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AFLP markers for the study of somatic recombination in Phytophthora infestans

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AFLP markers for the study of somatic recombination in *Phytophthora infestans*

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Abstract

Somatic recombination is the exchange of nuclei or cytoplasm between two fungal isolates without the need for any specialised sexual structures and its detection requires the use of phenotypic or nuclear markers. Previous studies using virulence or drug-resistant markers suggested that somatic recombination occurred between some isolates of *P. infestans*, but recombinants could not be identified from mutants without molecular markers. The aims of the study were to evaluate the usefulness of AFLPs for detecting variation in *P. infestans* and then use the AFLP technique to identify somatic recombination in self-fertile (A1/A2) and self-sterile (A1/A1) pairings.

The ability of Restriction Fragment Length Polymorphisms (RFLPs) (probe RG57) and Amplified Fragment Length Polymorphisms (AFLPs) to detect polymorphisms in *Phytophthora infestans* was compared. Ninety-eight isolates, representing 35 RG57 fingerprint patterns and mitochondrial haplotypes Ia and IIa were fingerprinted for AFLPs. UPGMA analysis of AFLP and RG57 fingerprints suggested that sexual reproduction may be uncommon or isolated in *P. infestans* populations in the UK as isolates of A2 mating type or mitochondrial haplotype IIa had similar fingerprints. Some isolates with the same RG57 fingerprint had quite different AFLP fingerprints, suggesting convergence of the RG57 fingerprint.

Somatic recombination was studied using various approaches. Asexual progeny from three self-fertile isolates (one synthesised in the lab with known parents, the others isolated from the field) were investigated. No evidence was obtained in support of somatic recombination in the synthesised self-fertile using the RG57 probe; the 180 hyphal-tips and single-sporangia isolated from one self-fertile all had the fingerprint pattern of one parent. Twenty-six of the 92 progeny fingerprinted for AFLPs had non-parental AFLP fingerprints. This and further AFLP variation from two field self-fertiles could have been the result of mitotic crossing-over or somatic recombination.

Forty-three pairings were constructed (mainly A1xA1) with different drug-resistant markers (metalaxyl, streptomycin, geneticin or hygromycin). Progeny from two pairings exhibited a combination of parental phenotypes but these were unstable and quickly lost their ability to grow on double-drug amended media. Protoplast fusion between drug-resistant strains yielded some hyphal-tip or single-sporangial lines able to grow on double-drug amended media. Single-zoospores isolated from these strains displayed the double-drug phenotype and also contained AFLP bands from both parents, suggesting that karyogamy had occurred in these pairings.

Co-cultivating two A1 isolates on leaves, allowed single-sporangia to be selected with race phenotypes appearing as a combination of the two A1 strains. AFLP analysis of these strains suggested that these apparent "recombinants" were possibly the result of mutation in one of the parental isolates.

The AFLP technique proved to be useful in identifying somatic recombinants in protoplast fusions. The lack of definite evidence for somatic recombination in other pairings is discussed.

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Definitions

AFLP	Amplified Fragment Length Polymorphism
AF	Genotype derived from AFLP DNA fingerprint
Cluster	Group of isolates with similar DNA fingerprints
DNA	Deoxyribose Nucelic Acid
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
Heterokaryon	Mycelium containing nuclei with different genotypes
Heteroplasmon	Mycelium containing mitochondria with different genotypes
Homokaryon	Mycelium containing identical nuclei
Locus	Position of a gene or DNA marker on a chromosome
MT	Mating Type
mtDNA	Mitochondrial DNA
Polymorphism	DNA marker that is present in some and absent in other individuals
RF	Genotype derived from RG57 DNA fingerprint
RGR	Relative Growth Rate
RNA	Ribose Nucleic Acid
UPGMA	Unweighted Paired Group with Arithmetic Averages

1 Introduction

1.1 THE GENUS *PHYTOPHTHORA*

The genus *Phytophthora* is closely related to the genus *Pythium*. Both genera are classified in the family Pythiaceae and order Pythiales within the class Oomycota (Erwin & Ribeiro, 1996). There are currently 64 morphological species of *Phytophthora*. Most are plant pathogens responsible for some of the world's most destructive diseases of crops and natural vegetation.

Biologically, members of the Oomycota have unique physiological and biochemical properties. Their cell walls are composed of β-glucans (cellulose-like polymers) instead of the chitin present in the walls of true fungi (Sietsma *et al.*, 1969; Bartnicki-Garcia & Hemmes, 1974) and they require sterols for sexual reproduction (Hendrix, 1970; Elliott & Glen, 1981; Ko, 1982). They also store β-glucans such as mycolaminarin as their principal carbohydrate reserve (Bartnicki-Garcia & Hemmes, 1974). The flagellar rootlet structure, mitotic apparatus and cytochrome systems resemble those of plants and animals rather than those of true fungi (reviewed in Erwin & Ribeiro, 1996). *Phytophthora* and other oomycetous fungi have a diploid life cycle (Figure 1.1) (Gallegly & Galindo, 1958; Sansome, 1963 & 1966; Sansome & Brasier, 1973; Shaw & Khaki, 1971; Sansome, 1977; Shaw, 1983a) unlike true fungi which have a zygotic meiosis and thus a haploid life cycle (Shaw, 1991). Ribosomal RNA sequence data (Förster *et al.*, 1990) suggest that oomycetous fungi represent a monophyletic group that evolved along different lines to ascomycetes and basidiomycetes, probably from heterokont photosynthetic algae (Gunderson *et al.*, 1987; Förster *et al.*, 1990). Indeed, recent classification schemes place the Oomycota in the kingdom Chromista (Hawksworth *et al.*, 1995; Erwin & Ribeiro, 1996). Although members of the Oomycota are closely related to the golden algae, they superficially resemble fungi, and are thus referred to here and elsewhere as such.

1.1.1 Life history of *Phytophthora infestans*

Phytophthora infestans causes the late-blight disease of potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*). In the UK, £13 M is spent annually on fungicide applications to control the disease (Anon, 1995), mainly on potatoes. In the USA, $100 M was spent in efforts to suppress the disease in 1994, but even so, losses

amounted to a further $100 M (Fry & Goodwin, 1995; Daly, 1996). *P. infestans* shows a high degree of biotrophy and host specificity with successful infections mainly limited to potato and tomato, although up to 100 species of host are known to support growth of the fungus when leaves are inoculated in laboratory experiments (Erwin & Ribeiro, 1996). Infections typically begin when sporangia land on host tissue and release motile biflagellate uni-nucleate zoospores that encyst after contact with the leaf. A germ tube from either a sporangium or a zoospore, forms an appressorium and a penetration peg emerges, which pierces the cuticle and penetrates the epidermal cell. This marks the beginning of the biotrophic stage of the disease cycle. Hyphae can then grow into the mesophyll cell layers both intra- and intercellularly and occasionally, haustoria are formed. After 3-4 days, *P. infestans* starts to grow saprophytically in the necrotised centre of the growing lesion. Sporangiophores emerge through the stomata and new sporangia are produced on their tips. These can be deposited in the soil or water and can also be wind-dispersed for tens of kilometres (Fry *et al.*, 1992). This is known as the asexual cycle (Figure 1.1) and is responsible for the rapid spread of the fungus in epidemics.

P. infestans has a well defined sexual cycle (Figure 1.1) which requires an interaction between two parental strains of different mating type, designated A1 and A2, for sexually produced oospores to form (Smoot *et al.*, 1958; Galindo & Gallegly, 1960; Savage *et al.*, 1968; Shaw, 1983a; Brasier, 1992). Isolates can act as the male or female donor or both, depending on the relative sexuality of the other isolate (Galindo & Gallegly, 1960; Judelson, 1997b). Oospores are thick-walled resting structures that can overwinter in the field for at least one year (Pittis & Shattock, 1994; Drenth *et al.*, 1995) and may also act as a primary source of inoculum. In sexual matings, the large size of the oogonium means that the female parent contributes a large volume of cytoplasm, which seems to be inherited uni-parentally (Whittaker *et al.*, 1994).

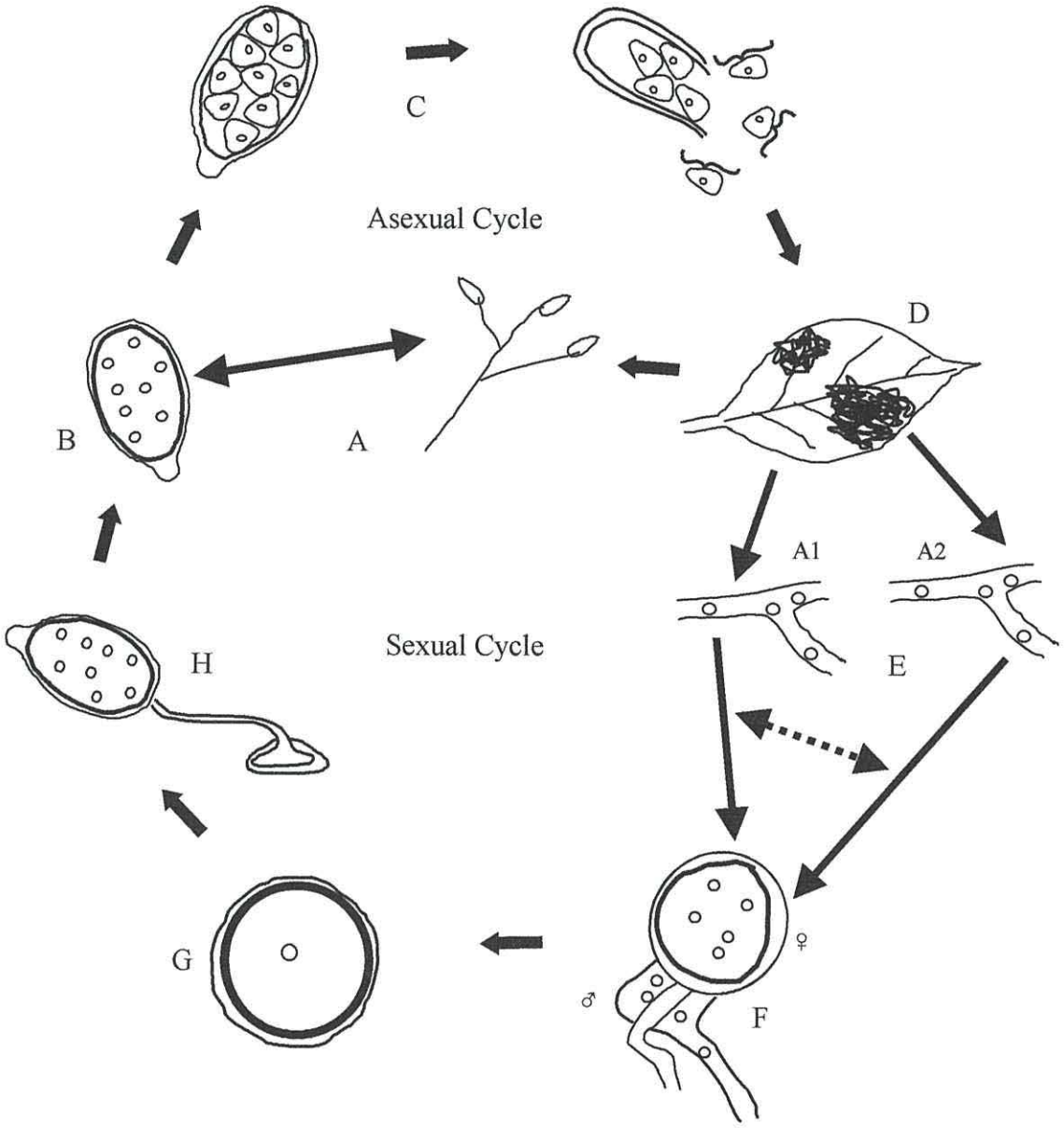


Figure 1.1. Diagramatical representation of the asexual and sexual cycles of *P. infestans* (not to scale). A: Diploid coenocytic mycelium of A1 or A2 mating type. B: Asexual diploid multinucleate sporangium. May germinate directly or produce zoospores. C: Diploid uninucleate biflagellate zoospores, motile and without a cell wall. D: zoospores infect foliage, lesion grows radially and produces more sporangia. When A1 and A2 hyphae make contact sexual reproduction can occur. E: Production of mating hormones (Ko, 1978 & 1988) stimulates beginning of sexual reproduction. F: Formation and association of antheridium (♂) and oogonium (♀). Amphigynous antheridium penetrated by developing oogonium, followed by mitosis, nuclear abortion and meiosis. Migration of haploid male gametic nucleus into the oogonium from antheridium. G: Karyogamy results in a diploid zygotic nucleus followed by development of thick spore wall of the oospore. H: Germination of oospore often through development of germ sporangium. Based on Judelson (1997a).

1.2 MARKERS USEFUL FOR THE STUDY OF *P. INFESTANS*

Numerous phenotypic and molecular markers are available to study the biology of *P. infestans*.

1.2.1 Mating type

P. infestans has a mating system that is representative of many heterothallic oomycetous fungi (Michelmore & Sansome, 1982; Shaw, 1991; Brasier, 1992). The two mating types, A1 and A2, represent compatibility types rather than dimorphic sexual forms (Galindo & Gallegly, 1960; Judelson *et al.*, 1995) as they differ in the production of a mating-specific (α) hormone (Ko, 1978 & 1988). Each mating type is bisexual but self-incompatible; responses of each mating type to the hormone of the opposite type are similar. They include suppression of aerial mycelium and sporangia *in vitro* as well as the formation of both antheridia and oogonia in the sexual cycle (Figure 1.1) (Galindo & Gallegly, 1960; Shaw, 1987).

Mating type in *P. infestans* is governed by a single locus (Gallegly, 1970; Shaw, 1991; Judelson *et al.*, 1995; Judelson, 1996). The region linked to the mating type locus displays non-Mendelian segregation and may be linked to two recessive lethal loci (Judelson, 1996), resulting in the exclusion of certain genotypes in the progeny. Several models have been erected to explain the inheritance of mating type in sexual progeny. Using DNA markers, Judelson *et al.* (1995) proposed that A1 and A2 mating types are fixed for heterozygosity at the mating type locus with one allele determining mating type and the other being neutral or non-functional. By fixing heterozygosity between active and neutral alleles at the locus, certain ambiguous or sterile genotypes would be excluded. Such a model was supported with data from molecular markers absolutely, or closely linked to the mating type locus.

Alternatively, it is proposed that mating type is determined by the ratio between alternate alleles at the locus, with A1 types normally being heterozygous (*A/a*) and A2 types homozygous (*a/a*) (Gallegly, 1970; Shaw, 1991; Judelson, 1996). This model explains the occasional observation of self-fertile (A1-A2) progeny (*Aaa*) (Shattock *et al.*, 1986; Judelson, 1996) from sexual crosses and could also explain the production of oospores in stressed cultures of single mating types (Chang & Ko, 1990). The observed non-Mendelian segregation of DNA markers at the mating type locus could be the result of a system designed to repress crossing over within the mating type locus (Sansome, 1980; Judelson, 1996) which may eliminate isolates with

ambiguous or deleterious combinations of alleles.

1.2.2 Drug resistance

Resistance to antibiotics often exhibit dominant or semi-dominant inheritance (Shaw & Khaki, 1971; Long & Keen, 1977a; Shattock, 1988). They can be used as appropriate phenotypic selectable markers for genetic analysis and have been used in positive selection systems to recover progeny with novel phenotypes (Shattock & Shaw, 1976; Lucas *et al.*, 1990). Mutants resistant to streptomycin and chloramphenicol have been used together and in combination with metalaxyl, as selectable markers in *P. infestans* (Shattock & Shaw, 1976; El Refai, 1990; Whittaker *et al.*, 1996; chapters 5 and 6). Other antibiotics or compounds used as selective markers include acriflavine, oxytetracycline and blasticidin (Poedinok *et al.*, 1988) and fluorotryptophan (Long & Keen, 1977b; Layton & Kuhn, 1988a; Lucas *et al.*, 1990), although resistance to metalaxyl, streptomycin and chloramphenicol have been most widely used.

1.2.3 Virulence phenotype

Many single resistance-genes (R) in *Solanum demissum* confer resistance to some isolates of *P. infestans* (Umaerus *et al.*, 1983). Eleven of these genes were bred into lines of *S. tuberosum* to provide a differential series (R1, R2, R3...R11) (Black *et al.*, 1953). R-genes conferring specific resistance usually behave as single gene traits and their inheritance in the potato is dominant (Umaerus *et al.*, 1983). For each individual R-gene, isolates of *P. infestans* are described as being avirulent if inoculated leaflets are symptom-less showing necrotic hypersensitive, non-sporulating lesions, or virulent if lesions bearing sporangia are observed (Gallegly, 1970; Shaw, 1991). The combined virulence phenotype (race) is the spectrum of pathogenicity on a range of differential potato lines, (Black, 1952; Denward, 1970).

Attempts to understand the genetic basis of inheritance of avirulence genes in *Phytophthora* (Smoot *et al.*, 1958; Niederhauser, 1961; Romero & Erwin, 1969; Sweigard *et al.*, 1987) suggested that each avirulence gene in the fungus was controlled by a single locus, similar to a gene-for-gene relationship (Flor, 1956). Avirulence may exhibit dominant or recessive inheritance, depending on the locus (Spielman *et al.*, 1989; Al-Kherb *et al.*, 1995; Tyler *et al.*, 1995). Some loci may also be controlled by additional modifying loci (Spielman *et al.*, 1990) or by separate loci

in different isolates (Al-Kherb *et al.*, 1995) as in some isolates of *Bremia lactucae* (Crute, 1987).

1.2.4 Isozymes

Prior to 1992, the most widely available molecular markers were isozymes (Shattock *et al.*, 1986; Goodwin *et al.*, 1992b) which are single gene, co-dominant chromosomal markers. Two loci, glucose phosphate isomerase (*Gpi-1*) and peptidase (*Pep-1*), have been most useful (Tooley *et al.*, 1985; Spielman, 1991; Goodwin *et al.*, 1992b). Out of seventeen loci investigated, these were the only polymorphic isozymes in Mexican populations (Tooley *et al.*, 1985) and the limited number of loci and low level of variability hindered population studies (Drenth *et al.*, 1993). Isozymes have proven useful for confirmation of diploid Mendelian inheritance and for identifying hybrid offspring (Tooley *et al.*, 1985; Shattock *et al.*, 1986) but suffer a lack of sensitivity as more than one nucleotide change can lead to the same isozyme polymorphism. More sensitive techniques that reveal direct DNA polymorphisms have subsequently been developed.

1.2.5 Mitochondrial DNA RFLPs

Mitochondrial DNA (mtDNA) Restriction Fragment Length Polymorphisms (RFLPs) were first detected in *P. infestans* from a sample of Israeli isolates as a 1.5 kb insert (Goodwin & Fry, 1988). Carter *et al.* (1990) devised a mtDNA RFLP technique using a range of restriction enzymes which digest nuclear DNA into small fragments, leaving the A-T rich mtDNA as a series of polymorphic bands. They found that in some isolates, the mtDNA contained a 1.7-2 kb insert but in others this was absent. Those without the insert were termed type I (*sensu* Carter *et al.*, 1990) or A (*sensu* Goodwin, 1991) and those with the insert were termed type II or B. Each of these types could be further divided by digesting with more enzymes (Carter *et al.*, 1990; Goodwin, 1991) giving four known haplotypes. These same mtDNA haplotypes can now be identified using the Polymerase Chain Reaction (PCR) (Griffith & Shaw, 1998) with combinations of four primer-pairs that can distinguish between types I and II or a and b. Types Ia and IIa are currently the most widely distributed and are found in Europe, Mexico, USA and Asia (Carter *et al.*, 1990; Goodwin, 1991; Griffith & Shaw, 1998; section 1.3.4). Some mtDNA types may be associated with isolates of particular mating types or genotypes (chapter 3; Goodwin, 1991).

1.2.6 Random Amplified Polymorphic DNA (RAPD)

RAPD markers (Williams *et al.*, 1990) have been used to investigate sexual recombination in *P. infestans* (Maufrand *et al.*, 1995). They have also been used to find markers linked to the mating type locus (section 1.2.1) and for investigating the stability of transgenic isolates of *P. infestans* (Judelson, 1991; Judelson *et al.*, 1995). However, they have not been used extensively for population studies due to problems concerning their reproducibility. RAPD fingerprint patterns are sensitive to concentration differences in PCR reagents (Ellsworth *et al.*, 1993; Kernodle *et al.*, 1993) but Maufrand *et al.* (1995) found that RAPD patterns were stable after optimising the reaction conditions. Even though some RAPD primers reveal equal or more variation than RFLP probes (Maufrand *et al.*, 1995), they have not been used for population analysis as frequently as RFLP techniques.

1.2.7 Nuclear RFLPs

The detection of nuclear RFLPs is technically more difficult than the detection of mtRFLPs (Goodwin, 1991; Shaw, 1991) due to the large size of the nuclear genome, estimated to be 250 Mb (Tooley & Therrein, 1987; van der Lee *et al.*, 1997). The need for independent, well-characterised loci for population and recombination studies (Goodwin, 1991) fuelled attempts to identify single-locus (Carter *et al.*, 1991 & 1999) or multi-locus (Goodwin *et al.*, 1992a; Pipe & Shaw, 1997) nuclear probes.

Single-locus nuclear RFLPs (Carter *et al.*, 1991) are useful because heterozygotes can be distinguished from homozygotes. Certain probes reveal a high degree of polymorphism amongst isolates from different countries (Carter *et al.*, 1991) and can also identify triploid and tetraploid isolates (Carter *et al.*, 1999). However, single-locus RFLPs do not reveal enough information for them to be used individually in population studies.

The most widely used DNA probe for population studies in *P. infestans* has been RG57 (Goodwin *et al.*, 1992a). RG57 is a 1.2 kb genomic fragment from a partial genomic library of *P. infestans* DNA, which may hybridise to 25 or more *Eco*RI fragments in Southern blots of genomic DNA. Nine fragments are thought to segregate independently in sexual progeny and two pairs show linked segregation. Fingerprint patterns are somatically stable through single-zoospore propagation and are transmitted to sexual progeny in Mendelian fashion (Goodwin *et al.*, 1992a). RG57 is a dominant marker so homozygote (+/+) bands are rarely distinguishable

from heterozygote (+/-) bands and the absence of a band may not necessarily be due to the same mutation. As it is a dominant marker, the fingerprint is therefore a phenotype, although it is here and elsewhere referred to as a genotype. The RG57 probe has been used extensively since 1992 to investigate the genetic diversity of *P. infestans* populations in many countries (Forbes *et al.*, 1998). Somatic stability and high levels of conservation distinguish RG57 from the variable number tandem repeat loci, which have been used for fingerprinting in other organisms (Jeffreys *et al.*, 1985).

A multilocus telomeric probe, pLT11, has also been developed (Pipe & Shaw, 1997). The probe is based on a telomeric repeat from *Arabidopsis thaliana* and reveals >24 end-fragment bands when hybridised to digested genomic DNA of *P. infestans*. Many of these bands are polymorphic within and between populations of *P. infestans* and the probe has proven useful in identifying variation undetected by RG57 (chapter 3).

1.2.8 AFLP DNA fingerprinting

Amplified Fragment Length Polymorphisms (AFLPs) were developed by Keygene in 1994 (Vos *et al.*, 1995). The technique involves selective amplification of restriction fragments by PCR, yielding polymorphic, dominant markers similar to those generated by multilocus RFLPs. The technique has been widely used, for example, in the molecular typing of bacteria (Lin *et al.*, 1996), investigating introgression of *Salix* species along a hybrid zone (Beismann *et al.*, 1997) and construction of linkage maps in *P. infestans* (van der Lee *et al.*, 1997). Being PCR-based, AFLP analysis requires less DNA than RFLP analysis. Unlike RAPDs (Williams *et al.*, 1990; Ellsworth *et al.*, 1993), AFLPs are less sensitive to variations in DNA concentration or PCR reagents (Majer *et al.*, 1996; van der Lee *et al.*, 1997; Duncan *et al.*, 1998). One AFLP reaction separated on a single gel can distinguish many more bands than RFLP or RAPD techniques (Majer *et al.*, 1996). Polymorphic AFLP markers have been shown to segregate in Mendelian ratios in *P. infestans*, except those linked to the mating type locus (van der Lee *et al.*, 1997). AFLP markers are likely to be neutral and variation is revealed in any part of the genome (Majer *et al.*, 1996). These attributes make AFLPs a useful marker for identifying variation and assessing diversity in plant pathogenic fungi (Majer *et al.*, 1996).

1.2.8.1 *Theory of the AFLP Technique*

The AFLP method involves several stages (Figure 1.2).

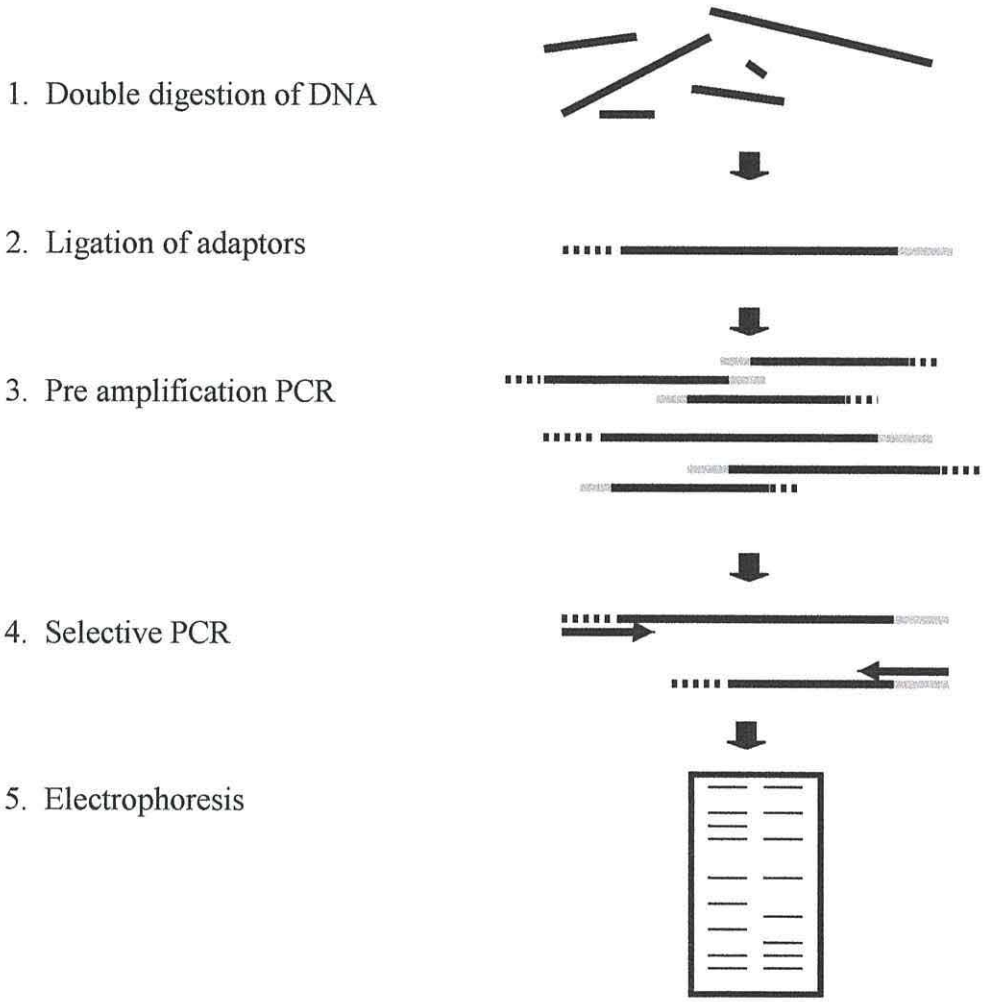


Figure 1.2. The principle steps involved in the AFLP process. See text for details.

First, DNA is digested with two enzymes, one that cuts at four, the other six base pairs. *Eco*RI (6) and *Mse*I (4) produce fragments of the appropriate size (<1000bp) with *P. infestans* DNA (van der Lee *et al.*, 1997) and are probably the most common pair used for DNA digestion. The second step is the ligation of double-stranded adaptors to the ends of the restriction fragments, followed by two rounds of PCR. The first round involves amplification of all digested fragments using non-selective primers complimentary in sequence to the adaptors (Table 2.1). The second round involves amplification of a subset of fragments using selective primers which have between one and four nucleotide extensions to the 3' end extending into the genomic DNA between the two adaptors (Table 2.1). One primer (usually the *Eco*RI)

is labelled by attaching a radioactive nucleotide [^{33}P] to the 5' end of the primer. This allows amplified fragments to be visualised after electrophoresis through a polyacrylamide gel. Two types of polymorphism can be observed in this way; point mutations at the primer or restriction sites and insertions or deletions between them (Vos *et al.*, 1995).

Majer *et al.* (1996) used AFLPs to study variation in two fungal species that have few RFLP polymorphisms and found a mean number of between 3 and 4 polymorphisms per primer-pair. The almost unlimited combination of primer-pairs (Vos *et al.*, 1995) means that polymorphisms should be detectable between almost any two non-clonal individuals of the same species if enough primer-pairs are used.

