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Parasexual Analysis of Fungicide Sensitivity and Pathogenicity in the Eyespot Fungus *Pseudocercosporella herpotrichoides*

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ABSTRACT

The two major pathotypes (W & R) of the cereal eyespot fungus, *Pseudocercosporella herpotrichoides*, show variation of major agronomic importance, specifically in sensitivity to fungicides and in differences in host species specificity. This thesis describes the genetic analysis of these characters using the parasexual cycle to generate recombinant progeny between W and R pathotypes. Interspecific hybrid progeny from a parasexual cross between *P. herpotrichoides* and *P. anguioides* were included in the analysis. Marker differences present in the parental isolates including auxotrophic requirements, isozyme banding patterns, sensitivity towards benzimidazole fungicides and spore length have been used as proof of recombination in the parasexual progeny, and to characterise the pattern of genetic segregation in the crosses.

Analysis of fungicide sensitivity focussed on the ergosterol biosynthesis inhibitors (EBI), where it is found that W-types are significantly more sensitive to the majority of the demethylation inhibiting (DMI) fungicides than are the R-types. Determination of sensitivity of progeny to EBI fungicides revealed the presence of 'major' and 'minor' gene(s) controlling the expression of fungicide resistance. Major gene segregation resulting in a substantial difference in sensitivity to triadimenol, was found to occur prior to exposure of the diploid fusion products to haploidizing agents with the result that progeny generated from any one fusion product were either sensitive or resistant to triadimenol. Segregation of additional minor genes occurred following exposure to haploidizing agents and these modified the level of resistance expression within the progeny. These analyses provided the first genetic evidence for cross-resistance to these fungicides in this fungus. Cross-resistance relationships were shown to depend on the mode of action of the chemicals and also varied within the chemical groups. Interaction was seen between major and minor gene(s) in the expression of cross-resistance relationships.

Pathogenicity towards wheat and rye was shown to be considerably disrupted by parasexual recombination. Although most paraexual progeny were not pathogenic a minority of isolates were able to infect and produce typical disease symptoms on wheat (14%) and / or rye (21%).

Pathogenicity to wheat and rye was not associated indicating that the inheritance of pathogenic ability towards wheat and rye is genetically separable and that new 'pathotypes' can be generated through recombination. Within the non-pathogenic progeny a subset of recombinant strains were found which were able to colonise host tissues demonstrating a clear parasitic ability. The remaining progeny appeared to be deficient in both pathogenic and parasitic abilities. These three groups of recombinants : pathogenic, parasitic/non-pathogenic and non-parasitic will allow the future investigation of both pathogenicity and parasitism in this fungus.

Parasexual progeny were used to investigate the inheritance of vegetative incompatibility in *P. herpotrichoides*. However, detection of vegetative incompatibility between W- and R-type isolates or between recombinant progeny was not successfull in spite of using a number of different techniques. Further refinement of the assay system is required.

DECLARATION

This thesis has been composed by me and is based on the results of investigation carried out by myself. The thesis has not been accepted for any application for any other degree. All information and assistance obtained from other sources has been specifically acknowledged by means of reference.

Janice Elizabeth McNaughton

February 1996

To my family and friends

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ABBREVIATIONS

ANS	8-Anilino napthylene sulphonic acid
arg	Arginine auxotrophy
CH	Chloral Hydrate
CMR	Complete Regeneration Media
cys	Cysteine auxotrophy
DAPI	4,6-Diamidino-2-phenylindol
DMI	Demethylation inhibitor
DTT	DL-dithiothreitol
EBI	Ergosterol biosynthesis inhibitor
ED50	Effective dose 50%
EDTA	Ethylenediaminetetraacetic acid
EMYG	Enriched MYG
EST	Esterase
FP	Fusion product
FPA	ρ-fluorylphenylalanine
GDH	Glutamate Dehydrogenase
glu	Glutamate auxotrophy
gly	Glycine auxotrophy
GPI	Glucose Phosphate Isomerase
his	Histidine auxotrophy
ino	Inositol auxotrophy
LMYG	Liquid MYG
lys	Lysine axuotrophy
MDH	Malate Dehydrogenase
MDPC	Methyl 3,5-dichlorophenyl carbamate
met	Methionine auxotrophy
MM	Minimal Medium
MMR	Minimal Regeneration Media
MPI	Mannose Phosphate Isomerase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MYG	Malt extract, Yeast extract, Glucose Agar
NADH	Nicotinamide adenine dinucleatide, reduced form
NADP	Nicotinamide adenine dinucleatide phosphate
nia	Nitrate non-utilizing
nic	Nicotinic acid auxotrophy
NUV	Near Ultraviolet Light
PDA	Potato Dextrose Agar
PMSF	Phenylmethylsulphonyl fluoride
R	R pathotype or R-type
TEMED	N,N,N',N'-tetramethylethylenediamine
TWA	Tap Water Agar
W	W pathotype or W-type

1 INTRODUCTION

1.1 THE PATHOGEN

The disease cereal eyespot is fairly common on cereals grown in cool climates (Sprague, 1934; Bruehl *et al.*, 1968) and is caused by the fungus *Pseudocercosporella herpotrichoides*. This fungus is able to infect a number of cereal and grass species although the main agricultural losses occur on winter wheat. The fungus infects the basal area of the shoot and the resulting symptoms of the disease are typically elliptical shaped lesions on leaf sheaths and stems. The disease is traditionally regarded as monocyclic in nature, evidence being found by Rowe & Powelson (1973). Unlike root and foliar diseases, eyespot infects a relatively small area of the plant and therefore the development of severity of infection depends less on the total area of the plant that is affected than on the depth of penetration of the lesion at the base of the stem. Yield loss, which occurs only when severe lesions develop, is caused not only because there is a blockage in the flow of metabolites from the shoot to the root but also because lodging of the stem occurs (Ponchet, 1959; Bruehl *et al.*, 1968; Scott & Hollins, 1974). The infection is initiated by spores dispersed in rain splash droplets from infected debris of previous crops (Fitt & Bainbridge, 1983) and frequently infects crops via the coleoptile (Bateman & Taylor, 1976). After infection is initiated the disease is considered to consist of three stages including: (1) penetration of successive leaf sheaths by the fungus; (2) spread of lesions from the leaf sheaths to the stem, this is considered to be the initial stage in the development of stem lesions and finally (3) the development of established lesions in the stem (Fitt, 1985). Daniels *et al.* (1995) showed that ascospores of the sexual stage of eyespot, *Tapesia yallundae*, were able to infect and produce typical disease symptoms on wheat seedlings *in vitro*. Under ideal conditions ascospores would become airborne and could implicate ascospores in the long range dispersal of inoculum of eyespot. (Sanderson & King, 1988). It is also possible that ascospores of *T. yallundae* may be produced all year round if environmental conditions are favourable

(Nicholson *et al.*, 1991b) and this could have significant consequences for the epidemiology of the disease.

Although both spore dispersal and leaf sheath infection can be affected by both environmental conditions and the time of sowing of the crop influencing the progression of the fungus through the plant, it is widely believed that the development of stem lesions is crucial as to whether the infection develops into a severe epidemic (Jorgensen, 1964; Scott & Hollins, 1974; Clarkson, 1981; Higgins *et al.*, 1986).

In general the occurrence of severe lesions at harvest is favoured by an early autumn sowing, dense crops (Sprague, 1937) wet, mild winters (Bruehl *et al.*, 1968) and wet, cool springs (Glynne & Salt, 1958). Although there is a need for cool temperatures the small rise in temperature occurring between autumn and spring is reflected by the rise in optimum temperature required for successive stages in the infection process. The optimum temperature required for sporulation of infected debris is 5°C (Higgins & Fitt, 1984) which rises to 8 - 9°C for spore dispersal and initiation of infection (Polley & Clarkson, 1978). There is again a further increase in temperature required for penetration of successive seedling leaf sheaths, where the required optimum temperature has risen to 6 - 18°C (Ponchet, 1959; Scott, 1971).

However, even if optimal conditions prevail during the stages where the fungus progresses through the leaf sheaths it does not necessarily dictate that severe stem lesions will develop as it has been shown that the development of stem lesions in adult wheat plants does not relate clearly to the penetration of seedling leaf sheaths (Higgins & Fitt, 1985b). Like other stages in the infection cycle the development of severe stem lesions and hence yield loss in the crop can be affected by a number of factors. The rate of death of leaf sheaths after stem elongation must be one of the most crucial. This determines the length of time where both the basal leaf sheaths and the stem are present together and hence the length of time available for the progression of

the fungus from the leaf sheath into the stem (Fitt, 1985). Higgins *et al.* (1986) have shown that this period is crucial by following the progress of disease in three consecutive seasons between 1981-84. In each of these three years disease incidence at growth stage (GS) 31 exceeded the threshold level and led to the use of fungicides to control this disease in all three seasons. However, only in 1982 did severe eyespot develop by harvest. It was shown that in this year the basal leaf sheaths and the stem were together for longer than in the other seasons.

It is recommended that an assessment of the incidence of eyespot is made at GS30/31. If there is greater than 20% disease incidence in the crop spraying with fungicides is recommended in the hope of preventing epidemics. However, as has been shown it is events occurring after this stage that are crucial in the development of severe lesions and hence yield loss in the crop. Therefore, it has been suggested by Higgins *et al* (1986) that an assessment of the disease when the basal leaf sheaths are withering, at which time the infection is spreading from the leaf sheaths into the stem would be more appropriate. It has also been suggested that an assessment of disease progression during grain filling would be essential in the prediction of epidemics of eyespot. However, whether this would allow enough time for sufficient control measures to be taken remains to be seen.

Over the last 15 -16 years there have been large changes in the population structure of *P. herpotrichoides* in both the UK (King & Griffin, 1985) and elsewhere in Europe (Cavelier *et al.*, 1985). The two main pathotypes of *P. hepotrichoides*, which can be routinely isolated from winter wheat, have been distinguished on the basis of seedling pathogenicity on rye (Scott *et al.*, 1975; Nirenberg, 1985; Fitt *et al.*, 1987) and cultural characteristics (Lange - de la Camp, 1966a, 1966b, 1967; Scott *et al.*, 1975; Hollins *et al.*, 1985). In the UK these two types are referred to as W- (wheat) and R- (rye) types. The W-types are faster growing and have a smooth/even colony margin in culture. The R-types are slower growing and have a feathery/uneven colony margin. It is now found that the R-types are dominant in the eyespot

population in cereal crops while before the early 1980's this pathotype was rarely encountered in arable fields other than where rye was routinely grown. Nirenberg (1981) recognized two varieties of the fungus *P.h.* var. *herpotrichoides* and *P.h.* var. *acuformis* which have been correlated with the W-and R-types respectively. No pathogenicity data were, however, reported on the varieties and therefore the validity of equating pathotypes with varieties has been questioned (Mauler & Fehrmann, 1987). More recently, biochemical markers such as isozyme banding patterns (Julian & Lucas, 1990; Priestley *et al.*, 1992) and DNA polymorphisms (Nicholson *et al.*, 1991a, b) have been developed which not only distinguish between the different pathotypes of *P. herpotrichoides* but can also distinguish between the different species *P. herpotrichoides*, *P. anguioides* and *P. aestiva*.

W-type and R-type isolates are also distinguished on the basis of their pathogenicity to wheat and rye in glasshouse experiments. In these experiments W-type isolates are typically more pathogenic to wheat than to rye, while R-type isolates pathogenic to a similar extent to both hosts. Work on the pathogenicity of W- and R-type isolates towards wheat seedlings has provided conflicting results. It has been shown that there were no differences in pathogenicity to wheat between the two pathotypes (Brown *et al.*, 1984), that R-types were more pathogenic to wheat than W-types (Hollins *et al.*, 1985), and elsewhere that W-types were more pathogenic to wheat than R-types (Higgins & Fitt, 1985b; Sanders *et al.*, 1986; Fitt *et al.*, 1987). However, it is known that within W- and R-types there are large differences in the level of pathogenicity of individual isolates (Fitt *et al.*, 1987) and this may help to explain why conflicting results are obtained.

Two further pathotypes of *P. herpotrichoides* have been recognised, the C- and S-types. The C-type has been shown to be pathogenic to wheat, *Aegilops squarrosa* and *Agropyron repens* while the S-type is pathogenic only to wheat and *Ae. squarrosa* (Cunningham, 1965; 1981; Scott *et al.*, 1976). The C-type is almost exclusively been isolated from Ireland, while the S-

type has been found in both France and the UK (Nicholson *et al.*, 1991a) Two other species of *Pseudocercosporella* have also been identified on cereal hosts: *P. anguioides* and *P. aestiva* (Nirenberg, 1981). *P. anguioides* has been isolated from the seedlings of winter wheat in the UK and has been shown to be relatively weakly pathogenic (Bateman, 1988). *P. aestiva* has been isolated from wheat in Germany (Nirenberg, 1981) and to date there have been no reports identifying the occurrence of this species in the UK. These two species can be readily distinguished from *P. herpotrichoides* on the basis of spore characteristics with *P. anguioides* producing longer and *P. aestiva* much shorter spores than *P. herpotrichoides* (Nirenberg, 1984). Differences between the species can also be found in isozyme banding patterns (Julian & Lucas, 1990)

Eyespot was successfully controlled by the benzimidazole fungicides, such as carbendazim between 1971, until resistance developed in the early 1980's. Resistance towards these fungicides was first reported in the Federal Republic of Germany (Rashid & Schlösser 1975) and carbendazim resistant strains were subsequently discovered in the UK in 1981 (Griffin & Yarham, 1983; Brown *et al.*, 1984). A majority of strains isolated from the field nowadays will be resistant to this group of fungicides.

Currently the disease is controlled using two ergosterol biosynthesis inhibiting fungicides, prochloraz and flusilazole, which although not giving as good a level of control as the benzimidazoles originally afforded, are still able to control the disease at a sufficient economic level. As with benzimidazoles, prochloraz and flusilazole are sprayed at GS 30/31 which coincides with stem elongation. The reason for this is that it is important for the fungicide to reach the base of the stem. Later in the growing season the crop canopy prevents effective chemical treatment of the stem base. The decision whether to apply fungicides for eyespot control has to be based on disease incidence at this early stage in crop development. The action threshold (more than 20% of stems infected) developed for use with benzimidazole fungicides if

still used for prochloraz and flusilazole. It has also been suggested that prochloraz may give effective control of eyespot when applied between GS 30 to GS 37 whereas with MBC fungicides it was necessary to spray at GS 30/31 (Fitt, 1988) which was not always the best time for assessing levels of infection.

The sexual stage of *P. herpotrichoides*, *Tapesia yallundae*, was discovered recently in Australia in 1987 (Wallwork & Spooner, 1987) and apothecia of this fungus can now be found in most major wheat growing areas in Europe (Dyer *et al.*, 1993a, b, 1994; Hunter, 1989; King, 1990; Nicholson *et al.*, 1991b). *T. yallundae* is a heterothallic ascomycete with two mating types. Sexual crosses appear possible within each pathotype and between the W, C and S pathotypes. However crosses between the W- (or C- and S-) type and R-types are not successful, indicating a barrier for sexual gene exchange between the two main pathotypes. Parasexual recombination can occur between W- and R-types following fusion of protoplasts of the strains (Hocart *et al.*, 1993b). This system allows the genetic analysis of differences between these two pathotypes of agronomically important traits.

1.2 PARASEXUAL RECOMBINATION

The parasexual cycle was first discovered in *Aspergillus nidulans* and made the genetic analysis of asexual species possible (Pontecorvo *et al.*, 1953). A parasexual cycle has subsequently been described in a wide range of fungi such as *Aspergillus sojae* (Bradley, 1962), *Verticillium dahliae* (Puhalla & Mayfield, 1974), *V. albo-atrum* (Typas & Heale, 1976), *Pyricularia oryzae* (Genovesi & Magill, 1976), *Gibberella fujikuroi* (Puhalla & Speith, 1985) and *Fusarium oxysporum* f.sp. *apii* (Puhalla, 1983) and suggests therefore that a parasexual cycle may be common to all fungal groups (Tinline & MacNeil, 1969; Caten, 1981). The system described for *A. nidulans* has been regarded as the standard parasexual cycle (Pontecorvo, 1956; Fincham *et al.*, 1979; Caten, 1981) in which four basic steps occur:

1. Hyphal fusion occurs between neighbouring hyphae to allow mixing of the two cytoplasms and organelle exchange. In particular, exchange of nuclei can occur, leading to heterokaryon formation.

2. Heterokaryon formation. Cells of *A. nidulans*, which have multinucleate vegetative cells are able to support the introduction of 'new' nuclei into the cell with the result that a balanced heterokaryon is formed (Jinks *et al.*, 1966). Phenotypic flexibility if possible, under selection, due to the changes proportion of nuclei of each genotype in the heterokaryon. Migration of nuclei can occur between the site of formation of the heterokaryon and the fungal hyphal tips, and the heterokaryon can be maintained indefinitely, in theory, by the subculture of individual hyphal tips. Sporulation from the heterokaryons of species that produce uninucleate conidia will again only reveal parental types (Hastie & Heale, 1984). Alternatively, if the fungal species produces multinucleate conidia the heterokaryon can be maintained by through the conidia, as is seen with *A. sojae* (Bradley, 1962).

3. Nuclear fusion can occur within these multinucleate heterokaryons. This is a random process and leads to the production of diploid nuclei, which can either be homozygous or heterozygous. The potential exists within the heterozygous diploid nuclei for the recovery of recombinant genotypes.

4. The last stage of the cycle can involve two separate events: (i) mitotic recombination, where crossing over can occur between non-sister chromatids during mitosis (Hastie, 1968), with the result that linked genes are separated and recombinant diploids formed. (ii) Non-disjunction within the diploid nucleus during mitosis leads to the formation of daughter nuclei whose genetic complement is either 2n+1 or 2n-1. Within nuclei which are 2n+1 the extra chromosome may be lost in subsequent mitotic divisions to give diploid nuclei again,

some of which can be homozygous for the trisomic chromosome, allowing expression of any recessive alleles present on that chromosome. Nuclei which are 2n-1 are also unstable and breakdown occurs during mitosis, with the random loss of chromosomes until recombinant haploids are generated. Work by Käfer (1961, 1977) suggested that within *A. nidulans* a sequential loss of chromosomes through non-disjunction was responsible for reduction at the diploid to the haploid state. Under ideal conditions the loss of chromosomes would be a random process with the result that haploid segregations would show independent assortment of the chromosomes. Usually non-disjunction is induced by exposing diploids to a haploidizing agent.

Exceptions are found to the standard parasexual cycle seen in *A. nidulans* and can be seen for each of the stages of the cycle described above. Hyphal fusion, the first step in the standard parasexual cycle may be precluded by the presence of barriers to anastomosis. Such barriers are recognised as vegetative incompatibility between fungal strains and is typically associated with differences in their genotypes for incompatibility, or *Het*, genes. Vegetative incompatibility is found in many fungal species and prevents the formation of hyphal anastomosis (Caten & Jinks, 1966; Leach & Yoder, 1983; Hastie & Heale, 1984; Typas, 1983). A study of the extent and distribution of vegetative incompatibility within fungal species should be useful for dissecting population structure, and assessing the extent of sexual recombination within the population. Where little vegetative incompatibility is evident it is likely that the fungal population is clonal. Where extensive vegetative incompatibility is present it is possible that sexual recombination plays a significant role in genetic variability within the fungal species. Every time sexual recombination occurs it would generate new combinations of the *Het* genes and hence generate new incompatibility groups (Puhalla & Speith, 1985).

Variations in the form and stability of heterokaryons have been recognised. Many fungal species contain uninucleate vegetative cells which are not able to sustain heterokaryosis where genetically different nuclei are present in each cell. Instead hyphal anastomosis occurs between neighbouring hyphae to form isolated heterokaryotic cells linked by short hyphal bridges, while the remaining hyphal cells are homokaryotic. This type of heterokaryon is found in *Verticillium* (Puhalla & Mayfield, 1974; Typas & Heale, 1976) and *Fusarium* (Puhalla & Speith, 1985) and is termed a 'mosaic heterokaryon'. Repeated hyphal fusions are required to sustain the heterokaryon. Nuclear migration does not occur between the heterokaryotic cells and the hyphal tips, with the result that if hyphal tips are subcultured then only parental homokaryon type colonies are recovered. Spore formation by mosaic heterokaryons will typically produce homokaryotic parental conidia because the majority of cells in the mycelium will be uninucleate, including the conidiogenous mother cells.

Variation in the duration and stability of the diploid stage is also recognised. For some fungal species the diploid that is formed following nuclear fusion is highly unstable. This is in contrast to the situation that is seen with *A. nidulans*, where stable diploids are formed. In *Cephalosporium acremonium* (=*Acremonium chrysogenum*) no detectable diploid stage was seen, only aneuploids and recombinant haploids are recovered after nuclear fusion (Hamlyn & Ball, 1979). The recombinant nature of the aneuploids and the haploids is the only proof that a diploid stage was present, however transient. In these cases systems have be developed to enable direct recovery of the aneuploids and haploids from crosses. In *C. acremonium* fusion products, produced by protoplast fusion of complementary auxotrophic strains, are plated directly onto minimal medium which contains combinations of the nutritional requirements of the parental stains in order to maximise the potential to recover the full complement of recombinant types (Norman, 1988).

In the recovery of parasexual recombinants from diploids problems may arise in the selection and recognition of stable haploids. In *A. nidulans* each of the eight monosomic aneuploid stages can be distinguished visually on the basis of colony morphology and growth. Indirect evidence from other fungi suggests that typically aneuploids are slow growing and morphologically unstable. Direct observation of fungal chromosomes is rarely possible due to their small size so determination of nuclear status, particularly of the haploid nature of parasexual recombinants can be problematic.

A parasexual cycle has been demonstrated to occur in *P. herpotrichoides* (Hocart, 1987) and each of the four stages in the cycle can be recognised. Heterokaryosis was shown to occur between complementary auxotrophic mutants derived from the same isolate. *P. herpotrichoides* mycelium is composed of uninucleate, vegetative cells with the result that an unstable heterokaryon similar to that of *Verticillium* is formed. Isolation of conidia or hyphal tips from the heterokaryon yielded only the parental homokaryon type colonies (Hocart *et al.*, 1993a). Hocart *et al.* (1993a) concluded therefore that a mosaic heterokaryon is formed in this fungus. Heterokaryosis between unrelated strains was not always possible due to the presence of vegetative incompatibility (Hocart *et al.*, 1989; Magnano di San Lio *et al.*, 1994). Vegetative incompatibility in *P. herpotrichoides* appears common both within and between pathotypes and would restrict the potential for parasexual recombination in the field. This incompatibility in *P. herpotrichoides* can be overcome by protoplast fusion (Hocart *et al.*, 1993b) for all W and R pathotype combinations (i.e. WxW, RxR & WxR) (Magnano di San Lio *et al.*, 1994). No heterokaryon phase is detected following protoplast fusion of vegetatively incompatible strains. The colonies recovered on selective media, from fusions of protoplasts from complementary auxotrophs, have been regarded as somatic diploids on the basis of their phenotype and the subsequent recovery of stable recombinants with novel marker combinations following exposure of 'fusion products' to haploidizing agents (Hocart *et al.*, 1993b). Protoplast fusion even allows interspecific parasexual crosses in *Pseudocercosporella*, and has

been used to gauge the degree of relatedness between *P. herpotrichoides* and *P. anguioides* (Hocart & McNaughton, 1994), a weakly pathogenic species occasionally found colonising cereal stem bases (Nirenberg, 1984; Bateman, 1988). Evidence for parasexual, or sexual, recombination between species is taken as indicating similarity in the organisation of the genome, and hence relatedness of species (Kevei & Peberdy, 1984; Sipiczki, 1987).

In intrastrain crosses with *P. herpotrichoides* the diploids recovered appear relatively stable. As in crosses between vegetatively incompatible strains, the diploid status of these colonies is presumed from the subsequent recovery of recombinants. Intrastrain diploids described by Hocart *et al.* (1993a) failed to sporulate and therefore spore measurements, which in other species have shown a significant increase in cell volume (Fincham & Day, 1971; Pontecorvo *et al.*, 1953), could not be performed. In addition, measurements of apical cell volume were too variable to allow distinction between haploids and diploids (Hocart *et al.*, 1993a).

Protoplast fusion between vegetatively incompatible strain combinations produces putative diploids directly with no detectable heterokaryon phase. These diploids are apparently stable for several generations of subculture (Hocart *et al.* 1993b). However, not all fusion products were identical with variation evident in morphology pigmentation, and growth rate. In addition, morphological heterogeneity is also found within single colonies (Magnano di San Lio *et al.*, 1994).

Linked genes are required in order to detect mitotic recombination. In the published literature for the eyespot fungus no evidence for linkage has been reported between markers. This could possibly be due to the markers being located on separate chromosomes or alternatively that lots of mitotic recombination is occurring. During breakdown of the diploid nucleus relatively persistent putative aneuploid stages are formed before a stable 'haploid' recombinant is recovered (Hocart *et al.*, 1993a). In intrastrain crosses it can be difficult to generate

recombinant haploids and attempts to induce aneuploid stages, using haploidizing agents, frequently only results in the recovery of diploids (M. J. Hocart, pers. comm.). Haploids are recognised on the basis of morphological stability and the failure to generate new marker combinations even after repeated exposure to haploidizing agents. Aneuploids typically appear as sectoring colonies (i.e. slow growing colonies with more rapid growing outgrowths). It is these outgrowths that are subcultured for the recovery of stable haploids (Hocart *et al.*, 1993a, b). In interspecific protoplast fusions between *P. herpotrichoides* and *P. anguioides* the fusion products which are obtained were very unstable and yielded stable recombinants spontaneously (Hocart & McNaughton, 1994).

1.3 MODE OF ACTION OF ERGOSTEROL BIOSYNTHESIS INHIBITING FUNGICIDES

Sterols are known to be major structural components of cell membranes where they may contribute to their selective permeability properties and modify membrane fluidity. Therefore, inhibition of biosynthesis of sterols would be detrimental to fungal growth and development. It is found that interference with sterol biosynthesis results in cell or hyphal membranes becoming disorganised and losing their ability to regulate the movement of substances in and out of the cell or hyphae (Carlisle, 1995).

Within most fungi ergosterol is the main membrane sterol. The general principles of the biosynthetic pathway to the sterols are common to all organisms able to synthesize sterols. In simplified form they involve a) the formation of mevalonate from acetate, b) the formation of squalene from mevalonate, c) the cyclization of squalene via 2,3-oxidosqualene to lanosterol and d) in fungi, the conversion of lanosterol into ergosterol by a series of nuclear demethylations, side chain alkylation, hydride shifts and reduction/dehydrogenations (Langcake *et al.*, 1983).

The greater proportion of fungicides that have been commercialized during the 1970's have been identified as sterol biosynthesis inhibitors and are found to act on the post squalene segment of the ergosterol biosynthesis pathway. Fungicides having this mode of action are found to be a chemically diverse group. However, they have two common characteristics. They all have at least one nitrogen containing heterocyclic ring: in addition, with two exceptions (buthiobate and prochloraz) they contain at least one asymmetric carbon atom. On the basis of the chemical nature of the nitrogen containing heterocyclic ring the following subgroups can be distinguished: piperazines (triforine), pyridines (buthiobate), pyrimidines (fenarimol and nuarimol), triazoles (triadimefon, triadimenol, fluotrimazole, bitertanol, diclobutrazol, propiconazole and etaconazole), imidazoles (imazalil, miconazole, clotrimazole, ketaconazole and prochloraz) and morpholines (fenpropimorph and tridemorph). All these fungicides inhibit primarily sterol C-14 demethylation, except for the morpholine fungicides, which have been convincingly demonstrated to inhibit sterol C-14(15) double bond reduction in fungi (Kerkenaar *et al.*, 1981; Kerkenaar *et al.*, 1984) and sterol Δ^8 - Δ^7 isomerization (Mercer, 1991).

Studies on the mode of action of ergosterol biosynthesis inhibiting fungicides were pioneered by Ragsdale with triarimol and by Kato with buthiobate and have been regarded as models by subsequent workers.(Langcake *et al.*, 1983) It was found in work on *Monilinia fructigena* with buthiobate that there was very little effect on the germination of conidia but that the development of germ tubes was strongly inhibited. In addition, hyphae were distorted, excessively branched and some had swollen hyphal tips (Kato *et al.*, 1974). Similar effects were observed with cucumber powdery mildew, *Sphaerotheca fuliginea*, where hyphal tips stained abnormally with vital dyes on exposure to the fungicide, suggesting an effect on membrane permeability and function (Kato *et al* 1975b).

With *M. fructigena* the major sterol fractions found are the steryl esters (esters of ergosterol), the desmethyl sterols (ergosterol) and the 4,4-dimethyl sterols (24-methylene dihydrolanosterol).

Treatment of fungal cells with low concentration of buthiobate (10 μM) caused an almost total inhibition of the incorporation of acetate into desmethyl sterols and an accumulation of label in their methylated sterol precursors (both 4-methyl and 4,4- dimethyl sterols). Based on the fact that the sterols in buthiobate treated cells all retain the methyl group at C-14, Kato *et al.* (1975a) concluded that buthiobate specifically inhibits demethylation.

To confirm this finding Kato and Kawase (1976) studied ergosterol biosynthesis in a cell-free multi-enzyme system from *Saccharomyces cerevisiae* using 14^{C}-labelled mevalonate. In the presence of buthiobate at 10^{-4}M, the formation of C-4 and C-14 desmethyl sterols was strongly inhibited and label accumulated in lanosterol. This parallels the accumulation of 24-methylene dihydrolanosterol in whole cells of *M. fructigena*. These findings therefore provide evidence for the specific action of buthiobate against sterol C-14 demethylation.

Ragsdale & Sisler (1972) confirmed that triarimol acts as a sterol biosynthesis inhibiting fungicide using sporidia of *Ustilago maydis*, where they confirmed the lack of effects against major metabolic processes other than lipid biosynthesis (Ragsdale & Sisler, 1972). Inhibition of C-14 demethylation was confirmed using gas chromatography - mass spectrometry analysis of the sterols accumulated in treated cells. C-14 demethylation which involves hydroxylation and elimination of the carbon atom as formic acid rather than carbon dioxide involves a mixed function oxidase in the initial hydroxylation and is mediated by cyctochrome P-450. Kato and Kawase (1976) have suggested that it is this initial hydroxylation that is inhibited by buthiobate, and presumably the other fungicides, and that it is possible that the nitrogen atom of the aromatic heterocycle (present in all compounds except triforine) interacts directly with the haem of the cytochrome.

Work on *Botrytis cinerea* and *Ustilago maydis* elucidated that mainly the morpholines had a dual mode of action, in that they inhibited two steps in the ergosterol biosynthetic pathway, i.e. Δ^{8-7} isomerization and Δ^{14-15} reductase. However, it was found that different morpholine

fungicides inhibit these steps to varying degrees and whilst tridemorph was found to have greatest activity against Δ^{8-7} isomerization, fenpropidin was found to be most active against the Δ^{14-15} reductase. In addition, further steps in the ergosterol biosynthetic pathway have also been shown to be inhibited by the morpholine fungicides, including $\Delta^{24(28)}$ reductase, Δ^{24} transmethylation and squalene cyclization. Each of these different target sites is found to involve high energy carbocationic intermediates, which are mimicked by the morpholines (Hollomon, 1994).

Although some of the effects of fungicides on metabolism are consequences of the action on sterol demethylation and the subsequent cessation of fungal growth, there is evidence that these fungicides have additional sites of action. This was suggested from various observations.

1) Gram-positive bacteria which do not synthesize sterols are sensitive to several of the fungicides (Langcake *et al.*, 1983).

2) Some pythiaceous fungi, which also do not synthesize sterols, are also sensitive to several of the fungicides (Buchenauer, 1979).

3) Addition of ergosterol and other sterols to fungicide-treated cells results in a poor reversal of inhibition (Langcake *et al.*, 1983).

Control of eyespot, *P. herpotrichoides*, is now based primarily on the use of two fungicides, prochloraz and flusilazole. Prochloraz is the exception amongst the EBI fungicides in that it mostly acts as a protectant fungicide with very little systemic activity. It is also reported that there is no difference in sensitivity between W- and R-type isolates of the pathogen to this fungicide (Leroux & Gredt, 1985; Cavelier *et al.*, 1987). However, a reduction in the efficiency of field control of eyespot with prochloraz has been reported in France (Leroux & Marchegay, 1991). Strains which are isolated from these sites are found to have a reduced sensitivity *in vitro* to prochloraz. Typically it was found that these isolates belonged to the R pathotype. With many other EBI fungicides, in particular the triazoles, it is found that there is differential

sensitivity within eyespot pathotypes, with the R-type isolates being found to be less sensitive than the W-type isolates.

1.4 RESISTANCE TO ERGOSTEROL BIOSYNTHESIS INHIBITING FUNGICIDES

Development of resistance to ergosterol biosynthesis-inhibiting fungicides has been relatively slow. This differs from the situation found with many other site-specific fungicides such as the benzimidazoles and phenylamides (Köller & Scheinflug, 1987) and could be due to a reported decrease in fitness or pathogenicity in fungi resistant to ergosterol biosynthesis inhibitors. Reduced fitness associated with EBI resistance was demonstrated first with triforine resistant mutants of *Cladosporium cucumerinum*, where it was found that the level of fungicide resistance was inversely proportional to the pathogenicity of the mutant (Fuchs *et al.*, 1977). This would suggest that EBI resistant strains would be unlikely to survive in the absence of the selection effect of the fungicide (de Waard & Fuchs, 1982). Consequently, the triazoles have been classified in the low risk category with the respect to development of resistance under field conditions, particularly when compared to other site-specific antifungal compounds (Dekker, 1984). The pleiotrophic effects of mutant genes would also include their influence on an organism's biochemistry. Differences in biochemistry between wild-type and fungicide resistant mutants can therefore be used to discover the biochemical mechanism of resistance to specific fungicides and may help to elucidate their mode of action (Grindle,1987)

A number of suggestions have been put forward as to the mechanism involved in the development of fungicide resistance and include: decreased permeability, increase in detoxification, reduced conversion to a fungitoxic compound, reduced affinity at the site of action, circumvention of the site of action and compensation at the site of action (Dekker, 1977). Work by de Waard & Fuchs (1982) and de Waard & van Nistelrooy (1980) has suggested that differences in efflux mechanisms may explain resistance to fenarimol in

Aspergillus nidulans mutants. In wild-type strains of *A. nidulans* there is a rapid accumulation of fungicide during the first ten minutes of incubation of the fungus with fenarimol with a subsequent gradual release with time (de Waard & Fuchs, 1982). This pattern of fungicide uptake and excretion was due to a passive influx and energy dependent efflux of the fungicide in this fungus (de Waard & van Nistelrooy, 1980). The efflux mechanism was also shown to be inducible. However, in mutants of *A. nidulans* possessing the *ima*B gene for resistance to uptake of fenarimol was relatively low in comparison to wild-type strains due to an energy-dependent efflux mechanism which was expressed constitutively in the mutant. This suggests that the only difference between the wild-type and resistant strains of *A. nidulans* is the efficiency of the fungus to expel fenarimol from the mycelium (de Waard & van Nistelrooy, 1982).

In contrast, analysis of resistant mutants of *Ustilago avenae* (Hippe, 1987) and *Candida albicans* (Vanden Bossche *et al.*, 1987) have indicated that the mechanism of resistance involved in these species is not due to a decreased intracellular concentration of fungicide. Differences in uptake and metabolism do not appear to be the mechanism responsible for resistance to triadimenol, and presumably other DMI's in *Rhynchosporium secalis*. In this fungus it is the sensitivity of the target enzyme which appears altered. Kendall & Holloman (1990) showed that the 14-demethylase enzyme of resistant strains is less inhibited by triadimenol than that of sensitive strains and higher doses of the fungicide were needed to achieve the same level of inhibition of enzyme activity as seen with lower doses applied to sensitive strains.

Laboratory mutants with reduced sensitivity to prochloraz have been induced in *P. herpotrichoides* (Julian *et al.*, 1994). These mutants displayed patterns of cross-resistance to related demethylation inhibitors that have not previously been reported within eyespot.. In addition, high levels of tolerance were reported to unrelated compounds, such as methanol (J.

Hardy, Pers Comm). This broad range of resistance has been taken as indicative of uptake mutants in other systems (Welker & Williams, 1983) However it also suggests that there is a lack of specificity of the resistance mechanism.

1.5 PATHOGENICITY

Pathogenicity of plant pathogens to host plants can be expressed at three levels:

1. Basic compatibility
2. Host species-specificity
3. Gene-for gene specific interactions.

Basic compatibility includes all the features which enable an organisms to be pathogenic or possess the potential to be pathogenic on host plants. Some fungal species are found to have a very broad host range. For example *Phytophthora palmivora* and *P. cinnamomi* are plurivorous species, able to infect and cause disease in many host species from diverse taxonomic groups (Holiday, 1992). These species presumably have basic compatibility with many host genera. Other pathogens are restricted to particular host families or orders (e.g. Graminae). Several pathogens are more or less restricted to parasitism of the Graminae, including *Gaeumannomyces graminis, Phialophora* spp., *Pythium graminicola* and *Hymenella cerealis* (Deacon, 1996). Interestingly the Graminae are not found to be hosts for *Phytophthora* species in nature (Zentmeyer, 1980).

Differences in host defence mechanisms, such as phytoalexin production, may help to explain family-specific parasitism (Bailey & Mansfield, 1982). In the Graminae there is an additional factor present in that the primary cell wall is composed mainly of arabinose, xylose and mixed glucans to which pathogens of the Graminae would appear to be enzymatically adapted. Cooper *et al.* (1988) found that *P. herpotrichoides, Rhizoctonia cerealis* and *Fusarium culmorum* produced large quantities of arabinase, xylanase and laminarinase when grown on cereal cell walls and therefore the ability to produce these enzymes would appear to be part of

the basic compatibility mechanisms required by these pathogens in order to parasitise graminaceous hosts.

Host species specificity defines the species that can act as hosts as distinct from the species that cannot. Often specialization for particular host species have been recognised within pathogen species and is typically used to define *formae speciales* (i.e. f.sp.), or pathotype. For example, *Fusarium oxysporum*, in which approximately 80 different form species have been recognised; *F. solani* and *Erysiphe graminis* (Smith *et al.*, 1988). In each of these species *formae speciales* are designated on the basis of pathogenicity to different hosts species (e.g. *Erysiphe graminis* f.sp. *hordei* - barley; *E.g.* f.sp. *tritici* - wheat; *E.g.* f.sp. *secale* - rye).

Gene-for-gene interactions are commonly seen in biotrophic organisms or species that have a biotrophic phase in the host-plant interaction (e.g. *Erysiphe* spp., *Puccinia* spp., *Phtyophthora infestans*, *Cladosporium fulvum*). Flor (1942) described the genetic basis of these interactions in which a gene for resistance (R) in the host species is opposed by a gene for avirulence (A) in the pathogen. The interactions of the products of these two genes will lead to resistance expression. This system for gene-for-gene interactions is superimposed on basic compatibility and host species specificity. In *E. graminis* each level of pathogenic ability is evident, with basic compatibility being expressed to the *Graminae*; different *formae speciales* are recognised for different host species (Host species specificity) and, additionally, differences are seen between isolates in their ability to infect different host genotypes (Gene-for-gene interactions).

Pathogenicity in *P. herpotrichoides* is found to be a combination of basic compatibility and host species-specificity. Basic compatibility is expressed to members of the *Graminae* with the fungus found to be pathogenic to a wide range of grass species including *Agropyron*, *Agrostis*, *Alopecuris*, *Avena*, *Bromus*, *Cynosurus*, *Festuca*, *Lolium*, *Phleum* and *Poa* (Oort, 1936; Sprague, 1934; Cunningham, 1965; Davies, 1970). In addition, host species specificity is also

found for different cereal and grass hosts in the degree of symptom severity. Isolates from wheat, barley, rye and oats were used in pathogenicity trials involving each of these four host species (Scott *et al.*, 1975). The results showed that isolates from each cereal were pathogenic to each of four cereals, although differences were found in the levels of pathogenicity observed. In general, all isolates were most pathogenic to wheat, less so to barley, and oats and rye were only slightly affected. Further pathogenic distinctions were also made on the basis of pathogenicity to wheat, rye, *Aegilops squarrosa* and *Agropyron repens* of different isolates of the fungus. These differences have been used to recognise separate pathotypes of the pathogen. Table 1.1 shows the pathogenic interactions characteristic of the currently recognised pathotypes in *P. herpotrichoides*. As mentioned in Section 1.1 the predominant pathotypes in cereal crops are the W- and R-types. The difference between the pathogenic and non-pathogenic interactions in this host pathogen system is not absolute but one of degree of disease severity. For example W-type typically cause far less severe symptoms on rye than R-types, while on wheat both pathotypes are usually considered equally pathogenic.

Table 1.1 Distinction of pathotypes of *P. herpotrichoides* on the basis of host species specific pathogenicity. Pathogenic interactions indicated by +, 'non-pathogenic' combinations indicated by -. Data compiled from Cunningham (1965, 1981) and Scott *et al.* (1976).

	Host Species			
Pathotype	**Wheat**	**Rye**	**Ae. squarrosa**	**A. repens**
W	+	-	-	-
R	+	+	-	-
C	+	-	+	+
S	+	-	+	-

Although variation in the level of pathogenicity in the eyespot fungus are not host cultivar specific, differences in host susceptibility are found between susceptible and resistant cultivars.

MATERIALS AND METHODS

YG, g l^{-1}; malt extract, 5; yeast extract, 2.5; glucose, 10; Davis
ro dextrose agar (PDA, Oxoid, 39 g l^{-1}) were the main complete
ture of *P. herpotrichoides*. For the identification of auxotrophic
r minimal medium was used (MM; g l^{-1}: K_2HPO_4, 0.1; $NaNO_3$,
l. 0.5; NaCl, 0.5; glucose, 10; trace element solution; 1ml of a
mine HCl 0.5 ml (1% stock solution); Oxoid No. 3 agar, 20). The
on contained the following salts (per 100ml): $Na_2B_4O_7.H_2O$,
g; $FePo_4.2H_2O$, 20mg; $MnSo_4.5H_2O$, 20mg; $Na_2MoO_4.2H_2O$,
. Spore production by *P. herpotrichoides* requires the use of a
a such as tap water agar (TWA: 20 g l^{-1} Davis standard agar
uid culture of *P. herpotrichoides* for the production of mycelium
loyed liquid MYG (LMYG, recipe as for MYG but omitting Davis
celium for isozyme preparation an enriched liquid MYG media was
xtract, 7.5; yeast extract, 2.5; glucose, 20; casamino acids, 0.5).
s and fusion products required the use of complete regeneration
that of MYG but with 0.6 M sucrose in place of glucose) and
(MMR; recipe as for that of MM but with 0.6 M sucrose instead

l isolates in protoplast fusion experiments, as control isolates in
esting, fungicide sensitivity testing and pathogenicity experiments
Hocart. Details on the origins of these isolates are presented in
re abstracted from Hocart (1987).

2.3 Spore Production

Conidia of *P. herpotrichoides* were produced by spreading a suspension of hyphal fragments of the required isolates over the surface of TWA plates. Hyphal suspensions were produced by gently scraping the surface of colonies growing on complete medium in a small volume of water with a round bladed scalpel. These plates were then incubated at 14°C under near ultraviolet (NUV) light for between 10 and 14 days for optimal conidia production.

