaryosis as the basis of variation in the virulence of this fungus, subcultures that were obtained from the zone of hyphal fusion (meld) were assayed for the expression of resistance to both iprodione and hygromycin B as well

as for virulence on both the cultivar Glenlea and line 6B365.

4.2 MATERIALS AND METHODS

4.2.1. Isolates

Mutant subcultures of isolates 86-124 and D308 of *P. tritici-repentis* resistant to either iprodione or hygromycin B (McCallum *et al.* 1994) were used in this study. Both isolates [86-124 (nec+chl-) and D308 (nec-chl+)] had previously been characterized for virulence (Lamari and Bernier 1989b). The virulence of the mutants is similar to that of their wild type parent. Isolate D308 is morphologically unstable in culture and frequently sectors. Isolate 86-124, although relatively more stable than D308, can sector to a fast growing, white colony that produces an orange pigment in the culture medium.

4.2.2 Media

V8PDA (150 mL of V8 vegetable juice, 10 g Potato dextrose agar, 3 g calcium carbonate, 10 g Bacto agar, 850 mL water) was prepared as described by Lamari and Bernier (1989a, b). The hygromycin B (hgm+) and iprodione (ipr+) resistant mutants were assayed for growth on V8PDA amended with hygromycin B (30 mg/L) and iprodione (60 mg/L) respectively (McCallum et al. 1994). Neither wild-type isolate 86-124 nor D308 could grow on V8PDA amended with these chemicals at these concentrations.

4.2.3 **Pairing**

Subcultures of each of the four mutants were grown on each of the following media: V8PDA alone, V8PDA plus iprodione alone, V8PDA plus hygromycin B alone and V8PDA plus iprodione and hygromycin B. Expectations were that all would grow on unamended V8PDA, only mutant ipr+ would grow on V8PDA plus iprodione, only mutant hgm+ on V8PDA plus hygromycin B and only a heterokaryon would grow on the doubly amended medium (V8PDA plus iprodione and hygromycin B). Once it was established that the mutants could grow only on unamended V8PDA and V8PDA amended with the chemical to which each was resistant, subcultures of each of the four mutants were paired in all combinations on unamended V8PDA.

There were four plates per pair of strains for each medium. The plates were incubated for 4 d at 22-25^{0}C in the dark and examined for the presence of dense white aerial hyphae (the meld) at the line of junction of the two colonies as an indication of vegetative compatibility. The meld is a term that was coined by Volk and Leonard (1989) to depict a mycelial interaction in *Morchella* spp. (morels) which is distinct from the "barrage" reaction. Barrage, as characterized in *N. crassa*, refers to a clear aversion zone where hyphae simply pile up in opposed regions (Burnett 1975; Esser and Meinhardt 1984). The meld, by contrast, is initiated as soon as contact is made between the two colonies in the centre of the plate and it involves hyphal fusion.

Analysis of heterokaryons

To test whether the meld was indicative of anastomosis and heterokaryon formation, the zone was well marked beneath the plate and the mycelia flattened with

the bottom of a sterile test-tube carrying a drop of sterile distilled water. The four plates were incubated at 25^{0}C under light provided by 3 fluorescent tubes for 18 h to induce formation of conidiophores and then transferred to 10-15^{0}C in the dark for 18 h for conidiation. To isolate single conidia, 6 x 1 mm pieces of agar were cut from the line of junction, inverted, and used to gently spread and thus separate conidia on the surface of V8PDA amended with both iprodione and hygromycin B. Then, under a dissecting microscope, pieces of agar carrying single conidia were cut, transferred to similarly amended V8PDA and spatially separated. The germinated conidia were counted, rescued by transferring to unamended V8PDA to assess growth characteristics, stability and virulence.

Anastomosis

To ascertain whether hyphal fusion occurs between hyphae of this fungus, a droplet of conidial suspension (3 x 10^3 conidia/ml of water) from each of the mutants 86-124ipr+ and D308hgm+ was initially paired 1 cm apart on glass slides coated with a thin layer of water agar, and the pairs were then incubated in transparent plastic boxes to maintain high moisture levels in the agar. The slides were observed every 2 h for conidial germination, germ-tube elongation and hyphal fusion. The high level of moisture required for conidial germination wets the agar, causing conidia to run-off of the slide before the mycelium is established. And because of the run-off, most of the conidia were lost or comingled, making it difficult to trace the origin of the fusing hyphae. Similar conidial pairs were placed 1 cm apart on the surface of leaf pieces of cultivar Glenlea, and then incubated in moist plastic chambers. Conidia adhere

firmly on leaves after 2 h of incubation and because of this, they are retained for prolonged periods of time at the inoculated sites even when placed in a staining solution. The leaf pieces were sampled every 2 h and stained in cotton blue (8 ml 95% ethanol, 1 ml 85% lactic acid, 1 ml glycerine, 1 ml water and 4 mg cotton blue). The leaf pieces were then mounted in lactophenol and observed with a compound microscope for conidial germination and hyphal fusion.

Test for Stability.

After 7 d of mycelial growth, the heterokaryotic colony was divided into 3 equal pie-shaped sections and assayed by taking mycelial plugs 3 mm in diameter at one cm intervals from the colony edge towards the centre of the colony, a method slightly modified from that of Puhalla and Mayfield (1974). A total of twenty hyphal plugs from each section were taken from the 1 cm band from the edge of the colony, 15 plugs from each section were taken from the 2 cm band and 10 plugs from each section were taken from the 3 cm band from the edge of the colony. All the plugs from the one cm band of the first section were transferred to V8PDA plus iprodione, all those from the 1 cm band of the second section were transferred to V8PDA plus hygromycin B, and all those of the 1 cm band of the third section to V8PDA plus iprodione and hygromycin B. The same procedure was repeated for the plugs obtained from 2 cm and 3 cm bands from the edge of the colony of each of the three sections. At least 3 replicate heterokaryotic colonies were sampled.

4.2.4 **Test for virulence**

Subcultures that were obtained from the meld and shown to be resistant to both iprodione and hygromycin B, their mutant parents and wild type progenitors were grown on unamended V8PDA and incubated for 4 d at 25^{0}C in the dark or until the colonies were 3-4 cm diameter. The plates were flooded with sterile distilled water and the mycelia flattened with the bottom of a flamed test-tube. The water was then decanted and the cultures subjected to a regime of light (18 h) and dark (18 h) as described by Lamari and Bernier 1989a, b). Sterile distilled water was added to each culture and the conidia dislodged using a wire loop. The resulting spore suspension was adjusted to 3.5 x 10^3 conidia/mL using a cell counter (Hauser Sc.Blue Bell, Pa). Ten drops of Tween 20 (polyoxyethylenesorbitan monolaurate) were added per L of conidial suspension to reduce surface tension. Seedlings of cv. Glenlea and line 6B365 at two leaf stage were sprayed with the conidial suspension until runoff, using a Devilbis sprayer at approximately 69 Kpa (10 psi). After inoculation, the seedlings were incubated for 24 h under continuous leaf wetness at 22^{0}C. Leaf wetness was provided by two computer controlled ultrasonic humidifiers filled with distilled water. The plants were then transferred to a growth room bench and kept at 22/18^{0}C (day/night) with a 16 h photoperiod (ca :180μE m.$^{-2}$ s^{-1}) for 7 d at which time they were rated for presence or absence of tan necrosis or extensive chlorosis. Conidia were induced to form on infected leaves by placing leaf pieces under fluorescent light and then in the dark, as previously described. Leaf pieces were then rubbed over V8PDA to dislodge conidia which were individually transferred to separate V8PDA

plates for growth. Subcultures from these colonies were assayed for resistance to the chemicals on V8PDA plates amended with either iprodione or hygromycin B or both.

4.3. RESULTS

4.3.1 Formation of the meld.

When mutants 86-124ipr+ and D308hgm+ were paired on unamended V8PDA, the meld formed at the line of junction of the two colonies (Fig.1). Formation of the meld was initiated as soon as contact was made between the two colonies. The meld grew from the initial point of contact at the centre towards the edges of the plates along the line of junction. The meld never formed in pairings between the same isolates (selfs) but instead, the colonies simply intermingled, with the separation line between them being barely visible.

Anastomosis.

Following initial hyphal contact, an opening occurred in the hyphal cell of mutant D308hgm+ in 90% of conidial pairs, and there appeared to be unidirectional protoplasmic movement from the apical cell of mutant 86-124ipr+ into the conidial cell of mutant D308hgm+. The hyphal cell of mutant 86-124ipr+ became vacuolated while the hyphal cell of mutant D308hgm+ became granular, and later, formed hyphal protrusions (Fig. 2). Hyphal fusion was observed between selfs of mutant 86-124ipr+ as well as between selfs of mutant D308hgm+, but no changes in protoplasmic movements and appearance of the cells were observed.

Growth characteristics of the subcultures from the meld.

When conidia from the meld were sampled and transferred to V8PDA plates amended with both iprodione and hygromycin B, only 11% of the conidia germinated (Table 1). None of the conidia from the various individual mutants nor from their parent progenitors grew on this medium whereas they all grew on unamended V8PDA. The conidia that germinated on V8PDA amended with both iprodione and hygromycin B were rescued and transferred to unamended V8PDA to assess growth characteristics, stability and virulence. The morphological features and sporulation ability of the mycelium that developed was distinguishable from that of parental isolates. Forty percent of the subcultures obtained from the meld and shown to be resistant to both iprodione and hygromycin B included cultures with clearly restricted growth, which seemed arrested after attaining a diameter of 3 cm and were non-sporulating (Table 2). The remaining were similar to parental types in growth, but sectored and sporulated sparsely at the centre of the colony unlike the mutant parents and wild-type progenitors which sporulated abundantly on the entire surface of the culture (Fig. 3).

Fig 1: The meld, a region of white aerial hyphae formed between colonies of the mutants 86-124ipr+ and D308hgm+ of *P. tritici-repentis.* meld = region between the arrows.

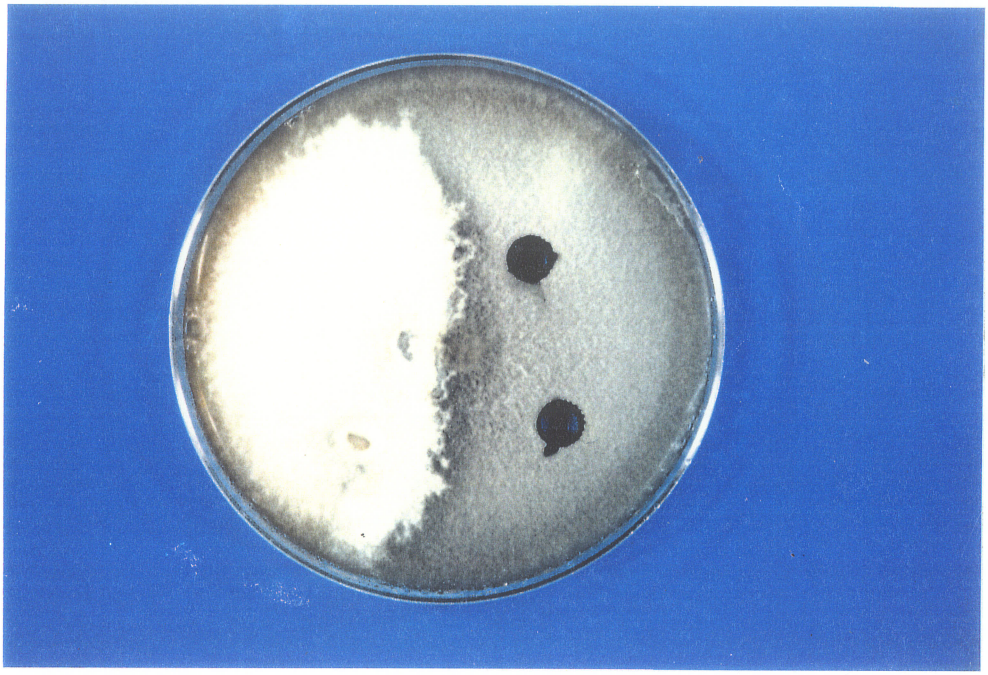


Table 1: Number of germinated conidia (%) obtained from wild -type cultures, mutants, and from the meld formed between mutants 86-124ipr+ and D308hgm+ of *P. tritici-repentis* on V8PDA amended with either iprodione or hygromycin B or both.

	Germination (%) on V8PDA amended with:			
ISOLATE	V8PDA alone	ipr	hgm	ipr & hgm
D308 wild type	100	-	-	-
D308ipr+	100	100	-	-
D308hgm+	100	-	95	-
86-124 wild type	100	-	-	-
86-124ipr+	100	100	-	-
86-124hgm+	100	-	100	-
86-124ipr+*D308hgm+	100	25	40	11

ipr = iprodione, hgm = hygromycin B, ipr&hgm = both iprodione and hygromycin B.
ipr+ = resistant to iprodione, hgm+ = resistant to hygromycin B, * = from the meld
- = no germination.
% = Of a total of 150 conidia obtained from 3 replicate heterokaryotic colonies.

Table 2: Mean growth in diameter (mm) after 7 days of wild type cultures, mutants, and subcultures with restricted mycelial growth obtained from the meld between mutants 86-124ipr+ and D308hgm+ of *Pyrenophora tritici-repentis* on V8PDA amended with either iprodione or hygromycin B or both.

	Diameter (mm)[a] on V8PDA amended with:			
ISOLATE	V8PDA alone	ipr	hgm	ipr & hgm
D308 wild type	74	0	0	0
D308ipr+	19	19	0	0
D308hgm+	44	0	44	0
86-124 wild type	69	0	0	0
86-124ipr+	29	28	0	0
86-124hgm+	64	0	60	0
86-124ipr+*D308hgm+	26	-	-	20

ipr = iprodione, hgm = hygromycin B, ipr & hgm = both iprodione and hygromycin B.
ipr+ = resistant to iprodione, hgm+ = resistant to hygromycin B, * = from the meld
- = no visible mycelial growth.
[a] mean of three replicate colonies.