1.3 POPULATION BIOLOGY

1.3.1 Mexican populations

The *P. infestans* – *Solanum* pathosystem is believed to have evolved in Central Mexico. Until the 1980s, this was the only location in which both mating types were known to occur (Niederhauser, 1961) and they were present in nearly equal frequencies of A1 and A2 mating types (Gallegly & Galindo, 1958; Fry & Spielman, 1991). The population in central Mexico has characteristics of a random-mating sexual population with diverse isozyme genotypes (Tooley *et al.*, 1985; Goodwin *et al.*, 1992b). Also, there is huge diversity in virulence phenotype (Fry & Spielman, 1991) and it is not uncommon to find many isolates with unique RG57 fingerprints in a single field (Goodwin *et al.*, 1992b). Sexual reproduction is undoubtedly an important and common stage of the life cycle in Mexico.

DNA Contents of 2C (diploid) (9-10 chromosomes) have been found in the majority of Mexican isolates (Sansome & Brasier, 1973; Tooley & Therrein, 1987). This is in contrast to other areas of the world where DNA contents ranging from 2C to 4C (tetraploid) have been found in European, Egyptian, Peruvian and American isolates (Tooley & Therrein, 1987; Tooley *et al.*, 1989; Whittaker *et al.*, 1991a). Sexual reproduction may select for diploidy in Mexico but, in areas where sex is rare, other factors (colder climate or higher fitness) may select for isolates with higher ploidy (Sansome, 1977; Fry & Spielman, 1991).

1.3.2 Migrations from Mexico

Historical outbreaks of late-blight in South America (reviewed in Andrivon, 1996) have fuelled suggestions that *P. infestans* originated in Peru or Bolivia, the same part of the world as its host. However, race-specific resistance genes, isozymes and RFLP fingerprint patterns are more diverse in Mexico than in South America (Andrivon, 1996) suggesting the latter is only a secondary home to *P. infestans* and that Mexico is probably the ancestral home of the pathogen.

During the 1840s, populations in the USA, Canada and Europe are thought to have been derived from the Mexican population, probably as a consequence of transporting the fungus from the central highlands of Mexico (Niederhauser, 1991; Spielman *et al.*, 1991). However, it is not clear whether isolates migrated from Mexico to the USA and from there to other parts of the world (Goodwin & Fry, 1991; Goodwin, 1997) or were transported directly from Mexico (Andrivon, 1996). It is thought that all populations outside of Mexico before the mid 1970s were dominated by a single clonal lineage, termed US-1 (Goodwin *et al.*, 1994), which had a characteristic RG57 fingerprint pattern, mitochondrial haplotype (Ib) and isozyme genotype (*Gpi-1 86/100 & Pep-1 92/100*). Any genetic diversity in US-1 was probably caused by mutation or mitotic recombination (Shaw, 1983a & b) within the lineage (Goodwin & Fry, 1991; Goodwin *et al.*, 1994). The result of the migrations became evident in the 1840s with the potato famine in Ireland and epidemics in North America (Bourke, 1991).

In the 1970s, a further migration is thought to have introduced more A1 and, crucially, A2 strains, into Europe (Spielman, *et al.*, 1991). This probably coincided with the transport of 25,000 tones of ware potatoes from Mexico to Europe in 1976 (Niederhauser, 1991). In the 1980s, the A2 mating type was detected in most European countries and elsewhere if enough samples were examined (Hohl & Iselin, 1984; Shaw *et al.*, 1985; Tantius *et al.*, 1986; Mosa *et al.*, 1989; Shaw, 1991; Sujkowski *et al.*, 1994). The presence of both A1 and A2 mating types in the same populations in Europe, the USA and elsewhere brought with it the potential for sexual reproduction with the accompanied threat of long-lived oospore inoculum in the soil.

Other hypotheses have been proposed to explain the origin of the A2 mating type outside of Mexico. It is possible that the A2 arose by mutation, mitotic recombination or mating type change either from exposure to fungicides or induced

selfing (Ko, 1994). However, using cluster analysis from previously published molecular data, Goodwin & Drenth (1997) showed that populations of the fungus were quite different before and after the detection of the first A2 isolates in the 1980s. These A2 isolates had distinctly different DNA fingerprint patterns from the local populations (they were "new" strains) suggesting that they were migrants rather than mutants within an essentially monomorphic population (Goodwin & Drenth, 1997). As such, the migrant origin of the A2 isolates is the only hypothesis with any scientific support.

1.3.3 Population structure

1.3.3.1 *Europe*

In Europe, the "new" strains revealed previously undetected isozyme alleles (Shattock *et al.*, 1990; Spielman *et al.*, 1991) and much greater diversity of DNA fingerprints (Drenth *et al.*, 1993 & 1994; Sujkowski *et al.*, 1994). Two new mtDNA haplotypes, Ia and IIa, were also observed and populations quickly developed resistance to the fungicide metalaxyl (Davidse *et al.*, 1981; Shattock *et al.*, 1990; Daggett & Gotz, 1991) which is now widespread amongst British isolates (Day & Shattock, 1997; Day *et al.*, unpublished). Shattock *et al.* (1990) reported the disappearance of the old *Gpi-1 86/100* genotypes from British collections during 1987 & 1988.

The population structure in Europe no longer consists of a single clonal lineage. Studies in England and Wales (N. Pipe, unpublished; Day *et al.*, unpublished) revealed that several clonal lineages (at least four to six) are widely distributed but many other fingerprints have only been detected at a single site or in a single year. RG57 variation has been identified in many European countries such as France (Lebreton & Andrivon, 1998), Norway & Finland (Brurberg *et al.*, 1999), The Netherlands (Drenth *et al.*, 1994) and Poland (Sujkowski *et al.*, 1994). More recently, Zwankhuizen *et al.* (in press) identified 146 RG57 genotypes among 981 isolates, 79% of which were unique. Further (linked?) changes were also observed in the frequency mtDNA haplotypes. In 1982, the "old" type Ib haplotype was recorded in 67% of UK isolates (Shattock & Day, 1996). Thereafter, apart from single isolates in 1986 and 1995, type Ib has been replaced by "new" isolates of type Ia or type IIa (Shattock & Day, 1996; Day & Shattock, 1997). A similar situation seems to be occurring in France, type Ib not having been detected since 1991 (Lebreton &

Andrivon, 1998). Variation of nuclear and mtDNA fingerprints amongst European isolates could be the result of somatic recombination (section 1.4.1) and/or sexual reproduction.

1.3.3.2 *USA*

At least two major migrations may have contributed to the variation detected in the USA and Canada (Goodwin *et al.*, 1994) and dramatic changes have occurred recently in the population (Goodwin *et al.*, 1998). Prior to 1992, most populations from commercial fields in the USA were monomorphic for RG57, isozymes and mating-type, and several strains (thought to be clonal lineages) were distributed widely throughout North America (Goodwin *et al.*, 1995). A migration from Mexico in the late 1970s of a highly pathogenic genotype, US-6, resulted in the appearance of new late-blight epidemics in the USA (Goodwin, 1997). In 1992, the introduction (from Mexico) of two further genotypes (US-7 and US-8), both A2 and resistant to metalaxyl (Goodwin *et al.*, 1995; 1998), changed the population structure. Both genotypes spread throughout the USA and moved into Canada by 1994 (Goodwin & Fry, 1994). US-1, the previously dominant genotype throughout the USA, made up only 8% of sampled isolates between 1994-1996 (Goodwin *et al.*, 1998). As there was little overlap between the distributions of the A1 and A2 isolates, opportunities for sexual recombination were thought to be rare. Even so, possible sexual recombinants have been found in Washington and Oregon (Miller *et al.*, 1997) although clonal reproduction probably predominates throughout the USA (Goodwin *et al.*, 1998).

1.3.4 Displacement of "old" with "new" strains

Following the migration in the 1970s, "new" strains of the pathogen have largely replaced the "old" A1 strains wherever they have appeared (Hohl & Iselin, 1984; Shattock *et al.*, 1990; Spielman *et al.*, 1991; Fry *et al.*, 1993; Drenth *et al.*, 1994). There are several possible reasons to account for the displacement of "old" with "new" strains.

The "new" populations may have been more aggressive (non host-specific pathogenicity) or may have had increased host-specific virulence. Differences in aggressiveness between "old" and "new" populations have been observed (Day & Shattock, 1997). Surprisingly, metalaxyl-resistant ("new") strains may be less

aggressive than metalaxyl-sensitive isolates on certain cultivars, but isolates with type Ia mtDNA haplotype may be more aggressive than isolates with IIa or Ib. This may be a consequence of asexual reproduction in the "old" non-Mexican population and consequently, a build up of detrimental mutations (Day & Shattock, 1997; Duncan *et al.*, 1998).

There is evidence from mtDNA and RG57 fingerprint analysis that some of the "old" type Ib isolates may have recombined either sexually or somatically with the "new" migrant strains (Day & Shattock, 1997) which may have resulted in introgression, rather than displacement, of the "old" and "new" populations. However, it is also possible that most sexual reproduction occurred between the "new" strains and not between "old" and "new" strains (Drenth *et al.*, 1994). Recombination between "old" and "new" strains may have been hindered by differences in ploidy as the "old" strains were 3C or 4C and the "new" strains may have been mainly 2C (Tooley & Therrein, 1987). Recombination between strains with different ploidy is, however, possible (Whittaker *et al.*, 1991b).

1.3.5 Self-fertile isolates of *P. infestans*

The appearance of self-fertile isolates in Europe and the USA seems to be linked with the migration of A2 isolates from Mexico. Most isolates of *P. infestans* are bisexual but are able to form oospores only when paired with the opposite mating type on a suitable medium (Shaw *et al.*, 1985). They display relative sexuality, preferentially forming antheridia or oogonia depending on their mating partner (Galindo & Gallegly, 1960; Judelson, 1997b). Self-fertile isolates able to produce oospores in single culture have been found in various countries, including the USA (Smoot *et al.*, 1958), Mexico (Niederhauser, 1956) and the UK (Tantius *et al.*, 1986; Fyfe & Shaw, 1992; Pipe *et al.*, in press). Self-fertile phenotypes can sometimes be transmitted through single hyphal-tips and sporangia (Fyfe & Shaw, 1992; Pipe *et al.*, in press) or zoospores (Niederhauser, 1991), suggesting some may be heterokaryons (section 1.4.1) or products of nuclear fusion between A1 and A2 nuclei. Self-fertile isolates are discussed extensively in chapter 4.

1.3.6 Evidence for sexual reproduction in populations of *P. infestans*

Oospores have been found in leaves and stems of plants exposed to normal weather conditions after inoculation with A1 and A2 strains (Drenth *et al.*, 1995)

demonstrating that sexual reproduction is possible in the field. Variation in RG57 fingerprints (Drenth *et al.*, 1994; Sujkowski *et al.*, 1994) suggest that sexual recombination has been occurring in populations of *P. infestans* since migration of the A2 mating type from Mexico (section 1.3.2). Experimental crosses of the two most common A1 and A2 field strains in the UK also revealed some recombinant progeny with genotypes identical to some of those observed in the field (Day *et al.*; unpublished), suggesting that some genotypes may well be sexual recombinants. However, in some countries (e.g. the UK) the A2 makes up a small proportion (2-6%) of the population and may only be detected at fewer than 1% of sites (Day *et al.*, unpublished; Zwankhuizen *et al.*, in press) in any one year. Opportunities for extensive sexual recombination may therefore be limited.

It is possible that mechanisms such as somatic recombination may occur in parallel with sexual recombination in *P. infestans* (Shaw, 1983a; Kuhn, 1991). Many true fungi, such as *A. nidulans* have well defined sexual and parasexual cycles (Roper, 1966; Burnett, 1975) so it is not unreasonable to suggest that both cycles may occur in *P. infestans*. However, it is difficult to obtain convincing evidence for the occurrence of somatic recombination in field situations where there are many fungal genotypes in a population (Burdon & Silk, 1997), as is currently found in populations of *P. infestans*.

1.4 SOMATIC RECOMBINATION

1.4.1 Somatic recombination and the parasexual cycle in haploid fungi

Somatic recombination is the recombination of genetic information without specialised sexual structures and includes exchange of nuclei and exchange of mtDNA between two strains (Bos, 1996). It is a mechanism adopted by sexual and asexual species of haploid fungi and can result in a significant amount of genetic exchange (Tinline & MacNeill, 1969). Somatic recombination has been demonstrated in most filamentous fungi where it has been looked for, such as in *Aspergillus* spp, *Fusarium* spp, *Verticillium* spp, *Puccinia* spp and *Colletotricum* spp (Buxton, 1962; Parmeter *et al.*, 1963; Tinline & MacNeil, 1969; Debets, 1998).

The parasexual cycle as reviewed by Pontecorvo (1956) consists of the following sequence of events (Figure 1.3). (1) hyphal anastomosis between homokaryons of different genotypes resulting in heterokaryotic mycelium; (2) fusion

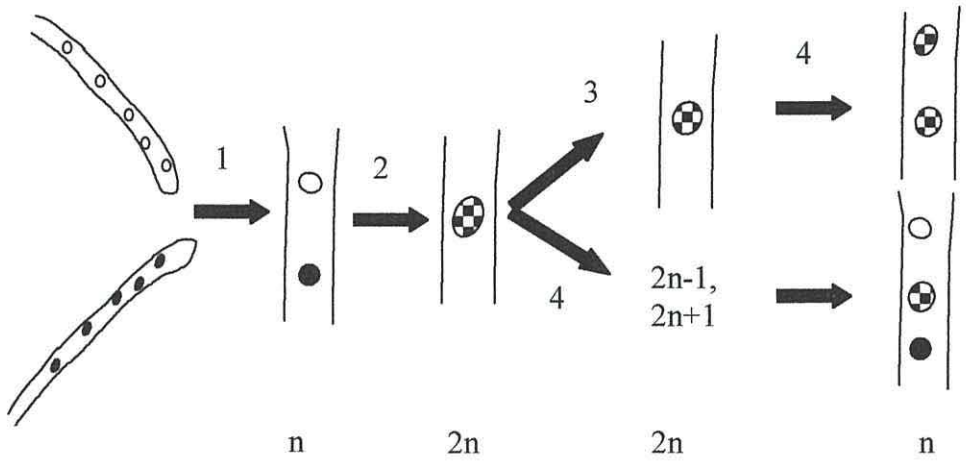


Figure 1.3. Diagrammatic representation of the parasexual cycle in *Aspergillus nidulans*. 1. hyphal anastomosis results in a heterokaryon. 2. nuclear fusion results in heterozygous diploid nuclei. 3. mitotic crossing-over results in recombinant diploids. 4. Non-disjunction of chromosomes generates aneuploids and recombinant haploids. After Roper (1966), Burnett (1975), Gu & Ko, (1998) and Debets (1998). For details see text.

of two unlike haploid nuclei generating a heterozygous diploid; (3) occasional mitotic crossing-over during multiplication of the diploid nuclei; and/or (4) non-disjunction of chromosomes leading occasionally, via successive aneuploid states, to a recombinant haploid. As in meiotic recombination there is both intrachromosomal (crossing-over) and interchromosomal (non-disjunction) recombination but unlike in meiosis, the stages of the cycle are independent of each other and the frequency of each is much lower (Clutterbuck, 1996; Debets, 1998).

Heterokaryosis, the first stage of the parasexual cycle, is the simplest form of somatic recombination. Heterokaryons can be selected for by using strains with different auxotrophic or drug-resistant markers and is often maintained only under selective pressure. Anastomosis of compatible strains allows nuclei to migrate into the opposite strain, where they can disperse throughout the hyphae via pores in the septa (Bos, 1996) or, as in the oomycetous fungi, migrate through the cytoplasm, the mycelium being coenocytic. Accordingly in *Phytophthora*, hyphal fusion at any point could lead to widespread mixing of nuclei and cytoplasm (Kuhn, 1991). Within a heterokaryon, nuclei from different strains can fuse (karyogamy), resulting in parts of the colony having heterozygous diploid nuclei (Bos, 1996).

Two processes can occur in diploid nuclei after karyogamy; non-disjunction and/or mitotic crossing over. These processes have been studied extensively in

haploid fungi where linkage groups and recombination can be investigated without the hindrance of recessive mutations being masked (Roper, 1966; Burnett, 1975; Bos, 1996). Mitotic crossing-over occurs at, or just after, chromosome duplication or nuclear fusion and results in homozygosity of all markers distal to the crossover point on sister chromatids. During haploidisation, chromosomes re-assort but do not recombine so that linked genes rarely re-associate during this process. The main feature of mitosis is that sister chromatids are separated and go to different daughter cells. Mitotic non-disjunction of sister chromatids leads to aneuploids (2n+1, 2n-1, 2n-1-1 etc.) and by successive loss of chromosomes, back to haploid nuclei but depending on which homologues are maintained, nuclei reduced through non-disjunction could be recombinant. Diploid nuclei are usually reduced to haploid nuclei at a low frequency through non-disjunction and hyphae may contain a variety of aneuploid nuclei (Bos, 1996). There is a wealth of experimental evidence concerning somatic recombination in haploid fungi. However, the role of somatic nuclear exchange in field populations is difficult to determine in many species due a lack of molecular markers (Burdon & Silk, 1997) and the extent to which it occurs may be limited by incompatibility factors.

1.4.2 Heterokaryon incompatibility

Although parasexual recombination is widespread amongst fungi, some barriers prevent indiscriminate exchange. Isolates of a species often show vegetative incompatibility and this is probably as widespread as the parasexual cycle (Caten, 1971). Jinks *et al.* (1966) found several incompatibility groups in *A. nidulans*. Eight *het*-genes are responsible for incompatibility reactions in *A nidulans* and only isolates with identical alleles at all *het* loci are compatible. In other (nonallelic) incompatibility systems, the existence of genetic differences at two separate loci can also result in incompatibility (Glass & Kuldau, 1992). The ability to form heterokaryons may therefore be determined by the degree of genome similarity; isolates with identical alleles at certain loci are more likely to form stable heterokaryons than those with more differences (Jinks *et al.*, 1966). Incompatibility may have different causes. Sometimes anastomosis does not occur or if it does, it may be followed by an incompatible reaction which results in cell death, such as in *N. crassa* (Garnjobst & Wilson, 1956).

In *P. infestans*, variation in ability to form heterokaryons between different

isolates (Poedinok & Dyakov, 1981, in Brasier, 1992) suggests that vegetative incompatibility factors may determine the extent of somatic recombination. Co-culturing A1 isolates on plates of rye A agar, Bagirova & Dyakov (1993) noticed that some isolates would grow into each other whereas others would leave a barrage zone and stop growing as if inhibited by the presence of the other strain. The genetic basis of these observations and their implications is still uncertain. Cytoplasmic incompatibility may have been observed by Gu & Ko (1998) who found that stable heterokaryons could be generated through nuclear transfer in *P. nicotianae* var. *parasitica* but not through protoplast fusion. This may be similar to heterokaryon incompatibility in *N. crassa* which is controlled by soluble cytoplasmic proteins (Garnjobst & Wilson, 1956; Wilson *et al.*, 1961). Vegetative incompatibility barriers (physical or biochemical) can also prevent cell fusion in fungi (Kuhn, 1991) and can in some cases be overcome by removing the cell wall with enzymatic treatments which liberate protoplasts (chapter 6). Protoplast fusion has been used to overcome vegetative incompatibility factors and to induce somatic recombination in many haploid fungi and in yeast (Kavanagh & Whittaker, 1996) as well as allowing interspecific hybridisation amongst *Phytophthora* species (Brasier *et al.*, 1999). The true extent and genetic basis of incompatibility still remains to be determined in *Phytophthora.*

1.5 OBJECTIVES OF THE PRESENT STUDY

Little is known about parasexual processes in the oomycetous fungi and only recently has direct evidence been produced that recombination outside of the sexual cycle does occur in some species of *Phytophthora* (Judelson & Yang, 1998) (chapter 6). Previous studies using self-fertile isolates (chapter 4), drug-resistance (chapter 6) or virulence phenotype (chapter 7) produced evidence in support of heterokaryon formation and recombination in *P. infestans.* However, some of these studies did not have neutral mitochondrial or chromosomal markers, so somatic recombination could not be confirmed. These studies need to be repeated with parental isolates having several distinctive and reproducible molecular markers, allowing somatic hybrids to be distinguished from variants originating from a single parent. This was approached, by asking the following questions:

1) Do RFLP and AFLP techniques provide suitable markers for detecting hybrids?

(chapter 3).

2) Are asexual progeny from mixed A1/A2 (self-fertile) cultures heterokaryotic or hybrid? (chapter 4)

3) Can drug resistant mutants be selected for on drug-amended media? (chapter 5) Can double-drug amended media be used to select for isolates which are somatic hybrids of two single-drug resistant parents? (chapter 6)

4) Are strains with wider virulence selected on detached leaflets of potato lines with several R-genes, heterokaryotic or recombinant? (chapter 7)

2 Materials and Methods

2.1 FUNGAL MATERIAL

2.1.1 Isolation and incubation

The origins of all wild-type isolates used in this study are given in the appropriate chapter. All isolates were derived from single-lesions on potato or tomato. Unless otherwise indicated, D. Shaw, N. Pipe or J. Day collected the isolates as part of a *P. infestans* population genetics project.

Pure cultures of mycelia were isolated from leaf or stem lesions by removing 10 mm^2 sporulating lesions and inoculating plates of rye A agar (Caten & Jinks, 1968) amended with RAN antibiotics (appendix 1), modified from Tantius *et al.* (1986), to prevent the growth of fungal and bacterial contaminants. Isolates were further sub-cultured to fresh rye A media once they had grown free of the contaminants.

2.1.2 Storage of isolates and sub-culturing

Unless otherwise indicated, cultures were maintained on rye A agar at 18°C in the dark or for longer term storage, in 0.5 ml tubes containing three mycelial plugs from clean, actively growing colonies, and sterile water. Mycelia were still viable after 30 months. Plugs of contaminated cultures were rescued onto rye A + RAN and isolates were further sub-cultured onto rye A + RAN once they had grown free of the contaminants. Routine sub-culturing involved the removal of a 10 mm^2 plug of hyphae onto fresh rye A or rye A + RAN. All inoculations were carried out in a laminar flow cabinet (Micro-flow).

2.1.3 Mating Types

Mating types were determined by placing a 10 mm^2 plug of hyphae centrally on rye A agar in a 9 cm Petri-dish. Two isolates of known mating type (E14C2, A2 and 95.161.5, A1) were then inoculated either side of the unknown isolate. After seven days incubation, the pairings were examined for the presence of oospores between the unknown plug and the tester isolates (Smoot *et al.*, 1958; Shattock *et al.*, 1986). Oospores usually form only in the presence of strains with the opposite mating type.

2.2 CULTURE TECHNIQUES

2.2.1 Propagation from single hyphal-tips

To obtain single hypha-tips, colonies were grown on 2-3 mm-deep plates of one-tenth pea agar (appendix 1) for 2 d. The low density of hyphae and the clarity of the agar allowed single hyphal-tips to be isolated after puncturing the agar surface with the sterile tip of a glass Pasteur pipette (1 mm diameter). Only hyphal tips below the agar surface could be reliably isolated as those on the surface were displaced by free water on the media surface. Punctured single hyphal-tips were then excised from the agar with a scalpel and placed into one square of a repli-dish (Sterilin) containing 2.5 ml of rye A agar and examined for germination after 7-10 d. Hyphal tips were generally 0.5 mm in length (half the diameter of the Pasteur pipette). The number of hyphal-tips removed and the number germinating into colonies was recorded microscopically.

2.2.2 Propagation from single-sporangia

Plates of rye A agar were inoculated with the appropriate isolate. After 10-14 d, cultures were flooded with 10 ml^{-1} of sterile water and sporangia were removed by gently agitating the surface of the mycelium with a sterile glass spreader. The resulting sporangial suspension was transferred to a sterile Universal bottle and the concentration determined using a haemocytometer (Neubauer) by obtaining an average of at least four replicates. At concentrations of 500 sporangia ml^{-1}, 0.5 ml of sporangial suspension was pipetted onto 2-3 mm-deep plates of one-tenth pea agar (appendix 1) and incubated for 24-48 h. Single germinating sporangia were isolated with the sterile tip of a Pasteur pipette (1 mm diameter) and microscopically removed with a scalpel, to a square of a repli-dish containing 2.5 ml of rye A media. Germination was recorded microscopically after 7-10 d.

2.2.3 Propagation from single zoospores

Single zoospores were obtained from 10 day-old colonies using a modified method of that used to obtain single sporangia. Sporangial suspensions in water (section 2.2.2) were incubated at 10°C in Universal bottles for 2 h. Zoospore release was confirmed microscopically and concentrations were determined using a haemocytometer (Neubauer) by obtaining an average at least four replicates. Ten plates of 2-3 mm-deep one-tenth pea agar (appendix 1) were each inoculated with approximately 1000-

1500 zoospores and incubated for 24-48 h. Germinating zoospores could be distinguished from germinating sporangia by their smaller size and spherical, rather than elliptical, shape. Single germinating zoospores could be isolated with the tip of a glass Pasteur pipette (1mm diameter), excised from the agar with a scalpel and placed into a repli-dish square containing 2.5 ml of rye A agar. Germination was recorded microscopically after 7-10 d.

2.2.4 Oospore production, extraction and germination

Hyphal strips (20 mm x 5 mm) of each parent isolate were placed 30 mm apart on rye A agar. Isolates were incubated for up to 4 weeks, after which time, oospores were extracted using a method based on those reported previously (Pittis & Shattock, 1994).

The culture strip between the two isolates containing most of the oospores was removed and blended in 5 ml distilled water in a glass grinder (Fisons). Hyphae were destroyed but the majority of oospores remained intact. The suspension was filtered through 20 μm-pore nylon filters (Lockertex) and washed with two changes of distilled water. The oospores were re-suspended in 10 ml of sterile deionised water containing ¼ RAN and 1 mg NovoZym 234 (Novo Biolabs). After incubation for 24-36 h, oospores were collected on 20 μm pore nylon filters, washed with 100 ml sterile deionised water and re-suspended in sterile deionised water to give the desired concentration of approximately 10^4 oospores ml^{-1} (J. Day, personal communication).

Oospore germination was induced by pipetting 0.5-1 ml of oospore suspension onto 0.8% water-agar plates amended with ¼ RAN (appendix 1). Plates were sealed with parafilm (BDH) and incubated at 18°C with a photoperiod of 16 h light supplied by eight 1.2 m 36 W white fluorescent tubes, which provided 120-140 $\mu moles/m^2/s^2$ photosynthetically active radiation. Plates were microscopically examined for germinated oospores every 3-4 days. After one week, 200 μl of rye A agar was added to the plates centrally to stimulate germination. Germinated oospores were dissected from the agar with a scalpel and removed to fresh rye A agar + RAN in a sterile repli-dish. Growing colonies were removed to fresh plates of rye A agar.

2.2.5 Protoplast isolation

Protoplasts were isolated using a modification of the protocol described by van West *et al.* (1999). Sporangia were removed from 14-day-old cultures (section 2.2.2). A

sporangial suspension (2 x 10^5 sporangia ml^{-1}) was mixed with two-times-concentrated pea-broth (appendix 1). After incubation for 30-48 h, young mycelia were harvested and washed in 10 ml of KC osmoticum (0.64 M KCl and 0.2 M $CaCl_2$) for 5 min and centrifuged at 1200 rpm for 2 min. Mycelia were re-suspended in 10 ml KC containing 2.5 mg ml^{-1} NovoZym 234 and 2.5 mg ml^{-1} cellulase (Sigma) at room temperature for 15-20 min to liberate protoplasts. Cell debris was removed by filtration through a 50 μm mesh nylon filter. After centrifugation at 1200 rpm for 2 min, the soft pellet was washed (without resuspending) in KC for 30 s, centrifuged at 1200 rpm for 2 min, washed in KC-MT (0.32 M KCL, 0.1 M $CaCl_2$, 0.5 M mannitol, and 5 mM Tris/HCl, pH 7.5) for 30 s and centrifuged for 2 min at 1200 rpm. The pellet was then washed in MT (1 M mannitol and 10 mM Tris/HCl, pH 7.5) for 30 s and centrifuged for 2 min at 1200 rpm. Protoplast concentrations were determined using a haemocytometer (Neubauer) by obtaining an average at least four replicates.

2.2.6 Protoplast fusion

Protoplast suspensions of two strains were diluted to an equal concentration of 10^6-10^7 protoplasts ml^{-1} and 0.5 ml of each parental strain were mixed. To 1 ml of the protoplast mix, 1 ml of 50% PEG 3350-4000 (Sigma) in 20 mM $CaCl_2$ and 10 mM Tris/HCl (pH 7.5) was added slowly, over a period of 2 min. The mixture was then inverted gently six times, left to stand at room temperature for 2 min and 25 ml of rye-sucrose-mannitol medium (RSM) (appendix 1) was then added. The mix was poured into petri dishes and left for 24 h for protoplasts to begin regenerating. Once germinating hyphae could be seen growing, the protoplast mixture was centrifuged at 1200 rpm and re-suspended in 3 ml of RSM. 0.5 ml of the suspension was plated onto petri dishes containing rye A amended with appropriate double-selective media (appendix 1). Any growing colonies were removed to fresh double-selective media.

2.2.7 Leaflet inoculation of potato plants

2.2.7.1 *Growth of potato differentials*

Tubers were raised by D. Shaw at Pen-y-Ffridd field station, Bangor, in 7 dm^3 pots containing a peat-sand compost, treated with 26.5 g of Chempack potting base (Pre-packed fertilizers) per dm^3 of compost as described previously (Al-Kherb, 1988).

Potting base contained $CaCO_3$, nitrogen, phosphorous and potassium in the ratio 7.5 : 3.6 : 5.2% respectively, and other minor nutrients required by the plants. In the first three weeks, plants were watered every two days and were grown on a capillary mat which was wetted every day. Three weeks after planting, Chempak liquid fertiliser (No. 3) was supplied weekly at the recommended concentration in water. Plants were grown in a 16 h day with illumination at Pen y Ffridd producing a day-night temperature of approximately 12-18°C.