2.4 Protoplast Production and Fusion

Spore suspensions of the two parental isolates were produced from 10 inoculated TWA plates which were harvested by gently scraping the agar surface into sterile distilled water. The spores were washed by centrifugation with distilled water to remove any remaining agar and divided between 10 flasks of 40 ml each of LMYG (approximately 2 x 10^5 spores per flask). The flasks were then incubated on a refrigerated orbital shaker for between 44 and 48 h at 19°C (44 h for W-type isolates and 48 h for R-type isolates). The resulting mycelium was harvested by centrifugation at 10,000 *g* for approximately 20 min before placing in a lytic enzyme solution (Rhozyme HP150N; Driselase; Cellulase CP 10 mg ml^{-1} of each in a 0.05 M Na maleate buffer, pH5.8. The buffer was prepared as follows: 50mls of 0.2M solution of acid sodium maleate, 8.0 g NaOH and 23.2 g maleate per litre with 20.8 mls, 0.2 M NaOH, 8.0 g NaOH per litre and 0.4 M $MgCl_2$, made up to 200 mls with distilled water to give a final pH of 5.8. Lytic suspensions were incubated with gentle shaking at room temperature for approximately 4.5 hours after which the suspension was filtered through sinter glass porosity 1 to separate undigested mycelium from the protoplasts. The protoplasts were washed twice in 0.4 M $MgCl_2$ before resuspension in 1 ml $MgCl_2$.

Protoplasts of the two isolates to be fused (approximately 10^7 protoplasts of each strain is desirable for successful fusions, Magnano *et al.*, (1994)) were mixed together, centrifuged at 3,000 *g* and then resuspended in 2ml polyethylene glycol (PEG: 30% PEG M.W. 8,000; glycine, 0.05 M; $CaCl_2$, 0.01 M, pH 7.5) and incubated at room temperature for 10 min. After dilution with 6ml 0.4 M $CaCl_2$, centrifugation at 3,000 *g* and re-suspension in 2 ml $MgCl_2$ the

protoplasts could then be plated out on minimal regeneration medium for recovery of putative diploids. Additionally, fusion mixture and protoplasts of both isolates were plated onto complete regeneration medium to assess viability.

2.5 Purification and Production of Progeny

Colonies growing on minimal regeneration medium were identified as putative diploids. These colonies were twice macerated and plated onto MM to try and eliminate any parental material contaminating the fusion products. Colonies were macerated by grinding a small piece of inoculum in water using a sterile Pasteur pipette. Diploids were typically very slow growing on MM and had dark aerial mycelium. These colonies were then subcultured, by maceration, onto a variety of haploidising agents, at varying concentrations depending on the fusion, including carbendazim, ρ-fluorylphenylalanine (FPA), or chloral hydrate (CH) dissolved in TWA containing the nutritional supplements required by the parents of the cross to induce mitotic haploidization and breakdown of the putative diploids. After approximately two weeks growth at 19°C a visual assessment of the plates showed morphological variation amongst the colonies. A selection of these were subcultured onto both MM and MYG by hyphal maceration to induce further breakdown of the fusion products to yield haploid progeny. A further two cycles of sub culturing were generally required before stable colony morphologies were recovered. At this stage, when no further instability in colony morphology was observed and colonies growing on any one plate were morphologically similar the colonies were presumed to be haploids.

3 PROTOPLAST FUSION, SELECTION OF PROGENY AND PROOF OF RECOMBINATION

3.1 INTRODUCTION

The production of recombinant progeny from protoplast fusion of vegetatively incompatible W- and R-type isolates of *P. herpotrichoides* has been demonstrated previously (Hocart *et al.*, 1993b). Evidence from this cross suggested that, in contrast to parasexual crosses involving vegetatively compatible strains, no detectable heterokaryon phase was formed following protoplast fusion and that relatively stable diploids were produced which generated haploid progeny. Analysis of the segregation of markers confirmed the recombinant status of the progeny and also indicated that non-Mendelian segregation for some markers had taken place in the progeny. The work described in this chapter used parasexual recombination following protoplast fusion to generate recombinant progeny from crosses between W- and R-type strains and W- and W-type strains. Characterization of the segregation of a number of parental markers including differing auxotrophic requirements, isozyme banding patterns, sensitivity to benzimidazole fungicides and spore characteristics was carried out to confirm the recombinant status of progeny and to analyse segregation patterns.

3.2 MATERIALS AND METHODS

3.2.1 Protoplast Production and Fusion

Protoplast production of a number of W- and R-type isolates to allow both W x R and W x W type crosses to be performed was carried out following the technique outlined in Chapter 2, Materials and Methods. Table 3.1 outlines the parasexual crosses which were attempted, the auxotrophic requirements of the parental strains and details of the field isolates from which they were derived.

Table 3.1 Details of crosses carried out between W- and R-type strains , the markers carried by the parental strains and the field isolates from which they were derived.

Cross	W-type Field Isolate	Auxotrophic Parent Strain	R-type Field Isolate	Auxotrophic Parent Strain
D	22-20	22-136 *his*1-1, *arg*2-3, *ben*S	23-2	23-2/9 *met*-9, *ben*S
E	22-1 *glu*-1	22-1/7 *glu*-1, *gly*-1, *ben*S	22-119 BEN-73R	22-119/5 *arg*-7, BEN-73R
G	22-20	22-402 *his*1-1, *lys*2-4, BEN-17R	22-12	22-228 *cys*-71, *ben*S
H	22-20	22-402 *his*1-1, *lys*2-4, BEN-17R	23-2	23-2/9 *met*-9, *ben*S
I	22-20	22-409 *his*1-1, *lys*2-4, *cys*3-6, BEN-17R	22-8 BEN-5R	22-342 *ino*1-1, *nia*1-15, BEN-5R
	W-type Field Isolate	**Auxotrophic Parent Strain**	**W-type Field Isolate**	**Auxotrophic Parent Strain**
F	22-1 *glu*-1	22-1/7 *glu*-1, *gly*-1, *ben*S	22-2 BEN-1R	22-2/2 *met*-5, BEN-1R

Key to markers: *arg*2-3, *arg*-7, arginine requiring; *his*1-1, histidine requiring; *met*-9, *met*-5, methionine requiring; *glu*-1, glutamine requiring; *gly*-1, glycine requiring; *lys*2-4, lysine requiring; *cys*3-6, *cys*-71, cysteine requiring; *ino*1-1, inositol requiring; *nia*1-15, nitrate non-utilizing

3.2.2 Production and Purification of Progeny

A number of isolation and selection systems were employed for the production of recombinant progeny from the fusion products produced in each of the crosses. The methods used for each cross shall be described individually.

Cross D

Fusion products were first macerated in sterile distilled water and the mycelial suspension plated onto MM to remove any contaminating parental material that may have been present. From these plates 20 colonies were selected from each fusion product, macerated in sterile distilled water, and 10 plated on MYG containing 0.02 μM carbendazim and the remaining 10 plated on TWA containing 25 μg ml^{-1} ρ-fluorophenylalanine (FPA). A further two rounds of maceration onto MYG were required to generate stable putative haploid colonies.

Cross E

The fusion product obtained from this cross was macerated and plated onto MM as above. Colony morphology on this media was uniform and therefore one colony was selected and macerated in sterile distilled water. Aliquots of the mycelial suspension were transferred to 1.5 ml Eppendorf tubes and treated separately with one of the following concentrations of haploidizing agents: FPA 25 μg ml^{-1}, carbendazim; 2.5, 25 and 100 μM and MDPC; 10 μM. After 24 h incubation at 19°C the suspensions were plated onto MYG. From these plates a number of colonies were selected showing morphological variation. These were macerated in sterile distilled water and plated onto MYG to yield morphologically stable colonies after two more subcultures. In addition, a hyphal suspension of the original fusion product was also macerated onto TWA and incubated under near ultraviolet light (NUV) at 14°C to induce sporulation. From plates where spores were produced these were harvested and used to produce single spore isolates to test for spontaneous haploidization.

Cross F

The fusion product obtained in this cross was firstly macerated onto MM as above. Colonies were allowed to grow until sufficient inoculum was produced that could be used to generate isolated protoplasts. The objective of this procedure was to attempt to recover colonies derived from individual protoplasts and hence single nuclei. Twelve blocks of agar, approximately 0.5 cm^2 cut from the fusion product derived colonies, were inoculated onto two plates of MYG covered in sterile cellophane. After 48 h growth the blocks of agar had a region of furry mycelial growth around them. The agar blocks were split into two aliquots from each MYG plate and incubated in the standard protoplasting enzyme mixture (Chapter 2, Materials and Methods) for approximately 4.5 h. Protoplasts were harvested in the usual way. Protoplasts from each of the four samples were counted separately before the samples were pooled and plated onto 10 MMR and 10 CMR plates at 100 protoplasts per plate.

Colonies were recovered from the MMR plates, which should by that stage be free of any parental material, and inoculated onto haploidizing plates including TWA containing 25 μg ml^{-1} FPA; TWA plus 10 μM carbendazim, or TWA plus 1 μM carbendazim and 2.5 μM MDPC. All TWA plates contained the nutritional requirements of the parental isolates used in the fusion. A number of colonies were selected from each of these haploidizing plates which after two further macerations onto MYG yielded stable uniform colonies.

Cross G and Cross H

Fusion products were macerated onto MM to eliminate parental material. From these MM plates four colonies were selected for each fusion product. Hyphal suspensions from two of these colonies were each plated onto MM containing 25 μg ml^{-1} FPA and MM containing 25 μg ml^{-1} FPA plus the nutritional supplements of the parental isolates of the fusion. The remaining two colonies were macerated and suspensions plated onto MM containing 10 μM

chloral hydrate and MM containing 10 μM chloral hydrate plus the nutritional supplements of the parental isolates of the fusion. A single colony was selected from each of these haploidizing plates and subcultured by maceration a further two times onto MYG to give morphologically stable colonies.

Cross I

Maceration of the fusion products onto MM was first carried out to remove any parental material which may have been present. Considerable variability resulted on these plates. In total 16 colonies were selected for each fusion product. These were macerated onto MM and MM supplemented individually with each of the nutritional requirements of the parental isolates: histidine, lysine, cysteine, and inositol. One colony was selected from each plate in this set, macerated in sterile distilled water and plated onto MYG amended with either FPA (25 μg ml^{-1}) or chloral hydrate (10 μM). A single colony was again selected from each of these plates and subcultured by maceration two further times, onto MYG, to yield uniform colonies.

3.2.3 Proof of Recombination

A variety of experiments were carried out on the recombinant progeny from each of the above fusions to determine whether these were true recombinants. In addition, parasexual progeny from a cross between W- and R-type isolates, supplied by Dr. M. J. Hocart (Hocart *et al.*, 1993b; Hocart & McNaughton, 1994), were also included in some of these tests for the determination of the status of the progeny.

Detection of Auxotrophic Markers

Characterization of the auxotrophic status of recombinant progeny was determined by testing progeny on a set of diagnostic plates including complete medium (MYG), minimal medium (MM) and minimal medium supplemented with different combinations of the

nutritional supplements required by the parental isolates of each fusion. Progeny and the control isolates were point inoculated onto the diagnostic plates, incubated at 19°C and assessed visually for growth after 7 and 14 days. Table 3.2 details the range of diagnostic plates that were used for each of the crosses to determine the nutritional requirements of the progeny. In addition, progeny from the interspecific fusion between *P. herpotrichoides* and *P. anguioides* (Hocart & McNaughton, 1994) were also included. Initially, progeny from crosses E, F, G & H were tested on MM and MYG. Progeny from crosses G & H which had not grown on MM were then tested on the individual nutritional supplements required by the parents of the cross. Cross I progeny were initially tested on MM, MYG and MM amended with different combinations of the nutritional requirements of the parental strains. Auxotrophic isolates were then further tested on individual supplements.

Isozyme Banding Patterns

A hyphal scraping of the isolates to be tested was inoculated into 25 mls of enriched malt, yeast, glucose agar (EMYG) and incubated at 19°C in a refrigerated orbital shaker at 100 rpm. After 19 days the mycelium was harvested by filtration and collected on a 9 cm Whatman No. 1 filter paper disk in a Buchner funnel. The filter papers and mycelium were then frozen at -70°C, before being freeze dried overnight and then stored at -70°C. Freeze dried mycelium was ground in a pestle and mortar using liquid nitrogen and the resulting powder used to half fill a 1.5 ml Eppendorf tube. One ml of sample buffer, containing Tris-HCl, 50 mM; glycerol, 18% v/v; sucrose, 17% w/v; Pepstatin A, 1 μM; PMSF, 200 μM; leupeptin 1 μM; EDTA, 100 μM; DTT, 0.1 mM and the volume adjusted to 10 ml with the addition of 5.95 ml H_2O. The contents of each Eppendorf tube were vortex-mixed for 2-3 min, incubated on ice for 30 min, re-mixed and left to stand on ice for a further 15 min. After this time the samples were centrifuged for 5 min before the supernatant was dispensed into 40 μl aliquots in Eppendorf tubes. Samples were then stored at -70°C until used.

Table 3.2 Details of the diagnostic plates used to determine the nutritional requirements of progeny from W x R crosses D, E, G, H and I, W x W cross F and also the interspecific hybrids obtained between *P. herpotrichoides* and *P. anguioides*.

Cross			Minimal Medium Amended With
D	MYG	MM	MET/CIT MET/HIS CIT/HIS
E	MYG	MM	
F	MYG	MM	
G	MYG	MM	HIS LYS CYS
H	MYG	MM	HIS LYS MET
I	MYG	MM	H/L/C/I H/L/C/A H/L/I/A H/C/I/A L/C/I/A H/L/C/I/A HIS LYS CYS INO ASN
Interspecific Hybrids	MYG	MM	CIT, NIC

where MET = methionine allows growth of *met*$^-$ strains

CIT = citrulline allows growth of *arg*$^-$ strains

HIS/H = Histidine allows growth of *his*$^-$ strains

LYS/L = Lysine allows growth of *lys*$^-$ strains

CYS/C = Cysteine allows growth of *cys*$^-$ strains

INO/I = Inositol allows growth of *ino*$^-$ strains

ASN/A = Asparagine allows growth of nitrate non-utilizing strains

NIC = Nicotinic Acid allows growth of *nic*$^-$ strains

Polyacrylamide gel electrophoresis, using the BioRad Mini-protean II gel system, was used for the separation of proteins. A 4% (w/v) polyacrylamide stacking gel (29.2 : 0.8 acrylamide to N', N'-bis-methylene-acrylamide in 0.125 M Tris-HCl buffer, pH 6.8, with 0.055% (w/v) ammonium persulphate and 0.1% (w/v) TEMED) was used on top of a 12% (w/v) resolving gel (29.2 : 0.8 acrylamide to N', N'-bis-methylene-acrylamide in 0.375 M Tris-HCl buffer, pH 8.8, with 0.05% (w/v) ammonium persulphate and 0.05% (w/v) TEMED). The running buffer, pH 6.3, contained 0.025 M Tris base and 0.19 M glycine. Twenty microlitre aliquots of each sample were loaded into each lane of the gel and the gel run at 150 V for approximately 50 min at room temperature.

Glucose phosphate isomerase (GPI: EC No. 5.3.1.9), glutamate dehydrogenase (GDH: EC No. 1.4.1.3), malate dehydrogenase (MDH: EC No. 1.1.1.37) and mannose phosphate isomerase (MPI: EC No. 5.3.1.8) activities were detected using the staining procedures as described by Tanksley and Orton (1983) (Appendix 3.1). The technique of Brewbaker *et al.* (1968) (Appendix 3.1) was used to detect esterase activity. It was shown by Julian and Lucas (1990) that these enzymes would give repeatable and consistent isozyme differences characteristic of W and R pathotypes and also of *P. anguioides*.

Isozyme analysis was carried out on a range of progeny produced from a number of intraspecific crosses and one interspecific cross. The interspecific hybrid recombinants (Hocart & McNaughton, 1994) were most extensively analysed by isozyme staining. Additionally, progeny from cross D and the 'diploid' fusion products from crosses G, H and I were also analysed for esterase banding patterns.

Table 3.3 lists the progeny, fusion products and the parental isolates of each cross which were analysed from each fusion and the enzyme stains used to characterize them.

Table 3.3 Parasexual progeny, fusion products and the parental isolates of each cross analysed by isozyme banding patterns.

Cross	Fusion Product	Progeny	Stains Used
Interspecific Hybrids *P. herpotrichoides* (R-type) x *P. anguioides*	FPt	BU1, BU2, BH, AM, FW, GD, AY, BD, EQ, EO, FP, AJ, AK, FD, FH	MDH, GDH, GPI, MPI, Esterase
	FPc	DM	
	FPe	DQ	
Interspecific Hybrids *P. herpotrichoides* (W-type) x *P. anguioides*	FPiii	CF2, CA	As Above
D	FP1	D1/18a, D1/14, D1/107, D1/8, D1/7	Esterase
22-136 (W)	FP2	D2/36, D2/27, D2/22, D2/26, D2/35	
x	FP3	D3/45, D3/46, D3/58, D3/41, D3/48,	
23-2/9 (R)		D3/42	
	FP4	D4/75, D4/78, D4/63, D4/64	
	FP5	D5/82, D5/83b, D5/100, D5/94, D5/85, D5/97	
G	FP1	original fusion product only tested	Esterase
22-402 (W)	FP2	"	
x	FP8	"	
22-228 (R)	FP10	"	
	FP12	"	
H	FP1	original fusion product only tested	Esterase
22-402 (W)	FP2	"	
x	FP3	"	
23-2/9 (R)	FP4	"	
	FP5	"	
I	FP1	original fusion product only tested	Esterase
22-409 (W)	FP6	"	
x			
22-342 (R)			

where MDH = Malate dehydrogenase

GDH = Glucose dehydrogenase

GPI = Glucose phosphate isomerase

MPI = Mannose phosphate isomerase

Inheritance of Benzimidazole Resistance

Difference in sensitivity levels towards carbendazim and MDPC was present between the parental isolates of crosses E, F, G & H (Table 3.1) and as such can be used as a further marker by which to test the progeny derived from these crosses as proof of recombination. Progeny from each of the four crosses were inoculated on to MYG media amended with 10 μ M carbendazim or 20 μM MDPC. After 14 days incubation at 19°C colonies were assessed visually for growth and scored as either growing (resistant) or not growing (sensitive).

Spore Analysis

Spore length and nuclear number was determined for a sample of the interspecific hybrid progeny and their parental isolates. An additional, two W- and two R-type isolates were included for comparison. Spores were produced for each isolate by inoculation onto TWA and incubation under near ultraviolet light (Chapter 2, Materials and Methods outlines the procedure used for spore production).

Blocks of agar of each isolate to be tested were cut from the TWA plates and mounted on glass microscope slides. Spore measurements were taken using an eyepiece graticule and viewing under a light microscope. At least fifty measurements were taken for each isolate. Conidia were fixed with 70% ethanol and stained with the cell wall-specific fluorescent brightener Uvitex MST (0.01%) and the DNA specific fluorochrome DAPI (0.1%) to allow an estimation of cell number and nuclear content. Differences in spore length were compared using analysis of variance on untransformed data. Cell number data were transformed using Cochran's (1938) square root transformation for small whole numbers ($X' = \sqrt{[x + 0.5]}$).

3.3 RESULTS

3.3.1 Protoplast Production and Fusion

Protoplast isolation using the technique outlined in Chapter 2, Materials and Methods, was successful in producing viable protoplasts from each of the auxotrophic strains listed in Table 3.1. Generally protoplast numbers isolated were found to range from approximately 5×10^6 to 9×10^7 (Table 3.4) giving sufficient numbers to determine protoplast viabilities as well as carry out the fusion experiments. Protoplast viabilities were relatively low for each strain with values falling between < 0.2% and 14%. This figure was further reduced after mixture of the protoplasts of the two strains to be fused and treatment with PEG, the viability of which ranged from 0.16 to 3.5%. Each of the strain combinations in Table 3.1 was successful in yielding prototrophic fusion products. Fusion frequencies are shown in Table 3.4 and were found to vary from 1.47×10^{-5} to 2.7×10^{-4}.

3.3.2 Production and Purification of Progeny

Cross D

Five putative diploid fusion products were obtained in this cross which, unlike the parental strains, were able to grow on the MM selection plates. Figure 3.1 illustrates two of the putative diploid fusion products subcultured onto MM. Macerates of the fusion products plated onto the haploidization plates yielded a variety of colony morphology types. Figure 3.2 illustrates the range of colony morphologies typically obtained by maceration of diploid fusion products prior to exposure to haploidizing agents. After two rounds of maceration of these colonies onto MYG uniform colony morphologies were yielded from each of the five fusion products. In total 20 colonies were selected from each of the fusion products after this final maceration stage, 10 from the carbendazim treated plates, and 10 from the FPA treated colonies, and makes up the fusion product set 20 of progeny.

Table 3.4 Production of prototrophic fusion products from W x R and W x W strain combinations. The number or protoplasts isolated for each strain and their viability are presented below in addition to fusion viability and frequency.

	Parental Strains	
	W-type 22-136	**R-type 23-2/9**
No. protoplasts Produced	9×10^6 ml^{-1}	3×10^7 ml^{-1}
Protoplast Viability	< 0.2%	6.3 % (100/plate)
Fusion Viability	1.8%	
Fusion Frequency	1.4×10^{-4}	
	Parental Strains	
	W-type 22-1/7	**R-type 22-119/5**
No. Protoplasts Produced	1×10^7	1×10^7
Protoplast Viability	0.25 % (10/plate)	14.25% (100/plate)
Fusion Viability	0.16% (10^{-2} protoplast per plate)	
Fusion Frequency	2.7×10^{-4}	
	Parental Strains	
	W-type 22-1/7	**W-type 22-2/2**
No. Protoplast Produced	9.31×10^6	1.145×10^7
Protoplast Viability	8.6% (100/plate)	1.6% (100/plate)
Fusion Viability	1.7% (10^{-2} protoplasts per plate)	
Fusion Frequency	1.9×10^{-5}	

Table 3.4 contd.

	Parental Strains	
	W-type 22-402	**R-type 22-228**
No. Protoplasts Produced	9.6×10^7	4.76×10^6
Protoplast Viability	< 0.2%	14.2 % (100/plate)
Fusion Viability	1.46% (10^{-2} protoplasts per plate)	
Fusion Frequency	5.99×10^{-5}	
	Parental Strains	
	W-type 22-402	**R-type 23-2/9**
No. Protoplasts Produced	9.6×10^7	5.42×10^6
Protoplast Viability	< 0.2%	3.5% (500/plate)
Fusion Viability	0.85% (10^{-2} protoplasts per plate)	
Fusion Frequency	1.47×10^{-5}	
	Parental Strains	
	W-type 22-409	**R-type 22-342**
No. Protoplasts Produced	7.3×10^6	1×10^7
Protoplast Viability	1.2% (100/plate)	6.6% (100/plate)
Fusion Viability	0.4% (10^{-2} protoplasts per plate)	
Fusion Frequency	6.9×10^{-4}	

Protoplast viability was calculated as the percentage of regenerating colonies.

Fusion viability was calculated as the percentage of the total protoplast mixture which regenerated on complete regeneration plates after treatment with PEG.

Fusion frequency was calculated as the proportion of colonies regenerated on minimal regeneration media in comparison to fusion viability.

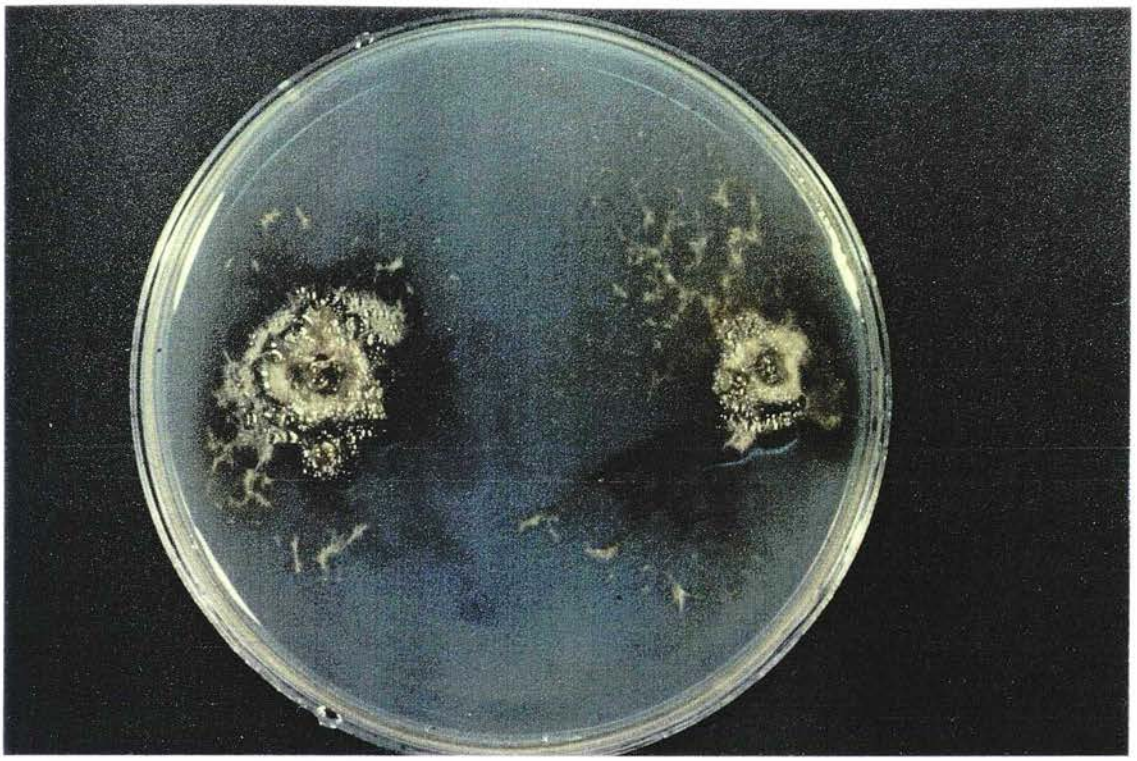


Fig. 3.1 Two of the putative diploid fusion products, generated in cross D, subcultured onto minimal medium.

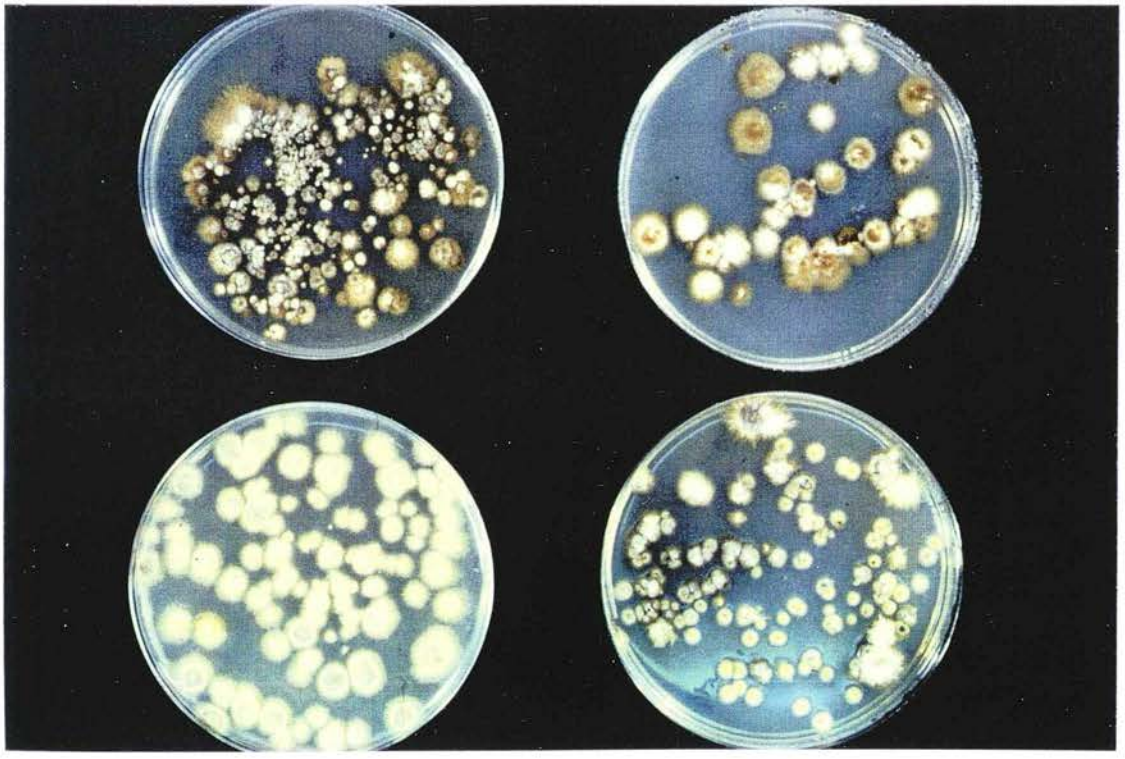


Fig. 3.2 Morphological variability typically obtained by maceration of diploid fusion products onto minimal medium, prior to exposure to haploidizing agents.

Cross E

Considerable background growth was present on the minimal regeneration plates (i.e. those plates that should yield fusion products), the morphology of which resembled that of the two parental strains 22-1/7 and 22-119/5. However, one colony was isolated which resembled a putative fusion product. Not much variation in colony morphology was evident from this fusion product prior to incubation in the presence of the different haploidizing agents. After incubation of hyphal suspensions in the haploidizing agents solution there was no growth from the macerates which had been incubated in the presence of 100 μM carbendazim and 10 μM MDPC. Growth was, however, found on all other plates. Some differences in colony morphology were evident from these plates and a range of colony types were selected for further purification until stable colony morphologies were generated. Spores were obtained on approximately half of the TWA plates, incubated under NUV light, and were used to produce single spore isolates.

Cross F

Four potential fusion products were isolated from the fusion products. After maceration onto MM, only one of these was sufficient to be treated further, due to contamination of the plates. The one fusion product was inoculated onto MYG with cellophane for the production of inoculum for protoplast isolation. Counts of protoplast numbers of each of the four aliquots were made which gave an average of 1.26×10^5 protoplasts ml^{-1}. Good regeneration of the protoplasts was found on both MMR and CMR with 63.8 and 59.2% regeneration respectively. Some variation in colony morphology was evident at this stage. Incubation of carbendazim-treated and FPA-treated colonies did not further increase this variation and stable colony morphologies were generated after a further two round of maceration.

Cross G and Cross H

Twelve putative fusion products were isolated from cross G, and five were produced in cross H. After maceration onto MM only five of the 12 colonies from cross G looked sufficiently like fusion products to be treated further, while all five colonies from cross H were considered sufficiently like fusion products. The colonies rejected from cross G all showed a morphology resembling one or other of the parental strains when macerated onto MM. At this stage there was variation in colony morphology evident for the remaining fusion products and four different colonies were selected from each fusion product. The different range of haploidizing plates were selected to try and optimise the range of auxotrophic markers that could be found in the haploid progeny. After exposure to the haploidizing agents two further rounds of maceration were required before stable colony morphologies were generated. A total of 40 progeny were selected for each of these crosses, eight from each fusion product.

Cross I

Eight colonies were selected from the fusion plates of cross I for maceration onto MM. From these plates only two of these eight colonies had a morphology sufficiently dissimilar to that of the parental strains to warrant any further treatment. The majority of the other colonies all displayed a morphology resembling that of the R-type parent in the cross, 22-342. The next stage in the purification process, the inoculation of 16 colonies from the macerates of each fusion product onto MM and MM supplemented individually with each of the nutritional requirements of the parental isolates, was again carried out to try and optimise the recovery of auxotrophic haploid progeny from the cross. Maceration of one colony from each of these plates onto haploidizing agents did not increase the variability in colony morphology observed and a further two rounds of maceration onto MYG was required to yield stable colony morphologies. In total 80 colonies were produced from each fusion product.

3.3.3 PROOF OF RECOMBINATION

Detection of Auxotrophic Markers

Characterization of auxotrophic markers for each of the crosses revealed a range of recombinant phenotypes (Table 3.5). However, clearly the majority of progeny recovered displayed a prototrophic phenotype. For cross G and cross H progeny subculture onto different media did not influence the recovery of auxotrophic progeny. Similarly with cross I, although more auxotrophic progeny were recovered from the fusion products, this was not influenced by which media type the colonies were selected on, as many auxotrophic strains were recovered from the MM plates as from any of the nutritionally-supplemented plates. Characterisation of progeny from FP1 and FP6 from cross I showed that the majority of progeny recovered were prototrophic. The majority of the auxotrophic progeny recovered were found to carry only one of the markers of either parent. Only a few progeny from FP6 were found to require more than one nutritional supplement, with only two progeny having nutritional requirements that were a combination of markers from both parents.

Auxotrophic characterization of progeny from the three fusion products of the interspecific fusion indicated a bias in favour of the auxotrophic marker of the R-type *P. herpotrichoides* parent (*cys*-71). More than 80% of the progeny from fusion product FPt were cysteine auxotrophs, while none showed the nicotinic acid requirement of the *P. anguioides* parent. Prototrophic progeny were recovered from two of the fusion products, FPt and FPe, while both parental markers were recognised in progeny from FPc.

Isozyme Banding Patterns

Polyacrylamide gel electrophoresis of soluble protein extracts confirmed the status of progeny from cross D and the interspecific fusion as recombinants and hybrids respectively. Figure 3.3 illustrates the typical esterase banding patterns of a *P. herpotrichoides* W-type (Lane 3), R-type (Lane 5) and a *P. anguioides* (Lane 10) isolate with major bands labelled for

Table 3.5 Auxotrophic phenotype of progeny derived from the W x R crosses D, E, G, H and I, the W x W cross, F as well as the interspecific hybrids cross.

	Fusion Product				
Cross D	FP1	FP2	FP3	FP4	FP5
Prototrophic	20	20	20		
met-9				20	20
Total Colonies Screened	20	20	20	20	20
Cross E	FP1				
Prototrophs	14				
Total Colonies Screened	14				
Cross F	FP1				
Prototrophs	28				
Total Colonies Screened	28				
Cross G	FP1	FP2	FP8	FP10	FP12
Prototrophic	7	3	5	4	4
Total Colonies Screened	7	3	5	4	4
Cross H	FP1	FP2	FP3	FP4	FP5
Prototrophic	7	2	6	2	7
Auxotrophs	0	0	1	1	
Total Colonies Screened	7	2	7	3	7
Cross I	FP1	FP6			
Prototrophic	36	47			
*his*1-1	11	10			
*lys*2-4	3	5			
*cys*3-6	0	4			
*ino*1-1	1	0			
*lys2-4, cys*3-6	0	2			
*cys*3-6, *ino*-1	0	1			
*his*1-1, *lys*2-4	0	1			
*his*1-1, *cys*3-6, *ino*-1	0	1			
Total Colonies Screened	51	73			
Interspecific Hybrids	FPt	FPc	FPe		
Prototrophs	22	0	2		
Cys^-	100	3	16		
Nic^-	0	4	0		
$Cys^- Nic^-$	0	0	0		
Total Colonies Screened	122	7	18		

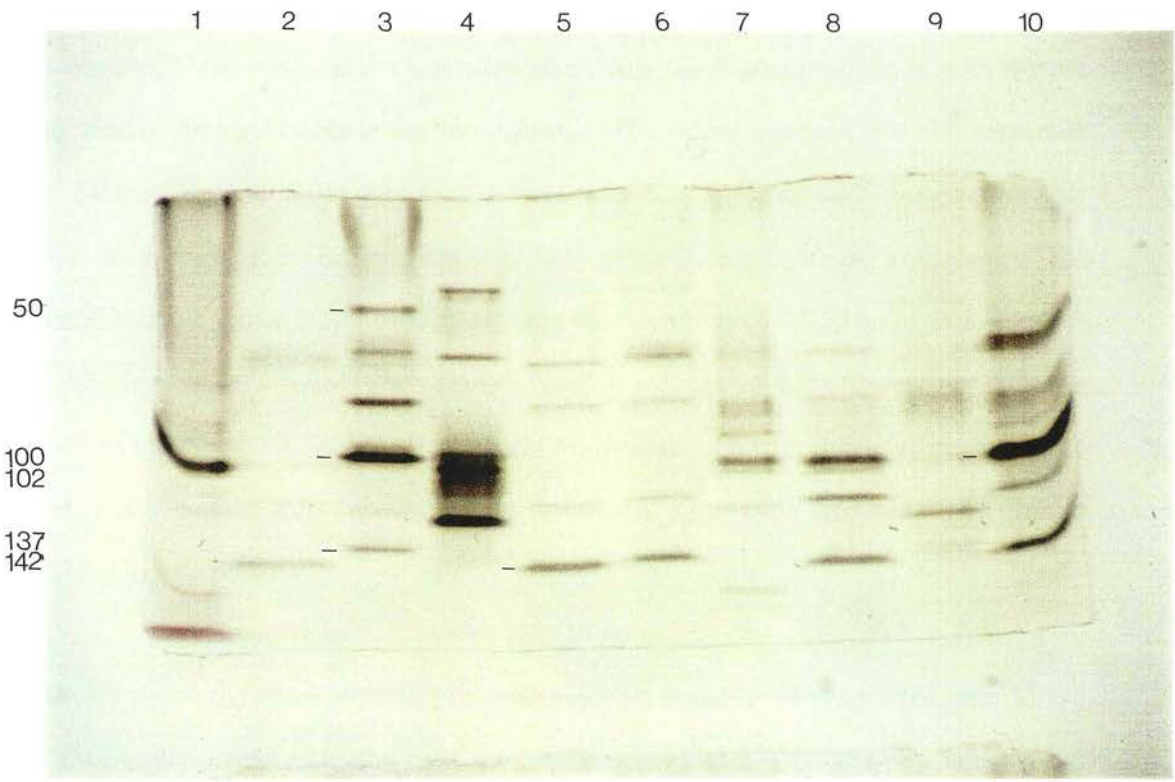


Fig. 3.3 Esterase banding patterns of a *P. herpotrichoides* W-type (Lane 3), R-type (Lane 5) and a *P. anguioides* isolate (Lane 10) with major bands labelled for each. Lanes 1, 2, 4, and 6-9 show recombinant esterase banding patterns that are typical of progeny produced by parasexual recombination between *P. herpotrichoides* (R-type) and *P. anguioides*. Bands are identified on their mobility relative to the major characteristic W-type band, which was designated esterase 100 (EST100).

each. Lanes 1,2, 4 and 6-9 show recombinant esterase banding patterns that are typical of progeny produced by parasexual recombination. Table 3.6 shows the presence or absence of major bands present within the interspecific hybrids and their parental isolates. Within the interspecific hybrids obtained between the R-type *P. herpotrichoides* (22-228) and *P. anguioides* (24-9) segregation of isozyme phenotypes for MDH and GPI was observed in progeny derived from fusion product FPt. Esterase banding patterns in many of the progeny also confirmed that hybridization had taken place, with bands characteristic of both parents being readily distinguishable in the recombinants. The hybrid status of progeny recovered from the cross between the W-type *P. herpotrichoides* (22-138) strain and *P. anguioides* (24-9) was also revealed by isozyme analysis. Both progeny tested showed a mixture of the parental isozyme forms. Novel banding patterns were observed for MDH and GDH in one of the two recombinants tested. The esterase banding patterns of these strains most clearly resembled the *P. anguioides* parent, except for the presence of a typical W-type esterase band EST129 in one of the recombinants, and the presence of a pair of W-type bands (EST162 & EST164) in both progeny.

Table 3.7 shows the esterase banding patterns that were found for progeny from cross D and the auxotrophic parentals of the cross 22-136 (W-type) and 23-2/9 (R-type). Progeny are scored in this table on the basis of presence or absence of bands found consistently for W- and R-type isolates. Esterase banding patterns of progeny from cross D were found to mostly resemble that of the R-type parent 23-2/9. Some isolates were however found that contained the typical W-type bands EST60, EST63, and EST127. Additionally, most progeny also displayed some novel banding patterns. Consistently these novel bands were found to be EST112 and EST131 (data not shown).

Table 3.8 displays the presence or absence of W- and R-type esterase bands for the fusion products produced in crosses G, H and I and the parental isolates used in the crosses.

Table 3.6 Isozyme phenotype of progeny derived in the interspecific crosses between *P. herpotrichoides* (W- and R-type) and *P. anguioides* (A) and the parental isolates used in the cross. Strain symbols (W, R and A) are used to indicate the phenotype of strains to MDH, GDH, GPI and MPI. Presence of particular esterase bands is denoted by a '+'.

Table 3.6

	Fusion Product	Strain No.	Auxotrophic Markers	Isozyme Phenotype MDH	GDH	GPI	MPI	Esterase 62	80	84	85	92	100	102	107	116	127	129	137	142	148	162	164
Parental Strains																							
P. herpotrichoides (R-type)		22-22	*CYS71*	R	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
P. anguioides		24~9	*NIC-7*	A	A	A	A	.	.	+	+	+	.	+	+	.	.	+	.	.	+	.	.
P. herpotrichoides (W-type)		22-13	*HIS3-3*	W	W	W	W	.	.	.	.	.	+	.	.	.	+	.	+	.	.	+	+
Recombinants 22-228 x 24~9	FPt	BU1	*cys-71*	R	R	R	R	+	+	.	.	.	.	+	.	+	.	.	.	+	.	.	.
		BU2	*cys-71*	R	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
		BH	*cys-71*	R	R	A	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
		AM	*cys-71*	R	R	R	R	+	+	.	.	.	.	+	.	+	.	.	.	+	.	.	.
		FW	*cys-71*	A	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
		GD	*cys-71*	R	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
		AY	*cys-71*	R	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
		BD	*cys-71*	R	R	A	R	+	+	.	+	+	.	.	.	+	.	+	.	+	.	.	.
		EQ	*cys-71*	R	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
		EO	Prototroph	R	R	R	R	+	+	+	+	.	.	.	+	+	.	.	.	+	.	.	.
		FP	Prototroph	R	R	A	R	+	+	+	+	.	.	.	.	+	.	+	.	+	.	.	.
		AJ	Prototroph	R	R	-	R	+	+	+	+	+	.	.	.	+	.	.	.	+	.	.	.
		AK	*cys-71*	R	-	R	R	+	+	+	+	.	.	.	.	+	.	.	.	+	.	.	.
		FD	Prototroph	R	R	R	R	+	+	+	+	+	.	+	+	+	.	.	.	+	.	.	.
		FH	Prototroph	R	R	R	R	+	+	.	+	+	.	.	.	+	.	.	.	+	.	.	.
	FPc	DM	*cys-71*	R	R	R	R	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
	FPe	DQ	prototroph	R	R	R	R	+	+	.	+	.	.	.	.	+	.	+	.	+	.	.	.
22-138 X 24~9	FPiii	CF2	*nic-7*	Novel	Novel	A	W	.	.	.	+	+	.	+	+	.	+	.	.	.	.	+	+
		CA	*nic-7*	W	A	A	A	.	.	.	+	+	.	+	+	.	.	+	.	.	+	+	+

Table 3.7 Isozyme phenotype of progeny produced in cross D, and the parental isolates of the cross which are scored on the basis of presence, +, or absence, -, of particular esterase bands.

