Generation and Utilization of Phenotypic Mutants in the Tan Spot Fungus (*Pyrenophora tritici-repentis*)

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Brent D. McCallum

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Forward

This thesis is written in manuscript style as outlined by the Department of Plant Science of the University of Manitoba. It contains one manuscript which includes an abstract, introduction, materials and methods, results and discussion. The manuscript is preceded by a general introduction and literature review, and followed by a general discussion, literature cited and appendices.

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General Introduction

Tan spot of wheat is caused by the ascomycete Pyrenophora tritici-repentis (Died.) Drech. (anamorph Drechlera tritici-repentis (Died.) Shoem.). Tan spot is widespread throughout the wheat growing areas of Manitoba and Saskatchewan (Tekauz, 1976), and is a major leaf spotting disease world wide (Dubin, 1983; Hosford, 1971; Mehta and Almeida, 1977; Misra and Singh, 1972; Tekauz, 1976; Valder and Shaw, 1952). P. tritici-repentis has the widest host range of any species within the genus Pyrenophora (Shoemaker, 1962), being pathogenic on 33 plant species (da Luz and Hosford, 1980), and is the most common leaf spot pathogen of the native prairie grasses (Krupinsky, 1987). Yield losses due to tan spot vary with the inoculum level, the degree of host resistance, duration and quantity of the post inoculation moisture, and the growth stage of the host at the time of inoculation (Shabeer and Bockus, 1988; Hosford et al., 1987). Wheat yields are reduced between 5% and 15% where inoculum levels are adequate and up to 49% in epidemics (Hosford and Busch, 1974; Rees et al., 1982).

P. tritici-repentis overwinters and produces ascospores, the primary source of inoculum, on wheat stubble. With the recent shift from conventional to conservation tillage, inoculum levels and disease incidence

of tan spot have been increasing (Pfender et al., 1988). One possible control measure for tan spot is burying infected crop residue because the fungus survives poorly on buried stubble (Pfender and Wootke, 1987). This practice, however, leads to soil erosion (Raymond et al., 1985). Tan spot control can also be achieved by the use of fungicides, but the cost may be prohibitive (Tekauz et al., 1983).

Genetically controlled resistance has been reported in a number of different wheat lines (Cox and Hosford, 1987; Hosford, 1982; Lamari and Bernier, 1989b; Raymond et al., 1985). It is difficult to measure the cost of producing resistant varieties, but breeding wheat for rust resistance has proven to be a cost effective means of control over many years (Green and Campbell, 1979).

Understanding the genetics of P. tritici-repentis may provide both an insight into breeding for durable resistance and promote a basic understanding of the mechanisms of variation in this fungus. Genetic studies on P. tritici-repentis require easily-scored genetic markers in order to distinguish hybrid progeny from selfed progeny because all isolates are homothallic.

Chemical resistance was the chosen marker system because large populations of mutagen treated spores can be rapidly screened for chemical resistance. Chemicals were selected that completely inhibited the growth of P. tritici-repentis in culture, then mutants resistant to these

chemicals were generated by U.V. mutagenesis. Mutants were tested for stability of chemical resistance, and virulence and toxin production to ensure that the mutagen treatment did not affect pathogenicity. Mutant and wild type isolates were paired in different combinations to determine if hybrid pseudothecia could be identified using genetic markers, and to determine the genetic basis of the chemical resistance markers.

Literature Review

2.1 Taxonomy Pyrenophora tritici-repentis is grouped in the kingdom Fungi, division Eumycota, subdivision Ascomycotina, class Loculoascomycetes, order Pleosporales, and family Pleosporaceae (Ainsworth et al., 1973). Conidia of its anamorph, Dreschlera tritici-repentis, are cylindrical, divided into five to seven cells, have a conical shaped basal cell, and measure (75)95-165(255) X (12)14-18(20) μm. Pseudothecia of P. tritici-repentis are black and produce abundant eight-spored asci, and vary in size from 200-700 μm (Pfender et al., 1988). Asci are bitunicate, cylindrical, and are narrow near the base. Ascospores are hyaline at maturity, have three transverse septa and one longitudinal septum, and measure (42)47-65(69) X (15)20-26(29) μm (Shoemaker, 1962).

2.2 Disease Cycle

P. tritici-repentis overwinters as pseudothecia on straw and stubble of wheat and other gramineae hosts, and these produce ascospores in the spring that act as the primary inoculum source (Hosford, 1971). Early season infection is largely initiated from inoculum formed on nearby gramineous stubble because ascospores are not readily wind dispersed. However, throughout the growing season conidia are produced from sporulating lesions on infected

plants, and these are wind dispersed to virtually all fields, but the severity of the disease is greatest in crops planted on gramineous stubble (Rees and Platz, 1980). Maximum conidial production occurs in a temperature range between 10-25^{0}C (Platt et al., 1977), with high relative humidity being essential for conidiation (Platt and Morrall, 1980); light is required for the formation of conidiophores, but not for conidia formation (Kahn, 1971).

2.3 Ascospore Production

P. tritici-repentis survives poorly in buried straw, but it persists and produces numerous pseudothecia in straw on and above the soil surface (Pfender and Wootke, 1988).

High relative humidity promotes the formation of pseudothecia and ascospores (Summerell and Burgess, 1988), with maximum pseudothecial formation being attained at -0.5 MPa and production ceasing under -2.5 MPa (Pfender et al., 1988). Temperature has little direct influence on the initiation of pseudothecia, but ascospores mature fastest at 15^{0}C (Ovody et al., 1982; Summerell and Burgess, 1988). Using a cellulose-based medium amended with macronutrients, Pfender and Wootke (1987) found the numbers of pseudothecia produced were proportional to the amount of nitrate available to the fungus, except that at concentrations above 900 ppm, nitrate inhibited ascospore production. Diaz de Ackermann and Hosford (1988) found pseudothecia developed on

autoclaved wheat seeds or corn leaves placed on water agar.

The glyphosate containing herbicide Roundup significantly suppressed the formation of pseudothecia and ascospores under field conditions (Sharma et al., 1989). Ascocarp production was also reduced by 50-99% when plant tissue infected with P. tritici-repentis was inoculated with the soil saprophyte Limonomyces roseipellis (Pfender, 1988).

2.4 Pathogen Variability

Plant pathologists are interested in using fungal pathogens, such as P. tritici-repentis for genetic studies of host-parasite interactions. Classical genetic analysis of crosses are important in establishing the genetic basis of phenotypic differences which exist between fungal strains as a preliminary to molecular analysis (Courtice and Ingram, 1987).

Genetic studies of any organism require precise, easily scored characters or markers (Michelmore and Hulbert, 1987). Markers which are used to study plant pathogens may be defined as a set of characteristics that provide information about an organism's genotype (Newton, 1987).

Few studies on virulence and pathogen variability have been undertaken with the tan spot fungus due to the difficulty in producing abundant inoculum in culture. Misra and Singh (1972) and Gilchrist et al. (1984) found significant differences between isolates based on the

severity of the reaction that various isolates induced on host plants. Luz and Hosford (1980) tested 40 isolates from the central great plains region of the U.S. and Canada on one barley and six wheat cultivars, and found they were separable into 12 races based on percent leaf area infected, and number of lesions formed per square centimetre on the host leaves. Krupinsky (1987) tested 27 isolates of P.tritici-repentis from smooth bromegrass to assess its potential to serve as an alternate source of inoculum for wheat in nearby fields, but found he could not differentiate between isolates on the basis of reaction induced on the wheat leaves.

Hunger and Brown (1987) searched for naturally occurring phenotypic markers in P. tritici-repentis. They characterized nine single-ascospore isolates (seven from the same ascus) in detail with respect to colony color, growth, sporulation, sensitivity to fungicides, and pathogenicity. They reported that differences occurred between isolates in terms of these traits, but they did not show that these traits were stable, nor demonstrate their genetic basis.

Symptom expression on host leaves is the result of a host-parasite interaction. Lamari and Bernier (1989c) inoculated 695 wheat lines, and separated the symptoms of the susceptible reaction in wheat lines into tan necrosis (nec) and chlorosis (chl), rather than one complex as had previously been believed; wheat lines demonstrating tan

necrosis were termed necrotic, those showing chlorosis were termed chlorotic. It was subsequently demonstrated that certain susceptible lines can show both tan necrosis and extensive chlorosis in response to infection (Lamari, 1991).

Lamari and Bernier (1989b) tested 92 isolates of P. tritici-repentis from western Canada. Some isolates would induce only tan necrosis (nec+ chl-)in susceptible cultivars, while others induced only chlorosis (nec- chl+). The majority of the isolates tested however had the ability to induced both necrosis and chlorosis (nec+ chl+). As a consequence of their studies, they proposed the pathogen existed as three pathotypes based on the individual isolate's ability to induce tan necrosis and/or extensive chlorosis on susceptible host lines. Pathotype one induced both chlorosis and necrosis (nec+ chl+), pathotype two only induced necrosis (nec+ chl-), and pathotype three only induced chlorosis (nec- chl+). Of the 92 isolates 81 were of pathotype one, two were of pathotype two, and nine isolates were found of pathotype three. Subsequently a fourth pathotype has been found which fails to induce either chlorosis or necrosis (nec- chl-) and is therefore avirulent (Lamari, personal communication).

2.5 Ptr Toxin

P. tritici-repentis produces a cultivar specific toxin in liquid culture (Tomas and Bockus, 1987; Lamari and

leaves, it produces tan-coloured necrotic lesions similar to those induced by the necrosis-inducing (nec+) isolates on susceptible host lines. All necrosis-inducing (nec+) isolates tested produced toxin, and all the isolates that lacked the ability to induce necrosis (nec-) did not produce toxin (Lamari and Bernier 1989a).

Lamari and Bernier (1989a) crossed toxin sensitive and toxin insensitive wheat lines, and determined that the formation of necrosis in response to infection by nec+ isolates, and sensitivity to Ptr toxin were controlled by the same dominant gene.