Figure 2: Germination of conidia derived from mutants 86-124ipr+ (left) and D308hgm+ (right) of *Pyrenophora tritici-repentis* and the fusion of a germ tube with a conidial cell 6 h after incubation.

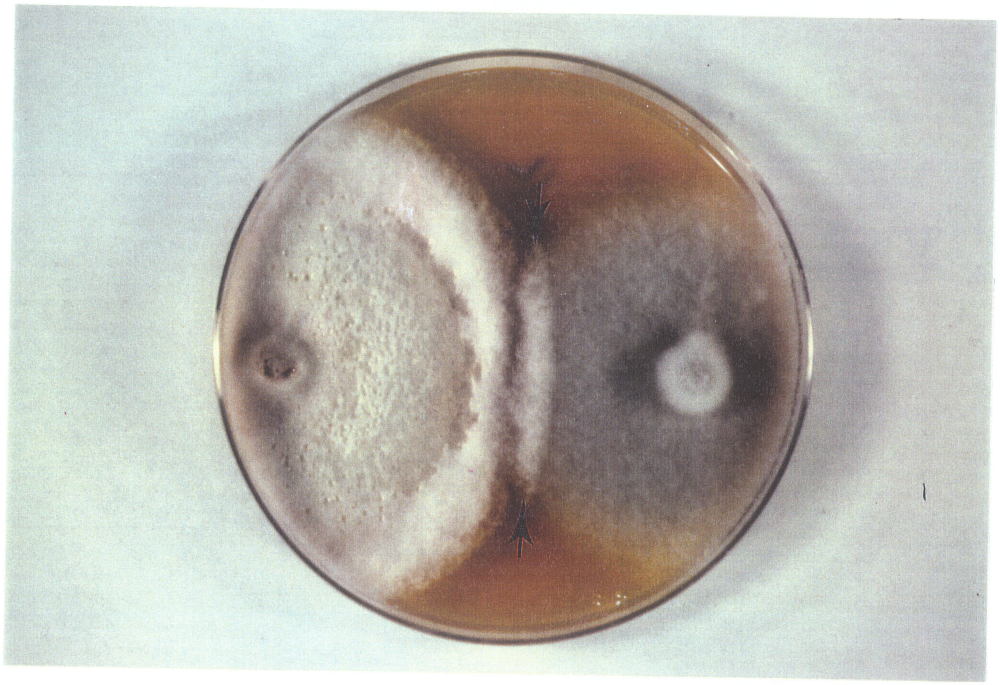
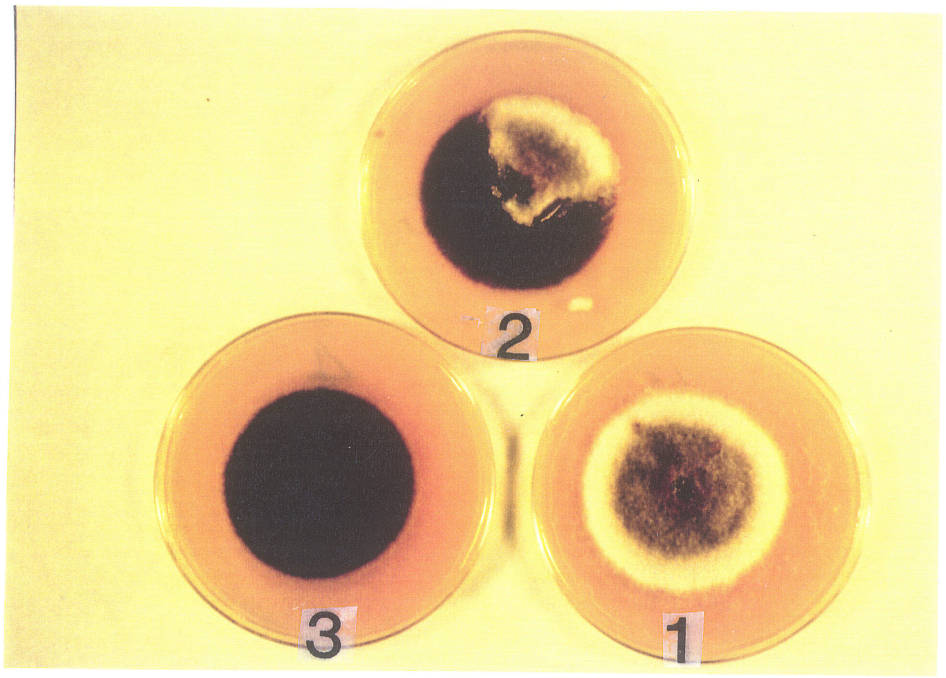


Fig 3: Mycelial characteristics and sporulation patterns of wild type isolates of *Pyrenophora tritici-repentis*, parental mutants involved in the formation of the meld and subcultures derived from the meld.

1) Heterokaryotic colony with sparse sporulation at the centre of the colony.

2) Heterokaryotic colony with sector

3) Illustration of both a wild-type and a mutant colony; such colonies have similar characteristics with the sporulation over the entire surface of the plate.


2
3
1

Stability

All hyphal plugs obtained from the subcultures with restricted mycelial growth consisted of a single component that grew on V8PDA amended with both iprodione and hygromycin B, and were heterokaryons. Hyphal plugs that were taken from the one cm band from the edge of the colony of all subcultures with normal mycelial growth grew only on V8PDA plus hygromycin B (Table 3). Hyphal plugs that were taken from the 1.5-2 cm band from the edge of the colony of all subcultures grew on V8PDA plus both iprodione and hygromycin B. Hyphal plugs that were taken from the 3 cm band from the edge of the colony were a mosaic that grew on V8PDA plus iprodione, V8PDA plus hygromycin B and V8PDA plus both hygromycin B and iprodione. The hyphal plugs that grew on V8PDA plus iprodione, did so after 7-10 days delay.

Table 3: Distribution of component genotypes in the subcultures with normal mycelial growth obtained from the meld between mutants 86-124 ipr+ and D308 hgm+ of *P. tritici-repentis* on V8PDA after 7 days of growth at 23-24°C.

distance from colony edge to centre (cm)	No. of Plugs tested/band	Hyphal plugs (No.)growing on V8PDA† amended with: ipr	hgm	ipr & hgm
1	20	0	20	0
2	15	0	0	15
3	10	10*	10	10

ipr = iprodione, hgm = hygromycin B, ipr & hgm = both iprodione and hygromycin B.
† = numbers are means of 3 replicate heterokaryotic colonies.
* = 7-10 days delay in growth.

4.4 Test for virulence

Subcultures with reduced growth did not sporulate and were not tested for virulence. Since subcultures with normal mycelial growth sporulated poorly, conidia from several plates were combined to provide an adequate inoculum concentration for testing. The conidia induced both extensive chlorosis on line 6B365 and small, dark-brown spots on cv. Glenlea similar to that induced by D308hgm+ (Table.4). Conidia that were reisolated from line 6B365 and cultured on variously amended media, grew on V8PDA plus hygromycin B, but not on V8PDA plus iprodione, or on V8PDA plus both iprodione and hygromycin B. They also were similar to D308hgm+ in their ability to grow on these media.

Table 4 : Virulence on cultivar Glenlea and line 6B365 of the subcultures with normal mycelial growth obtained from the meld formed between mutants 86-124 ipr+ and D308 hgm+ of *P. tritici -repentis* (A) and the ability of conidia reisolated from line 6B365 to grow on amended media (B).

	A		B		
	Cultivar reaction		growth on media amended with:		
Mutant	Glenlea	6B365	ipr	hgm	ipr & hgm
D308hgm+	-	+	-	+	-
HB	-	+	-	+	-

ipr = iprodione, hgm = hygromycin B, ipr & hgm = both iprodione and hygromycin B.
ipr+ = resistant to iprodione, hgm+ = resistant to hygromycin B.
HB = Heterokaryon with normal mycelial growth
\+ = virulent (A) or observed hyphal growth (B)
\- = small dark-spots (A) or no visible hyphal growth (B)

4:5 Discussion

The development of the meld between mutants 86-124ipr+ and D308hgm+ and the isolation of conidia from the meld that were able to grow on V8PDA amended with both iprodione and hygromycin B demonstrate that anastomosis and heterokaryon formation did occur between the paired strains. However, the incidence of heterokaryosis was low since only 11% of the conidia grew on the doubly amended medium. Similar findings were reported for *C. sativus* (Tinline 1962, 1988), where the heterokaryotic condition was carried by only 6-10 % of the conidia.

Isolation of conidia with combined resistance to both iprodione and hygromycin B suggests that the meld is associated with the transfer of genetic factors between hyphal types, and the formation of heterokaryons in this fungus. The dissociation of the initial cultures established by conidial transfer from the meld indicated that the heterokaryon was not stable. The dissociation was demonstrated by the mosaic pattern exhibited at the 3 cm band from the edge of the heterokaryotic colony, and the absence of the 86-124ipr+ component from the 1 cm bands. The delayed growth exhibited by component 86-124ipr+ at the 3 cm band would explain why it is eliminated from the 1 cm band. Since hyphae, conidia and ascospores of this fungus are multinucleate (Whitehead and Dickson 1952; Wehmeyer 1954), the instability could be due to differences in genetic make-up of the component nuclei or to cultural conditions.

Several factors that alone or in combination could lead to the formation of unstable heterokaryons are: nuclear ratio imbalance; influence of cultural conditions;

limited anastomoses brought about by either a relatively slow radial growth of strains; or allelic differences at single vegetative incompatibility loci (Pontecorvo *et al.* 1946; Burnett 1975). Balanced heterokaryons are easily lost for a number of reasons including back mutations, non genetic adaptation, recombination through sexual reproduction, formation of heterozygous diploid nuclei and accumulation in the medium of metabolites enabling either or both components to grow independently (Pontecorvo *et al.* 1953; Hartly and Williams 1971). Whether any of these reasons is applicable in *P. tritici-repentis* is not known and needs to be investigated.

The results of this study have demonstrated that two or more different nuclear types can be associated in a mycelium by anastomosis between vegetatively compatible hyphal types. The dissociation of the heterokaryon is indicative of the constant association and dissociation required for propagation of heterokaryons in vegetatively compatible hyphae. The phenotype of a heterokaryon is expressed as a sum total of all nuclear types present in a mycelium and this suppresses the expression of individual genotypes. The dissociation of the heterokaryon would allow the expression of individual genotypes. These nuclear associations ensure exchange of beneficial genotypes within the population through anastomosis, while vegetative incompatibility would restrict such exchanges, leading to the formation of different clones that would eventually differ through natural selection.

Heterokaryotic conidia or any other propagules are useful in ensuring survival of an individual fungus or as a mechanism of bridging differences in environments. For example, if a heterokaryotic mycelium is carrying virulence genes to certain

susceptible host genotypes that are currently out of season or not grown for some specified reason, the virulence genes would be held within the heterokaryon until such a time that favourable conditions do occur. Then, due to any of the reasons stated above, the heterokaryon could dissociate and release the virulence genes within the vicinity of the susceptible host cultivars. The occurrence of such a phenomenon in nature would provide the pathogen with susceptible host tissues, on which it would reproduce abundantly and cause severe epidemics, particularly in those crops which are grown as monocultures of homozygous susceptible genotypes. Transient heterokaryosis may therefore allow the pathogen to adapt to changes in the environment.

There is no simple explanation for the failure of the heterokaryotic colonies to induce both tan necrosis and extensive chlorosis on the respective differentials. In this study, subcultures with normal mycelial growth sporulated sparsely at the centre of the colony, and the resultant conidia induced only extensive chlorosis. Conidia that were reisolated from line 6B365, could only grow on V8PDA plus hygromycin B but not on either V8PDA plus iprodione or V8PDA plus both iprodione and hygromycin B, indicating that they were similar to D308 hgm+ but not 86-124 ipr+.

These results may be explained by assuming that hyphae of the heterokaryotic subcultures are homokaryotic diploids or haploids. If the two component nuclear types fused to form diploids, then one type (D308hgm+) is dominant or epistatic to the other. However, since the 86-124ipr+ component is screened out in the 2 and 1 cm bands, there is no possibility of these nuclei being diploids. The presence of haploid

nuclei would mean that there are post-fusion incompatibility factors in the protoplasmic portion of the mycelium contributing to the instability of the heterokaryon (Garnjobst and Wilson 1956; Boucherie *et al.* 1976). Future research to elucidate the incompatibility systems in this fungus will need to address these issues.

The results of this study suggests that heterokaryosis can occur in *P. tritici-repentis*, that the process has the potential of creating and concealing genotypic variation and/or is a temporary reservoir of genetic potential whereby genotypes are released in appropriate selective environments through sectoring, and distributed via conidia.