Progeny Family	Progeny #	Auxotrophy Markers	Esterase 50	60	62	63	80	83	100	116	127	137	142
Parental Strains													
W-type	22-136	*his1-1, arg2-3*	.	+	.	+	.	+	+	.	.	+	.
R-type	23~2/9	*met-9*	.	.	+	.	+	.	.	+	.	.	+
Fusion Product 1	D1/18a		.	+	.	+	+	+	.	+	+	.	+
	D1/14		.	.	+	.	+	.	.	+	.	.	+
	D1/107		.	.	+	.	+	.	.	+	.	.	+
	D1/8		.	.	+	.	+	.	.	+	.	.	+
	D1/7		.	.	+	.	+	.	.	.	.	.	+
Fusion Product 2	D2/26		.	+	.	+	+	.	.	.	+	.	+
	D2/36		.	+	.	+	+	.	.	+	+	.	+
	D2/27		.	+	.	+	+	.	.	+	.	.	+
	D2/22		.	.	+	.	.	.	.	.	+	.	+
	D2/37		.	.	+	.	+	.	.	+	.	.	+
	D2/35		.	.	+	.	+	.	.	+	.	.	+
Fusion Product 3	D3/45		.	+	.	+	+	.	.	+	+	.	+
	D3/46		.	.	+	.	+	.	.	+	.	.	+
	D3/58		.	.	+	.	+	.	.	+	.	.	+
	D3/41		.	.	+	.	+	.	.	+	.	.	+
	D3/48		.	.	+	.	+	.	.	+	.	.	+
	D3/42		.	.	+	.	+	.	.	+	.	.	+
Fusion Product 4	D4/75		.	+	.	+	+	.	.	+	+	.	+
	D4/78		.	.	+	.	+	.	.	+	.	.	+
	D4/63		.	.	+	.	+	.	.	+	.	.	+
	D4/64		.	.	+	.	+	.	.	+	.	.	+
Fusion Product 5	D5/82		.	+	.	+	+	.	.	+	+	.	+
	D5/83b		.	.	+	.	+	.	.	+	.	.	+
	D5/100		.	.	+	.	+	.	.	+	.	.	+
	D5/94		.	+	+	.	.	.	.	.	.	.	+
	D5/85		.	.	+	.	+	.	.	+	.	.	+
	D5/97		.	+	.	+	+	.	.	+	.	.	+

Table 3.8 Isozyme phenotype of fusion products from crosses G, H and I and the field isolates from which the parents of the crosses were produced. Presence of a particular esterase band is denoted as a '+'.

	Fusion Product	Esterase 50	60	62	63	80	83	100	116	137	142
Parental Strains											
22-20 (W-type)		+	+	.	+	.	+	+	.	+	.
23~2 (R-type)		.	.	+	.	+	.	.	+	.	+
22~8 (R-type)		.	.	+	.	+	.	.	+	.	+
Cross G	FP1	+	.	+	.	+	.	+	+	.	+
	FP2	.	.	+	.	+	.	.	+	.	+
	FP8	.	.	+	.	+	.	.	+	.	+
	FP10	+	.	+	.	+	.	.	+	.	+
	FP12	.	.	+	.	+	.	+	+	.	+
Cross H	FP1	+	+	.	+	+	.	+	+	+	+
	FP2	.	.	+	.	+	.	+	.	.	+
	FP3	.	.	.	.	+	.	.	.	.	+
	FP4	.	.	+	.	+	.	+	.	.	+
	FP5	.	.	+	.	+	.	+	.	.	+
Cross I	FP1	+	.	+	.	+	.	+	.	+	+
	FP6	+	.	+	.	+	.	+	.	.	+

Although not all of the major bands typical of W- and R-type strains are present in the fusion products, most fusion products did show an esterase banding pattern which contained major bands typical of both parental strains and confirmed that these fusion products were a combination of both W- and R-type strains.

Inheritance of Benzimidazle Resistance

Segregation of sensitivity towards carbendazim and MDPC are presented in Table 3.9. From the table it can be clearly seen that, with the exception of cross G FP1, within a fusion product all the progeny were either all carbendazim-sensitive/MDPC-resistant or carbendazim-resistant/MDPC-sensitive and that both phenotypes did not appear together in any one fusion product set.

Spore Size and Nuclear Number

Mean spore length data, with 95% confidence intervals, measured for seven hybrid recombinant progeny, six from a fusion between *P. herpotrichoides* R-type and *P. anguioides* and one from a fusion between *P. herpotrichoides* W-type and *P. herpotrichoides* are shown in Figure 3.4 along with the data from the parental isolates 22-8 (R-type), 22-20 (W-type) and 24-1 (*P. anguioides*). Data from three additional control isolates (22-119, 22-12; R-types and 22-2; W-type) are also presented.

In each case it was found that cell compartments were uninucleate and no measurable differences in spore width were found. From Figure 3.4 it can be seen that the mean spore length data of the hybrids fall between those of the parental isolates with the hybrids spores being found to be significantly longer than those of *P. herpotrichoides* and significantly shorter than those of *P. anguioides*. Significant differences in mean spore length data was also found between the hybrids. *P. herpotrichoides* and *P. anguioides* spore length measurements

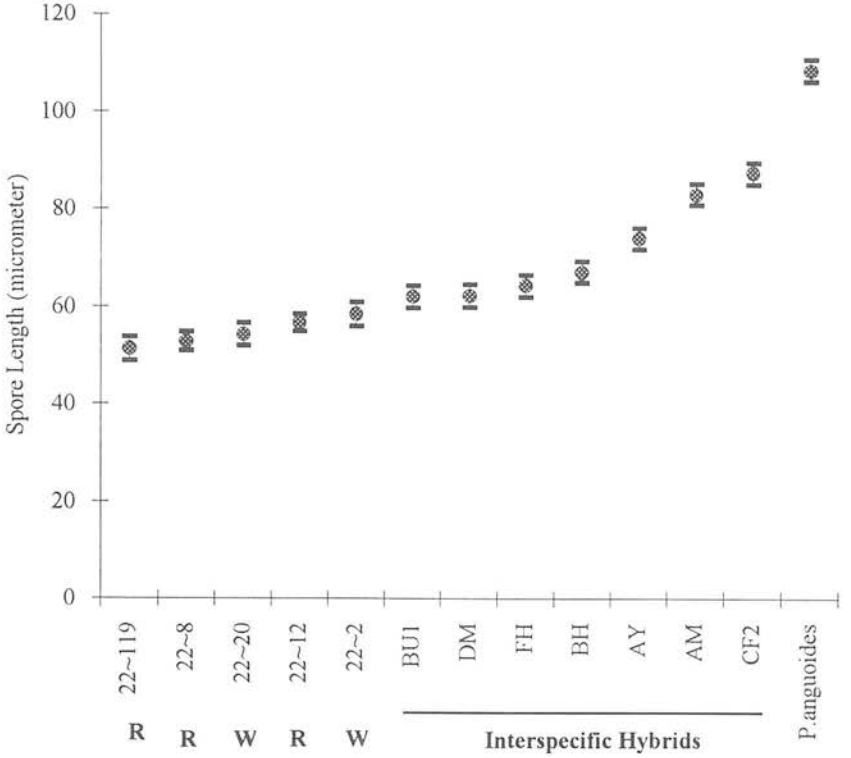
Table 3.9 Sensitivity of progeny from crosses E, F, G and H to carbendazim and MDPC. Progeny from each cross tested are split into the fusion product from which they were derived, the number of progeny exhibiting each phenotype and the total number of progeny tested from each fusion product.

Cross	Parental Strains		Fusion Product (FP)	Resistance Phenotypes* Carbendazim-S/MDPC-R	Carbendazim-R/MDPC-S	Total Progeny Tested
E	22-1 (W-type)	22-119 R-type)	1	0	8	8
F	22-1 (W-type)	22-2 (W-type)	1	8	0	8
G	22-20 (W-type)	22-12 (R-type)	1	6	1	7
			2	3	0	3
			8	5	0	5
			10	4	0	4
			12	4	0	4
H	22-20 (W-type)	23-2 (R-type)	1	0	7	7
			2	2	0	2
			3	6	0	6
			4	2	0	2
			5	7	0	7

* Progeny phenotypes are either carbendazim sensitive / MDPC resistant or carbendazim resistant/MDPC sensitive.

Fig. 3.4 Mean length (micrometer) of conidia produced by *P. herpotrichoides*, *P. anguioides* and a number of their somatic hybrids. The W and R pathotype isolates of *P. herpotrichoides* used in the crosses are represented by strains 22-20 and 22-12 respectively. The error bars represent the 95% confidence intervals for the means.

Fig. 3.4


120
100
80
60
40
20
0
Spore Length (micrometer)
22~119
22~8
22~20
22~12
22~2
BU1
DM
FH
BH
AY
AM
CF2
P.anguoides
R
R
W
R
W
Interspecific Hybrids

were found to be comparable with those previously published for these species (Deighton, 1973; Nirenberg, 1981).

The number of cells per spore and the average spore length of each cell, calculated by dividing total spore length by cell number for each isolate are presented in Table 3.10. Conidial cell number averaged 5.7 cells spore^{-1} for *P. herpotrichoides* (five strains) and 9.7 cells spore^{-1} for the *P. anguioides* isolate tested. Recombinants values ranged between 5.8 and 9.7 cells spore^{-1}. The average length of each spore cell was found to remain relatively constant, regardless of the length of the spore with a mean ± standard error of 9.7 ± 0.2 microns. Therefore, it can be concluded that longer spores contain more and not larger cells.

3.4 DISCUSSION

The work described in this chapter confirms the ability of protoplast fusion to overcome vegetative incompatibility in *P. herpotrichoides* for the production of recombinant haploid progeny from normally incompatible strain combinations. Previously, it had been shown that in contrast to compatible, intrastrain crosses, no detectable heterokaryon was formed where the parental strains were vegetatively incompatible. Subculture of fusion products typically gave rise to a high proportion of prototrophic colonies, which was shown to be transmissible through the production of protoplasts from the fusion product. In addition, it was shown that stable haploid recombinant progeny could be generated. This leads to the conclusion by Hocart *et al.* (1993b) that the viable fusion products formed in incompatible strain combinations were probably diploid. Similar results were found in the incompatible W x R crosses carried out in the present work, where the majority of colonies produced from the fusion products were prototrophic, from which stable recombinant progeny were produced. These progeny were presumed to be haploids since they subsequently remained uniform in culture with no further segregations occurring. It seems likely therefore that the products of protoplast fusion in these crosses were also diploids. The one W x W cross which was

Table 3.10 Number of cells in conidia produced by *P. herpotrichoides*, *P. anguioides* and the interspecific hybrids produced by protoplast fusion between *P. anguioides* and either R-type (BU1, DM, FH, BH, AY, AM) or W-type (CF2) *P. herpotrichoides*. Standard errors were calculated after first transforming cell data using Cochran's (1938) square root transformation for small whole numbers.

Species	Pathotype	Strain #	Cells/ spore	Square root data mean	SE	Sample size	Length / cell (um)
P. herpotrichoides	R	22~119	5.8	2.504	0.040	58	8.9
"	R	22~8	5.4	2.422	0.027	50	9.8
"	W	22~20	5.9	2.527	0.029	56	9.2
"	R	22~12	6.3	2.607	0.038	58	9.0
"	W	22~2	6.4	2.627	0.038	60	9.1
Hybrid	R x A	BU1	5.8	2.506	0.029	58	10.7
"	R x A	DM	6.1	2.562	0.027	57	10.3
"	R x A	FH	6.7	2.683	0.045	58	9.6
"	R x A	BH	6.9	2.722	0.055	30	9.7
"	R x A	AY	nd	nd	nd		nd
"	R x A	AM	8.4	2.978	0.040	60	9.9
"	W x A	CF2	9.7	3.199	0.044	58	9.0
P.anguoides	A	24~1	11.1	3.399	0.052	58	9.8

performed also involved vegetatively incompatible strains. In this cross prototrophic colonies were also produced with the recovery of stable recombinant progeny. However, much less variation in colony morphology was evident.

The frequency of fusion for the various crosses was relatively low, being 1.9 $x10^{-5}$ for the W x W cross and ranging between 1.47 $x10^{-5}$ and 6.9 $x10^{-4}$ for W x R crosses. These frequencies, while low, are comparable with those reported previously. Hocart *et al.* (1993b) obtained a fusion frequency of between 2 $x10^{-4}$ and $9x10^{-4}$ for a cross between W- and R-type strains and Magnano di San Lio *et al.* (1994) observed an average fusion frequency for incompatible W x W crosses of 4 $x10^{-5}$ and for W x R combinations of 3 $x10^{-5}$.

Marker segregation in progeny from each of the crosses performed here and also from the interspecific cross (Hocart & McNaughton, 1994) confirmed the status of progeny from each of the crosses as recombinants. Frequently with these marker systems it was observed that all the progeny derived from a single fusion product displayed an identical phenotype, suggesting that pre-segregation of these markers had occurred (i.e. segregation of certain markers prior to induced segregation from fusion products). This is especially clear in crosses where progeny were tested for sensitivity to carbendazim and MDPC. Had the fusion products all been still heterozygous diploids it would be expected that progeny from individual 'diploid' fusion products would have shown random segregation for this resistance marker.

Analysis of the segregation patterns of the nutritional requirements in the progeny showed that for four of the five crosses (Cross D, E, F & G) all the progeny derived from any one fusion product exhibited the same phenotype. The majority of progeny generated in these crosses were found to be prototrophic. Where auxotrophic progeny were obtained, as in cross D, then all the progeny from these fusion products were auxotrophic and had the same nutritional requirement. These results suggest that within these crosses pre-segregation of auxotrophic

markers had occurred within the fusion product and that there was no further modification of nutritional phenotype even after exposure to haploidizing agents. Only in progeny from crosses H, I and the interspecific hybrids were both auxotrophic and prototrophic progeny recovered from the same fusion product. Even in these crosses the majority of the progeny were still found to be prototrophs, with the exception of the hybrid progeny recovered from fusion product FPt, which were mostly cysteine auxotrophs. Although auxotrophic progeny were recovered from each of the three fusion products generated in a W x R parasexual cross reported by Hocart *et al.* (1993b) the results described in this thesis are in agreement with the previous findings in the inequality and non-random pattern of segregation with which auxotrophic phenotypes were recovered in progeny. Non-random marker segregation patterns are commonly found in interspecific crosses (Bradshaw *et al.*, 1983; Croft & Dales, 1983). This could be due to structural differences between the genomes of the two species or the expression of incompatibility in the heterozygous hybrid nucleus.

A comparison of sensitivity towards carbendazim and MDPC showed that, with the exception of FP1 progeny from cross G, all the progeny within a fusion product set displayed the same phenotype. i.e. They were either sensitive to carbendazim and resistant to MDPC or were resistant to carbendazim and sensitive to MDPC. This suggests that pre-segregation of carbendazim sensitivity had occurred in the fusion products prior to exposure to haploidizing agents. Whether further segregation had occurred to modify the levels of sensitivity of progeny could be determined by testing the progeny on a wider range of concentrations of these fungicides. Fusion products from two of the crosses, E and F, were exposed to a combination of both carbendazim and MDPC as haploidizing agents. However, it would appear that the resistance phenotype of progeny from each cross differ dramatically as progeny from cross E were carbendazim resistant / MDPC sensitive while those from Cross F were carbendazim sensitive / MDPC resistant. These data, taken together would support the suggestion that resistance to these two fungicides in controlled by the same gene.

Isozyme banding patterns confirmed the status of progeny from cross D and the interspecific fusion between *P. herpotrichoides* and *P. anguioides* as parasexual recombinants. Although bands characteristic of both parental types were found for some progeny from cross D, it was clear that there was a bias in favour of an R-type banding pattern. In progeny from the interspecific cross between *P. herpotrichoides* (R-type) and *P. anguioides* segregation for malate dehydrogenase and glucose phosphate isomerase occurred. Recombinant patterns of esterase bands were also found for 10 of the 17 recombinants tested from this cross. Banding patterns of the two progeny from the cross of *P. herpotrichoides* (W-type) x *P. anguioides* showed recombination for malate dehydrogenase, glutamate dehydrogenase, mannose phosphate isomerase and esterase. For both cross D and the interspecific progeny novel bands were also evident within the esterase banding patterns. Certain multimeric enzymes can be formed by the combinations of subunits of different origin. It has been suggested by Kelman *et al.* (1990), who showed the appearance of novel banding patterns in interspecific progeny obtained following protoplast fusion in *Aspergillus nidulans*, that the appearance of new isozymes can be explained by the presence of the genes in a new genetic background as a result of recombination between the parental chromosomes.

Isozyme banding patterns of the majority of fusion products G, H, and I confirmed the presence of both parental types within these fusion products. However, the results could not confirm that the fusion products were still diploid as not all of the major bands of both parental types were present, presumably reflecting the pre-segregation phenomenon observed for other markers in these crosses. The results do allow the suggestion that a diploid must have been formed during protoplast fusion. Segregation of spore length was evident in interspecific progeny with the spore lengths of hybrids falling between those of the parental species, *P. herpotrichoides* and *P. anguioides*. While the number of strains included in this

analysis was small the results would indicate that spore length is under additive genetic control.

4 INHERITANCE OF RESISTANCE TO ERGOSTEROL BIOSYNTHESIS INHIBITING FUNGICIDES

4.1 INTRODUCTION

Currently, eyespot is controlled using the ergosterol biosynthesis inhibiting fungicides prochloraz, an imidazole, and flusilazole, a triazole. It has been reported that no differences in sensitivity levels between W- and R-type isolates are seen towards prochloraz (Leroux & Gredt, 1985; Cavelier *et al.*, 1987). However, a reduction in the efficiency of prochloraz in France has been reported (Leroux & Marchegay, 1991). Eyespot strains isolated from these sites which had reduced sensitivity to prochloraz *in vitro* exhibited an R-type morphology. Differential sensitivity of W- and R-type isolates is frequently found to many of the triazole fungicides with the R-type isolates being found to be less sensitive than W-type isolates (Leroux & Gredt, 1985). The work described in this chapter was carried out to investigate the inheritance of differential sensitivity to demethylation inhibiting (DMI) fungicides exhibited by different pathotypes of *P. herpotrichoides*. The aim was to determine the genetic basis to DMI resistance in this pathogen and also to analyse the expression of cross-resistance to a range of ergosterol biosynthesis inhibiting fungicides including both DMI's and morpholines. This chapter describes the results obtained from *in vitro* fungicide dose response tests using progeny derived from six separate parasexual crosses between W- and R-type isolates and one interspecific cross between *P. herpotrichoides* (R-type) and *P. anguioides*.

4.2 MATERIALS AND METHODS

4.2.1 Characterization of Fungicide Sensitivity

In vitro preliminary characterization, using a single concentration of triadimenol, and full dose response tests using a range of concentrations for 16 different ergosterol biosynthesis inhibiting fungicides, were carried out to study the inheritance of differential sensitivity

exhibited by W- and R-type isolates towards DMI fungicides, and the cross resistance relationships within and between DMI's and morpholine fungicides.

Both the preliminary characterization and the full dose response tests followed the same protocol. In most cases technical grade samples were used, generously supplied by the producing company (Appendix 4.1). Fungicides were first dissolved in acetone before adding to autoclaved MYG to give a final solvent concentration of not greater than 0.5% v/v. Where only formulations of the fungicides were available these were initially dispersed in sterile distilled water before incorporation into the media. Control plates containing only the solvent (at 0.5% v/v) were used to assess fungal growth in the absence of the fungicide. Table 4.1 below shows the full list of fungicides that were employed, the chemical group to which they belong and the resistance factor for each, calculated from the ED50 values obtained for the W- and R-type parental isolates used in two of the crosses (A & D). The chemicals used as formulations are indicated by an asterisk after the chemical name and the product name is given below.

Preliminary Characterization of Fungicide Sensitivity

Initially a preliminary investigation of the sensitivity of the progeny to triazole fungicides was carried out by testing all the progeny produced from crosses A, D, E, G, H and I on one concentration of triadimenol. This fungicide was chosen for the magnitude of the difference in sensitivity between W- and R-type isolates, as indicated by the resistance factors for this compound. This allowed single concentrations of the chemical to be selected which clearly differentiate 'resistant' from 'sensitive' progeny (25 μM, cross A; 50 μM, cross D and E; 30 μM, cross G, cross H and cross I progeny). Colonies were point inoculated six per plate in a clockwise direction and a visual assessment of growth was made after 10 days incubation at 19°C. Preliminary characterization of cross A progeny was carried out by Dr M. J. Hocart and the results are reproduced with his permission (M. J. Hocart, unpublished data).

Table 4.1. Details of ergosterol biosynthesis inhibiting fungicides used in sensitivity analysis of parasexual progeny and the resistance factor for each obtained by dividing the ED50 values of the resistant (R-type) by that of the sensitive (W-type) field isolate used in the dose response test of cross A and cross D progeny.

CHEMICAL NAME	RESISTANCE FACTOR		CHEMICAL GROUP
	FUSION A	FUSION D	
Azaconazole	50		Triazole
Difenconazole	17	7.8	Triazole
Flusilazole * *Punch C*	5		Triazole
Flutriafol	19		Triazole
Imibenconazole	125		Triazole
Penconazole	126		Triazole
Propiconazole	7		Triazole
Triadimefon * *Bayleton*	28		Triazole
Triadimenol * *Bayfidan*	196	64.0	Triazole
Triflumizole	26		Triazole
Fenarimol	6	4.6	Pyrimidine
Nuarimol	4		Pyrimidine
Imazalil	4		Imidazole
Prochloraz	5	0.2	Imidazole
Fenpropimorph * *Corbel*	6	0.4	Morpholine
Tridemorph * *Calixin*	34		Morpholine

An asterisk placed after the chemical name represents those fungicides which were supplied as formulations. The product used is presented below in italics. The R-type was, in the majority of cases, more resistant than the W-type giving resistance factors greater than one. The two exceptions were for the W- and R-types tested in the cross D progeny experiment for prochloraz and fenpropimorph where the R-type was found to be less sensitive than the W-type and resulted in a resistance factor of less than one.

Dose Response Test: Cross A Progeny

A dose response test using 16 ergosterol biosynthesis inhibiting fungicides, and a subset of progeny representing each of the three fusion product (FP) families of cross A was conducted. A preliminary experiment employing just a few concentrations of fungicide covering a wide range was first carried out for each compound to enable an effective concentration to be identified. This allowed a number of concentrations to be identified that would potentially differentiate any differences in sensitivity present in the progeny.

Isolates were point inoculated using sterile cocktail sticks six per plate in a clockwise direction. After 10 days incubation at 19°C, assessments of fungal growth were made by taking two perpendicular measurements of colony diameter relative to a pre-determined point, using micrometer callipers. Three replicates were used giving six diameter readings per isolate for each fungicide concentration.

From the parental dose response results four fungicide concentrations were chosen that straddled the anticipated ED50 value. These were chosen from the middle of the plotted values of the parental isolates, where generally the straightest area of the plotted line was, and should allow all four points to be used in the regression.

Dose Response Test: Cross D Progeny

Following the full dose response test of fusion A progeny, a smaller scale experiment was carried out with cross D progeny involving a smaller number of fungicides selected to represent each of the different classes of fungicides tested for cross A progeny. The fungicides selected were: triadimenol, difenconazole (triazoles); fenarimol (pyrimidine); prochloraz (imidazole); and fenpropimorph (morpholine). Fungicide plates were inoculated and scored as those of cross A progeny described earlier.

Preliminary Segregation: Interspecific Hybrid Progeny

Although no experiment was carried out to determine initial fungicide sensitivity within the interspecific hybrid progeny produced from a cross between *P. herpotrichoides* and *P. anguioides*, it could be determined whether progeny within a fusion product set were either sensitive or resistant by picking a concentration of triadimenol that discriminates between the parents 22-228 (R-type) and 24-9 (*P. anguioides)* in the full dose response test and assessing the phenotype of the progeny at this concentration. 50 μM triadimenol was chosen as a concentration that would clearly differentiate between the sensitivities of the parental isolates 22-228 and 24-9. This concentration was also comparable with the concentration of triadimenol chosen for other crosses as being able to differentiate the parental isolates.

Dose Response Test: Interspecific Hybrid Progeny

A small scale *in vitro* fungicide dose response test was carried out using *P. herpotrichoides* (R-type) x *P. anguioides* interspecific parasexual progeny (Hocart & McNaughton, 1994) in which the sensitivity of a number of recombinants generated from three separate fusion products was tested towards the morpholine fungicide fenpropimorph and the triazoles triadimenol and difenconazole. Fungicide plates were inoculated and scored as those of cross A progeny described earlier.

Data Analysis

Log transformed fungicide concentrations were plotted against colony diameter measurements for each strain and fungicide combination. The data points around the 50% reduction in colony diameter were used for regression analysis for the estimation of a log ED50 value. In most cases reasonable lines were obtained. Some strains however, showed a marked increase in growth at low concentrations compared to the control. These points were typically excluded from the analysis. Analysis of variance (ANOVA) was used to detect deviations from linearity and 95% confidence intervals were calculated for log ED50's which

were then back transformed to give a confidence range for the ED50 value (Zar, 1974). For the detection of cross-resistance between fungicides log ED50 values for pairs of fungicides were compared by correlation analysis and correlation coefficients tested for significance by t-testing. All fungicide combinations were tested in this way.

In addition, within the triadimenol sensitive (FP1) and resistant (FP2 & FP5) progeny subsets a χ^2 analysis was carried out to assess the frequency of finding cross-resistance within each of these groupings based on the classifications made using cross-resistance data where

$$\chi^2 = \Sigma(f_i - F_i) / F_i$$

and f_i is the observed value for each category and F_i is the expected value for each category with (C-1)(R-1) degrees of freedom where C and R are the number of columns and rows respectively. The expected frequency of each category is calculated by multiplying the observed total of each row by the observed total of each column and dividing by the total number of observations.

A t-test for the comparison of the slopes and intercepts of regression lines was employed to assess whether there were significant differences amongst the cross D progeny. Progeny giving the highest and lowest ED50 values were compared with the appropriate parental isolate, whose ED50 value they most closely resembled. The following formulae were used (Zar, 1974):

$$t = b_1 - b_2 / S_{b1-b2}$$

with $n_1 + n_2 - 4$ degrees of freedom to test for significant differences between slopes where

$$S_{b1-b2} = \sqrt{(S^2_{y.x})_p / (\Sigma\chi^2)_1 + (S^2_{y.x})_p / (\Sigma\chi^2)_2}$$

and the pooled residual mean square is calculated as

$$(S^2_{y.x})_p = (\text{residual SS})_1 + (\text{residual SS})_2 / (\text{residual DF})_1 + (\text{residual DF})_2$$

and

$$b = \Sigma xy / \Sigma x^2$$

and

$$t = (Y_1-Y_2) - b_c(X_1-X_2)/\sqrt{(S^2_{y.x})c[1/n_1+1/n_2 + (X_1-X_2)_2/(\Sigma\chi^2)_1+(\Sigma\chi^2)_2]}$$

with $n_1 + n_2 - 3$ degrees of freedom to test for significant differences between intercept values where

$$(S^2_{y.x})_c \text{ is } SS_c / DF_c$$

and SS_c is the 'common regression' calculated using the formulae

$$SS_c = C_c - B^2_c / A_c$$

where A_c, B_c and C_c are the sums of $\Sigma\chi^2$, $\Sigma\chi y$ and Σy^2 for each of the regression analysis respectively.

4.3 RESULTS

4.3.1 Preliminary Characterisation of Fungicide Sensitivity

The method chosen to assess preliminary segregation was successful in that the fungicide concentrations chosen allowed the progeny to be scored as either growing (resistant) or not growing (sensitive). The results for the preliminary characterisation of progeny sensitivity towards triadimenol are illustrated in Table 4.2. Progeny from cross A all segregated as either 'sensitive' or 'resistant'. Furthermore, these phenotypes did not appear together in the progeny derived from any one fusion product. The progeny from FP1 were all more sensitive than the progeny from both FP2 and FP5. On this basis the progeny from FP1 were identified as triadimenol sensitive and those from FP2 & FP5 as triadimenol resistant.

The results of the progeny from each of the crosses D, E, G, H and I also showed that progeny segregated as either sensitive or resistant and only one of these phenotypes was found within the progeny of any one fusion product, confirming the findings for cross A progeny. From these crosses it was found that progeny from crosses D, E and I all showed a triadimenol resistant phenotype, cross G progeny exhibited a triadimenol sensitive phenotype, whilst

Table 4.2. Triadimenol sensitivity of progeny obtained from six interpathotype parasexual crosses tested using one discriminatory concentration of fungicide.

	Parental Strains		Fusion	Triadimenol Sensitivity		Total Progeny
Cross	W-Type	R-Type	Product	Sensitive	Resistant	Tested
A	22-20	22-8	1	65	0	65
			2	0	7	7
			5	0	25	25
D	22-20	23-2	1	0	17	17
			2	0	17	17
			3	0	15	15
			4	0	15	15
			5	0	18	18
E	22-1	22-119	1	0	14	14
G	22-20	22-122	1	0	7	7
			2	0	3	3
			8	0	5	5
			10	0	4	4
			12	0	4	4
H	22-20	23-2	1	7	0	7
			2	0	2	2
			3	0	6	6
			4	0	2	2
			5	0	7	7
I	22-20	22-8	1	62	0	62
			6	77	0	77

progeny from cross H behaved more like cross A generating both sensitive and resistant progeny but still with only one phenotype appearing in the progeny from each fusion product.

4.3.2 Dose Response Test: Cross A Progeny

For each of the ergosterol biosynthesis inhibiting fungicides tested it was found, as expected, that the W-type parent in the cross, 22-20, was more sensitive than the R-type parent, 22-8. Resistance factors were calculated for each fungicide using the ED50 values of the parental isolates and substituting into the formulae ED50 resistant / ED50 sensitive. The results of this calculation are presented in Table 4.1 from which it can be seen that there was considerable variation in the difference in sensitivity levels between 22-20 and 22-8 for different chemicals with there bring almost a 50 fold difference between the lowest and highest resistance factor calculated. From these resistance factors the fungicides can be split into three groups dependent on the magnitude of their resistance factor (Table 4.3).

From these groupings it can be seen that there is a clear distinction in terms of resistance factor magnitude between each of the groups. Also, the triazoles, with the exception of flusilazole and propiconazole, are all found to have either a moderate to high resistance factor magnitude. Tridemorph is the exception amongst the non-triazole fungicides which were all found to have a low resistance factor magnitude.

The results presented for the dose response test and for cross-resistance are split into subsections which generally typify the response seen. Within each section the figures that are illustrated are representative of all the possible examples and typify the results.

Table 4.3 Grouping of EBI fungicides based on the magnitude of their resistance factor.

Resistance Factor Magnitude	Range *	Fungicide
Low	4-7	Flusilazole, Propiconazole, Fenarimol, Nuarimol, Imazalil, Prochloraz, Fenpropimorph
Moderate	17-50	Azaconazole, Difenconazole, Flutriafol, Triadimefon, Triflumizole, Tridemorph
High	>120	Imibenconazole, Penconazole, Triadimenol

* The range represents a grouping of the resistance factors calculated for these chemicals (Table 4.1) by dividing the ED50 of the R-type isolate by that of the W-type isolate.

DMI's (excluding prochloraz)

The dose response test with triadimenol (Fig. 4.1a) confirmed the findings of the preliminary screen of fusion A progeny, with the progeny from FP1 being more sensitive than the progeny from both FP2 and FP5. Not all the progeny within a fusion product family were however, sensitive or resistant to the same degree and there was variation in sensitivity between progeny derived from individual fusion products. On the whole this pattern of segregation was repeated amongst the other triazoles tested and with imazalil, an imidazole fungicide.

Table 4.4 shows the ED50 values calculated to all the fungicides for 22-20 (W-type parent), 22-8 (R-type parent) and the mean ED50 values obtained for progeny derived from the three fusion products FP1, FP2 and FP5. From the table it can be seen that for nine out of the ten triazoles tested the mean resistance level (i.e. ED50 values) for the FP5 progeny was greater than the fungicide resistant R-type parent. Progeny with a greater mean resistance than 22-8 were also found amongst the FP2 progeny for five of these nine triazoles. In addition, for the imidazole imazalil the mean progeny ED50 values from both FP2 and FP5 were greater than that for 22-8. A comparison with progeny ED50 values calculated towards each fungicide (Appendix 4.2) shows that those mean ED50 values which were greater than 22-8 were generally due to at least two progeny ED50 values being greater than that of 22-8. This table of ED50 values (Appendix 4.2) also shows that on the whole 22-20 and 22-8 were at opposite ends of the spectrum, 22-20 being most sensitive and 22-8 one of the most resistant, with ED50 values for the majority of progeny falling between the two.

The pyrimidines, fenarimol and nuarimol, showed a different pattern of segregation from the triazoles. For fenarimol (Fig 4.1b) there was not such a large difference in the sensitivity level between the triadimenol sensitive progeny (FP1) and the triadimenol resistant progeny (FP2 &

Fig. 4.1 (a-d). Fungicide sensitivity response of progeny derived from three fusion products produced in a *P. herpotrichoides* interpathotype (W x R) parasexual cross, and the parental isolates towards four different ergosterol biosynthesis inhibiting fungicides, 4.1a) Triadimenol, 4.1b) Fenarimol, 4.1c) Fenpropimorph and 4.1d) Prochloraz. ED50 values are expressed as μ M active ingredient.

FIG 4.1a

TRIADIMENOL

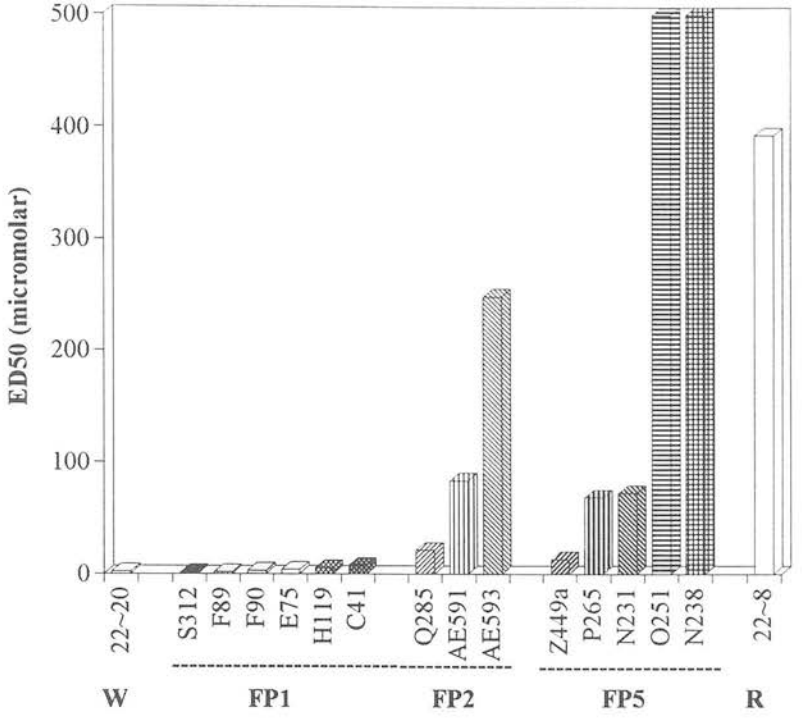


FIG 4.1b

FENARIMOL

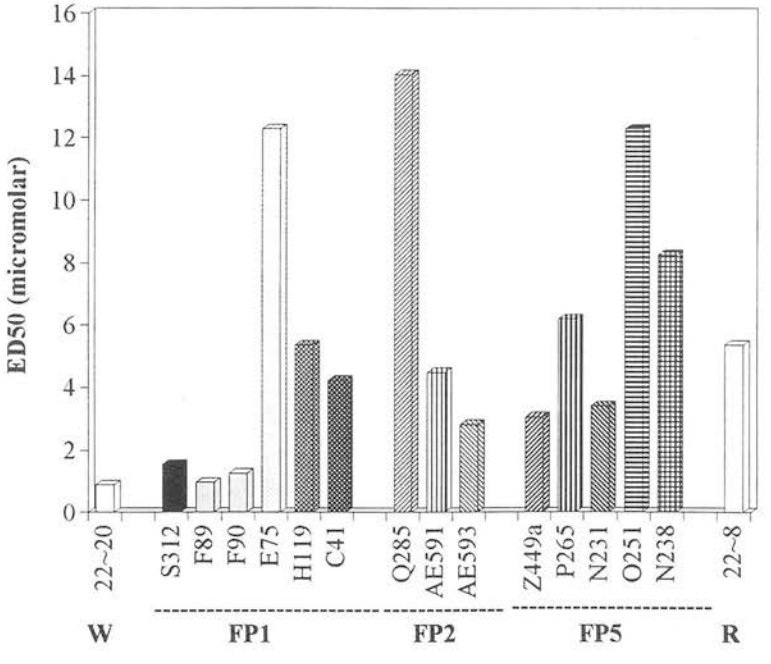


FIG 4.1c

FENPROPIMORPH

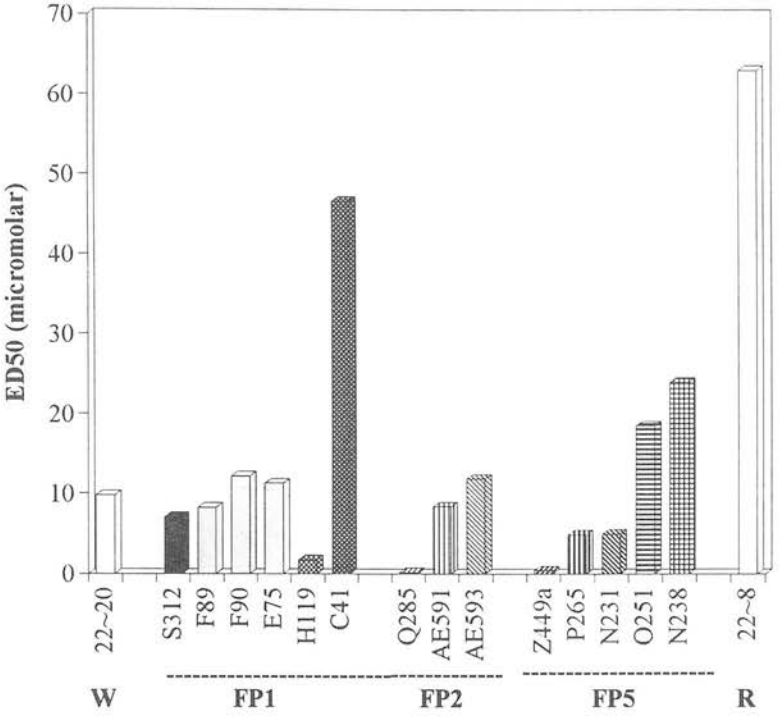


FIG 4.1d

PROCHLORAZ

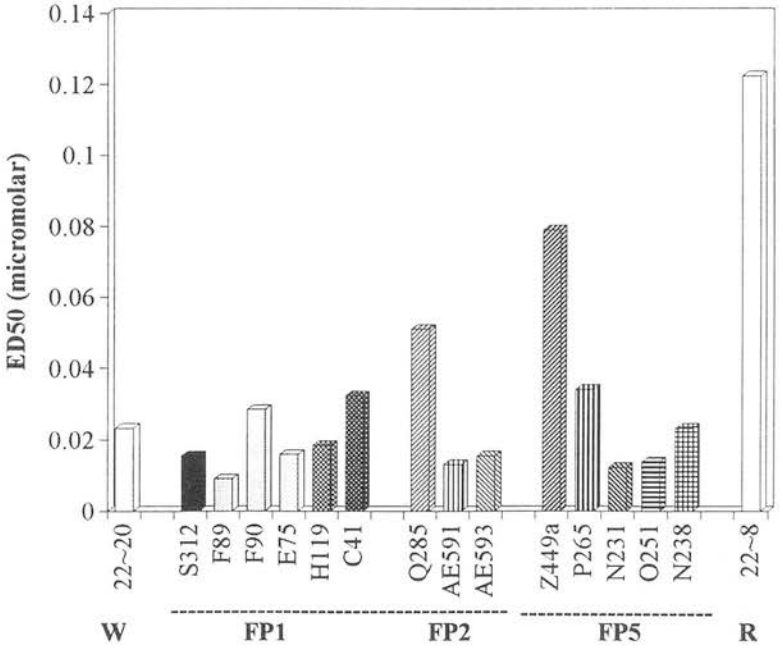


Table 4.4. A comparison of mean ED50 values calculated for progeny from each of the three fusion products, with the ED50 values for the parental isolates 22-20 (W-type) and 22-8 (R-type). Sample sizes are presented in brackets below the column labels.

	W TYPE	MEAN PROGENY ED50			R TYPE
	22-20	FP1	FP2	FP5	22-8
	(1)	(6)	(3)	(5)	(1)
Azaconazole	0.4	0.8	23	59	21
Difenconazole	0.2	0.1	1.5	2.2	3
Flusilazole	0.05	0.6	3	4.5	2.3
Flutriafol	0.02	0.2	0.8	0.7	0.3
Imibenconazole	0.07	0.1	7	43	9
Penconazole	0.04	0.2	4	8	5
Propiconazole	0.2	0.3	1.4	1.6	1.3
Triadimefon	12	17	>100	>100	>100
Triadimenol	2	3	>100	>100	>100
Triflumizole	0.5	0.7	6	33	13
Fenarimol	0.9	2	7	6	5
Nuarimol	1	3	7	7	4
Imazalil	0.01	0.2	0.6	0.8	0.5
Prochloraz	0.02	0.02	0.03	0.03	0.1
Fenpropimorph	10	14	7	9	63
Tridemorph	1.5	2	2	24	49

FP5). A few strains were more resistant than 22-8 and these were distributed amongst all three fusion product sets.

Morpholines

A large difference in sensitivity levels was seen between the parental isolates of the cross with 22-20 (W-type) found to be sensitive and 22-8 (R-type) resistant. From a spread of ED50 values for fenpropimorph (Fig. 4.1c) clearly many of the progeny from FP2 and FP5 were as sensitive, if not more so, than the progeny from FP1. There also appeared to be a super-sensitivity of some progeny, with their ED50 values to fenpropimorph substantially lower than that of 22-20, the W-type parental isolate.

Prochloraz

Clear differences in sensitivity levels between progeny from FP1 and FP2, FP5 were not found for prochloraz (Fig 4.1d), where it can be seen that the sensitivity of the majority of progeny was similar to that of 22-20 (W-type).This segregation pattern was different to what was found for the triazoles and imazalil but similar to the pyrimidines and morpholines in the lack of distinction between the FP1 and FP2, FP5 progeny sets.

4.3.3 Cross Resistance Relationships

DMI's

Correlations of ED50 values between pairs of fungicides indicated the occurrence of cross-resistance. To prove this statistically and to see if any patterns of cross-resistance were formed within and between the different chemical groups, significance testing of the correlations was carried out using a t-test. Analysis of ED50 values for all fungicide combinations by correlation analysis and significance testing was successful in recognising particular cross-resistance relationships between and within chemical groups. However, this wasn't sufficient to identify the distribution of ED50 values of the progeny and hence identify

any possible patterns of cross-resistance which graphs of paired ED50 values for test fungicide combinations was able to.

Table 4.5 shows the correlation values that were obtained for all possible combinations of fungicides. The values highlighted in Table 4.5 were significant to $P < 0.05$ for 15 degrees of freedom. The triazoles, the imidazole imazalil and the pyrimidines all showed a positive correlation to one another for all combinations indicating cross-resistance between these compounds. Two distinct patterns of cross-resistance were formed with the progeny analysed. In the first the progeny were split into two distinct subgroups representing the 'sensitive' and 'resistant' progeny types recognised previously. This pattern was seen with approximately half of the DMI's tested. Figure 4.2a showing ED50 values to azaconazole plotted against those for imibenconazole, is a typical example of this pattern. This pattern of segregation was found for azaconazole, flusilazole, imibenconazole, triadimenol, difenconazole and penconazole and is classed here as type I segregation.