The toxin was purified by gel filtration and ion exchange chromatography, and found to be a protein of approximately 13,900 mw, which is large in comparison to most other fungal toxins (Balance et al., 1989). Necrosis has been induced by infiltrating host leaves with 26 femtomoles of protein, making it one of the most potent toxins produced by a plant pathogen.

2.6 Genetics

2.6.1 Pathogen Genetics

Genetic investigation of plant-parasite interaction has been based primarily on the genetics of the host, but pathogen genetics are equally important (Crute, 1986).

H.H. Flor (1959) stated:

"At least two organisms, the host and the

> "At least two organisms, the host and the parasite, are involved in infectious diseases. The genes in the host that condition reaction can be identified only by their interaction with specific cultures of the parasite; the genes in the parasite that condition pathogenicity can be identified only by their interaction with specific varieties of the host. Thus, parallel studies of the genetics of disease reaction in the host and of genetics of pathogenicity in the parasite are a requisite to the determination of host-parasite interaction."

The key to understanding variability in fungi lies in their reproductive systems (Webster, 1974). The ability to sexually cross the pathogen is important as a means of recognizing the genes responsible for variation within the pathogen population.

2.6.2 Genetics Studies of Pyrenophora

The only Pyrenophora species that have been analyzed genetically in detail are P. teres and P. graminea. Both species have bipolar heterothallic mating systems (McDonald, 1963; Smedegard-Peterson, 1978).

P. teres, the causal agent of net blotch of barley, occurs in two forms; the net form produces a net like pattern of necrotic streaks, and the spot form produces

crosses between spot and net forms, four classes based on lesion type were found in the progeny; the parental spot-inducing and net-inducing lesion types, and two recombinant classes. The two recombinant classes are comprised of one class whose strains induce lesions intermediate between spot-type and net-type lesions, while the strains of the second recombinant class induce fleck or non-pathogenic lesions (Smedegard-Peterson, 1977). The two parental and two recombinant strains occurred approximately in a 1:1:1:1 ratio, and parental types occurred in a 1:1 ratio with recombinant types indicating the ability to produce spot and net lesions is conditioned by two independent allelic pairs.

In crosses between the spot form of P. teres and the barley stripe pathogen P. graminea, the ascospore progeny were again grouped into four classes based on symptom expression on barley leaves. The occurrence of two parental classes (the spot lesions induced by P. teres and the "gram" lesions characteristically induced by P. graminea) and two recombinant classes (strains which induced intermediate and fleck lesions), indicated the ability to induce spot lesions in P. teres and gram lesions in P. graminea is controlled by two allelic pairs at two loci. Parental types far exceeded recombinants, indicating close linkage of the loci responsible for the induction of spot and gram lesions on the test hosts.

2.6.3 Genetics of Homothallic Ascomycetes

P. tritici-repentis is a homothallic fungus, in which every thallus is sexually self-fertile and can reproduce sexually without the aid of another thallus. Heterothallic fungi are those in which every thallus is sexually self-sterile, and requires mating with another compatible thallus of a different mating type to facilitate sexual reproduction (Alexopoulos and Mims, 1979). 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 crossed zygote (Pontecorvo, 1953). For genetic analysis the interest is primarily in crossed asci, but morphologically the asci of crossed and selfed zygotes appear identical. Genetic analysis of other homothallic ascomycetous fungi may provide insight for the study of P. tritici-repentis.

The most widely studied homothallic ascomycete is Aspergillus nidulans. Wild type isolates of A. nidulans have green conidia. Two color mutants were isolated, one of which produced white conidia, the other formed yellow conidia (Pontecorvo, 1953). Since a cleistothecium is formed as a result of single fertilization event, the ascospores which form will either all be of one of the two parental types if self fertilization occurred, or it will show segregation for parental characters if cross fertilization occurred. If the two partners of a cross form

conidia of different colors the hybrid cleistothecia will have progeny which segregate for the color markers (Cove, 1977). Different pairings of isolates yielded different proportions of selfed and crossed cleistothecia, and the term relative heterothallism was used to describe combinations that yielded in excess of 50% crossed asci (Pontecorvo, 1953).

A similar phenomenon has been observed in the plant pathogenic fungus Glomerella cingulata. Wild type strains from nature are homothallic, but mutants can be readily isolated that are partially or completely self-infertile due to a block in the normal process of sexual development (Wheeler et al., 1959). Pairing of different mutants together or with the wild type will complement the mutation and ascospores will be produced (Wheeler, 1954).

Ascospore color has been widely utilized because progeny can be analyzed directly by viewing the progeny with a microscope. Ascospore color mutants have been utilized in the homothallic ascomycetes Sordaria fimicola (Olive, 1956) and Ascobolus immersus (Leblon, 1972), as well as in the heterothallic plant pathogen Venturia inaequalis (Boone and Keitt, 1956).

The wild type ascospore color in S. fimicola is black. Olive (1956) used ascospore color mutants (grey, white, and yellow) to directly identify hybrid perithecia. Since the ascospores of this species are produced in an ordered

series, spore colour mutants make it possible to visually analyze the segregation of the spore colour locus. Ascospore color is an ideal marker for homothallic species because there is no need to germinate ascospores and form thalli to detect hybrid perithecia. Olive found that of the 19 strains paired, some would cross and others could not. Only matings between wild type and mutant mycelia of the same isolate, or two mutants derived from the same isolate, produced abundant crossed perithecia (up to 50% at the line of contact).

The ascospores of P. tritici-repentis are hyaline, therefore it would be difficult to obtain ascospore color mutants to use as visible markers for this fungus.

2.7 Ultraviolet Light (UV) Mutagenesis

The advancement of fungal genetics has been based on the use of mutations at specific chromosomal loci. Mutations occur spontaneously at low frequency in all cell populations. Mutagens such as X and gamma rays, U.V. light, and mutagenic chemicals have been used to increase the mutation rate. Genetic markers created by mutagenesis have been used to develop several Ascomycete fungi (Saccharomyces cerevisiae, Aspergillus nidulans, and Neurospora crassa) as genetic model systems (Fincham et al., 1979).

U.V. light was first used to generate morphological markers in fungi (Hollaender and Emmons, 1941), and then

auxotrophic mutants in Neurospora (Beadle and Tatum, 1945). Demerec (1951) used U.V. mutagenesis to create the first mutants resistant to antibiotics in Escherichea coli, and ultraviolet light has subsequently been used as a mutagen in numerous systems.

Nucleic acids specifically absorb U.V. light in the range of 250-270 nm, producing a number of alterations in the DNA structure called photoproducts (Apler, 1979). Mutagenesis by U.V. light is a complex process involving a variety of photoproducts and a variety of repair mechanisms to deal with them (Auerbach, 1979). Mutagenic and lethal effects of U.V. light are mainly due to the formation of pyrimidine dimers, particularly thymine dimers which are the most frequent and stable.

Pyrimidine dimers may be split into monomers by a photoreactivating enzyme, which is activated by visible light (Parry and Cox, 1965). Pyrimidine dimers are also removed by excision repair, where the section of DNA bearing the dimer is cut out by nuclease activity and the gap is repaired by DNA polymerase using the opposite strand as a template (Setlow and Carrier, 1964). Generally, excision gaps are filled accurately, however a small fraction of gaps are filled incorrectly giving rise to mutations. DNA repair can occur before or after DNA replication. In bacteria only a small fraction of U.V. induced mutations arise from prereplicative repair, the majority are produced by error

prone post-replicative repair (Witkin, 1969). In yeast, error prone repair can occur prior to, or after DNA replication (James and Kilbey, 1977). Inaccurate excision of thymine dimers produces both frameshift mutations and base changes in DNA, although single base pair substitutions are the most common mutation (Resnick, 1969).

2.8 Resistance Markers

Resistance to chemical inhibitors has been used along with auxotrophic, morphological, and molecular markers to facilitate genetic analysis of plant pathogenic fungi (Michelmore and Hulbert, 1987). Inhibitors used include fungicides, antibiotics, and toxic analogues of normal metabolites. But while resistance may be developed to many of the antibiotics and systemic fungicides which act at specific sites in fungal metabolism, it rarely develops to conventional fungicides with nonspecific modes of action (Dekker, 1976).

Resistance to various fungicides which are used under field conditions has developed naturally in a number of fungal species, and in almost all cases where such resistance has been analyzed genetically, it resulted from mutation of a single chromosomal gene (Dekker, 1976). In Phytophthora species, selection has yielded a range of drug-resistance mutations, many of which appear to be useful single gene markers (Shaw, 1988).

Iprodione, vinclozolin, and procymidone all belong to the dicarboximide group of systemic fungicides which are used predominately to control Botrytis, Sclerotinia, and Monilinia species (Pommer and Lorenz, 1987). Resistance to dicarboximide fungicides is easily generated in vitro and in vivo and has been reported to occur in many plant pathogenic fungi including Sclerotinia minor, Pyrenopeziza brassicae, Alternaria alternata, and Botrytis squamosa (Lorenz, 1988). In general if an isolate is resistant to one dicarboximide fungicide it will be cross-resistant to many different dicarboximides.

The specific mode of action of the dicarboximides is not yet known, but the main effect is on the chromosomes and, possibly the mitotic spindle. Georgopoulos et al. (1979) found that dicarboximides induce mitotic instability in diploid colonies of A. nidulans. The mode of fungal resistance to these compounds is also not known (Sisler, 1988).

Resistance to antibiotics in plant pathogenic fungi has also been used as a class of selectable markers. Kasugamycin resistance has been utilized extensively in genetic analysis of the rice blast fungus, Magnaporthe grisea. Taga et al. (1979) demonstrated the existence of three unlinked loci (kas-1, kas-2, and kas-3), each of which determined kasugamycin resistance independently.

Hygromycin B produced by Streptomyces hygroscopicus is

an aminocyctitol antibiotic. It strongly inhibits both the 70S and the 80S ribosomes, and specifically blocks the translocation step of protein synthesis (Cabanas et al., 1978).