2.2.7.2 *Sporangial inoculation of detached leaflets*

Fully expanded leaflets were detached from 6-week-old potato plants 2-3 h before being inoculated with sporangia. Once detached, 2-3 leaflets were placed in inverted petri-dishes filled with 2% water agar (appendix 1). Sporangia were harvested from growing cultures (section 2.2.2) and concentrations altered to 10^3-10^5 ml^{-1} with addition of sterile water, depending on the experiment (see chapters 6 & 7).

Leaflets were inoculated by pipetting 15-20 10 µl droplets of the sporangial suspension on the abaxial surface of the leaflets. Zoospore release was induced by incubation at 10°C for 2 h, then at 18°C, with a photoperiod of 16 h light supplied by eight 1.2 m 36 W white fluorescent tubes, which provided 120-140 µmoles/m^2/s^2 photosynthetically active radiation. Leaflets were observed every day for the development of necrosis and sporulation.

2.2.7.3 *Virulence assay*

Virulence phenotypes were determined using a modified method of Al-Kherb *et al.* (1995). Disease assessment was made after 3-5 days on cultivars with different combinations of R-genes (chapter 7). Disease interactions were scored as virulent if at least two of the three inoculated leaflets had sporulating lesions. Non-sporulating lesions or hypersensitive necrotic reactions were considered to indicate avirulence. The reactions were recorded as the percentage of the leaves that were necrotic and the percentage of the leaf surface on which sporangia were produced.

2.2.8 Drug sensitivities and mutant isolation

2.2.8.1 *Sensitivity to metalaxyl, streptomycin and chloramphenicol*

The extensive culture collection at Bangor contains many isolates insensitive to 5-200

$\mu g\ ml^{-1}$ of the fungicide metalaxyl (Ciba Geigy). Isolates (chapter 5) were tested on a range of concentrations of metalaxyl, streptomycin (Sigma) and chloramphenicol (Sigma) by placing a 10 mm^2 plug onto plates of drug-amended agar. The growth of these isolates as linear extension from the plug was measured at several concentrations of the drug (chapter 5) after 7 and 13 d, as the distance from the inoculum to the advancing edge of the colony margin. Four radii from each of four replicate plates were measured for each drug concentration and colony growth rate calculated as both mean linear hyphal extension and relative growth rate (RGR). RGR was calculated as linear growth rate on the drug as a percentage of growth on drug-free rye A agar. Isolates with an RGR of 80% or more of the drug-free control were deemed to be resistant at that concentration. Isolates with an RGR of less than 10% were deemed to be sensitive to the drug at that concentration. Isolates with an RGR between these two values were considered to show intermediate resistance. The stability of any drug-resistant colonies was examined as described in section 2.2.8.3.

2.2.8.2 *Obtaining spontaneous drug-resistant mutants*

Hyphal plugs (10 mm^2) were removed from the colony edge of metalaxyl-sensitive isolates growing on rye A agar. These were used to inoculate plates of rye A agar amended with concentrations of streptomycin that just inhibited radial extension of sensitive isolates but would allow growth of resistant isolates (Table 5.2.5). In addition acriflavine (Sigma) blasticidin (donated by A. Dolgova) or phosphorous acid (Sigma) were used to select for mutants (Table 5.2.5). Each drug-amended plate was inoculated with eight evenly spaced plugs (10 mm^2). Isolates were incubated for 2-4 weeks after which time, plugs from any fast-growing sectors were removed to fresh media containing the drug on which the mutant was selected. After three sub-cultures on this drug, the growth rates and stability of mutant phenotypes were compared to original wild-type isolates (Figure 5.2.1)

2.2.8.3 *Stability of drug-resistant phenotypes*

Stability of the drug-resistant phenotype was assessed in two ways. Each drug-resistant isolate was purified through single-zoospore isolation to confirm that the drug-resistant phenotype could be transmitted asexually. Single-zoospore lines were also sub-cultured on drug-free media at weekly intervals for up to four weeks. At the end of each week, the mutant was challenged on media amended with a range of drug

concentrations (Figure 2.1).

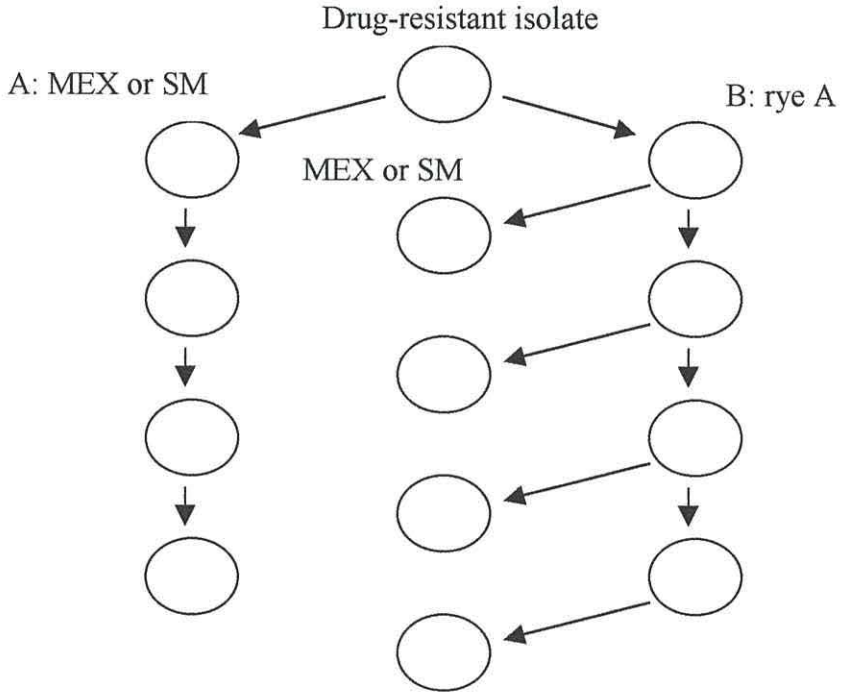


Figure 2.1. Scheme for testing the viability (A) and stability (B) of drug-resistant isolates. Each arrowhead represents a single sub-culture. Plates were inoculated centrally and growth measured from the inoculum to the advancing colony edge along 4 radii. Concentrations of metalaxyl (Mex) ranged from 10-200 μg $ml^{-1}$and concentrations of streptomycin (SM) were 10-1000 μg ml^{-1}.

2.2.9 Transformed drug-resistant isolates

In addition to mutant strains, three isolates (N18, Y1 & W1) were stable transformants to either of the antibiotics Geneticin (G418) (Judelson, 1991; van West *et al.*, 1998) or Hygromycin (van West *et al.*, 1998 & 1999). Y1 and W1 were different lines of the same isolate, transformed with hygromycin (W1) or G418 (Y1) resistance genes. N18 was transformed with the G418 resistance gene.

2.2.10 Synthesis of self-fertile colonies

Self-fertile colonies were synthesised as described previously (Fyfe & Shaw, 1992; Pipe *et al.*, in press). Plates of rye A agar were inoculated with an A1 and A2 isolate, 4 cm apart. After incubation for 10-15 d, 10 plugs of 10 mm^2 were taken from the oosporic region between the inocula and colonies were established from these on fresh plates of rye A agar. Colonies were microscopically examined for the presence of oospores and therefore the maintenance of the self-fertile phenotype. Colonies that

were self-fertile after further sub-culture were used in subsequent analysis (chapter 4).

2.2.10.1 *Stability of the self-fertile phenotype on agar*

The production of oospores and the stability of the self-fertile phenotype was examined by transferring 10 mm^2 plugs to the centre of fresh rye A agar, allowing the colonies to fill the agar plate and monitoring microscopically for the production of oospores. Progeny were classed as being self-fertile (producing oospores over the whole colony), self-sterile (producing no oospores) or intermediate (producing sectors bearing either oospores or sporangia).

2.3 DNA EXTRACTION

2.3.1 Mycelium for DNA Extraction

Petri dishes (9 cm) containing pea-broth and ¼ RAN (appendix 1) were inoculated with a 20 mm^2 plug of hyphae. Plates were incubated for approximately 10 d. Mycelium was harvested and the culture medium removed by filtering through No. 1 filter paper (Whatman) in a Buchner funnel attached to a vacuum. The mycelium was rinsed with water, placed in a 15 ml centrifuge tube and lyophilised. Two steel ball bearings (5 mm diameter) were then added, and the tubes placed into liquid nitrogen for 5 min. The tubes were then vortexed for 30 s until the mycelium was shattered into a fine powder.

2.3.2 DNA Extraction Protocol

The following methods were adapted from those of Raeder & Broda (1985). Ground, lyophilised mycelium from one Petri-dish was re-suspended in 3.3 ml DNA extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Samples were homogenised by gentle inversion and incubated at 55°C for 15 min. 2.2 ml of liquefied TRIS-buffered phenol (Fisher) was added, followed by 3 ml chloroform-isoamyl alcohol (24:1) (BDH). Tubes were then centrifuged at 15,000 rpm (Beckman) for 60 min at 4°C. The aqueous layer was removed to a fresh tube and 1 mg RNAase A (Sigma) was added to each tube followed by incubation at 37°C for 15 min. 3.3 ml chloroform-isoamyl alcohol (24:1) was then added and the tubes centrifuged at 10,000 rpm for 10 min at 4°C. To the aqueous layer, 0.54 volumes of isopropanol were added and the tubes left for up to 60 min at room temperature. Precipitated DNA was centrifuged at 10,000 rpm for 5 min at 4°C, washed in 70%

ethanol for 5 min, and centrifuged again for 5 min under the same conditions. The pellet was dried briefly, and re-suspended in 300 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA concentration was determined for each DNA sample using a dedicated DNA flourimeter (Hoefer Scientific) following the manufacturer's instructions.

2.4 MOLECULAR TESTING

2.4.1 Mitochondrial haplotypes

DNA samples for PCR were pre-diluted to 10 µg μl^{-1} in TE buffer. Griffith & Shaw (1998) describe how, using up to four pairs of primers, four mitochondrial haplotypes (Ia, Ib, IIa & IIb) can be detected in *P. infestans*. Three of these haplotypes are found in the UK and these can be distinguished using the primers P2 and P4 in a PCR-RFLP assay. PCR amplification was achieved as a multiplex reaction containing both pairs of primers. Each reaction contained 4 pM each of primers P2F, P2R, P4F and P4R (Cruachem), 200 µM dNTP, 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.75 mM $MgCl_2$, 1 U of Taq DNA polymerase, 10 ng DNA and H_2O up to a total volume of 25 µl. The reactions were then overlaid with 20 µl of mineral oil (Sigma) and subjected to 1 cycle of 94°C for 90 s then 40 cycles of 94°C for 40 s, 55°C for 60 s and 72°C for 90 s on a PCR machine (Hybaid Omnigene).

Primer-pairs P2 and P4 are predicted to give amplified products of 1070 bp and 964 bp respectively and this was checked by electrophoresis of 10 µl on a 1% agarose gel. Amplified products were then digested with *Eco*RI and *Msp*I (Promega). A further 10 µl of amplified product were digested in 25 µl volumes containing 1x Promega buffer B (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) and 2 U *Msp*I and 2 U *Eco*RI at 37°C for 3 h. Restricted products were then electrophoresed through a 1.4% agarose gel in 1 x TAE (4 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 80 V for 2-3 h and visualised under UV at 254 nm. Fragment size was determined by comparing mobility with a 100 bp marker (GIBCO BRL) and mtDNA haplotypes were determined as described previously (Griffith & Shaw, 1998).

2.4.2 RG57 fingerprinting

2.4.2.1 *Southern blotting*

8.0 µg genomic DNA was digested overnight with 8-15 U *Eco*RI in Promega buffer B (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) at 37°C, in a volume of 100 µl. DNA fragments were precipitated by mixing with 10 µl 3 M NaAc, pH 5.8, 2.5 volumes 100% ethanol, and incubating at –20°C for 2-3 h. Following centrifugation at 13,000 rpm for 15 min, pellets were washed with 50 µl 70% ethanol, dried briefly and resuspended in 20 µl TE buffer. The DNA fragments were then separated on a 0.8% agarose gel (GIBCO BRL) in 1 x TAE. DNA was then transferred to Hybond N+ membrane using a Vacugene XL vacuum blotter (Pharmacia). The membrane was then baked at 120°C for 30 min to link the DNA to the cellulose.

2.4.2.2 *RG57 probe DNA preparation*

The moderately repetitive probe RG57 has been previously cloned as a 1.2 kb fragment in P. Bluescript (Stratagene) (Goodwin *et al.*, 1992a). The probe (insert) was isolated by digesting 10 µg of plasmid with 1 U *Eco*RI in Promega buffer B (6 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) in 50 µl volumes for 3 h and electrophoresing on a 1.4% agarose gel in 1 x TAE. The probe, appearing as a 1.2 kb fragment, was dissected from the gel using a sterile scalpel and purified using Wizard Columns (Promega). The dissected gel slice was boiled in an Eppendorf tube for 5 min to completely melt the agarose and then 1 ml of resin mixture (Promega) was added and mixed for 30 s. The mixture was filtered through a Wizard Column under vacuum. 2 ml of 80% isopropanol was passed through the column, which was then dried by centrifugation at 15,000 rpm for 30 s. 50 µl of TE buffer, pre-heated to 68°C, was added to the column and the eluted DNA was collected and stored at –20°C.

2.4.2.3 *Probe labelling with digoxygenin-11-dUTP*

A Digoxygenin (DIG) DNA labeling kit (Boehringer) was used for random-primed labelling. A 15 µl volume containing 3 µg of DNA was denatured by boiling for 5 min. 2 µl of hexanucleotides, 2 µl of dNTP labelling mixture and 1 U klenow polymerase were added and the reaction mixture was incubated for 20 h at 37°C. The

reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0). The probe was precipitated with 2 µl 4 M LiCl and 60 µl of ice-cold ethanol (-20°C) for 2 h. After centrifugation at 14,000 rpm for 15 min, the supernatant was removed, the pellet washed with 70% ethanol, and resuspended in 50 µl TE buffer. Control DIG-labelling reactions and tests of the reaction efficiency were carried out as described in the product information protocol.

2.4.2.4 *Southern hybridisation*

Hybridisation followed the DIG nucleic acid detection protocol (Boehringer). 50 ml 5 x SSC pre-hybridisation buffer (0.75 M NaCl, 0.075 M NaCitrate, pH 7.0), 1% blocking reagent, 0.1% N-lauryl sarcosine, 0.02% SDS) was added to the filter containing the immobilised DNA in a hybridisation bag for 60 min at 55°C. The solution was replaced with 50 ml hybridisation solution containing 25 ng freshly denatured probe. After hybridisation at 55°C overnight, the filters were washed twice in 250 ml wash buffer (2 x SSC, 0.1% SDS) at room temperature for 15 min, then four times in 250 ml of wash buffer containing a lower salt concentration (0.1x SSC, 0.1% SDS) at 55°C for 15 min. The membrane was not allowed to dry out before the DIG detection assay.

2.4.2.5 *DIG detection Assay*

Standard immunological detection with CSPD® (chemi-luminescent alkane substrate; TROPIX) was performed following the DIG detection procedure (Boehringer). After the hybridisation washes, the membrane was equilibrated for 5 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.2 M NaOH, 0.3% Tween 20; pH 7.5) at room temperature. The filter was then incubated for 30 min in 100 ml of blocking solution (0.1 M maleic acid, 0.15 M NaCl, 0.2 M NaOH, 0.3% Tween 20, 2% blocking reagent; pH 7.5). Anti-DIG-AP conjugate was diluted to 75 U ml^{-1} in 1 x blocking solution before adding to the filter and incubating at room temperature for 30 min. The filter was then washed twice (15 min each) in 250 ml washing buffer. The membrane was equilibrated for 5 min in 250 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.1 M MgCl; pH 9.5). The membrane was incubated for a final 5 min in 25 ml CSPD diluted 1:100 in detection buffer. Excess liquid was squeezed from the top of the bag and the damp membrane was sealed in the hybridisation bag before incubation at 37°C for 15 min to enhance the luminescent reaction. The membrane

was exposed to Kodak XOMAT-AR X-ray film in an X-ray cassette for 1 h (up to 3 h). The film was developed manually using Kodak photochemicals.

2.4.3 AFLP DNA Fingerprinting

AFLP requires digestion of genomic DNA (extracted as in section 2.3) with two restriction enzymes, *Eco*RI (Promega) and *Mse*I (New England Biolabs). *Eco*RI, cuts at sites with the sequence 5'-G$^{\blacktriangledown}$AATTC-3' and *Mse*I cuts more frequently at the sequence 5'-T$^{\blacktriangledown}$TAA-3'. Oligonucleotide adaptors and primers were synthesised by GIBCO BRL (Table 2.1). The theory behind the AFLP technique is explained in section 1.2.8.

2.4.3.1 *Restriction digestion and ligation of adaptors*

0.5 μg of genomic DNA was incubated for 3 h at 37°C with 0.5 U each of *Eco*RI and *Mse*I in 20 μl volumes containing 1 x multi-core buffer (Promega) (25 mM Tris-Ac, 100 mM KAc, 10 mM MgAc, 1 mM DTT). After digestion for 3 h at 37°C, 5 pM *Eco*RI adaptor, 50 pM *Mse*I adaptor, 1 U T4 DNA Ligase (Promega) and 1 μl 10 mM ATP were added to the digest. The reaction was then incubated for a further 3 h at 37°C to allow adaptor ligation.

Table 2.1. Sequence of AFLP adaptors and PCR primers used.

Adaptors	*Eco*RI	5'-CTCGTAGACTGCGTACC 3'-CATCTGACGCATGGTTAA
	*Mse*I	5'-GACGATGAGTCCTGAG 3'-TACTCAGGACTCAT
Primers	*Eco*RI	5'-CTCGTAGACTGCGTACCAATTC(NNN)
	*Mse*I	5'-GACGATGAGTCCTGAGTAA(NNN)
Extensions	*Eco*RI	E11 + AA; E19 + GA; E54 + CCT
	*Mse*I	M14 + AT; M16 + CC; M40 + AGC

AFLP PCR primers consist of two parts: a core sequence (underlined) and an enzyme-specific sequence. These primers were used for pre-amplification (section 2.4.3.2). Selective PCR used primers of the same sequence, but with two or three nucleotide extensions (NNN) added to the 3' end of the primer. Nomenclature of these selective primers (e.g. primers E11, M14) is described in Vos *et al.* (1995).

2.4.3.2 *Pre-amplification PCR*

Each reaction contained 50 ng of both the *Eco*RI and the *Mse*I universal primers, 1 U of Taq DNA Polymerase, 200 μM dNTP, 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$ and H_2O up to a total volume of 25 μl. The pre-

amplification PCR programme consisted of 30 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. The product was checked on a 1% agarose gel (TAE) for a 0.1-1 kb smear of digested, amplified genomic DNA. 25 µl pre-amplified PCR product was mixed with 50 µl 0.1 x TE. This was known as template and was used for subsequent selective amplifications.

2.4.3.3 *Primer labelling*

The *Eco*RI selective primer was 5'end-labelled with $\gamma[^{33}P]$-ATP (Amersham) using T4 polynucleotide kinase (PNK) (GIBCO BRL). Labeling reactions contained (per sample) 3.3 ng of *Eco*RI selective primer, 1 x forward reaction buffer (50 mM imidazole HCl, pH 6.4, 12 mM $MgCl_2$, 1 mM β-mercaptoethanol, 70 µM ADT), 0.1 U T4 PNK, 0.1 µl $\gamma[^{33}P]$-ATP (50 µCi) (Amersham) and H_2O up to a final volume of 0.5 µl. Labeling was performed at 37°C for 1 h and was stopped by heating to 94°C for 2 min.

2.4.3.4 *AFLP amplification with selective primers*

Each PCR reaction contained 17 ng of the *Mse*I selective primer, 200 µM dNTPs, 1 U Taq DNA polymerase, 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) and 2.5 mM $MgCl_2$, 0.5 µl of labeled *Eco*RI primer, 2 µl of template DNA and H_2O up to a final volume of 10 µl. The selective amplification PCR programme consisted of 1 cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 60 s followed by 11 cycles over which the annealing temperature was decreased by 0.7°C per cycle, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

After PCR, samples were immediately mixed with 10 µl of formamide loading buffer (98% formamide, 10mM EDTA, 1% bromophenol blue and 1% xylene cyanol) and denatured at 94°C for 120 s.

2.4.3.5 *Polyacrylamide electrophoresis*

Polyacrylamide gels contained 6% polyacrylamide (National Diagnostics) and 7.5 M urea in 1 x TBE (90 mM Tris-borate, 2 mM EDTA). To catalyse gel matrix formation, 150 µl of 25% ammonium persulphate (Sigma) and 80 µl TEMED (Sigma) were added to 100 ml of gel solution. Gels were immediately cast using SequiGen gel apparatus (BIORAD). The gel was pre-run for an hour in 1 x TBE to achieve a gel

temperature of 50°C. 4-10 µl of each denatured sample was then loaded onto the gel depending on the age of the radioactive label. Electrophoresis was performed at 90-100 W (50°C constant temperature) for between 3.5 and 4 h. The gel was dried onto Whatman 3 MM paper using a vacuum gel dryer (BIORAD) at 80°C and exposed to high-sensitivity Kodak XOMAT-AR film for 24–96 h. Films were developed manually using Kodak photochemicals.

2.4.3.6 *Nomenclature of AFLP markers*

The sizes of the amplified bands were estimated by comparing mobility with *Msp*I restricted pBR322 (GIBCO BRL) labeled as in section 2.4.3.3 in an exchange reaction. No faint bands were included in any analysis. AFLP markers were designated by the primer combination that was used to amplify the fragment and amplified bands were numbered from 1 (smallest) upwards. The approximate sizes of all polymorphic bands identified in the present study can be found in appendix Table A3.1 and characteristic appearance of the bands can be found in appendix Figures A3.1 and A3.2.

2.5 DATA ANALYSIS

For estimating the relatedness of isolates (chapter 3), monomorphic loci were excluded from the analysis of the AFLP data (but not from the RG57 data). The AFLP data from two (or six) primer pairs were combined and an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis was performed based on the Jaccard's coefficient (Sokal & Sneath, 1963) using the SAHN algorithm of the statistical package NTSYS-PC, release 1.80 (Rohlf, 1993). Dendrograms (trees) showing similarity amongst isolates were generated from these coefficients using the TREE program of NTSYS-PC. Mating type and mtDNA haplotype data were not used in constructing the dendrograms.

3 Investigating the usefulness of AFLPs for analysis of genetic variation in field isolates of *Phytophthora infestans*

3.1 INTRODUCTION

In Mexico, *Phytophthora infestans* exhibits a high level of molecular variation, apparently as a result of sexual reproduction (Tooley *et al.*, 1985; Goodwin *et al.*, 1992b; Fry & Goodwin, 1995). Migrations of A1 and A2 mating types from Mexico to other parts of the world beginning in the late 1970s (section 1.3.2) have resulted in populations with differing levels of variation (section 1.3.3; Goodwin *et al.*, 1994). This has been demonstrated using isozyme markers (Spielman *et al.*, 1991; section 1.2.4) and a multi-locus RFLP fingerprinting probe, RG57 (section 1.2.7).

Populations in Mexico are highly polymorphic for RG57 and it is common to find many isolates with unique RG57 fingerprints in a single field (section 1.3.1; Goodwin *et al.*, 1992b). In contrast, most populations from commercial fields in the USA are monomorphic for RG57, isozymes and mating-type, and several strains (thought to be clonal lineages) are distributed widely throughout North America (section 1.3.3.2; Goodwin *et al.*, 1994; Goodwin *et al.*, 1995). The population structure in Europe seems to be intermediate to those of Mexico and the USA; many RG57 fingerprints are detected and certain of these occur widely and re-occur in different seasons (section 1.3.3.1). In the UK, there is evidence that isolates with certain RG57 genotypes are widely distributed; genotypes such as RF39 and RF26 (appendix 2) were detected during each of three years at many sites throughout England and Wales during 1995-1998 (Day *et al.*, unpublished). Indeed, 46% of the 2614 isolates sampled over the three years were of the RF39 genotype (Day *et al.*, unpublished) even though 51 genotypes were identified in total. An intensive study in North Wales revealed 68 RG57 genotypes, some of which were unique and occurred at only one site in a single season (N. Pipe, unpublished). Similar findings have been reported for other European countries (Drenth *et al.*, 1994; Sujkowski *et al.*, 1994; Lebreton & Andrivon, 1998; Brurberg *et al.*, 1999; Zwankhuizen *et al.*, in press).

The RG57 probe (section 1.2.7) has been used extensively since 1992 to investigate the genetic diversity of *P. infestans* populations in many countries (Forbes *et al.*, 1998). However, when using molecular markers to detect genetic variability, it is

important to establish whether different molecular markers give rise to consistent conclusions regarding the genetic similarity of isolates. Individuals with identical fingerprints using one technique are not necessarily identical or clonal, there is just no evidence to say they are different. Therefore, fingerprinting results obtained may depend on the regions of the genome targeted by the particular technique and different techniques could produce different conclusions relating to the similarity of a group of isolates. For instance, isozyme and mating type analysis of 20 isolates indicated that they had identical genotypes but fingerprinting 18 of these isolates with RG57 revealed 12 dissimilar genotypes (Fry *et al.*, 1992). Isolates with identical RG57 fingerprints could also be distinguished from each other using a telomeric probe (Pipe & Shaw, 1997). A further sample of 18 US-1 isolates from Germany with no known isozyme or RFLP polymorphisms revealed 49 polymorphic AFLP bands suggesting divergence within a clonal lineage (Kamoun *et al.*, 1998).

3.1.1 Aims

RG57 often gives too few polymorphisms between any two isolates to detect somatic or sexual recombination. AFLP can potentially reveal as many polymorphisms as required by fingerprinting with as many primer-pairs as needed. The aim of this chapter was to determine whether conclusions drawn about relatedness of isolates based on their RG57 fingerprints were supported using the AFLP technique. In doing this, the usefulness of particular AFLP primers for detecting somatic recombination (variation) between any two isolates could also be estimated.

3.2 RESULTS AND DISCUSSION

3.2.1 Choice of isolates

Ninety-eight isolates were chosen from a core collection of single-lesion isolates collected between 1995-1997 (Table 3.2.1).

Table 3.2.1. Description of 98 isolates used for RG57 AFLP comparison. The RF numbers refer to the RG57 genotype designation as in Figure 3.2.1 and the AF numbers refer to AFLP genotype designation as in Figure 3.2.2.