5. Inheritance of virulence and toxin production in *Pyrenophora tritici-repentis.*

ABSTRACT

Isolates of *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat, are currently grouped into four pathotypes [(nec+chl+, nec+chl-, nec-chl+ and nec-chl-)], based on their ability to cause tan necrosis (nec) and chlorosis (chl) on differential cultivars of wheat. Isolates that induce tan necrosis also produce Ptr-necrosis toxin. To investigate the inheritance of virulence and toxin production, twelve isolates, at least one from each pathotype, were paired in all combinations. The isolate pairs were observed for the formation of fertile pseudothecia at the line of junction. Fertile pseudothecia were obtained only from the pair of isolates 86-124 (nec+chl-) and Hy331-9 (nec-chl+). Ascospores from individual pseudothecia and complements of 8 ascospores from single asci were analysed for virulence and for the production of Ptr-necrosis toxin on differential cultivars of wheat. Sixteen percent of the 101 pseudothecia sampled contained ascospores that segregated in a ratio of 1:1 (parental : recombinants). Complements of 8 ascospores from each of five individual asci segregated in a ratio of 1:1:1:1 for the four disease phenotypes and in a ratio of 1:1 (Ptr-necrosis toxin producer : non-producer). These data suggested the involvement of two genes, one controlling the ability to induce tan necrosis, the other extensive chlorosis. The factor for Ptr-necrosis toxin was associated with the factor for tan necrosis. Progeny from the inter-cross of F_1 belonging to pathotype 1 and 4 segregated in a ratio of 1:1 (parental : recombinants) and three single asci segregated in a ratio of 1:1:1:1 for the four disease phenotypes and in a ratio of 1:1 for (Ptr-

necrosis producer : non-producer) further supporting the involvement of two genes.

5.1. INTRODUCTION

Pyrenophora tritici-repentis (Died.) Drechs (Conidial state : *Drechslera tritici-repentis* (Died.) Shoem.) the cause of tan spot of wheat (*Triticum aestivum* L.) is now wide spread throughout the world (Duff 1954; Hosford 1971; Morrall and Howard 1975; Summerell and Burgess 1989). *P. tritici-repentis* infects many grass species (Hosford 1971; Krupinsky 1982, 1987; Morrall and Howard 1975) and has the widest host range of any *Pyrenophora* species (Shoemaker 1962).

In recent years, the prevalence and severity of tan spot have increased to damaging levels and yield losses in spring wheat of 12.9 to 49% have been reported in moist weather (Hosford and Busch 1974; Rees *et al.* 1981). Ascospores discharged from pseudothecia on wheat stubble are the main source of primary inoculum. Increase in incidence and severity of tan spot in recent years has been associated with the adoption of conservation tillage practices in North America and Australia whereby stubble remains on the surface of the soil. In Australia, the high susceptibility of most modern wheat cultivars has also been reported to be a contributing factor to disease severity (Rees *et al.* 1988).

Recent studies have shown that the tan spot syndrome consists of two host phenotypes which are tan necrosis and chlorosis (Lamari and Bernier 1989a,b). These are each the result of specific interactions between individual isolates of the fungus and wheat genotypes. Susceptible wheat lines develop tan necrosis and/or extensive chlorosis, whereas resistant lines develop small, dark-brown lesions without

accompanying tan necrosis or extensive chlorosis (Lamari *et al.* 1991). Resistance to tan necrosis is recessive and resistance to extensive chlorosis varies from dominant to partially dominant. Each of these reactions is controlled by one independent gene (Lamari and Bernier 1991). However, preliminary results from an on-going study (Duguid, S.; personal comm.) and other reports (Nagle *et al* . 1982; Sykes and Bernier 1991) indicate that resistance to tan necrosis in some cultivars of wheat is controlled by more than one gene.

The existence of variability in aggressiveness and virulence has been reported among isolates of *P. tritici-repentis* (Krupinsky 1992b). Luz and Hosford (1980) tested 40 isolates from a wide geographic area and differentiated 12 races on the basis of the amount of spotting caused on a differential set of six wheat and one barley cultivar. Misra and Singh (1972) tested three isolates on 50 wheat genotypes and found significant differences in the amount of disease caused by the different isolates.

More recently, isolates of *P. tritici-repentis* have been assigned to one of four pathotypes based on the ability of isolates to induce in appropriate differential cultivars either tan necrosis or extensive chlorosis (compatible/virulent phenotypes) or small, dark-brown lesions (incompatible/avirulent phenotypes) (Lamari and Bernier 1989b). Isolates of pathotype 1 induce both tan necrosis and extensive chlorosis (nec+chl+), pathotype 2 only tan necrosis (nec+ chl-), pathotype 3 only extensive chlorosis (nec-chl+) and pathotype 4, classified avirulent (nec-chl-), induces small, dark-brown lesions without accompanying tan necrosis or chlorosis.

Susceptibility of wheat genotypes to necrosis inducing isolates and sensitivity

to the Ptr-necrosis toxin released specifically by these isolates are controlled by the same dominant gene (Lamari and Bernier 1989c). Isolates that induce only extensive chlorosis do not produce Ptr-necrosis toxin *in-vitro.*

The ability to sexually cross a pathogen is important as a means of determining the genetic control of virulence within the population. The only *Pyrenophora* species that have been crossed and genetically analysed for virulence are *P. teres* and *P. graminea* . Both species have bipolar (two mating types required for fertility) heterothallic mating systems (McDonald 1963; Smedegard-Peterson 1977) whereas *P. tritici-repentis* is homothallic.

Most isolates of *P. tritici-repentis* form fertile pseudothecia when grown singly or in pairs. If there is any sexual differentiation of nuclei, then the multinucleate character of the hyphae may distribute both types of nuclei to all cells equally (Whitehead and Dickson 1952; Wehmeyer 1954). In an attempt to cross *P. tritici-repentis*, Diaz de Ackerman *et al.* (1988) obtained fertile pseudothecia by culturing isolates singly and in pairs on wheat seeds and corn leaves placed on water agar in petri plates, and with one exception they found that all isolates formed pseudothecia. However, they did not indicate whether all pseudothecia contained asci or whether the ascospores they obtained from the pairings were hybrids or selfs.

Genetic analysis of this homothallic fungus was difficult until recently when mutants resistant to the fungicide iprodione or to the antibiotic hygromycin B were created in isolates 86-124 (nec+chl-) and D308 (nec-chl+) using ultraviolet light mutagenesis, and these were then used in sexual crosses (McCallum *et al.* 1994). The

mutants were crossed in all possible combinations and the progeny analysed to determine the genetic basis of chemical resistance. Hybrid progenies were obtained in crosses involving mutants of isolate 86-124 but not in crosses involving mutants of isolate D308 nor in crosses between mutants of isolates 86-124 and D308 which belong to different pathotypes. The results indicated that one locus controls resistance to iprodione and a second independent locus controls resistance to hygromycin B. In a subsequent study, analysis of conidia from the meld formed between mutants of the two isolates (86-124 x Hy331-9) indicated that heterokaryons formed but dissociated (In thesis, manuscript 1). The objectives of this study were to investigate the possibility of crossing isolates from different pathotypes and to determine the genetic control of virulence and toxin production in isolates of *P. tritici-repentis* using specific host resistance/susceptibility phenotypes as genetic markers. The results of this work are intended to complement studies on the inheritance of resistance to tan spot in the host and to provide information on the potential for development of isolates with increased virulence. Both are critical for the design of effective breeding programs searching for durable resistance to tan spot in wheat.

5.2. MATERIALS AND METHODS

5.2.1 Matings

The isolates of *P. tritici-repentis* used in this study were previously characterized for virulence and production of Ptr-necrosis toxin by Lamari and Bernier (1989b). The colony characteristics and virulence of the isolates used in this study are shown in Tables 5 and 6. Each isolate was cultured on V8PDA (150 ml of V8

vegetable juice, 10 g potato dextrose agar, 3 g calcium carbonate, 10 g of Bacto agar, 850 ml distilled water) by inoculating the medium with a single conidium as described by Lamari and Bernier (1989a, b). Matings were set up in polystyrene petri dishes on autoclaved wheat leaves placed on Sachs' nutrient agar (Appendix 1), a method slightly modified from McCallum *et al.* (1994). All of the twelve isolates comprising at least one from each pathotype, were paired in all possible combinations by placing a 4- d-old mycelial plug (4 mm diameter) of each isolate pair at opposite ends of the leaves. The plates were sealed with parafilm and incubated at 22-25^{0}C in the dark for 7 d and then transferred to 10-15^{0}C in the dark for 8-12 weeks. The plates were observed for formation of fertile pseudothecia at the line of junction of the paired isolates.

5.2.2 **Isolation of ascospores**

Pseudothecia were transferred singly with a sterile forceps to plates of double strength water agar (20 g agar/L water). Individual pseudothecia were crushed by applying gentle pressure with the forceps to release asci. Mature asci were broken by this process thus releasing ascospores randomly on the surface of the medium. Less mature asci have harder bitunicate membranes which were not readily broken by the pressure of the forceps. Under a dissecting microscope, these intact asci were selected and separated from each other with a fine-tipped needle. To release ascospores from such asci, a drop of cell-wall degrading enzyme (5% β-glucuronidase) was applied over the ascus (Yoder 1988) and allowed to digest the ascus wall until the ascospores were released (approximately 30 minutes). Forty random ascospores were picked from

individual pseudothecia (pseudothecial analysis) and, whenever possible, complements of eight ascospores from a single ascus were also obtained (ascal analysis). Individual ascospores were subcultured on numbered V8PDA plates to identify the pseudothecium and/or the ascus from which each was isolated. The numbering of the spores from a single ascus was random since they are not produced in ordered fashion. One half of each pseudothecium with a few intact asci and some ascospores was retained and stored at -17^{0}C as described by McCallum *et al.* (1994) for possible further analysis, if some of the ascospores sampled were found to be recombinant types.

5.2.3 **Analysis of progeny for virulence**

Seedlings of the differential wheat cv. Glenlea (susceptible only to tan necrosis) and line 6B365 (susceptible only to extensive chlorosis) and of the resistant cvs. Erik and Salamouni, were grown in 7.5 X 7.5 cm milk cartons containing soil-less mix (Metromix 200, W. R. Grace and Co: Ajax, Ontario) in growth chambers at 18-20^{0}C with 16 h photoperiod (ca: 180μE.m.$^{-2}$s^{-1}). Three seedlings of each cultivar were planted opposite each other in each of three cartons..

Subcultures of individual ascospores were incubated in the dark at 20^{0}C until the colonies reached about 4-5 cm in diameter as described by Lamari and Bernier (1989b). They were then flooded with sterile distilled water, the mycelium was flattened with a flamed test tube bottom, and excess water decanted. The cultures were incubated for 18-24 h at room temperature (20-24^{0}C) under light (ca: 90 μE.m.$^{-2}$.s^{-1}) provided by three cool white fluorescent tubes, followed by 18-24 h in the dark

at 15 ^{0}C. Spores were suspended in distilled water by using a wire loop and, inoculum concentration was measured with a cell counter (Hauser Scientific, Blue Bell, PA) and adjusted to 3.5 x 10^3 per millimeter with distilled water.

Test seedlings of each of the four cultivars or line at the two leaf stage, were inoculated with the conidial suspension containing ten drops of Tween 20 (polyoxyethlenesorbitan monolaurate) per litre of spore suspension, and the inoculum applied with a Devilbis sprayer at 69 Kpa (10 psi) to the point of impending run-off as described by Lamari and Bernier (1989a, b, 1991).

The seedlings were incubated for 24 h at 22^{0}C in a chamber under continuous leaf wetness provided by two computer controlled ultrasonic humidifiers filled with distilled water. The plants were then transferred to a growth room bench kept at 22/18^{0}C (day/night) and a 16 h photoperiod (ca: 180 $\mu E.m.^{-2}.s^{-1}$), and scored after 6-7 d for their reaction. Seedlings inoculated with conidia from parental isolates and non-inoculated seedlings were used as checks.

Avirulence on cv. Glenlea was expressed as small, dark-brown lesions and virulence as tan necrotic lesions. Avirulence on line 6B365 was expressed as small, dark-brown lesions and virulence as extensive chlorosis (Fig. 4). Progenies found to be virulent or avirulent on both differentials were considered to be recombinants and the result of fertilization rather than selfing. A pseudothecium or ascus was considered to be selfed if no segregation was observed amongst their ascospore progenies and were therefore omitted from the analysis. The test for independence of virulence factors was based on the hypothesis that if the factors are inherited

independently of each other, half of the progeny will be like the parents and half will exhibit new combinations of factors. Chi-square values were calculated for the deviations of the observed number of recombinants from the expected.

Table 5: Colony characteristics of the *P. tritici-repentis* isolates used in the study.

ISOLATE	COLOUR	PIGMENT	TISSUE[a]	STABILITY[b]
ASC1	dark-grey	dark-grey	-	stable
86-124	grey	dark-grey	-	stable
D308	white-grey	grey-orange	-	stable
331-1	white	cream	-	moderate
331-2	white-grey	cream	+	moderate
331-3	white-grey	cream	+	unstable
331-4	white-grey	dark-grey	-	moderate
331-5	white-grey	cream	+	moderate
331-6	white-grey	grey-orange	-	unstable
331-8	grey	dark-grey	-	unstable
331-9	white	cream	+	moderate
331-12	white	grey	-	moderate

a) +/- = presence or absence of sclerotial tissue in culture
[b] refers to frequency of forming sectors.
stable = no sectors
moderate = a few sectors
unstable = many sectors

Table 6: Virulence patterns of the isolates of *P. tritici-repentis* on the two differential cultivars of wheat.

ISOLATE	CULTIVAR	
	Glenlea	6B365
ASC1	nec+	chl+
86-124	nec+	chl-
D308	nec-	chl+
331-1	nec-	chl+
331-2	nec-	chl+
331-3	nec+	chl-
331-4	nec+	chl+
331-5	nec-	chl+
331-6	nec-	chl+
331-8	nec+	chl+
331-9	nec-	chl+
331-12	nec-	chl+

nec+ = presence of tan necrosis on Glenlea
chl+ = presence of chlorosis on 6B365
nec- = absence of tan necrosis on Glenlea
chl- = absence of chlorosis on 6B365

5.2.4 Analysis of progeny for the production of Ptr-necrosis toxin.

A one ml suspension of ca. 10^3 conidia/mL of each of the individual ascospore subcultures was transferred to a 250 ml Erlenmeyer flask containing 50 ml of Fries medium (Appendix 2) amended with 0.1% yeast extract. The flasks were incubated at 20^{0}C in the dark without agitation for three weeks. The cultures were then vacuum filtered through Whatman # 1 filter paper, and the filtrates then stored at 4^{0}C for immediate use or kept in a freezer at -17^{0}C for long-term storage. Each culture filtrate was diluted 1:50 prior to use, and the suspension was then infiltrated into three seedlings each of the cultivars Glenlea, Erik, Salamouni and line 6B365 at the 4 leaf stage using a Hagborg device (Lamari and Bernier 1989c). Sterile distilled water and sterile Fries medium were used as controls. Seedlings were examined daily for 4 d for development of tan necrosis.