Resistance to hygromycin B was not reported in plant pathogenic fungi until recent advances in the technology for gene transfer made it possible to clone, transfer, and gain expression of genes for chemical resistance (Greaves et al., 1989). The gene hph from S. hygrosconicus (Pardo et al., 1985) or from E. coli (Gritz and Davies, 1983) codes for hygromycin B phosphotransferase, an enzyme that inactivates hygromycin B. The hph gene was used as a selectable marker to transform a number of plant pathogens including Glomerella cingulata f.sp. phaseoli (Rodriguez and Yoder, 1987), Magnaporthe grisea (Leung et al., 1990), Ustilago maydis (Wang et al., 1988), Ustilago hordei, and Ustilago nigra (Holden et al., 1988).

Generation and Utilization Of Chemical Resistant Mutants In The Tan Spot Fungus (Pyrenophora tritici-repentis)

3.1 Abstract

Tan spot is a major leaf-spotting disease of wheat, distributed worldwide. Pyrenophora tritici-repentis, the causal organism of this disease, was the most common leaf spotting pathogen in Manitoba in 1990. To facilitate genetic analysis of this homothallic fungus, mutants resistant to iprodione (Ipr) or hygromycin B (Hyg) were created through ultraviolet light mutagenesis and used in sexual crosses. Approximately 8 X 10^6 conidia from two isolates of P. tritici-repentis sensitive to both chemicals (IprS HygS), were exposed to U.V. light to obtain four mutants resistant to iprodione but sensitive to hygromycin B (IprR HygS) and three mutants resistant to hygromycin B and sensitive to iprodione (IprS HygR).

The mutants were paired in all combinations and the markers allowed crossed progeny to be distinguished from selfed progeny. Crossed ascospore progeny from pairings between IprR HygS isolates and IprS HygR isolates segregated in a 1:1:1:1 ratio (IprS HygR : IprR HygS : IprR HygR : IprS HygS). Crossed progeny of a pairing between an isolate resistant to both chemicals (IprR HygR) and an isolate sensitive to both (IprS HygS) produced a similar 1:1:1:1 ratio, indicating one locus controls iprodione resistance

(IprR) and a second unlinked locus (HygR) controls hygromycin B resistance. This study should facilitate further genetic research on the tan spot fungus by providing a simple marker system.

3.2 Introduction

Tan spot of wheat is caused by the ascomycete Pyrenophora tritici-repentis (Died.) Drech. (anamorph Drechslera tritici-repentis (Died.) Shoem.)(Shoemaker, 1962). Tan spot is widespread throughout the wheat growing areas of Manitoba and Saskatchewan (Tekauz, 1976), and is a major leaf-spotting disease worldwide (Dubin, 1985; Hosford, 1971; Mehta and Almeida, 1977; Misra and Singh, 1972; Tekauz, 1976; Valder and Shaw, 1952).

The pathogen population has been divided into four pathotypes based on the ability to induce tan necrosis and/or extensive chlorosis on susceptible host lines (Lamari and Bernier, 1989b). Pathotype one induces both chlorosis and necrosis (nec+ chl+), pathotype two only necrosis (nec+ chl-), pathotype three only chlorosis (nec- chl+), and pathotype four induces neither (nec- chl-) and is therefore avirulent. Necrosis-inducing isolates of P. tritici-repentis produce a cultivar specific proteinaceous toxin (Ptr toxin), that induces necrosis when infiltrated into susceptible host leaves (Balance et al., 1990; Lamari and

Bernier, 1989a). In wheat, resistance to necrosis and insensitivity to Ptr toxin is controlled by a single gene, and resistance to chlorosis is controlled by another independent gene (Lamari and Bernier, 1991). Crossing isolates from different pathotypes may enable researchers to identify genes controlling virulence and Ptr toxin production. Genetic analysis of this homothallic species requires easily scored phenotypic markers to distinguish crosses from selfed progeny. The objective of this research was to establish a genetic marker system that could be used to identify hybrid pseudothecia, and to determine the genetic basis of this system.

3.3 Materials and Methods

Isolates and Media. Isolates used were provided by Dr. L. Lamari of the Department of Plant Science, University of Manitoba. One isolate representative of each pathotype of P. tritici-repentis was obtained; ASC1 (pathotype one, nec+ chl+), 86-124 (pathotype two, nec+ chl-), D308 (pathotype three, nec- chl+), and 190288 (pathotype four, nec- chl-).

A chemically-defined medium was required to facilitate the study of auxotrophs and chemically resistant mutants in P. tritici-repentis. After testing several media, one employed by Newton and Caton (1988) for Septoria nodorum was employed, except three grams of yeast extract were added per

litre (SYE medium, Appendix 1a). SYE medium was unsatisfactory for conidial production however, and V-8 vegetable juice/Potatoe Dextrose Agar (V-8 PDA medium, appendix 1b) was used to generate conidia by the method of Lamari and Bernier (1989a). Isolates were maintained by cutting 0.9 cm diameter plugs of mycelium from the outer edge of a thallus growing on V-8 PDA, and transferring to 3.5 cm petri dishes which were then sealed with parafilm and frozen at -17° C.

Sensitivity of P. tritici-repentis to Selected Chemicals. A number of fungicides and antibiotics were tested individually for their ability to inhibit the growth of P. tritici-repentis in culture. The effect of each chemical on the growth of isolates 86-124 and D308 were tested by adding 10, 20, 30, 40, 50, and 60mg a.i./l to SYE medium, then dispensing 20ml of the amended medium into each Petri plate. Mycelial plugs (0.5 cm dia.) of cultures growing on SYE medium were plated onto five plates of each concentration plus five plates of SYE medium to act as a control. The plates were then incubated at room temperature for seven days, then colony diameter was measured.

Chemicals tested included the fungicides benomyl, iprodione, vinclozolin, prochloraz, chloroneb, dichloran, and carbendazim, as well as the antibiotics cyclohexamide, kanamycin, and hygromycin B. Minimum inhibitory concentration (MIC) was the lowest concentration tested that

completely inhibited growth. Chemicals were rejected if the MIC was greater than 30 mg/l, but if the MIC of a chemical was less than 30 mg/l attempts were made to generate mutants resistant to the chemical.

Generation of Mutants. One isolate of pathotype two (86-124 nec+ chl-) and one isolate of pathotype three (D308 nec- chl+) were subjected to U.V. mutagenesis to generate chemical resistant mutants. The sensitivity of P. tritici-repentis to U.V. light was determined to identify the optimum dosage to use for mutagenesis. Conidia were harvested aseptically from colonies growing on V-8 PDA agar by adding sterile water, then dislodging the spores with a flamed wire loop. Twelve ml. of spore suspension was pipetted into each of seven glass petri dishes. Six of these dishes with the lids removed were irradiated, and one served as the control. The U.V. light source was a 30 watt bulb (GTE Sylvania G30T8) with a rated irradiance at 253.7nm of 80 $\mu W/cm^2$ at one meter from the bulb (GTE Sylvania Engineering Bulletin). During irradiation the plates were positioned at 122 cm from the bulb, and the treatments consisted of 10, 20, 30, 40, 50, and 60 minutes of exposure. From each treatment 0.5 ml aliquots of irradiated spore suspension were transferred to and spread on each of three Petri plates containing SYE medium. Plates were incubated for 24 hours in the dark to avoid exposure to other light sources (room lights and sunlight), and thus diminish the

action of photoreactivating light (Parry and Cox, 1965). After incubation, the spores were checked for germination using a dissecting microscope. Although the relationship between mutation yield and radiation dose depends on the organism and the gene in question, generally the treatment which kills approximately 95% of the spores will yield the most mutants (Anagnostakis, 1988; Clutterbuck, 1974). Since the 60 minute treatment yielded approximately 4% germinated spores by the following day, this exposure interval was chosen to maximize the number of mutants obtained (Appendix 2).

To generate mutants resistant to a given inhibitory chemical, 12 ml of a conidial suspension in sterile water was irradiated for 60 minutes, then 0.5 ml aliquots were plated onto SYE plates supplemented with the MIC of the chosen inhibitor. Spores were also plated onto control plates of non-supplemented SYE to check the viability of the spores, and ensure that spontaneous mutation to chemical resistance was not occurring. Non-irradiated spore suspensions were also plated onto amended and non-amended media to serve as controls.

The plates were incubated at room temperature for seven days, and any colonies that developed on the inhibitor amended media were subcultured onto fresh SYE plates supplemented with the same concentration of inhibitor. If an isolate continued to grow following such transfer, it was

increased on V-8 PDA and stored at -17°C as previously described.

Mutant Stability and Characterization. Mutant isolates demonstrating high levels of resistance to specific chemicals were subcultured on V-8 PDA medium. To verify that chemical resistance was a stable trait, apparently resistant colonies were incubated at room temperature for seven days, then a single conidium was transferred to fresh V-8 PDA medium. After five successive subculturings, each isolate was tested for chemical sensitivity along with the original mutant isolate, retrieved from frozen storage, using the procedure described above for the wild type isolates. If the isolate's level of resistance had not changed after subculturing, it was retained and stored at -17°C. When all the mutant isolates had been obtained they were again tested for sensitivity to both iprodione and hygromycin B, along with the wild type isolates from which they were derived.

Each mutant was designated by adding an extension of a three letter symbol and a number to the name of the wild type isolate from which the mutant was derived (Yoder et al., 1986). The extensions Ipr and Hyg were added to the name of the wild type isolate for iprodione resistant mutants, and hygromycin B resistant mutants respectively. Each isolate was also given a phenotypic designation based on its sensitivity to both chemicals, with S and R

indicating chemical sensitivity or resistance respectively.

Each stable chemical-resistant mutant was assessed to determine whether changes in virulence and toxin production had occurred as a result of the mutagen treatment. To test virulence host lines [6B365 (chlorosis sensitive, nec- chl+), BH1146 (resistant, nec- chl-), Glenlea (necrosis sensitive nec+ chl-), and Salamouni (resistant, nec- chl-)] were inoculated at the two to three leaf stage as described by Lamari and Bernier (1989b). Final conidial concentration, as measured by a cell counter (Hausser Scientific, Blue Bell, Pa.), was 1.5-3.0 X 10^3 spores/ml. The wild type isolates from which the mutants were derived were also tested to serve as controls.