Number	Bangor code	Origin	Mating type	mtDNA	RG57 type (RF)	AFLP type (AF)
1	96.126.16	Bryn Farm	A1	Ia	40	01
2	96.126.18	Bryn Farm	A1	Ia	40	01
3	96.126.5	Bryn Farm	A2	Ia	26	01
4	96.70	Beaumaris	A2	Ia	40	01
5	95.205.20	Pen y Ffridd	A2	Ia	40	01
6	96.89.41	Pant y Ddolen	A2	Ia	34	02
7	95.206	Norfolk	A2	Ia	65	02
8	96.90.29	Siambra Gwynion	A2	Ia	30	03
9	96.126.23	Bryn Farm	A2	Ia	40	03
10	96.89.40	Pant y Ddolen	A2	Ia	34	04
11	96.52.12	Siambra Gwynion	A2	Ia	40	04
12	96.54.5	Siambra Gwynion	A2	Ia	40	04
13	96.51.1	Siambra Gwynion	A2	Ia	40	04
14	96.91.2	Pant y Ddolen	A2	Ia	40	04
15	93.82.5	Cambridge	A2	Ia	42	05
16	96.93.22	Bryn Farm	A2	Ia	34	06
17	96.54.27	Siambra Gwynion	A2	Ia	30	07
18	96.126.35	Bryn Farm	SF	Ia	32	08
19	82.18.2	Brynsiencyn	A2	Ia	64	09
20	97.274.19	Siambra Gwynion	A1	IIa	11	10
21	97.283.7	Siambra Gwynion	A1	IIa	06	10
22	96.113	Aberteifi, Cardigan	A1	Ia	39	10
23	96.88.1	Caernarfon	A1	IIa	06	10
24	96.42.1	Hampshire	A1	IIa	06	10
25	96.93.24	Bryn Farm	A1	IIa	09	10
26	96.154.23	Rhiw Goch	A1	IIa	06	10
27	97.312.1	Rhiw Goch	A1	IIa	39	11
28	97.358.3	Pant y Ddolen	A1	IIa	03	11
29	97.358.7	Pant y Ddolen	A1	IIa	11	11
30	97.284.4	Llanfairfechan	A1	IIa	06	11
31	82.6.6	Brynsiencyn	A2	IIa	08	11
32	96.11.2	Yorkshire	A1	IIa	06	11
33	96.93.6	Bryn Farm	A1	IIa	06	11
34	96.93.5	Bryn Farm	A1	IIa	06	11
35	96.126.40	Bryn Farm	A1	IIa	06	11
36	96.96.1	Carneddi	A1	Ia	11	11
37	96.127.15	Siambra Gwynion	A1	IIa	11	11
38	96.126.43	Bryn Farm	A1	IIa	11	11
39	96.127.5	Siambra Gwynion	A1	IIa	12	11
40	96.126.41	Bryn Farm	A1	IIa	06	12
41	96.154.24	Rhiw Goch	A1	IIa	22	13
42	96.104.2[#]	Bryn Hethog	A1	IIa	48	14
43	96.102.6	Tregarth	A2	IIa	13	15
44	96.91.3	Pant y Ddolen	A2	Ia	26	16
45	96.126.17	Bryn Farm	A2	Ia	26	17
46	95.190.1	Kent	A1	Ia	39	17

47	96.154.20	Rhiw Goch	A1	Ia	26	18
48	96.91.25	Pant y Ddolen	A1	Ia	34	18
49	96.148.1	Siambra Gwynion	A1	Ia	26	18
50	96.89.31	Pant y Ddolen	A1	Ia	26	18
51	96.95.18	Tyddyn y Berth	A1	Ia	33	18
52	96.110.49	Tyddyn y Berth	A1	Ia	18	19
53	96.93.8#	Bryn Farm	A1	Ia	26	20
54	96.110.11	Tyddyn y Berth	A1	Ia	35	20
55	96.95.19	Tyddyn y Berth	A1	Ia	33	20
56	96.95.11	Tyddyn y Berth	A1	Ia	33	20
57	96.110.21	Tyddyn y Berth	A1	Ia	32	20
58	96.89.43	Pant y Ddolen	A1	Ia	32	20
59	96.95.25	Tyddyn y Berth	A1	Ia	26	20
60	96.110.4	Tyddyn y Berth	A1	Ia	26	20
61	96.93.21	Bryn Farm	A1	Ia	36	20
62	96.95.4	Tyddyn y Berth	A1	Ia	28	21
63	96.89.35	Pant y Ddolen	A1	Ia	27	22
64	96.127.7	Siambra Gwynion	A1	Ia	31	22
65	96.91.24	Pant y Ddolen	A1	Ia	26	22
66	96.130.6	Abergwyngregyn	A1	Ia	31	23
67	96.105.1	Pentir	A1	Ia	37	23
68	96.110.56	Tyddyn y Berth	A1	Ia	26	23
69	97.312.21	Rhiw Goch	A1	IIa	39	24
70	96.60.1	Somerset	A1	Ia	39	24
71	95.147.1	Aberystwth	A1	Ia	39	24
72	96.11.3	Yorkshire	A1	IIa	39	24
73	95.83	Cornwall	A1	Ia	39	25
74	96.159a	Exeter	A1	Ia	39	26
75	96.133.1a	Nottingham	A1	Ia	39	27
76	96.76	Llandona	A1	Ia	39	27
77	96.74	Beaumaris	A1	Ia	39	27
78	95.207	Norfolk	A1	Ia	39	27
79	96.175	Wiltshire	A1	Ia	39	27
80	96.36.1	Hampshire	A1	Ia	39	28
81	96.161.1	Hampshire	A1	Ia	39	28
82	96.102.7	Tregarth	A1	Ia	39	29
83	96.89.45	Pant y Ddolen	A1	Ia	32	30
84	96.90.33	Siambra Gwynion	A1	Ia	37	31
85	96.110.36	Tyddyn y Berth	A1	Ia	50	32
86	96.110.34	Tyddyn y Berth	A1	Ia	52	32
87	96.110.48	Tyddyn y Berth	A1	Ia	52	33
88	96.110.43	Tyddyn y Berth	A1	Ia	42	34
89	96.95.16	Tyddyn y Berth	A1	Ia	49	34
90	96.110.38	Tyddyn y Berth	A1	Ia	52	35
91	96.110.47	Tyddyn y Berth	A1	Ia	49	36
92	96.149a	Surrey *	A2	Ia	67	37
93	96.95.6	Tyddyn y Berth	A1	Ia	41	38
94	96.110.12	Tyddyn y Berth	A1	Ia	02	39
95	96.101.5	Siambra Gwynion	A1	Ia	05	40
96	95.162.15	Rhiw Goch	A2	IIa	01	41
97	95.164.2	Rhiw Goch	A2	Ia	01	42
98	95.189.1	Siambra Gwynion *	A2	Ia	45	43

Origins and genotypes of isolates used. Abergwyngregyn, Beaumaris, Bryn Farm, Bryn Hethog, Brynsiencyn, Caernarfon, Llandona, Llanfairfechan, Pant y Ddolen, Pen y Ffridd, Pentir, Rhiw Goch, Siambra Gwynion, Tregarth and Tyddyn y Berth are sites within a 50 km radius in North Wales. SF, self-fertile. * Isolates collected from tomato. RG57 (RF) fingerprints can be found in appendix Table A2.1. AFLP (AF) fingerprints can be found in appendix Table A3.2.

Forty isolates with the same RG57 fingerprint pattern (RF39) were chosen primarily from isolates collected in England and Wales during 1997 (Table 3.2.2).

Table 3.2.2. Description of isolates with RG57 fingerprint pattern RF39 used for AFLP RG57 comparison. Isolate numbers are the same as those in Figure 3.2.4.

Isolate		Geographic origin
Number	Bangor Code	
01	97.43.1	Farnsfield, Nottinghamshire
02	97.248.5	Penbronydd, Tregarth
03	97.247.4	Llandegai
04	97.250.35	Old Rectory, Abergwyngregyn
05	97.282.1	Village, Abergwyngregyn
06	97.250.23	Old Rectory, Abergwyngregyn
07	97.325.5	Bryn Farm
08	97.361.3	Pentir
09	97.46.1	Clipstone, Nottinghamshire
10	97.146.1	Siambra Gwynion
11	97.147.1	Tyddyn y Berth
12	97.241.1	Pant y Ddolen
13	97.250.34	Old Rectory, Abergwyngregyn
14	97.303.1	Spain.
15	97.347.1	Pen y Ffridd
16	97.250.22	Old Rectory, Abergwyngregyn
17	97.250.37	Old Rectory, Abergwyngregyn
18	97.283.10	Llanfairfechan
19	97.351.1	Port Dinorwic
20	97.250.38	Old Rectory, Abergwyngregyn
21	97.250.28	Old Rectory, Abergwyngregyn
22	97.205.11	Old Rectory, Abergwyngregyn
23	97.250.39	Old Rectory, Abergwyngregyn
24	97.250.26	Old Rectory, Abergwyngregyn
25	97.250.18	Old Rectory, Abergwyngregyn
26	97.353.1	Bethesda
27	97.250.16	Old Rectory, Abergwyngregyn
28	97.250.9	Old Rectory, Abergwyngregyn
29	97.250.40	Old Rectory, Abergwyngregyn
30	97.48.1	Hayle, Cornwall
31	97.356.1	Tregarth
32	97.250.25	Old Rectory, Abergwyngregyn
33	97.52.4	St. Mary's, Isles of Scilly
34	97.267.1	Selbourne, Hampshire
35	97.81.1	Monnington, Hampshire
36	97.82.3	Taylor, Hampshire
37	97.250.33	Old Rectory, Abergwyngregyn
38	97.249.5	Bryn Cul, Tregarth
39	97.295.1	Village, Abergwyngregyn
40	97.312.1	Rhiw Goch

Origin of isolates used. Abergwyngregyn, Bethesda, Bryn Farm, Llanfairfechan, Pant y Ddolen, Pen y Ffridd, Pentir, Rhiw Goch, Llandegai, Port Dinorwic, Siambra Gwynion, Tregarth and Tyddyn y Berth are sites within a 50 km radius in North Wales. All isolates A1 mating type and all type Ia mtDNA except sample 40 (Rhiw Goch), type IIa. The RF39 fingerprint pattern can be found in appendix Table A2.1. The AFLP fingerprints of these isolates can be found in appendix Table A3.3.

Isolates were selected for comparison of RFLPs and AFLPs primarily on the basis of their previously determined RG57 genotypes (J. Day & N. Pipe, unpublished). The 98 isolates (Table 3.2.1) represented 35 different RG57 genotypes (prefixed "RF" for

RFLP Fingerprint using RG57"). The fingerprint patterns of these "RF" genotypes are shown in appendix Table A2.1. Multiple isolates were included from each of the RG57 genotypes RF06, RF26, RF39 and RF40 since these are common genotypes found in successive years throughout England and Wales. Other RF genotypes chosen for the study have been observed at only a few sites (e.g. RF34, found at two sites) or a single site (e.g. genotypes RF41 and RF45) (N. Pipe, unpublished). The selection included isolates of both mating-types and mitochondrial DNA (mtDNA) haplotypes Ia and IIa (Carter *et al.*, 1990). A further 40 isolates with the same RF39 fingerprint (Table 3.2.2) were chosen to investigate the extent of variation in an apparently clonal lineage of the fungus and to compare AFLP variation amongst isolates with the same RG57 fingerprint from the same population and from widely scattered populations.

3.2.2 Similarity of RG57 genotypes

Nineteen loci were detected amongst the 98 isolates with the RG57 probe, of which 13 (68%) were polymorphic. UPGMA analysis (section 2.5) using the RG57 data showed that the four common genotypes were less similar to each other than they were to some of the less common genotypes (Figure 3.2.1). For example, genotype RF26 clustered on the UPGMA tree with genotypes RF27, RF28 and RF31 which differ from each other by only one band, whereas the four common genotypes differ from each other at from two to six bands (Table 3.2.3).

Table 3.2.3. RG57 fingerprints of the four most common RF genotypes. "1" and "0" indicate presence and absence of bands 1-25 recognised by the RG57 probe (Goodwin *et al.*, 1992a). The 13 underlined bands were polymorphic amongst the 98 isolates.

RG57 genotype	Fingerprint
RF06	10001 00011 00110 10001 10011
RF26	11101 11011 00110 10011 11011
RF39	10101 11111 00110 10011 11011
RF40	11101 01001 00110 10011 11011

3.2.3 Similarity of AFLP genotypes using two or six primer-pairs

The 98 isolates were fingerprinted with AFLP primers E19-M16 and E19-M40. Fifty-eight of these isolates were fingerprinted with a further four AFLP primer-pairs (Table 3.2.4). A total of 142 scorable bands were amplified with E19-M16 and E19-M40 from the 98 isolates and 24 of these were polymorphic (Table 3.2.4). Using a subset of 58

isolates and six AFLP primer-pairs, a further 157 loci could be scored and a further 33 (a total of 57) loci were polymorphic.

Primer-pairs E19-M16 and E19-M40 resolved 43 AFLP genotypes (prefixed "AF" for "AFLP Fingerprint") amongst the 98 isolates (Table 3.2.1). UPGMA analysis revealed a tree (Figure 3.2.2) in which the primary branches were more clearly defined compared to the RG57 tree (Figure 3.2.1). Twenty-seven AFLP fingerprints were unique to individual isolates and six groups of isolates were identified which contained five or more individuals with identical AFLP fingerprints. Ten of the isolates with unique AFLP genotypes also had unique RG57 genotypes (e.g. isolates 93 and 98 with genotypes RF41, RF45 respectively).

Table 3.2.4. Number of bands amplified and the proportion of polymorphic bands for each primer pair used in the AFLP analysis of 98 (58) isolates of numerous "RF" genotypes.

Primer-pair	Extension bases (primer 1 + primer 2)	Number of isolates	Total no. bands amplified	Number of polymorphic bands	Polymorphic bands as % of total
E19 - M16	2 + 2	98	80	8	10
E19 - M40	2 + 3	98	62	16 (21)*	26 (34)*
E19 - M14	2 + 2	58	38	7	18
E54 - M16	3 + 2	58	27	9	33
E11 - M16	2 + 2	58	65	5	8
E54 - M14	3 + 2	58	46	7	15

* 21 polymorphic bands could be scored in the subset of 58 isolates but only 16 could be accurately scored throughout the total sample of 98. Isolates fingerprinted with six primer-pairs are in Figure 3.2.3.

UPGMA analysis using the 57 polymorphic loci from six primer-pairs produced a highly structured tree (Figure 3.2.3) in which all but three isolates (10, 11 & 14) had a unique AFLP fingerprint. The use of extra AFLP primers demonstrated that increasing the number of primer-pairs enables more polymorphisms, if they are there, to be detected. However, the overall topology of the AFLP trees with two (Figure 3.2.2) or six (Figure 3.2.3) primer-pairs were similar when clusters corresponding to mtDNA and mating type were examined. This suggests that in subsequent analyses it may only be worthwhile fingerprinting isolates with no more than two AFLP primer-pairs. Consequently, two primer-pairs were used for analysis of the total sample of 98 isolates and unless otherwise stated, the following discussion relates to this data (Figure 3.2.2) and not that using six primer-pairs (Figure 3.2.3).

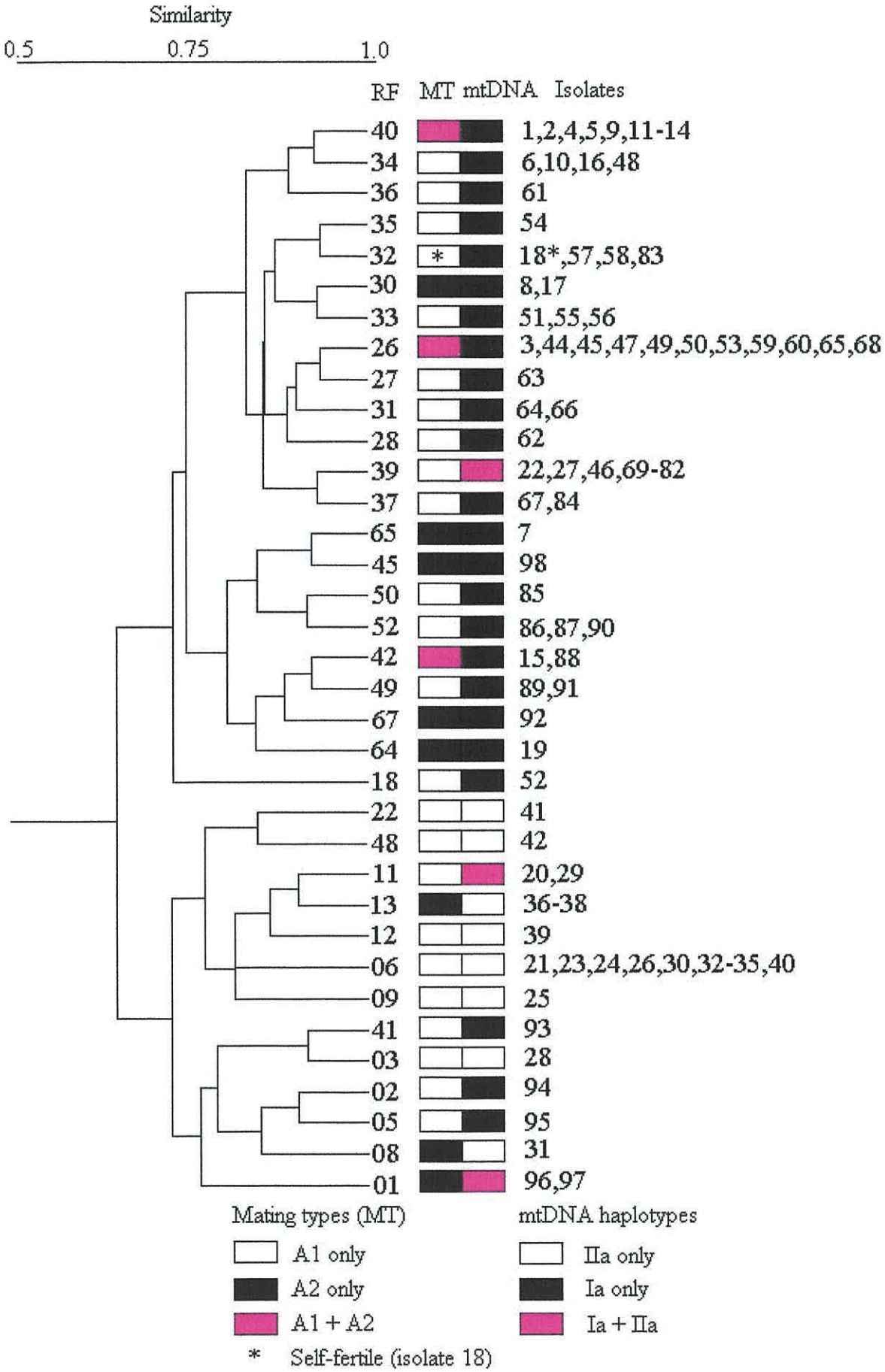


Figure 3.2.1. UPGMA tree constructed for 98 isolates using 25 RG57 loci. The RF and isolate numbers are as described in Table 3.2.1.

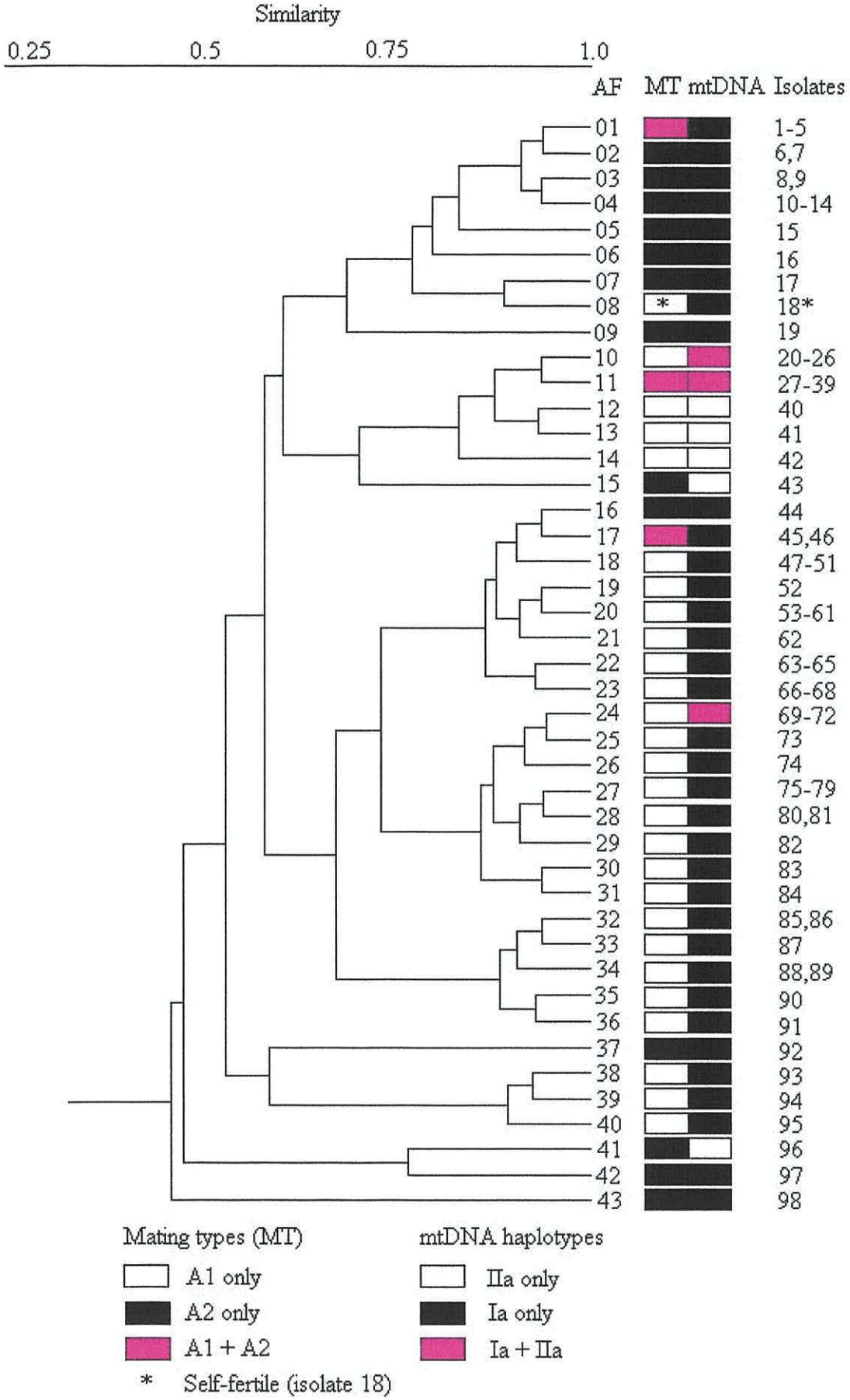


Figure 3.2.2. UPGMA tree constructed for 98 isolates using AFLP fingerprints from two primer-pairs. The Isolate numbers indicated are as described in Table 3.2.1.

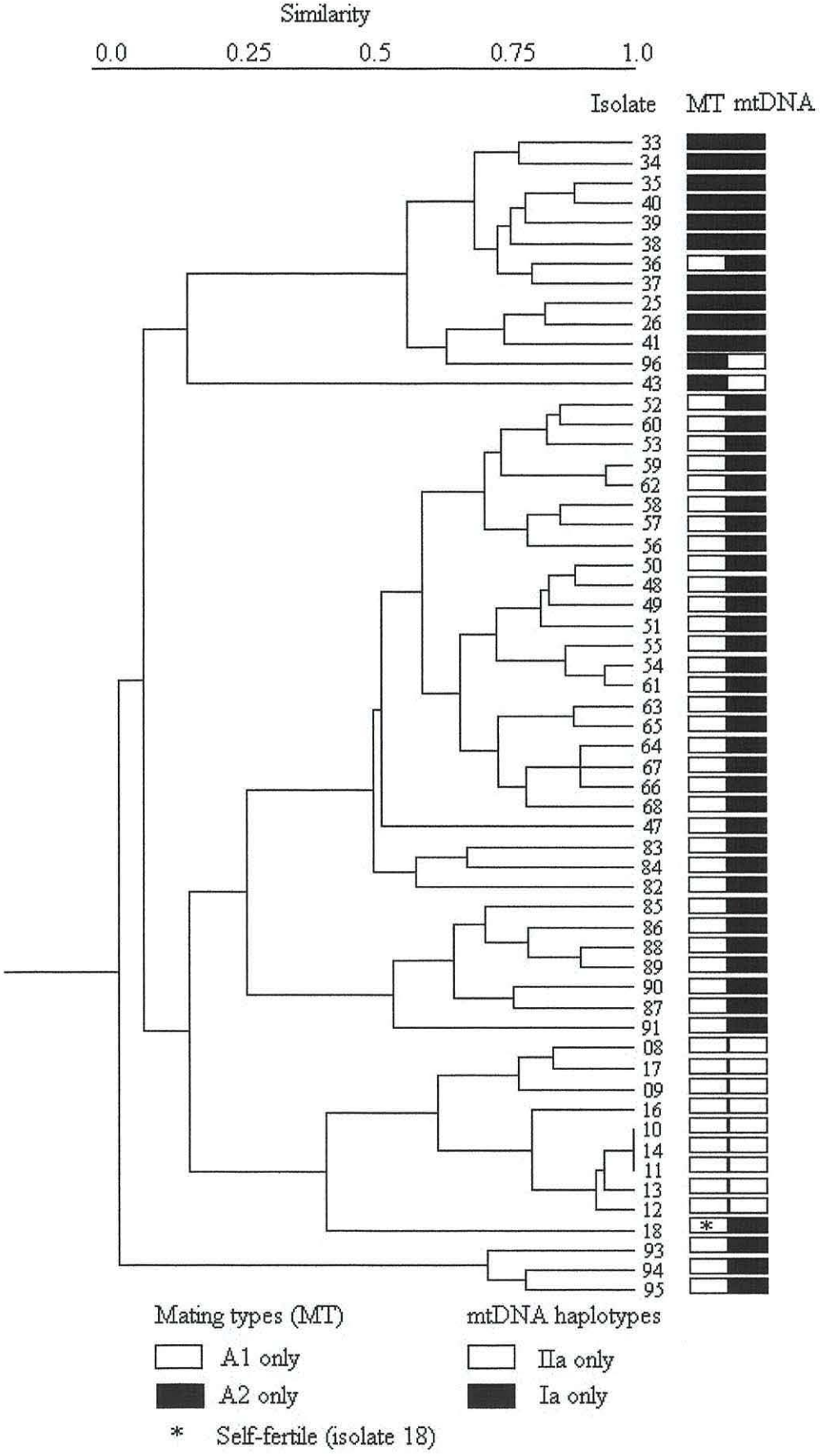


Figure 3.2.3. UPGMA tree constructed for 58 isolates using AFLP fingerprints from six primer pairs. The Isolate numbers indicated are as described in Table 3.2.1.

3.2.4 Comparison of AFLP and RFLP analyses

Phenetic and taxonomic studies comparing AFLPs and RFLPs in plants (Ma & Lapitan, 1998) and bacteria (Clerc *et al.*, 1998) have shown them to be generally in good agreement. The working hypothesis in the present study was that isolates with identical RG57 fingerprints would have AFLP fingerprints which, if not the same, were at least closely related. Although some isolates with identical RG57 fingerprints were found to also be identical or very similar using AFLP analysis, others showed variation at up to 10 of the 24 polymorphic loci scored. The levels of AFLP variation within each of the four common RG57 genotypes are summarised in Table 3.2.5. For example, the ten isolates of RG57 genotype RF06 divided into only three closely related AFLP genotypes (AF10–12) which differed from each other at no more than two AFLP loci (Table 3.2.5; Figure 3.2.2).

Table 3.2.5. Origins and characteristics of isolates having one of the four common RG57 genotypes from the sample of 98 isolates (Table 3.2.1) and the number of AFLP genotypes amongst isolates having the same RG57 genotype

RG57 Genotype	Number of isolates	Geographical origin	No. of sites	mtDNA	Mating type	No. AFLP genotypes
RF06	10	England & Wales	7	IIa	A1	3
RF26	11	North Wales	5	Ia	A1 + A2	7
RF39	17	England & Wales	16	Ia & IIa	A1	9
RF40	9	North Wales	5	Ia	A1 + A2	3

In contrast, the 17 RF39 isolates, from 16 sites, were split into nine AFLP genotypes. Six of these genotypes (comprising 14 of the isolates) clustered on the AFLP tree (AF24 – AF29) (Figure 3.2.2) and differed from each other at no more than four AFLP loci. The remaining three RF39 isolates (22, 27 & 46) appeared in more distant parts of the tree. A similar result was observed for the 11 RF26 isolates from North Wales. Ten of these isolates clustered into six AFLP genotypes (AF16, AF17, AF18, AF20, AF22 and AF23) (Figure 3.2.2) differing at not more than three AFLP loci. However, the eleventh isolate (isolate 03) was quite different, having genotype AF01 and differing from the other RF26 isolates by up to eight bands.

Isolates with closely related RG57 genotypes sometimes had closely related AFLP fingerprints. For example, the 11 isolates of genotype RF26 clustered on the RG57

tree with four isolates (62, 63, 64, 66) of three closely related RG57 genotypes (Figure 3.2.1). These four isolates also clustered with the main group of RF26 isolates on the AFLP tree (Figure 3.2.2). Three isolates had identical AFLP genotypes using 57 AFLP loci (Figure 3.2.3). These isolates were of RF40 or RF34 genotypes, and also clustered on the RG57 tree as they differed from each other at only one RG57 band. However, AFLP analysis also identified other isolates, such as isolates 48 & 61, more distantly related to RF26 by RG57 fingerprinting (Figure 3.2.1), which clustered with the RF26 isolates in the AFLP tree (Figure 3.2.2).

There was no clear relationship between the similarity of isolates and the location of the sites from which they were collected. Of four RF06 isolates with the same AFLP fingerprint (AF10), three were from North Wales whilst the fourth was from the south of England. Similarly, three RF06 isolates collected at the same site in Wales (Bryn Farm) had the same AFLP genotype (AF11) but this was also shared by an isolate from Yorkshire. In the most extreme example, five RF39 isolates of the same AFLP genotype (AF27) were collected from widely scattered sites up to 400 km apart in England and Wales. The AFLP analysis also detected variation amongst isolates with the same RG57 fingerprint collected at the same site; for example, the RF26 isolates 44 and 50 were collected from Pant y Ddolen but had different AFLP fingerprints.

The occurrence of AFLP variation among isolates with the same RG57 genotype may be due to mutations accumulating in clonal lineages, or convergence of RG57 fingerprints (recombination between isolates of RF26 and RF39 could yield some RF40 progeny). The convergence of RFLP fingerprints is well known in populations of other fungi (Kohli & Kohn, 1998) and is a problem when using a single RFLP probe. It is less likely that AFLP genotypes converge in a similar way since the probability of convergence using more polymorphic loci would be much less. From RG57 population data, Zwankhuizen *et al.* (in press) calculated the probability of certain RG57 fingerprint patterns being generated through convergence. They suggested that isolates collected from different areas of The Netherlands with the same RG57 fingerprints were likely to have the same RG57 fingerprint through convergence as these isolates differed at 14 or more AFLP loci. AFLP analysis of 55 US-1 genotypes from Eurasia, America and Africa (Kamoun *et al.*, 1998) with no known isozyme or RFLP polymorphisms revealed 29 AFLP genotypes. A further 18 US-1 isolates from Europe were fingerprinted with four

AFLP primer-pairs revealing 46 polymorphic AFLP bands amongst the clonal lineage. The observation in the present study that isolates with the same RG57 fingerprint can have similar or sometimes quite dissimilar AFLP fingerprints (e.g. RF39, above) agrees with previous data (Zwankhuizen *et al.*, in press). It is possible that isolates which have the same or similar RG57 and AFLP genotypes are clones that have accumulated mutations detected by AFLP. However, those that share RG57 genotypes but have quite different AFLP genotypes have the same RG57 fingerprint as a result of convergence through sexual or asexual recombination.