For the remaining DMI's showing cross-resistance, it was found that there was considerable overlap between the ED50 values of the triadimenol sensitive and resistant progeny groups. Figure 4.2b shows a typical example of this pattern which is classified here as type II segregation. It was also seen that within the chemicals for which a type II segregation was found there could be a further classification depending on the extent of overlap of the ED50 values of the progeny. For three out of the four remaining triazoles: propiconazole; triadimefon and triflumizole, there was only a slight overlap of one or two values (type IIa segregation) but for the last triazole: flutriafol; the imidazole imazalil, and the pyrimidines, nuarimol and fenarimol, there was considerable overlap of triadimenol sensitive and triadimenol resistant progeny (Fig 4.2b) (type IIb segregation).

Table 4.5. Correlation analysis of Log ED50 data for cross A progeny and parental isolates, 22-20 and 22-8, where n=15 and any value greater than 0.482 is significant at the 5% level (bold figures).

	Azaconazole	Difenconazole	Flusilazole	Flutriafol	Imibenconaozle	Penconazole	Propiconazole	Triadimefon
Azaconazole	1.00							
Difenconazole	**0.91**	1.00						
Flusilazole	**0.84**	**0.90**	1.00					
Flutriafol	**0.71**	**0.71**	**0.78**	1.00				
Imibenconazole	**0.97**	**0.89**	**0.82**	**0.63**	1.00			
Penconazole	**0.95**	**0.91**	**0.87**	**0.72**	**0.95**	1.00		
Propiconazole	**0.88**	**0.87**	**0.79**	**0.71**	**0.87**	**0.86**	1.00	
Triadimefon	**0.92**	**0.79**	**0.62**	**0.51**	**0.92**	**0.84**	**0.84**	1.00
Triadimenol	**0.94**	**0.86**	**0.69**	**0.56**	**0.93**	**0.89**	**0.82**	**0.97**
Triflumizole	**0.90**	**0.77**	**0.62**	**0.52**	**0.93**	**0.85**	**0.80**	**0.96**
Fenarimol	**0.84**	**0.84**	**0.76**	**0.88**	**0.76**	**0.81**	**0.83**	**0.74**
Nuarimol	**0.72**	**0.72**	**0.72**	**0.96**	**0.63**	**0.69**	**0.74**	**0.57**
Imazalil	**0.84**	**0.86**	**0.83**	**0.87**	**0.76**	**0.84**	**0.77**	**0.61**
Prochloraz	0.17	0.42	0.48	0.38	0.12	0.21	0.20	−0.02
Fenpropimorph	−0.02	−0.08	−0.35	−0.29	0.12	−0.06	0.07	0.31
Tridemorph	**0.53**	**0.53**	0.23	0.28	**0.60**	**0.53**	**0.55**	**0.66**

	triadimenol	Triflumizole	Fenarimol	Nuarimol	Imazalil	Prochloraz	Fenpropimorph	Tridemorph
Triadimenol	1.00							
Triflumizole	**0.94**	1.00						
fenarimol	**0.77**	**0.72**	1.00					
Nuarimol	**0.60**	**0.58**	**0.94**	1.00				
Imazalil	**0.69**	**0.63**	**0.83**	**0.84**	1.00			
Prochloraz	0.17	0.00	0.31	0.35	0.33	1.00		
Fenpropimorph	0.26	0.35	−0.12	−0.19	−0.29	−0.24	1.00	
Tridemorph	**0.67**	**0.74**	0.47	0.37	0.40	0.01	**0.67**	1.00

Fig. 4.2 (a-c). Detection of cross-resistance between EBI fungicides through correlation of log ED50 (μM) values. Parental strains (W & R) are indicated as shaded triangles with interpathotype progeny shown as diamonds (sensitive) and squares (resistant). Illustration of three correlations are given; 4.2a) Azaconazole v. Imibenconazole, 4.2b) Flutriafol v. Nuarimol and 4.2c) Prochloraz v. Flutriafol.

FIG 4.2a

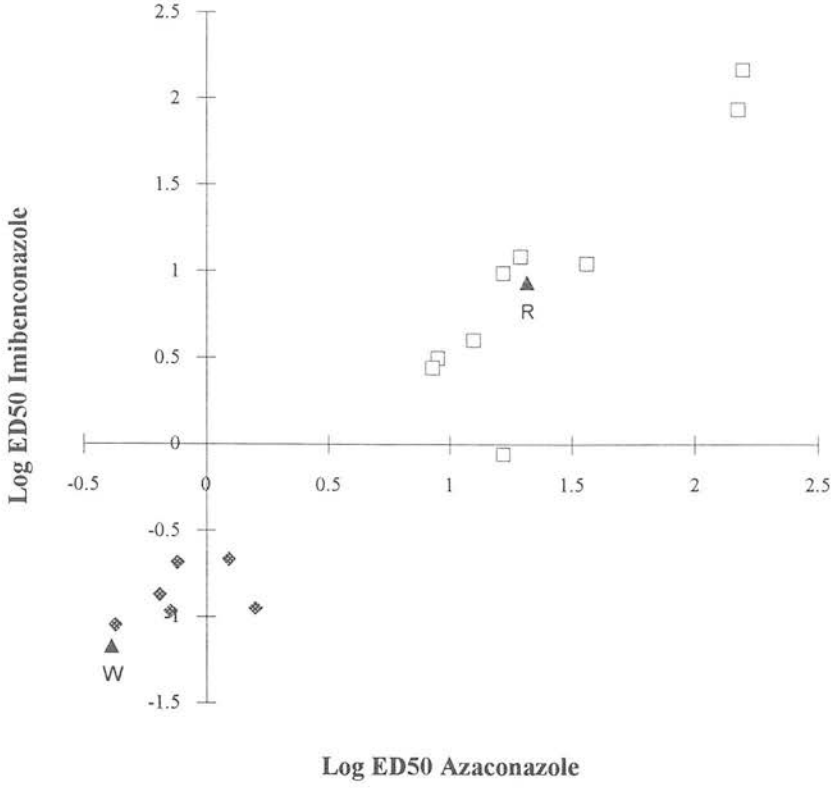


FIG 4.2b

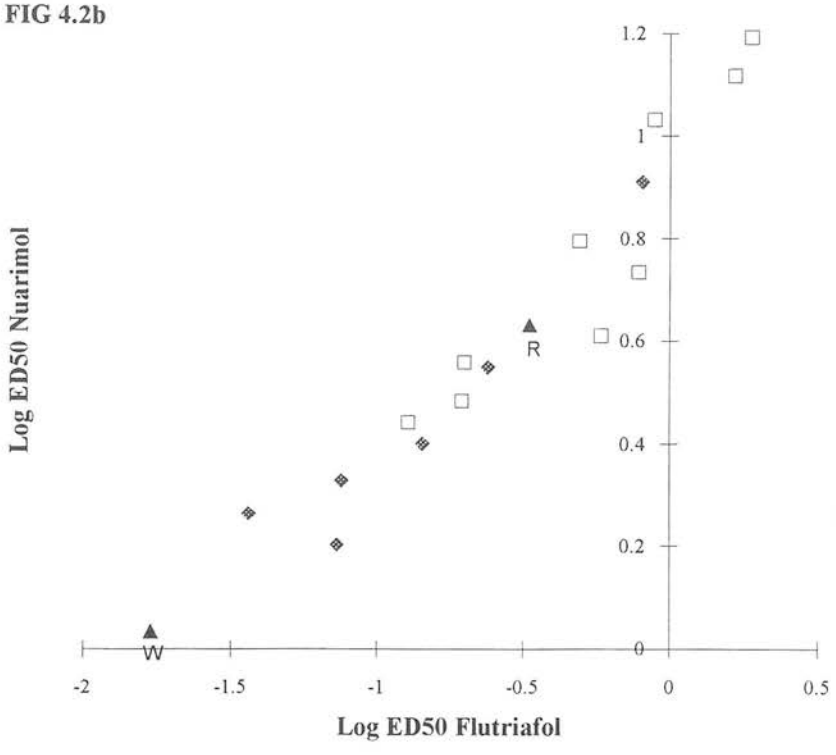
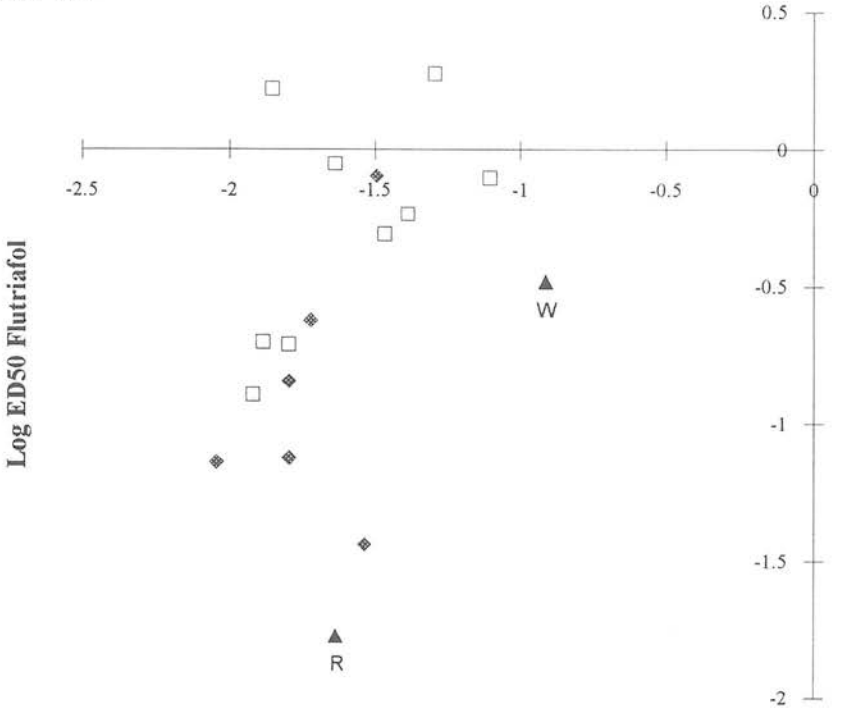


FIG 4.2c


0.5
0
-2.5
-2
-1.5
-1
-0.5
0
-0.5
-1
-1.5
-2
W
R
Log ED50 Flutriafol
Log ED50 Prochloraz

Prochloraz

Interestingly, prochloraz, although being a DMI did not show a significant correlation, and hence cross resistance, to any of the other DMI's. There was no cross resistance between prochloraz and either of the morpholines, fenpropimorph and tridemorph. Figure 4.2c shows log ED50 values to prochloraz plotted against those to flutriafol, and is typical of the pattern found for prochloraz where there was considerable overlap of ED50 values of progeny from each of the three fusion product sets and no evidence for cross-resistance.

Morpholines

The morpholines, fenpropimorph and tridemorph showed resistance to one another (r = 0.67) but neither showed cross-resistance to the imidazole and pyrimidine fungicides. When tested for cross-resistance to the triazole fungicides the two morpholines behaved differently. Fenpropimorph was not cross resistant to any of the triazoles. Tridemorph on the other hand showed a positive correlation to all the triazoles (r = 0.53 to 0.74) except flutriafol and flusilazole (r = 0.28 and 0.23 respectively). These positive correlations were usually just significant and at values generally lower than triazole combinations.

Correlation Analysis of Triadimenol Sensitive and Resistant Progeny Subsets

ED50 data for the 'sensitive' (FP1) and 'resistant' (FP2 & FP5) progeny subsets were re-analysed separately for every fungicide combination using correlation analysis. This was to determine if cross-resistance could be detected between pairs of fungicides within these two progeny subsets or was only detected when the complete data set was analysed. Correlation analysis was successful in illustrating cross-resistance relationships between chemicals within the triadimenol sensitive (FP1) and resistant (FP2, FP5) subsets, although not as uniformly as when the whole data set was analysed (Tables 4.6a & 4.6b). Figures in bold represent those correlations that were significant at $P < 0.05$ for five (sensitive subset) and eight (resistant

Table 4.6 (a & b). Correlation analysis using Log ED50 data for cross A progeny where the triadimenol sensitive (4.6a) and resistant (4.6b) progeny were analysed individually with n=7 (sensitive) and n=10 (resistant) and any value highlighted in bold was significant at the 5% level.

Triadimenol Sensitive Progeny (Table 4.6a)

	Azaconazole	Difenconazole	Flusilazole	Flutriafol	Imibenconazole	Penconazole	Propiconazole	Triadimefon
Azaconazole	1.00							
Difenconazole	0.13	1.00						
Flusilazole	0.51	**0.87**	1.00					
Flutriafol	**0.81**	0.13	0.59	1.00				
Imibenconazole	0.59	0.42	**0.74**	**0.74**	1.00			
Penconazole	0.36	0.22	0.47	0.44	**0.72**	1.00		
Propiconazole	0.05	0.26	0.34	0.37	0.54	0.11	1.00	
Triadimefon	0.59	0.46	**0.72**	**0.82**	0.63	0.10	0.65	1.00
Triadimenol	**0.75**	**0.72**	**0.91**	0.63	**0.68**	0.40	0.07	0.64
Triflumizole	**0.78**	0.43	**0.77**	**0.81**	**0.78**	**0.76**	0.19	0.61
Fenarimol	**0.72**	0.39	**0.76**	**0.94**	**0.77**	0.38	0.34	**0.86**
Nuarimol	**0.77**	0.28	**0.69**	**0.96**	**0.74**	0.31	0.37	**0.88**
Imazalil	**0.94**	0.19	0.52	**0.71**	0.62	0.53	0.11	0.49
Prochloraz	0.17	**0.74**	0.67	0.17	0.26	-0.22	0.06	0.48
Fenpropimorph	-0.13	0.58	0.54	0.23	0.51	0.06	**0.88**	0.63
Tridemorph	-0.32	0.41	0.45	0.20	0.48	0.40	0.50	0.31

	Triadimenol	Triflumizole	Fenarimol	Nuarimol	imazalil	Prochloraz	Fenpropimorph	Tridemorph
Tradimenol	1.00							
Triflumizole	**0.79**	1.00						
Fenarimol	**0.77**	**0.77**	1.00					
Nuarimol	**0.72**	**0.74**	**0.99**	1.00				
imazalil	**0.70**	**0.84**	0.58	0.62	1.00			
Prochloraz	**0.68**	0.14	0.48	0.41	0.01	1.00		
Fenpropimorph	0.22	0.14	0.37	0.34	-0.11	0.43	1.00	
Tridemorph	0.10	0.20	0.37	0.25	-0.30	0.24	**0.73**	1.00

Triadimenol Resistant Progeny (Table 4.6b)

	Azaconazole	Difenconazole	Flusilazole	Flutriafol	Imibenconazole	Penconazole	Propiconazole	Triadimefon
Azaconazole	1.00							
Difenconazole	**0.62**	1.00						
Flusilazole	0.07	0.40	1.00					
Flutriafol	0.36	**0.66**	**0.75**	1.00				
Imibenconazole	**0.89**	0.50	0.07	0.14	1.00			
Penconazole	**0.96**	**0.75**	0.24	0.48	**0.88**	1.00		
Propiconazole	**0.89**	**0.78**	0.15	0.56	**0.71**	**0.85**	1.00	
Triadimefon	**0.84**	0.35	-0.37	-0.13	**0.82**	**0.71**	**0.72**	1.00
Triadimenol	**0.82**	0.48	-0.36	-0.10	**0.80**	**0.75**	**0.71**	**0.95**
Triflumizole	**0.90**	0.44	-0.14	0.05	**0.92**	**0.82**	**0.76**	**0.93**
Fenarimol	0.58	**0.67**	0.21	**0.73**	0.32	0.58	**0.82**	0.34
Nuarimol	0.54	**0.67**	0.55	**0.93**	0.27	0.58	**0.75**	0.14
Imazalil	0.56	**0.78**	0.59	**0.90**	0.31	**0.66**	**0.66**	0.06
Prochloraz	-0.39	0.20	-0.42	0.32	-0.43	-0.21	-0.22	-0.54
Fenpropimorph	0.60	0.27	-0.43	-0.38	**0.76**	0.56	0.43	**0.85**
Tridemorph	**0.83**	**0.72**	-0.09	0.16	**0.82**	**0.86**	**0.74**	**0.76**

	Triadimenol	Triflumizole	Fenarimol	Nuarimol	Imazalil	Prochloraz	Fenpropimorph	Tridemorph
Triadimenol	1.00							
Triflumizole	**0.92**	1.00						
Fenarimol	0.34	0.43	1.00					
Nuarimol	0.13	0.29	**0.91**	1.00				
Imazalil	0.12	0.19	**0.66**	**0.84**	1.00			
Prochloraz	-0.31	-0.39	0.01	0.14	0.18	1.00		
Fenpropimorph	**0.92**	**0.84**	0.04	-0.19	-0.17	-0.27	1.00	
Tridemorph	**0.89**	**0.85**	0.46	0.31	0.39	-0.12	0.80	1.00

subset) degrees of freedom. This cross-resistance was mainly restricted to being found within the triazoles, the pyrimidines and the imidazole imazalil. In addition, pairs of fungicides for which cross-resistance was found within one subset of progeny were not necessarily found to be cross-resistant for the other progeny subset. Cross-resistance was found between fenpropimorph and tridemorph in both the triadimenol 'sensitive' and 'resistant' progeny subsets. Cross-resistance was still found between tridemorph and some of the triazoles, but only within the triadimenol resistant subset. Fenpropimorph was found to be cross-resistant with triadimenol and imibenconazole in the resistant subset, which was not found previously in the analysis of the whole data set. In addition, cross-resistance was also found for prochloraz within the resistant subset to propiconazole and flusilazole. The results obtained can be presented in a generalised diagram showing the different patterns of cross resistance that could be found within the 'sensitive' and 'resistant' progeny subsets where either no cross resistance was found in either subset (Fig. 4.3a), cross-resistance was detected within only one of the subsets (Fig. 4.3b & 4.3c) or cross-resistance was detected within both subsets (Fig. 4.3d). Table 4.7a and 4.7b represent diagrammatically, for each fungicide combination, where cross-resistance was detected within each of the triadimenol sensitive and resistant subsets. Black squares represent where a significant correlation and hence cross-resistance was found, whilst the shaded areas indicate a lack of cross-resistance between that particular fungicide pairing.

The two tables have been split, by dotted lines, into the groupings recognised in the correlation analysis of the whole data set with type I chemicals being those chemicals for which there was a clear difference between the 'sensitive' and 'resistant' progeny subsets. Type IIa and IIb chemicals are those fungicides for which there were one or two points of overlap, or considerable overlap of the ED50 values respectively. From these tables it can be seen that there are no clear patterns of cross-resistance within the class I, class IIa or class IIb fungicides. Clearly, behaviour within the groups is not consistent and individual differences

Fig. 4.3 (a - d) Different patterns of cross-resistance found within the triadimenol sensitive and resistant subsets of cross A progeny. The sensitive progeny subsets are represented by closed diamonds, the resistant progeny subset by the open squares and the triangles represent the parental isolates.

Fig 4.3a represents where neither the 'sensitive' or 'resistant' subsets were cross-resistant.

Fig 4.3b Cross-resistance was detected within only the 'sensitive' progeny subset.

Fig 4.3c Cross-resistance was detected within only the 'resistant' progeny subset.

Fig4.3d Both the 'sensitive' and 'resistant' progeny subsets were found to be cross-resistant.

Fig 4.3a

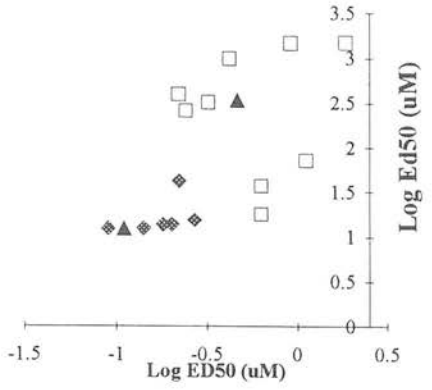


Fig 4.3b

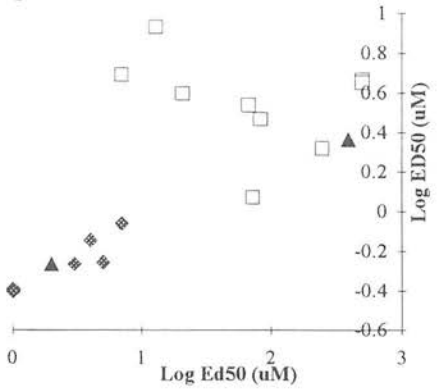


Fig 4.3c

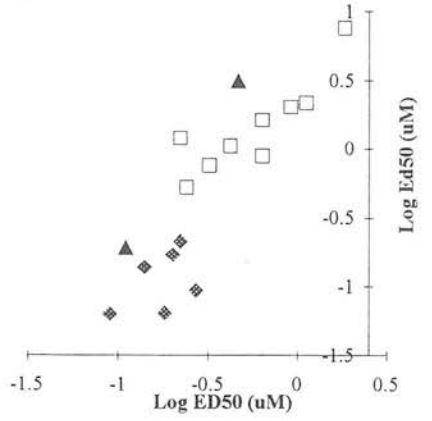


Fig 4.3d

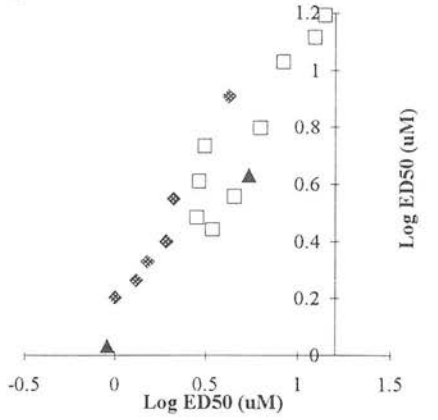


Table 4.7a & 4.7b Diagrammatic representation of incidence of cross-resistance within the triadimenol sensitive (4.7a) and resistant (4.7b) progeny subsets of cross A. Black squares represent where cross-resistance was detected, whilst shaded areas indicate a lack of cross-resistance. Fungicides are split by dotted lines into class I, class IIa and class IIb chemicals based on cross-resistance patterns within the whole data. Prochloraz and the morpholines, fenpropimorph[h and tridemorph, are separated from the other chemicals.

Table 4.7a

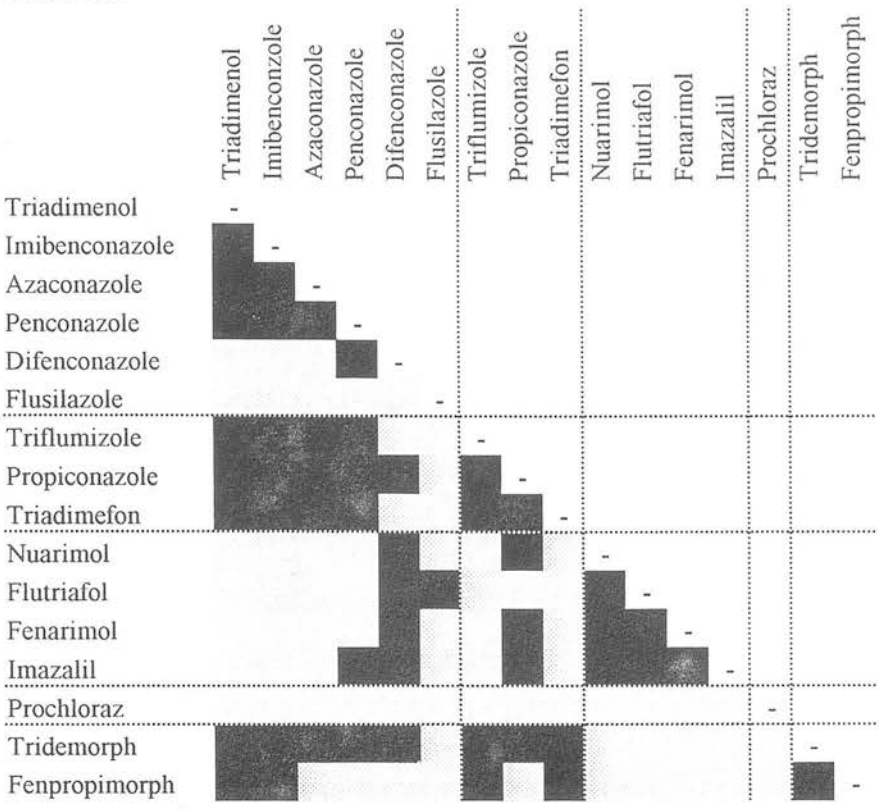
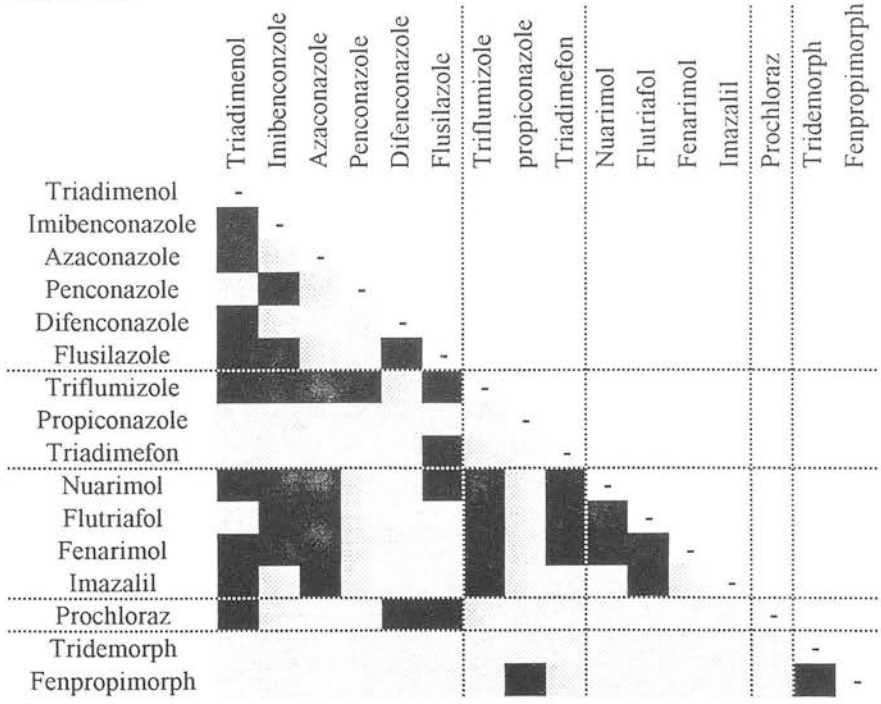


Table 4.7b



within groups could be due to other factors including differences in resistance factor, although no clear pattern could be found between the two.

A χ^2 analysis was carried out to test the frequency of finding cross-resistance within each of the 'sensitive' and 'resistant' progeny subsets when the interaction of type IIa and type IIb chemicals with type I chemicals was compared. Tables 4.8a and 4.8b show the contingency tables that would be constructed for each of the 'sensitive' and 'resistant' subsets. Within the 'resistant' progeny subset the Null Hypothesis, that it is as likely that pairs of fungicides would exhibit cross-resistance as not, would be rejected. From the numbers in the contingency table it can be seen that you are more likely to find cross-resistance within pairings of fungicides from the class I and class IIa segregation type, whilst the pairings of fungicides from class I and class IIb are more likely not to be cross-resistant.

Within the sensitive subset, however, the χ^2 was not significant. It is as likely, therefore, that within pairings of fungicides from class I and either class IIa or class IIb grouping there will be as many pairings that are cross-resistant as those that do not exhibit cross-resistance.

4.3.4 Dose Response Test: Cross D Progeny

The sensitivity of the two parental isolates in this cross, 22-20 (W-type) and 23-2 (R-type), towards the tested DMI's is shown in Figures 4.4a-4.4e. When tested against the majority of the fungicides the two parental isolates, 22-20 and 22-8, exhibited clear differences in their sensitivity levels. The W-type isolate, 22-20, was found to be more sensitive than the R-type isolate, 22-8, towards triadimenol, difenconazole (triazoles) and fenarimol (pyrimidine) and is reflected in the resistance factors calculated for these isolates and shown in Table 4.1. For prochloraz and fenpropimorph 22-20 was found to be less sensitive than 22-8 and is reflected in the resistance factors which are less than one (Table 4.1).

Table 4.8a & 4.8b Contingency tables for χ^2 analysis of the frequency of finding cross-resistance within the 'sensitive' and 'resistant' progeny subsets of cross A when the interaction of class I chemicals with both class IIa and class IIb chemicals is analysed. Expected values are presented in brackets after the observed values.

	No cross resistance	Yes cross resistance	Total
Class IIa	5 (9.86)	13 (8.14)	18
Class IIb	18 (13.14)	6 (10.86)	24
Total	23	19	42

$\chi^2 = 9.26$ with $\chi^2_{0.05,1} = 3.841$

	No cross resistance	Yes cross resistance	Total
Class IIa	12 (10.71)	6 (7.28)	18
Class IIb	13 (14.28)	11 (9.71)	24
Total	25	17	42

$\chi^2 = 0.66$ with $\chi^2_{0.05,1} = 3.841$

Fig. 4.4 (a-e). Fungicide sensitivity towards five different EBI fungicides of *P. herpotrichoides* (W x R) interpathotype progeny, split into the five FP sets, compared with the parental isolates. ED50 values are expressed as μM active ingredient for each of the fungicides, 4.4a).Triadimenol, 4.4b) Difenconazole, 4.4c) Fenarimol, 4.4d) Prochloraz, 4.4e) Fenpropimorph.

FIG 4.4a

TRIADIMENOL

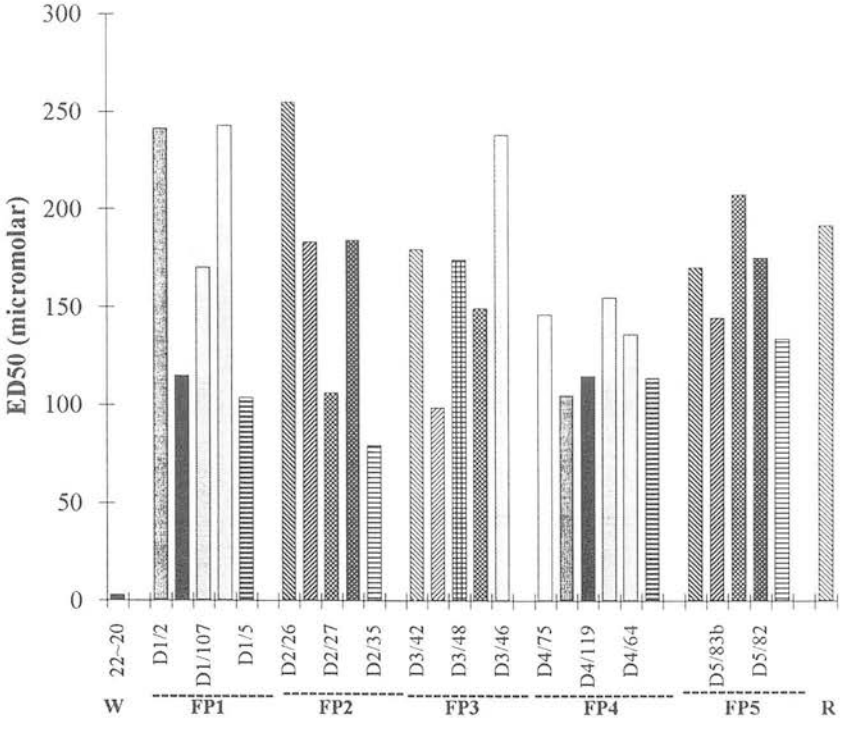


FIG 4.4b

DIFENCONAZOLE

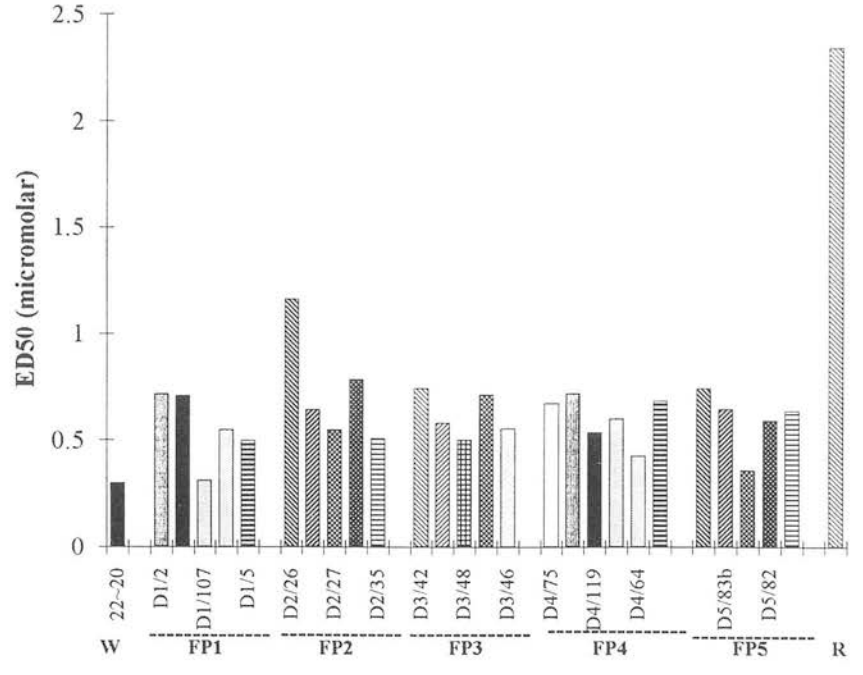


FIG 4.4c

FENARIMOL

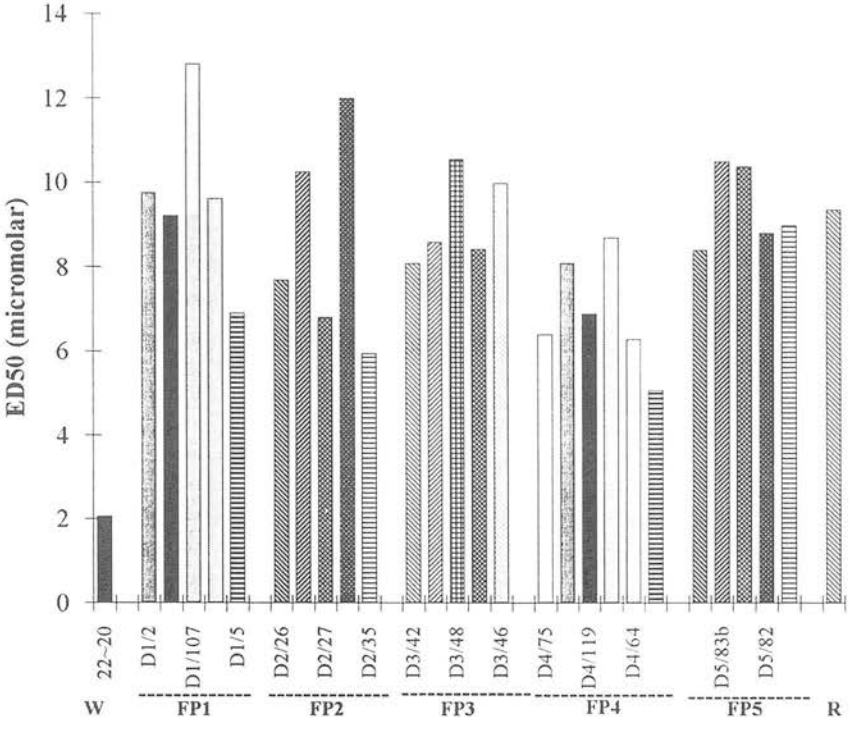


FIG 4.4d

PROCHLORAZ

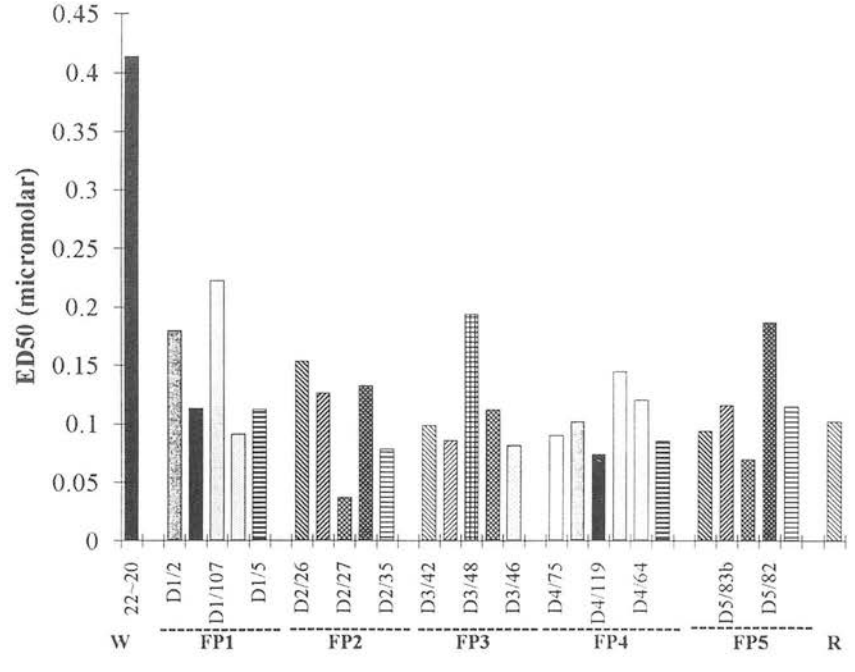
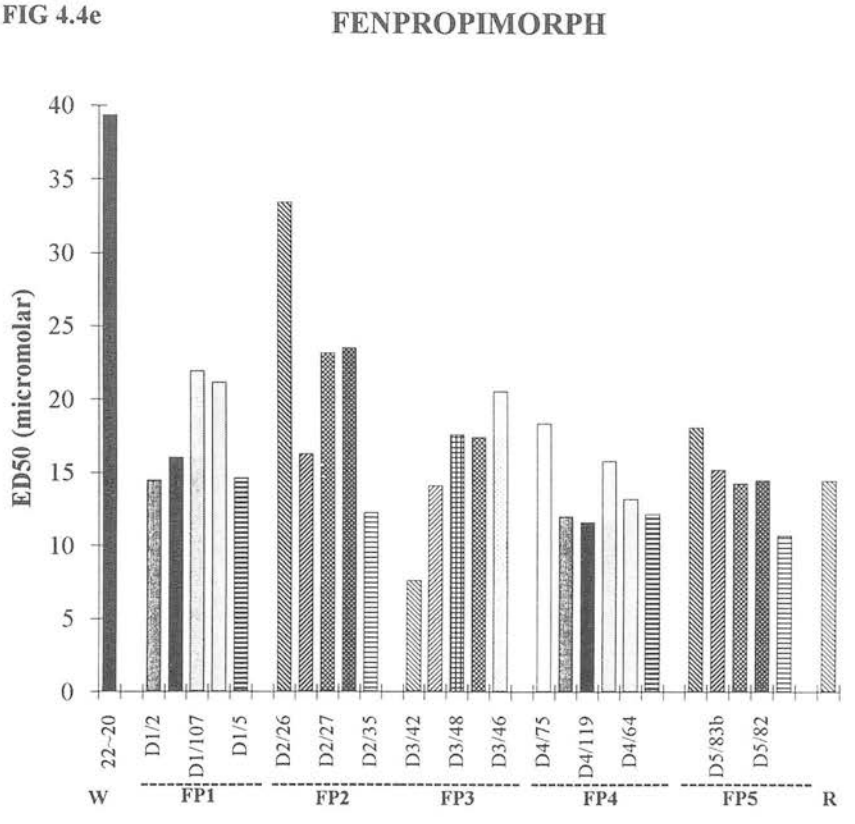


FIG 4.4e


FENPROPIMORPH
ED50 (micromolar)
40
35
30
25
20
15
10
5
0
22~20
D1/2
D1/107
D1/5
D2/26
D2/27
D2/35
D3/42
D3/48
D3/46
D4/75
D4/119
D4/64
D5/83b
D5/82
W
FP1
FP2
FP3
FP4
FP5
R

Due to there being only a representative sample of fungicides tested for cross D the dose response data for each chemical are presented separately. Correlation analysis results shall, however, be presented together.

Triadimenol

The ED50 values of the progeny confirmed the finding of the preliminary screen that all the progeny from the five fusion products were resistant towards triadimenol (Fig. 4.4a & Table 4.2). However, clearly there were differences between the resistance levels of the progeny representing each of the fusion products. These differences in sensitivity levels were exhibited within fusion product sets as much as between them. It was also seen that four of the five fusion products (FP1, FP2, FP3 & FP5) generated some recombinants having ED50 values greater than the resistant R-type parent 23-2.

Difenconazole

Large differences in sensitivity levels were found between 22-20 (W) and 23-2 (R). In this instance the progeny behaved more like 22-20 and were relatively sensitive to difenconazole (Fig 4.4b). Again there was variation in the levels of sensitivity, although generally this variation was restricted to a relatively narrow range.

Fenarimol

Resistance levels of the progeny were comparable with the R-type parent 23-2, i.e. resistant (Fig 4.4c), with quite a lot of variation in the ED50 values. For progeny derived from the fusion products FP1, 2, 3 & 5 some were found to have ED50 values marginally greater than 23-2. Those are the same four progeny sets which gave ED50 values to triadimenol greater than the resistant parent.

Prochloraz

Against prochloraz, progeny in each of the fusion product sets gave ED50 values comparable with 23-2, displaying a 'sensitive' phenotype (Fig 4.4d). Again some variation in ED50 values was found within each set of progeny.

Fenpropimorph

Variation in the ED50 values was seen towards fenpropimorph (Fig. 4.4e). The majority of the isolates behaved like 23-2 and were less sensitive than 22-20, the W-type, although there was one exception, D2/26, whose ED50 value was closer to that of 22-20 than 23-2.

Comparison of Slope and Intercept Data

When fusion D progeny were ranked in order of increasing ED50 values for each of the fungicides tested it was clear that there was a gradual increase in ED50 values amongst the progeny. It was also found that progeny derived from a particular fusion product did not congregate together in terms of their ranked ED50 values, but rather that progeny representing each of the fusion product sets were dispersed throughout the range of ED50 values. The analysis to test for differences between the slopes and intercepts of progeny giving the highest and lowest ED50 values for each fungicide with the parental isolate they most closely resembled would determine whether there were any differences between the progeny or they were infact a single population. The results for the analysis of differences between slopes and intercepts are presented in Table 4.9 where a "+" indicates a significant difference was present and a "-" indicates that there was no significant difference between the progeny and the parental isolate being tested. It is clear from the table that the results vary depending on the fungicide being tested. Only for difenconazole, fenarimol and the isolate giving the largest ED50 on triadimenol, D2/26, did the results show that the isolates tested gave different regression lines from the parental isolate whose ED50 they most closely resembled. Both

Table 4.9 Comparison of the slopes and intercept values of cross D progeny having the highest and lowest Ed50 values and the control isolates they most closely resembled where a '+' indicates that a significant difference was found between the two values and a '-' indicates that no diference was found.

	DIFFERENCE FROM 22-20	
DIFENCONAZOLE	**SLOPE**	**ELEVATION**
D1/107	+	-
D2/26	+	-

	DIFFERENCE TO 23-2	
FENARIMOL	**SLOPE**	**ELEVATION**
D4/67b	+	+
D1/107	+	+

	DIFFERENCE TO 23-2	
PROCHLORAZ	**SLOPE**	**ELEVATION**
D2/27	-	+
D1/107	-	+

	DIFFERENCE TO 23-2	
FENPROPIMORPH	**SLOPE**	**ELEVATION**
D3/42	-	+
D2/26	-	-

	DIFFERENCE TO 23-2	
TRIADIMENOL	**SLOPE**	**ELEVATION**
D2/35	-	+
D2/26	+	-

isolates analysed for prochloraz gave significantly different elevations but not slopes and show therefore that the regression lines were parallel. The most sensitive isolate tested for fenpropimorph, D3/42, and triadimenol, D2/35, also illustrated regression lines parallel to the parental isolate, 23-2, having significantly different elevations but not slopes, whereas the isolate most resistant on fenpropimorph, D2/26 had neither a significantly different slope or elevation to 23-2 and is therefore the same line. These results would seem to suggest that for difenconazole, fenarimol and triadimenol there are differences in sensitivity levels present between the progeny and the parental isolate, indicating that the progeny do not represent a single population. However, for prochloraz and fenpropimorph the differences in ED50 values seen between the progeny and the parental isolates could just be due to experimental error or biological variation.