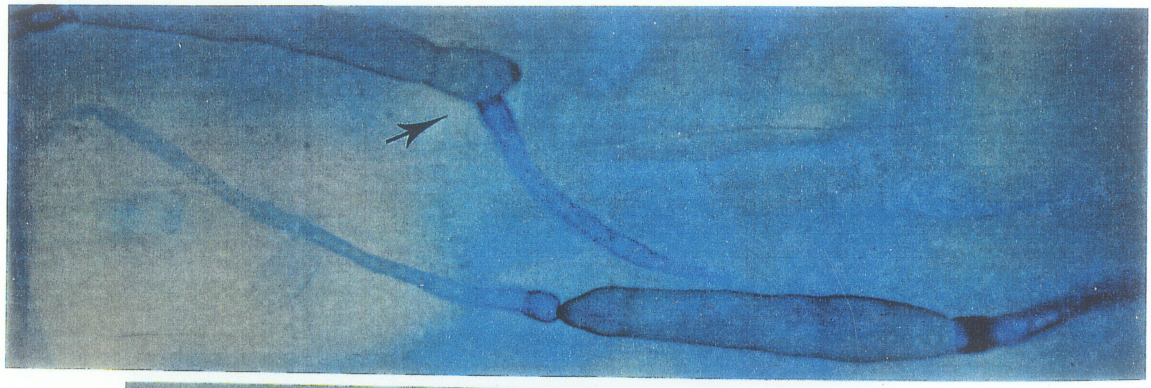
Fig. 4. Reactions of the two differential cultivars of wheat to the isolates of *P. tritici-repentis.*

a) Tan necrotic lesions induced by isolates of pathotype 1 (nec+chl+) (left) and pathotype 2 (nec+chl-) (right) on cultivar Glenlea.

b) Extensive chlorosis induced by isolates of pathotype 1 (nec+chl+) (left) and 3 (nec-chl+) (right) on line 6B365.

c) Small, dark-brown lesions induced by isolates of pathotype 4 (avirulent) on cultivar Glenlea (left) and line 6B365 (right).

a



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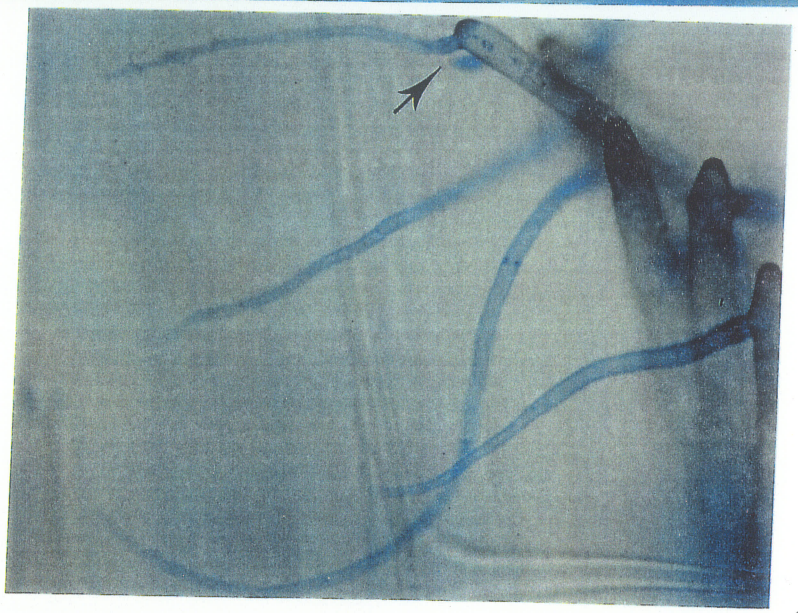

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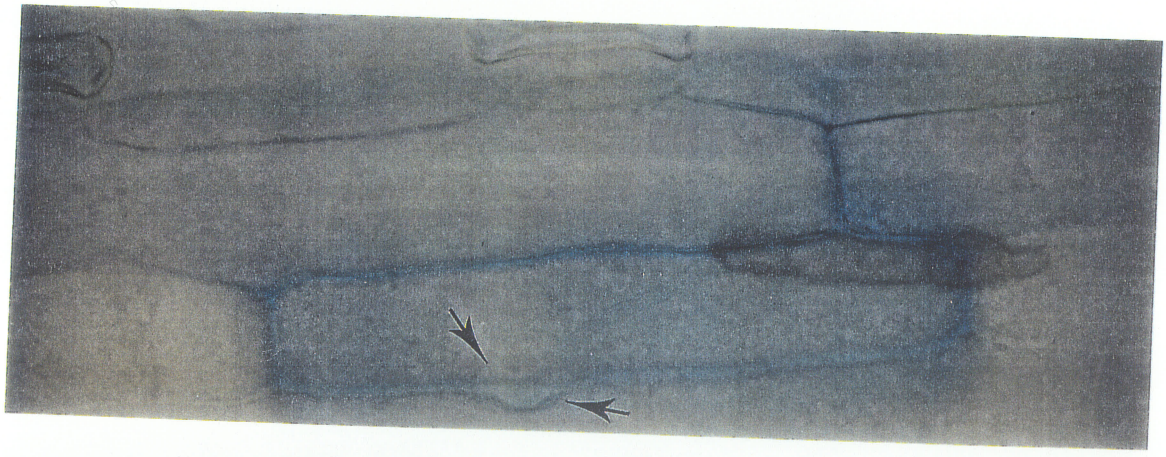


5.3. RESULTS

5.3.1 Formation of pseudothecia

Most isolates formed pseudothecia over the entire surface of the plate when grown singly, but a few did not form pseudothecia at all. Among the isolate pairs, only isolates 86-124 (nec+chl-) and Hy331-9 (nec-chl+) formed pseudothecia consistently at the line of junction of the two colonies (Fig 5). Formation of pseudothecia in all other pairs was not consistent between replicates and tests, often falling into one or more of the following patterns :

i) A single line of pseudothecia at the line of junction of the two colonies (isolates 86-124 and Hy331-9).

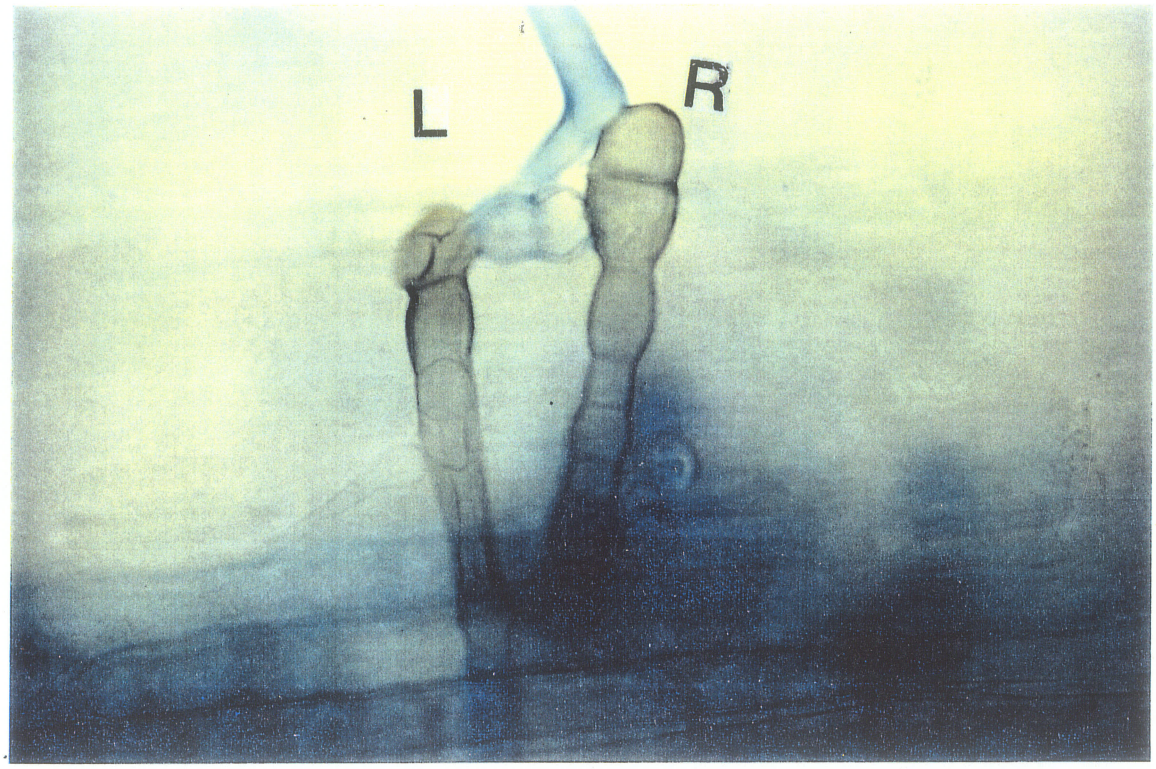
ii) Pseudothecia around the plug of inoculum but not close to the line of junction (isolates D308 x 331-4, 331-1 x ASC1, 331-12 x 331-8).

iii) A ring of pseudothecia around each plug of the pair and a single line of pseudothecia at the line of junction with a clear space on either side of the line of pseudothecia (86-124 x ASC1, 86-124 x 331-3).

iv) Uniform development of pseudothecia all over the plate (86-124 x D308)

v) Pseudothecia surrounding one of the two plugs of a pair only, the other remaining sterile, without ascocarp initials (86-124 x 331-5, 331-6 x 86-124).

Fig 5: A single line of pseudothecia formed at the line of junction of isolates 86-124 (nec+chl-) and Hy331-9 (nec-chl+).


L
R

5.3.2 Analysis of pseudothecia

A total of 101 pseudothecia obtained from the line of junction of isolates 86-124 and Hy331-9 were analysed for fertility (presence of asci and ascospores in a pseudothecium) and for the virulence of individual ascospore progenies on the tan spot differential cultivars of wheat. About 62.4% of the pseudothecia were sterile, forming immature ascocarps or pseudothecia-like bodies without asci (Table 7). Only 16% of the examined pseudothecia (42% of fertile pseudothecia) contained ascospores that induced tan necrosis on cv. Glenlea and extensive chlorosis on line 6B365 (nec+chl+) or small, dark-brown lesions (nec-chl-) on both differentials, and were therefore recombinants. Twenty two percent of all the pseudothecia (57.9% of the fertile) contained ascospores that were virulent only on cv. Glenlea or line 6B365 and were therefore selfs. None of the ascospore progenies were virulent on cvs. Erik and Salamouni. Analysis of the data indicated that random ascospores obtained from individual hybrid pseudothecia segregated in a ratio of 1:1 parentals : recombinants (X^2 = 1.10, P = 0.50-0.10) except for ascospores from pseudothecium P8 (Table 8) which did not fit a 1:1 ratio.

Table 7: Frequency of hybrid pseudothecia from a cross between isolates 86-124 and Hy331-9 of *P. tritici-repentis* as indicated by the virulence phenotypes.

	Number of pseudothecia.			
Plate #	ASSAYED	STERILE†	HYBRID*	SELFED‡
1	30	19	4	7
2	12	8	2	2
3	15	10	2	3
4	13	7	3	3
5	17	12	2	3
6	14	7	3	4
TOTAL	101	62.4%	16%	21.8%

† Absence of asci and ascospores

* Contained ascospores that were virulent and avirulent to both cultivar Glenlea and line 6B365.

‡ Contained ascospores that were virulent to either cultivar Glenlea or line 6B365.

5.3.3 Analysis of asci

A total of twenty asci were selected from the hybrid pseudothecia, and full complements of eight ascospores were recovered from seven of these asci. Ascospores from each of the five asci (P8A5, P8A6, P8A9, P13A3, P15A1), three of which were from a single pseudothecium, segregated in a ratio of 1:1:1:1, for nec+chl+:nec+chl-: nec- chl+:nec-chl-, (Table 9), indicating segregation patterns for two gene pairs, each with two alleles. Two asci were selfs, containing ascospores that induced only tan necrosis. Full complements of eight ascospores were not obtained in the 13 other asci due to difficulties in separating ascospores from each other on the agar surface or to difficulties in picking up and successfully transferring the ascospores via the needle tip to V8PDA for sub-culturing. Many ascospores were pressed into the agar while attempting to pick them or fell off the tip of the needle when being transfered. Success in picking ascospores was increased by flaming the needle slightly and, after cooling, it was placed close to an ascospore which was then lifted electrostatically. Sometimes, separated ascospores were dragged to a clear section of the plate and a piece of medium carrying the spore was cut and transferred to V8PDA. Even when successfully transferred, some ascospores formed cultures which eventually sectored or formed colonies that did not produce conidia (Fig 6), thereby reducing the number of ascospores that could be tested for virulence. If less than 6 ascospores were recovered from an individual ascus, they were included in the pseudothecial analysis not in the ascal.