One set of seedlings was inoculated per isolate tested. Seedlings were sprayed until runoff with spore suspensions containing one drop of Tween 20 per 100ml of suspension. Inoculated plants were incubated for 24 hours under complete leaf wetness, then transferred to a growth room for seven days at 20/17°C (day/night). After seven days, the reaction on each of the host lines was rated for the development of chlorosis or necrosis, and the virulence phenotype of each isolate determined by observing the reactions on all the host lines.

Each mutant was also tested for its ability to produce Ptr necrosis toxin following the procedure of Lamari and Bernier (1989a). One ml aliquots of an aqueous conidial

suspension were used to inoculate two flasks of 50ml of Fries medium (Appendix 1c) for each isolate tested. Cultures were incubated at 17°C for 14 days, then filtered through #4 Whatman filter paper and frozen at -17°C. The culture filtrate was thawed 14 days later and refiltered through a #4 Whatman filter, then through an 8.0 μm millipore filter to obtain a cell free extract. The filtrate was diluted 1:10, 1:100, and 1:200 with sterile distilled water and each dilution was infiltrated into 8-12 seedling leaves of the wheat cultivars Glenlea (toxin sensitive) and Erik (toxin insensitive) using a Hagborg device (Hagborg, 1970). Two days after infiltratation, these leaves were scored for presence or absence of necrosis.

Inheritance of Chemical Resistance. Mutant and wild-type isolates were paired in all possible combinations to determine if the markers could be used to identify hybrid asci, and to determine the genetic basis of the chemical resistance. Two series of pairings were conducted: in series one pairings mycelial agar plugs of the two isolates to be paired were placed on opposite sides of a V-8 PDA agar plate; and in series two pairings strips of autoclaved wheat leaves were placed on the surface of the V-8 PDA agar, so that the developing colonies grew along the length of the strips.

All paired cultures were incubated at room temperature

until the colonies converged in the middle of the plate. The plates were then flooded with sterile water, and the mycelium flattened with a flamed test tube before the water was decanted. These plates were then wrapped with parafilm and incubated at 15°C under constant fluorescent light until ascospores matured within the pseudothecia (6 to 10 weeks).

Pseudothecia were picked from the junction of the two colonies and crushed with sterile forceps. The asci were then spread on the surface of an SYE plate containing double strength agar, separated using a sterile dissecting needle, and intact asci with fully mature spores were isolated individually on the plate. A drop of 5% ß-glucoronidase enzyme was placed onto each isolated ascus (Yoder 1988), and allowed to digest the ascus wall until the ascospores were released from the ascus (approximately 30 minutes). The released ascospores were then spread out on the plate and agar blocks containing single spores were cut and transferred to a fresh SYE plate. Each ascospore was identified by a number, which also denoted the ascus and pseudothecium of origin; for example P1A2S6 is the sixth spore, from the second ascus, in the first pseudothecium.

The colonies that developed from the ascospores were tested for resistance to iprodione and hygromycin B. One mycelial plug (0.5 cm dia.) from each colony was plated on SYE containing 40 mg/l iprodione and another on SYE with 40 mg/l hygromycin B. The plates were incubated for seven days

after which the isolate was rated resistant (R) if a colony developed or sensitive (S) if the mycelium was completely inhibited by the chemical.

The technique used to sample pseudothecia was improved in the second series of pairings to allow sampling of pseudothecia more rapidly and efficiently. In the first series of pairings, five or more asci were analyzed from each pseudothecium, and the rest discarded. In the second series pairings, only two to four asci were analyzed per pseudothecium, but the rest of the asci were labelled and frozen at -17°C on agar plugs in 35mm petri dishes. Thus, if the sampled progeny showed that a pseudothecium contained hybrid asci, it was retrieved, thawed, and the rest of the asci analyzed.

A sample of hybrid progeny were tested for virulence using the procedure described above, except the final conidial concentration of the inoculum was adjusted to 3.5-4.0 X 10^3 spores/ml for each isolate. This progeny subset was also tested for Ptr toxin production using the procedure described for the mutant isolates. However, the culture filtrate was not passed through the millipore filter, only the 1:100 dilution was used, and only seedlings of the cultivar Glenlea were infiltrated.

3.4 Results

Sensitivity of P. tritici-repentis to Selected Chemicals. Isolates D308 and 86-124 of P. tritici-repentis were completely inhibited by less than 30 mg/l of the fungicides iprodione, prochloraz, and vinclozolin, and the antibiotics hygromycin B, and cyclohexamide (Appendix 3). Since iprodione and vinclozolin are closely related dicarboximides, and cross resistance between the dicarboximides is common (Lorenz, 1988) only iprodione was subsequently used to develop resistant mutants.

Generation of Mutants. From approximately 8 X 10^6 conidia irradiated with U.V. light, four isolates were obtained which were resistant to iprodione (IprR), and three to hygromycin B (HygR) (Table 3.1). No mutants resistant to cyclohexamide or prochloraz were recovered and chemical-resistant isolates were not recovered from any of the non-irradiated control plates. Wild type isolates were inhibited by both iprodione and hygromycin B at less than 40 mg/l, and were given the phenotypic designation IprS HygS. Each mutant and wild-type isolate was morphologically unique, in terms of colony color, topography, and margin. Colony morphology was a very stable phenotypic character for all the isolates.

Mutant Stability and Characterization. Chemical resistance was stable through five successive single conidial transfers for each of the four mutants resistant to

iprodione (Appendix 4) and each of the three mutants resistant to hygromycin B (Appendix 5). The minimum inhibitory concentration (MIC) remained above 40 mg/l for all resistant mutants after the conidial transfers. The comparison of all isolates for chemical sensitivity demonstrated that iprodione mutants have enhanced resistance to iprodione (MIC > 40 mg/l) (Figs. 3.1 and 3.2) and hygromycin B mutants have enhanced resistance to hygromycin B (MIC > 40 mg/l) (Figs. 3.3 and 3.4), but cross resistance was not evident. An iprodione resistant mutant (86-124Ipr1) was tested for sensitivity to the fungicide vinclozolin, and demonstrated a high level of cross resistance (MIC > 40 mg/l).

U.V. mutagenesis did not alter either the virulence phenotype (Appendix 6) nor the Ptr toxin production (Appendix 7) of any of the mutant isolates when compared to the wild type isolate from which they were derived. All mutants derived from wild type isolate D308 (pathotype three), were nec- chl+ and did not produce toxin. Similarly, all isolates derived from wild type isolate 86-124 (pathotype two) were nec+ chl- and did produce toxin.

Inheritance of Chemical Resistance. Hybrid pseudothecia were identified by segregation of resistance to iprodione and hygromycin B in the ascospore progeny; in the absence of segregation, a pseudothecium was considered to be selfed (Table 3.2).

From the series one pairings twenty nine pseudothecia were analyzed; twenty-seven were selfed and two were hybrid. The two hybrid progeny were recovered from the same pairing (cross 1), 86-124Ipr2 [IprR HygS] X 86-124Hyg2 [IprS HygR]). The ascospores in both hybrid pseudothecia of this pairing showed segregation for both iprodione and hygromycin B resistance (Table 3.3). The ascospore progeny could be divided into four phenotypic classes; IprS HygR, IprR HygS, IprS HygS, and IprR HygR. Progeny segregated 1:1:1:1 for these four classes, 1:1 for sensitivity/resistance to iprodione, and 1:1 for sensitivity/resistance to hygromycin B. This ratio indicates that a single gene controls resistance to iprodione and another independent gene controls resistance to hygromycin B. This hypothesis is supported using a chi-square test at the $\alpha = 0.05$ confidence level (Appendix 8).

Forty pseudothecia were analyzed from series two pairings; thirty eight were selfed and two were hybrid. The two hybrid pseudothecia (P1 table 3.4 and P4 table 3.5) were both recovered from the same pairing (cross 2), 86-124 [IprS HygS] X 86-124I2-H2 [IprR HygR progeny P1A2S5 from cross 1]. Since the remaining frozen asci from these hybrid pseudothecia could be thawed and analyzed many more asci were processed. The ratio obtained in the hybrid progeny was not significantly different from a 1:1:1:1 ratio for the four phenotypic classes; 1:1 for sensitivity/resistance to

iprodione, and 1:1 for sensitivity/resistance to hygromycin B (Appendix 9). Ascospores frozen in the series 2 pairing remained viable for more than five months. Samples of hybrid ascospore progeny from both cross one and cross two were stored as mycelial plugs at -17°C.

Colony morphology co-segregated exactly with chemical resistance. In hybrid pseudothecia, the two parental and two recombinant types of colony morphology corresponding precisely with the chemical resistance markers. Selfed asci showed no segregation for neither chemical resistance nor colony morphology. Thus, segregation for colony morphology also indicated hybridization.

Tetrad analysis of the eight-spored asci for both crosses are summarized in Table 3.6. Tetratype asci represented the largest class, with parental ditypes and nonparental ditypes approximately equal in number. In cross 2 there was a large proportion of aberrant eight-spored asci, where one or both genes did not segregate in the Mendelian ratio of 4 mutant : 4 wild type, but instead segregated either 6:2, 2:6, 3:5, or 5:3.

From the sample hybrid progeny tested from cross 1 (Appendix 10) and cross 2 (Appendix 11) there was no segregation for virulence or toxin production. Progeny tested produced necrosis on a susceptible host line and had the ability to produce Ptr toxin.

3.5 Discussion

Iprodione and hygromycin B completely inhibited the growth of P. tritici-repentis at low concentrations. Mutants resistant to either chemical, which were generated through U.V. mutagenesis, retained the virulence and Ptr toxin phenotype of the wild type isolate from which they were derived. All mutants proved to have stable chemical resistance through both conidial transfers and ascospore production.