4.3.5 Cross Resistance

Figure 4.5, triadimenol ED50 values plotted against fenarimol ED50 values, represents an example amongst the cross D progeny where a significant positive correlation and hence cross-resistance was detected. From the graph it can be seen that the W-type parent, 22-20, which was sensitive to both triadimenol and fenarimol is positioned considerably below the progeny and the R-type parent for both of these chemicals on the concentration range. This outlying position of 22-20 may have a considerable effect on the correlation figures that were found. However, as was demonstrated by the analysis of slope and intercept data it was not always 22-20 that was the outlying parent. Therefore, the correlation data presented in Table 4.10 represents where only the cross D progeny ED50 values were analysed to detect cross-resistance, without including the parental isolates. Those values highlighted in bold were significant to $P < 0.05$ for 24 degrees of freedom. From this analysis it can be seen that significant correlations and hence cross-resistance, was detected between triadimenol and fenarimol and fenpropimorph and also between prochloraz and fenarimol.

Fig. 4.5 Detection of cross resistance between EBI fungicides fenarimol and triadimenol through correlation of ED50 (μM) values. Parental strains (W & R) are labelled on the figure.

FIG 4.5

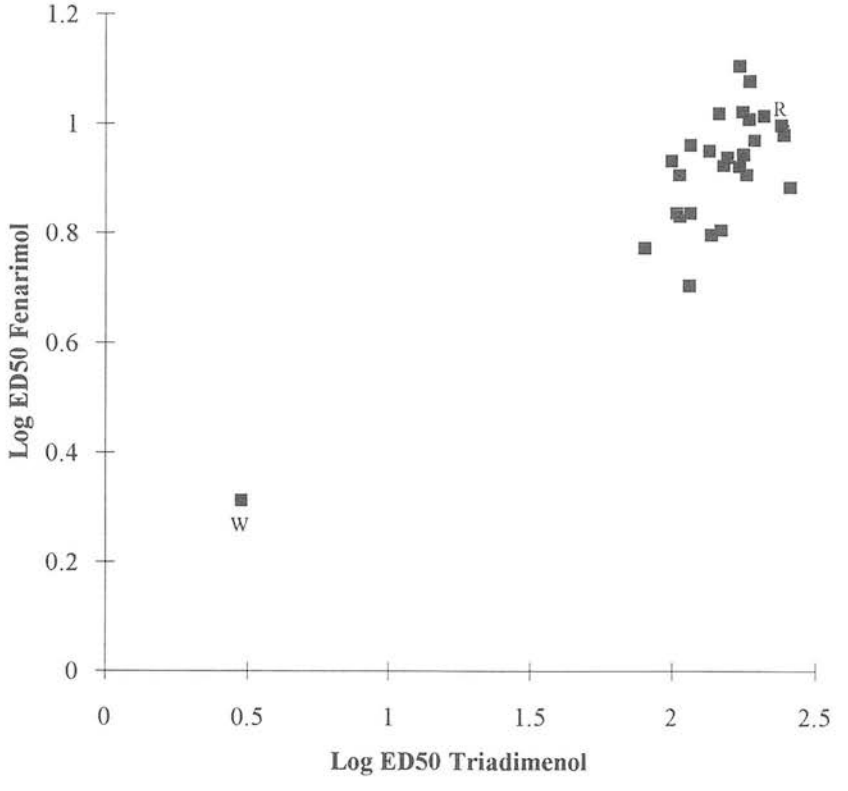

1.2
1
0.8
0.6
0.4
0.2
0
Log ED50 Fenarimol
R
W
0
0.5
1
1.5
2
2.5
Log ED50 Triadimenol

Table 4.10. Correlation analysis using Log ED50 data for cross D progeny where n = 26 and values highlighted in bold are significant at the 5% level.

	Triadimenol	*Prochloraz*	*Difenconazole*	*Fenarimol*	*Fenpropimorph*
Triadimenol	1.00				
Prochloraz	**0.41**	1.00			
Difenconazole	0.17	0.05	1.00		
Fenarimol	**0.57**	**0.49**	-0.15	1.00	
Fenpropimorph	**0.43**	0.12	0.15	0.31	1.00

Preliminary Screen: Interspecific Hybrid Progeny

Selecting a concentration from the full dose response test was successful in demonstrating that there was a pre-segregation event occurring within the hybrid progeny where it was found that progeny from each of the three fusion product sets (FPt, FPc & FPe) tested all exhibited a triadimenol resistant phenotype being all much less sensitive to 50 μM triadimenol than the sensitive parent (*P. anguioides*).

Dose response Test: Interspecific Hybrid Progeny

When tested against each of the three fungicides, triadimenol, difenconazole and fenpropimorph it was found that the R-type parent, 22-228, was more resistant than the *P. anguioides* parent, 24-9 (Fig 4.6a-4.6c). The hybrid progeny exhibited a range of sensitivities to the fungicides, and the majority of the progeny were all more resistant than *P. anguioides* (24-9). In addition, a few hybrids were seen to be more resistant to each chemical than 22-228 (R-type), and these hybrids could be found amongst progeny tested for each of the three fusion product sets, FPt, FPe and FPc. However, apart from the hybrid DY which was highly resistant on each of the fungicides, it was not necessarily the same hybrids which were most resistant towards the different fungicides tested. The progeny could be ranked in terms of sensitivity towards fenpropimorph but this same pattern would not be repeated with triadimenol and difenconazole if the progeny were put into the same order as for fenpropimorph.

4.3.7 Cross Resistance Relationships of Hybrid Progeny

Evidence of a significant correlation and hence cross-resistance was only found between difenconazole and fenpropimorph (Fig 4.7). This is in contrast to the cross A correlation analysis of the whole data set where there was no cross resistance found between fenpropimorph and any of the DMI's tested.

Fig 4.6 (a - c). Fungicide sensitivity of interspecific hybrid progeny, from a cross between *P. herpotrichoides* (R-type 22-228) and *P. anguioides* (24-9), representing three fusion product sets compared with the parental isolates. ED50 values are expressed as μM active ingredient for three different fungicides, 4.6a) Triadimenol, 4.6b) Difenconazole, 4.6c) Fenpropimorph.

FIG 4.6a

TRIADIMENOL

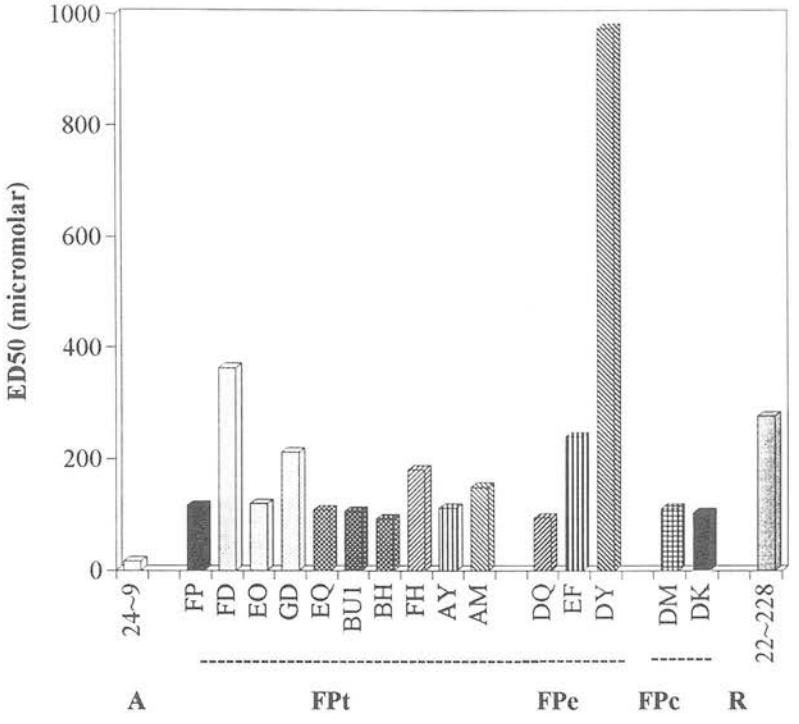


FIG 4.6b

DIFENCONAZOLE

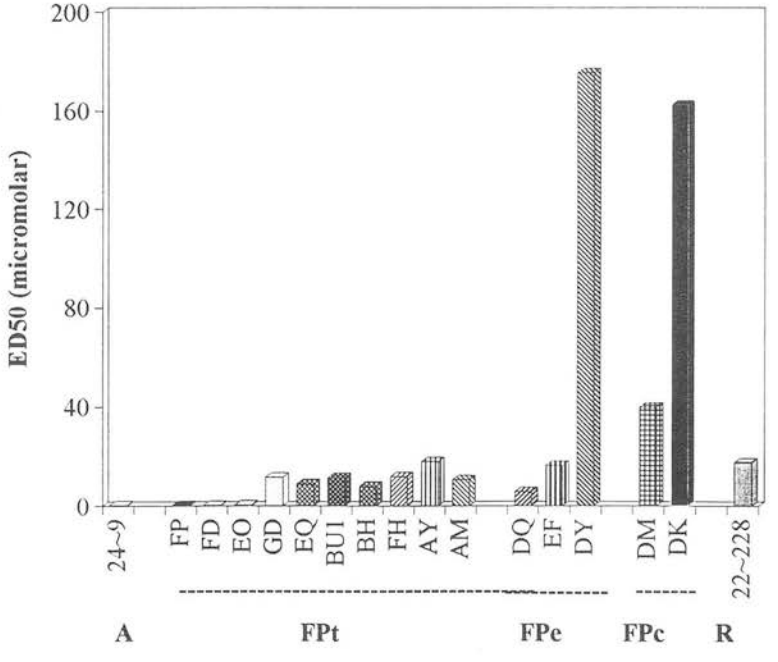


FIG 4.6c **FENPROPIMORPH**

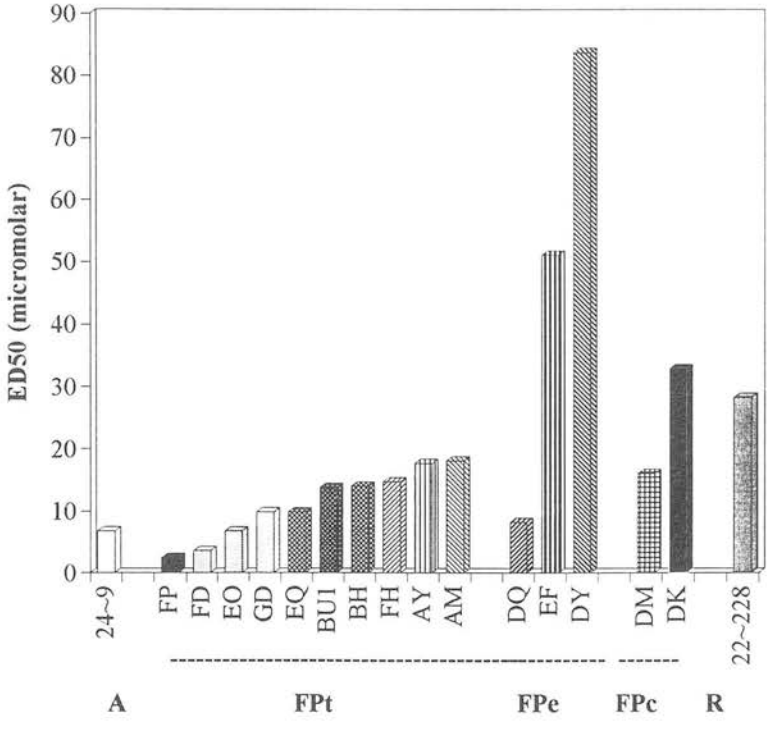
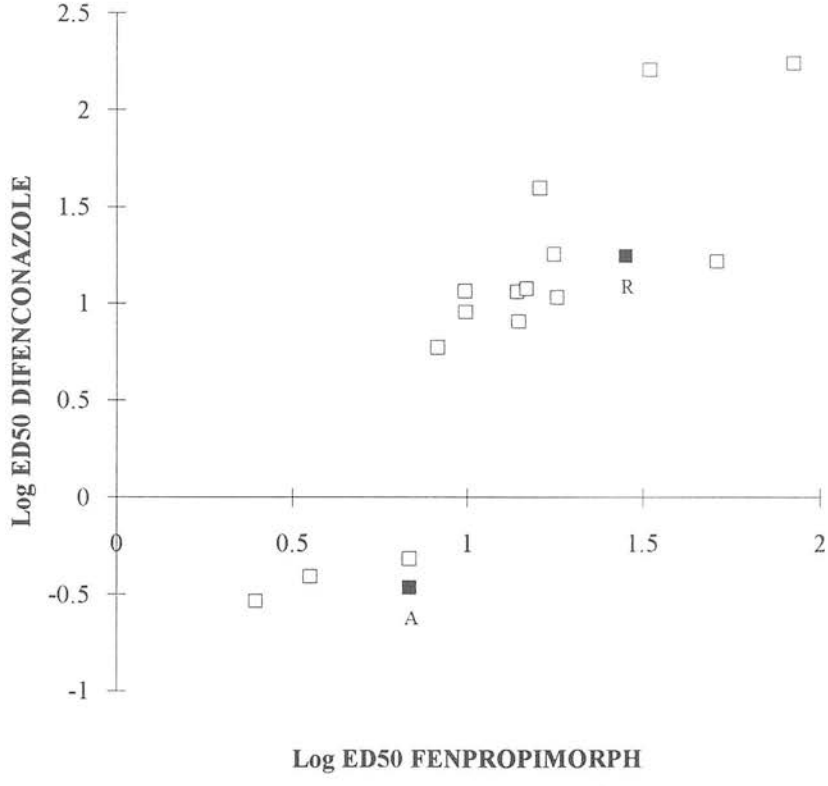


Fig 4.7. Detection of cross-resistance between the EBI fungicides difenconazole and fenpropimorph through correlation of Log ED50 (μM) values for cross D progeny (open squares). Parental strains (R & A) are labelled on the figure (closed squares).

Fig 4.7


2.5
2
1.5
1
0.5
0
-0.5
-1
0
0.5
1
1.5
2
R
A
Log ED50 DIFENCONAZOLE
Log ED50 FENPROPIMORPH

4.4 DISCUSSION

This work produces the first clear genetic evidence for the inheritance of resistance to ergosterol biosynthesis inhibiting fungicides in *P. herpotrichoides* and provides evidence for the involvement of several genes in determining sensitivity towards the ergosterol biosynthesis inhibiting fungicides. Evidence was obtained for the segregation of one or more genes having a large effect on EBI sensitivity even before haploidization of fusion products was induced. This pattern of pre-segregation has been observed for other genetic markers in these crosses, as described in Chapter 3. As a result of this pre-segregation it is not possible to state how many genes controlling EBI resistance segregate at this point. However these gene(s) clearly exert a profound effect on resistance expression and consequently are here termed 'major' gene(s) for resistance. The major gene(s) involved in fungicide resistance expression determined whether all the progeny within a fusion product set were either sensitive or resistant to the DMI's. The resistant phenotype of progeny within the two subgroups which were classified as either triadimenol sensitive (cross A: FP1; cross H: FP1; cross I: FP1, FP6) or triadimenol resistant (cross A: FP2, FP5; cross D: FP1, FP2, FP3, FP4, FP5; cross E: FP1; cross G: FP1, FP2, FP8, FP10, FP12; cross H: FP2, FP3, FP4, FP5; interspecific hybrids: FPt, FPc, FPe) was not homogeneous and a range of sensitivities were observed providing strong evidence of segregation of additional genes affecting sensitivity to EBI fungicides during haploidization.

When haploidization of the fusion product was induced there was further segregation of other genes able to modify the level of resistance expression and are here termed minor genes because their effect on resistance level is less than the major gene(s). The involvement of several genes with major and minor effects on fungicide resistance has been demonstrated by several others in a variety of fungal species. For example, Sanoamung *et al.* (1993) demonstrated that resistance inheritance in *Monilinia fructicola* was conferred by a mutation

in a single gene but was affected by modifying genes. This is also confirmed by Ishii *et al.* (1993) who demonstrated a single major gene controlling fenarimol resistance in *Venturia inaequalis* but suggested that additional subtle genetic methods may modify the level of resistance expression.

For cross A where both 'sensitive' and 'resistant' progeny were obtained the relative magnitude of the effect of the major and minor gene(s) could be assessed by the distribution pattern of the plotted ED50 values for the individual fungicides. It would appear that where the effect of the major gene(s) was greatest the effect of the minor genes was smallest and vice versa. The effect of both major and minor gene(s) appeared to have a different effect depending on which ergosterol biosynthesis inhibiting fungicide was being tested. For a group of triazoles including azaconazole, flusilazole, imibenconazole, triadimenol, difenconazole and penconazole the progeny were clearly differentiated into two sub-classes. Here the minor genes had little effect and the segregation pattern found was due largely to the major gene(s). For other groups of fungicides the major gene(s) did not have such a great effect over the expression of resistance with the minor genes having more of an effect in determining the resistance level. For propiconazole, triadimefon and triflumizole where there were one or two points of overlap between the triadimenol classified sensitive and resistant progeny the major gene(s) was having some effect but not as great as in the previous group. For the group of fungicides including imazalil, flutriafol, fenarimol, nuarimol, prochloraz, fenpropimorph and tridemorph a spread of ED50 values showed that there was considerable overlap of the triadimenol sensitive and resistant progeny. For this group the effect of the minor genes was greatest and they were probably having more of an effect on resistance levels than the major gene(s).

Progeny generated in cross D were all classified as triadimenol resistant in the preliminary screen and are directly comparable to the triadimenol resistant progeny derived from two of

the three fusion products in cross A, FP2 and FP5. A comparison of ED50 segregation values for the fungicides selected for cross D illustrate that the results of the cross D progeny were not as would have been expected from cross A FP2 and FP5 progeny. However, it should be remembered that the W-type isolate, 22-20, is the only parent in common in cross A and cross D. Both crosses had different R-type isolates as parents in the cross and the sensitivity of these two isolates, 22-8 (cross A) and 23-2 (cross D), differed when comparing ED50 values for the fungicides tested for cross D progeny. This difference in sensitivity may be due to different genes acting (Grindle,1987) and may explain different patterns of segregation between the cross A triadimenol resistant progeny sets, FP2 and FP5, and cross D progeny. The analysis and therefore the comparison between cross A and cross D could be extended by testing the cross D progeny against the other fungicides tested for cross A.

Within fusion D it is hard to determine the relative effects of the major and minor gene(s) without the presence of a sensitive subset of progeny. Therefore, any differences seen within the cross D progeny are only due to either minor gene differences or environmental effects. Analysis of differences in slopes and differences in elevation of the regression lines between fusion D progeny and the parental isolate they most resembled indicated that there were significant differences in sensitivity within these groups providing evidence that the 'minor' genes are 'real' and segregating in this cross.

Progeny were produced from crosses E, G, H and I where for three of these crosses (E, G & I) there were only either sensitive or resistant progeny and one cross (H) where there was both sensitive and resistant progeny. Full dose response tests which could be carried out using these progeny sets should provide further evidence on the segregation of major and minor gene(s). How typically the segregation of these genes for sensitivity towards a particular fungicide can be predicted by looking at segregation towards a fungicide of the same chemical group and

mode of action could also be investigated. It may also provide more information on how segregation is affected by particular strain combinations in the crosses.

Given the difference in ED50 values of 24-9 (*P. anguioides*) and 22-228 (*P. herpotrichoides*, R-type), the parental isolates of the interspecific hybrid progeny, the triadimenol concentration chosen from the full dose response test to indicate preliminary segregation would appear to be appropriate. This concentration was of a similar magnitude to the concentration of triadimenol chosen for the other fusions and illustrates that for the interspecific hybrid progeny there is also a pre-segregation event occurring for sensitivity towards triadimenol.

Clear evidence of cross-resistance was obtained for 13 of the demethylation inhibiting fungicides tested in all combinations for cross A progeny. A comparison of the three sets of correlation analysis carried out on these isolates, i.e. whole data sets and triadimenol sensitive and resistant subsets individually, reveals that the major gene(s) and the minor genes all conferred cross-resistance, but where 'sensitive' and 'resistant' progeny subsets were examined separately then examples were found where there was no cross-resistance between pairs of fungicides due to minor genes.

A comparison could be made between the correlation analysis of cross D progeny and the triadimenol resistant subset of cross A progeny. On the whole it was found that the pattern of cross-resistance found within the fungicides tested for cross D progeny was a repeat of what was found for these chemicals and the resistant subset of cross A progeny. The only exceptions to this were found to be fenarimol correlated with difenconazole and the correlation of fenarimol and prochloraz which within the cross D progeny were found not to be cross-resistant and to exhibit cross-resistance respectively. This was not what was found for the triadimenol resistant progeny subset of cross A.

Significantly, within the demethylation inhibiting fungicides the only compound for which there was consistently a different pattern of cross-resistance from either cross A or cross D was prochloraz. This result is important as it suggests that variation for sensitivity to prochloraz has a different basis than that for the other DMI fungicides. There was some indication that there was a significant positive correlation and hence cross-resistance between prochloraz and the triazoles triadimenol, difenconazole and flusilazole within the triadimenol sensitive subset of cross A progeny and also between prochloraz and fenarimol within the cross D progeny, but no significant correlation for any of the other DMI's. Full dose response data from progeny derived from crosses E, G, H and I should enable this to be further investigated.

J. Hardy (Pers Comm.) has been able to generate eyespot mutants with reduced sensitivity to prochloraz by exposure to UV irradiation. These laboratory mutants showed cross-resistance to several other DMI fungicides, and also to other unrelated inhibitors such as methanol. These mutations were generated by repeated exposure to UV irradiation and gave additional levels of resistance to what was present naturally in the field isolates. Additionally, new cross-resistance patterns were detected which were not present amongst wild type isolates. These induced cross-resistance patterns found by J. Hardy using prochloraz resistant mutants differs from the present work where only restricted cross-resistance was detected between prochloraz and the other DMI's, based on natural differences in fungicide sensitivity. This would suggest that the biochemical - and presumably the genetic basis - of the induced resistance is different to the natural variation in sensitivity seen to this fungicide.

A number of R-type isolates showing resistance *in vitro* to prochloraz were isolated from France in 1990 (Leroux & Marchegay, 1991). When compared to previously isolated R-types these new prochloraz resistant isolates had altered sensitivity to a number of other DMI's, including reduced sensitivity to imazalil, flusilazole, flutriafol, propiconazole and

tebuconazole and increased sensitivity to triflumizole, fenarimol, cyproconazole, hexaconazole and triadimenol which could be interpreted as cross-resistance and negative cross-resistance respectively. In addition, no cross-resistance was detected between prochloraz and the morpholines turbinafine and fenpropimorph for these prochloraz resistant R-type isolates. This pattern of positive and negative cross-resistance demonstrated by Leroux and Marchegay (1991) between DMI fungicides differs from the present work where only positive cross-resistance was detected amongst the tested DMI's.

It has been suggested that the form of selection for resistance to DMI's and which would have led to the isolation of these prochloraz resistant isolates tends to be directional and is characterized by a gradual shift in the population (Julian *et al.*, 1994). Isolation of resistant isolates is also often associated with multigenic forms of resistance, which has been suggested in the present work with the presence of major and minor gene(s) and also by that of J. Hardy who noted increased levels of resistance to prochloraz through successive rounds of mutagenesis suggestive of a multigenic trait.

Sensitivity to the two morpholines, fenpropimorph and tridemorph, was significantly correlated for cross A progeny, for both the complete data set and also when the triadimenol sensitive and resistant progeny subsets were analysed individually. Significant correlations, and hence cross-resistance, were found between fenpropimorph and the triazoles triadimenol, imibenconazole, triflumizole and triadimefon within the cross A resistant progeny subset, with propiconazole in the cross A sensitive progeny subset, as well as with triadimenol within the cross D progeny. The cross-resistance detected here is due to the effect of the minor genes. No significant correlations were, however, found between fenpropimorph and any of the tested DMI fungicides within the cross A complete data set. Tridemorph, however, did show some cross-resistance to all of the triazoles with the exception of flusilazole and flutriafol, but no cross-resistance to the pyrimidines, fenarimol and nuarimol, and the imidazoles, imazalil and

prochloraz. Cross-resistance between tridemorph and the triazoles was only found within the triadimenol resistant progeny subset from cross A. This was found between tridemorph and each of the triazoles tested, with the exception again of flusilazole and flutriafol. Again no cross-resistance was detected between tridemorph and either the pyrimidines or the imidazoles. The positive correlations shown between tridemorph and the triazoles in the complete data set were however, lower than those of triazole-triazole combinations.

5 PATHOGENIC ABILITY OF INTRA- AND INTERSPECIFIC RECOMBINANTS

5.1 INTRODUCTION

Two main pathotypes of *P. herpotrichoides* have been recognized in the field, W- and R-types (Scott *et al.*, 1975; Fitt *et al.*, 1987). These two pathotypes are both equally pathogenic to wheat, which represents a common host to both, whereas the R-types are more pathogenic towards rye than are W-types. A second species of *Pseudocercosporella*, *P. anguioides*, found on cereals in the UK is only weakly pathogenic towards wheat (Bateman, 1988). This chapter describes the assessment of pathogenicity to wheat and rye of parasexual recombinants from intraspecific W x R progney and interspecific *P. herpotrichoides* x *P. anguioides* hybrids. The objective of the pathogenicity assessments was to investigate the inheritance and expression of host-specific pathogenicity in these crosses. Three different test systems were employed for the investigation of pathogenicity. The first of these was carried out in a controlled environment cabinet in which pathogenicity was assessed eight weeks after inoculation of the host plants. In the second, pathogenicity was assessed under glasshouse conditions where again symptoms were assessed eight weeks after inoculation. In both of these experimental systems expression of pathogenicity was assessed visually on the basis of symptom development, and are regarded here as standard pathogenicity trials. In the last test system, carried out in a controlled environment cabinet, pathogenicity was assessed microscopically three weeks after inoculation. This third method is here termed the short assay system.

5.2 MATERIALS AND METHODS

5.2.1 Controlled Environment Cabinet assessment of Pathogenicity

A controlled environment cabinet (Sanyo Fi-totron PG660) was used for both standard and short assay assessments of pathogenicity of progeny generated from intra- and inter-specific

crosses. For both types of experiments the conditions inside the cabinet were maintained at a 16 h, 12°C day; 8 h, 9°C night with 80% relative humidity throughout.

Two standard pathogenicity experiments were set up in the controlled environment cabinet. Seeds of wheat cv. Beaver and rye cv. Halo were sown four per 5 cm diameter pot, filled to within 1 cm of the rim with peat-based potting compost (120 g fritted trace elements; 120 g ammonium nitrate; 120 g potassium nitrate; 450 g superphosphate; 675 g ground limestone & 675 g dolomite limestone per 300 L bale of peat). Three weeks post-emergence the seedlings were inoculated with fungal infested filter paper disks. The inoculum was grown on TWA plates inoculated by spreading a hyphal suspension of each isolate over the surface of two agar plates. These were incubated at 19°C for 24 h before 5 mm sterile filter paper disks (Whatman No.1) were spread over the surface. The plates were then placed under near ultraviolet light (NUV, 360 nm λ) light at 14°C for 14 d to induce sporulation. A single filter paper disk was placed between the coleoptile and the first leaf sheath of each seedling before the pots were topped up to the rim with vermiculite to maintain a high humidity around the stem base.

Three pots of each of winter wheat cv. Beaver and winter rye cv. Halo were used for each isolate and after inoculation were placed randomly in a block design in the cabinet. The plants were watered from below, using a liquid feed (nutrient ratio NPK 2:1:2), to prevent cross contamination between pots. Plants were maintained in these conditions for eight weeks before visual assessment of symptoms using the method of Scott (1971). This involved peeling back successive leaf sheaths to determine the progression of infection which was then scored using the following criteria:

0 = No Infection

1 = Coleoptile Infected

2 = Coleoptile Penetrated

3 = 1st Leaf Sheath Infected

4 = 1st Leaf Sheath Penetrated

5 = 2nd Leaf Sheath Infected .....

Typically between six and eight leaf sheaths were found per plant giving a maximum leaf sheath infection score of 13 or 17 respectively. Where the disease had progressed as far as the stem this was treated as the next leaf sheath and the disease scored accordingly.

Cross A Progeny

An experiment was set up to determine the pathogenicity towards wheat (cv. Beaver) and rye (cv. Halo) of a random sample of prototrophic progeny generated in cross A between 22-136 (W-type; *his*1-1, *arg*2-3) and 22-342 (R-type; *ino*-1, *nia*1-15, BEN-5R) (Hocart *et al.* 1993b). A total of 14 isolates were tested including 12 prototrophic recombinants from this cross and the two *P. herpotrichoides* isolates (22-20, W-type and 22-8, R-type) from which the parentals of cross A were generated. The recombinant progeny tested were derived from three fusion products: FP1 (J166, H112, B22, I143, I131, F90), FP2 (AE591, AE586, Q285) and FP5 (M218, X397, Z449a). A set of uninoculated pots were included as pathogen-free controls. The isolates 22-20 and 22-8 were used instead of the auxotrophic parental strains and only prototrophic progeny generated in this cross were included in the pathogenicity test in order to rule out any possible effect of the auxotrophic markers on pathogenicity. Auxotrophic strains typically show reduced pathogenicity.

Interspecific Hybrid Recombinants

The pathogenicity to wheat (cv. Beaver) and rye (cv. Halo) of a sample of progeny generated by interspecific hybridization between *P. herpotrichoides* and *P. anguioides* (Hocart & McNaughton, 1994) was investigated. Eight progeny were selected which had been derived from three separate fusion products. In addition, six control isolates consisting of two W- and two R-types of *P. herpotrichoides* as well as two *P. anguioides* isolates were tested,

and included the isolates from which the parent strains in the interspecific cross were generated. Table 5.1 lists all the isolates tested in this experiment. A set of uninoculated pots of both host species were included as pathogen-free controls.

5.2.2 Pathogenicity Assessment under Glasshouse Conditions

Two large scale pathogenicity experiments were carried out under glasshouse conditions at the Bush Estate, Roslin. Both experiments followed the same procedure. Seeds of wheat cv. Beaver and rye cv. Halo were sown eight per 12.5 cm diameter pots which were filled to within 2 cm of the rim with peat-based potting compost. Three weeks post-emergence the seedlings were inoculated with fungal infested filter paper disks (5 mm diameter; Whatman No. 1). These were produced by growing each isolate to be tested from an inoculum of hyphal fragments in 10 ml of liquid MYG (1/5th normal strength LMYG) in 90 mm Petri dishes containing between 30 and 40 filter paper disks for three weeks at 19°C. Single infested paper disks were placed against the seedling base and were held in place using filter paper collars with a 5 mm hole in the center (Fig. 5.1). Three pots each of Beaver and Halo were allowed for each isolate being tested. After inoculation the pots were topped up with vermiculite to maintain a high humidity around the stem base. The pots were positioned in a randomized block design in the glasshouse and watered from below using capillary matting to prevent cross-contamination between pots. The pots were grown for a further eight weeks during which period air temperature measurements were taken using a thermohygrograph placed next to the pots. After this time the plants were assessed for visual symptoms of disease using the method of Scott (1971) by peeling back successive leaf sheaths to assess the extent of infection as described above in section 5.2.1.

Fig. 5.1 Inoculation procedure for glasshouse based assessment of pathogenicity. Single infested paper disks were placed against the seedling base and held in place using paper collars before covering with vermiculite.

Fig. 5.2 Inoculation procedure for short assay pathogenicity trial. Two centimetre long pieces of polyvinyl tubing were placed over each seedling before addition of vermiculite (up to the rim of the tubes. Individual plants were inoculated by pipetting spore suspensions into each tube.

Table 5.1 Interspecific hybrid recombinants and control isolates tested in the controlled environment cabinet assessment of pathogenicity. The parental isolates from this cross are represented in this experiment by 24-1 (*P. anguioides*) and 22-12 (*P. herpotrichoides*, R-type).

ISOLATE	PATHOTYPE / FUSION PRODUCT	AUXOTROPHIC MARKERS *
24-1	*P. anguioides*	
24-12	*P. anguioides*	
22-1	*P. herpotrichoides* W-TYPE	
22-2	*P. herpotrichoides* W-TYPE	
23-2	*P. herpotrichoides* R-TYPE	
22-12	*P. herpotrichoides* R-TYPE	
FD	FPt	Prototroph
FH	FPt	Prototroph
DM	FPc	*cys*-71
FW	FPt	*cys*-71
DQ	FPe	Prototroph
AJ	FPt	*cys*-71
FP	FPt	Prototroph
EO	FPt	*cys*-71

* *cys*-71 - cysteine auxotrophy derived from *P. herpotrichoides* R-type parental strain

Intraspecific W x R Progeny

A pathogenicity trial was set up to determine the pathogenicity of progeny generated in four intraspecific W x R crosses in comparison with that of the parental strains used in each of the crosses and the field isolates from which they were derived (Table 5.2). Seedlings of wheat cv. Beaver, rye cv. Halo and, in addition, the eyespot resistant winter wheat cv. Rendezvous were inoculated and assessed between the 11th January and 8th March 1993. A total of 60 different isolates including 26 cross D progeny (selected from each of the five fusion products), 6 cross E progeny, 13 cross F progeny, 4 progeny from cross A and 10 control isolates were screened. An uninoculated set of pots was included as pathogen-free controls. The cross A progeny were selected from those tested under controlled environment conditions and provides a comparison of the two experimental systems.

Interspecific Hybrid Recombinants

The pathogenicity of a number of interspecific hybrids was assessed towards wheat (cv. Beaver) and rye (cv. Halo). These hybrids were produced by protoplast fusion between isolates of *P. herpotrichoides* (R type) and *P. anguioides* (Hocart & McNaughton, 1994). In addition, the pathogenicity of nine control isolates, including prototrophs and auxotrophs of both *P. herpotrichoides* and *P. anguioides*, was determined. Details of all isolates tested are presented in Table 5.3. Two sets of uninoculated pots were included in this experiment as controls. The experiment was carried out between 8th March and 10th June 1993. The mean air temperature in the glasshouse during that time was found to be 12.5 ± 0.4°C, with average daily maxima and minima of 22.7 ± 0.8°C and 7.8 ± 0.3°C respectively..

Microscopic Examination

Tissue samples from all of the standard pathogenicity trials under both greenhouse and controlled environment conditions were viewed microscopically to look for the presence of infection structures. Tissue samples were stained with 0.01% 8-anilino sulphonic acid (ANS)

Table 5.2 Control isolates and intraspecific parasexual progeny from crosses A, D, E and F tested in the glasshouse assessment of pathogenicity.

Cross	Original Isolates	Auxotrophic Parental Strains *	Parasexual progeny +
A	22-20 (W) 22-8 (R)	22-136 *his*1-1, *arg*2-3 22-342 *ino*1-1, *nia*1-15	FP1: F90, I131 FP2: Q285 FP5: Z449a
D	22-20 (W) 23-2 (R)	22-136 *his*1-1, *arg*2-3 23-2/9 *met*-9	FP1: D1/107, D1/2, D1/18a, D1/7, D1/5 FP2: D2/22, D2/27, D2/26, D2/36 D2/35 FP3: D3/46, D3/45, D3/48, D3/42 FP4: D4/119, D4/64, D4/63, D4/67b, D4/78, D4/75 FP5: D5/100, D5/97, D5/85, D5/82, D5/83b
E	22-1 (W) 22-119 (R)	22-1/7 *glu*-1, *gly*-2 22-119/5 *arg*-7	FP1: E1F24/1, E1F26/1, E1M485/1, Spores4.2, E1F23/2, E1F25/1, E1F26/3, Spores2/2
F	22-1 (W) 22-2 (W)	22-1/7 *glu*-1, *gly*-2 22-2/2	FP1: FF3/2, FM3/4, FM22/2, FR5, FM2/1, FM1/3, FM22/3, FF1/1, FM21/3, FF2/2, FM2/1

* auxotrophic marker symbols: *his*1-1, histidine requiring; *arg*2-3, *arg*-7, arginine requiring; *ino*1-1, inositol requiring; *nia*1-15, nitrate non-utilizing; *met*-9, methionine requiring; *glu*-1, glutamate requiring; *gly*-2, glycine requiring

+ FP1, FP2, etc. Fusion products from which recombinant progeny were derived following haploidization.

Table 5.3 Control isolates and interspecific hybrid recombinants from parasexual crosses between *P. herpotrichoides* and *P. anguioides* tested in the glasshouse assessment of pathogenicity.

Cross	Original Isolate	Auxotrophic Parental Strains *	Parasexual Progeny
P. anguioides x R-type	24-1 (*P. anguioides*) 22-12 (R)	24-9 *nic*-7 22-228 *cys*-71	FPt: EO (P) FD (P), FP (P), AJ (P), GD (*cys*-71), AM (*cys*-71), FW (*cys*71), AW(*cys*-71), BH (*cys*-71), BE (*cys*-71), BU1 (*cys*-71), EQ (*cys*-71), AY (*cys*-71) FPc: DM (*cys*-71), DK (*cys*-71) FPe: DY (*cys*-71), EF (*cys*-71)
P. anguioides x W-type Additional Strains:	24-1 (*P. anguioides*) 22-20 (W) 24-12 (*P.anguioides*) 22-5 (W) 22-119 (R) CT (R) *cys*-71 **	24-9 *nic*-7 22-138 *his*3-3, BEN-21R	FPiii: CF2 (*nic*-7)

* auxotrophic marker symbols: *nic*-7, nicotinic acid requiring; *cys*-71, cysteine requiring; (P) represents prototrophic progeny.

** strain CT represents an internal control for the R-type parental strain 22-228 having been recovered from a regenerated protoplast following PEG treatment of the parental strain and otherwise is identical to this R-type isolate.

in 0.05 M potassium phosphate buffer (0.93 g of citric acid and 2.12 g of potassium phosphate disolved in 100 ml H_2O and the pH adjusted to 5) before viewing with fluorescence microscopy using a Leitz orthoplan II microscope fitted with a 100W mercury vapour lamp, a dichroic mirror and Biener filters.

5.2.3 Data Analysis

Pathogenicity scores are represented graphically as median disease scores with maxima and minima for each isolate. Mean pathogenicity scores for each isolate are also illustrated with graphs to show how the mean and median relate to each other for each isolate. In determining whether an isolate was pathogenic or not median values were found to be a more accurate representation of pathogenicity scores, particularly for isolates where the majority, i.e. greater than 50%, of the individual plant scores were zero. To test for the difference between isolates, pathogenicity results were analysed by Kruskal - Wallis analysis of variance by ranks (Zar, 1974). This method has been used as it avoids the assumptions implicit in standard parametric ANOVA that the data was normally distributed and that the sample variances are the same, which they are unlikely to be in these experiments. The 24 score values for each strain (3 replicates x 8 plants per pot) were placed in ascending order before assigning each data point a rank value. The following formula was used to test whether there were any significant differences in pathogenicity between isolates :

$$H = (12/N(N+1))\Sigma R^2_i/n_i - 3(N+1)$$

where H is the Kruskal-Wallis test statistic; N is the total number of ranks; R is the sum of the assigned rank values and n is the number of ranks per sample. Additionally this method can also take into account any tied ranks that appear in the data set by calculating the following:

$$C = 1 - \Sigma T/(N^3 - N)$$

where

$$\Sigma T = m_i\Sigma(t^3_i - t_i)$$

and t_i is the number of ties in the ith group of ties and m is the number of groups of tied ranks.

This value is then used to calculate a corrected Kruskal - Wallis statistic by

$$H_c = H/C$$

The critical value for these tests was taken from a table of χ^2 values with k-1 degrees of freedom. This method of analysis was used to test for any differences within the whole data set, to test for differences between strains once the uninoculated control data had been removed, i.e. to assess whether there were any differences within the test isolates, and lastly to test for significant differences amongst those isolates whose median was greater than zero. These latter isolates were classified as pathogenic as at least 50% of the plants scored were diseased. Correlation analysis was also carried out for each test by comparing median data from wheat and rye to assess for any association in pathogenicity on these two host species.

5.2.4 Short Pathogenicity Assay

This protocol was adapted from that of Daniels & Lucas (1990) and used as a 'rapid' method for determining the pathogenic potential of strains. Seeds of Beaver and Halo sown in 5 cm diameter pots filled to within 1 cm of the rim with potting compost were grown until the two leaf stage before 1 inch long pieces of polyvinyl tubing (internal diameter 3 mm) washed in a dilute solution of Triton-X-100 and then autoclaved, were placed over each seedling. The pots were then filled to the rim with vermiculite (Fig. 5.2). A 1 ml spore suspension (2×10^5 spores/ml) was slowly pipetted down the inside of the tubing. Individual pots were covered with polythene bags for the first 48 h to maintain a high humidity.

An experiment was set up to assess the infection behaviour of a range of progeny from cross D which were selected from those tested in the large glasshouse-based pathogenicity trial described in section 5.2.2 above and represented both those recombinants which gave visible disease symptoms and also those which did not give any visual symptoms of infection in the earlier experiment. Growth conditions were the same for earlier controlled environment experiments (section 5.2.1). After three weeks tissue samples, representing the entire leaf

sheath adjacent to the point of inoculation, from the first and second leaf sheaths were assessed microscopically by staining the tissue with a fluorochrome (8-anilino sulphonic acid, ANS, see section 5.2.2 above) and examined for the presence of eyespot infection structures (i.e. infection plaques). The abundance of infection structures was scored on a 1 to 5 scale:

Score	Description	Parameters
1	None	(0)
2	Few	(1-4) on entire tissue
3	Moderate	(5-20) on entire tissue
4	Many	(21-50) on entire tissue
5	Abundant	(>50) on entire tissue

5.3 RESULTS

5.3.1 Controlled Environment Cabinet Assessment of Pathogenicity

Cross A Progeny

The results of the assessment of pathogenicity of selected parasexual progeny from cross A (W x R) are shown in figures 5.3a and 5.3b. Both 22-8 (R-type) and 22-20 (W-type), the parental isolates used in cross A, were pathogenic on both host species. The pathogenicity of these two isolates to wheat was similar while the R-type (22-8) showed greater pathogenicity and achieved a greater disease rating on rye, as expected. Of the recombinant progeny the majority of strains (10 out of 12 or 83%) were classified as non-pathogenic, having median disease scores of zero. Only Z449a was pathogenic on both hosts. Strain F90 was pathogenic on wheat but non-pathogenic on rye, while I131 was deemed pathogenic on rye but non-pathogenic on wheat on the basis of the median disease scores for these progeny. Generally it was found that the mean and median values were a close approximation for each other. For strains giving a median disease score of zero but with a range greater than zero, the mean disease score was typically small (i.e. < 1.0) indicating the occurrence only of one or two symptomatic plants in the population of plants inoculated with that strain (e.g. Q285 on

Fig 5.3a & 5.3b Median (black squares) and mean (open diamonds) pathogenicity values with maxima and minima for cross A progeny towards wheat (Fig 5.3a) and rye (Fig 5.3b) when tested under controlled environment conditions.