3.2.5 Similarity of AFLP fingerprints from 40 isolates of the RF39 genotype

To investigate isolates with the same RG57 fingerprint further, 40 isolates of RF39 (Table 3.2.2) were fingerprinted for AFLPs. 139 AFLP bands could be scored with two primer pairs and 17 of these were polymorphic, dividing the 40 isolates into 16 genotypes (Figure 3.2.4) on UPGMA analysis. Of these 16 genotypes, 10 were unique, three were represented by two isolates and a further three genotypes contained five or more isolates. Of the 26 sites represented by the forty isolates, only eight were not in North Wales (sites in Hampshire, Cornwall & Nottinghamshire, UK and one site in Spain, Table 3.2.2; Figure 3.2.4). Replicate samples were obtained only from the Old Rectory population, Abergwyngregyn, North Wales, where 16 isolates were sampled.

The distribution of the 40 isolates throughout the tree (Figure 3.2.4) suggested there was no clear distinction between isolates from North Wales and isolates from elsewhere. Seven AFLP genotypes were identified amongst the 16 isolates from Abergwyngregyn, and three of these genotypes were represented by more than one isolate (7, 3 & 2 isolates respectively). Isolates 13, 16 & 17 from Abergwyngregyn, had the same AFLP fingerprint as isolates from Nottinghamshire (09) and Spain (14). Most of the isolates (1-39) had similar AFLP fingerprints, differing from each other at from 0-4 loci. Indeed, isolates 1-27 only differed from each other at 1 AFLP locus and are probably representative of the RF39 clonal lineage, especially as the Spanish isolate was included in this group (isolate 14). However, isolate 40, from Rhiw Goch, North Wales, revealed a vastly different fingerprint from all other isolates, differing from them at up to 13 of the 17 polymorphic bands. This isolate could be a product of convergence as discussed above (section 3.2.4). If the majority of these RF39 isolates are representatives of a widely dispersed, clonal lineage, it is possible that 25 years have elapsed since

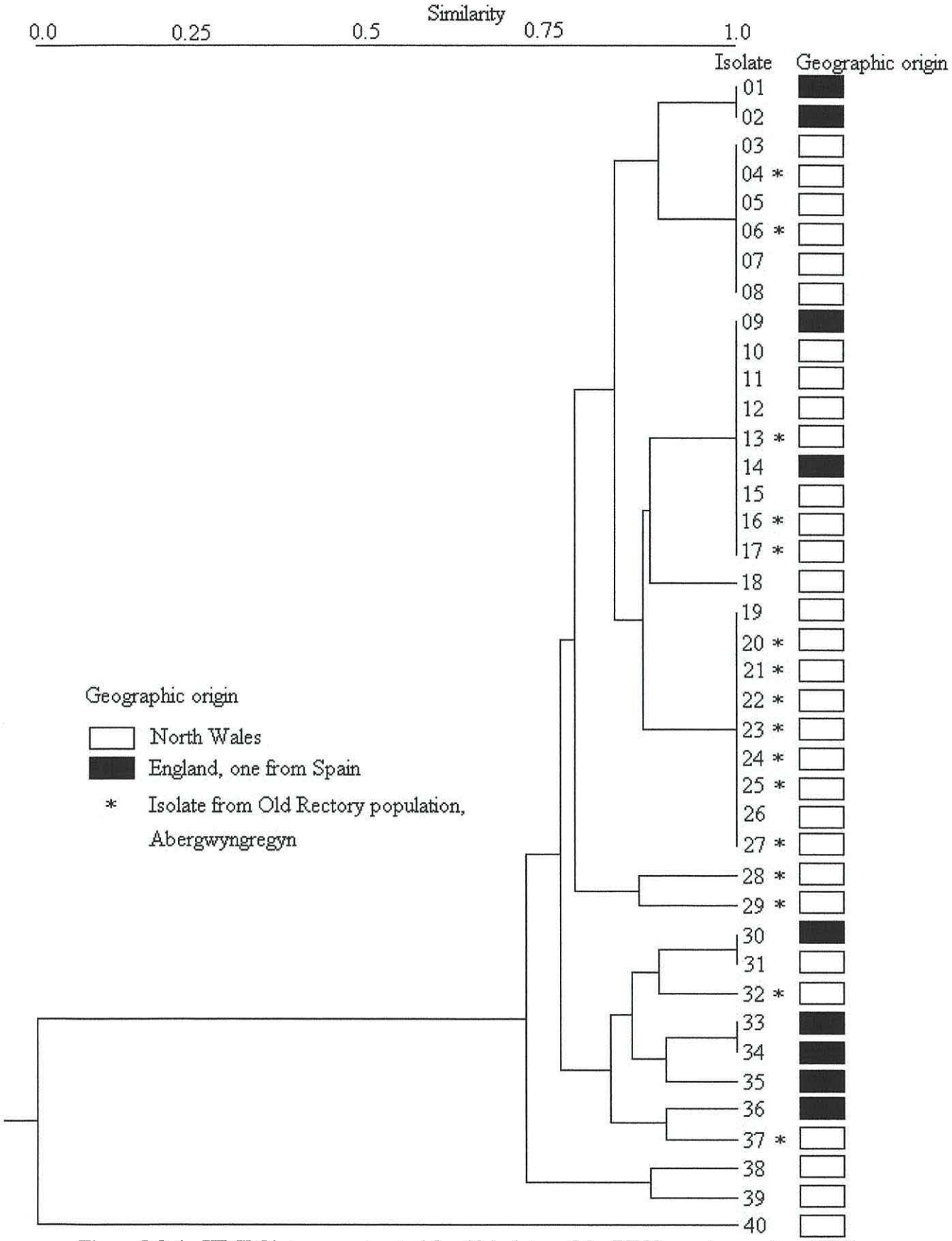


Figure 3.2.4. UPGMA tree constructed for 40 isolates of the RF39 genotype using AFLP fingerprints from two primer-pairs. The isolate numbers indicated are as described in Table 3.2.2. Isolates from North Wales and other areas are indicated, as are isolates from the Abergwyngregyn population. All isolates A1 mating type. Isolates 1-39 mtDNA type Ia, isolate 40 type IIa.

isolates had a common ancestor (Shattock *et al.*, 1990) and mutations, resulting in AFLP variation, could have become established in populations.

3.2.6 Similarity of isolates with the A2 mating type

The 25 isolates of A2 mating type included in the sample of 98 isolates (Table 3.2.1) were distributed throughout the RG57 tree (Figure 3.2.1) in 12 genotypes, four of which also included A1 isolates. Although there was some clustering of A2 genotypes (RF45, RF64, RF65, RF67), this branch also included three genotypes containing only A1 isolates and one containing both mating-types. The AFLP tree showed a more obvious clustering pattern in relation to the mating type of the isolates (Figure 3.2.2). Sixteen of the AFLP genotypes contained isolates of A2 mating type and only three of these also included A1 isolates. A striking feature of the AFLP tree was that 17 of the 25 A2 isolates formed a major branch (AF1-AF9) in which only genotype AF01 included isolates of both mating types. Two A2 isolates (31, 43) were among 44 isolates in the adjacent branch (AF10-AF15) and two further A2 isolates (44, 45) were in the largest AFLP branch (AF16 – AF36). Similarly, using six primer-pairs and 57 loci, all 12 A2 isolates formed a tight cluster (Figure 3.2.3) similar to the data using two primer-pairs (Figure 3.2.2).

Mating-type in *P. infestans* appears to be determined by a single locus with the A1 being the heterozygote and the A2 the homozygote (Judelson *et al.*, 1995; Judelson, 1997a; section 1.2.1). Since no RG57 bands show evidence of linkage with mating type (Goodwin *et al.*, 1992a), the mating type locus would be expected to segregate independently of most markers in populations of *P. infestans* undergoing regular sexual outcrossing. Under such circumstances, one would expect to find isolates of both mating types with the same or related RG57 fingerprints. That most A2 isolates clustered away from the A1 isolates on the AFLP tree suggests that A1 and A2 isolates are not recombining freely with each other, despite the fact that at sites where the A2 mating type was detected, A1 isolates were also present. Although oospores have been observed *in planta* at sites containing both mating types (Drenth *et al.*, 1995; J. Day, unpublished), it is possible that sexual progeny are less fit than those arising asexually, or that observed oospores are not viable or are non-hybrid.

Exceptional A2 isolates such as 44 and 45 which did not cluster with the majority of the A2 isolates may have an alternative origin. Whilst it is possible that these are the

products of sexual recombination, an alternative hypothesis is that A1 isolates heterozygous at the mating type locus have mutated and/or undergone mitotic crossing over (Shaw, 1983a & b) to yield homozygous A2 genotypes. There is some evidence that mating type change in both directions can be induced by treatment with certain fungicides (Ko, 1981 & 1994) although the mechanism involved is obscure. The same argument can be advanced to account for the co-occurrence at the Bryn Farm site of A1 and A2 isolates with the same AFLP genotype (AF1), although it is also possible that these are products of recombination. The four "unrelated" A2 isolates (92, 96, 97, 98) may be members of rare clonal lineages, which are reproductively isolated from other genotypes, perhaps because they are recent immigrants.

3.2.7 Similarity of isolates with haplotype IIa

Twenty-five of the isolates were of mtDNA haplotype IIa (Table 3.2.1) which is less common in western Europe than the predominant haplotype Ia (Griffith & Shaw, 1997). These isolates were of 12 RG57 genotypes, three of which (RF39, RF01, RF11) also included isolates of haplotype Ia. Most haplotype IIa isolates clustered on the RG57 tree (Figure 3.2.1), with the exception of three RF39 isolates (27, 69, 72). Using six AFLP primer-pairs, 11 of the 13 haplotype IIa isolates, formed a tight cluster (Figure 3.2.3). Using two AFLP primer pairs the 25 haplotype IIa isolates were divided into eight AFLP genotypes, three of which also contained isolates with haplotype Ia. Twenty-two of the haplotype IIa isolates formed a major cluster (AF10-AF15), 18 of them having the closely related genotypes AF10 or AF11 (Figure 3.2.2). The cluster contained isolates from the north (32) and the south (24) of England. Isolate 27, which was excluded from the IIa cluster in the RG57 analysis, fell within the AFLP IIa cluster. In Figure 3.2.4, the one isolate of mtDNA haplotype IIa (isolate 40) had a vastly different fingerprint from all other isolates.

The fact that haplotypes Ia and IIa assorted mainly with certain related nuclear genotypes argues against free sexual exchange which would allow re-assortment amongst the nuclear and mitochondrial genomes, and argues for clonal propagation in these lines. Alternatively, if there is sexual reproduction between isolates with haplotype Ia and IIa mtDNA, there may be selection against re-assortment of mitochondrial and nuclear genomes. The three isolates of haplotype IIa which did not group within the AFLP IIa cluster came from Rhiw Goch (69, 96) and Yorkshire (72), sites at which IIa isolates

which did belong to the cluster were also found. These three may be examples of recent reassortment of mitochondria and nuclei following heterokaryosis or heteroplasmosis. Indeed, somatic fusion and heterokaryosis has been demonstrated in the laboratory between isolates originating from the Rhiw Goch site (Pipe *et al.*, in press; chapter 4).

3.2.8 Conclusions regarding similarity of isolates using RG57 and AFLP

UPGMA analysis using the RG57 data divided the sample into two major but loose clusters, one of which contained most of the haplotype IIa isolates (Figure 3.2.1). The same analysis using AFLPs gave a more structured tree (Figure 3.2.2), confirmed and intensified the haplotype IIa cluster (AF10-AF15) and also defined two additional clusters, one containing most of the A2-mating type isolates (AF01-AF09) and the other containing isolates with A1 mating type and haplotype Ia (AF16-AF36). The seven isolates excluded from these clusters (isolates 92-98) showed little similarity to the other isolates, and four of them little similarity amongst themselves.

Siambra Gwynion, Pant y Ddolen and Bryn Farm are garden / smallholder sites less than 5 km apart. The 8-10 RG57 genotypes chosen from each site comprised a similar number of AFLP genotypes and both methods of analysis were able to demonstrate that certain common genotypes were present at two or at all three sites. However, the AFLP tree showed more clearly that the RG57 tree that the isolates at all three sites belonged to at least three clusters based on A2 mating type, mtDNA haplotype IIa and A1 mating type / haplotype Ia. The larger sample of 19 isolates from Tyddyn y Berth, an organic farm approximately 10 km from the other sites, contained 12 RG57 genotypes, and an equal number of AFLP genotypes. In 1996, when all of the samples from this site were collected, no isolates of A2 mating type or haplotype IIa were detected (N. D. Pipe, unpublished). Accordingly, most of the isolates fell into the A1 / haplotype Ia cluster, although it was possible to identify two distinct groups of similar AFLP genotypes within this grouping, AF18 - AF23 and AF32 - AF36 (Figure 3.2.2). Examining the RG57 tree (Figure 3.2.1), it is possible to discern a similar picture. However, clustering on the RG57 tree was less obvious than on the AFLP tree and it is possible that such clustering patterns would have been overlooked in the absence of the AFLP data.

3.2.9 Do clonal lineages exist in England and Wales?

RG57 analysis has shown that at least four genotypes are common and widespread in England and Wales (Day *et al.*, unpublished). Although most isolates within each of the RG57 genotypes had similar AFLP fingerprints, isolates with the same RG57 genotype but different AFLP genotypes (and *vice versa*) were also detected. Taken alone, the RG57 data suggests that some genotypes in England and Wales are clonal lineages that propagate vegetatively and may survive the winter as mycelium within infected tubers. This was supported by some, but not all, of the AFLP data. It appears from the AFLP analysis that some isolates assigned to a lineage on the basis of their RG57 genotype have the same genotype through convergence and not descent and are therefore only distantly related to other members of the lineage. This supports previous findings using RG57 and AFLP in The Netherlands (Zwankhuizen *et al.*, in press) and other countries (Kamoun *et al.*, 1998). It is also unclear which mechanisms are generating variation within (and between) the lineages, although it is possible that these include mutation and parasexual recombination in additional to sexual exchange.

3.2.10 Reproducibility of the AFLP technique

For any of these conclusions to be valid, and to be confident in using the technique in subsequent analysis, it is important to investigate the reproducibility of the AFLP technique. AFLP analysis seems to be insensitive to variation in template DNA concentration unlike techniques such as RAPD (section 1.2.6). Above a certain absolute concentration (approximately 1 pg), AFLP fingerprint patterns were "very similar" using template quantities that ranged 1000-fold (Vos *et al.*, 1995). Slight variations in replicate AFLP fingerprints at low DNA concentrations are probably the result of non-random distribution of digested DNA molecules (Vos *et al.*, 1995). However, it was important to determine whether the AFLP fingerprints produced in the present study were reliable, especially through asexual propagation. This was determined by the experiments described below.

3.2.10.1 *Multiple DNA extractions and PCR reactions*

Six single-zoospore isolates were grown in liquid pea-broth (section 2.3.1). After two weeks, the liquid cultures were divided into three with a scalpel. DNA was then extracted from each section independently (section 2.3.2) except that the ground hyphae

were vortexed for two minutes in extraction buffer as opposed to the tubes being inverted by hand (as in all other extractions in the present study). The AFLP fingerprint of each of the three replicates from the six single-zoospore isolates was then determined as described (section 2.4.3) using primer-pair E19-M40.

In total, seventy-two loci could be scored amongst the 18 (3 x 6) samples and seven of these loci revealed variation within some of the isolate-replicates (Figures 3.2.5 & 3.2.6). Three replicates of two isolates (97.123.1 & 97.185.5) were identical at all loci and a further two isolates (96.89.41 & 96.93.22) were identical at all but one locus. Three (96.93.8) and five (96.161.5) loci were not identical across the three replicates in the remaining isolates. Variation amongst replicates of these last two isolates revealed that either one (96.93.8) or two (95.161.5) of the replicates were responsible for the observed variation. Of the seven bands that varied amongst the six isolates, four (41, 62, 67 & 68) were not found to be polymorphic in any other isolates fingerprinted for AFLPs.

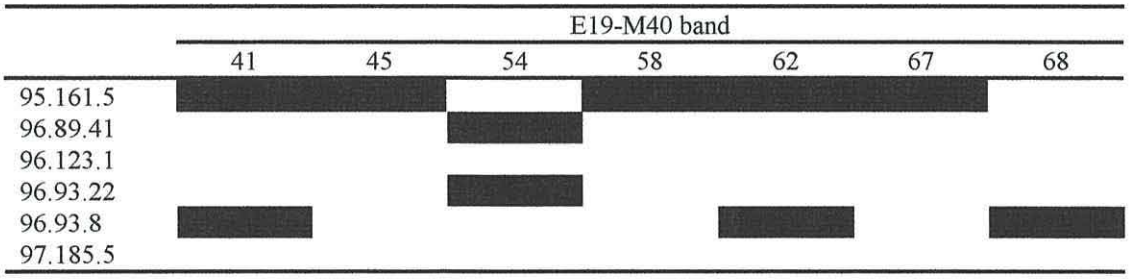


Figure 3.2.5. The seven loci that showed variation within replicates of some isolates. Shaded squares indicated that one replicate had gained or lost a band at the locus. Clear squares indicate that each replicate was monomorphic at that locus. Bands were numbered as in section 2.4.3.6.

The variation observed amongst the replicates could have been a result of the DNA extraction procedure. The ground hyphae were vortexed in extraction buffer, which may have caused more shearing of DNA than if the extraction buffer was mixed by gentle inversion. Shearing of DNA should result in loss rather than gain of bands if it occurs between two of the enzyme recognition sequences and would result in that fragment not being amplified, as the target would be absent. However, it is unlikely that all the fragments of a particular locus would be sheared so unless there was a very low initial copy number, shearing of bands should not result in complete absence of that band on the gel. Incomplete restriction of DNA, through salt or protein contaminants or methylation (Young *et al.*, 1999) could produce partially digested fragments and therefore additional bands, predominantly of higher molecular weight (Vos *et al.*, 1995).

Also, sub-optimal NaCl or Mg^{2+} concentrations can alter the specificity of restriction enzymes so that cleavage occurs at additional, non-standard restriction sites (Brown, 1990) and could result in both loss and gain of fragments. As further variation was not detected in replicate isolates (section 3.2.11.1) when the extraction mix was inverted instead of being vortexed, shearing of DNA may explain the difference in DNA fingerprint of some replicates.

To further examine whether the variation observed in replicates was a result of variation in PCR amplification, rather than differences in the quality of the DNA, the three replicates of the six isolates were amplified again with the same primers, E19-M40. All fingerprints were identical to those from the original amplifications. There was no extra variation in the form of bands appearing or disappearing in any isolate, confirming that the PCR reaction of the AFLP procedure was highly reproducible and that variation among replicates was due to differences in target DNA.

3.2.11 AFLP variation in isolates collected from the field

Field isolates (such as those in Tables 3.2.1 & 3.2.2) may be heterokaryotic and this may be detected as variation by AFLPs. Furthermore, heterokaryotic areas may sector on growth in the laboratory (Fyfe & Shaw, 1992) and could result in different fingerprint patterns if certain areas of the mycelium or certain sub-cultures were used for DNA extraction. To investigate this, two isolates (99.02 and 99.03) were freshly collected from the field as single-lesion isolates.

Three replicates of hyphal inocula (section 3.2.10.1) and ten single-sporangial isolates (section 2.2.2) were isolated from 99.02 and 99.03. One hyphal replicate was contaminated from each of 99.02 and 99.03 and of the ten single-sporangial cultures of each isolate, only three from each isolate grew into colonies. The two hyphal and three single-sporangial replicates were fingerprinted for AFLPs (section 2.4.3) using primer pairs E19-M16 and E19-M40.

Table 3.2.6. AFLP variation revealed in two hyphal-tip and three single-sporangial replicates of 99.02 and 99.03 and the number of bands (band numbers in parentheses) polymorphic amongst replicates

	Primer-Pair	Bands amplified	Present in all isolates	Variable bands	Number of genotypes
99.02	E19-M16	65	64	1 (15)	2
	E19-M40	51	50	1 (34)	2
99.03	E19-M16	65	64	1 (15)	2
	E19-M40	51	50	1 (34)	2

Two bands varied between the hyphal and sporangial replicates from each isolate (99.02 & 99.03). For each primer-pair, two different genotypes were observed (Table 3.2.6) which suggests that the colonies could have been heterokaryons composed of two genetically distinct nuclei. Without the analysis of single-zoospores from these cultures, this cannot be confirmed. It is also possible (as in section 3.2.10.1) that variation between subcultures of the same isolate could have been a result of the technique, such as incomplete digestion and not a consequence of different genotypes being present. Nonetheless, only small amounts of variation were detected amongst replicate subcultures, so on the basis of these data, AFLPs from field isolates that have not been purified through single-zoospore isolation are reproducible and worthwhile.

3.2.11.1 *Analysis of single zoospores from one isolate*

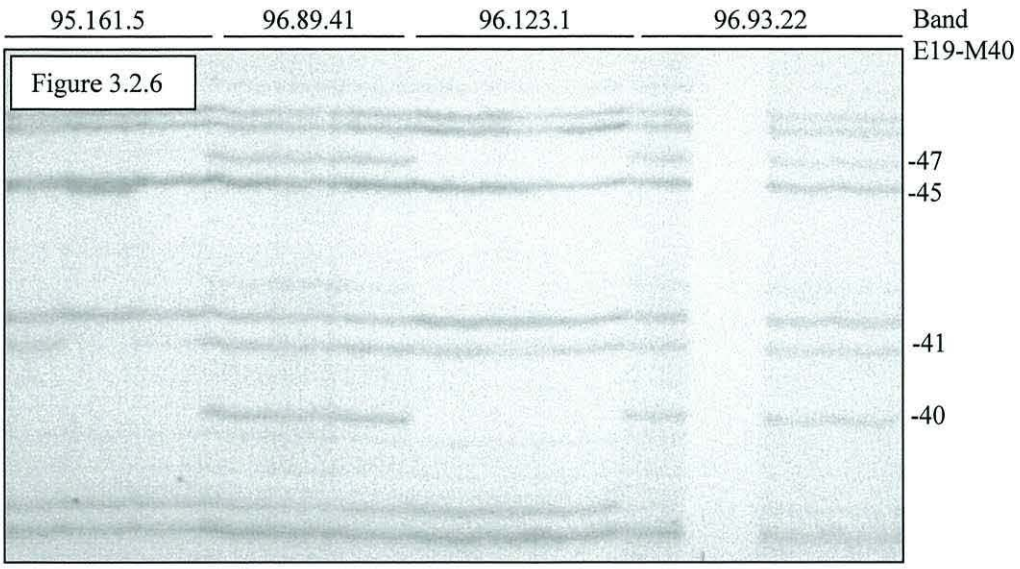
To establish whether AFLP fingerprints were stable through asexual reproduction, zoospores were obtained from an isolate (97.185.5), previously established from a single-zoospore (section 2.2.3) and grown for 12 months. Twenty single-zoospores were isolated from 97.185.5 and termed 97.185.5/1-20. Ten single-zoospore lines were immediately isolated from 97.185.5/1 and termed 95.185.5.1/1-10. DNA was extracted from all of these isolates (section 2.3.2) by inverting, not vortexing the samples in extraction buffer. All lines were then fingerprinted for AFLPs using primer pair E19-M40 (section 2.4.3).

Single-zoospore isolate 97.185.5 and the 20 single-zoospore lines established from it after a period of 12 months in the laboratory, differed at one out of over 60 legible loci (Figure 3.2.7a). This band (54) was present in 97.185.5 but was found in only half of the single-zoospore lines 97.185.5/1-20. This variation could be due either to mutation within 97.185.5 during prolonged storage, differences in the degree of DNA shearing during DNA extraction or incomplete digestion. The single-zoospore isolate 97.185.5/1 and the 10 single-zoospore lines established from it all lacked band 54, suggesting that mutation of 97.185.5 is the more likely explanation for the polymorphism (Figure 3.2.7b). The identical fingerprints obtained with these multiple single-zoospore isolates confirms that AFLP fingerprints are stable through asexual reproduction and that the DNA extraction procedure and AFLP-PCR were stable and reproducible. This is consistent with a previous study. AFLP patterns of 12 single-zoospore isolates were identical to those of the parental isolates (van der Lee *et al.*, 1997). However, one of

these single zoospore isolates with the same fingerprint as the parental isolate had an additional fragment, probably as a result of point mutation, deletion of a restriction or priming site or DNA shearing (van der Lee *et al.*, 1997).

3.2.12 AFLPs for the investigation of somatic recombination

AFLP fingerprints are stable through asexual reproduction and are highly reproducible in replicate samples. More loci can be visualised on a single AFLP gel than using RG57 and theoretically, these are randomly distributed throughout the genome (Majer *et al.*, 1996). Techniques that reveal markers from as many chromosomes as possible are required to investigate somatic recombination as it is possible that only certain chromosomes will recombine during somatic recombination. By increasing the number of AFLP primers, an almost unlimited number of polymorphic bands (assuming they are present) can be visualised, allowing the detection of both heterokaryons and recombinant nuclei. The AFLP technique would seem to offer more potential than RG57 due to these advantages and consequently was used in the following chapters to determine whether somatic hybridisation is a factor in the life cycle of *P. infestans*.



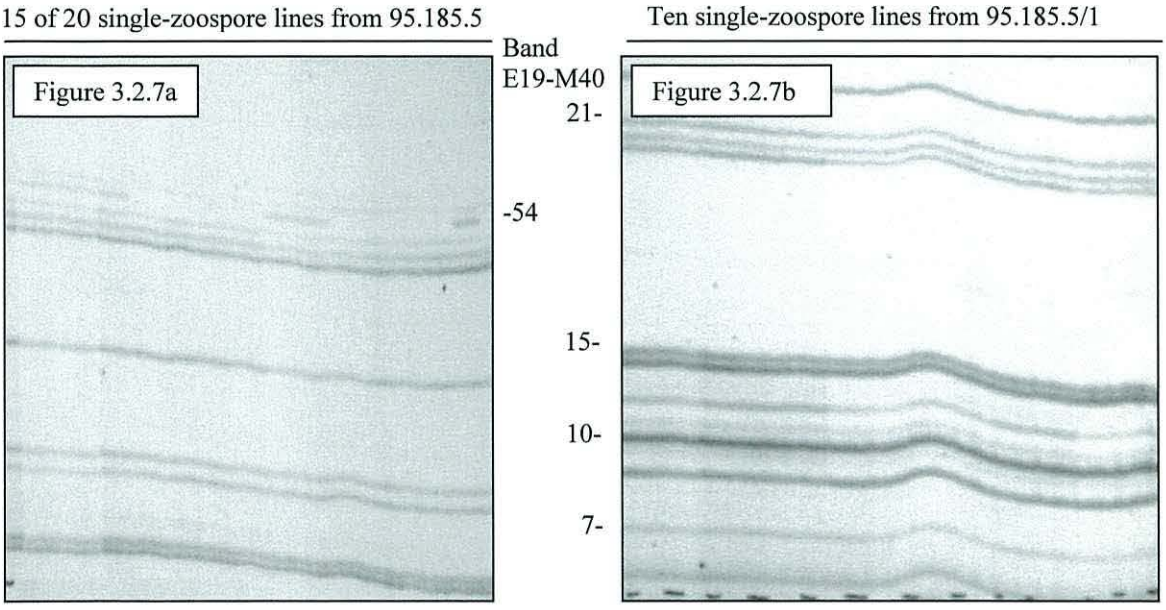


Figure 3.2.6. Three replicate DNA extractions and AFLP reactions of four isolates showing some of the bands that varied between replicates (section 3.2.10.1). Bands 45 and 41 revealed variation amongst replicates of isolate 95.161.5.

Figure 3.2.7a. Presence and absence of a single AFLP band (54) in single-zoospore lines of 95.185.5 (section 3.2.11.1).

Figure 3.2.7b. Reproducibility of the AFLP technique demonstrated by isolation of ten single-zoospore lines, 95.185.5/1/1-10 (section 3.2.11.1) with identical AFLP fingerprints.

4 Heterokaryosis and somatic recombination in self-fertile isolates of *Phytophthora infestans*

4.1 INTRODUCTION

Self-fertile isolates (section 1.3.5) are able to produce sexual oospores in single culture (Fyfe & Shaw, 1992) and display a characteristic flat, waxy appearance due to the inhibition of sporagiophore production. Some self-fertile isolates may be homokaryotic (have A1 and A2 determinants in a single nucleus) whereas others may be heterokaryons of A1 and A2 nuclei (Fyfe & Shaw, 1992).

Experiments investigating self-fertility in the Pythiales used self-fertile isolates of the normally heterothallic species, *Bremia lactucae* (Michelmore & Ingram, 1982; Michelmore & Sansome, 1982). Single-conidial lines (114) were established from a self-fertile isolate of which 92 were self-fertile, 22 were mating type B2 and two were mating type B1. Cytological investigations of the self-fertile progeny revealed that an extra chromosome was associated with the reciprocal translocation complex at meiosis (Sansome, 1980), indicating that these isolates were trisomic for the determinants of mating type (Michelmore & Ingram, 1982; Michelmore & Sansome, 1982).

In *P. drechsleri*, self-fertile oospore progeny from an A1 and A2 cross gave rise to both self-sterile (A1 and A2) and self-fertile progeny in single-zoospore lines (Khaki & Shaw, 1974; Mortimer *et al.*, 1977). Similarly, self-fertile isolates of *P. infestans* isolated from the field in Mexico (Niederhauser, 1991) readily formed sporangia and zoospores which could transmit either the self-sterile (A1 or A2) or self-fertile phenotype. Self-sterile progeny from these isolates were probably derived from heterokaryotic areas of the colonies but self-fertile single-zoospore cultures must have been derived from areas where karyogamy between A1 and A2 nuclei had occurred (Mortimer *et al.*, 1977; Niederhauser, 1991) as single-zoospores are usually uni-nucleate (Castro, 1963). The self-fertile phenotype of the *P. drechsleri* (and possibly the *P. infestans*) isolates may have been due to isolates being trisomic (*Aaa*) at the mating type locus (Sansome, 1977; section 1.2.1). A self-fertile single-oospore isolate of *P. infestans* has been confirmed as being trisomic at the mating type locus with molecular markers (Judelson, 1996).