With the exception of mutant 86-124Ipr1, the iprodione resistant mutants had a slower radial growth rate than the wild type isolate from which they were derived. This agrees with the finding that dicarboximide resistant isolates tend to be less fit than sensitive strains in the absence of the fungicide (Wade and Delp, 1990). Dicarboximide resistant isolates of Botrytis cinerea grew 25-30% slower than sensitive strains without fungicides in the growth medium (Katan, 1982), however resistant isolates of Pyrenopeziza brassicae grew and sporulated as well as sensitive isolates on non-amended media (Ilott et al., 1987).

Since P. tritici-repentis is homothallic, abundant numbers of selfed pseudothecia are produced along with any hybrids. Selfed pseudothecia were recovered from all pairings whereas hybrids were only recovered from pairings between 86-124 derived mutant isolates, or between 86-124 wild type and a mutant derived from 86-124. Hybrid progeny

were not recovered from pairings between mutants derived from D308 and 86-124 derived mutant isolates, nor from pairings amongst D308 derived mutant isolates. Similar results were observed by Olive (1956) with Sordaria fimicola, who found that of the 19 strains paired, some would cross and others could not. Only matings between wild type and mutant mycelia of the same isolate, or two mutants derived from the same isolate, produced abundant crossed perithecia (up to 50% at the line of contact).

The technique of freezing the remaining asci from all pseudothecia analyzed, used in series 2 pairings, greatly accelerated the recovery of hybrids. Many more pseudothecia were examined because only 3-5 asci from each pseudothecium were analyzed initially, thereby increasing the chances of finding hybrids. All the asci available in hybrid pseudothecia were thawed and evaluated later to obtain a larger population.

Two forms of genetic control of fungicide resistance are now known; in the most common form only a single gene is responsible, but in the second form many genes control resistance (Brent et al., 1990). When conditioned by a single gene, resistance may be dominant, semi-dominant, or recessive. In haploid cells mutations for resistance will be expressed immediately (Dekker, 1976).

Analyses of the hybrid progeny from crosses 1 and 2 reveal that iprodione resistance in 86-124Ipr2 is controlled

by a single gene, and hygromycin B resistance in 86-124Hyg2 is controlled by a second independent gene. The segregation from cross 1 (86-124Ipr2 X 86-124Hyg2) was not significantly different, at the $\alpha = 0.05$ level, from the 1:1:1:1 ratio expected if the traits are each controlled by single genes segregating independently (Appendix 8). The results of cross 2 (86-124I2-H2 X 86-124) confirm this hypothesis (Appendix 9). Hybrid progeny from the crosses involving the other mutants would have to be analyzed to determine if the same genetic control of chemical resistance is present in those mutants.

Grindle (1984) used genetic crosses to demonstrate that vinclozolin resistance in Neurospora crassa was controlled by a single gene in all mutants, but the mutation arose at several distinct loci in the different mutant isolates. Ilott et al. (1987) also used genetic analysis to determine that resistance to iprodione and vinclozolin is controlled by the same single gene in Pyrenopeziza brassicae.

Colony morphology co-segregated exactly with chemical resistance in both crosses. These morphological markers seem to be reliable indicators of hybridization for these mutant isolates of P. tritici-repentis. The pairing procedure may be accelerated by simply observing the colony morphology of the ascospore progeny to detect hybrid asci. Grindle (1984) also reported co-segregation of various phenotypic abnormalities such as slow radial growth rate,

and reduced sporulation with vinclozolin resistance in mutants of Neurospora crassa.

In the cross 86-124I2-H2 X 86-124 (cross 2) three selfed asci were obtained from pseudothecium 1, two from 86-124 (2 and 7) and one from 86-124I2-H2 (6), while the rest of the asci were hybrid. There are two hypotheses which might explain the origin of these selfed asci; (1) three pseudothecia may have been inadvertently picked off the crossing plate together, one with hybrid asci and two with selfed asci one from each parent; or (2) a single pseudothecium may have contained both hybrid and homozygous asci. The latter process has been reported in Sordaria fimicola (Olive 1974), and Aspergillus nidulans where "twinned" cleistothecia which are partly of selfed and partly of hybrid origin are common (Clutterbuck, 1974). The three selfed asci were excluded from the analysis of segregation for iprodione and hygromycin B resistance.

Tetrad analysis (Table 3.6) revealed the presence of aberrant eight-spored asci which could not be classified as either tetratype, nonparental ditype, or parental ditype. These asci demonstrated unusual segregation for one or both of the resistance genes. Instead of segregating in the typical Mendelian fashion of 4 wild type : 4 mutant, these ascospores segregated 6:2, 2:6, 3:5, or 5:3 for one or both of the genes. Possible explanations for this are; mixing of ascospores from different asci, mutation at the resistance

locus, or gene conversion.

If spores from different asci were mixed then unusual segregation ratios may result. To minimize this problem single asci were picked and moved to an isolated spot on the double strength agar plate before the ascus wall was degraded and the ascospores picked off. Thus, it is unlikely that mixing of ascospores occurred.

Mutation is not a likely explanation because both hygromycin B resistance and iprodione resistance were stable through five successive single conidial transfers. Chemical resistance was also stable through the ascospore stage. In the sixty five selfed asci from both series there was no segregation for chemical resistance. Forty one selfed pseudothecia recovered were derived from a mutant parent and no progeny from these pseudothecia reverted back to wild type. Twenty two selfed pseudothecia were derived from a wild type parent and no progeny from these were chemical resistant.

Lindegren (1952) first described gene conversion in Saccharomyces cerevisae where one allele is converted unilaterally to the type of the other allele. Crosses between mutant and wild types in some asci did not show the expected 2:2 wild type to mutant ratio, but gave 3 wild types to 1 mutant or 3 mutants to 1 wild type. The phenomenon has since been demonstrated in a number of other fungal systems including Neurospora crassa (Case and Giles,

1964), Ascobolus immersus (Leblon, 1972), and Sordaria fimicola (Kitani and Olive, 1967). Recently, Chumley and Valent (1990) proposed gene conversion as an explanation for aberrant tetrads in Magnaporthe grisea segregating for pigment production.

Gene conversion in yeast can also occur in mitotic cells, although the frequency is two to three orders of magnitude less than that of meiotic conversion (Roman and Ruzinski, 1990). Kitani and Olive (1967) used Sordaria fimicola to demonstrate that conversion is highly correlated with crossing over in the vicinity of the site converted. A number of models have been proposed to explain the mechanism of crossing over which eventually leads to gene conversion (Holliday, 1964; Meselson and Radding, 1975; and Szostak et al., 1983). Although these models propose different mechanisms of recombination between homologous DNA strands, they all account for the formation of heteroduplexes between different single strands from each homolog.

Heteroduplex or hybrid DNA formation is generally regarded as the basic mechanism underlying gene conversion (Roman and Ruzinski, 1990). In a heteroduplex region a mismatch of base pairs between the two strands may occur. A mismatch repair may then excise a portion of the DNA in one strand, and fill the gap using the complementary strand as a template (Rothwell, 1988). Using the Holliday (1964) model, which predicts reciprocal exchange of broken single strands

creating two heteroduplexes, the 5:3 and 3:5 asci can be explained on the basis of correction in one chromatid, and the 6:2 and 2:6 asci may be caused by correction in both chromatids in the same direction (Fincham et al., 1979).

Aberrant meiotic ratios are known in many species though at widely different frequencies. Essentially every allelic difference in Saccharomyces spp. shows a deviation from the normal 2:2 segregation (Fincham, 1983). Frequency of aberrant segregation varies depending on the species and the particular locus. Aberrant ratios occur in less than 1.0 percent of the progeny up to the 10.0 percent level in species of Saccharomyces, but occurs between 0.1 and 1.0 percent in Sordaria spp. depending on the locus. Although gene conversion may be a plausible explanation for the aberrant ratios in P. tritici-repentis the frequency of aberrant ratios, approximately 25% for cross 2, seems high in comparison to other species. Analysis of many more asci is required to determine the actual cause of these aberrant ratios.

Asci of P. tritici-repentis almost always contain eight ascospores (Pfender and Wootke, 1987). In cross 2, a number of asci were noted that contained only four spores instead of the normal number of eight. Three of these asci (P1A5, P1A7, and P4A18) were analyzed to determine the segregation of the resistance markers. There are a number of possible mechanisms for the formation of asci with half the expected

number of spores (Taga et al., 1985). If meiosis was normal but mitosis failed, four different products would have been produced and asci with two sets of twins (such as P1A5) would have been rare. Alternatively meiosis and mitosis may have occurred normally, but half the mitotic products aborted randomly. If the progeny of the three asci are taken together, equal numbers of ascospores fall into each of the four phenotypic classes for the resistance markers, and this suggests that ascospore abortion occurred post-mitotically and was a random process. However, a greater sample of four spored asci would have to be analyzed to confirm this hypothesis.

Along with base changes, U.V. light also induces chromosomal aberrations such as translocations. In Aspergillus nidulans, unusually high frequency of ascospore abortion in crosses may be due to chromosome aberrations as the result of U.V. mutagenesis (Kafer, 1965). This may be one possible explanation for ascospore abortion in P. tritici-repentis.

Chemical resistance markers have proven to be useful in identifying hybrid progeny in pairings of P. tritici-repentis. Two marker loci, HygR and IprR, have been characterized as single gene mutations and form the basis of the first two linkage groups. These markers should prove useful in crossing isolates from different pathotypes to investigate the genetic basis of pathogenicity and Ptr toxin

production.

More pairings should be attempted between isolates from different pathotypes to determine if pathotypes can be inter-mated. Genetically marked isolates could be paired with many wild-type isolates from different pathotypes to recover hybrids. Some isolates may have a higher frequency of outcrossing than the majority of isolates and these could be developed as laboratory strains.

Table 3.1. Recovery of chemical resistant mutants following U.V. irradiatation of conidia from isolates D308 and 86-124 of P. tritici-repentis.