Fig 5.3a

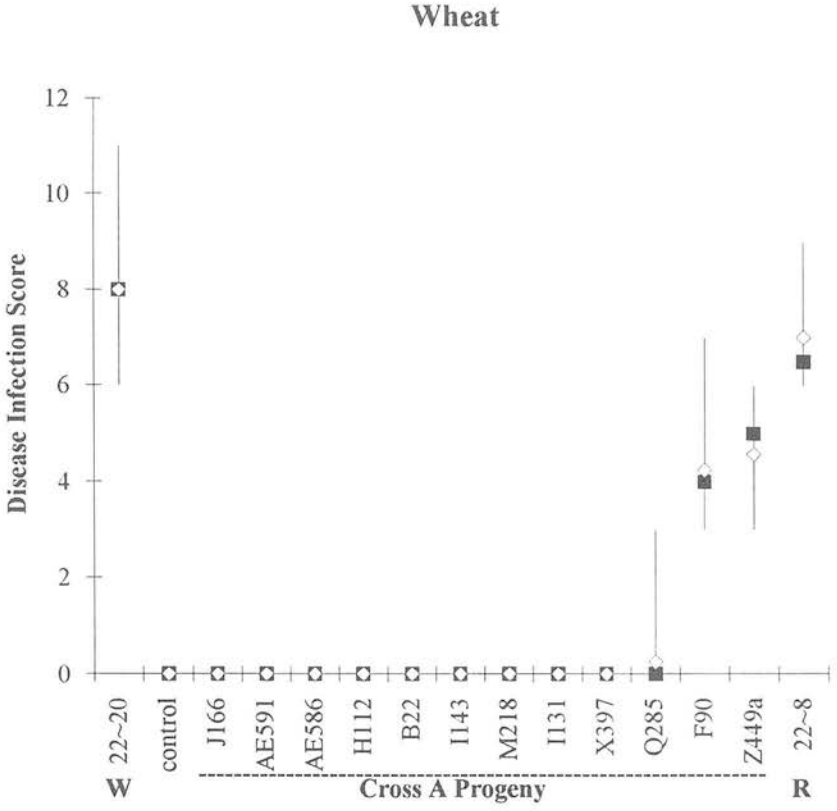
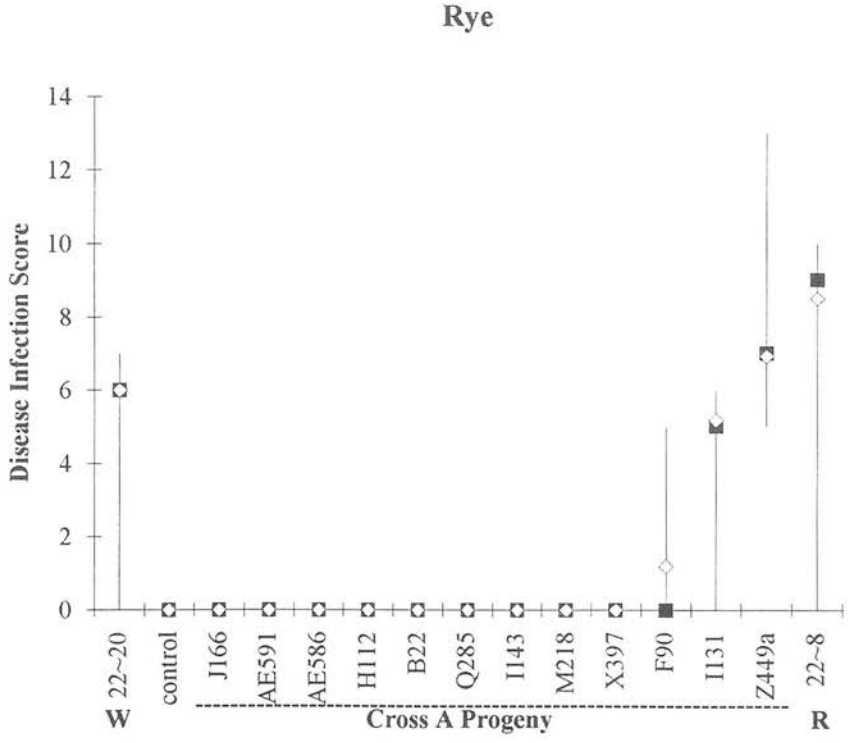


Fig. 5.3b



wheat, F90 on rye). Consequently, it was considered reasonable to assume that median disease scores gave a better indication of pathogenicity than mean values in these assessments as it more closely reflected the majority of the data.

The statistical analysis showed that the differences in pathogenicity between the strains to both wheat and rye were significant ($P < 0.01$). Similarly when only those strains deemed pathogenic were considered (i.e. those with median disease scores greater than zero) significant differences ($P < 0.01$) between strains were detected indicating variation in the level of pathogenicity expressed. The Kruskal-Wallis analyses indicate that the values were not randomly distributed between the isolates and this results when an isolate has a greater number of higher or lower ranks.

Interspecific Hybrid Recombinants

Figures 5.4a and 5.4b present the results for the assessment of pathogenicity of progeny from the interspecific fusion. Only 22-12, the R-type parent of the cross, was pathogenic on both hosts. The *P. anguioides* parent of the cross, 24-1, was not pathogenic on either host. Of the additional control isolates only 23-2, R-type, and 24-12, *P. anguioides*, were found to be pathogenic and then only on rye. The other strains of both *P. herpotrichoides* (W-type) and *P. anguioides* were classed as non-pathogenic, having median disease scores equal to zero. Unexpectedly, the uninoculated wheat control gave a median disease rating above zero. Of the hybrids, at least 50% were classified as being pathogenic on both hosts (6 out of 8 on rye and 4 out of 8 on wheat) having median disease scores greater than zero. The four hybrid progeny pathogenic on wheat were also found to be pathogenic towards rye. On both hosts the hybrid's pathogenic ability was generally found to lie between that of *P. anguioides* and *P. herpotrichoides* (R-type). There would appear to be no effect of auxotrophy on the pathogenic ability of the progeny. DM, FW, AJ and EO all of which are cysteine requiring (*cys*-71) were all found to be pathogenic either to both wheat and rye (DM,

Fig 5.4a & 5.4b Mean and Median pathogenicity values with maxima and minima of *P. herpotrichoides* x *P. anguioides* hybrids towards wheat (Fig. 5.4a) and rye (Fig 5.4b) under controlled environment conditions. Median pathogenicity scores are represented by black squares whilst the open diamonds represent mean infection scores.

Fig 5.4a

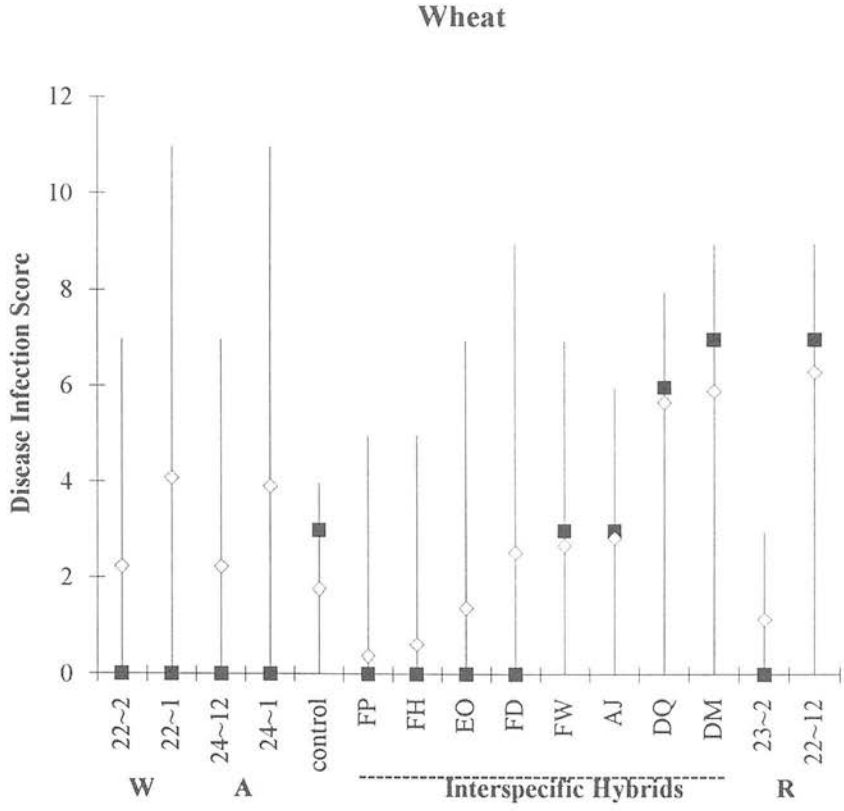
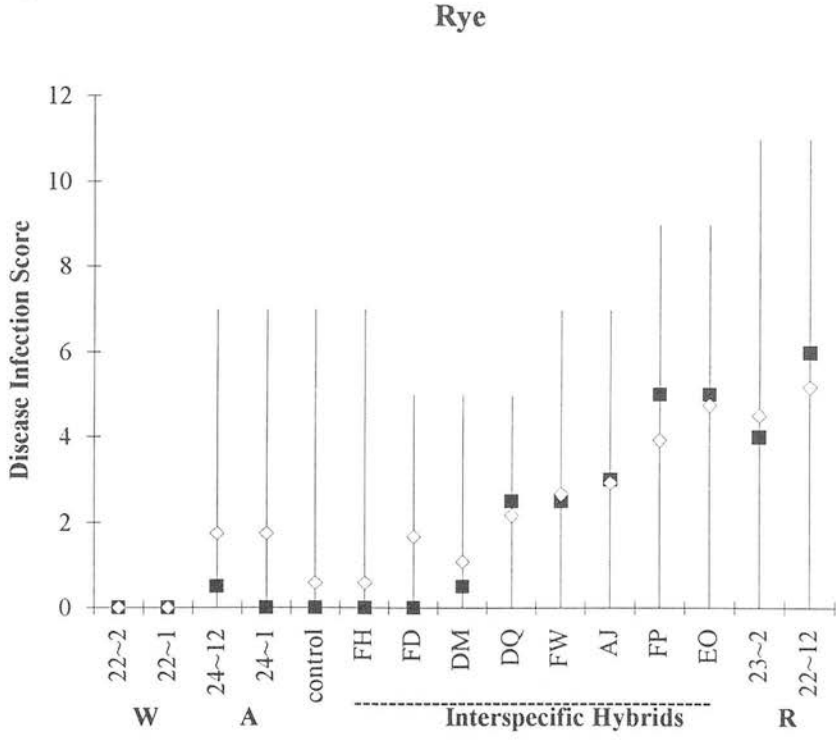


Fig 5.4b



FW, AJ) or only to rye (EO) and to a level comparable to the pathogenic prototrophic progeny (FP, DQ). Amongst isolates classed as pathogenic it was generally found that the mean and median disease scores were a good approximation for each other. The greatest difference in mean and median scores were seen in the control isolates on wheat. Again, the difference between mean and median scores was due to the occurrence of one or two symptomatic plants from those inoculated with that particular strain. The statistical analysis showed that the differences in pathogenicity were significant ($P < 0.01$) for both hosts. When only those strains classed as pathogenic, i.e. having a median disease score above zero, were analyzed significant differences were only found between strains on wheat ($P < 0.01$) indicating that only for wheat was there variation in the levels of pathogenicity.

5.3.2 Glasshouse Pathogenicity Assessment

Intraspecific W x R Progeny

The pathogenicity of progeny from the four intraspecific crosses (cross A: 22-136 (W-type) x 22-342 (R-type), cross D: 22-136 (W-type) x 23-2/9 (R-type), cross E: 22-1/7 (W-type) x 22-119/5 (R-type) & cross F: 22-1/7 (W-type) x 22-2/1 (W-type)) and the parental isolates used in each of these crosses was assessed in one large pathogenicity trial conducted under glasshouse conditions.

When assessing for disease symptoms the first pot was assessed as usual by peeling back successive leaf sheaths to assess the penetration of infection. However, in the assessment of plants from the second and third pots it was noticed that on some plants, even though there were no visible disease symptoms on the leaf sheaths, there were severe lesions on the stem base. On further examination of the lesions, by staining with ANS and viewing under a UV microscope, typical *P. herpotrichoides* infection plaques were seen to be present in abundance. As scoring of stem infection was not fully carried out during the scoring of the first replicate only the data from the second and third replicate pots were included in the

analysis of this pathogenicity trial. The behaviour of the control isolates on wheat and rye will be described initially before the pathogenicity of progeny from each of the intraspecific crosses is related individually.

Unexpectedly, very few of the W- and R-type control isolates were found to be pathogenic on both wheat and rye (Fig. 5.5a & 5.5b). When tested for pathogenicity to wheat cv. Beaver only the prototrophic field isolates 22-20, 22-2 (W-type) and 23-2, 22-119 (R-type) gave appreciable levels of infection. All auxotrophic mutant strains, of both W- and R-types were non-pathogenic, giving median disease scores of zero. Even fewer isolates were found to be pathogenic towards rye cv. Halo with only 22-1 (W-type) giving noticeable levels of infection. All other W- and R-type isolates, both prototrophic and auxotrophic were non-pathogenic.

Cross D Progeny

Of the progeny from cross D tested in this pathogenicity trial approximately 96% of the isolates on wheat and 73% of the isolates on rye were found to be non-pathogenic. On wheat only one recombinant isolate, D2/22, had a median value above zero, and only five strains had a range which extended beyond zero. This was due to the presence of only one or two symptomatic plants giving disease scores above zero for each of these isolates. On rye (Fig 5.6) seven isolates were found whose median pathogenicity score was greater than zero, with a further 15 isolates having a range greater than zero. For the majority of isolates on rye the maxima values recorded were comparable with those of the parental and control isolates. However, this was mainly only due to one or two disease score values in each case. The isolates pathogenic on rye were mostly less pathogenic than the control isolate 22-1 and came from four of the five fusion product sets of progeny tested (FP2 - FP5). Progeny from FP1 were all non-pathogenic on rye.

Fig. 5.5a & 5.5b. Mean (open diamonds) and median (black squares) infection scores towards wheat (Fig. 5.5a) and rye (Fig. 5.5b) of the control isolates from the glasshouse assessment of pathogencity of intraspecific recombinants.

Fig 5.5a

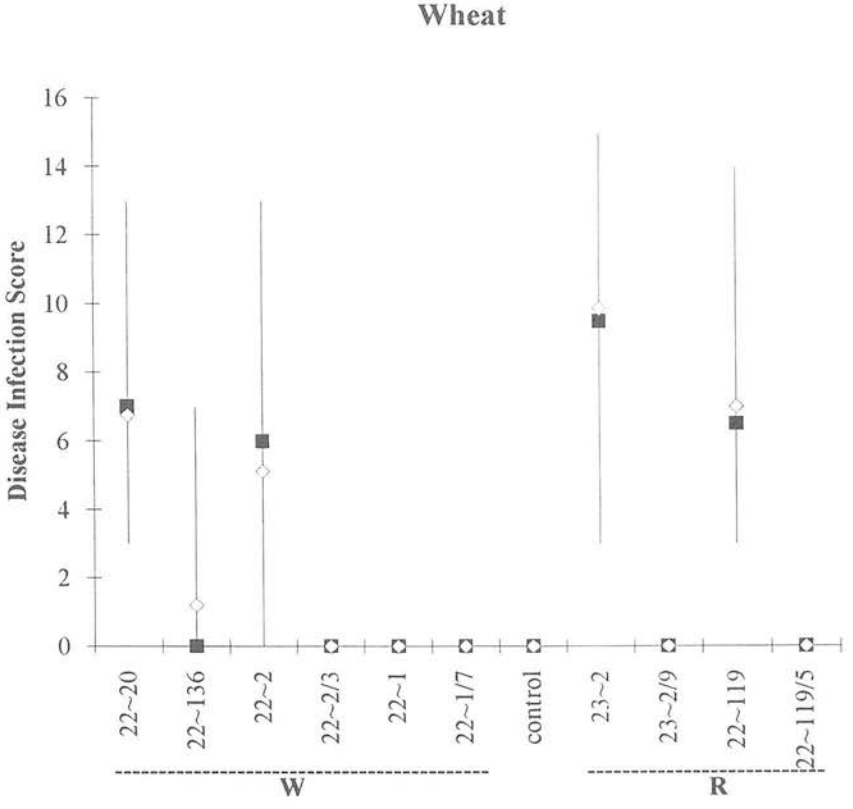


Fig. 5.5b

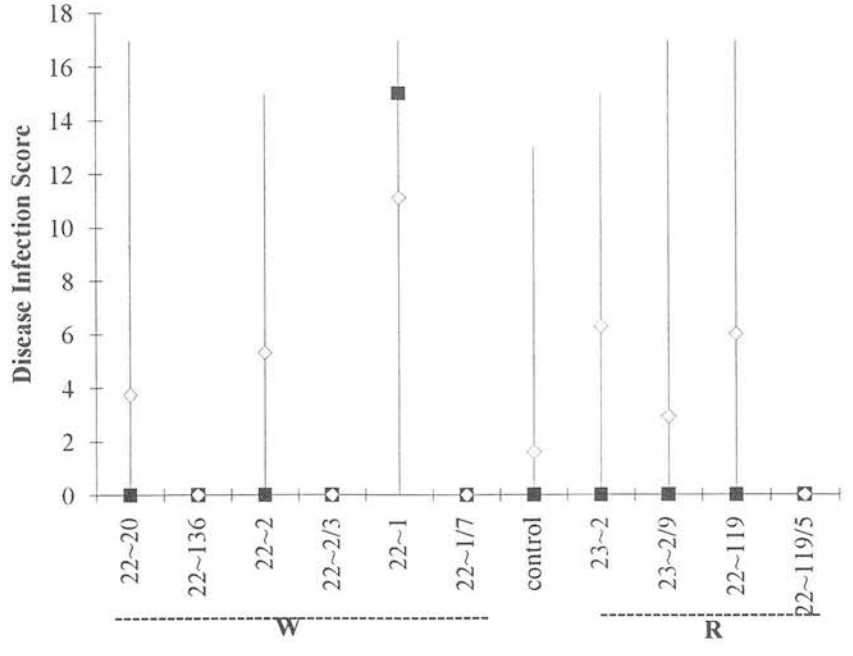
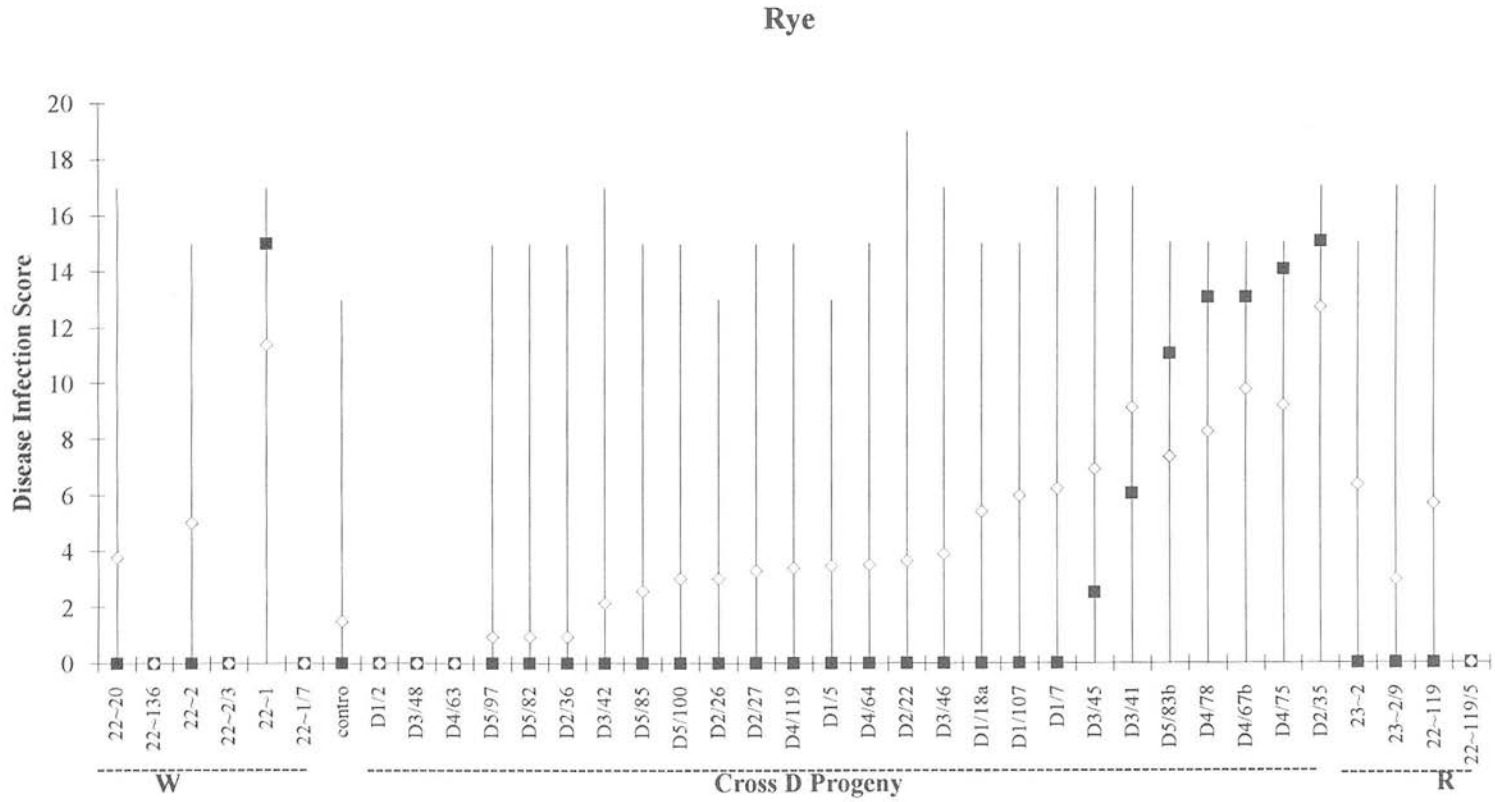


Fig 5.6 Mean (open diamonds) and median (black squares) pathogenicity values with maxima and minima for progeny derived from cross D towards rye cv. Halo when tested under glasshouse conditions.

Fig. 5.6


Rye
Disease Infection Score
0
2
4
6
8
10
12
14
16
18
20
22~20
22~136
22~2
22~2/3
22~1
22~1/7
contro
D1/2
D3/48
D4/63
D5/97
D5/82
D2/36
D3/42
D5/85
D5/100
D2/26
D2/27
D4/119
D1/5
D4/64
D2/22
D3/46
D1/18a
D1/107
D1/7
D3/45
D3/41
D5/83b
D4/78
D4/67b
D4/75
D2/35
23~2
23~2/9
22~119
22~119/5
W
Cross D Progeny
R

Significant differences ($P < 0.01$) were detected between the strains tested within both wheat and rye when both the complete data set and the data minus the uninoculated control were analyzed. An analysis of pathogenic strains only was carried out for rye where it was found that there were significant differences between these isolates. On wheat only one cross D progeny isolate, D2/22, was classed as pathogenic and the disease score for this strain was significantly higher than for the other progeny strains.

Cross E Progeny

Very few disease symptoms were observed on plants inoculated with cross E progeny. This was reflected by the fact that only one isolate, $E1F_26/3$ was pathogenic having a median pathogenicity score greater than zero, and then only for rye. However, some isolates (3 out of eight for wheat and 6 out of eight for rye) were seen to have maxima values greater than zero. Generally however, this was due to the presence of three or less plants with disease symptoms out of a possible 16.

Cross F Progeny

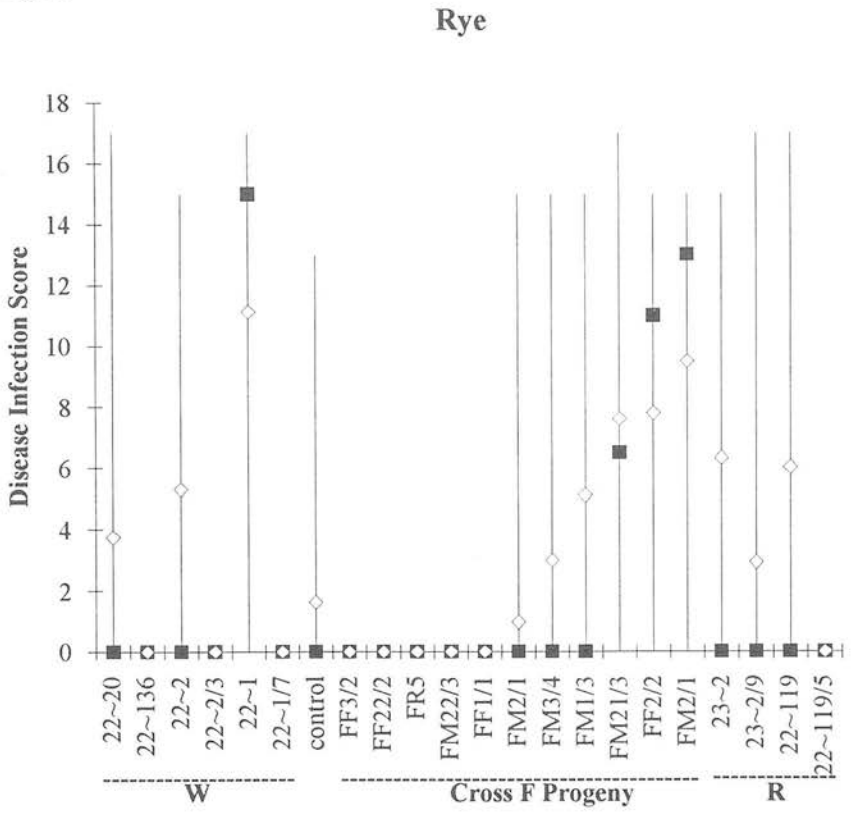
Very few cross F progeny isolates were classed as pathogenic. Those that were, were found only when tested on rye (Fig. 5.7). The small number of pathogenic recombinant isolates was similar to the situation found for cross D and cross E progeny. The median disease scores of those isolates classed as pathogenic on rye were seen to be below that of 22-1, although when these isolates were analyzed no significant differences were found between them indicating that the few pathogenic recombinants were as pathogenic as the parental isolate.

Cross A progeny

Similar results were observed for the cross A progeny as with the other crosses, D; E and F in that very little pathogenicity was observed from the progeny to either wheat or rye.

Fig 5.7 Mean (open diamonds) and median (black squares) pathogenicity values with maxima and minima for cross F progeny towards rye under glasshouse conditions.

Fig 5.7


Rye
Disease Infection Score
18
16
14
12
10
8
6
4
2
0
22~20
22~136
22~2
22~2/3
22~1
22~1/7
control
FF3/2
FF22/2
FR5
FM22/3
FF1/1
FM2/1
FM3/4
FM1/3
FM21/3
FF2/2
FM2/1
23~2
23~2/9
22~119
22~119/5
W
Cross F Progeny
R

As with cross E and F progeny the only pathogenic isolate was found on rye. These results did not confirm the findings of the controlled environment test in which the recombinants Z449a, F90 and I131 were found to be pathogenic on either wheat or rye or both.

Rendezvous

Analysis of disease infection scores towards wheat cv. Rendezvous using Kruskal-Wallis analysis of variance by ranks showed significant differences in the levels of pathogenicity by strains ($P < 0.01$). When the plants were assessed one or two plants were occasionally found to be symptomatic with eyespot lesions being observed on the stem base and hence gave mean infection scores greater than zero. As was found for wheat cv. Beaver and rye cv. Halo these symptomatic plants were in the minority for plants inoculated with a particular strain. It is probably sufficient to assume that these infection scores were enough to suggest that there were significant differences amongst the data. However, when median disease scores were plotted there were no isolates with a median above zero, and hence pathogenic and it can therefore be assumed that the resistance introduced from *Aegilops ventricosa* was still effective.

Microscopic Examination of Tissues

A random sample of tissue was taken from these trials for microscopic examination. Growth of *P. herpotrichoides* was found on the outer leaf sheaths of both symptomatic and non-symptomatic plants from each of Beaver, Halo and Rendezvous. on this material it was seen that there was considerable hyphal growth produced by the majority of isolates, both parental and progeny, on each of the three cultivars. However, there was little sporulation by the strains although on a few occasions conidiophores were seen to be present. Infection plaques were seen on the tissue produced by both progeny and parental isolates (Fig. 5.8). Additionally, 'pseudo-infection plaques' were produced by some progeny. These appeared to be aggregates of hyphae, some cells of which had a swollen appearance resembling standard

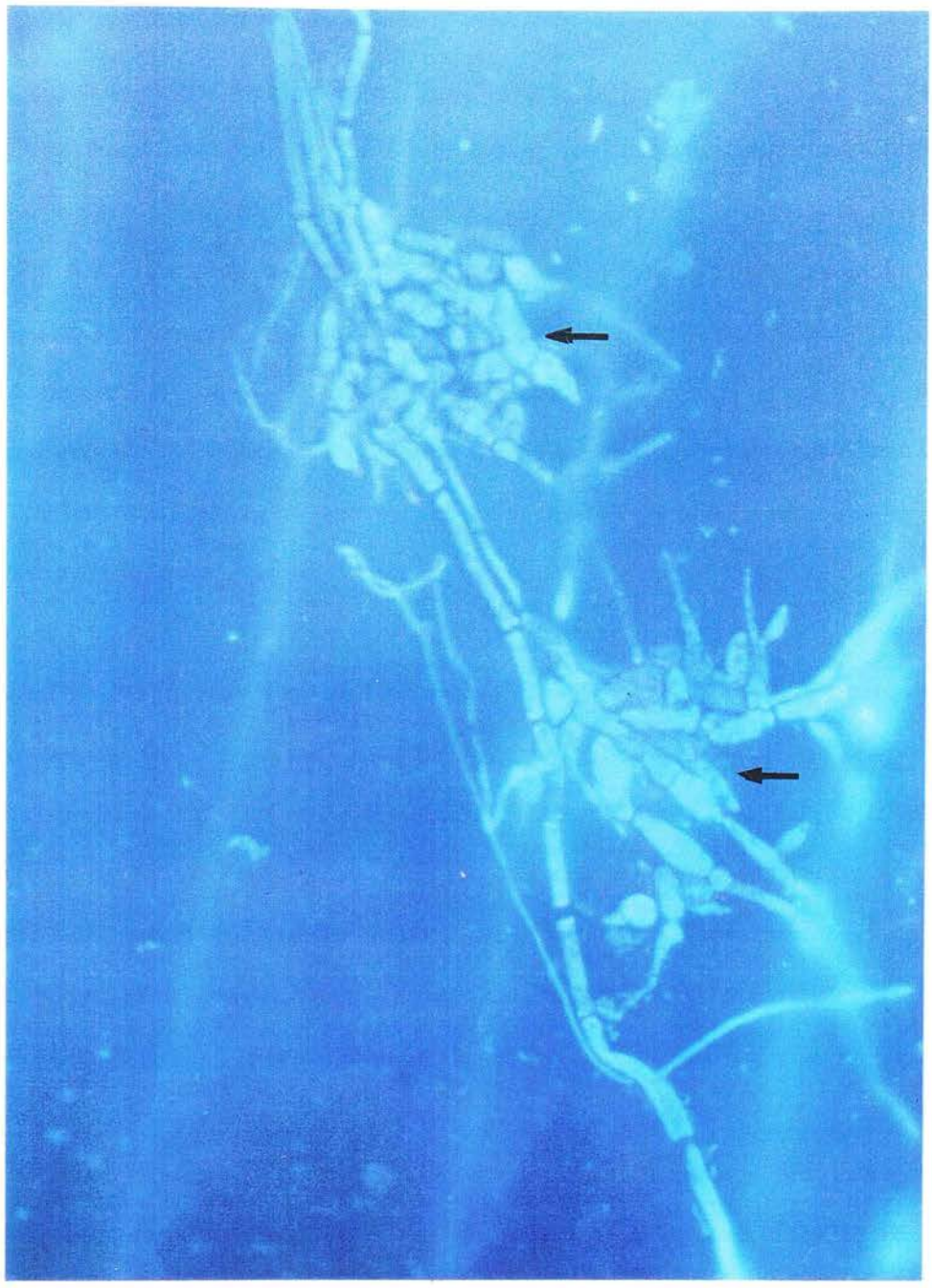


Fig. 5.8 Typical infection plaques (indicated by arrows) produced by both progeny and parental isolates on the leaf sheaths of host plants.

P. herpotrichoides infection plaques. Some recombinants, however, showed no obvious growth on the host tissues forming neither infection plaques or hyphae with the leaf sheaths.

Glasshouse assessment of Pathogenicity of Interspecific Hybrids

The pathogenicity to wheat of the W- and R-type field isolates was confirmed for each of the two W- and R-type field isolates which all gave typical disease symptoms on leaf sheaths (Fig. 5.9a). The cysteine auxotrophy marker carried by the R-type parental strain 22-228 (*cys*-71) did not impair the pathogenicity of the isolate which was as pathogenic as 22-12, the R-type field isolate from which it was derived. The two isolates of *P. anguioides*, 24-1 and 24-12, gave only slight disease symptoms on wheat and were classified as non-pathogenic, as expected for this weakly pathogenic species. The auxotrophic *P. anguioides* strain (24-9) did not produce symptoms on any of the plants.

When tested for pathogenicity to rye cv. Halo only the R-type isolate 22-119 gave appreciable levels of infection. The W-type isolates and the *P. anguioides* isolates were non-pathogenic, giving median disease scores of zero and mean disease scores of less than one (Fig 5.10b). Unexpectedly, the second R-type strain 22-12, and the auxotrophic parental strain derived from it (22-228), also showed a median disease score of zero to rye. The mean disease scores for these R-type strains was marginally higher than those of the W-type isolates and *P. anguioides* isolates.

Similarly, the recombinants follow the same pattern as the control isolates, with pathogenic hybrids only being found for wheat (Fig 5.10a). The progeny from the cross of the R-type 22-228 and *P. anguioides* isolate display a large variation in pathogenicity levels, which appears to be affected by which fusion product the progeny were derived from. Progeny derived from FPc were all highly pathogenic, those from FPt varied between low and high pathogenicity, whereas progeny from FPe were not pathogenic. Although no hybrid strains were pathogenic

Fig. 5.9 Eyespot disease symptoms, as indicated by arrows, produced on wheat cv. Beaver eight weeks post inoculation in glasshouse conditions. A control plant, lacking symptoms is situated on the far right.

Fig 5.10a & 5.10b Mean (open diamonds) and median (black squares) pathogenicity values with maxima and minima for *P. herpotrichoides* and *P. anguioides* recombinants towards wheat (Fig. 5.10a) and rye (Fig. 5.10b), tested under glasshouse conditions.

Fig. 5.10a

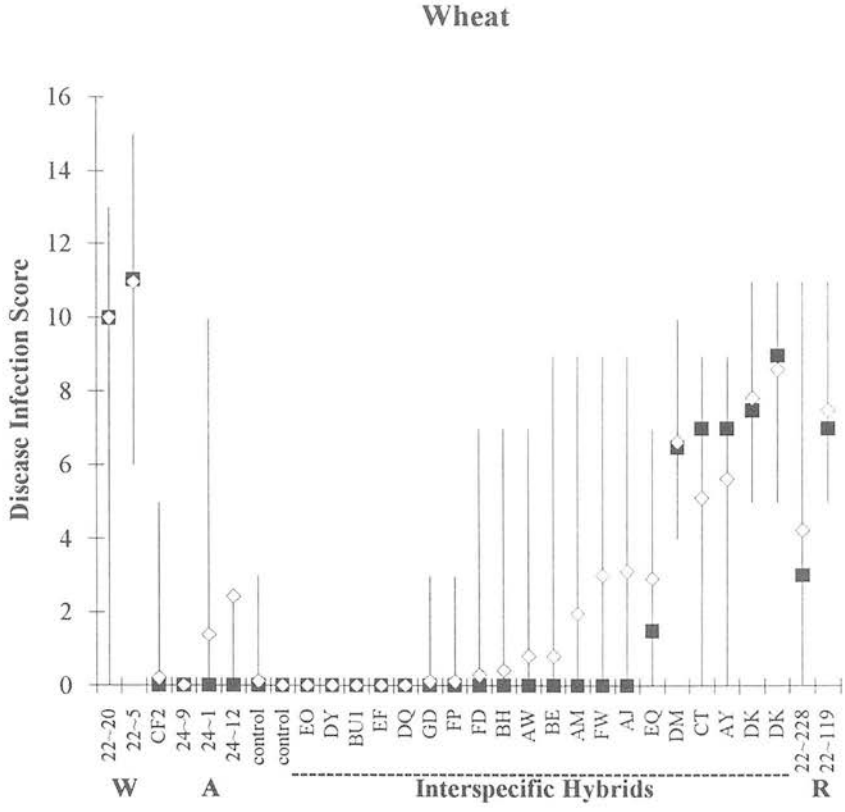
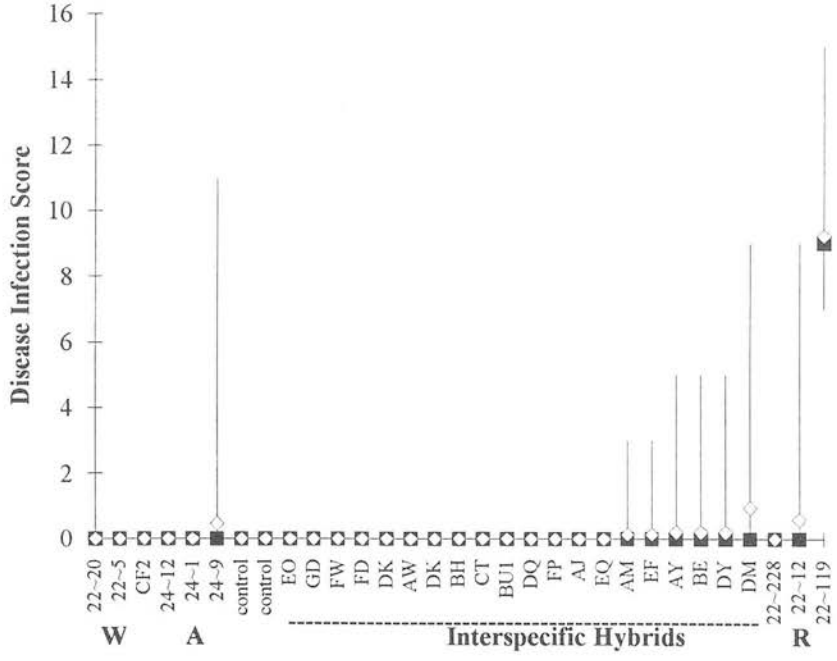


Fig 5.10b



on rye a few strains (6 out of 21) did produce symptoms on a minority of the plants inoculated with that particular strain. This resulted in a range of infection scores greater than zero, but was only due to one or two values, and is reflected in the mean disease scores values which for these isolates were all less than one.

When the wheat pathogenicity data were analyzed using the Kruskal-Wallis test, significant differences were found amongst the isolates at $P < 0.01$. These differences were found for the analyses carried out on the complete data set, when the uninoculated control was omitted and also when only those strains classed as pathogenic were analyzed. These results indicated not only that the strains classed as pathogenic were different from the non-pathogenic strains, but also that there were differences in the levels of pathogenicity expressed.

Tissue samples were taken from wheat cv. Beaver for viewing microscopically of both progeny and parentals. From these all isolates showed considerable hyphal growth on the outer leaf sheaths, although no sporulation was seen from the hyphae. The majority of isolates also formed infection plaques on the leaf sheaths and shows that both pathogenic and non-pathogenic strains were able to infect and colonize the host tissue.

Correlation Analysis

Comparison of median data for wheat and rye showed that there was no significant correlation present. This shows that being either pathogenic or non-pathogenic on one host did not necessarily determine an isolates pathogenicity on another host. This situation was found for all pathogenicity experiments carried out with the exception of the assessment of pathogenicity of cross A progeny under controlled environment conditions where a significant correlation ($r = 0.79$) was found between pathogenic ability to wheat and rye although this was found on a small sample size of progeny.

5.3.3 SHORT ASSAY

The majority of the progeny tested in the short assay were found to be non-pathogenic in the standard pathogenicity trial in the glasshouse i.e. they did not test significantly different from the uninoculated control. There were only two isolates included in the short assay which were pathogenic in the standard pathogenicity trial and on observation were able to produce typical eyespot infection structures. The non-pathogenic isolates were split on their ability to colonize the host tissue. Evidence of colonization of Beaver and Halo was found in the production of typical eyespot infection structures on the outer leaf sheaths by isolates. These infection structures were generally more abundant on Beaver than Halo. In contrast, for the remaining non-pathogenic strains no fungal structures indicative of infection were found, indicating an inability to colonize host tissues.

5.4 DISCUSSION

Variability was found in the pathogenic ability of control strains in that some of the controls did not always behave as expected. For example, in the glasshouse assessment of pathogenicity of intraspecific (W x R) recombinants to wheat cvs. Beaver and Rendezvous and rye cv. Halo the levels of pathogenicity observed for 22-1 on rye were higher than expected. The R-type isolates 23-2 and 22-119 gave lower levels of infection in the same experiment, and yet it is generally reported that R-type isolates are more pathogenic to rye than are W-type isolates (Scott *et al.*, 1975; Nirenberg, 1985; Fitt *et al.*, 1987). In addition, some of the strains, 22-1, 22-2 and 23-2, on wheat in the controlled environment cabinet assessment of pathogenicity of interspecific hybrids also gave lower levels of infection than expected.

This reduction in pathogenic ability of some isolates may have occurred for a number of reasons, including a loss of pathogenicity of isolates in culture Fitt *et al.* (1987). It is also possible that the source of inoculum was poor and variable or that the inoculation procedure

was inadequate in ensuring the contact of the inoculum with the host plant. Alternatively it is also possible that there was unsuitable environmental conditions for infection of the host species with eyespot or for development of disease symptoms during the course of these experiments.

It is possible that for some isolates there was a reduction in pathogenic ability due to the length of time in culture. However, other isolates, such as 22-20 which was isolated in 1971 still retain the ability to be highly pathogenic, as was evident from the disease score recorded in the controlled environemt assessment of pathogeicity of cross A progeny (Fig. 5.3a). Poor or variable inoculum or failure of the technique could help to explain why particularly for the glasshouse assessment of the pathogenicity of intraspecific progeny some plants were found to be highly diseased while others were completely clean. However, the fact that considerable hyphal growth and production of infection plaques was found on the leaf sheaths of non-symptomatic plants would suggest that the problem is not due to the failure of the inoculation procedure. It was found in a comparison of the two glasshouse pathogenicity experiments that the mean night temperatures recorded in the assessment of interspecific hybrid pathogenicity was lower than that recorded for the intraspecific recombinants, even though the assessment was carried out at a later date when temperatures might have been expected to have risen. It has however, been observed that under certain environmental conditions eg. high night temperatures, infection of winter wheat with eyespot could progress as far as the stem base where typical lesions would be found without any disease symptoms being expressed the leaf sheaths (R. Bayles, Pers. Comm.).

This work demonstrates that parasexual recombination between W- and R-type isolates reduces the pathogenic ability of around 75 to 80% of recombinant progeny. Of the remaining progeny that were classed as pathogenic variation was detected in the levels of pathogenicity expressed. No correlation was found amongst progeny between pathogenic ability to wheat

and rye indicating that the ability to cause disease on these two host species was separable. The lack of association between pathogenicity to wheat and rye within eyespot has been demonstrated by Creighton *et al.* (1989) who were investigating the pathogenicity of *P. herpotrichoides* isolates from France and the UK to wheat and rye. Hence wheat pathogenicity is not simply a 'default' ability possessed by all strains, with rye pathogenicity being an 'add on' character in R-type isolates. Both phenotypes are 'extras'. The recombination of W and R pathotypes have generated a novel 'pathotype' where rye pathogenicity is not associated with wheat pathogenicity.