Theoretically, trisomic isolates should be self-fertile only if mating type in

heterothallic species of *Phytophthora* is determined by two alleles at a single locus (Gallegly, 1970; Mortimer *et al.*, 1977; Judelson *et al.*, 1995; section 1.2.1) at which the ratio of A:a alleles determines mating type (Judelson, 1996).

Fyfe & Shaw (1992) isolated colonies of *P. infestans* from the field that resembled the mid-line of a sexual mating between two compatible isolates (A1 & A2) when grown on agar. Seven self-fertile isolates were analysed and these were generally stable when transferred by mass culture to fresh media. Occasionally, some sub-cultures or isolates would revert to the self-sterile phenotype and form self-sterile sectors of either A1 or A2 mating type. Most propagations of single hyphal-tips and single-sporangia produced self-sterile A1 or A2 colonies but some (up to 2%) resulted in self-fertile colonies. These self-fertile isolates produced fewer sporangia than their self-sterile derivatives and the few sporangia obtained could not be induced to differentiate zoospores. Self-fertility in these isolates was therefore not equivalent to the homothallism observed previously Niederhauser (1991) and was probably due to their being a mixture or mosaic of A1 and A2 hyphae, similar to that observed in a sexual cross. The small percentage of propagations that developed into self-fertile colonies were presumed to be derivatives of heterokaryotic hyphae and sporangia, which contained both A1 and A2 nuclei.

Fyfe & Shaw (1992) also demonstrated that self-fertile colonies could be regenerated by pairing self-sterile A1 and A2 lines derived from a self-fertile colony. Even randomly chosen A1 and A2 isolates had a high probability of interacting to form a stable mating and develop into a self-fertile colony. However, in similar studies with *P. infestans* (Anikina *et al*, 1997) or *B. lactucae*, (Michelmore & Ingram, 1982), attempts to restore the self-fertile phenotype by co-culturing self-sterile lines have not been as successful.

Many self-fertile isolates of *P. infestans* collected from the field may be mixtures of A1 and A2 isolates. The recent introduction of the A2 into Europe would allow interactions of both mating types on host tissue and could explain the recent increase in observations of self-fertile isolates on host tissue (Tantius *et al.*, 1986; Shattock *et al.*, 1990; Fyfe & Shaw, 1992; J. Day & N. Pipe, unpublished). Evidence suggesting that A1 and A2 isolates interact to form self-fertile isolates in the field was obtained by Pipe *et al.* (in press). Using mtDNA and nuclear probes, they found that two self-fertile isolates from a site in North Wales both had a composite nuclear fingerprint pattern of the two (A1 and A2 mating type) common genotypes present

there; these were therefore regarded as putative parents of the self-fertile isolates. Of 40 single hyphal-tips and single sporangia isolated from one of the self-fertiles, 16 had the same genotype as the A2 putative parent (the common A2 strain in the population) and one was identical to the A1 parent (the common A1 strain). The remainder germinated into self-fertile (four) or A1 (17) progeny but revealed nuclear markers from both the A1 and A2 putative parents and mitochondrial markers from the A2. Single-zoospores from these A1 lines were either A1 or A2 mating type, but all had the A2 nuclear and mitochondrial genotypes. The data suggest that the original self-fertile isolates were probably composed of heterokaryotic hyphae and sporangia containing A1 and A2 nuclei. These nuclei may have fused to produce tetraploid nuclei from which non-parental zoospore genotypes segregated. Recent experimental evidence suggests that such recombination between A1 and A2 nuclei can occur outside of the sexual cycle (section 6.1) and may result in somatic hybrids (Judelson & Yang, 1998).

4.1.1 Aims

The aim of the present study was to determine the extent of heterokaryon formation and somatic recombination in self-fertile colonies of *P. infestans*. In the work of Pipe *et al.* (in press), the parents of the self-fertile colony were unknown but could be implied from the common strains present in the field. To extend this work, a self-fertile colony was synthesised from A1 and A2 segregants derived from the self-fertile in Pipe *et al.* (in press) which had known RFLP and AFLP polymorphisms. Asexual progeny were investigated using these markers to identify recombinants. Results were compared to those obtained from two natural self-fertile isolates obtained directly from the field.

4.2 RESULTS

4.2.1 Isolates

HT7 (hyphal-tip derivative) and 12SS5 (single-sporangial derivative) were obtained from a natural self-fertile, 95.161.9, isolated from the Rhiw Goch population, North Wales (Pipe *et al.*, in press). From HT7 and 12SS5, a new self-fertile (RE-SF) was synthesised (Figure 4.2.1 and section 2.2.10).

Table 4.2.1. Origins and genotypes of isolates used in chapter 4.

Isolate	Origin	Mating type	mtDNA	RG57 type (RF)
HT7	Rhiw Goch	A2	IIa	01
12SS5	Rhiw Goch	A1	Ia	02
98.01	Llanfairfechan	SF	Ia	ND
98.02	Llanfairfechan	SF	IIa	ND

Llanfairfechan and Rhiw Goch are sites within a 50 km radius in North Wales. ND, not determined. RF and AFLP fingerprints can be found in appendix Table A2.1 and Table A3.4 respectively.

Two other two isolates (98.01 and 98.02) were self-fertiles isolated from the Llanfairfechan allotment, North Wales, in 1998 (Table 4.2.1). Both isolates produced oospores in single culture and also with either A1 or A2 tester isolates. RE-SF, 98.01 and 98.02 were maintained on rye A agar and sub-cultured through mass hyphal transfer before they reached the edge of the Petri dishes (roughly every 8-10 days).

4.2.2 Removal of hyphal-tips and sporangia

The use of one-tenth pea-broth agar (appendix 1) facilitated the removal of hyphal-tips and sporangia. This medium was clear and any contaminating hyphae or spores could easily be identified and avoided. Single hyphal-tips and hyphae from germinating single-sporangia were isolated below the surface of the medium as those on the surface were dislodged by the pipette tip, making it difficult to remove these accurately. It was important to sub-culture the edge of the growing self-fertile colonies every 8-10 days) to avoid sampling germinating oospores. These were never observed although the density of the surrounding hyphae may have obscured such germination.

The three self-fertile isolates formed abundant oospores though there was some variation in their ability to produce sporangia and zoospores. Oospores were always formed at least 1 cm behind the advancing edge of the colony but once the

colony had reached the edge of the plate, oospore production extended to the colony margin.

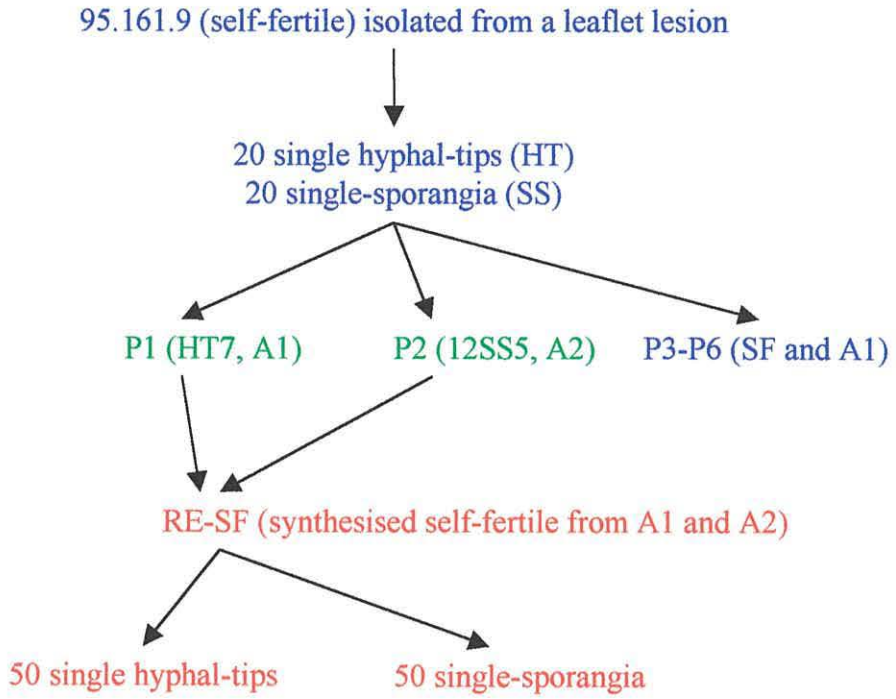


Figure 4.2.1. Origins of the starting material used for the synthesis and analysis of self-fertile RE-SF. Isolates and progeny in Pipe *et al.* (in press) are in blue. Progeny obtained in Pipe *et al.* (in press) and used in the present study are in green and those isolated in the present study are in red. P1-P6 (Pipe *et al.*, in press): phenotypes of progeny obtained from the single hyphal-tips and single-sporangia.

4.2.3 Synthesis of RE-SF and stability of the self-fertile phenotype

The synthetic self-fertile, RE-SF, was easily regenerated by pairing self-sterile segregants from self-fertile isolate 95.161.9. The self-sterile A1 and A2 isolates collided on the plate after seven days and formed a dense oosporic region across the plate,confirming that A1 and A2 mating types were present. Characteristic of all self-fertile colonies growing on rye A agar + RAN, this media turned pink under the oosporic regions as opposed to yellow throughout the self-sterile areas of the colony (Figure 4.2.5). The three plugs removed from RE-SF to fresh media produced oospores and each plug resulted in a self-fertile colony (RE-SF1-3) stable for between 6-8 serial transfers taken from the advancing edge of the colonies (Figure 4.2.2). After each fortnight, four sub-cultures were tested from RE-SF1-3 for mating type (section 2.1.3). After six sub-cultures, oospore production became patchy in RE-SF1

and some areas of the colony were devoid of oospores. Correspondingly, sporangial production increased in those areas without oospores and eventually, RE-SF1-3 lost the ability to produce oospores and reverted to self-sterile colonies (Figure 4.2.2). The original culture RE-SF also lost the self-fertile phenotype after 9-10 transfers.

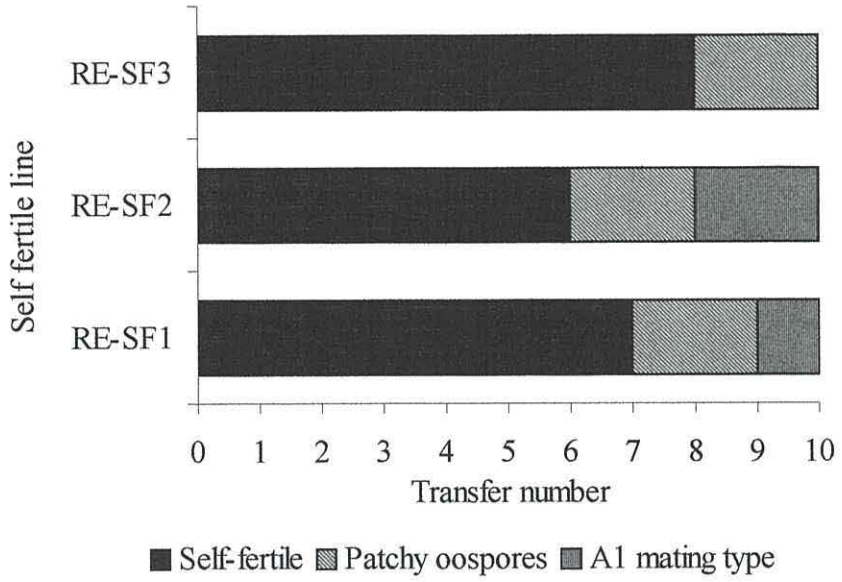


Figure 4.2.2. The fates of three lines of RE-SF over ten serial transfers on rye A agar

4.2.4 Analysis of asexual progeny from RE-SF

To determine if RE-SF was a mixture of hyphae, a heterokaryon or a recombinant, 50 single hyphal-tip and 50 single-sporangial cultures were established from RE-SF at monthly intervals for six months (sections 2.2.1 & 2.2.2). During this period, RE-SF was routinely sub-cultured from the colony edge before it reached the edge of the agar plate. Fifty-seven percent (171) of the isolated sporangia and 48% (148) of the isolated single hyphal-tips developed into colonies and 97% of these reacted as A2 mating type (Table 4.2.2). The exceptions were six A1 progeny detected in month 1 and three during month 4, all from single hyphal-tip propagations. No single hyphal-tips or single-sporangia germinated into self-fertile colonies.

4.2.5 Polymorphisms in parental isolates and asexual progeny

HT7 and 12SS5 were selected because they were originally derived from a natural self-fertile isolate, 95.161.9, and also had polymorphisms for both nuclear (RG57) and mtDNA markers (Pipe *et al.*, in press). The genotypes of the two parental isolates

as determined by RG57, mtDNA PCR and AFLP using primers E19-M16 and E19-M40 are shown in Table 4.2.2. DNA was also extracted (section 2.3.1) from 30 single hyphal-tip and 30 single-sporangial isolates (selected at random but including all the A1 progeny) from each monthly sample and these were typed for RG57, mating type and mtDNA haplotype. Fifteen isolates from each monthly sample were further tested for AFLPs using primers E19-M16 and E19-M40 (Table 4.2.2; Figures 4.2.6 & 4.2.7a & b).

Table 4.2.2. Polymorphisms revealed amongst the parental isolates and 30 hyphal-tip and single-sporangial progeny in six monthly samples from RE-SF.

Phenotype	Mating type	mtDNA	RG57 Fingerprint	AFLP Fingerprint		Number of progeny-phenotypes per month					
				E19-M16	E19-M40	1	2	3	4	5	6
A (12SS5)	A1	Ia	**110**	**111**	**10**						
B (HT7)	A2	IIa	**001**	**110**	**01**	9	13	12	12	5	15
AB (RE-SF)	SF	Ia/IIa	**111**	**111**	**11**						
Progeny											
C	A1	IIa	**001**	**111**	**01**	1					
D	A1	IIa	**001**	**010**	**11**	5			3		
E	A2	IIa	**001**	**111**	**10**		2			2	
F	A2	IIa	**001**	**110**	**11**			2			
G	A2	IIa	**001**	**100**	**11**			1			
H	A2	IIa	**001**	**111**	**00**					8	

RG57 fingerprint after Goodwin *et al*, 1992a. Only polymorphic bands 2, 16 & 19 are shown. AFLP fingerprint: only bands 9, 47 & 53 (E19-M16) or bands 45 & 54 (E19-M40) are shown. Bands in green were present in 12SS5 only, bands in red were present in HT7 only and bands in blue were present in both parents but were polymorphic amongst the progeny.

Combining the RG57 and AFLP data, 12SS5 had four markers not present in HT7 and HT7 had two markers not present in 12SS5. Additionally, 12SS5 and HT7 had different mtDNA haplotypes. All single hyphal-tip and single-sporangial propagations from RE-SF were of mtDNA haplotype IIa, the same as the A2 parent. In agreement with this, all single hyphal tip and single sporangial progeny had RG57 band 19 but not 2 or 16, and therefore had the same nuclear fingerprint as the A2 parent (Figure 4.2.6). Amongst the 90 progeny fingerprinted for AFLPs, seven genotypes (B to H) could be identified in the single hyphal-tip and single-sporangial progeny (Table 4.2.2). Type A was the phenotype of the A1 parent 12SS5, and type B the A2 parent HT7. Type A was not detected in the progeny.

The most common genotype detected amongst the 90 progeny was type B, the same fingerprint pattern as the A2 parental isolate HT7; this was found in 66 of the 90 progeny and was found in each monthly sample. The other 24 progeny had the A2 RG57 fingerprint pattern but exhibited A1 and/or A2 parental AFLP bands revealing a

further five genotypes C-H (Table 4.2.2). Further analysis of these progeny through single zoospores was impossible as neither the self-fertile RE-SF or any of the asexual progeny would release viable zoospores from sporangial suspensions.

4.2.6 Stability *in vitro* of self-fertile isolates from the field

Three separate sub-cultures (10 mm^2) of 98.01 and 98.02 were removed from the edge of the growing colony (each separated by at least 3 cm) and were grown separately into individual self-fertiles to investigate the stability of the self-fertile phenotypes. After 10-14 days, each of the three colonies was examined microscopically for the presence of oospores and further sub-cultured (10 mm^2) from the edge of the colony.

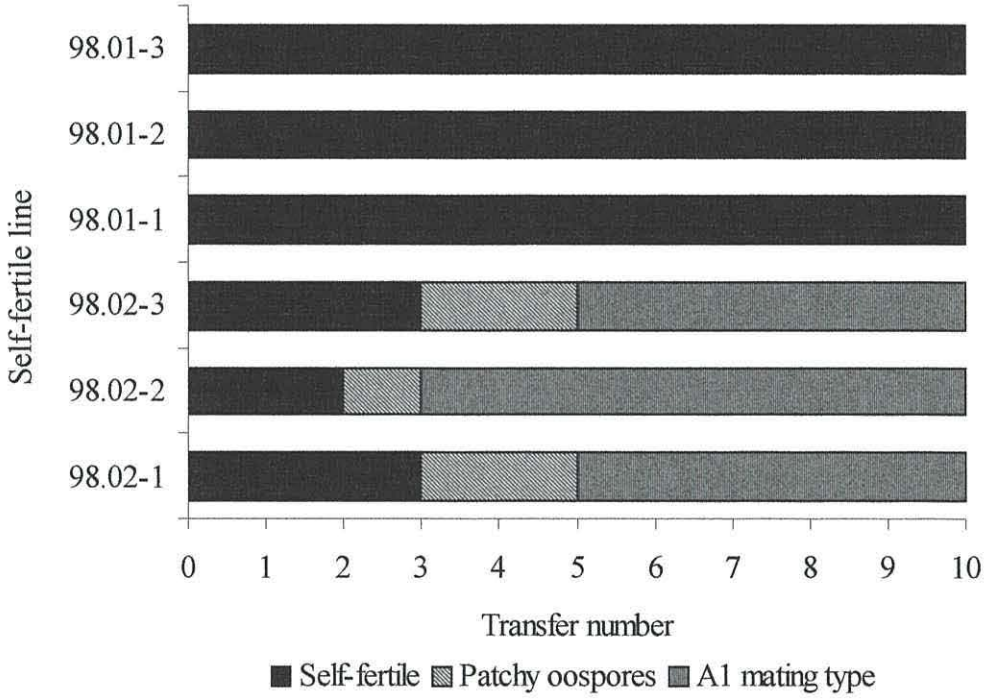


Figure 4.2.3. The fates of three lines of 98.01 and 98.02 over ten serial transfers on rye A agar

Self-fertiles 98.02 and 98.01 could be transferred with no loss of the self-fertile phenotype for at least two sub-cultures (98.02) or over ten sub-cultures (98.01) to fresh rye A agar (Figure 4.2.3). 98.02 always produced more aerial mycelia and sporangia than 98.01 and did not have the classic waxy phenotype of 98.01 and other self-fertile isolates. It was inherently less stable than 98.01 and developed self-sterile sectors within the first three sub-cultures.

Production of sporangia varied amongst the two self-fertiles and their single-sporangial or single hyphal-tip derivatives (Figure 4.2.4). Self-fertile colony 98.01 produced far fewer sporangia than any of its derivatives, indicating that 98.01 was a stable, fertile, self-fertile (Fyfe & Shaw, 1992). 98.02 produced numbers of sporangia similar to those produced by its single-sporangial and single hyphal-tip derivatives.

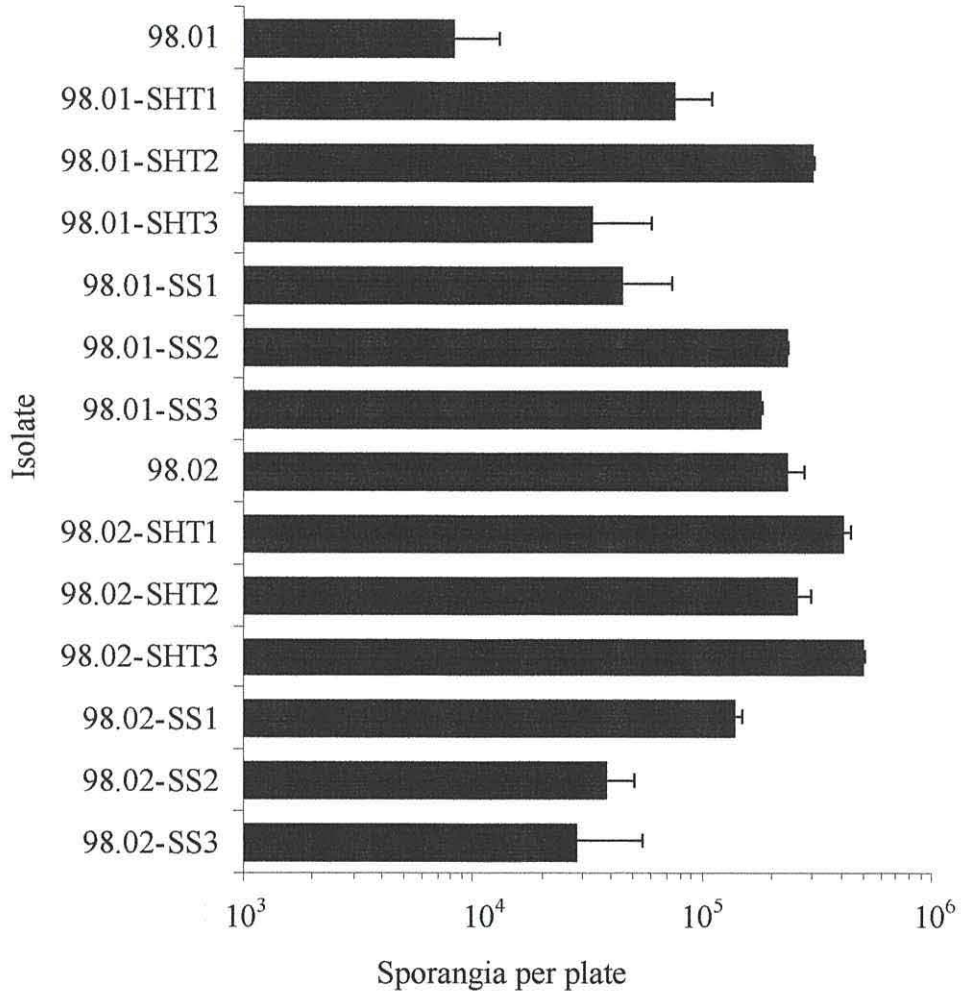


Figure 4.2.4. Yield of sporangia from four replicate plates of parental cultures 98.01 and 98.02 and self-sterile derivatives. Error bars indicate SEM. SS, single-sporangia, SHT, single hyphal-tip.

4.2.7 Analysis of asexual progeny from 98.01 and 98.02

All colonies propagated from single-sporangia or single hyphal-tips from 98.01 were self-sterile and mainly of A1 mating type (Table 4.2.3). From 98.01, 22 progeny were A1 and three single hyphal-tip colonies were A2. All had type Ia mtDNA, the same as the parental self-fertile colony.

Table 4.2.3. Fingerprint patterns of the parental self-fertile isolate 98.01 and the A1 and A2 self-sterile progeny from single sporangia (SS) and single hyphal-tips (SHT). Single zoospores (SZ) were isolated from SHT (B, D) or SS (F) progeny of 98.01.

			Mating type	AFLP fingerprint E19-M40	Genotype designation
98.01 (parent)			SF	110	A
Number of progeny					
SS	SHT	SZ			
4	6	4	A1	010	B
0	1	0	A2	010	C
2	5		A1	011	D
		7	A1	010	B
0	2	0	A2	011	E
8	0	4	A1	000	F

AFLP fingerprint with E19-M40 bands 40, 43 & 45.

AFLP analysis of progeny from 98.01 (Table 4.2.3) revealed three bands to be polymorphic. One locus, E19-M40 (40) was present only in the parental self-fertile culture. The other loci (43 & 45) were present in both single hyphal-tip and single-sporangial progeny. No polymorphisms could be observed with primers E19-M16. Six genotypes were identified (A-F) and two of these, A (self-fertile parent) & C, were unique. The remaining four types had 10 (B), seven (D), two (E) and eight (F) representatives found in either or both single hyphal-tip and single-sporangial progeny. All progeny were A1 except C and E, which were A2. Single-zoospore cultures were established (section 2.2.3) from progeny with B, D and F genotypes. All of the single zoospores from B or F had the same genotype as the hyphal-tip (B) or sporangial culture (F) from which they were derived. However, zoospores from a culture of type D, did not reveal band E19-M40 (45) and so had a different genotype (B) compared to the progenitor hyphal-tip strain.

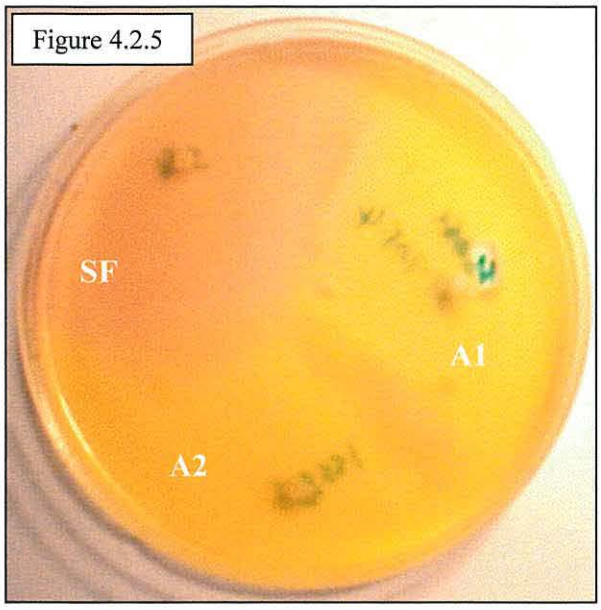
All asexual progeny of 98.02 were A1 mating type and mtDNA haplotype IIa (Table 4.2.4). AFLP analysis (E19-M40) revealed that only two loci (E19-M40 43 & 45) were polymorphic amongst the 20 progeny and the parental self-fertile isolate. These loci were present in representatives of both single-sporangial and single hyphal-tip progeny and in total, three different genotypes were identified including the parental self-fertile and its asexual derivatives (Table 4.2.4)

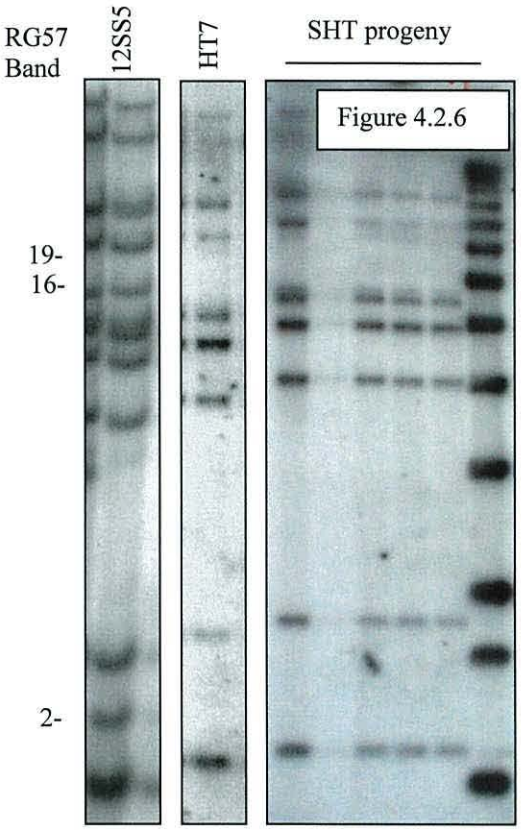
Single-zoospore cultures (section 2.2.3) were established from three single-sporangial cultures (Table 4.2.4) of each observed genotype (A-C). All of the single zoospore colonies had the same genotype (B) regardless of the genotype of the single-sporangial parent from which they were obtained.

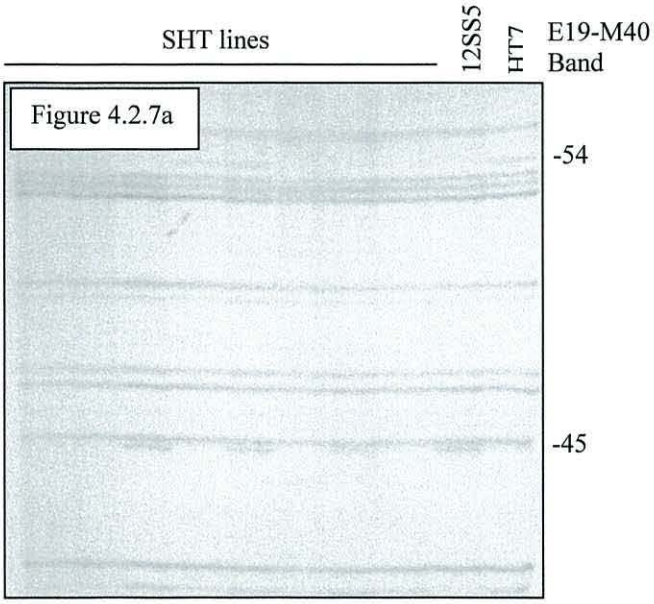
Table 4.2.4. Fingerprint patterns of the parental self-fertile isolate 98.02 and the self-sterile progeny from single sporangia (SS) or single hyphal-tips (SHT). Single zoospores (SZ) were isolated from SS progeny of each genotype (A-C).