Table 8: Segregation for virulence amongst randomly isolated ascospores from hybrid pseudothecia in the cross between isolates 86-124 (nec+chl-) and Hy331-9 (nec-chl+) of *P. tritici-repentis.*

PSEUD[a]. PROB. #	PARENTAL		RECOMBINANTS		X^2	
	(nec+chl-)	(nec-chl+)	(nec+chl+)	(nec-chl-)		
P2	14	2	11	7	0.11	0.50-0.95
P3	16	3	12	4	0.25	0.50-0.95
P6	9	10	16	3	0.00	0.95-0.99
P8	9	11	6	2	5.14	0.01-0.05*
P10	8	10	14	1	0.27	0.50-0.95
P13	13	1	15	3	0.25	0.50-0.95
P15	6	12	9	5	0.50	0.10-0.50
Total	75	49	83	25	1.10	0.10-0.50

* Ratio of parental : recombinants differ significantly (5 % level) from the expected 1 : 1 ratio. Some, not all, ascospores did produce conidia.

[a] = pseudothecium

Table 9: Segregation for virulence amongst ascospores from five single asci in a cross between isolates 86-124 and Hy331-9 of *P. tritici-repentis.*

ASCUS P8A6

ASCOSPORE#	1	2	3	4	5	6	7	8
NECROSIS	+	-	-	+	+	-	+	-
CHLOROSIS	-	+	-	+	-	-	+	+

ASCUS P8A9

ASCOSPORE #	1	2	3	4	5	6	7	8
NECROSIS	+	+	+	-	-	-	-	+
CHLOROSIS	-	+	+	+	-	+	-	-

ASCUS P13A3

ASCOSPORE #	1	2	3	4	5	6	7	8
NECROSIS	+	-	-	+	+	+	-	-
CHLOROSIS	-	+	-	-	+	+	-	+

ASCUS P15A1

ASCOSPORE #	1	2	3	4	5	6	7	8
NECROSIS	-	+	-	+	-	+	+	-
CHLOROSIS	+	-	-	+	+	+	-	-

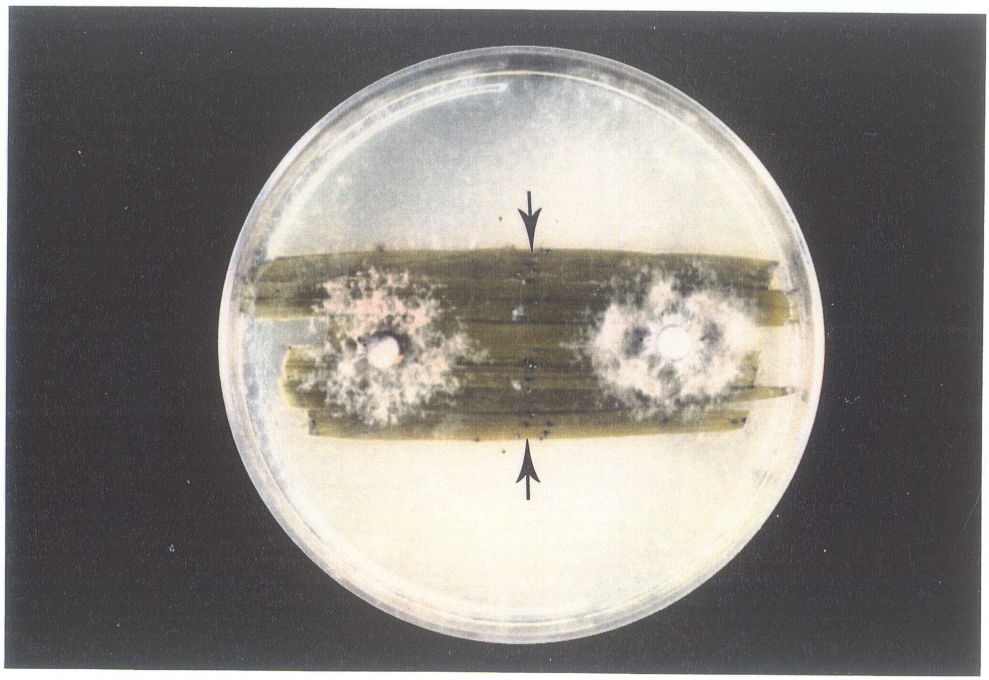
ASCUS P8A5

ASCOSPORE #	1	2	3	4	5	6	7	8
NECROSIS	+	-	-	+	-	+	+	-
CHLOROSIS	+	-	+	-	+	+	-	-

indicate ascospore number (un-ordered), + = Virulent reaction.
- = Avirulent reaction.

Fig. 6: Sectoring and degeneration (white area) in the otherwise normal gray appearing culture of one of the progeny derived from the cross of isolates 86-124 and Hy331-9 of *P. tritici-repentis.*

The two circles are areas where hyphal plugs were removed for subculturing.



5.3.4 **Backcross and inter-cross of F_1 progeny.**

Backcrosses of F_1 progeny types nec+chl+ and nec-chl- to either parent (86-124 nec+chl- or Hy331-9 nec-chl+) never formed a single line of pseudothecia at the line of junction. Pseudothecia were scattered over the entire surface of the plate with no clear pattern. Some replicates of the inter-crosses of the two types of progeny (nec+chl+ x nec-chl-) formed a single line of pseudothecia at the junction, but others did not. To improve on the chances of recovering recombinant ascospores, only the pseudothecia that formed along such lines of junction were analysed for ascospore fertility and virulence. Eighty nine percent of these pseudothecia were sterile as compared to 62.4% in the initial cross of isolates 86-124 x Hy331-9, and only 25% of the fertile pseudothecia were hybrids compared to 42% in the initial cross (Table 10). This indicates a possible increase in sterility and a decrease in number of hybrid pseudothecia with continued subculture of the strains of this fungus.

Analysis of the progeny from the inter-cross for virulence showed that the same four disease phenotypes as in the initial cross of isolates 86-124 and Hy331-9 were expressed. Random ascospores obtained from these pseudothecia segregated in a 1:1 ratio, parental (nec+chl+, nec-chl-) : recombinant (nec-chl+, nec+chl-) (Table 11) and ascospores from 3 individual asci segregated in 1:1:1:1 ratio for the four disease phenotypes (Table 12), supporting the previous interpretation that each of the two isolates has two loci, one controlling the ability to induce tan necrosis and a second locus controlling the ability to induce extensive chlorosis.

Table 10: Frequency of hybrid pseudothecia in the inter-cross of F_1 progenies (pathotype 1 and 4) from the cross of isolates 86-124 and Hy331-9 of *P. tritici-repentis* as indicated by the virulence phenotypes.

	Number of Pseudothecia.			
Plate #	Assayed	Sterile†	Hybrid‡	Selfed*
1	60	49	2	9
2	57	54	1	3
3	39	37	0	2
4	48	41	3	4
Total	204	89%	3%	9%

† Absence of asci and ascospores.

‡ Contained ascospores that were virulent and avirulent to both cultivar Glenlea and line 6B365.

* Contained ascospores that were virulent to either cultivar Glenlea or line 6B365.

Table 11 : Segregation for virulence amongst randomly isolated ascospores from hybrid pseudothecia in the inter-cross of F_1 progenies (pathotype 1 and 4) from the cross of isolates 86-124 and Hy331-9 of *P. tritici-repentis.*

Pseud#†.	Parental type		Recombinant type		Ratio 1:1	
	(nec+chl+)	(nec-chl-)	(nec+chl-)	(nec-chl+)	X^2	Prob.
P1	16	4	10	9	0.50	.50 - .70
P2	11	9	12	6	2.00	.10 - .20
P3	12	6	14	3	0.50	.50 - .70
P4	7	10	8	10	0.50	.50 - .70
P5	18	5	17	7	0.50	.50 - .70
Total	64	34	64	35	0.50	.50 - .70

† = Pseudothecium

Table 12 : Segregation for virulence amongst ascospores from three single asci in the inter-cross of F_1 progenies (pathotype 1 and 4) from the cross of isolates 86-124 and Hy331-9 of *P. tritici-repentis.*

ASCUS (IC) P1A4

Ascospore#	1	2	3	4	5	6	7	8
Necrosis	-	-	+	-	+	+	+	-
Chlorosis	-	+	+	-	+	-	-	+

ASCUS (IC) P3A7

Ascospore #	1	2	3	4	5	6	7	8
Necrosis	+	+	-	-	+	-	-	+
Chlorosis	+	+	+	-	-	+	-	-

ASCUS (IC) P3A1

Ascospore#	1	2	3	4	5	6	7	8
Necrosis	+	+	+	-	+	-	-	-
Chlorosis	-	-	+	-	+	+	-	+

indicate ascospore number (un-ordered), + = Virulent reaction
- = Avirulent reaction

5.3.5 Analysis of progeny for the production of Ptr-necrosis toxin.

To assess whether the factor for tan necrosis in recombinant progenies possessing virulence to both cv. Glenlea and line 6B365 (nec+chl+) was associated with the ability to produce Ptr-necrosis toxin in culture, cultures derived from individual ascospores from each of the eight asci (P8A5, P8A9, P13A3, P15A1, P8A6, IC P1A4, IC P3A7 and IC P3A1) were assayed for the production of Ptr-necrosis toxin. For each of the eight asci, four of the eight ascospore progenies tested produced Ptr-necrosis toxin (Tables 13 and 14). Each ascospore progeny that produced Ptr-necrosis toxin also induced tan necrosis (Tables 9, 12, 13 and 14). Those that did not produce Ptr-necrosis toxin did not induce tan necrosis. The Ptr-necrosis toxin produced by these progenies had no effect on either cv. Erik or Salamouni nor on line 6B365. The 64 ascospores segregated in a ratio of 1:1, Ptr-necrosis toxin producers : non-producers, indicating that a single gene pair controls the production of Ptr-necrosis toxin and that the ability to produce Ptr-necrosis toxin is associated with the ability to induce tan necrosis. This confirms previous findings by Lamari and Bernier (1989c) that sensitivity to the Ptr-necrosis toxin in the host is closely associated with susceptibility to tan necrosis.

Table 13: Segregation for production of Ptr-necrosis toxin amongst the ascospore progeny from five asci in a cross between isolates 86-124 and Hy331-9 of *P. tritici-repentis.*

ASCUS P8A6

ASCOSPORE #	1	2	3	4	5	6	7	8
TOXIN	+	-	-	+	+	-	+	-

ASCUS P8A5

ASCOSPORE #	1	2	3	4	5	6	7	8
TOXIN	+	-	-	+	-	+	+	-

ASCUS P8A9

ASCOSPORE #	1	2	3	4	5	6	7	8
TOXIN	+	+	+	-	-	-	-	+

ASCUS P13A3

ASCOSPORE #	1	2	3	4	5	6	7	8
TOXIN	+	-	-	+	+	+	-	-

ASCUS P15A1

ASCOSPORE #	1	2	3	4	5	6	7	8
TOXIN	-	+	-	+	-	+	+	-

+/- = presence or absence of Ptr-necrosis toxin in a culture filtrate when tested on cultivar Glenlea. # indicate ascospore number (un-ordered)

Table 14 : Segregation for production of Ptr-necrosis toxin amongst the ascospore progeny from three single asci in the inter-cross of F_1 progenies (pathotype 1 and 4) from the cross of isolates 86-124 and Hy331-9 of *P. tritici-repentis.*

ASCUS (IC) P1A4

Ascospore#	1	2	3	4	5	6	7	8
Toxin	-	-	+	-	+	+	+	-

ASCUS (IC) P3A7

Ascospore #	1	2	3	4	5	6	7	8
Toxin	+	+	-	-	+	-	-	+

ASCUS (IC) P3A1

Ascospore#	1	2	3	4	5	6	7	8
Toxin	+	+	+	-	+	-	-	-

indicate ascospore number (un-ordered)
+/- = Presence/absence of Ptr-necrosis toxin when tested on cultivar Glenlea.

5.4 Discussion

The development of a single line of pseudothecia at the line of junction of isolates 86-124 and Hy331-9 and the subsequent recovery of both recombinant and parental type ascospore progenies from individual asci and pseudothecia is significant in that it indicates the existence of mechanisms that regulate out-crossing and selfing in the population of this homothallic species. Analysis of ascospores from twenty individual asci indicated that five asci contained progenies that were either virulent to both cv. Glenlea and line 6B365 or avirulent to both, and that the virulence of the other ascospores was like that of the the parental strains. This shows that both nec+chl+ and nec-chl- progeny were present in individual asci as well as in individual pseudothecia and indicates conclusively that fertilization occurred between the two parental isolates. This is important since the presence of both selfed and crossed asci within a pseudothecium has been reported in *P. tritici repentis* (McCallum *et al.* 1994) and within a perithecium in *Cryphonectria parasitica* (Anagnostakis 1984, 1988) and a cleistothecium in *Aspergillus nidulans* (Pontecorvo *et al.* 1953).

Matings amongst the 12 isolates showed that isolates 86-124 and Hy331-9 formed pseudothecia consistently at the line of junction, from which progenies with recombined virulence were obtained. The inconsistent patterns of pseudothecia formation between most pairs of isolates indicated that there is an incompatibilty system operating in the population of this fungus.

The recovery of both the nec-chl- and nec+chl+ progeny in addition to the parental types from the cross of two virulent isolates 86-124 (nec+chl-) and Hy331-9

(nec-chl+), indicates that the parental isolates differ at two loci, and that there are two genes for virulence in each isolate. An allele of one gene pair induces tan necrosis (virulent) on cultivar Glenlea and the other induces small, dark-brown lesions (avirulent) on line 6B365. One allele of the other gene pair induces extensive chlorosis (virulent) on line 6B365 and the other induces small, dark-brown lesions (avirulent) on cultivar Glenlea. This explains the recovery of four different disease phenotypes from one meiotic event in the individual asci. Recombinant progenies that were obtained from the initial cross of the parental isolates and those that were obtained from the inter-cross of the F_1 progeny indicate that the four pathotypes can be reconstituted by sexual recombination.