The level of resistance expressed by the wheat resistant cv. Rendezvous was unaffected. No recombinant strains were tested which showed significantly greater ability to cause disease on this cultivar than the control isolates. Therefore the resistance due to *Aegilops ventricosa* remains unbroken.

From both the short assay assessment and microscopic observation of tissue samples randomly selected from the standard pathogenicity tests it was apparent that even though the majority of the recombinants tested were not pathogenic, i.e. did not display a median value greater than zero, a considerable number of these isolates were still able to colonize the leaf sheaths of both Beaver and Halo producing typical eyespot infection structures in the absence of disease symptoms such as tissue necrosis. It would therefore appear that as well as pathogenic or non-pathogenic ability of progeny identified from standard pathogenicity experiments there also appeared to be a parasitic response of isolates where they were able to colonize the host tissue but do not induce the production of disease symptoms. Therefore, in order to fully understand pathogenicity within the eyespot fungus it would seem reasonable to carry out microscopic as well as visual assessments of host plants including both symptomatic and non-symptomatic plants.

P. anguioides isolates included in the pathogenicity trials of the interspecific hybrid progeny were found to be, on the basis of median pathogenicity scores, less pathogenic than the majority of the W- and R-type isolates and confirmed the finding of Bateman (1988) that *P. anguioides* is a weakly pathogenic species. Successful isolations of this fungus could be made from leaf sheaths of seedlings but not from adult plants (Bateman, 1988). This would be consistent with the findings of Schreiber & Prillwitz (1985) who reported a decline in *P. anguioides* between spring and summer in Germany. In most cases it was found that pathogenic ability was eliminated in strains carrying an auxotrophic marker. The exception was with the *cys*-71 marker which had no effect in the test of pathogenicity of either the parental R-type interspecific hybrid progeny or the *met*-9 marker of some of the cross D progeny. Both prototrophic and auxotrophic hybrids were tested for pathogenic ability in the glasshouse assessment of pathogenicity with the *cys*$^-$ progeny being as pathogenic as the prototrophic progeny. Previously, pathogenicity experiments were carried out without including auxotrophic isolates as it was thought that it may impair the pathogenic ability of strains. However, the interspecific cross produced mostly auxotrophic recombinants and to exclude them from the experiments would not have allowed an adequate sample of progeny to be chosen. The pathogenic ability of auxotrophic isolates has been demonstrated previously by Clarkson & Heale (1985) where auxotrophic mutants of *Verticillium albo-atrum* were shown to be as pathogenic as wild type strains. Assessment of the pathogenicity of a range of interspecific hybrids on wheat suggests that the method by which the inoculum was produced, i.e. by allowing the fungus to colonize filter paper disks whilst being grown in dilute media, would give the fungus a sufficient supply of the amino acid it was lacking to allow infection of wheat (Hocart & McNaughton, 1994). However, although this method of inoculation may help in some circumstances to allow auxotrophic isolates to be pathogenic it is clearly not always sufficient as some isolates inoculated using these pre-colonized filter paper disks were pathogenic in some experiments and not pathogenic in others. Work by Wood (1967) demonstrated that the pathogenicity of auxotrophic strains of *Venturia inaequalis* was

restored by the addition of the required amino acid or vitamins. Presumably, this could be how the inoculation procedure using pre-colonized filter paper disks enables auxotrophic strains to be pathogenic.

6 VEGETATIVE INCOMPATIBILITY TESTING

6.1 INTRODUCTION

Vegetative incompatibility within the eyspot fungus has been shown to occur both between and within the W- and R-types (Hocart *et al.*, 1989; Magnano di San Lio *et al.*, 1994). Investigation of the recombination of genes controlling vegetative incompatibility will not only permit a study of the number of genetic controls for vegetative incompatibility within this fungus, but it will also give an indication of the extent of sexual recombination within this fungus. As would be expected, every generation of sexual recombination would increase the number of possible combinations of vegetative incompatibility genes and therefore if a large number of vegetative incompatibilty groups are found it may suuggest that sexual recombination is occurring. Alternatively, if very few different vegetative incompatibility groups are detected it may be that the population is clonal.

Three different techniques were used to try and demonstrate vegetative incompatibility (V.I.) between isolates of *P. herpotrichoides* and also progeny produced in intra- and inter- specific crosses. These experiments would firstly establish the pattern of vegetative incompatibility between the different field isolates (parental) used in the protoplast fusions. Additionally, this work would show the effect that this, protoplast fusion, had on the derived progeny and also how the progeny related to the parental isolates in terms of vegetative incompatibility groups. The first two methods used, based on the use of either mycelial or spore inoculation, required that isolates being tested were auxotrophs. The final method did not require for isolates to be auxotrophic and was to be developed in the hope of testing a wider range of isolates without the need to induce mutations.

6.2 MATERIALS AND METHODS

6.2.1 Interspecific Hybrid Recombinants - Hyphal Block Inoculation Assessment of Vegetative Incompatibility

A sample of 19 recombinants carrying the *cys*$^-$ auxotrophic marker from the interspecific fusion of 22-228 (R-type, *cys-71*) and 24-9 (*P. anguioides, nic-7*) were selected from two fusion product sets (FPt: BD, AY, EQ, GD, AM, FW, BW, AP, BU1, BU2, BK, BH, BL, BP, AZ, AK, AT & FPc: DK). In this experiment each recombinant was tested for complementation only with the two parental strains using complementary markers. Strain 24-6 (*ade-6*) was included in the experiment in place of 24-9 since the vitamin requirement of the latter is not a good marker for vegetative incompatibility testing as it cross feeds very easily. Strain 22-228 and an additional auxotrophic mutant 22-186 (*lys-5*) were also included in the test. No assessment was made between hybrid recombinants as suitable marker compatibility was lacking and new markers would have to be introduced into these strains before they could be tested. All strains were grown on MYG incubated at 19°C to produce inoculum for assessment.

Assessment of possible compatibility between each of the hybrids and both parental isolates was carried out on minimal medium (MM). Inoculum plugs approximately 1 mm^2 of each isolate combination to be tested were placed roughly 5 mm apart on the agar plate. Each plate was inoculated with a pairing of the test isolate (recombinant) with each of the two control isolates (22-228 & 24-6). In addition an inoculum plug of each recombinant being tested was inoculated on the agar plate to assess its growth and degree of pigmentation on MM. Two replicate plates were inoculated for each set of isolate combinations. Complementation between isolates would be visible as a line of dark pigmented growth where the hyphae of each isolate meet (Hocart *et al.*, 1987). Plates were incubated at 19°C for up to six weeks before a visual assessment of growth was made.

6.2.2 Intraspecific Parasexual Progeny - Assessment of Vegetative Incompatibility Using Spore Inoculum

A sample of recombinants from two intraspecific (W x R) crosses 22-136 (W-type, *his1-1, arg2-3*) x 22-342 (R-type, *ino-1, nia-15*) (cross A) and 22-136 (W-type, *his1-1, arg2-3*) x 23-2/9 (R-type, *met-9*) (cross D) were selected for testing in two separate vegetative incompatibility experiments. Twenty-one cross A progeny were chosen from three fusion product sets (FP 1,2 & 5) carrying a range of auxotrophic markers were included in the first experiment. In addition, two W-type strains, 22-136 (*his1-1, arg2-3*) and 22-404 (*lys2-4, cys3-6*) and two R-type isolates, 22-119/5 (*arg-7*) and 22-228 (*cys-71*) which vary in their auxotrophic requirements were included as control isolates. Details of the relationship of these auxotrophic control strains in terms of the field isolate from which they were derived is presented in Appendix 2.2. Due to the differences in auxotrophic requirements amongst the fusion A progeny, isolates could be tested not only against the control isolates but also against progeny with different auxotrophic requirements (Table 6.1) allowing a degree of between progeny comparisons to be made.

In the second test of vegetative incompatibility twelve progeny from FP4 (D4/63, D4/62, D4/119, D4/64, D4/78, D4/75) and FP5 (D5/97, D5/85, D5/83b, D5/82, D5/100, D5/94) of cross D were tested. These two of the five fusion products recovered from cross D yielded auxotrophic progeny, all carrying the *met*$^-$ (methionine) requirement of the R-type parental strain. Two control isolates were also included: 22-136 (*his1-1, arg2-3*) which was the W-type parent in the cross and had different auxotrophic requirements from the progeny and the R-type strain $23\text{-}2_{8\text{-}3}$ (*met-8*) which was not used in this fusion but was derived from the same field isolate as 23-2/9 and hence was fully vegetatively compatible with it. Due to the lack of differences in auxotrophic requirement amongst the cross D progeny incompatibility interactions could only be carried out between progeny and control isolates.

Table 6.1

Cross A progeny and control isolates to be tested for vegetative incompatibility and the interactions tested. Included are auxotrophic requirements of each isolate where:

A arginine requiring (Arg^-)

C cysteine requiring (Cys^-)

H histidine requiring (His^-)

I Inositol requiring (Ino^-)

N nitrate non-utilising (Nia^-)

and + indicates where complementation was tested between isolates.

Each of the cross A progeny strains was derived from FP1 with the exception of N231 which was derived from FP5.

TABLE 6.1		PARENTAL STRAINS										PARASEXUAL RECOMBINANT PROGENY													
		HA	C	A	LC	HIN	AN	H	A	A	IN	H	A	A	HN	AI	A	A	H	AN	HA	AIN	AN	H	AN
		22~136	22~228	22~119/5	22~404	B27	B28	F84	E78	T357	T356	AI386	W373	A1	C40	D55	D59	U345	G108	J160	J161	W369	J173	T362	N231
HA	22~136																								
C	22~228	+																							
A	22~119/5		+																						
LC	22~404	+		+																					
HIN	B27		+	+	+																				
AN	B28		+	+	+	+																			
H	F84		+	+	+																				
A	E78		+	+	+			+																	
A	T357		+	+	+																				
IN	T356		+	+	+					+															
H	AI386		+	+	+																				
A	W373		+	+	+							+													
A	A1		+		+	+		+			+	+													
HN	C40		+	+	+									+											
AI	D55		+		+	+		+				+			+										
A	D59		+		+	+		+			+	+			+										
A	U345		+		+	+		+			+	+			+										
H	G108		+	+	+									+		+									
AI	J160		+		+			+				+							+						
HA	J161		+		+												+	+							
AIN	W369		+		+			+																	
AN	J173		+		+			+				+							+						
H	T362		+	+	+				+		+		+	+	+	+	+	+		+		+	+		
AN	N231		+	+	+			+				+							+		+			+	
A	P265		+	+	+	+		+			+	+			+				+		+			+	

Interactions between isolates were carried out on minimal media (MM). Twenty microlitre samples of a spore suspension of each isolate (approximately 10^6 spores/ml) were placed in each well of a 25 well compartmented Petri dish containing approximately 3ml of minimal medium per well. In addition to the compatibility testing, isolates were inoculated individually in wells to allow an assessment of growth on MM. Petri dishes were incubated at 19°C for six weeks. A visual assessment of growth was made at both three and six weeks post inoculation. It was expected that where complementation had occurred growth in the wells would be similar to wild type growth on MM as opposed to incompatibility interactions where no or very restricted, hyaline growth was expected.

6.2.3 Assessment of Vegetative Incompatibility Using Complementary Staining

A small sample of W- and R-type field isolates whose vegetative compatibility status was known, (Hocart *et al.*, 1987; Magnano di San Lio *et al.*, 1994), were to be used to develop a third method of vegetative compatibility testing. The isolates to be tested included: 22-8; 23-2; 22-119 (R-type) and 22-20; 22-2 (W-type). Within these isolates it has been found that incompatibility would be present between the two W-type isolates abd also between all possible combinations of the W- and R-type isolates. Within the R-type isolates it was shown that 22-8 and 23-2 are compatible. In addition, limited compatibility was found between 22-119 and 22-8/23-2, but this was found to vary depending on the exact combination of mutants and isolates being tested.

A method for staining conidia with different fluorochromes to enable hyphal interactions between stains to be scored visually was developed. The advantage of such a method is that it avoids the need to first introduce auxotrophic, or other, mutations into the strains to be used in vegetative compatibility tests. The approach was adapted from that of Stewart & Deacon (1995). Spores of each isolate were harvested from tap water agar (TWA) and diluted to give a concentration of approximately 2×10^6 spores ml^{-1}. Spores of each isolate were stained

with each of three fluorochromes: Nile red (50μg ml^{-1}) which is highly specific for neutral lipids and will fluoresce yellow-gold under UV light, carboxyfluorescein diacetate (CFDA, 150μg ml^{-1}) which is hydrolysed in living cells to give carboxyfluorescein, which fluoresces bright yellow-green and Cellufluor (100μg ml^{-1}) which gives a bright blue fluorescence when bound to β1-4 glucans or their derivatives in fungal cell walls (Stewart & Deacon, 1995) . Spores were incubated in stain solutions for approximately 24 hours at 4°C in the dark with continual mixing to ensure an even distribution of the stain. Excess stain was removed from the spores by washing the spores with sterile distilled water (SDW) by centrifugation at 3000*g* for five minutes to obtain a pellet of spores. This process was repeated to ensure the complete removal of all excess stain. The spores were then resuspended in 1ml of sterile distilled water. A sample of each spore suspension was observed microscopically under UV light to determine the intensity of staining of the spores. In addition, control samples of unstained spores were incubated for 24 hours at 4°C in the dark. Twenty microlitre samples of both stained and unstained spores were inoculated onto glass microscope slides coated with a thin layer of minimal medium and incubated at 19°C in Petri dishes containing TWA to maintain a high humidity. Mixtures of spores representing both vegetatively compatible and incompatible combinations of isolates were also inoculated onto the agar slides. A time course study was carried out to assess germination rate and the ability to form compatible interactions of stained spores compared to unstained spores.

6.3 RESULTS

6.3.1 Interspecific Hybrid Recombinants - Hyphal Block Inoculation Assessment of Vegetative Incompatibility

Significant background growth developed from the single pieces of inoculum of each isolate on the MM agar plates as expected with these relatively leaky auxotrophic strains. This growth was hyaline and sparse, characteristic of the growth of the strains on unsupplemented MM. There was no evidence of complementation for any isolate combination in the paired

samples even after 3 weeks. What was noticeable, however, was that the auxotrophic growth of the *P. anguioides* strain 24-6 (*ade-6*) was in the majority of isolate combinations engulfed by the background growth produced by the recombinant strain. In combinations between the R-type strain 22-186 (*lys-5*) and the recombinant progeny the background growth of the two strains was more or less equal, and usually met in the space between the two inoculum plugs. Even so no dark line of pigmented hyphae was observed to indicate complementation and vegetative compatibility. Where the background growth did not meet, or where it was engulfed, i.e. 24-6 was entirely surrounded but not overgrown, it appeared that the two isolates were inhibited from growing too close to one another. No evidence of vegetative compatibility was seen in any strain combination.

6.3.2 Intraspecific Fusion Progeny - Assessment of Vegetative Incompatibility Using Spore Inoculum

No complementation was observed for any of the isolate combinations when spore suspensions were inoculated onto MM contained in 3ml wells. However, it was noticed that for some of the cross D recombinants there was considerably more than just hyaline growth in the wells, suggesting a very leaky auxotrophic requirement. This would suggest that this method of assessment would not be suitable for some of the recombinants and an assessment of the suitability of the auxotrophic requirements of strains would be necessary before carrying out this method in future. Unfortunately at the time of this experiment there was also considerable contamination of the cross A complementation combinations which could therefore not be incubated further to assess the occurrence of vegetative compatibility between strains.

6.3.3 Assessment of Vegetative Incompatibility Using Complementary Staining of Field Isolates

Due to the inability to detect complementation between isolate combinations in the previous two methods and the apparent leaky behaviour of some recombinants it was decided to devise a method of assessing complementation that would not need the presence of auxotrophic requirements in the strains to be tested. This method has not yet been fully developed to allow the routine determination of complementation ability between paired isolates. From the work carried out so far preliminary information has been obtained on the uptake of stains by the fungus. Spores of the test isolates were able to take up each of the stains tested. There also was no apparent reduction in spore germination in comparison to unstained spores. However, due to contamination, primarily by bacteria, the experiment was unable to proceed any further. Therefore, further investigation is needed to develop a system which would keep the inoculations sterile to allow fuller time course observations to assess the suitability of this technique for testing vegetative incompatibility.

6.4 DISCUSSION

Vegetative incompatibility has been demonstrated successfully in a wide range of fungal pathogens using a variety of techniques including: the use of paired blocks of hyphal inoculum of auxotrophic mutants on minimal medium in *P. herpotrichoides* (Magnano di San Lio *et al.*, 1994) and *Verticillium* (Heale, 1966); paired nitrate non-utilizing mutants (Nit^-) in *P. herpotrichoides* (Hocart *et al.*, 1987), *Fusarium oxysporum* (Puhalla, 1983) and *Giberella fujikoroi* (Sidhu, 1985); complementary spore colour mutants in *Fusarium oxysporum* f.sp. *apii* (Puhalla, 1983) and *Aspergillus nidulans* (Grindle, 1963a, b).

In the present work vegetative incompatibility relationships within *P. herpotrichoides*, as demonstrated by Hocart *et al.*, (1987) and Magnano di San Lio *et al.*, (1995), were to be exploited to demonstrate relationships between intraspecific hybrids, interspecific

recombinants and the parental isolates. Firstly, paired blocks of inoculum were inoculated onto MM to assess the segregation of VCG's in the hybrid progeny. However, this experimental design was not successful and although in some instances mixing of hyphae took place there was no obvious complementation and hence hyphal anastomosis. It was therefore decided that an alternative inoculation method such as mixing spore suspensions of test isolates inoculated onto MM, which would use the existing auxotrophic mutants, should be tried to investigate vegetative compatibility within cross A and cross D progeny. It has been previously shown by Heale (1966) that anastomosis commonly occurs between germinating conidia of *Verticillium albo-atrum*. However, what was noticed in the present work was the particularly leaky nature of the auxotrophic fusion D progeny and it perhaps can be concluded that the auxotrophic requirements needed by the hybrid and recombinant progeny used in these experiments were not sufficiently tight as to force the formation of hyphal anastomosis. Alternatively, it may also have been possible that cross feeding occurred between isolates and therefore it would be necessary to test this as well as the suitability of auxotrophic markers for use in future vegetative incompatibility testing.

Although experiments to look at vegetative incompatibility within hybrid and recombinant progeny were unsuccessful, the likelihood of finding any compatibility within such a small sample size is probably small and would ultimately depend on the number of genes and their frequency controlling vegetative incompatibility. Within *Giberrella fujikoroi* Sidhu (1985) estimated that there were nine *het* genes controlling vegetative incompatibility which could generate 512 possible vegetative compatibility groups if segregation occurred at each *het* gene. In the progeny sets used in the current work the parental isolates belonged either to different distinct, and biologically isolated, pathotypes or belonged to different species. It is probably reasonable to assume that these parental strains differed at several *het* loci and consequently this would limit the possibility of finding complementary strain combinations in a small sample size.

It has also been reported that often there are discrepancies between the patterns of vegetative incompatibility found when either auxotrophic or non-utilising mutants are used. Within *P. herpotrichoides* additional complementation between R-type isolates was detected using auxotrophic mutants (Magnano di San Lio *et al.*, 1994) which had not previously been detected when nitrate non-utilizing mutants had been tested (Hocart *et al.*, 1987). This highlights the need to develop a suitable method for testing vegetative incompatibility without the need to induce auxotrophic or non-utilising mutations in all strains.

The success of using vital stains in the staining of fungi for use in time course studies (Stewart & Deacon 1995) and also for complementary staining of nuclei in *Pyricularia oryzae* and *Colletotrichum lagenarium* (Butt *et al.*, 1989) has been demonstrated previously. Therefore the possibility exists to use counter staining of *P. herpotrichoides* spores to assess vegetative compatibility within this fungus. Although experiments were still at a very preliminary stage the success of uptake of the stains and no obvious reduction in spore viability would indicate that it would be worth further developing this technique. Although no obvious reduction in spore viability was reported here or by Stewart & Deacon (1995) it has been reported that there may be more subtle effects such as the disruption of the chitin cytoskeleton by Calcofluor (Jackson & Heath 1990), or an alteration of the normal assembly of chitin microfibrils in the cell walls (Elorza *et al.*, 1983; Roberts *et al.*, 1987), which could have an effect on physiological responses or molecular recognition responses by the fungi thereby affecting the formation of anastomosis and the patterns of vegetative incompatibility between isolates. These effects would need to be determined by comparing the vegetative compatibility relationships of isolates using a range of staining methods.

It is clear from the experiments carried out in this present work that further investigation is required to characterise vegetative incompatibility within *P. herpotrichoides*. Although first

impressions are that a system needs to be developed which does not rely solely on the use of either auxotrophic or non-utilising mutants, it is clear that a variety of techniques should be used to assess the suitability of each before relying on only one method.

7 GENERAL DISCUSSION

The results presented in this thesis provide additional evidence for the parasexual cycle following protoplast fusion in the eyespot fungus and in particular that viable recombinant progeny can be generated from crosses between the two main pathotypes of *P. herpotrichoides*. In addition, evidence for the interspecific hybridization of *P. herpotrichoides* and *P. anguioides* is presented. This aspect of the work has been published.

The thesis further describes the use of the parasexual cycle to analyse the inheritance of EBI fungicide sensitivity and host species specificity in this fungus. Resistance to EBI fungicides was shown to be controlled by several genes with differing effects on resistance level and showing differences in their pattern of segregation. One or more major genes were seen to segregate in the early stages of the parasexual cycle and additional genes with a lesser effect on resistance level segregated subsequently, after exposure of the fusion products to haploidizing agents.

Pathogenicity to wheat and rye was largely disrupted following protoplast fusion and recombination within eyespot. However, sufficient numbers of pathogenic recombinants were obtained to show that W- and R-type pathogenic ability could be separated and also that they could be inherited separately.

Fusion products formed following protoplast fusion in *P. herpotrichoides* are presumed to be relatively stable diploids. However, from the evidence presented in the current work and that previously published (Hocart *et al.*, 1993b; Magnano di San Lio *et al.*, 1994) it is difficult to confirm the status of these fusion products. Fusion products of *P. herpotrichoides* do not sporulate, and unlike other species where a measurement of spore cell volume of conidia produced by the diploid has shown a significant increase in comparison to the haploid parents (Fincham & Day, 1971; Pontecorvo, 1956) this cannot be demonstrated in eyespot. At the other extreme, it is found in *Cephalosporium acremonium* that recombinant haploids are

isolated directly upon selection of the fusion product (Hamlyn & Ball, 1979). What is likely to be occurring in *P. herpotrichoides* would be an intermediate between these two extremes. Pre-segregation of markers has been shown to occur in fusion products, but the ability remains for further segregation to occur, with markers appearing that were not previously seen e.g. changes in morphology and auxotrophy.

Parasexual recombination within eyespot is limited in fully investigating the inheritance of markers in that to date random segregation with the production of all possible combinations of parental markers has not been demonstrated. Sexual recombination, which is possible between the W, C and S pathotypes, should allow independent assortment of markers and could allow for a better investigation of the inheritance of marker differences between these pathotypes. To date, however, sexual recombination between W and R pathotypes and between different *Pseudocercosporella* species has not been possible. These intraspecific crosses were shown here to be relatively easy to carry out between different W- and R-type isolates. Interspecific crosses between *P. herpotrichoides* and *P. anguioides* are also possible (Hocart & McNaughton, 1994). Therefore parasexual recombination is still the only possible mechanism available for investigating differences between the two major pathotypes of the fungus and also different species.

Fungicide resistance within eyespot was shown here to be controlled multigenically. A similar conclusion was reached by Julian *et al.* (1994) looking at induced levels of resistance within eyespot. This involvement of several genes in the control of fungicide resistance has also been shown for other fungal species. For example, Sanoamung *et al.* (1993) demonstrated that resistance inheritance in *Monilinia fructicola* was conferred by a mutation in a single gene but was affected by modifying genes. This is also confirmed by Ishii *et al.* (1993) who demonstrated a single major gene controlling fenarimol resistance in *Venturia inaequalis* but suggested that additional subtle genetic methods may modify the level of resistance expression. Cross-resistance was shown to be extensive within eyespot between the majority of the demethylation inhibiting fungicides tested. This pattern of cross-resistance was found when the

triadimenol sensitive and resistant progeny subsets of cross A were tested together. Cross-resistance could also be detected when the sensitive and resistant progeny subsets were tested individually, although the same pattern of cross-resistance was not found as above for either subset. Additional patterns of cross-resistance were also detected within the sensitive and resistant progeny subsets that were not found previously, when the whole data was analyzed. Here it was found that cross-resistance was detected within the sensitive subset between the morpholines, tridemorph and fenpropimorph, and some of the triazoles tested. Within the resistant subset, cross-resistance was found between prochloraz and a few triazoles. This could have implications on population structure and future control of the disease. The use of non-target fungicides, such as the morpholines, may select for that portion of the eyespot population i.e. W-type (sensitive) or R-type (resistant), to which cross-resistance was detected. In addition, it may be found that continued use of prochloraz for the control of eyespot may select for R-type isolates, which showed resistance to the other demethylation inhibiting fungicides.

Generally it was found that pathogenicity was disrupted in the recombinant progeny. The possibility exists that protoplast production, fusion and recovery of recombinants was responsible for this effect. However, in the glasshouse assessment of pathogenicity of interspecific hybrids, isolate CT, which is equivalent to the R-type parent 22-228, is found to be pathogenic. This isolate was recovered after protoplast isolation, PEG treatment and exposure to haploidizing agents and provides a control strain to asses the effect of these treatments on pathogenicity. The validity of the methods of assessing pathogenicity could be questioned in that variable and unpredictable responses were achieved with the control isolates. In a further assessment of pathogenicity of intraspecific recombinants which used pathogen infested straw collars to inoculate the host species, good levels of infection were achieved for the control isolates. The recombinant progeny, however, were still found to give variable levels of infection (Ritchie *et al.*, unpublished data). Therefore, it is reasonable to assume that the methods of testing pathogenicity were not completely inappropriate. The standard method of assessing pathogenicity i.e. visual assessment of symptoms eight weeks after inoculation, provide considerable limitations on the extent of the analysis that can be carried out. Not only

are the experiments fairly time consuming but the number of strains and host species/cultivars which can be screened at any one time are also restricted. The results presented here indicate that a visual assessment of pathogenicity is not sufficient as it was possible to find hyphal growth and production of typical infection structures on non-symptomatic plant-strain interactions. A better approach may be to combine an assay of colonization of the host tissues by ELISA or quantitative PCR with a visual assessment of symptom expression.

Analysis of the segregation of vegetative compatibility groups in recombinant progeny could prove invaluable in the investigation of the number of het alleles involved in vegetative incompatibility within eyespot. The number of het loci found helps to indicate the degree of relatedness of clonal species and also estimating the extent of sexual reproduction in the population. Limitations are found within the first two methods of evaluating vegetative incompatibility in the current work as both rely on the presence on nutritional markers within strains to be tested. However, Magnano di San Lio *et al.* (1994) revealed that different patterns of vegetative compatibility were possible within eyespot depending whether nitrate non-utilizing or auxotrophic mutants were used. The potential value in developing the counter staining of spores to estimate the occurrence of hyphal anastomosis and hence vegetative incompatibility may prove to be invaluable as it avoids the need to first induce markers in the strains to be tested.

There are a few areas which would be worth further investigation to develop the current work. It would be interesting to see whether the pre-segregation occurring in the fusion products could be manipulated to increase the number of segregant types recovered in the progeny. This would allow a fuller analysis of genetic control of important characters. Fungicide resistance could be further investigated by not only assessing sensitivity of additional progeny from the crosses already studied but also by analysing progeny from additional crosses.The dose response work could be extended to look for the presence of modifying 'minor' genes. Pathogenicity testing of additional progeny from each of the crosses could be carried out using

both visual and microscopic assessment to further investigate the differences between pathogenic and parasitic ability displayed in recombinant progeny.

APPENDIX 2.1

Origin of field isolates of *P. herpotrichoides*, *P. anguioides* and *P. aestiva* used throughout the current work.

Isolate Number	Other Numbers	Origin	Type
22-1	S1 (MD 9)	ADAS (Harpenden)	W-type
22-2	SD (PBI 265)	""	W-type
22-8		wheat 'Avalon' (1)	R-type
22-12		wheat 'Rapier' (0)	R-type
22-20	C71/8	NIAB, Cockle Park (wheat)	W-type
22-21	C71/67	PBI, Cambridge (oats)	W-type
22-119	PCB85/382/2	ADAS (Bristol)	R-type
23-1	64002	Manchester University	*P. aestiva*
24-1		FBC Ltd Chesterford Park	*P. anguioides*

APPENDIX 2.2

Isolation of 22-20 (W-type) mutants

22-20 W-type

22-108 *his*1-1

22-135 *his*1-1, *cys*3-6

22-136 *his*1-1, *arg*2-3

22-402 *his*1-1, *lys*2-2, Ben^r -17

22-409 *his*1-1, *lys*2-4, *cys*3-6, Ben^r -17

22-29 Ben^r-17

22-127 Ben^r-17, *lys*2-4

24-9 Ben^r -21

22-138 Ben^r-21, *his*3-3

22-311 *bio*1-1

22-311/1 *bio*1-1, *ima*-1

22-311/11 *bio*1-1, *ima*-1, *sel*-2

22-311/12 *bio*1-1, *ima*-1, *sel*-3

22-311/13 *bio*1-1, *ima*-1, *sel*-7

22-311/2 *bio*1-1, *ima*-2

22-311/21 *bio*1-1, *ima*-2, *sel*-8

22-311/22 *bio*1-1, *ima*-2, *sel*-11

22-312 *bio*1-2

APPENDIX 2.2

Isolation of mutants of *P. herpotrichoides*, W and R types, and *P. anguioides*

22-12 R-type

22-186 *lys*-5

22-228 *cys*-71

22-1 W-type

22-1/4 *glu*-1, *arg*-6

22-1/7 *glu*-1, *gly*-2

22-2 W-type

22-2/1 Ben^r-1, *met*-5 (cys)

23-2 R-type

23-2/8 *met*-8 (Thr)

23-2/9 *met*-9

24-1 *P.anguioides*

24-9 *nic*-7

APPENDIX 3.1 Recipes for stains tested in determining isozyme phenotypes.

Glucose Phosphate Isomerase (GPI)

Tris-HCL 0.1 M pH7.5	100 ml
$MgCl_2.6H_2O$ (1 M)	1 ml
Fructose-6-P (Na)	80 mg
$NADP^+$	20 mg
MTT	20 mg
PMS	4 mg
Glucose-6-P dehydrogenase	20 units

Mannose Phosphate Isomerase (MPI)

Tris-HCL 0.2 M pH7.5	10 ml 1M plus 40 ml distilled water
D-mannose-6-phosphate	20 mg
pyruvate	20 mg
NAD^+	10 mg
$NADP^+$	5 mg
glucose phosphate isomerase	10 units
glucose phosphate dehydrogenase	17 units
MTT	10 mg
PMS	3 mg

Esterase

Na H_2PO_4 . $2H_2O$	0.1 M pH6.2	(10 ml 1 M plus 90 ml H_2O)
Na_2HPO_4 (anhydrous)		
α-naphthyl acetate	30 mg dissolved in acetone	
fast blue RR salt	100 mg	

Malate Dehydrogenase (MDH)

Tris-HCl 0.1M pH7.5 (10 ml 1 M stock)

DL-malate 0.5 M pH7.5 (see below for preparation)

β-NAD^+ 30 mg

MTT 20 mg

PMS 4 mg

Make up to 100 ml with H_2O, keep out of light by screening flask and staining box with foil.

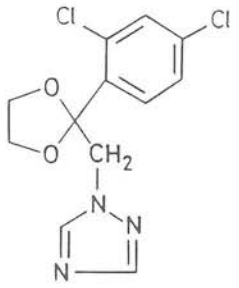
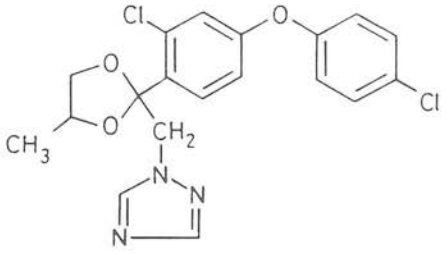
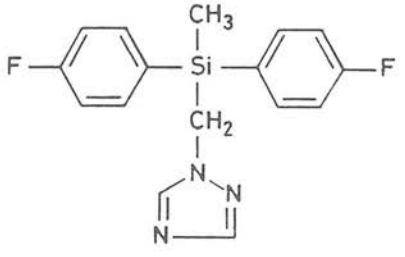
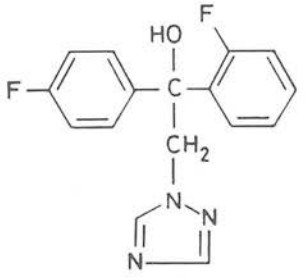
0.5 M DL-malate

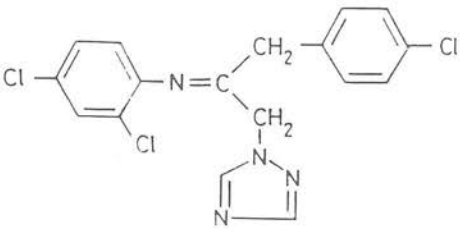
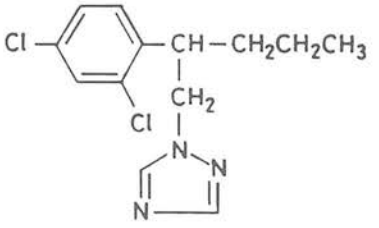
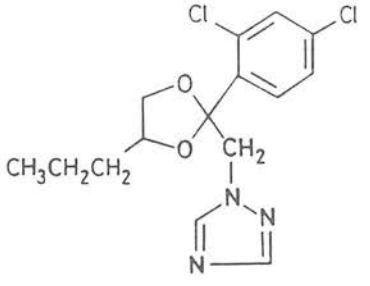
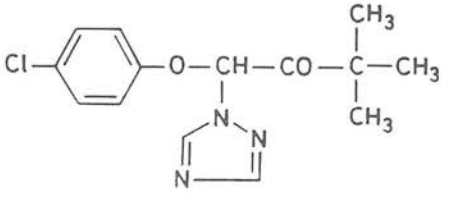
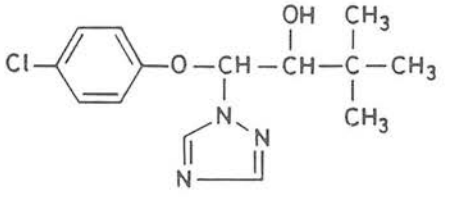
Add 5.36 g malic acid to ~70 ml H_2O and gradually add solid sodium hydrogen carbonate to bring pH up to 7.5.

Glutamate Dehydrogenase (GDH)

Tris-HCl 0.1 M pH7.5	10 ml
MTT	10 mg
PMS	8 mg
NADP	10 mg
0.5 M malate pH7.5	10 ml
H_2O	80 ml

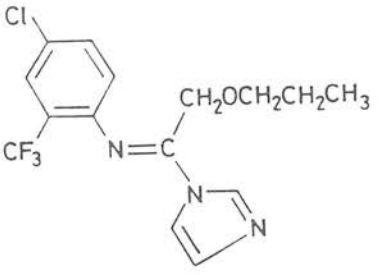
APPENDIX 4.1 Molecular weight, chemical strucutre and supplying company of the EBI fungicides tested in dose response experiments.

Fungicide and Molecular Weight	Chemical Structure	Supplying Company
Azaconazole 300.1		Janssen Pharmaceutical
Difenconazole 406.3		Ciba-Geigy
Flusilazole 315.4		EI du pont de Nemours & Co
Flutriafol 301.3		ICI Agrochemicals

Imibenconazole 411.7		Hokko Chemical Ind. Co. Ltd
Penconazole 284.2		Ciba-Geigy AG
Propiconazole 342.2		Ciba-Geigy AG
Triadimefon 293.8		Bayer AG
Triadimenol 295.8		Bayer AG

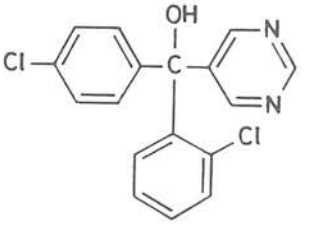
Triflumizole

345.7



Fenarimol

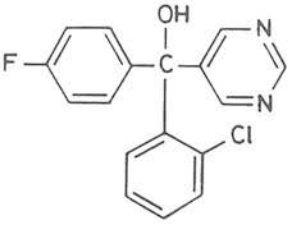
331.2



Dow Elanco

Nuarimol

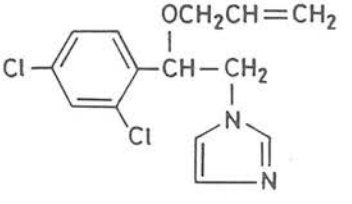
314.7



Dow Elanco

Imazalil

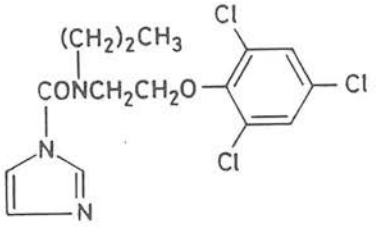
297.2



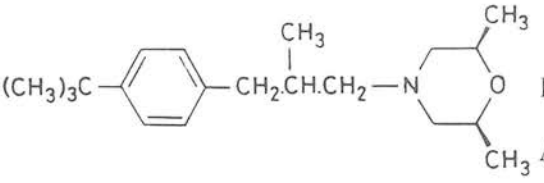
Janssen Pharmaceutical

Prochloraz

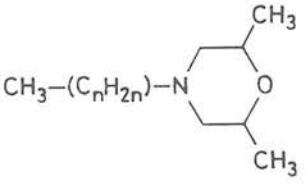
376.6



Schering Agrochemicals Ltd

Fenpropimorph  BASF Rhone-Poulenc

303.5 Agrochemie

Tridemorph  BASF

297.5

Appendix 4.2 Mean Ed50 data for Cross A progeny for each of the EBI fungicides tested.

	22-20	S312	F89	F90	E75	H119	C41	Q285	AE591	AE583	Z449a	P265	N231	O251	N238	22~8
Azaconazole	0.4	0.4	0.7	0.6	0.8	1.6	1.2	16.7	16.6	36.2	8.9	19.5	12.6	156.0	149.1	20.7
Difenconazole	0.2	0.1	0.1	0.1	0.2	0.1	0.2	2.1	1.2	1.1	1.6	0.8	0.5	7.5	2.0	3.18/
Flusilazole	0.5	0.4	0.4	0.5	0.7	0.6	0.9	4.0	2.9	2.1	8.5	3.4	1.2	4.5	4.6	2.3
Flutriafol	0.0	0.1	0.1	0.0	0.1	0.2	0.8	1.9	0.2	0.2	0.8	0.5	0.1	1.7	0.9	0.3
Imibenconazole	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.9	9.9	11.2	3.2	12.2	4.0	148.8	86.7	8.7
Penconazole	0.0	0.1	0.1	0.1	0.9	0.1	0.1	2.9	3.0	4.9	2.1	3.3	1.5	22.6	17.6	5.0
Propiconazole	0.2	0.2	0.5	0.3	0.3	0.1	0.8	1.7	1.1	1.2	1.0	1.2	1.0	3.5	2.3	1.3
Triadimefon	12.4	12.4	13.7	12.5	13.8	15.3	41.8	71.0	387.6	977.7	36.8	320.2	257.0	1500	1436	343.7
Triadimenol	2.0	1.0	1.0	3.0	4.0	5.0	7.0	21.0	83.0	248.0	13.0	68.0	72.0	782.0	1300	393.0
Triflumizole	0.5	0.5	0.6	0.5	1.0	0.8	0.9	1.4	8.6	9.1	1.5	13.0	5.2	58.9	120.3	12.9
Fenarimol	0.9	1.5	1.0	1.3	1.9	2.1	4.2	14.0	4.5	2.8	3.1	6.2	3.4	12.3	8.3	5.4
Nuarimol	1.1	2.1	1.6	1.8	2.5	3.6	8.1	15.6	3.6	3.0	5.4	6.5	2.8	13.1	10.8	4.3
Imazalil	0.1	0.1	0.2	0.1	0.2	0.3	0.2	1.1	0.2	0.4	0.6	0.3	0.2	1.9	0.9	0.5
Prochloraz	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1
Fenpropimorph	9.8	7.1	8.2	12.2	11.3	1.7	46.6	0.1	8.4	11.8	0.4	4.9	4.9	18.5	24.0	63.0
Tridemorph	1.5	2.8	0.9	1.4	4.1	0.5	3.3	0.8	3.0	2.9	0.5	1.0	1.7	88.1	50.7	49.2

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Interspecific hybridisation between *Pseudocercosporella herpotrichoides* and *P. anguioides* achieved through protoplast fusion

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The relatedness of the cereal eyespot pathogen, *Pseudocercosporella herpotrichoides*, to the weakly pathogenic species *P. anguioides* was investigated using protoplast fusion to produce somatic hybrids. Crosses between *P. anguioides* and strains representing the two main pathotypes of *P. herpotrichoides* (W and R) were conducted. Procedures for protoplast isolation and regeneration developed for *P. herpotrichoides* were successfully used, without modification, with *P. anguioides*. PEG-induced fusion of protoplasts, isolated from auxotrophic mutants of *P. herpotrichoides* and *P. anguioides*, generated prototrophic fusion products on minimal regeneration medium at low frequency (0·00016% — 0·0016%). Subculture of fusion products on non-selective media generated spontaneous segregants with a range of morphological phenotypes. Characterization of parental nutritional and isozyme markers revealed novel marker combinations, confirming that genetic recombination between the species had occurred, presumably by parasexual processes following karyogamy. Both pathotypes of *P. herpotrichoides* appeared able to hybridize with *P. anguioides* under these conditions. Marker segregation was not random, suggesting structural differences in the genomes of the two species of the expression of genomic incompatibility. Determination of spore sizes of species and hybrids showed that the recombinants formed conidia intermediate in length between the parental species. Mean cell length of conidia remained relatively constant at 9·7 ± 0·2 μm (standard error) per cell, with longer spores containing more cell compartments. No evidence to indicate that the hybrid recombinants were diploid was obtained from the spore analysis and these strains remained stable after exposure to the haplodizing agent carbendazim. Clear evidence for the segregation of pathogenicity to wheat cv. Beaver was obtained from a 9 wk pathogenicity test with a range of hybrids and parental isolates. *P. anguioides* was only weakly pathogenic to wheat, whereas both pathotypes of *P. herpotrichoides* caused severe lesion development. Interspecific hybrids varied in pathogenicity. Some were non-pathogenic, like the *P. anguioides* parent, while others gave infection levels comparable to the *P. herpotrichoides* R-type parent. Pathogenicity was not impaired by the presence of the cysteine auxotrophic marker of the R-type. The successful recovery of interspecific hybrids indicates that these two species are relatively closely related.

Interspecific hybridization of fungi has been used for a number of purposes. In taxonomic studies the use of hybrids has allowed estimation of the degree of relatedness of species (Kevei & Peberdy, 1984; Sipiczki, 1987), and can help to establish the taxonomic status of species within a genus (Kawchuk, Kim & Nielsen, 1988; Trail & Mills, 1990). Hybridization has also been used to introduce desirable characteristics from related fungi into commercially important species such as edible mushrooms (Wu, 1990; Yang *et al.*, 1990) or fungi used in food processing (Reymond & Fevre, 1986; Ushijima *et al.*, 1990). Similarly, strain improvement through hybridization has been used to improve the performance of biological control fungi by combining characteristics of both species (Stasz *et al.*, 1987, 1989). Corner & Poulter (1989) used hybridization between *Candida tropicalis* and *C. albicans* to test for allelism of adenine auxotrophic mutants in the development of a transformation selection system.