			Mating type	AFLP Fingerprint E19-M40	Genotype designation
98.02 (parent)			SF	11	A
Type of progeny					
SS	SHT	SZ			
1	9		A1	11	A
		4	A1	00	B
5	0	4	A1	00	B
3	1		A1	10	C
		4	A1	00	B

AFLP fingerprint with E19-M40 bands 43 & 45.







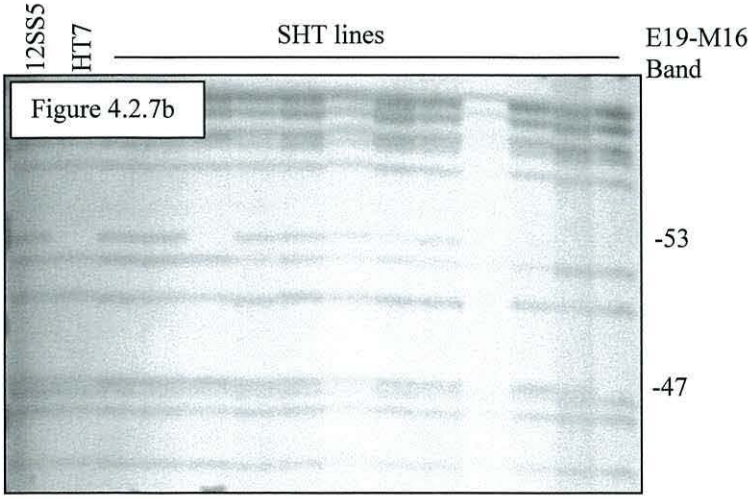


Figure 4.2.5. SF, A1 and A2 isolates growing on RAN media.

Figure 4.2.6. Isolates HT7 and 12SS, parents of RE-SF and hyphal-tip progeny, fingerprinted with RG57. Polymorphic bands are indicated.

Figure 4.2.7a & b. AFLP fingerprints of HT7, 12SS5 and hyphal-tip progeny with primers two pairs of AFLP primers. Polymorphic bands are indicated.

4.3 DISCUSSION

The aim of this chapter was to determine whether somatic recombination could occur in self-fertile isolates if *P. infestans*. This work extended that of Fyfe & Shaw (1992) investigating the stability of self-fertile colonies and also extended the work of Pipe *et al.* (in press) as isolates with known molecular markers were used to synthesise a self-fertile colony before fingerprinting asexual progeny. Zoospores, often difficult to induce in self-fertile lines or their derivatives, were obtained from two natural self-fertile isolates.

4.3.1 The cause of self-fertility in the three self-fertiles

Propagations were made from single hyphal-tips and single-sporangia to determine whether the synthesised self-fertile colony RE-SF and the field self-fertiles 98.01 and 98.02 were a mixture of A1 and A2 hyphae or could have been somatic recombinants. Any germinating hyphal-tips or sporangia producing a self-fertile colony would potentially be A1 x A2 heterokaryons or if A1 and A2 nuclei had fused in a heterokaryon, possibly recombinants. Unlike other studies (Pipe *et al.*, in press; Fyfe & Shaw, 1992), all propagations from the three self-fertiles were self-sterile, the mating types (A1 or A2) of the progeny depending on the particular self-fertile isolate. This suggests that no (or few) heterokaryotic hyphal-tips or sporangia were present within any of the self-fertile isolates; self-fertility was probably due to a mixture of parental hyphae, similar to the isolates of Fyfe & Shaw (1992).

If a self-fertile colony were an uneven mosaic of A1 and A2 hyphae, randomly removing plugs to fresh media (during sub-culture) may result in the loss of the self-fertile phenotype and may have accelerated the breakdown of two of the self-fertiles (Figures 4.2.2 & 4.2.3). Most of the hyphal-tip or sporangial progeny from a particular self-fertile were of the same mating type suggesting that hyphae of one or other mating type predominated. Hyphal-tips would select for fast growth and sporangia may only be produced by one of the isolates. Consequently, these techniques may have selected for self-sterile rather than self-fertile propagations, if they were present.

4.3.2 Analysis of RE-SF

4.3.2.1 *Fingerprints of self-fertile RE-SF and asexual progeny*

Polymorphisms in mtDNA, 3 RG57 and 3 AFLP loci should have allowed the detection of somatic recombination. In the self-fertile culture RE-SF, from which the hyphal-tips and sporangia were obtained, all polymorphic RG57, AFLP and mtDNA markers could be detected, indicating both A1 and A2 nuclei were present in RE-SF.

The mtDNA, and RG57 genotypes of all hyphal-tips and sporangia obtained in the present study were identical to the A2 parental type. However, AFLP analysis allowed both A1 and A2 progeny to be resolved into seven different genotypes (B-H) based on which polymorphic bands were detected from 12SS5 and HT7 (Table 4.2.2). Type B accounted for 73% of the progeny and was identical to the A2 parent for all markers (mating type, mtDNA, RG57 and AFLPs). These progeny probably represented parental A2 propagules within the self-fertile colony.

The nine A1 mating type progeny obtained from RE-SF revealed AFLP markers from both 12SS5 and HT7. Previous analysis of self-fertile 95.161.9 from which HT7 and 12SS5 were derived, revealed two types of asexual progeny; A1 mating type with markers from the A1 and A2 putative parents and A2 mating type with markers only from the putative A2 parent (Pipe *et al.*, in press). Through single-zoospore analysis, Pipe *et al.* (in press) concluded that at least three nuclear genotypes (based on mtDNA, RG57 and telomeric probes) made up the self-fertile.

In the present study, the nine A1 mating type progeny (types C & D) revealed mtDNA and RG57 markers only from the A2 parent, and these had same RG57 genotype as some of the A1 mating type isolates obtained in previous work (Pipe *et al.*, in press). Nuclei with such genotypes were not present in HT7 or 12SS5, the parents of RE-SF (Table 4.2.2) and there are several possible origins for the appearance of these "recombinant" A1 isolates in RE-SF. It is possible that mutation from an A2 to A1 mating type may have occurred in some hyphal-tip or sporangial lines (section 1.2.1) as suggested previously in derivatives from self-fertile isolates of *P. infestans* (Niederhauser, 1991) and *P. drechsleri* (Mortimer *et al.*, 1977). Another possibility is that AFLP variation was generated in these cultures by mitotic crossing-over (Shaw 1983a & b). Crossing-over at mitosis by breakage and rejoining of chromosomes is known to occur in diploid strains of the higher fungi and could result in loss or gain of bands within a single isolate. However, there is little evidence to

suggest that such a phenomenon would occur at such a high frequency to generate the levels of variation observed in these isolates. Furthermore, both this and the mating type mutation hypotheses do not account for the presence of AFLP markers from A1 and A2 parents in asexual progeny. A further possibility is that hyphal anastomosis within the self-fertile colony allowed somatic nuclear fusion to generate a tetraploid nucleus that subsequently lost chromosomes and became diploid during growth. Pipe *et al.* (in press) suggested that A1 lines bearing both A1 and A2 markers were the result of an A1 + A2 tetraploid in the self-fertile colony. Loss of A1 markers in their single-zoospore progeny suggested that chromosomes bearing markers from the A1 putative parent were preferentially lost during segregation of the tetraploid nucleus. Indeed, it is possible that types C (1%), D (9%), F (2%) and G (1%), which had mtDNA and RG57 markers from the A2 parent only but revealed AFLP markers from both the A1 *and* A2 parents, represented intermediate segregation through unstable mitoses. Judelson & Yang (1998) also identified stable tetraploid nuclei from an A1 x A2 cross. Their progeny revealed molecular markers from both parents, yet expressed A1 mating type due to the dominance of the A1 allele (Judelson, 1996; Judelson & Yang, 1998; section 1.2.1). It is possible that such a mechanism produced the observed variation in progeny from RE-SF but the inability to induce single-zoospores prevented further analysis. Telomeric markers (Pipe & Shaw, 1997), single-locus RFLPs (Carter *et al.*, 1999) or microsatellites could allow the identification of hyperploid strains and thus somatic recombinants in further studies.

4.3.2.2 *Detection of heteroplasmons in RE-SF*

During anastomosis, both nuclei and mitochondria could be exchanged between strains and so it is possible that heterokaryon-heteroplasmons could be generated. As well as being a heterokaryon, the original self-fertile, 95.161.9, was also a heteroplasmon since both mtDNA types (Ia & IIa) were detected (Pipe *et al.*, in press). No heteroplasmons were observed in asexual progeny in the original analysis (Pipe *et al.*, in press) or in the present study; only isolates with type IIa mtDNA were obtained. Gu & Ko (1998) found that incompatibility factors develop when foreign cytoplasm (mitochondria) is inserted into a different strain; incompatibility was not observed when single isolated nuclei were inserted. If incompatibility systems exist in *P. infestans*, heteroplasmons could be selected against. Inheritance of mitochondria during somatic recombination would then be similar to sexual reproduction, where the

mitochondrion is uniparentally inherited (Whittaker *et al.*, 1994).

4.3.3 Analysis of self-fertiles 98.01 and 98.02

The advantage of synthesising a self-fertile from two known parental isolates is that the starting genotypes are known and can be compared to genotypes observed in asexual progeny. Self-fertile isolates obtained directly from the field have unknown origins but nevertheless, investigating asexual progeny from such isolates can still yield valuable data especially if, as in the case of 98.01 and 98.02, single-zoospores can be induced from their self-sterile derivatives.

Both A1 and A2 single hyphal-tip progeny were observed from 98.01, expected if the self-fertile was a mixture of two genotypes of A1 and A2 mating type. Only A1 single hyphal-tips and single-sporangia were observed from 98.02. This may reflect that self-fertility in 98.02 was not as stable as in 98.01, the A1 possibly having a faster growth rate than the A2 therefore being selected on sub-culture. Only three (98.01) or two (98.02) AFLP loci were polymorphic amongst asexual progeny obtained from these two isolates. One band E19-M40 (45) was detected in one of the progeny genotypes of 98.01 (C) but was not detected in the self-fertile 98.01 or the other two progeny genotypes (B & D). Single-zoospores from hyphal-tips or sporangial cultures either had the same AFLP fingerprint as the parent strain or lost a band present in the parental hyphal-tip strain (D) resulting in zoospore progeny with a different AFLP fingerprint (Table 4.2.3). Hyphal-tip or sporangial derivatives revealed both, one or neither of the two polymorphic bands in 98.02 (Table 4.2.4). Like 98.01, single-zoospores from different single-sporangial lines either had the same fingerprint as the parent sporangial culture (B) or did not reveal either of the two polymorphic bands (A & C).

Both 98.01 and 98.02 could have been heterokaryons composed of several nuclear genotypes; single-zoospore lines often revealed fewer bands than the parental hyphal-tip or sporangial strain and this could represent segregation of nuclei. However, as actual segregation of different genotypes was not observed from a single hyphal-tip or sporangial culture, this cannot be confirmed. Alternatively, variation in these progeny could be a result of chromosome loss from polyploid nuclei. Many UK isolates may be diploid-tetraploid heterokaryons (Whittaker *et al.*, 1991a) and it is possible that 98.01 and 98.02 contained hyperploid nuclei, although this was not determined. Interestingly, progeny types B and C from 98.01 (Table 4.2.3) had the

same AFLP fingerprint but different mating types which, as discussed in section 4.3.2.1 may indicate that an A1 + A2 tetraploid may have formed, followed by loss of certain chromosomes. This is a hypothesis that could be tested with further experiments using these strains.

4.3.4 Why should self-fertiles recombine outside of the sexual cycle?

Oospores often germinate slowly or erratically and in many crosses, no oospores will germinate (Al-Kherb *et al.*, 1995). Some A1 and A2 isolates that produce infertile oospores produce recombinants through asexual mechanisms (Judelson & Yang, 1998) and isolates that are sexually incompatible may be compatible through asexual reproduction. In the field, somatic recombinants could be established faster than sexual recombinants and the formation of heterokaryons rather than sexual recombinants could allow an isolate to be more plastic in its ability to adapt to changing environmental factors.

It is possible that the intimate mixing and coiling of A1 and A2 hyphae within self-fertile colonies (Fyfe & Shaw, 1992) and mating cultures (Shaw, 1987) provides the opportunity for hyphal anastomosis. However, it is not known what factors are involved in determining whether a self-fertile will remain self-fertile or segregate into its constituent parts, as some "pairings" are more stable than others (Fyfe & Shaw, 1992). It is possible that the stability of a self-fertile isolate may depend on the ratio and distribution of A1 and A2 hyphae. Isolates with similar growth rates may also produce more stable self-fertile colonies and this could be tested experimentally.

4.3.5 Conclusions and further work

No evidence was obtained using RG57 that heterokaryons or somatic recombinants had formed in RE-SF. With the AFLP technique, more polymorphisms could be detected but definitive evidence was not obtained for the occurrence of heterokaryosis and recombination in the self-fertile colony as single-zoospore lines could not be induced from hyphal-tip or sporangial lines of RE-SF and the observed variation could have had alternative origins. Similarly, no definitive evidence of heterokaryons was obtained through analysis of 98.01 or 98.02 as only single genotypes were identified in zoospore progeny from a single hyphal-tip or sporangial parental culture. Fingerprinting with more AFLPs and single-locus markers may identify any products of somatic recombination in future studies.

5 Isolation of drug-resistant mutants and inheritance of AFLP markers in sexual progeny

5.1 INTRODUCTION

5.1.1 Selectable markers

Selectable drug-resistant markers allow recombination to be detected between two strains with different drug-resistant phenotypes. Progeny with novel drug-resistant phenotypes (recombinants) can be selected for and then analysed. Selectable drug-resistant markers have been obtained by chemical mutagenesis of zoospores, mainly using *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG) followed by selection on a substrate to which resistance is required (Shattock & Shaw, 1975; Long & Keen, 1977b; Layton & Kuhn, 1988a; Lucas *et al.*, 1990). However, using chemical mutagenesis, there is a danger of inducing too many non-specific, semi-lethal mutations (Shaw, 1983b) with, for instance, impaired growth rate or pathogenicity (Khaki & Shaw, 1974; Long & Keen, 1977b; Lucas *et al.*, 1990). However, mutagenic agents are not necessarily required to obtain drug-resistant mutants. Spontaneous mutations occur at a high enough frequency to allow resistant sectors to be isolated from strains incubated on media with an inhibitory concentration of the drug to which resistance is required (Shaw & Elliott, 1968; Ann & Ko, 1986 & 1992; El Refai, 1990; Whittaker *et al.*, 1996). Such mutants will have fewer non-specific, semi-lethal mutations. The most commonly used selectable markers in *P. infestans* are described below.

5.1.1.1 *Metalaxyl*

Metalaxyl (Ridomil® *N*-[2,6-dimethylphenyl]-*N*-[methoxyacetyl]-alanine methyl ester) is used as a systemic fungicide to control many soilborne plant pathogens of the oomycetous fungi. Metalaxyl, along with other phenylamides, can persist in soil for many months (Bailey & Coffey, 1984) and has a high intrinsic toxicity (Schwin & Margot, 1991). Metalaxyl inhibits mycelial growth, reduces lesion size and also inhibits sporangial development (Bruck *et al.*, 1980; Schwin & Margot, 1991) but has little or no effect on sporangium or zoospore germination. Biochemical studies suggest that metalaxyl inhibits RNA synthesis in *P. infestans* and *P. megasperma* f.sp.

glycinea, by preventing the incorporation of uridine into RNA (Davidse *et al.*, 1983). The incorporation of thymidine into DNA and phenylalanine into protein may also be affected to a lesser extent (Davidse *et al.*, 1991).

Many isolates of *P. infestans* have developed resistance to metalaxyl (Day & Shattock, 1997; section 1.3.3.1) since the arrival, from Mexico, of new genotypes. Inheritance of metalaxyl-resistance may be semi-dominant in some isolates of *P infestans* (Shattock, 1988), *P. sojae* (Bhat *et al.*, 1993) and *Bremia lactucae* (Crute & Harrison, 1988). Inheritance through a single dominant gene has also been suggested for *P. nicotianae* var. *parasitica* (Chang & Ko, 1990) and some isolates of *P. infestans* (Lee *et al.*, 1999). There is also growing support that in some isolates, more than one major and several minor loci may be involved in determining metalaxyl resistance in *P. infestans* (Harrison, 1991; Fabritus *et al.*, 1997; Lee *et al.*, 1999; Judelson & Roberts, 1999).

5.1.1.2 *Streptomycin*

Streptomycin has been used as an experimental fungicide (Cohen & Perl, 1973) and stable resistance to streptomycin has been developed in the laboratory (Shattock & Shaw, 1975; Ann & Ko, 1986; Whittaker *et al.*, 1996). Streptomycin reacts with prokaryotic ribosomes, specifically the smaller subunit, where binding prevents normal interaction between mRNA and tRNA (Hammond & Lambert, 1978) resulting in nonsense-proteins. Bacterial resistance to streptomycin is thought to be a consequence of developing smaller subunits which cannot bind the antibiotic and also, the development of an additional permeability barrier which may prevent entry of the drug and reduce the leakage of low molecular weight ions through the membrane (Hammond & Lambert, 1978). High levels of streptomycin are fungicidal, resulting in irreversible decrease in growth rate (Rooke & Shattock, 1983).

Ann & Ko (1987), Chang & Ko (1990) and El Refai (1990) observed that segregation of streptomycin-resistance did not conform to ratios expected if the markers were nuclear and suggested that resistance may be encoded on mtDNA. With the advent of a reliable typing method for the mitochondrial genome (Carter *et al.*, 1990) and evidence that the mitochondrion was uni-parentally inherited (Whittaker *et al.*, 1994), Whittaker *et al.* (1996) demonstrated that streptomycin-resistance was inherited through the cytoplasm and probably the mitochondrion in some isolates of *P. infestans*.

5.1.1.3 *Chloramphenicol*

Chloramphenicol inhibits vegetative growth and sporulation of *P. infestans* (Shattock & Shaw, 1975; Rooke & Shattock, 1983) and mutants resistant to chloramphenicol have been obtained in the laboratory (Shattock & Shaw, 1975; Whittaker *et al.*, 1996). Chloramphenicol reacts with the large subunit of prokaryotic ribosomes, inhibiting peptide bond formation and preventing incorporation of amino acids (Hammond & Lambert, 1978). Resistance to chloramphenicol in *P. infestans* is not due to chemical modification of the antibiotic (Rooke & Shattock, 1984) or differences in the permeability of membranes to chloramphenicol, but is probably due to reduced uptake of the drug by resistant isolates. Inhibitory concentrations of chloramphenicol are fungistatic and inhibition of growth is reversible (Rooke & Shattock, 1983).

Ann & Ko (1987) and Chang & Ko (1990) found that segregation of chloramphenicol-resistance did not conform to ratios expected if the markers were nuclear. Whittaker *et al.* (1996) also found that inheritance patterns did not correlate well with the inheritance of mtDNA but nor was the inheritance obviously Mendelian in other studies (El Refai, 1990). It is possible that inheritance of chloramphenicol-resistance is determined by different genes in different isolates, some of which may be mitochondrial or nuclear.

5.1.1.4 *Acriflavine, blasticidin and phosphorous acid*

Acridine dyes, such as acriflavine, bind selectively and tightly to nucleic acids, intercalating between the adjacent base pairs in the double helix and inhibiting both RNA and DNA synthesis (Hammond & Lambert, 1978). Although not true antibiotics, some isolates exhibit resistance to these dyes (Poedinok *et al.*, 1988) and they have been used in positive selection experiments (Poedinok *et al.*, 1988). Mutations in *P. infestans* conferring resistance to acriflavine may be similar to mutations in the *acr-2* gene, which controls acriflavine sensitivity in *Neurospora crassa* (Akiyama & Nakashima, 1996).

Blasticidin is an aminoacylnucleoside antibiotic produced by some species of *Streptomyces*, and inhibits protein synthesis in a variety of organisms by interfering with the peptidyl transferase reaction (Tamura *et al.*, 1995). Blasticidin strongly inhibits the growth of *Magnaporthe grisea* (Tamura *et al.*, 1995) and has also been used for selecting resistant mutants of *P. infestans* (Poedinok *et al.*, 1988).

Phosphorous acid is a breakdown product of the systemic fungicide fosetyl-Al

(Bower & Coffey, 1985). Some mycelial growth occurs in the presence of phosphorous acid even in sensitive isolates of *P. capsici* (Lucas *et al.*, 1990) and many *P. infestans* isolates are resistant to high levels (>200 μg ml^{-1}) of phosphorous acid *in vitro* (Coffey & Bower, 1984). However, this inhibitor has been used as a drug-resistant marker (Lucas *et al.*, 1990).

5.1.1.5 *Transformed isolates*

Bacterial resistance genes to either geneticin (G418) or hygromycin have been isolated from bacteria and used to transform isolates of *P. infestans* (Judelson, 1991) using promotors and terminators from another Oomycete, *Bremia lactucae*. These integrative transformations have allowed mitotically stable, drug-resistant isolates to be used for investigating *P. infestans* biology (Judelson & Yang, 1998). The strains have an added advantage over drug resistant mutants for *in vitro* studies, as nuclei from individual transformed strains can be identified with PCR by amplifying the inserted fragments. They have proven useful in recombination studies (Judelson & Yang, 1998) and investigations into gene silencing (van West *et al.*, 1998 & 1999).

5.1.2 Aims

The aims of this chapter are to select antibiotic resistant mutants and cross these strains to establish sexual progeny. The inheritance of mtDNA, AFLP and the drug-resistant markers can then be investigated to determine their stability through asexual reproduction and determine whether they are located on the nuclear or mitochondrial genomes.

5.2 RESULTS

5.2.1 Isolates

Most isolates used in chapter 5 were also used in chapter 6. Table 5.2.1 describes isolates from both chapters.

Table 5.2.1. Origins and genotypes of isolates used in chapters 5 and 6.

Used in Chapter	Isolate	Origin	Mating type	mtDNA	RG57 type (RF)	AFLP Fingerprint
5	87.205.4	Cornwall	A2	Ia	63	Y
5	96.93.11	Bryn Farm	A1	IIa	06	Y
5	97.179.1	Yorkshire	A1	ND	ND	N
5	97.131.2	Devon	A1	ND	ND	N
5	97.120.8	Lincolnshire	A1	Ia	ND	N
5, 6	E14C2	Egypt	A2	IIa	ND	Y
5, 6	N18[#]	California	A1	IIa	ND	Y
5, 6	W1[$]	The Netherlands	A1	Ia	ND	Y
5, 6	Y1[$]	The Netherlands	A1	Ia	ND	Y
5, 6	95.205.1	Pen y Ffridd	A1	ND	ND	N
5, 6	95.161.5	Rhiw Goch	A1	Ia	02	Y
5, 6	96.127.15	Siambra Gwynion	A1	IIa	11	Y
5, 6	97.123.1	Cheshire	A1	Ia	ND	Y
5, 6	97.185.5	Nottinghamshire	A1	Ia	ND	Y
5, 6	98.64.1	England	A1	Ia	ND	Y
5, 6	98.71.1	England	A1	Ia	ND	N
5, 6	96.89.41	Pant y Ddolen	A2	Ia	34	Y
5, 6	96.93.8	Bryn Farm	A1	Ia	26	Y
5, 6	96.93.22	Bryn Farm	A2	Ia	34	Y
5, 6	98.75.13	England	A1	Ia	ND	Y
5, 6	98.70.2	England	A1	Ia	ND	Y
5, 6	98.74.7	England	A1	Ia	ND	Y
5, 6	98.57.9	England	A1	Ia	ND	Y
5, 6	98.88.8	England	A1	Ia	ND	Y
6	5Aue[¥]	Moscow	A1	ND	ND	N
6	18.2[¥]	Moscow	A1	ND	ND	N
6	81.9	Gwynedd	A2	Ia	39	N
6	95.205.11	Pen y Ffridd	A1	Ia	26	N
6	95.205.16	Pen y Ffridd	A1	IIa	09	N
6	95.155.22	Siambra Gwynion	A1	IIa	06	N
6	95.152.3	Rhiw Goch	A1	Ia	02	N
6	95.154.7	Rhiw Goch	A2	IIa	01	N
6	95.162.7	Rhiw Goch	A1	Ia	02	N
6	96.122.1	Derbyshire	A1	Ia	26	N
6	96.37.1	England	A1	ND	ND	N
6	97.128.1	England	A1	ND	ND	N
6	96.5.8	Norfolk	A1	Ia	24	N
6	98.78.4	England	A1	ND	ND	N

Y and N indicate whether isolate was fingerprinted for AFLPs. ND indicates no data for this isolate. The RG57 (RF) and AFLP fingerprints of these isolates can be found in appendix Table A2.1 & A3.4. Bryn Farm, Pen y Ffridd, Rhiw Goch and Siambra Gwynion are sites within a 50 km radius in North Wales. Isolate donated by Howard Judelson[#], University of California, Riverside; Francine Govers[$], Wageningen Agricultural University or Alina Dolgova[¥], Moscow State University.

5.2.2 Selection of naturally occurring drug resistance

The sensitivity of 17 single-zoospore isolates (Table 5.2.2) to metalaxyl, streptomycin and chloramphenicol was investigated by determining the relative growth rates on drug-amended media compared to drug-free media (section 2.2.8.1). Actual growth rates of these isolates on various drug-concentrations in mm d^{-1}±S.D. can be found in appendix Table A4.1

5.2.2.1 *Sensitivity to metalaxyl*

All of the isolates had been previously tested for their sensitivity to metalaxyl at 10 μg ml^{-1} as part of a population genetics project (J. Day, unpublished). Metalaxyl resistant and sensitive isolates were chosen from this collection and grown on three concentrations of metalaxyl (Table 5.2.2).

Table 5.2.2. Relative growth rates (RGR) of wild-type isolates on metalaxyl-amended rye A agar as a percentage of growth on drug-free media.

Isolate	Metalaxyl concentration (μg ml^{-1})		
	10	100	200
95.161.5	85	72	60
97.185.5	82	88	71
97.123.1	91	77	59
98.64.1	97	66	40
98.57.9	79	72	67
98.71.1	91	76	47
98.70.2	95	81	59
98.74.7	92	77	27
96.104.2	30	7	0
98.75.13	88	71	44
98.88.8	85	75	55
96.93.8	0	0	0
96.93.22	0	0	0
96.89.41	0	0	0
87.205.4	113	94	42
96.127.15	52	28	0
E14C2	0	0	0

All of the isolates able to grow on 10 μg ml^{-1} were also able to grow on 100 μg ml^{-1} metalaxyl. Two isolates (96.104.2 and 96.127.15) which had RGRs of 7% and 28% on 100 μg ml^{-1} metalaxyl could not grow on 200 μg ml^{-1}. All other isolates able to grow on 100 μg ml^{-1} could grow to some extent on 200 μg ml^{-1} but no tested isolates grew at 500 μg ml^{-1}. Four isolates were unable to grow on 10 μg ml^{-1}. All isolates were inhibited to some extent by metalaxyl but 87.205.4 had a faster growth rate on 10 μg ml^{-1} metalaxyl than on drug-free media. The morphology of all

metalaxyl-resistant isolates on metalaxyl-amended media was similar to the morphology on drug-free media; they sporulated well, showed no hyphal inhibition (such as curling back on themselves) and the sporangia would germinate on media amended with concentrations of metalaxyl up to 200 μg ml^{-1}.

5.2.2.2 *Sensitivity to streptomycin*

The same 17 isolates were grown on rye A agar with various concentrations of streptomycin (Table 5.2.3) to determine whether any of the isolates were naturally resistant and to determine appropriate concentrations for selecting spontaneous mutants.

Table 5.2.3. Relative growth rates (RGR) of wild-type isolates on streptomycin-amended rye A agar as a percentage of growth on drug-free media.

Isolate	Streptomycin concentration (μg ml^{-1})				
	10	100	200	500	1000
95.161.5	98	74	70	64	32
97.185.5	24	0	0	0	0
97.123.1	41	0	0	0	0
98.64.1	26	0	0	0	0
98.57.9	36	23	0	0	0
98.71.1	18	0	0	0	0
98.70.2	22	0	0	0	0
98.74.7	15	0	0	0	0
96.104.2	55	0	0	0	0
98.75.13	29	0	0	0	0
98.88.8	18	0	0	0	0
96.93.8	34	0	0	0	0
96.93.22	68	0	0	0	0
96.89.41	21	0	0	0	0
87.205.4	42	0	0	0	0
96.127.15	36	0	0	0	0
E14C2	0	0	0	0	0

All isolates except E14C2 grew on agar amended with 10 μg ml^{-1} streptomycin, ranging from an RGR of 98 (95.161.5) to 15 (98.74.7). Only two of these isolates were able to grow on 100 μg ml^{-1}, 98.75.9 (RGR of 23) and 95.161.5 (RGR of 74). Only isolate 95.161.5 could grow at higher concentrations of streptomycin, up to the highest tested concentration of 1000 μg ml^{-1} (RGR of 32). Only this isolate therefore seemed to show natural resistance to streptomycin and as this isolate was also resistant to metalaxyl, it could serve as a useful control for investigating growth on double-drug media (chapter 6). This isolate had similar healthy morphology to the metalaxyl resistant isolates when grown on streptomycin

amended media. Sporangia from this isolate were viable at all tested streptomycin concentrations except 1000 μg ml^{-1}.

5.2.2.3 *Sensitivity to chloramphenicol*

The 17 isolates were grown on rye A agar with various concentrations of chloramphenicol to determine appropriate concentrations for selecting spontaneous mutants. All except two isolates tested showed intermediate resistance to chloramphenicol at 400 μg ml^{-1}; their RGRs ranged from 26-76 (Table 5.2.4).