Conidia Irradiated	Screening Media (mg/l)	Number of Mutants	Designation	Phenotype
2.5 X 10^5	Ipr 20	4	86-124Ipr1 D308Ipr3 86-124Ipr2 D308Ipr4	IprR
1.0 X 10^6	Cyc 20	0		
1.0 X 10^6	Pro 20	0		
1.0 X 10^6	Hyg 20	0		
4.7 X 10^6	Hyg 5	3	86-124Hyg1 D308Hyg3 86-124Hyg2	HygR
7.95 X 10^6		7		

Note; the following chemical inhibitors were all added to SYE medium
Ipr = iprodione
Cyc = cyclohexamide
Pro = prochloraz
Hyg = hygromycin B

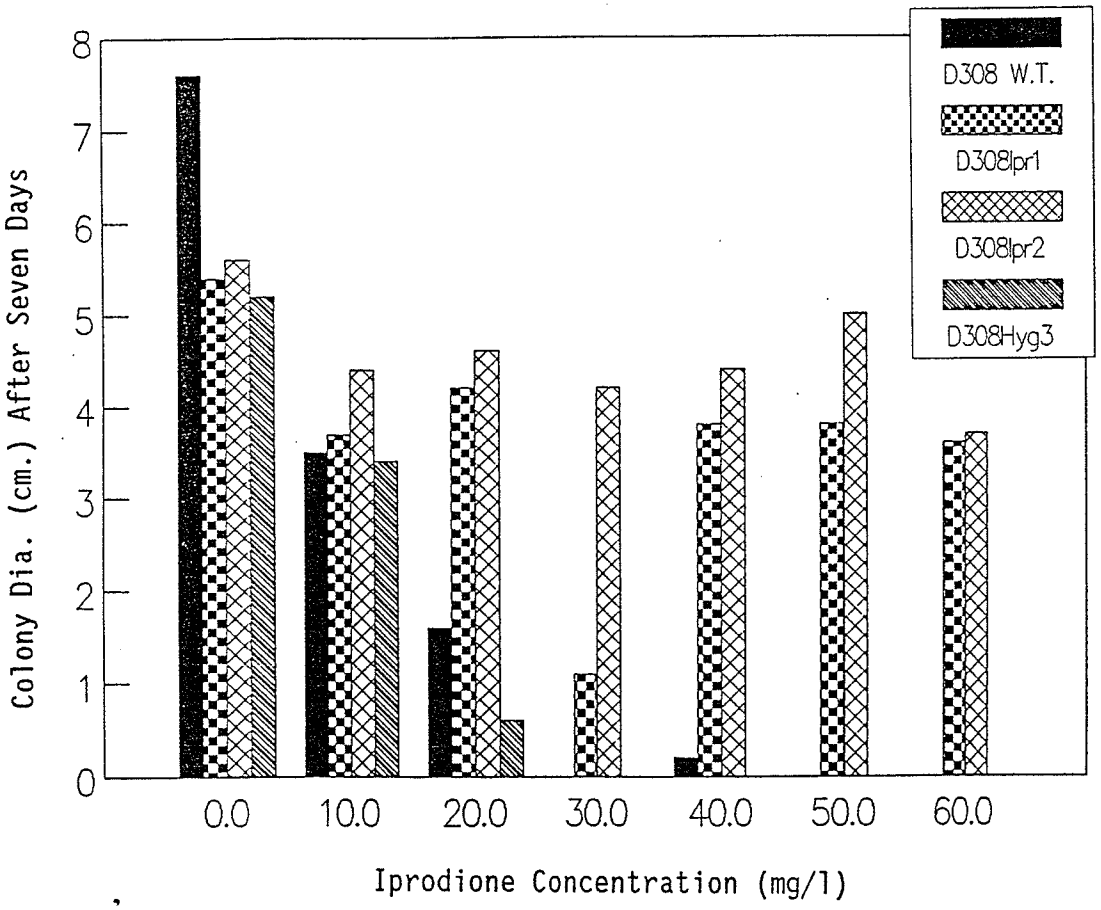

Figure 3.1 The effect of various concentrations of iprodione on the growth of the D308 wild type (W.T.) and mutant isolates of P. tritici-repentis

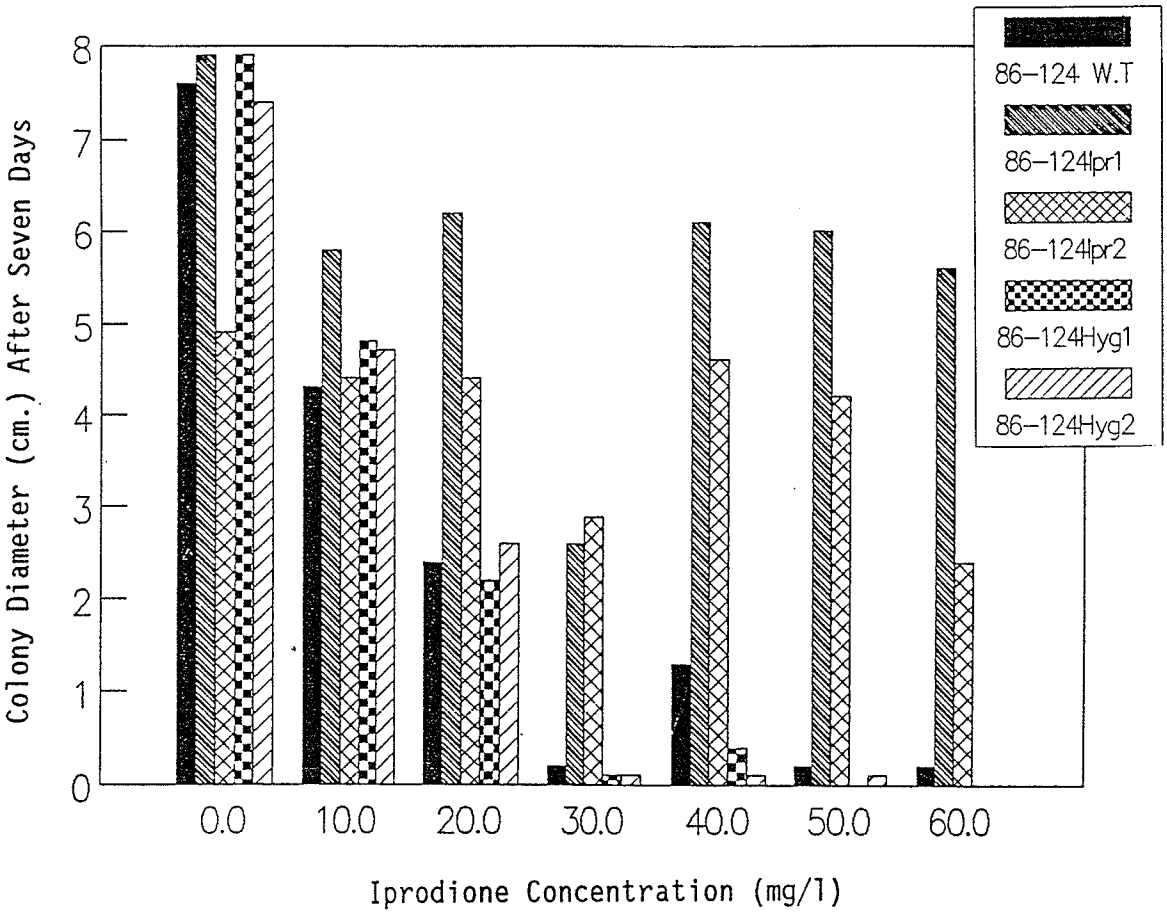

Figure 3.2 The effect of various concentrations of iprodione on the growth of the 86-124 wild type (W.T.) and mutant isolates of P. tritici-repentis

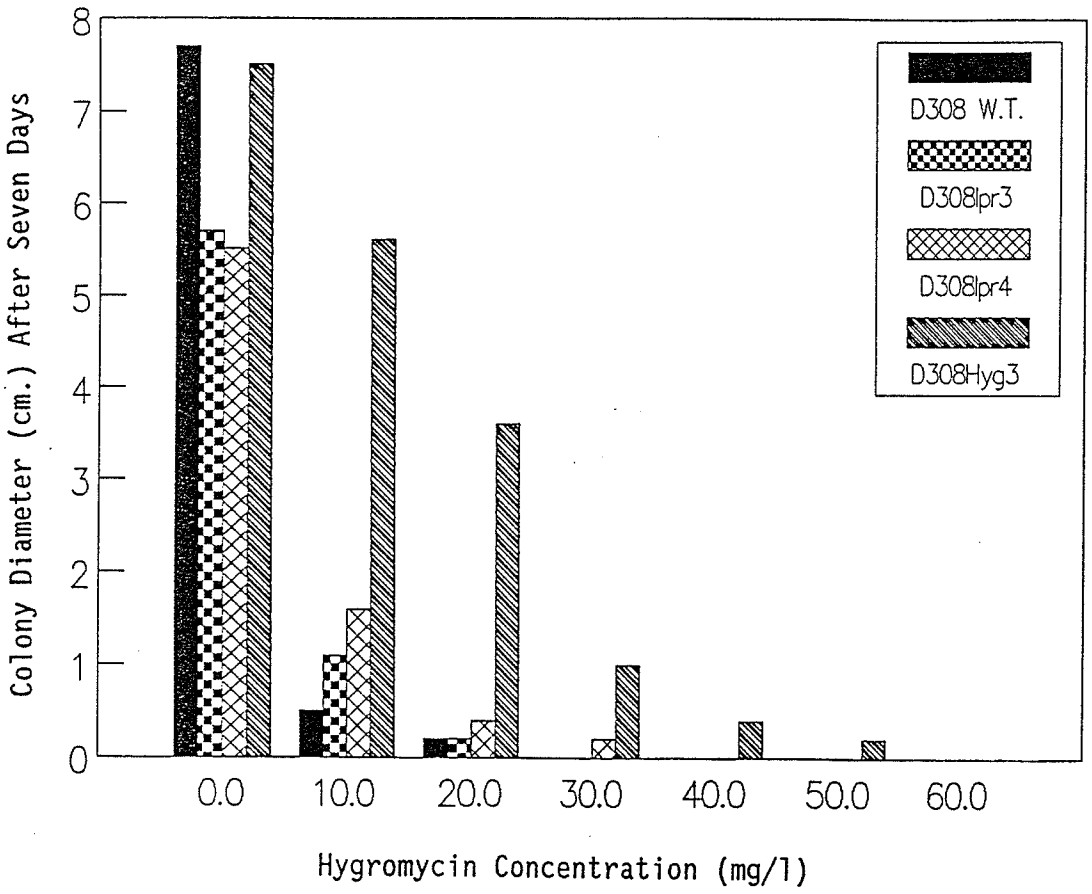

Figure 3.3 The effect of various concentrations of hygromycin B on the growth of the D308 wild type (W.T.) and mutant isolates of P. tritici-repentis