Interspecific hybrids can occasionally be obtained through sexual mating with the formation of hybrid sexual progeny. In plant pathogenic fungi sexual hybrids were used in taxonomic studies to analyse species relationships in *Tilletia* (Trial & Mills, 1990), while sexual hybridization between *Pyrenophora graminea* and *P. teres* allowed genetic characterization of components of pathogenicity and symptom development (Smedegaard-Petersen, 1983). The number of fungi which may be hybridized in this way is small, being limited by incompatibility barriers to sexual crossing or the absence of a perfect stage in the species of interest.

The use of protoplast fusion artificially to initiate heterokaryosis and stimulate interspecific recombination via the parasexual cycle has proved to be a fruitful method for hybrid formation in fungi. Protoplast fusion can overcome many of the non-sexual incompatibility barriers which preclude normal cell fusion between species (Croft, 1985). Taxonomic relationships can be analysed by this approach. Determination of fusion frequency, the nature of subsequent parasexual events and the extent of genetic recombination between the two genomes, allow an estimate of relatedness and similarity of genome organization in the two species. This approach was

used by Typas (1984) in conjunction with microinjection of nuclei, to obtain hybrids between the plant pathogens *Verticillium albo-atrum* and *V. dahliae*. The extent of recombination between these two species was used as evidence of similarity between their genomes.

The aim of the present work was to investigate the relatedness of the cereal eyespot pathogen *Pseudocercosporella herpotrichoides* (Fron) Deighton and the weakly pathogenic *Pseudocercosporella anguioides* Nirenberg. Both species are found associated with cereal stem bases. *P. anguioides* is not an economically important species but its presence in cereal crops is thought to represent a possible source of confusion when assessing crops for the presence of eyespot disease (Bateman, 1988). The research had three objectives: (1) to assess whether protoplast fusion could be used to generate interspecific hybrids: (2) to compare the nature of any subsequent parasexual events with those observed previously in intraspecific fusions (Hocart *et al.*, 1993*a*, *b*) and (3) to assess the pathogenicity of hybrids to wheat plants.

MATERIALS AND METHODS

Strains

Two isolates of *Pseudocercosporella herpotrichoides* and one of *P. anguioides* were used in fusion experiments. Auxotrophic mutants derived from these strains allowed selection of hybrid fusion products on a defined minimal medium. All auxotrophic strains were generated by uv mutagenesis of conidial suspensions (Hocart, 1987; Hocart *et al.*, 1993*a*). Strain 22-138 (*his3-3*, *BEN-21R*), a carbendazim resistant, histidine auxotroph, was produced from the *P. herpotrichoides* W-type isolate 22-20, used in previous studies (NIAB, Northumberland, No. C71/8; Hocart *et al.*, 1987, 1989, 1990). Strain 22-228 (*cys-71*), requiring cysteine, was obtained from the *P. herpotrichoides* R-type strain 22-12 originally isolated from wheat cv. Rapier (Hocart, 1987; Hocart *et al.*, 1989) and strain 24-9 (*nic-7*), a nicotinic acid requiring auxotroph, was produced from the *P. anguioides* isolate 24-1, originally from Germany (Nirenberg, 1981) and supplied by Schering Agrochemicals Ltd, Chesterford Park Research Station, Saffron Walden, Essex.

Additional strains used for comparison in the assessment of pathogenicity were *P. herpotrichoides*: W-type 22-5, isolated from wheat cv. Rapier (Hocart, 1987); R-type 22-119 originally from ADAS, Bristol (Strain PCB85/382/2) and *P. anguioides*: isolate 24-12, supplied by Schering Agrochemicals Ltd (Julian & Lucas, 1990).

Media

Complete (CM) and minimal (MM) media for routine maintenance and characterization of auxotrophs have been described previously (Hocart *et al.*, 1987, 1993*a*, *b*). Spores were produced using tap water agar (TWA; 20 g l^{-1} Davis standard agar in tap water) and incubation at 12 °C under continuous near uv light (black light). Mycelium for protoplast isolation was grown in shake culture using CM prepared without agar. Media for protoplast regeneration (CMR, MMR) were identical to the standard complete and minimal media except that 0·6 M sucrose was used as both osmoticant and carbon source in place of glucose. Mycelium for isozyme analysis was grown in an enriched form of complete medium containing malt extract (7·5 g l^{-1}), yeast extract (2·5 g l^{-1}), glucose (20 g l^{-1}) and casaminoacids (0·5 g l^{-1}), adjusted to pH 6·5 with NaOH. Unless otherwise stated all cultures were grown at 19°.

Protoplast isolation, fusion, regeneration and viability

Protoplasts were obtained from 42 h old shake culture grown mycelium of *P. herpotrichoides* following treatment with a lytic enzyme solution containing three commercial enzyme preparations: driselase, cellulase CP and rhozyme HP150N (10 mg ml^{-1} each, dissolved in 50 mM sodium maleate buffer, pH 5·8, containing 0·4 M-$MgCl_2$ as the osmotic stabilizer) (Hocart *et al.*, 1987). Protoplasts were collected after 3 h digestion by filtration through sinter glass (porosity 1), followed by slow centrifugation (10 min at 3000 ***g***: 1000 rpm). The same procedure was used without modification to generate protoplasts from *P. anguioides*.

Fusion of protoplasts was performed as follows: protoplasts of the two parental strains (10^7 protoplasts of each strain) were mixed, centrifuged for 10 min at 3000 ***g*** and the pellet resuspended in 2 ml polyethylene glycol solution (30% PEG, Sigma Chemical Co., MW 8000; 50 mM glycine, 10 mM-$CaCl_2$; pH 7·5). The suspension was left for 10 min at room temperature, then diluted with 6 ml 0·4 M-$MgCl_2$ and the protoplasts recovered by centrifugation as before. After removal of the supernatant, the protoplasts were resuspended in 2 ml 0·4 M-$MgCl_2$ and appropriate dilutions plated on complete (CMR) and minimal (MMR) regeneration media. Fusion frequency (%) was calculated as the ratio of colonies obtained on MMR compared with CMR.

Control platings on CMR of protoplasts of the parental strains, with and without PEG treatment, were made where protoplast numbers allowed. In addition, for the cross of 22-138 with 24-9, non-PEG treated mixtures of protoplasts were plated on MMR to estimate the frequency of spontaneous hybridization. The level of contamination of the protoplast preparations by conidia was determined by plating protoplasts, lysed by resuspension in sterile distilled water, on complete medium (CM). Regeneration frequencies were adjusted to take spore contamination into account. Regeneration frequencies and their standard errors were calculated using arcsine transformed data ($X = \sin^{-1}\sqrt{[P]}$) for the proportion [P] of regenerating protoplasts and the mean value back-transformed to obtain the percentage regeneration.

Recovery of recombinants

Mycelium from prototrophic fusion products was macerated by crushing small pieces of the colony against the inside of an Eppendorf tube with the tip of a sterile Pasteur pipette and suspended in 1 ml sterile distilled water. Drops of the suspension were plated on CM and MM. The growth and appearance of the colonies on the two media was visually assessed after 28 d. This procedure was repeated two further

times using mycelium taken from the preceding series of purification plates. Mycelial macerates were also plated on CM containing the mitotic inhibitors carbendazim (10 μM) or methyl-3,5-dichlorophenyl carbamate (MDPC, 50 μM) in an attempt to induced segregation of haploid recombinants and assess the mitotic stability of the fusion products.

Single spore isolations were used to recover genetically homogeneous colonies. Spores were produced by plating hyphal macerates on tap water agar (TWA) containing nicotinic acid (10 μg ml^{-1}) and either histidine or cysteine (50 μg ml^{-1}). After incubation at 12° under continuous near UV light for 28 d, spore suspensions were prepared by shaking a small piece of agar (approximately 2 cm^2) in 1–2 ml of sterile distilled water. Spore numbers were assessed using a counting chamber and diluted suspensions plated on CM to obtain single colonies. Colonies failing to show signs of sporulation on TWA were subcultured by transfer of hyphal tips onto CM. Finally, the colonies were characterized for auxotrophic phenotypes by transfer of mycelium to MM and MM supplemented with either histidine, nicotinic acid or cysteine.

Isozyme analysis

Protein extracts for isozyme analysis were prepared using the protocol developed for *P. herpotrichoides* by Julian & Lucas (1990) as modified by Hocart *et al.* (1993*b*). Soluble proteins were separated by polyacrylamide gel electrophoresis as described previously (Hocart *et al.*, 1993*b*). Enzyme activities for malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), glucose phosphate isomerase (GPI) and mannose phosphate isomerase (MPI) were detected using the staining procedures described by Tanksley and Orton (1983). Esterase (EST) activity was detected using the method of Brewbaker *et al.* (1968); 'black' and 'red' esterases were distinguished using a mixture of α- and β-naphthyl acetates in the staining solution.

Spore size determination

Spore length and cell number measurements were made for a sample of hybrid progeny and their parental isolates. Additional W and R type *P. herpotrichoides* strains were included for comparison. Spores were produced by plating hyphae on TWA containing appropriate nutritional supplements. Plates were incubated at 19° in the dark for the first 48 h, then transferred to 12° with continuous near uv illumination for 21 d. Single pieces of agar were cut from the plates, mounted on glass slides and viewed under a light microscope. Spore lengths were measured using an eyepiece graticule and at least fifty spores were measured for each strain. Conidia were fixed with 70% ethanol and stained with the cell wall specific fluorescent brightener Uvitex MST (0·01%) and the DNA-specific fluorochrome DAPI (0·1%) to allow an estimation of cell number and nuclear content. Differences in spore length were compared by analysis of variance using untransformed data. Cell number data were transformed for analysis using Cochran's (1938) square root transformation for small whole numbers ($X' = \sqrt{[X+0{\cdot}5]}$).

Pathogenicity assessment

The pathogenicity to wheat of a sample of the interspecific hybrids was assessed. Wheat seeds, cv. Beaver, were sown in 12·5 cm pots filled to within 2 cm of the rim with peat based compost. Fungal inoculum consisted of 5 mm filter paper disks (Whatman No. 1) infested with mycelium. These were produced by growing the strains for 3 wk in dilute liquid CM (10 ml $\frac{1}{5}$ normal concentration liquid CM in 90 mm Petri dishes) containing 30 to 40 sterile paper disks. At 3 wk of age the plants were inoculated with parental and recombinant strains of the pathogens by placing single infested filter paper disks against the stem base and holding them in position with paper collars placed over the plant. Collars consisted of 1 cm squares of filter paper with a 5 mm hole in the centre.

After inoculation the pots were filled with vermiculite to maintain a high humidity around the stem base. Plants were grown for 9 wk in an unheated glasshouse and watered from below using capillary mating to avoid cross contamination between pots. The experiment was conducted between 8 March and 10 June 1993 at the Bush Estate, Roslin, near Edinburgh. The air temperature in the glasshouse during that period was measured with a thermohygrograph placed next to the plants. Mean temperature (±standard error) during the experiment was 12·5 ± 0·4°, with average daily maxima and minima of 22·7 ± 0·8° and 7·8 ± 0·3°, respectively. Each pot contained eight plants and three pots were used for each strain. The position of pots on the glasshouse bench was assigned randomly. Two series of pots, inoculated with sterile filter paper disks were included as controls. Both the original isolates and the parental auxotrophic strains were tested. Additional W- (22-5) and R-type (22-119) *P. herpotrichoides* isolates and a second *P. anguioides* isolate (24-12) were included for comparison.

Assessment of pathogenicity was made using the infection scoring system described by Scott (1971). Uninfected plants scored 0; coleoptile infected, 1; coleoptile penetrated, 2; first leaf sheath infected, 3; first leaf sheath penetrated, 4; second leaf sheath infected, 5; and so on. Data were analysed by analysis of variance (ANOVA) and using the Kruskal–Wallis one-way ANOVA by ranks (Siegel & Castellan, 1988), to avoid problems associated with unequal variances among means. Both analyses gave essentially the same result so the data are shown with standard deviations and standard errors calculated from the parametric ANOVA.

RESULTS

Protoplast isolation from *P. anguioides* using the protocol developed for *P. herpotrichoides* was highly successful. In excess of 5×10^7 protoplasts were obtained from approximately 500 mg fresh weight of mycelium. A similar yield was obtained for the W-type *P. herpotrichoides* strain 22-138. Overall protoplast numbers from the R-type strain, 22-228, were lower ($1{\cdot}1 \times 10^7$ protoplasts in total) due to the poorer growth of this strain in liquid culture, which gave a lower yield of mycelium for protoplast isolation.

Protoplast viability before PEG treatment was relatively high, falling between 14·8% for the R-type strain and 25·4%

Table 1. Protoplast regeneration and fusion data: (*a*) Protoplast regeneration of parental strains on complete (CMR) regeneration media before and after PEG treatment. Regeneration frequencies shown exclude colonies derived from contaminating spores. (*b*) Fusion frequency for interspecific crosses between *P. herpotrichoides* (W-Type, 22-138; R-Type, 22-228) and *P. anguioides* (A, 24-9). Fusion mixtures contained 10^7 protoplasts in each case. Arcsin transformed data ± standard error based on 'n' replicates in table

	Medium	n	%	Arcsin ± S.E.	%	Arcsin ± S.E.	%	Arcsin ± S.E.
(*a*) Parental regeneration data			22-138 W-type		24-9 *P. anguioides* (A)		22-228 R-type	
Spore contamination	—	3	0	0·0 ± 0·0	0·002	0·2 ± 0·1	7·4	15·7 ± 0·2
Control viability	—	4	25·4	30·3 ± 1·6	20·3	26·8 ± 1·4	14·8	22·6 ± 2·4
PEG-treated control	—	3	4·7	12·5 ± 0·04	3·6	10·9 ± 0·6	nd	nd
(*b*) Fusion data			22-138 × 24-9 W × A		22-138 × 24-9 W × A		22-228 × 24-9 R × A	
Untreated mixture	CMR	5	20·3	26·8 ± 1·1	18·0	25·1 ± 0·5	nd	nd
	MMR	5	0	0·0 ± 0·0	0	0·0 ± 0·0	nd	nd
PEG-treated fusion	CMR	5	5·6	13·6 ± 0·3	4·0	11·6 ± 0·5	2·6	9·2 ± 0·6
	MMR	8	$2{\cdot}33 \times 10^{-5}$	0·028 ± 0·0012	$6{\cdot}25 \times 10^{-6}$	0·014 ± 0·007	$4{\cdot}04 \times 10^{-5}$	0·036 ± 0·009
Fusion frequency (%)			$4{\cdot}16 \times 10^{-4}$		$1{\cdot}56 \times 10^{-4}$		$1{\cdot}56 \times 10^{-3}$	

nd = not determined; MMR = minimal regeneration medium.

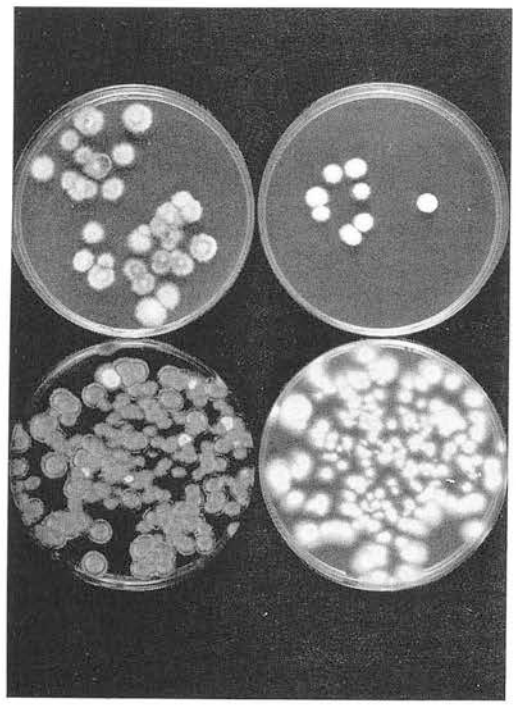

Fig. 1. Segregant colonies produced following protoplast fusion of *Pseudocercosporella anguioides* with the *P. herpotrichoides* W-type strain 22-138. (A) colonies derived from fusion product (FP) i, (B) colonies derived from FPii, (C) colonies derived from FPiii, (D) *P. anguioides*.

for the W-type strain (Table 1). PEG treatment reduced percentage regeneration, presumably by causing aggregation of protoplasts into clumps. Sufficient protoplasts of strains 22-138 and 24-9 were obtained to allow two separate fusions to be performed. Plating unfused mixtures of these two strains did not produce any prototrophic products, confirming the normal vegetative incompatibility between these two species and also demonstrating the absence of spontaneous reversion to prototrophy in either strain. Following PEG treatment of the fusion mixture a small number of putative hybrid fusion products were obtained on MMR. Comparison of colony numbers on MMR and CMR gave a fusion frequency of less than 5×10^{-4}% for the cross between the W-type *P. herpotrichoides* with *P. anguioides* and $1{\cdot}56 \times 10^{-3}$% between the R-type and *P. anguioides*.

W-type P. herpotrichoides *(22-138)* × P. anguioides *(24-9)*

Nine putative fusion products were obtained from this cross. Only three of these fusion products gave non-parental type colonies on sub-culture; the remainder generated auxotrophic colonies with an appearance typical of the *P. anguioides* parent. In the subsequent maceration steps the three novel fusion products continued to generate material which was distinct from either of the parental strains (Fig. 1). Stable, homogeneous phenotypes were recovered from these colonies spontaneously and, in each case, only one main colony type was obtained. Exposure to carbendazim or MDPC did not increase the range of colony types or speed up the recovery of stable progeny. The first of the fusion products (FPi) produced dark pigmented, prototrophic colonies on MM, and mid-grey colonies on CM. The second (FPii) formed stable, white, prototrophic colonies. The progeny from the third fusion product (FPiii) were all carbendazim sensitive, nicotinic acid auxotrophs, like the *P. anguioides* parent but differed in their pigmentation. On CM the colonies were chestnut brown and exuded a dark brown, soluble pigment into the surrounding agar.

R-type P. herpotrichoides *(22-228)* × P. anguioides *(24-9)*

Seven putative fusion products were obtained between the R-type strain and *P. anguioides*. Four of these, when macerated and plated on CM and MM, gave predominantly parental type colonies. On MM the sparse hyaline growth typical of 22-228 (Cys-) was occasionally associated with a denser grey mycelium at the colony centre. Subculture of this central region on CM generated only parental type colonies. The segregation into parental types, and the very restricted

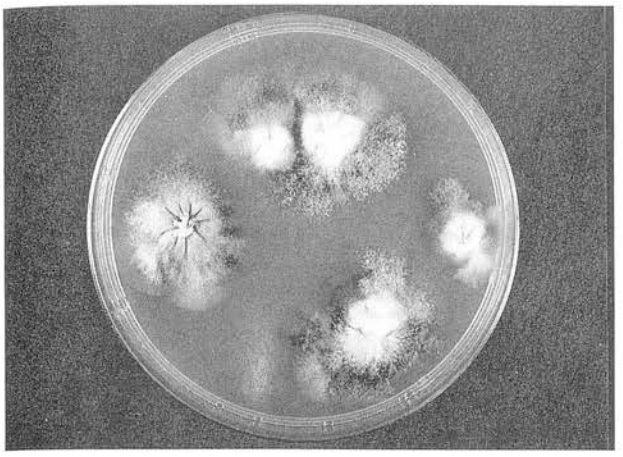

Fig. 2. Fusion product FPt formed between *P. anguioides* and *P. herpotrichoides* R-type strain 22-228, showing spontaneous sector formation on non-selective, complete medium. Photographed approximately 21 d after inoculation.

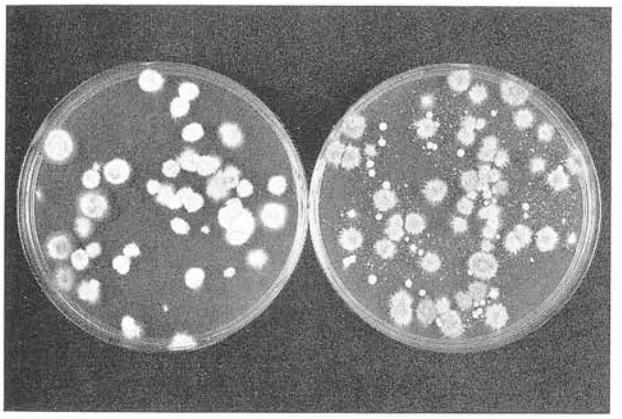

Fig. 3. Segregation of different morphological variants from fusion products FPc and FPe, formed between *P. anguioides* and *P. herpotrichoides* R-type strain 22-228, on non-selective medium. Plates photographed after 21 d incubation.

Table 2. Auxotrophic phenotypes of mitotically stable single spore and hyphal tip progeny derived from three independent fusion products from the cross of 22-228 (*P. herpotrichoides*, R-type; *cys-71*) and 24-9 (*P. anguioides*; *nic-7*)

Fusion product	Number of progeny colonies tested	Progeny phenotypes			
		Cys-	Nic-	Cys-, Nic-	Prototrophic
FPt	122	100	0	0	22
FPc	7	3	4	0	0
FPe	18	16	0	0	2

prototrophic growth of these colonies are consistent with the interpretation that they resulted from cross-feeding rather than genetic complementation.

Three of the original fusion products generated non-parental colonies; one (FPt) produced unstable prototrophic colonies, which sectored spontaneously on both CM and MM (Fig. 2). On CM the pigmentation of these sectors was seen to vary considerably, ranging from buff to chestnut and mouse grey (Anon., 1969). The two remaining fusion products (FPc & FPe) formed slow growing, unstable colonies on CM (Fig. 3), which resembled the 'aneuploid' stages observed in intraspecific parasexual crosses in *P. herpotrichoides* (Hocart *et al.*, 1993*a*).

Marker characterization of progeny from these three fusion products indicated a bias in favour of the auxotrophic marker of the R-type *P. herpotrichoides* parent (*cys-71*). More than 80% of the progeny from the first fusion product (FPt) were cysteine auxotrophs, while none showed the nicotinic acid requirement of the *P. anguioides* parent (Table 2). Prototrophic progeny were recovered from two of the fusion products (FPt & FPe), while both parental markers were recognized in progeny from FPc. The use of CM rather than MM during the previous subcultures did not appear to have had any clear effect on progeny phenotype.

Isozyme analysis

Analysis of isozyme phenotypes confirmed the hybrid status of progeny derived from the fusion of the R-type *P. herpotrichoides* and *P. anguioides* (Table 3). Segregation of isozyme phenotypes for MDH and GPI was observed in progeny derived from FPt. Esterase banding patterns in many of the progeny also showed that hybridization had taken place, with bands characteristic of both parents being readily distinguishable in the recombinants.

Esterase band identification was assisted by recognition of black and red esterases. For example, possible confusion between EST100 of W-type *P. herpotrichoides* and EST102 of *P. anguioides* was avoided, since the former is a black esterase while the latter stains red when the staining solution contains a mixture of α- and β-naphthyl acetates.

The hybrid status of progeny recovered from the cross between the W-type *P. herpotrichoides* (22-138) strain and *P. anguioides* was also revealed by isozyme analysis. Both progeny tested showed a mixture of the parental isozyme forms. Novel banding patterns were observed for MDH and GDH in one of the two recombinants tested. The esterase banding pattern of these strains most closely resembled the *P. anguioides* parent, except for the presence of a typical W-type esterase band EST127 in place of the *P. anguioides* band EST129 in one of the recombinants, and the presence of a pair of W-type bands (EST162 & EST164) in both progeny.

Spore size

The size and cell number of conidia from seven recombinants was measured, six derived from the cross of *P. anguioides* with the *P. herpotrichoides* R-type and one from the cross with the W-type. In all cases, cell compartments were uninucleate and there were no measurable differences in spore width. The mean spore lengths of the hybrids ranged between those of the two parental strains (Fig. 4). The hybrids produced spores that were significantly longer than those of *P. herpotrichoides* but shorter than *P. anguioides*; significant differences in spore length were also found between hybrids. Spore measurements for the parental *P. herpotrichoides* and *P. anguioides* were comparable with those published in the literature (Deighton, 1973; Nirenberg, 1981). Conidial cell number averaged 5·7

Table 3. Isozyme phenotypes of interspecific hybrids produced by protoplast fusion between *P. anguioides* (A) and R- and W-types of *P. herpotrichoides*

	Fusion product	Strain no.	Auxotrophic markers	Isozyme phenotype			
				MDH	GDH	GPI	MPI
Parental strains							
P. herpotrichoides (R-type)		22-228	*cys71*	R	R	R	R
P. anguioides		24-9	*nic-7*	A	A	A	A
P. herpotrichoides (W-type)		22-138	*his3-3*	W	W	W	W
Recombinants							
22-228 × 24-9	FPt	BU1	*cys-71*	R	R	R	R
		BU2	*cys-71*	R	R	R	R
		BH	*cys-71*	R	R	A	R
		AM	*cys-71*	R	R	R	R
		FW	*cys-71*	A	R	R	R
		GD	*cys-71*	R	R	R	R
		AY	*cys-71*	R	R	R	R
		BD	*cys-71*	R	R	A	R
		EQ	*cys-71*	R	R	R	R
		EO	Prototroph	R	R	R	R
		FP	Prototroph	R	R	A	R
		AJ	Prototroph	R	R	—	R
		AK	*cys-71*	R	—	R	R
		FD	Prototroph	R	R	R	R
		FH	Prototroph	R	R	R	R
	FPc	DM	*cys-71*	R	R	R	R
	FPe	DQ	Prototroph	R	R	R	R
22-138 × 24-9	FPiii	CF2	*nic-7*	Novel	Novel	A	W
		CA	*nic-7*	W	A	A	A

	Esterase															
	62	80	84	85	92	100	102	107	116	127	129	137	142	148	162	164
Parental strains																
P. herpotrichoides (R-type)	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
P. anguioides	.	.	+	+	+	.	+	+	.	.	+	.	.	+	.	.
P. herpotrichoides (W-type)	.	.	.	.	.	+	.	.	.	+	.	+	.	.	+	+
Recombinants																
22-228 × 24-9	+	+	.	.	.	.	+	.	+	.	.	.	+	.	.	.
	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
	+	+	.	.	.	.	+	.	+	.	.	.	+	.	.	.
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	+	+	.	+	+	.	.	.	+	.	+	.	+	.	.	.
	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
	+	+	+	+	.	.	.	+	+	.	.	.	+	.	.	.
	+	+	+	+	.	.	.	.	+	.	+	.	+	.	.	.
	+	+	+	+	+	.	.	.	+	.	.	.	+	.	.	.
	+	+	+	+	.	.	.	.	+	.	.	.	+	.	.	.
	+	+	+	+	+	.	+	+	+	.	.	.	+	.	.	.
	+	+	.	+	+	.	.	.	+	.	.	.	+	.	.	.
	+	+	.	.	.	.	.	.	+	.	.	.	+	.	.	.
	+	+	.	+	.	.	.	.	+	.	+	.	+	.	.	.
22-138 × 24-9	.	.	.	+	+	.	+	+	.	+	.	.	.	.	+	+
	.	.	.	+	+	.	+	+	.	.	+	.	.	+	+	+

cells spore^{-1} for *P. herpotrichoides* isolates (5 strains) and 11·1 cells spore^{-1} for the isolate of *P. anguioides* (Table 4); while that of the recombinants ranged from 5·8 to 9·7 cells spore^{-1}. The average length of each conidial cell, obtained by dividing total spore length by cell number, remained constant (mean $\pm$ standard error = 9·7 $\pm$ 0·2 μm) regardless of the spore length. Consequently, longer spores contain more, not larger, cells. No evidence for diploidy was obtained since there was no detectable increase in cell size to indicate a doubling in cell volume.

Pathogenicity assessment

Lesion development was generally advanced with many of the plants, particularly those inoculated with the *P. herpotrichoides* isolates, showing typical eye shaped lesions. Mean

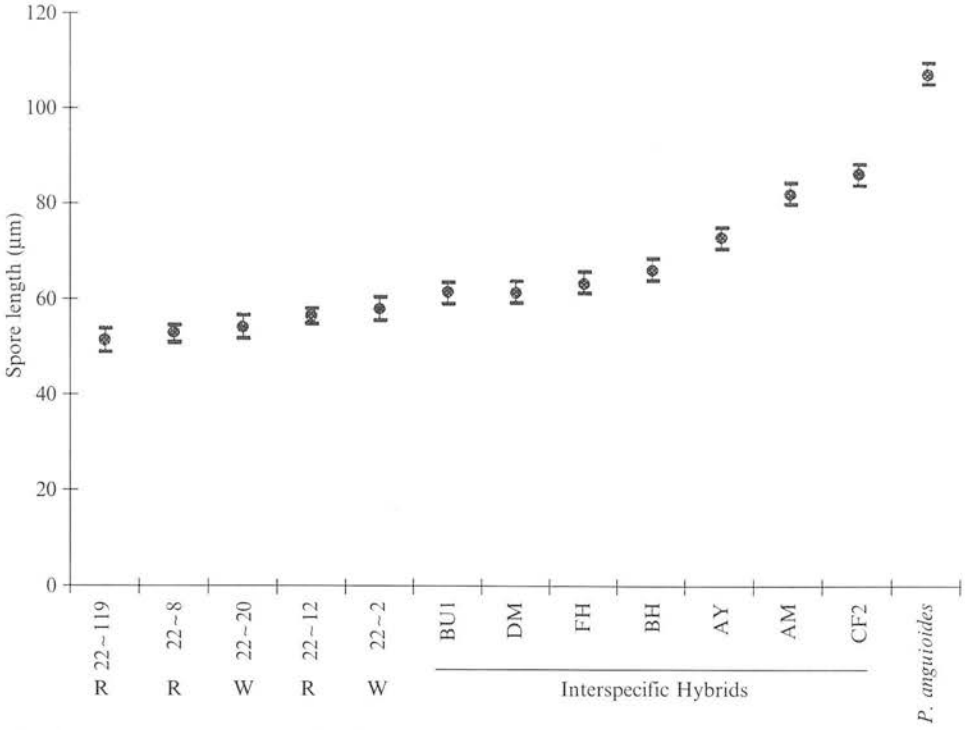

Fig. 4. Mean length (μm) of conidia produced by *P. herpotrichoides*, *P. anguioides* and a number of their somatic hybrids. The W and R pathotype isolates of *P. herpotrichoides* used in the crosses are represented by strains 22-20 and 22-12 respectively. The error bars shown represent the 95% confidence intervals for the means.

Table 4. Number of cells in conidia produced by *P. herpotrichoides*, *P. anguioides* and the interspecific hybrids produced by protoplast fusion between *P. anguioides* and either R-type (BU1, DM, FH, BH, AY, AM) or W-type (CF2) *P. herpotrichoides*. Standard errors were calculated after first transforming cell data using Cochran's (1938) square root transformation for small whole numbers

Species	Pathotype	Strain no.	Cells/ spore†	Square root data		Sample size	Length/cell (μm)
				Mean	S.E.		
P. herpotrichoides	R	22-119	5·8	2·504	0·040	58	8·9
P. herpotrichoides	R	22-8	5·4	2·422	0·027	50	9·8
P. herpotrichoides	W	22-20	5·9	2·527	0·029	56	9·2
P. herpotrichoides	R	22-12	6·3	2·607	0·038	58	9·0
P. herpotrichoides	W	22-2	6·4	2·627	0·038	60	9·1
Hybrid	R × A	BU1	5·8	2·506	0·029	58	10·7
Hybrid	R × A	DM	6·1	2·562	0·027	57	10·3
Hybrid	R × A	FH	6·7	2·683	0·045	58	9·6
Hybrid	R × A	BH	6·9	2·722	0·055	30	9·7
Hybrid	R × A	AM	8·4	2·978	0·040	60	9·9
Hybrid	W × A	CF2	9·7	3·199	0·044	58	9·0
P. anguioides	A	24-1	11·1	3·399	0·052	58	9·8

† Cell number/spore values obtained by back transformation of mean square root data.

infection scores, with their associated standard deviations and standard errors, are shown in Fig. 5. Significant differences were detected between strains ($P < 0{\cdot}05$). *P. herpotrichoides* isolates were all pathogenic to wheat, with the W-types showing somewhat greater infection scores than the R-types tested. *P. anguioides* was less pathogenic, with only the second isolate (24-12) showing mean infection significantly greater than the uninoculated control pots ($P < 0{\cdot}05$). This finding is consistent with the description of this species as a weak pathogen (Bateman, 1988).

The cysteine auxotrophy of 22-228 did not impair the pathogenicity of this strain, which was as pathogenic as the original R-type isolate 22-12. Presumably the method of inoculation, using precolonized filter paper disks, provided an adequate source of cysteine to enable the fungus to infect the host plant. The auxotrophic *P. anguioides* strain (24-9) did not produce symptoms on any of the plants. Interspecific recombinants showed a range of pathogenic abilities. The single recombinant tested from the cross of *P. anguioides* and the W-type *P. herpotrichoides* (CF2), which carried the Nic-marker of strain 24-9, was non-pathogenic. Recombinants derived from the cross between *P. herpotrichoides* R-type and *P. anguioides* varied in their ability to infect and induce disease symptoms. This variation was not dependent on the presence

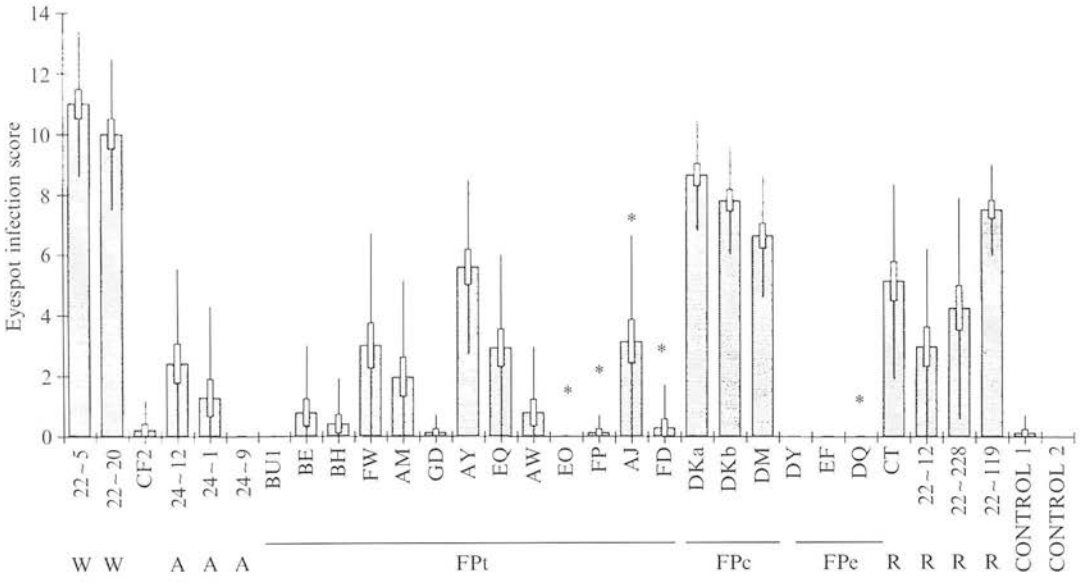

Fig. 5. Pathogenicity to wheat cv. Beaver of *P. herpotrichoides* W- and R-types, *P. anguioides* (A) and the interspecific hybrids obtained between them (W × A: strain CF2; R × A; strains BU1 to DQ inclusive). Two copies of strain DK and two uninoculated controls were included. Hybrids between R-type and *P. anguioides* were derived from three separate fusion products (FPt, FPc & FPe). Infection was scored using the system devised by Scott (1971). Columns represent mean infection scores with standard errors (open boxes) and standard deviations (solid lines) based on a sample size of 24 in each case. Prototrophic recombinants are indicated by an asterisk.

or absence of the Cys-auxotrophic marker. Six Cys-strains (FW, AY, EQ, AJ, DK and DM) gave infection scores significantly greater ($P < 0{\cdot}05$) than the control and comparable with the R-type parent 22-228. Only one (AJ) of the five prototrophic recombinants (EO, FP, AJ, FD and DQ) was significantly pathogenic. The fusion product from which the recombinants derived did appear to influence the pathogenic ability of the progeny. Both recombinants (DK, DM) derived from fusion product c (FPc) tested were highly pathogenic whereas those from FPe (DY, EF, DQ) were non-pathogenic. The recombinants obtained from FPt included both pathogenic and weakly pathogenic strains. Strain CT was included as an extra control. This strain is identical to strain 22-228 but was exposed to all the treatments given to the recombinants, including protoplast isolation and regeneration and exposure to the haploidizing agent carbendazim. The pathogenicity of this strain was unimpaired by these treatments and was no different to the R-type parent.

DISCUSSION

Genetic recombination between species has been interpreted as indicating taxonomic relatedness (Ferenczy, 1981, 1984; Toyama *et al.*, 1984; Typas, 1984; Park *et al.*, 1986). The extent of recombination achieved, measured by the range of progeny phenotypes recovered and the segregation of markers, allows an estimate of the similarity between the genomes of the two species. Closely related species will share much organizational similarity in their linkage groups and, consequently, a relatively high degree of segregation would be expected (Croft & Dales, 1983; Kevei & Peberdy, 1984). This paper provides evidence for parasexual genetic recombination between *Pseudocercosporella herpotrichoides* and *P. anguioides*. Hybrid progeny were obtained following protoplast fusion which revealed novel combinations of nutritional and/or isozyme markers. In addition, conidial size of the hybrids, a morphological characteristic distinguishing the species, appeared intermediate between those of the parental species.

The most extensive evidence for interspecific hybridization was obtained from the cross of *P. anguioides* with an R-type pathogenicity *P. herpotrichoides* strain. Three separate fusion products from this cross generated hybrid progeny. While no recombinants were recovered which combined the auxotrophic requirements of both parents, a number of the progeny from two of the original fusion products showed stable prototrophic growth. Segregation for isozyme markers was seen for both MDH and GPI, while esterase banding patterns for nine of the seventeen progeny showed bands characteristic of both parents. Attempts to cross *P. anguioides* with a W-type strain of *P. herpotrichoides* also generated colonies with novel morphological phenotypes. While phenotypic characterization was completed for only two hybrid recombinants, isozyme analysis was able to confirm the hybrid nature of these progeny. The appearance of novel isozyme banding patterns in interspecific progeny following protoplast fusion has also been seen in *Aspergillus* (Kelman, Varga & Kevei, 1991).

Fusion frequency, calculated from the ratio of colonies obtained on minimal and complete regeneration media, was low for interspecific hybridization compared with that recorded previously in intraspecific fusion experiments (Hocart *et al.*, 1993*b*). Fusion of auxotrophic mutants derived from W and R-type strains of *P. herpotrichoides* generated prototrophic fusion products at a frequency of between 0·002% and 0·009%. Fusion frequencies obtained here for interspecific fusions were 1·3 to 22 fold lower. The estimate of fusion

frequency in the present work is likely to be an overestimate of the actual frequency, since between one half and two-thirds of the putative fusion products obtained generated only parental type colonies on subculture. In view of the vitamin requirement of the *P. anguioides* parent strain and the very restricted prototrophic growth exhibited by these colonies it seems reasonable to conclude that a proportion of the original putative fusion products resulted from cross-feeding rather than cell fusion.

Segregation of fusion products took place spontaneously. In the cross between *P. anguioides* and the W-type *P. herpotrichoides* this segregation appeared to take place rapidly, since no sectoring colonies were seen and the first plating from fusion products onto non-selective complete medium generated apparently stable segregants. Segregation was slower with the cross of *P. anguioides* and the R-type *P. herpotrichoides* strain. Putative diploid colonies were recovered which broke down spontaneously, forming variously pigmented sectors which were stable on subculture. No additional segregation was seen following exposure to the haploidizing agent carbendazim, suggesting that the progeny recovered were mitotically stable and not diploid. This observation is supported by the spore size analysis, where no evidence was found for the increased volume of conidial cell compartments usually associated with diploidy in other species (Clutterbuck, 1974; Hastie, 1981).

Segregation of parental markers was not random but showed a bias in favour of one of the parental genotypes. For example more than 80% of the progeny from fusion product (FPt) from the cross of *P. anguioides* with the R-type *P. herpotrichoides*, showed the cysteine requirement of the *P. herpotrichoides* parent, the remainder were prototrophic and none showed the nicotinic acid requirement of the *P. anguioides* parent. Non-random segregation patterns are a common feature of interspecific crosses and presumably reflect the presence of structural differences between the genomes of the two parent species or the expression of incompatibility in the heterozygous hybrid nucleus (Bradshaw *et al.*, 1983; Croft & Dales, 1983). Alternatively, the mutagenic treatments involving uv radiation used to generate the original auxotrophic markers may have resulted in structural genome differences through uv-induced translocations in one or both parents (Käfer, 1977). The use of spontaneous mutant strains in repeat fusion experiments would allow discrimination between these alternatives. Similar non-random patterns of marker segregation have been found in interpathotype crosses within *P. herpotrichoides* (Hocart & McNaughton, unpublished). In interspecific crosses in *Pseudocercosporella* the recovery of a range of hybrid segregants clearly indicates a degree of similarity in the genomes of these two species.

Interspecific protoplast fusion in other species has generated novel phenotypes through non-parasexual processes (Croft & Dales, 1983; Harman & Stasz, 1991). Cybrids resulting from the transfer or recombination of cytoplasmic genetic factors between species have been shown to generate progeny with novel phenotypes, which may involve the altered expression of nuclear genes. Transfer of mitochondrial genomes in interspecific crosses in *Aspergillus* was common with a high probability of genetic recombination of the mitochondrial DNA, even in the absence of selection (Croft & Dales, 1983). Transfer of the mitochondrial genome was apparently restricted to crosses between species that could also show nuclear hybridization. No attempt was made in the present work to characterize the mitochondrial phenotype of the parents or hybrids, so the cytoplasmic genotype of the hybrids is unknown. However, recent work by Nicholson *et al.* (1993) indicates some homology in the mitochondrial DNA of the two species. The recombination data presented in the present work support the suggestion that *P. herpotrichoides* and *P. anguioides* are reasonably closely related.

Assessment of the pathogenicity of the two species confirmed the status of *P. anguioides* as a weak pathogen of wheat, whereas both W- and R-type *P. herpotrichoides* were highly pathogenic. The determination of pathogenicity of the hybrids revealed variation for this characteristic among recombinants. Differences in pathogenic ability were not correlated with auxotrophic phenotype, at least with respect to the Cys-phenotype of the R-type strain 22-228; nor were prototrophic recombinants automatically pathogenic. The loss or reduction in pathogenicity, typically associated with the introduction of auxotrophic markers into pathogens, was not apparent in this experiment, presumably due to the nature of the inoculation procedure. Similarly, some auxotrophic mutants of *Verticillium albo-atrum* were shown to be equally as pathogenic as the wild-type strains (Clarkson & Heale, 1985), while pathogenicity of auxotrophic mutants of *Venturia inaequalis* was restored by the addition of the appropriate exogenous amino acids or vitamins (Wood, 1967). In *Pseudocercosporella* the difference in pathogenic ability between the two parental species was clearly heritable. The successful recovery of interspecific hybrids of *P. herpotrichoides* and *P. anguioides*, showing variation for pathogenic ability, will greatly assist the characterization of features important in determining pathogenicity in this genus.

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