The ratio of 1:1 for (parental : recombinant) segregation into random ascospores of a pseudothecium would be expected on the basis of ascal analysis or if this fungus was heterothallic. Since *P. tritici-repentis* is homothallic, hybrid pseudothecia contain both crossed and selfed asci and pseudothecial analysis would therefore not be expected to show a 1:1 ratio. Pseudothecial analysis indicated a 2:1:2:1 ratio for the four disease phenotypes which is not consistent with the hypothesis of independent loci. This may be due to the large number of selfed asci in the individual pseudothecia. Ascal analysis was consistent with a 1:1:1:1 ratio for the four disease phenotypes suggesting the involvement of two genes, each with two alleles. The sample size of the asci that were to be analysed was reduced by the difficulty involved in isolating all the eight ascospores of an ascus. It is not possible to conclude from such a small sample whether the two genes are inherited

independently. An improvement in the methodology of isolating single ascospores is required to increase the number of asci that can be analysed.

The occurrence of both out-crossing and selfing populations in the same fungal species was reported in *Cerastocystis ulmi* (Brasier and Gibbs 1975), *A. nidulans* (Pontecorvo et al. 1953), *Cryphonectria parasitica* (Anagnostakis 1987) and *Colletotricum graminicola* (Vaillancourt and Hanau 1991). For the occassional out-crossing mechanism to be effective in generating variation within a predominantly selfing population, out-crossed progeny would have to be competetively superior to the selfed progeny. Ascospores with combined virulence could become more successful pathogens by attacking cultivars that were previously resistant to one or the other of the virulence genes. The recovery of recombinant progenies from the cross of isolates 86-124 and Hy331-9 indicates the potential of *P. tritici-repentis* to evolve new virulence patterns that could overcome resistance genes in the previously resistant cultivars. Theoretically, if a mutation from avirulence to virulence occurred, recombination could associate the mutated gene with other virulence genes. The resultant progenies could cause severe epidemics, if they encountered monocultures of susceptible host plants. The virulent isolates could reproduce abundantly on the susceptible cultivars and release more inocula to the detriment of the less fit isolates. The severe epidemics that develop following the release of new virulence genes usually calls for renewed efforts and strategies to improve the resistance of desirable cultivars.

Investigations into the inheritance of host resistance to tan spot indicate that

resistance to tan necrosis induced by isolate 86-124 on cultivar Glenlea is controlled by a single recessive gene and that resistance to extensive chlorosis induced by isolate D308 on line 6B365 is controlled by a dominant or partially dominant gene (Lamari and Bernier 1991). The finding that two genes are involved in the virulence of *P. tritici-repentis* on the two differential cultivars of wheat suggests that there is possibly a "gene-for-gene" relationship in the wheat : *P. tritici-repentis* system, whereby the genes for resistance in the host are matched by avirulence genes in the pathogen. However, preliminary results from an going study (Duguid, S.; personal comm.) and a previous report by Nagle *et al.* (1982), indicate that resistance to tan necrosis in some cultivars is controlled by more than one gene. In breeding for resistance to tan spot, resistance regulated by a single gene may not remain effective forever since haploid recombinants with wider virulence may be able to overcome resistance genes in the previously resistant cultivars. More crosses and larger populations of asci are required to fully assess the potential for increased virulence on a greater number of resistant wheat cultivars, and for aggressiveness on a range of susceptible cultivars.

The effect of Host-Selective Ptr-necrosis toxin on colonization of wheat leaves by isolates of *Pyrenophora tritici-repentis.*

6.1 ABSTRACT

Isolates of *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat, are ascribed to one of four pathotypes (nec+chl+, nec+chl-, nec-chl+, and nec-chl-) based on their ability to cause necrosis (nec), chlorosis (chl) or a resistant reaction (small, dark-brown spots) on the differential cultivars of wheat. Necrosis inducing isolates produce a cultivar specific toxin *in- vivo* and *in-vitro*. Ptr-necrosis toxin was purified from culture filtrates of isolate 86-124 (nec+chl-) and tested at several concentration for its effect on colonization of wheat leaves by isolates of *P. tritici-repentis*. Various concentrations of toxin, alone or added to suspensions of spores of an avirulent, non - toxin producing isolate P8A6 3, and a virulent, toxin-producing isolate 86-124 were sprayed on detached leaves of the susceptible cultivar Glenlea and the resistant cultivar Salamouni. The leaves were visually rated for the development of tan necrosis and whole leaf pieces were examined microscopically every 6 h for the extent of fungal growth in leaf tissues. Untreated leaves, those that were inoculated with the spores of the avirulent isolate alone, and those that were sprayed with the toxin alone at less than 0.1µg/ mL, remained symptomless. Ptr-necrosis toxin at 0.1µg/mL applied alone or with spores of the avirulent isolate induced within 48 h a necrosis which was similar to that of the virulent isolate on the susceptible cultivar Glenlea. The initial stages of fungal development were the same on both the susceptible and resistant

cultivars. Differences in development between virulent and avirulent isolates were observed after 24 h post-inoculation when hyphae of the virulent isolate became established in the intercellular spaces of mesophyll of the susceptible cultivar Glenlea but not in the resistant cultivar Salamouni. Hyphae of the avirulent isolate were generally restricted to the epidermal cells of both cultivars. When Ptr-necrosis toxin was present at 0.1μg/ml, the avirulent isolate invaded and colonized the intercellular spaces of the mesophyll in the susceptible cultivar Glenlea but not of the resistant cultivar Salamouni and induced symptoms as rapidly as the virulent isolate. Leaves of the resistant cultivar Salamouni were not colonized by either the virulent toxin-producing isolate 86-124 or the avirulent isolate P8A6 3 supplemented with Ptr-necrosis toxin. This suggests that host resistance to tan necrosis is based on insensitivity to the toxin produced by the necrosis inducing isolates. These results support the hypothesis that Ptr-necrosis toxin is a primary factor in the virulence of the necrosis inducing pathotypes.

6.2 INTRODUCTION

Pyrenophora tritici-repentis (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) is a stubble borne pathogen that causes tan spot of wheat in several regions of the world including the central plains of North America (Hosford 1971, 1982; Takauz 1976). *P. tritici-repentis* infects many grass species (Krupinsky 1982) and has the widest host range of any described *Pyrenophora sp.* (Shoemaker 1962).

Recent studies have shown that the tan spot syndrome consists of two phenotypes: tan necrosis and chlorosis (Lamari and Bernier 1989a, b). These are each the result of specific interactions between individual isolates of the fungus and wheat genotypes. Susceptible wheat lines develop tan necrosis and/or extensive chlorosis, whereas resistant lines develop small, dark-brown lesions without accompanying tan necrosis or extensive chlorosis. Resistance to tan necrosis is recessive and resistance to extensive chlorosis varies from dominant to partially dominant. Each of these reactions is controlled by a single gene (Lamari and Bernier 1991). Susceptibility of wheat genotypes to necrosis inducing isolates and sensitivity to the Ptr- necrosis toxin released specifically by these isolates are controlled by the same dominant gene (Lamari and Bernier 1989c, 1991).

Isolates of *P. tritici-repentis* are ascribed to one of four pathotypes based on their ability to induce, on appropriate differential cultivars, tan necrosis and extensive chlorosis (Lamari and Bernier 1989c). Isolates from pathotype 1 induce both chlorosis and necrosis (nec+chl+), pathotype 2 isolates only induce necrosis (nec+chl-),

pathotype 3 isolates only induce chlorosis (nec-chl+), and pathotype 4 isolates lack the ability to induce either symptom (nec-chl-) and are classified as avirulent.

Genetic analysis of this homothallic fungus was difficult until recently when mutants resistant to the fungicide iprodione or the antibiotic hygromycin B were generated in isolates 86-124 (nec+chl-) and D308 (nec-chl+) through ultraviolet light mutagenesis, and then used in sexual crosses (McCallum *et al.* 1994). The mutants were crossed in all possible combinations and the progeny analysed to determine the genetic basis of chemical resistance. Hybrid progenies were obtained in crosses involving mutants of isolate 86-124 but not of D308. The results indicated that one locus controlled iprodione resistance and a second locus controlled hygromycin B resistance.

Investigations on the possibility of crossing isolates from different pathotypes as well as the determination of the genetic basis of virulence and the production of Ptr-necrosis toxin, showed that sexual crosses were possible between isolate 86-124 (nec+chl-) and Hy331-9 (nec-chl+). In crosses between the necrotic and chlorotic isolates, four classes were found in the progeny on the basis of lesion type induced on differential cultivars; two parental and two recombinant classes. The two recombinant classes were comprised of one class whose strains induced both tan necrosis and extensive chlorosis (nec+ chl+) while the strains of the second class induced small, dark-brown spots (nec- chl-) (in section 2 of this thesis).

Previous investigations on the infection process of *P. tritici-repentis* (Lamari and Bernier 1989b; Larez *et al.* 1986; Loughman and Deverall 1986) have indicated

that a susceptible host reaction was expressed only after the fungus had grown intercellularly in the mesophyll tissue of the host. Larez *et al.* (1986) and Dushnicky (1993) reported that the fungus affected wheat host mesophyll cells beyond the hyphae long before the hyphae came into contact with mesophyll tissue. The effect was characterized by the disruption and deformation of mesophyll cells and chloroplast breakdown. The intercellular hyphae were never observed to penetrate the mesophyll cells. These observations suggested the involvement of toxic metabolites in the disruption of the mesophyll tissue. The above studies utilized combinations of virulent isolates alone with both susceptible and resistant host genotypes. Differences in infectivity of avirulent and virulent isolates and the effect of Ptr-necrosis toxin on pathogenicity of these isolates were not investigated.

Investigations on Ptr-necrosis toxin have been focussed on its purification and characterization (Ballance *et al.* 1989; Lamari and Bernier 1989; Tomas and Bockus 1987; Tomas *et al.* 1990). The Ptr-necrosis toxin has been isolated from culture filtrates of necrosis inducing isolates initiated from mycelia or conidia, and intercellular washing fluids of wheat leaves infected with isolates which induce tan necrosis but not from those which do not. It is not known whether Ptr- necrosis toxin is required in initiating early or late events of the infection process of nec+ isolates. Presently, there is no information as to whether avirulent recombinants, which lost the ability to produce toxin and induce tan necrosis, would exhibit virulence similar to that of the necrosis inducing parent, if provided with an exogenous supply of Ptr- necrosis toxin. The objective of this study was to determine the effect of Ptr-necrosis toxin on

colonization of wheat leaves by isolates of *P. tritici-repentis.*

6.3. MATERIALS AND METHODS

6.3.1 Wheat cultivar/lines

Seedlings of the differential wheat cultivars Glenlea (susceptible to necrosis) and Salamouni (resistant) were grown in 7.5 x 7.5 cm milk cartons containing soil-less mix (metromix 200, W.R Grace and Co: Ajax, Ontario) in growth chambers at 18-20 ^{0}C with a 16 h photoperiod. Seedlings were grown until they reached the two leaf stage. Leaves were detached and placed on moist paper towels placed in 5.5 x 7.5 cm transparent plastic boxes. Three leaves of the susceptible cultivar Glenlea were placed at one end of the box, and three leaves of the resistant cultivar Salamouni were placed at the opposed end. Each box was treated with a spore suspension and/or toxin, and was replicated three times.

6.3.2 The fungus

Avirulent recombinant strains P8A6 3 and P8A6 6 (nec-chl-) previously obtained from the cross of isolates 86-124 (nec+ chl-) and Hy331-9 (nec-chl+) and their two parents (thesis section 2) were selected for use in this experiment. Avirulent isolate 90-2 (nec-chl-), which induces small dark-brown spots on both differential wheat lines (Lamari *et al.* 1991) was included as a control.

6.3.3 Inoculum production

Mycelial plugs of isolates 86-124, 331-9, 90-2, P8A6 3 and P8A6 6 were transferred onto V8PDA (150 mL V8 vegetable juice, 10 g calcium carbonate, 10 g

Bacto agar, 850 mL water) and incubated for 4 d at 20 ^{0}C in the dark or until the colonies were 3-4 cm diameter. The plates were flooded with sterile distilled water and the mycelia flattened with the bottom of a flamed test-tube as described by Lamari and Bernier (1989a, b). The water was decanted and the cultures subjected to a regime of light (18 h) and dark (18 h). Sterile distilled water was added to each culture and the conidia dislodged using a wire loop. The resulting spore suspension was adjusted to $5x10^3$ conidia/mL using a cell counter (Hauser Sc. Blue, Bell, Pa). A drop of Tween 20 (Polyoxyethlenesorbitan monolaurate) was added to the spore suspension to reduce surface tension.

6.3.3 **Ptr-necrosis toxin**

Ptr-necrosis toxin was purified from culture filtrates of isolate 86-124 according to Ballance *et al.* (1989), and tested at several concentrations for its effect on colonization of wheat leaves by isolates of *P. tritici-repentis*. Ptr-necrosis toxin was added to water or to spore suspensions ($5x10^3$conidia/ml) of both nec+ and nec- isolates to yield a final toxin concentration of (0, 0.001, 0.01, 0.1 and 1 µg/mL). Two ml of each toxin concentration in water or spore suspension was atomized onto the surface of detached leaves placed on moist paper towels in transparent plastic boxes. Spore suspensions without the toxin and toxin without spores were used as controls. Inoculated leaves were incubated in moist plastic chambers at 22/18^{0}C (day/night) and a 16 h photoperiod. The reactions of the leaves were scored every 12 h. Treated leaf samples were taken every 6 h and stained in cotton blue (8 ml 95% ethanol, 1 ml 85% lactic acid, 1 ml 90% phenol, 1 ml glycerine, 1 ml water and 4 mg

cotton blue (Clark 1981) for microscopic observation. The leaf pieces were mounted in lactophenol and observed with a compound microscope for conidial germination, appressorium formation and for the extent of hyphal development into leaf tissues.