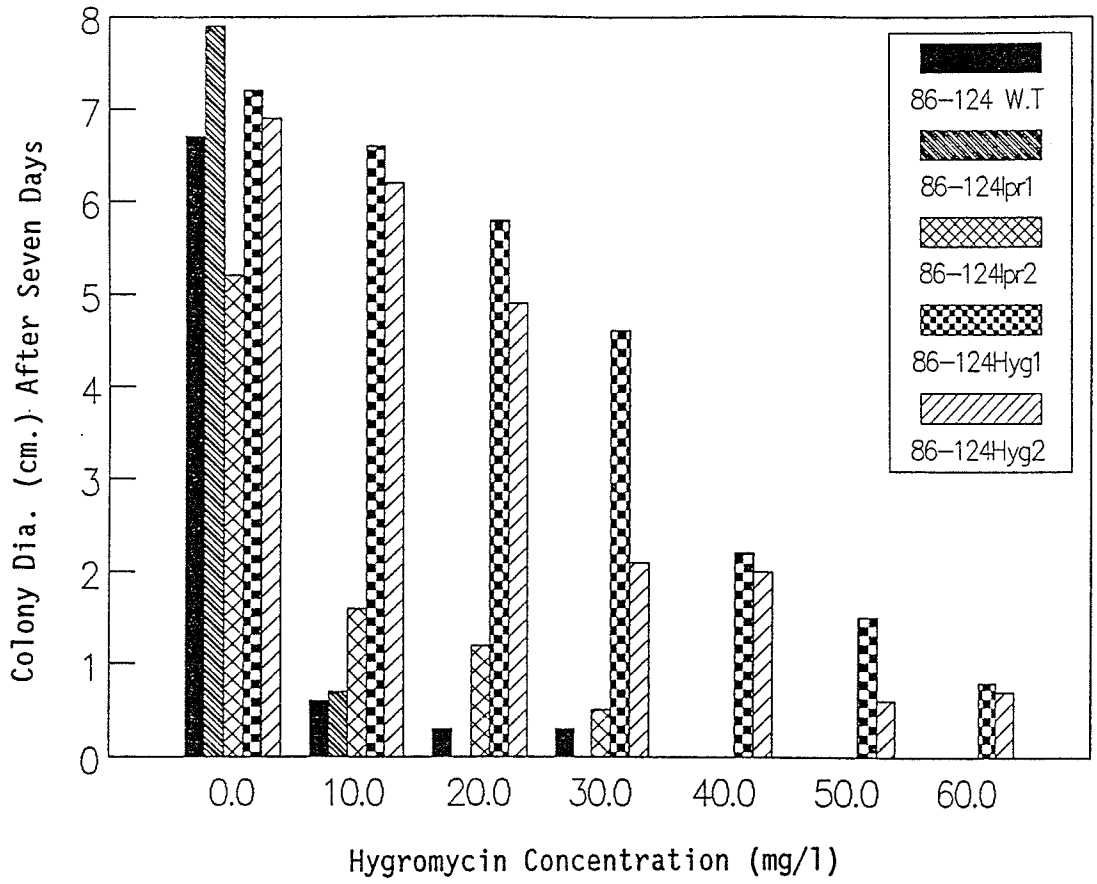

Figure 3.4 The effect of various concentrations of hygromycin B on the growth of the 86-124 wild type (W.T.) and mutant isolates of P. tritici-repentis

Table 3.2. Summary of the pairings of chemical resistant and wild type isolates of P. tritici-repentis

Parents		Number of Sampled Pseudothecia		
Parent 1 X Parent 2		Parental 1	Parental 2	Crossed
Series 1				
86-124	86-124Ipr2	5	5	0
D308	D308Ipr4	0	3	0
86-124	86-124Hyg2	2	3	0
86-124Ipr2	86-124Hyg2	5	0	2 a
D308Ipr4	86-124Hyg2	1	3	0
Series 2				
D308Hyg3	86-124Ipr1	6	5	0
D308Hyg3	86-124	0	2	0
D308Hyg3	86-124Ipr2	2	3	0
D308Ipr4	86-124Hyg2	5	0	0
86-124	86-124I2-H2	3	0	2 b
D308	86-124Hyg2	2	0	0
ASC1	D308Hyg3	3	0	0
ASC1	86-124I2-H2	7	0	0
Pseudothecia Selfed: 65		Pseudothecia Crossed: 4		

Note;
86-124I2-H2 = the progeny P1A2S5 [IprR HygR] of cross 86-124Ipr2 X 86-124Hyg2 from series 1
a = cross 1
b = cross 2

Table 3.3. Resistance phenotype of ascospore progeny from cross 1 (86-124Ipr2 [IprR HygS] X 86-124Hyg2 [IprS HygR])

Ipr	Hyg	P6A1	P6A2	P6A3	P6A4	P6A5	P6A6	P1A1	P1A2	Total
S	R	2	0	2	3	2	2	2	2	15
R	S	0	0	2	2	4	2	2	2	14
R	R	1	3	2	1	1	0	2	2	12
S	S	1	4	2	0	1	1	2	2	13
Totals		4	7	8	6	8	5	8	8	54

Table 3.4. Resistance phenotype of ascospores from asci of pseudothecium 1 from cross 2 (86-124I2-H2 [IprR HygR] X 86-124 [IprS HygS])

		Ascus Number								
Ipr	Hyg	1	2	5	6	7	8	9	10	Total
S	R	2	0	0	2	1	0	4	0	9
R	S	2	0	2	2	1	0	4	0	11
R	R	2	0	2	2	0	0	0	6	12
S	S	2	5	0	2	2	7	0	0	18
Totals		8	5	4	8	4	7	8	6	50

Table 3.5. Resistance phenotype of ascospores from asci of pseudothecium 4 from cross 2 (86-124I2-H2 [IprR HygR] X 86-124 [IprS HygS])

		Ascus Number																	
Ipr	Hyg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	Ra	Total
S	R	2	0	2	4	2	4	2	3	2	2	0	4	2	2	1	0	2	7	41
R	S	2	0	0	4	0	4	2	3	2	4	0	4	2	2	2	2	0	7	40
R	R	2	4	4	0	4	0	2	2	2	0	4	0	2	2	1	2	1	4	35
S	S	2	4	2	0	2	0	2	0	2	0	4	0	2	2	1	4	1	8	36
Totals		8	8	8	8	8	8	8	8	8	6	8	8	8	8	5	8	4	26	152

Ra = random ascospores

Table 3.6. Tetrad analysis of the eight-spored asci from crosses 1 and 2

Crosses	T	NPD	PD	Others
Cross 1				
86-124Ipr2 X 86-124Hyg2	3	0	0	1
Cross 2				
86-124I2-H2 X 86-124	7	3	2	4

T = tetratypes
NPD = nonparental ditypes
PD = parental ditypes

General Discussion

Chemical resistance genes IprR and HygR were useful for distinguishing hybrid pseudothecia from selfs. All the mutants were generated from one of the two wild type isolates; 86-124 from pathotype two, or D308 from pathotype three. Hybrid progeny were only recovered in pairings between different mutants derived from 86-124, or from pairings between 86-124 wild type and mutant mycelia derived from it. Pairings between different D308 mutants or between D308 and 86-124 derived mutants failed to yield any hybrid progeny. Isolate D308 may have a very low frequency of outcrossing and many more pseudothecia may need to be analyzed to obtain hybrids, or the isolate may have lost the ability to outcross completely. Lack of hybridization between 86-124 and D308 could be due to incompatibility between these two isolates, which may or may not be related to the pathotype system.

To determine if incompatibility is operating between pathotypes, existing mutants could be used in pairings with wild type isolates from different pathotypes. New marked isolates could be created through U.V. mutagenesis, using existing protocols, or by successful genetic crosses. By using the marker genes to identify hybrid progeny many isolates could be paired from different pathotypes to determine if mating between pathotypes is possible.

The ultimate goal of this research is to use these marked isolates to study the genetics of virulence and Ptr toxin production. Development of suitable strains of P. tritici-repentis for genetic studies will depend on knowledge of compatibility and fertility in this fungus. The effect of the marker loci on pathogen fitness will also be an important consideration.

Marker loci may be useful in field studies on the epidemiology of P. tritici-repentis by allowing the researcher to distinguish released isolates from the natural populations. Markers may also prevent accidental contamination of one isolate by another in the laboratory.

The anomaly of aberrant segregation ratios observed in hybrid asci has not been previously reported in P. tritici-repentis and may warrant further study. Aberrant segregation ratios in the ascospore progeny may be due to gene conversion and are interesting genetically. Since asci of P. tritici-repentis almost always contain eight ascospores (Pfender and Wootke, 1987) asci with less than eight ascospores (such as were observed in some hybrid asci) are a rarity and investigation of this phenomenon may reveal information on the sexual processes of this fungus.

Genetic investigation of the pathogen complements analysis of the host to develop the wheat/P. tritici-repentis system into a model system for host/parasite genetics.