6.4. RESULTS.

Cultivar response to Ptr-necrosis toxin and strains of the fungus

Untreated leaves, and those that were inoculated with spores of avirulent (nec-) isolates or toxin at less than 0.1 µg/mL, remained symptomless up to 96 h, when they turned chlorotic due to senescence. Leaves of the susceptible cultivar Glenlea inoculated with a suspension of spores of avirulent (nec-) isolate to which Ptr- necrosis toxin was added at a concentration of 0.1 µg/mL, developed necrosis within 48 h, similar to that induced by isolate 86-124 alone (Fig. 7, Table 15). Leaves of the resistant cultivar Salamouni developed small, dark-brown spots in response to infection by all isolates, and leaves of the susceptible cultivar Glenlea developed small, dark-brown spots in response to infection by nec- isolates only. Ptr-necrosis toxin alone at 1 µg/mL or spores of the virulent isolate (86-124) induced necrosis within 24 h in the susceptible but not in the resistant cultivar. Necrosis appeared 6 h earlier and was more severe in the susceptible cultivar when Ptr-necrosis toxin was added to the spore suspensions of the virulent isolate 86-124. Addition of Ptr-necrosis toxin to isolate 86-124 had no effect on the resistant cultivar Salamouni.

Microscopic observations.

Spores of both isolates (avirulent and virulent) had 100% germination rate on the leaf surfaces of both cultivars, and formed appressoria within 24 h. Most of the appressoria formed above the junctions of epidermal cell walls but some formed above epidermal cells or over stomata. In all combinations of fungal isolates and host cultivars, the initial stages of fungal development were the same in both the susceptible and resistant leaves. Differences between virulent and avirulent isolates were observed after 24 h post-inoculation when hyphae of the virulent isolates became established in the intercellular spaces of the mesophyll of the susceptible cultivar Glenlea and not in the resistant cultivar Salamouni. Hyphae of the avirulent isolates were confined to the epidermal cells of both the resistant and susceptible cultivars with very limited growth into the mesophyll (Fig. 8). Further growth in leaf tissues by avirulent isolates stopped less than 24 h after inoculation.

When Ptr-necrosis toxin was added at a concentration of 0.1 μg/mL or higher, hyphae of the avirulent isolates invaded and colonized intercellular spaces of the mesophyll of the susceptible cultivar within 24 h, and produced symptoms similar to those of the virulent isolate alone and/or toxin at 1μg/mL. Tan necrosis appeared on the leaves of the susceptible cultivar Glenlea exposed to Ptr-necrosis toxin at 0.1μg/ml or higher, but no mycelial growth was observed inside the leaf tissue (Table 16).

Table 15: Effect of concentration of Ptr-necrosis toxin and incubation period on induction of tan necrosis in the susceptible cultivar Glenlea and on the pathogenicity of avirulent isolate P8A6 of *P. tritici-repentis.*

Incubation period (hr)	Ptr-necrosis toxin concentration(μg/ml)							Isolate alone	
	0.001		0.01		0.1		1	86-124	P8A6
	W	WT	W	WT	W	WT	WT		
6	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	+	+	-
30	-	-	-	-	-	-	+	+	-
36	-	-	-	-	-	-	+	+	-
42	-	-	-	-	+	-	+	+	-
48	-	-	-	-	+	+	+	+	-

\+ = necrosis
\- = absence of necrosis
W = with spores of P8A6
WT = without spores of P8A6
86-124 (nec+chl-)
P8A6 (nec-chl-)

Table16: Effect of Ptr-necrosis toxin on virulence of isolates of *P. tritici-repentis.*

Isolate	Toxin (µg/ml)	Host Reaction		Extent of fungal growth in leaf tissues[b]	
		Glenlea	Salamouni	Glenlea	Salamouni
86-124	0.1	+++	+	++	+
86-124	0	++	+	++	+
331-9	0.1	+++	+	++	+
331-9	0	+	-	+	+
90-2	0.1	+++	-	++	+
90-2	0	-	-	+	+
P8A6	0.1	+++	-	++	+
P8A6	0	-	-	+	+
WS	0.1	++	-	-	-
water	0	-	-	-	-

- = no visible symptoms after 48 hrs
\+ = small dark-brown spots
++ = tan necrosis
+++ = very severe tan necrosis
WS = toxin alone

b) Evaluated by examining hyphal growth under light microscope.
- = no mycelial growth
\+ = spore germination and infection peg within epidermal cells.
++ = abundant intercellular mycelial development within leaf tissue.

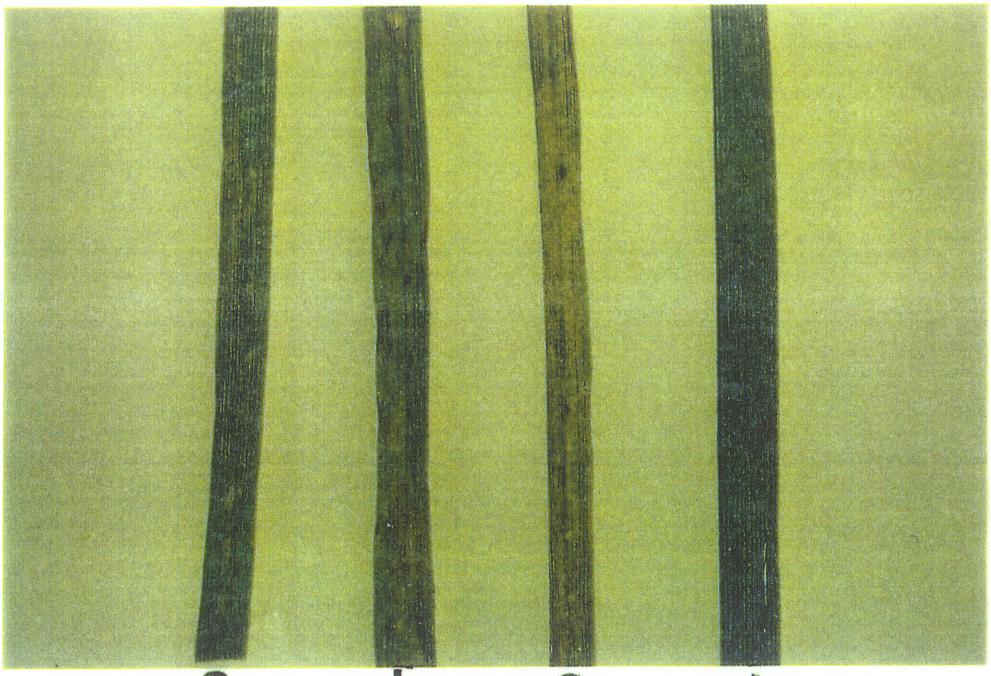
Fig 7: Effect of Ptr-necrosis toxin supplementation on the virulence of isolates of *P. tritici-repentis.*

a) Tan necrosis on the susceptible cultivar Glenlea, sprayed with 1 µg/ml of ptr-necrosis toxin alone, 48 h post-inoculation

b) Tan necrosis on the susceptible cultivar Glenlea, inoculated with spores of the necrosis inducing isolate 86-124 (nec+chl-) alone, 48 h post-inoculation.

c) Tan necrosis on the susceptible cultivar Glenlea, inoculated with a suspension of spores of the avirulent, non-toxin producing isolate (P8A6 3), together with Ptr-necrosis toxin at 0.1 µg/ml, 48 h post-inoculation.

d) Small, dark-brown spots on the susceptible cultivar Glenlea, inoculated with spores of the avirulent, non-toxin producing isolate (P8A6 3) alone, 48 h post-inoculation.



97g

Fig 8: Extent of hyphal growth of the avirulent (P8A6) and the virulent (86-124) isolates of *P. tritici-repentis* in the resistant and susceptible cultivars of wheat.

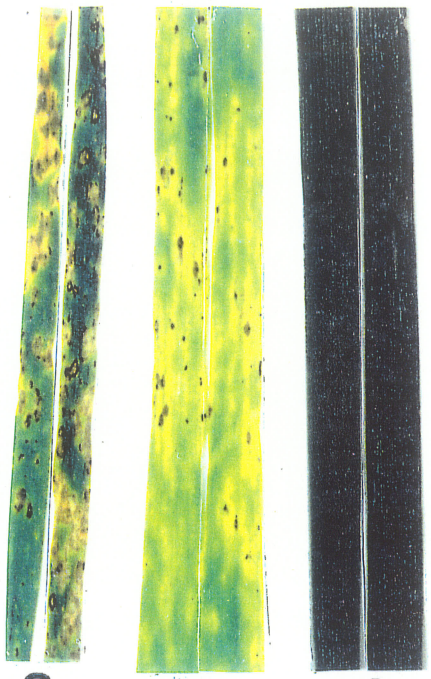
a) Whole leaf mount showing germinated conidia with germ tubes from basal cells of the avirulent isolate, b) the virulent isolate on the resistant cultivar Salamouni 6 h post-inoculation.

c) Whole leaf mount showing infection of epidermal cells of the susceptible cultivar Glenlea by the avirulent isolate, 12 h post-inoculation. Note formation of papilla beneath the epidermal cell in response to infection by avirulent isolate.

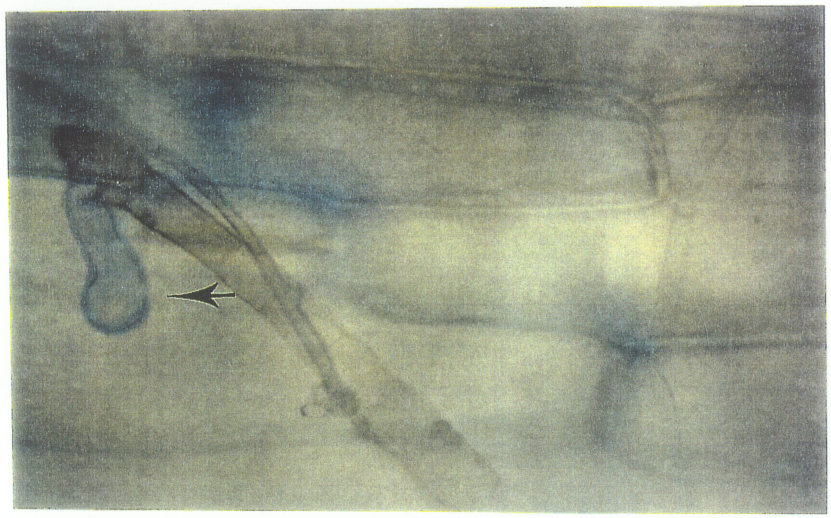
d) Whole leaf mount showing formation of appressorium by the virulent isolate 86-124 in epidermal cell of the susceptible cultivar Glenlea, 18 h post-inoculation.

e) Whole leaf mount showing hyphae of the avirulent isolate restricted to epidermal cells of the susceptible cultivar Glenlea in the absence of Ptr-necrosis toxin.

f) Whole leaf mount showing hyphae of the avirulent isolate growing beside the small midvein and through the epidermal layer into intercellular spaces of the mesophyll of the susceptible cultivar Glenlea in the presence of 1μg/ml of Ptr-necrosis toxin 24 h post-inoculation.


a
b
c

d



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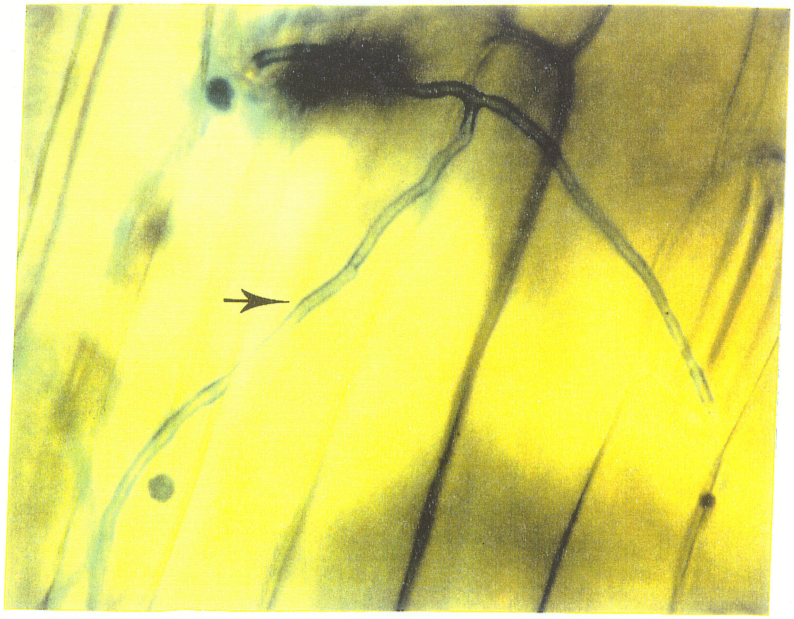

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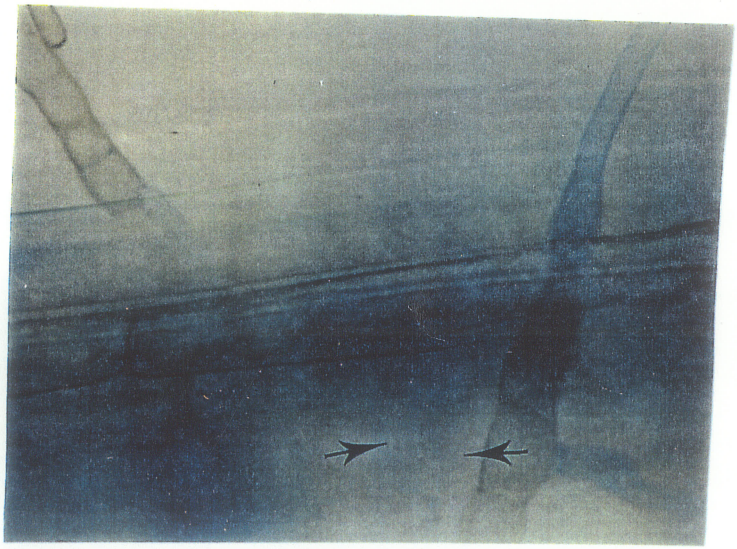

6.5 DISCUSSION

The major objective of this study was to determine the effect of Ptr-necrosis toxin on colonization of wheat leaves by isolates of *P. tritici-repentis*. The results of this study established that, in the absence of Ptr-necrosis toxin, avirulent (nec-) isolates failed to colonize the intercellular spaces of the mesophyll tissue of both the resistant and susceptible cultivars. However, by adding an exogenous supply of Ptr-necrosis toxin produced by the virulent isolate 86-124, the avirulent isolates became as virulent as the necrosis inducing isolate.