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Appendices

Appendix 1. Culture media
Appendix 1a. SYE defined minimal medium

Modified from;
Newton A.C.,and Caten C.E. auxotrophic mutants of Septoria nodorum isolation by direct screening and by selection for resistance to chlorate. 1988 Tran. Brit. Mycol. Soc. 90(2),199-207.

		g/l
	Sucrose	30.0
	$NaNO_3$	2.0
	KCl	0.5
	$MgSO_4$	0.5
	$ZnSO_4$	0.01
	$FeSO_4$	0.01
	$CuSO_4$	0.0025
	K_2HPO_4	1.0
	Agar	15.0
Added->	Yeast Extract	3.0

Appendix 1b. V-8 PDA sporulation and crossing medium

PDA	10.0gm
$CaCO_3$	3.0gm
Agar	10.0gm
V8	850.0ml
Water	150.0ml

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

Appendix 1c. Fries medium for toxin production, #66 in Dhingra and Sinclair (1985)

NH_4	5.0gm
NH_4NO_3	1.0gm
$MgSO_4.7H_2O$	0.5gm
KH_2PO_4	1.3gm
Sucrose	30.0gm
Yeast Extract	1.0gm
Trace Element Solution	2.0gm
Water	1.0l

Trace Element Solution

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

Reference :
Dhingra, O.D., and Sinclair, J.B. 1985. Basic Plant Pathology Methods. CRC Press, Boca Raton, Florida. pp.355.

Appendix 2. Effect of U.V. light exposure on the germination of P. tritici-repentis conidia.

U.V. Exposure (Min.)	Germinated Conidia	Total Conidia	Percent Germinated
10	116	118	98.3
20	71	92	77.2
30	131	261	50.2
40	108	325	33.2
50	38	160	23.7
60	86	2136	4.0

Note;
U.V. light source was a 30 watt bulb (GTE Sylvania G30T8)

Appendix 3. Minimum inhibitory concentration for various chemicals on the mycelial growth of isolate 86-124 of P. tritici-repentis.

Chemical Tested	Minimum Inhibitory Concentration (mg/l)
Benomyl	>600
Carbendazim	>200
Iprodione	10
Vinclozolin	30
Chloroneb	>100
Dichloran	>100
Prochloraz	20
Kanamycin	>100
Hygromycin B	10
Cyclohexamide	20

Appendix 4. Effect of five successive conidial transfer on iprodione sensitivity of P. tritici-repentis mutant isolates.

Iprodione Conc. (mg/l)	0.0	10.0	20.0	30.0	40.0	50.0	60.0
Isolate	Colony Diameter (cm.) After Seven Days						
[86-124Ipr1] Original	4.9	5.1	5.2	4.8	4.9	4.3	4.3
[86-124Ipr1] After Subculture	5.0	4.9	5.4	4.9	5.2	4.3	4.4
[86-124Ipr2] Original	4.4	4.6	4.8	1.9	3.2	1.0	1.0
[86-124Ipr2] After Subculture	4.4	4.4	4.6	1.7	3.1	1.0	1.0
[D308Ipr3] Original	4.1	3.0	3.7	3.8	3.8	3.6	2.8
[D308Ipr3] After Subculture	4.1	3.6	4.0	4.1	3.9	3.9	3.3
[D308Ipr4] Original	4.8	4.2	4.2	4.8	2.3	2.9	1.3
[D308Ipr4] After Subculture	4.9	3.9	4.7	4.7	2.4	2.9	1.4

Appendix 5. Effect of five successive conidial transfer on hygromycin B sensitivity P. tritici-repentis mutant isolates.

Hygromycin Conc. (mg/l)	0.0	10.0	20.0	30.0	40.0	50.0	60.0
Isolate	Colony Diameter (cm.) After Seven Days						
[86-124Hyg1] Original	6.5	5.7	3.8	3.3	2.2	1.4	0.5
[86-124Hyg1] After Subculture	7.2	6.6	5.8	4.6	2.2	1.5	0.8
[86-124Hyg2] Original	6.6	6.0	4.7	2.2	2.0	1.4	1.0
[86-124Hyg2] After Subculture	6.9	6.2	4.9	2.0	2.0	1.4	0.7
[D308Hyg3] Original	1.9	0.3	0.2	0.2	0.1	0.0	0.0
[D308Hyg3] After Subculture	2.4	6.6	0.3	1.5	0.2	0.0	0.0

Appendix 6. Virulence of seven chemical resistant mutants of P. tritici-repentis and the two wild type isolates from which they were derived.

Isolate	nec.	chl.	Reaction on Host Line; 6B365	BH1146	Glenlea	Salamouni
D308	-	+	chl.	n.s.	n.s.	n.s.
D308Ipr4	-	+	chl.	n.s.	n.s.	n.s.
D308Ipr3	-	+	chl.	n.s.	n.s.	n.s.
D308Hyg3	-	+	chl.	n.s.	n.s.	n.s.
86-124	+	-	n.s.	sl. nec.	nec.	n.s.
86-124Ipr1	+	-	n.s.	sl. nec.	nec.	n.s.
86-124Ipr2	+	-	n.s.	sl. nec.	nec.	n.s.
86-124Hyg1	+	-	n.s.	sl. nec.	nec.	n.s.
86-124Hyg2	+	-	n.s.	sl. nec.	nec.	n.s.

Note;
nec. = necrotic
sl. nec. = slightly necrotic
chl. = chlorotic
n.s. = no visible symptoms
+ = symptom inducing
- = non-symptom inducing

Appendix 7. Bioassay for Ptr toxin production by seven mutants and the two wild types they were derived from on one toxin sensitive and one toxin insensitive host line

		Erik			Glenlea		
Isolate	Dilution	1:10	1:100	1:200	1:10	1:100	1:200
86-124		-	-	-	+	+	+
86-124Ipr1		-	-	-	+	+	+
86-124Ipr2		-	-	-	+	+	+
86-124Hyg1		-	-	-	+	+	+
86-124Hyg2		-	-	-	+	+	+
D308		-	-	-	-	-	-
D308Ipr3		-	-	-	-	-	-
D308Ipr4		-	-	-	-	-	-
D308Hyg3		-	-	-	-	-	-

Note;
- = no visible symptoms
+ = necrosis

Appendix 8. Observed segregation and Chi-square test for cross 1 [86-124Ipr2 (IprR HygS) X 86-124Hyg2 (IprS HygR)] involving resistance and sensitivity to both iprodione and hygromycin B

	Observed	Expected	O-E	$(O-E)^2/E$
IprS HygR	15	13.50	1.50	0.166
IprR HygS	14	13.50	0.50	0.018
IprR HygR	12	13.50	-1.50	0.166
IprS HygS	13	13.50	0.50	0.018
Total	54	54.00		X^2= 0.368

P=0.94

Appendix 9. Observed segregation and Chi-square test for cross 2 [86-124 (IprS HygS) X 86-124I2-H2 (IprR HygR)] involving resistance and sensitivity to both iprodione and hygromycin B

	Observed	Expected	O-E	$(O-E)^2/E$
IprS HygR	50	46.00	4.00	0.348
IprR HygS	51	46.00	5.00	0.543
IprR HygR	41	46.00	-5.00	0.543
IprS HygS	42	46.00	-4.00	0.348
Total	184	184.00		X^2= 1.782

P=0.65

Note; Asci numbers 2, 8, and 10 from pseudothecium #1 have been omitted from this analysis because they are assumed to be selfs.

Appendix 10. Virulence and resistance phenotypes of mycelial cultures derived from eight ascospore progeny from cross 1 (86-124Ipr2 [IprR HygS] X 86-124Hyg2 [IprS HygR])

						Reaction on Host Line;			
Isolate	Ipr	Hyg	nec	chl	Toxin	6B365	BH1146	Glenlea	Salamouni
P1A2S1	S	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A2S2	R	S	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A2S3	S	S	+	-	+	n.s.	nec.	nec.	n.s.
P1A2S4	R	R	+	-	+	n.s.	n.s.	sl. nec.	n.s.
P6A3S8	S	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P6A3S5	R	S	+	-	+	n.s.	sl. nec.	nec.	n.s.
P6A3S7	S	S	+	-	+	n.s.	nec.	nec.	n.s.
P6A3S3	R	R	+	-	+	n.s.	n.s.	sl.nec.	n.s.

Note;
nec. = necrotic
sl. nec. = slightly necrotic
chl. = chlorotic
n.s. = no visible symptoms
+ = symptom inducing
- = non-symptom inducing
S = chemical sensitive
R = chemical resistant

Appendix 11. Virulence and resistance phenotypes of mycelial cultures derived from sixteen ascospore progeny from cross 2 (86-124 [IprS HygS] X 86-124I2-H2 [IprR HygR])

						Reaction on Host Line;			
Isolate	Ipr	Hyg	nec	chl	Toxin	6B365	BH1146	Glenlea	Salamouni
P6A5S1	R	S	+	-	+	n.s.	nec.	nec.	n.s.
P6A5S2	S	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P6A5S7	S	S	+	-	+	n.s.	nec.	nec.	n.s.
P6A5S8	R	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A1S5	R	S	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A1S2	S	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A1S1	S	S	+	-	+	n.s.	nec.	nec.	n.s.
P1A1S6	R	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A6S2	R	S	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A6S4	S	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A6S3	S	S	+	-	+	n.s.	sl. nec.	nec.	n.s.
P1A6S1	R	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P4A9S5	R	S	+	-	+	n.s.	sl. nec.	nec.	n.s.
P4A9S3	S	R	+	-	+	n.s.	sl. nec.	nec.	n.s.
P4A9S2	S	S	+	-	+	n.s.	nec.	nec.	n.s.
P4A9S1	R	R	+	-	+	n.s.	sl. nec.	nec.	n.s.

Note;
nec. = necrotic
sl. nec. = slightly necrotic
chl. = chlorotic
n.s. = no visible symptoms
+ = symptom inducing
- = non-symptom inducing
S = chemical sensitive
R = chemical resistant