When Ptr-necrosis toxin was sprayed alone on the susceptible cultivar Glenlea at a concentration as low as 0.1μg/ml, it induced a charateristic tan necrosis within 48 h, and at a higher concentration of 1 μg/ml, symptoms developed within 24 h. Applications of Ptr-necrosis toxin at 0.1μg/ml with spores of the virulent isolate enhanced the severity of the necrosis.

The present study and genetic evidence presented in thesis section 2, show that Ptr-necrosis toxin is required for the colonization of the susceptible cultivars. Cultivar Glenlea, susceptible to isolate 86-124 and sensitive to Ptr-necrosis toxin produced by this isolate, was colonized by the nec- isolate only when supplemented with Ptr-necrosis toxin. Leaves of cultivar Salamouni, resistant to *P. tritici-repentis* were not colonized by isolate 86-124 nor by the nec- isolates supplemented with Ptr-necrosis toxin, suggesting that resistance to the fungus is based on the insensitivity to the toxin produced by the fungus. These observations support the results of Lamari

and Bernier (1989c, 1991), that susceptibility of wheat genotypes to necrosis inducing isolates and sensitivity to the Ptr-necrosis toxin released specifically by these isolates are controlled by the same dominant gene.

The early steps of the infection process were reported to be non-specific events in the wheat/*P. tritici-repentis* system. No significant differences were observed between compatible and incompatible interaction, until the invading hyphae had reached the intercellular spaces of the mesophyll (Loughman and Deverall 1986; Lamari and Bernier 1989b, Larez *et al.* 1986). The effect was characterized by the disruption and deformation of the mesophyll cells and breakdown of chloroplasts. This disruption and breakdown was attributed to a metabolite suspected to be released by the fungal isolates (Dushnicky 1993). These studies utilized combinations of virulent isolates with susceptible and resistant host genotypes but did not investigate the differences in the virulence of avirulent and virulent isolates and the effect of Ptr-necrosis toxin on virulence of these isolates.

Results similar to those of the present study were reported by Comstock and Scheffer (1973) who showed that, when small amounts of *Helminthosporium carbonum* (HC) toxin were added to the nonpathogen (*H. victoriae*) or to an avirulent *H. carbonum* isolate which does not produce toxin, the isolates developed throughout the susceptible leaves just as rapidly as the pathogenic or virulent ones.

The results of the present study imply that, if nec- isolates were supplemented with Ptr-necrosis toxin, they could infect and colonize ptr-necrosis toxin sensitive cultivars to which they were previously avirulent. This could occur in nature if nec-

and nec+ isolates were co-inoculated on the sensitive host cultivars. The *in-vivo* production of Ptr-necrosis toxin by nec+ isolates could enable the nec- isolates to acquire the ability to infect and colonize cultivars to which they were previously avirulent. However, it is not known whether Ptr-necrosis toxin is required in initiating early or late events of the infection process by nec+ isolates. The search for and the production of anti-Ptr-necrosis toxin molecules could assist in the control of tan spot in wheat. Spraying of the susceptible host genotypes with anti-Ptr-necrosis toxin molecules as crop protectants could reduce the risk of epidemics caused by *P. tritici-repentis.*

7. GENERAL DISCUSSION

The major goal of this study was to provide information on the potential development of isolates of *P. tritici-repentis* with increased virulence. Two approaches were employed to obtain this information. The first was by examining the possibility of obtaining variants through asexual processes of anastomosis and heterokaryon formation, and the second was by sexual recombination. Results of the first study demonstrated that two mutants (86-124ipr+ and D308hgm+) of the four that were paired, were vegetatively compatible, and formed heterokaryons. Formation of the heterokaryon between these two mutants indicated the ability of two or more genetically different nuclear types to associate in the same mycelium. The phenotype of such a heterokaryotic mycelium would be expressed as the sum total of all the nuclear types present in the mycelium, and could suppress the expression of the individual genotypes. The presence of previous virulence genotypes or of new genotypes brought about by mutation in such heterokaryons would constitute a genetic pool from which new genotypes could be released by dissociation, in appropriate selective environments. Vegetative compatibility and heterokaryon formation ensures genetic exchange within the population, while vegetative incompatibility restricts such exchanges, resulting in the formation of different clones.

The results of this study demonstrated that a heterokaryon did form as a result of pairing two mutants of *P. tritici-repentis*, that it dissociated, but that new types were not obtained. Heterokaryosis as a mechanism of variation could be useful if different nuclear types associated in the same mycelium, and then through the

parasexual cycle, produced new genotypes. Dissociation of the heterokaryon would then allow the expression of the individual genotypes.

In the present study, two types of heterokaryons were obtained on the basis of mycelial growth and the ability to sporulate: one with reduced mycelial growth and no sporulation, and the other with normal mycelial growth and reduced sporulation. The relevance of these two types to the pathogen was not addressed but, the heterokaryon with reduced mycelial growth and no sporulation would not be expected to compete effectively with the heterokaryon with normal mycelial growth and reduced sporulation. The latter could out-compete the former for nutrients due to its faster growth rate and by colonization of large areas of the substrate including susceptible host cultivars. In both the pathogenic and saprophytic phases of *P. tritici-repentis*, the sporulating heterokaryon could reproduce and multiply to the exclusion of the non-sporulating type.

The large size of fungal populations coupled with their high rates of reproduction suggests that even a low frequency of anastomosis and heterokaryosis could lead to significant changes in variation within the population of a fungus. The formation of a heterokaryon in *P. tritici-repentis* followed by the release of a new virulence genotype through dissociation may not be advantageous to the pathogen if the new virulence genotype is released in an environment comprised of fully susceptible wheat cultivars or grass hosts. The new virulence genotype would have to compete against existing isolates to be able to infect the host. However, the presence of a newly introduced resistence genotype in the host population would offer a

selective advantage to a new virulence genotype over the existing genotypes of the pathogen which would not be able to infect the new host genotype. The new virulent genotype would grow and produce a large quantity of inoculum that could lead to a rapid buildup of disease particularly in crops which are grown as monocultures of uniformly susceptible genotypes, and over wide areas. The occurrence of such a process in nature would put added pressure on breeding and pathology programs to monitor the population of the pathogen for variation in virulence. Such information would be essential to the development of wheat cultivars with effective and durable resistance.

The study on the role of Ptr-necrosis toxin in the expression of virulence by isolates of *P. tritici-repentis* was based on the hypothesis that the toxin enhances the colonization of host tissues by isolates of this fungus. The results indicated that Ptr-necrosis toxin is a primary factor for infection and colonization of toxin sensitive cultivars. When supplemented with Ptr-necrosis toxin, nec- isolates acquired the ability to infect and colonize the toxin sensitive cultivar Glenlea, but not the toxin insensitive cultivar Salamouni. The enhancement of the colonization of host tissues by supplementation of nec- isolates with Ptr-necrosis toxin, indicated that the toxin is used to disrupt and disorganize living host tissues, so that nutrients leach out and become available for fungal growth and subsequent colonization of the host tissues. Ptr-necrosis toxin might also be used by the fungus to exclude other organisms including saprophytes from the substrate. *P. tritici-repentis* has a saprophytic phase, but the ecological significance of Ptr-necrosis toxin in saprophytism of this fungus has

not been investigated. This could be determined by assessing the sensitivity of other leaf spot pathogens of wheat as well as several saprophytic fungi normally found on wheat straw for sensitivity to Ptr-necrosis toxin in culture as well as on straw.

The results of this study also suggest another approach to assessing the potential of *P. tritici-repentis* to generate variation, that is by the co-inoculation of nec+ isolates with nec- isolates on a toxin sensitive host cultivar. The *in-vivo* production of Ptr-necrosis toxin by nec+ isolates could enable the nec- isolates to infect and colonize host cultivars to which they were previously avirulent. However, it is not known whether Ptr-necrosis toxin is required in initiating early or late events of the infection process by nec+ isolates. In nature, the chance co-inoculation of nec+ and nec- isolates of *P. tritici-repentis* could lead to the association of different nuclear types in the same mycelium and, thus to variation through either anastomosis and heterokaryon formation or through sexual recombination.

The possibility of obtaining variants in *P. tritici-repentis* through sexual recombination was addressed in a separate study. Anastomosis and heterokaryon formation provide an opportunity for the protoplasm and nuclei of different genotypes including mating types to associate in the same cell. Heterokaryon formation is the first step in out-crossing in homothallic and imperfecti fungi, whether in sexual or parasexual cycles (Pontercovo *et al.* 1953; Leslie 1993). Sexual recombination could fail due to either nuclear or cytoplasmic post-fusion incompatibility factors, resulting in sterility. Sterility in fungi is associated with a number of genetic factors known to block the sequence of sexual processes at various steps from vegetative mycelium to

ascospore formation (Wheeler 1954; Nelson 1959; Welz and Leonard 1994). Based on the dissociation of the heterokaryon as demonstrated in the first study, and the inconsistent formation of pseudothecia in most of the isolate pairs as shown in this study, it would appear that there is a multi-step or mult-faceted incompatibility system regulating out-crossing in the population of this fungus. The large proportion (65%) of sterile pseudothecia obtained from the pair of isolates 86-124 and Hy331-9 indicates that there are genetic factors influencing additional steps of ascospore formation in *P.tritici-repentis.*

In homothallic fungi including *P. tritici-repentis*, when two strains are grown in mixed culture, the ascospores from any single ascus may derive their nuclei from a selfed zygote of one strain or the other, or from crossed zygotes (Pontecorvo *et al.* 1953). In the present study, ascospores were analysed in two ways; by sampling random asospores from pseudothecia, and by isolating all the 8 ascospores of an ascus from a pseudothecium shown to contain hybrid ascospores on the basis of virulence phenotypes. Sixteeen percent only of pseudothecia taken from the line of junction between isolates 86-124 and Hy331-9 were hybrids, since they contained recombinant ascospores as evidenced by the recovery of new virulence phenotypes, and were thus the result of fertilization. In the same hybrid pseudothecia, even when some asci were truely hybrid, others were not. Sampling of random ascospores from a pseudothecium only indicates the presence of new virulence phenotypes, and the results can not be used to determine ratios of various phenotypes because of the presence of a large number of selfed asci. However, in fungi like *P. tritici-repentis* which have hyaline

ascospores and for which colour markers are not available, sampling of random ascospores from individual pseudothecia is a useful and necessary method for the identification of hybrid pseudothecia. Thus, analysis of ascospores from asci obtained from known hybrid pseudothecia is presently the only method that allows the determination of genetic ratios of virulence phenotypes and the number of genes involved.

The recovery of recombinant progeny (nec+chl+ and nec-chl-) from the initial cross of the parental isolates indicates that there is potential for *P. tritici-repentis* to evolve new virulence patterns through sexual recombination. These new virulence types could infect cultivars that were previously resistant to one or the other of the virulence genes. In the present study, recombinants that were virulent and avirulent to both cultivar Glenlea and line 6B365 were obtained from the cross of isolates 86-124 and 331-9, but progeny with increased virulence (i.e with ability to infect resistant cultivars, Erik and Salamouni) were not recovered, indicating that recombination may not necessarily create new genotypes (or does so at very low frequencies) but merely reshuffle existing ones. Unless there are suppressive or epistatic factors to mask the expression of some of the virulence genes, all genes that are constitutively available may be fully expressed. More crosses and larger populations of asci will need to be used to fully assess the potential for increased virulence in *P. tritici-repentis.* The development of fungal isolates with additional markers and the use of molecular techniques will be required to assist in identifying recombinant genotypes. Progenies will also need to be tested for new virulence patterns on a greater number of resistant

wheat cultivars, and for aggressiveness on a range of susceptible cultivars in order to effectively assess the generation of new genotypes.

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APPENDIX 1

SACHS' NUTRIENT AGAR

$CaCO_3$	4.0 g
$CaNO_3$	1.0 g
K_2HPO_4	0.25 g
$MgSO_4$	0.25 g
FeCl	trace
Agar	20 g
Dist. H_2O	1000 mL

Medium must have PH 5.3 after autoclaving.

Reference: Shoemaker, R. A. 1955. Can. J. Bot. 33: 562-576.

APPENDIX 2

FRIES MEDIUM

NH_4tartrate	5.0 g
NH_4NO_3	1.0 g
$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	2.6 g
KH_2PO_4	1.3 g
Sucrose	30.0 g
Yeast extract	1.0 g
Trace element soln.	2 mL
H_2O	1000 mL

Stock solution/L.

LiCl	167 mg
$CuCl_2.H_2O$	107 mg
H_2MoO_4	34 mg
$MnCl_2.4H_2O$	72 mg
$CoCl_2.4H_2O$	80 mg

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