Table 5.2.4. Relative growth rates (RGR) of wild-type isolates on chloramphenicol-amended rye A agar as a percentage of growth on drug-free media.

Isolate	Chloramphenicol concentration (μg ml^{-1})			
	10	100	200	400
95.161.5	83	62	32	30
97.185.5	85	70	56	59
97.123.1	111	82	57	70
98.64.1	106	83	66	54
98.57.9	87	64	59	46
98.71.1	88	62	59	56
98.70.2	100	81	54	54
98.74.7	88	50	42	38
96.104.2	100	45	20	0
98.75.13	88	53	38	47
98.88.8	85	63	38	45
96.93.8	93	66	C	46
96.93.22	100	78	62	57
96.89.41	95	61	18	26
87.205.4	123	83	0	0
96.127.15	96	112	84	76
E14C2	106	96	76	74

C, plates contaminated.

Most isolates growing at 400 μg ml^{-1} chloramphenicol produced unhealthy-looking growth and most strains lost viability after several weeks on this media. Very few sporangia were produced above 200 μg ml^{-1} and at 400 μg ml^{-1}, hyphae appeared to curl back on themselves in concentric circles. The lack of aerial hyphae and the shiny appearance of colonies suggested some hyphal lysis was occurring at this concentration.

Using the metalaxyl, streptomycin and chloramphenicol resistance phenotypes of these 17 isolates, it was not possible to pair isolates with different resistances so that recombinants (with a combination of the parental resistance phenotypes) could be selected. Isolates were therefore screened for resistance to other compounds (section 5.2.3) and mutant drug-resistant isolates were selected for (section 5.2.4).

5.2.3 Sensitivity to acriflavine, blasticidin and phosphorous acid

All isolates in Tables 5.3.1-5.3.3 were further examined for their growth on two concentrations each of acriflavine (10 or 20 μg ml^{-1}), blasticidin (140 or 280 μg ml^{-1}) and phosphorous acid (50 or 100 μg ml^{-1}). No growth was observed from plugs placed on these drugs suggesting that all isolates were completely inhibited by relatively low concentrations of these compounds. However, two isolates, 5 Aue (resistant to 20 μg ml^{-1} acriflavine) and 18.2 (resistant to 280 μg ml^{-1} blasticidin) were obtained from A. Dolgova, Moscow State University (Table 5.2.1).

5.2.4 Isolation and growth of spontaneous drug-resistant mutants

Numerous metalaxyl-resistant isolates were available to use in selection experiments. However, from the sample of isolates available, no metalaxyl-sensitive isolates were resistant to streptomycin and the majority of isolates exhibited abnormal growth on chloramphenicol. No isolates grew on acriflavine, blasticidin (except the donated resistant mutants) or phosphorous acid. To obtain mutants resistant to one of these drugs, 160 plugs of each of eight isolates were placed on rye A agar containing either of two concentrations of these drugs (Table 5.2.5). From the previous growth tests on streptomycin (Table 5.2.3), all isolates showed some growth on 10 μg ml^{-1} but were completely inhibited at 200 μg ml^{-1} (except resistant isolate 95.161.5), so 100 and 200 μg ml^{-1} were chosen to obtain streptomycin mutants, as previously suggested (Shaw, 1996). Concentrations of the other drugs (Table 5.2.5) were as recommended (A. Dolgova, personal communication).

Table 5.2.5. The number of spontaneous drug-resistant sectors obtained from culturing 160 plugs of each isolate on drug-amended media.

	Number of sectors			Sectors viable on sub culture		
Isolate	SM	AC	BL	SM	AC	BL
96.93.8	5	0	0	1	0	0
96.93.22	3	1	1	2	0	0
96.89.41	1	0	1	1	0	0
E14C2	0	0	0	0	0	0
97.120.8	1	2	1	0	1	1
97.131.2	0	1	1	0	1	0
97.179.1	0	0	1	0	0	0
95.205.1	0	0	0	0	0	0

Drug concentrations (streptomycin (SM), 100 and 200 μg ml^{-1}; acriflavine (AC), 10 and 20 μg ml^{-1}; blasticidin (BL), 140 and 280 μg ml^{-1}; phosphorous acid, 50 and 100 μg ml^{-1}. No growths were observed on phosphorous acid.

After two-four weeks, resistant sectors had developed on some of the plates as

dense sporulating regions growing from the original inoculum plug. The number of sectors obtained from each isolate–drug combination and the number of these viable on sub-culture to fresh media is shown in Table 5.2.5. Incubating isolates on inhibitory concentrations of drugs produced numerous mutant sectors (Table 5.2.5); all resistant sectors were obtained on the lower concentration of the drug used for mutant isolation. Most resistant sectors were obtained on streptomycin where 10 (0.8%) of the 1280 plugs formed resistant sectors; four of these sectors were able to grow after sub-culture to fresh drug-amended media. Four (0.3%) of the plugs on acriflavine and five (0.4%) on blasticidin resulted in resistant growths but only three of these grew on first sub-culture to drug-amended media. Neither of the two acriflavine mutants or the blasticidin mutant produced viable zoospores. These isolates did not sporulate well and had hyphal morphology similar to isolates grown on chloramphenicol-amended media (section 5.2.2.3). These isolates eventually stopped growing and could not be revived even on rye A agar.

A single zoospore line was isolated from each of the four streptomycin-resistant mutants able to grow after sub-culture and these were suffixed with Sr to identify them from wild-type. Growth rates of these isolates were reduced only slightly at 500 $\mu g\ ml^{-1}$ when compared to growth on drug-free media (Figure 5.2.1) and all four mutants could grow on higher concentrations of streptomycin than they were selected on. Isolates 96.93.22.Sr1 and 96.93.22.Sr2 are single-zoospore isolates from different mutant sectors of the same parental isolate.

5.2.5 Growth rates of transformed isolates

The relative growth rates (section 2.2.8.1) of the three transformed isolates (From H. Judelson and F. Govers, Table 5.2.1) were compared on a range of drugs as detailed in Table 5.2.6. Actual linear growth rates can be found in appendix Table A4.2. The three transformed isolates, when grown on the substrates for which they had the transformed resistance gene, had growth rates slightly less than growth on drug-free medium. None of the isolates would grow at even low concentrations (10 $\mu g\ ml^{-1}$) of metalaxyl or streptomycin. The G418-resistant isolates would not grow on low concentrations of hygromycin and the hygromycin-resistant isolate would not grow on low concentrations of G418.

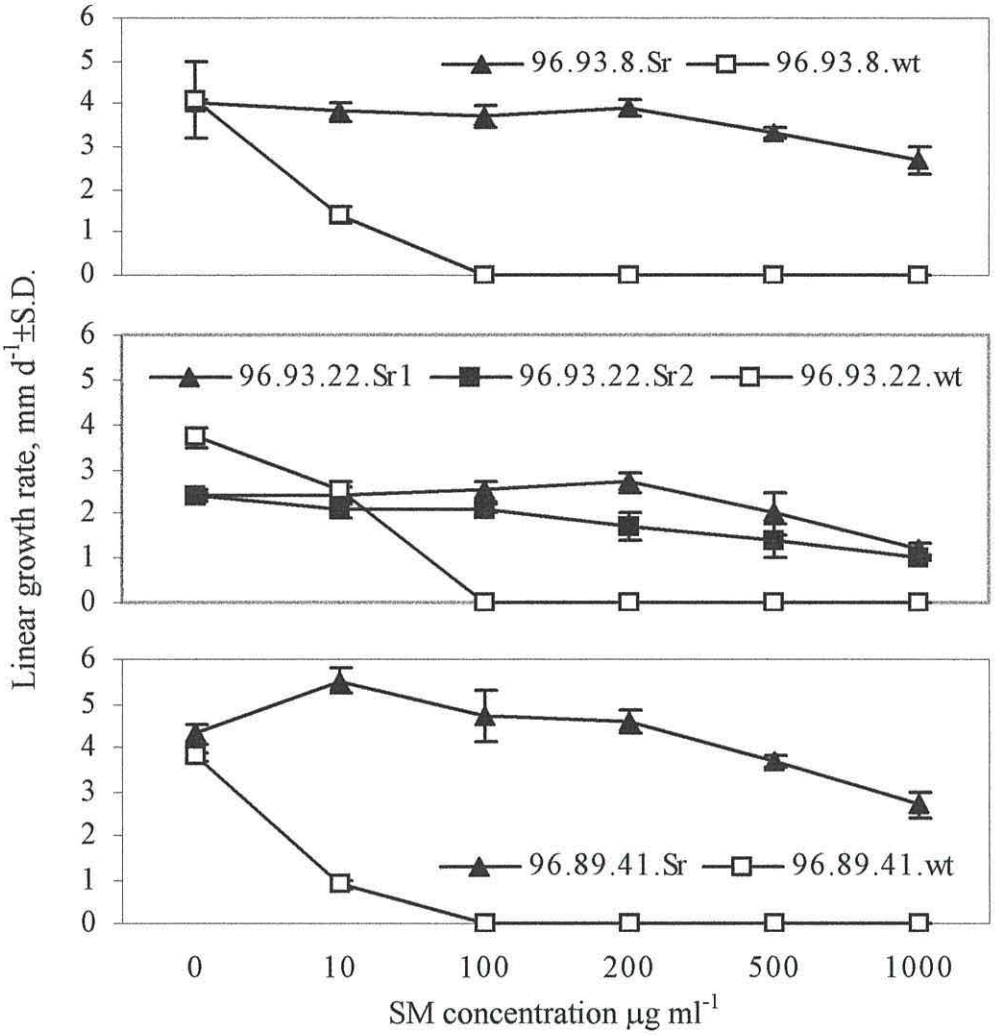


Figure 5.2.1. Linear growth rate (increase in colony diameter) of single-zoospore isolates from streptomycin-resistant and wild-type strains on rye A agar containing various concentrations of streptomycin (SM). Values (mm d^{-1}±S.D) are means of four replicate plates.

Table 5.2.6. Relative growth rates of transformed isolates on drug-free and drug-amended media. Means of four replicates.

Isolate	Drug concentration (µg ml^{-1})						
	Hygromycin			G418			
	5	10	20	5	10	20	50
N18	0	0	ND	98	98	94	84
Y1	0	0	ND	102	94	96	75
W1	87	100	83	0	0	ND	ND

ND, not determined

5.2.6 Stability of drug-resistant isolates

A single-zoospore line (section 2.2.3) was isolated from each metalaxyl-resistant isolate and along with the single-zoospore lines from the streptomycin resistant

mutants, (section 5.2.4) the stability of these drug resistant isolates was assessed as in section 2.2.8.3. None of the metalaxyl-resistant isolates lost their resistance after sub-culture on drug-free media. After four sub-cultures on drug-free media, the relative growth rates of metalaxyl-resistant isolates (on 100 μg ml^{-1}) ranged from 32-97, similar to the range of relative growth rates (57-100) observed after four sub-cultures on metalaxyl-media.

All streptomycin-resistant mutants were stable when grown on drug-free media. After four sub-cultures on rye A agar, relative growth rates (41–60) were similar to relative growth rates of the same lines that had not been removed from streptomycin medium (52–73). The three transformed isolates (Table 5.2.6) were supplied as single-zoospore isolates (F. Govers & H. Judelson, personal communication) and could also grown on drug-free media with no loss in growth rate compared to cultures of the same isolate grown on drug-amended media. The spontaneous mutants resistant to either blasticidin or acriflavine could not be cultivated for more than four sub-cultures on media amended with these drugs.

5.2.7 Summary of drug-resistant strains

The drug-sensitivity of all strains is summarised in Table 5.2.7.

Table 5.2.7. Sensitivity of wild-type, mutants and transformed isolates on various concentrations of drugs (μg ml^{-1}).

Isolate	Metalaxyl			Streptomycin		Chloramphenicol		Hygromycin	G418
	10	100	200	10	100	200	400	10	10
95.161.5	R	I	I	R	I	I	I	S	S
97.185.5	R	R	I	I	S	I	I	S	S
97.123.1	R	R	I	I	S	I	I	S	S
98.64.1	R	I	I	I	S	I	I	S	S
98.57.9	R	I	I	I	I	I	I	S	S
98.71.1	R	I	I	I	S	I	I	S	S
98.70.2	R	R	I	I	S	I	I	S	S
98.74.7	I	I	I	I	S	I	I	S	S
96.104.2	R	S	S	I	S	I	I	S	S
98.75.13	R	I	I	I	S	I	I	S	S
98.88.8	R	I	I	I	S	I	I	S	S
96.93.8	S	S	S	I	S	C	I	S	S
96.93.22	S	S	S	I	S	I	I	S	S
96.89.41	S	S	S	I	S	I	I	S	S
87.205.4	R	R	I	I	S	S	S	S	S
96.127.15	I	I	S	I	S	I	I	S	S
E14C2	S	S	S	S	S	I	I	S	S
96.93.8.Sr	S	S	S	R	R	I	I	S	S
96.93.22.Sr1	S	S	S	R	R	I	I	S	S
96.93.22.Sr2	S	S	S	R	R	I	I	S	S
96.89.41.Sr	S	S	S	R	R	I	I	S	S
W1	S	S	S	S	S	I	I	R	S
Y1	S	S	S	S	S	I	I	S	R
N18	S	S	S	S	S	I	I	S	R

R, resistant, S, sensitive, I, intermediate, C, contaminated

Isolates with a RGR of 80% or more were classed as resistant to the drug at that concentration and those with a RGR of 10% or less were sensitive. Isolates between these two values were intermediate.

Media containing streptomycin, hygromycin or G418 inhibited all metalaxyl-resistant isolates, except 95.161.5. Media containing metalaxyl, hygromycin or G418 inhibited the mutant strains resistant to streptomycin and the transformants resistant to hygromycin or G418 were inhibited by low concentrations of metalaxyl or streptomycin. Most isolates revealed growth on media containing chloramphenicol as described previously (section 5.2.2.3).

5.2.8 Germination of oospores

To investigate inheritance of AFLP bands and determine the origin of resistance to metalaxyl and streptomycin in these isolates, isolates were crossed (section 2.2.4) with drug-sensitive isolates having different mtDNA haplotypes. Three isolates transformed for resistance to G418 & hygromycin were not crossed as the origin of their resistance to these chemicals is known (Judelson, 1991; van West *et al.*, 1998). Resistance is probably through single-site transformation (Judelson, 1991) but this is not known for certain.

The six matings differed in respect to the numbers of oospores produced, the percentage germinating and the percentage of germinated oospores which when isolated to fresh media grew into colonies (Table 5.2.8). Germination of oospores from the six matings began on the water agar plates after 6-10 days. Germlings, some having a germ sporangium, were dissected from the plates every 2-3 days for up to 5 weeks although germination may have continued for longer. The number of germinated oospores which grew into colonies when incubated on rye A agar ranged from 4% (matings C & D) to 16% (mating A) (Table 5.2.8). Germlings of mating E did not grow into viable colonies.

Table 5.2.8. Fate of 50 germinated oospores removed to rye A agar from each of 6 matings (A-F)

A1 parent	A2 parent	Code	% developing into colonies
97.123.1	96.102.6	A	16
97.185.5	96.102.6	B	10
96.93.11	96.89.41	C	4
96.127.15	96.89.41	D	4
95.161.5	E14C2	E	0
95.161.5	96.102.6	F	14

5.2.9 Inheritance of resistance to metalaxyl in F1 progeny

5.2.9.1 *Mating A: 97.123.1 x 96.102.6*

Eight single-oospore cultures were obtained from mating A. Only one of these (P2) could grow on media amended with 100 μg ml^{-1} metalaxyl and on this medium it had a linear growth rate of 2.1±0.3 compared to 3.5± 0.24 (97.123.1) and 0 (96.102.6) (mm d^{-1}±S.D) of the parents. Parents and progeny were fingerprinted for AFLPs and typed for mtDNA haplotype (Table 5.2.9).

Table 5.2.9. Mating type (MT), metalaxyl sensitivity (MEX) and polymorphic AFLP bands of parents and progeny (P1-8) in cross A: 97.123.1 x 96.102.6.

Parent/ Progeny	MT	MEX	mtDNA	Polymorphic AFLP bands										
				E19-M16						E19-M40				
				15	42	45	46	51	64	6	17	29	38	49
97.123.1	A1	I	Ia	0	0	1	0	1	0	1	0	1	1	0
96.102.6	A2	S	IIa	1	1	1	1	1	1	0	1	0	0	1
P1	A1	S	Ia	1	1	1	1	0	1	0	1	0	1	0
P2	A1	I	Ia	1	0	1	0	0	1	1	0	1	1	0
P3	A1	S	Ia	1	1	0	1	0	1	1	1	0	1	0
P4	A1	S	Ia	1	0	1	1	1	1	1	1	1	1	0
P5	A1	S	Ia	1	0	1	0	0	1	1	1	0	1	0
P6	A1	S	Ia	1	1	1	1	0	1	0	1	1	1	0
P7	A1	S	Ia	1	0	1	0	0	1	1	0	0	1	0
P8	A1	S	Ia	1	1	1	1	0	1	1	1	0	1	0

Bands in green were only present in the A2 parent and bands in red were only present in the A1 parent. Bands in blue were present in both parents but were polymorphic in the progeny.

All progeny had the mtDNA haplotype of parent 97.123.1. With AFLP primers E19-M16, six bands were polymorphic amongst the parents and progeny and with E19-M40, five bands varied amongst parents and progeny (Table 5.2.9; Figure 5.2.3). Using E19-M16 alone, four genotypes could be resolved in the progeny. Using E19-M40 alone, seven different genotypes could be identified. Combining the data from both pairs of primers, all of the progeny had different genotypes from each other and from the parental isolates. Further, all progeny showed bands that must have been inherited from both parents, suggesting that they were all hybrids.

5.2.9.2 *Mating B: 97.185.5 x 96.102.6*

Five viable oospore progeny were obtained from mating B. Only one of these (P1) could grow on media amended with 100 μg ml^{-1} metalaxyl and on this medium it had a linear growth rate of 2.4±0.2 compared to 2.9± 0.27 (97.185.5) and 0 (96.102.6) (mm d^{-1}±S.D) of the parents.

All progeny had mtDNA haplotype Ia from the metalaxyl-resistant A1 parent. AFLP primers E19-M16 revealed six bands and E19-M40 seven bands that were polymorphic amongst the parents and progeny (Table 5.2.10; Figure 5.2.3). Combining the data from both primer-pairs, four different fingerprint patterns could be identified in the progeny. Four of the progeny (P1-P4) had AFLP bands from both parents and were probably hybrid. One of the progeny (P5) only had bands from the A1 parent although it differed from this isolate at 3 loci (Table 5.2.10) and it is possible that this isolate could be a product of selfing. Interestingly, P5 differed in its sensitivity to metalaxyl from that of the A1 parent, the parental isolate being resistant and the putative self (P5) being sensitive.

Table 5.2.10. Mating type (MT), metalaxyl sensitivity (MEX) and polymorphic AFLP bands of parents and progeny (P1-P5) in cross B, 97.185.5 x 96.102.6.

Parent/ Progeny	MT	MEX	mt DNA	Polymorphic AFLP bands												
				E19-M16						E19-M40						
				9	38	42	45	46	51	6	16	17	29	34	38	57
97.185.5	A1	R	Ia	1	1	1	1	1	0	1	0	0	1	1	1	0
96.102.6	A2	S	IIa	0	0	1	1	1	1	0	1	1	0	1	0	1
P1	A1	R	Ia	0	0	0	1	0	0	1	0	1	0	0	1	0
P2	A1	S	Ia	0	1	0	0	0	0	1	0	1	0	0	1	0
P3 & P4	A1	S	Ia	0	0	1	0	1	0	1	0	1	0	1	1	0
P5	A1	S	Ia	0	0	1	1	0	0	1	0	0	1	1	1	0

Bands in green were only present in the A2 parent and bands in red were only present in the A1 parent. Bands in blue were present in both parents but were polymorphic in the progeny.

5.2.10 Inheritance of resistance to streptomycin in F1 progeny

5.2.10.1 *Mating C: 96.93.11 x 96.89.41 Sr*

Only two viable oospore progeny were obtained from mating C (Table 5.2.11).

Table 5.2.11. Mating type (MT), streptomycin sensitivity (SM) and polymorphic AFLP bands of parents and progeny (P1-P2) in cross C, 96.93.11 x 96.89.41 Sr.

Parent/ Progeny	MT	SM	mtDNA	Polymorphic AFLP bands									
				E19-M16				E19-M40					
				2	13	42	45	6	16	38	47	49	57
96.93.11	A1	S	IIa	0	0	1	1	0	1	0	0	1	1
96.89.41 Sr	A2	R	Ia	1	1	0	0	1	0	1	1	1	1
P1	A1	S	IIa	0	0	0	1	0	1	0	1	0	0
P2	A1	S	IIa	0	0	0	1	1	1	1	1	0	0

Bands in green were only present in the A2 parent and bands in red were only present in the A1 parent. Bands in blue were present in both parents but were polymorphic in the progeny.

Both progeny had the same drug-resistant phenotype, mating type and mtDNA

haplotype as the streptomycin-sensitive parent (Table 5.2.11). Primers E19-M16 revealed four and E19-M40 revealed six bands that were polymorphic amongst the parents and two progeny (Table 5.2.11; Figure 5.2.3). Both progeny revealed bands from both parents and so were probably hybrids.

5.2.10.2 *Mating D: 98.127.15 x 96.89.41 Sr*

Two viable oospore progeny were obtained from mating D and both were streptomycin sensitive and had the same phenotype as the sensitive parent for mating type and mtDNA haplotype (Table 5.2.12).

Table 5.2.12. Mating type (MT), streptomycin sensitivity (SM) and polymorphic AFLP bands of parents and progeny (P1-P2) in cross D, 98.127.15 x 96.89.41 Sr.

Parent/ Progeny	MT	SM	mt DNA	Polymorphic AFLP bands											
				E19-M16					E19-M40						
				9	21	38	45	46	6	7	16	34	38	49	57
96.127.15	A1	S	IIa	0	0	1	1	1	0	1	1	1	0	1	1
96.89.41 Sr	A2	R	Ia	1	1	0	0	1	1	1	0	1	1	1	1
P1	A1	S	IIa	1	0	0	1	1	1	1	1	0	1	0	0
P2	A1	S	IIa	1	0	0	1	0	0	0	0	1	0	1	1

Bands in green were only present in the A2 parent and bands in red were only present in the A1 parent. Blue bands were present in both parents but were polymorphic in the progeny.

Using primers E19-M16, five bands and with E19-M40 seven bands varied amongst parents and progeny (Table 5.2.12; Figure 5.2.3). Using these primers, bands from both parents could be identified in the progeny and so both progeny were probably hybrid.

5.2.11 Inheritance of resistance to metalaxyl and streptomycin in F1 progeny

5.2.11.1 *Mating F: 95.161.5 x 96.102.6*

Colonies were established from six germinated oospores. All progeny were A2 mating type and inherited mtDNA from the A2 parent (Figure 5.2.4). All were sensitive to metalaxyl and only one (P2) showed intermediate resistance to streptomycin. On media amended with 100 μg ml^{-1} streptomycin, P2 had a linear growth rate of 0.7±0.1 compared to the parental rates of 3.5±0.3 (95.161.5) and 0 (96.102.6) mm d^{-1}±S.D.

With E19-M16, four bands and with E19-M40, five bands were polymorphic amongst the parents and progeny (Table 5.2.13). Five of the six progeny inherited bands from both parents and were probably hybrid. P1 only revealed bands from the

A2 parent and so may have been a self of this isolate.

Table 5.2.13. Mating type (MT), metalaxyl (MEX) or streptomycin (SM) sensitivity and polymorphic AFLP bands of parents and progeny in cross F 95.161.5 x 96.102.6.

Parent/ Progeny	MT	MEX	SM	mt DNA	Polymorphic AFLP bands								
					E19-M16				E19-M40				
					9	21	25	46	6	12	17	29	38
95.161.5	A1	R	R	Ia	1	0	0	0	1	0	0	1	1
96.102.6	A2	S	S	IIa	0	1	1	1	0	1	1	0	0
P1	A2	S	S	IIa	0	0	1	1	0	0	0	0	0
P2	A2	S	I	IIa	0	0	1	1	1	0	1	0	1
P3	A2	S	S	IIa	1	0	1	1	1	0	1	0	1
P4	A2	S	S	IIa	1	0	1	1	1	0	1	0	1
P5	A2	S	S	IIa	1	0	1	1	1	0	1	0	1
P6	A2	S	S	IIa	1	0	1	1	1	0	1	0	1

Bands in green were only present in the A2 parent and bands in red were only present in the A1 parent.

5.2.12 AFLP bands polymorphic in each pairing

The AFLP fingerprints were reproducible in all parents and progeny and no bands appeared in the progeny that were not present in the parents. Between 88 (mating F) and 110 (mating A) scorable loci were monomorphic and detected in all parents and progeny; the majority of AFLP loci were therefore monomorphic and were transmitted to sexual progeny (Table 5.2.14). From 9% (A, C & F) to 11% (B) of scorable loci were polymorphic amongst parents and progeny.

Table 5.2.14. The number of scorable and polymorphic AFLP bands from each mating-primer-pair combination.

Mating	No. of scorable bands			No. of polymorphic bands		
	E19-M16	E19-M40	Total	E19-M16	E19-M40	Total
A	54	67	121	6	5	11
B	56	66	122	6	7	13
C	54	58	112	4	6	10
D	55	63	118	5	7	12
F	41	56	97	4	5	9

5.2.13 Usefulness of AFLP loci in these matings

To compare the conclusions reached using both AFLP primers to those reached using only one or other of the primer pairs, Figure 5.2.2 shows AFLP bands scored and in which crosses they were polymorphic. Using both primer-pairs, 21 of the 23 progeny revealed bands from both parents and were thus hybrid progeny.

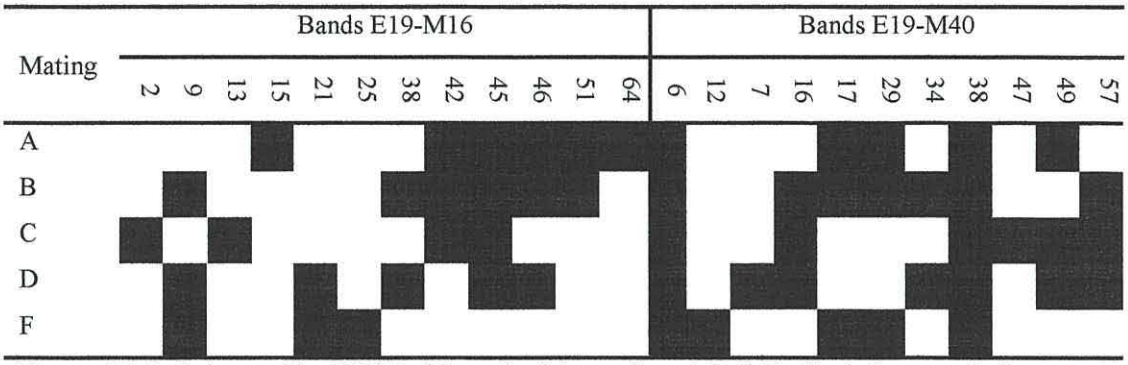
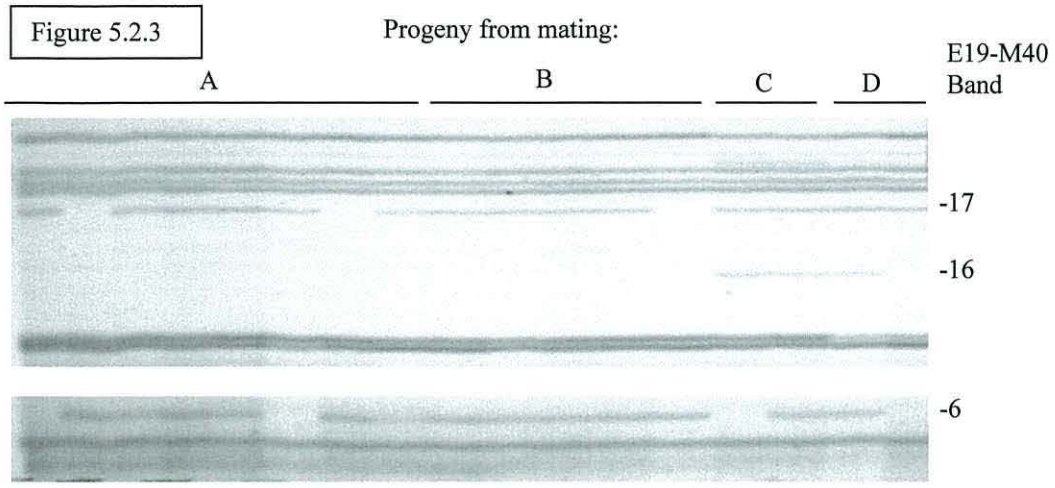


Figure 5.2.2. Polymorphic AFLP loci in each of the matings A-D & F. Shaded squares indicate band was polymorphic in parents or progeny in that mating.

Combining the data from all of the matings, 23 loci were polymorphic amongst parents and oospore progeny using primer-pairs E19-M16 and E19-M40. One locus (E19-M16 45) was polymorphic in all but one pairing and two loci (E19-M40 6 & 38) were polymorphic in all pairings (Figure 5.2.2). Alone, the E19-M40 data suggested that 16 of the 23 oospore progeny were hybrid as they had inherited bands from both parents. Only six of the 23 progeny appeared to be hybrid when the E19-M16 data was used alone, even though more polymorphic bands were observed using these primers.



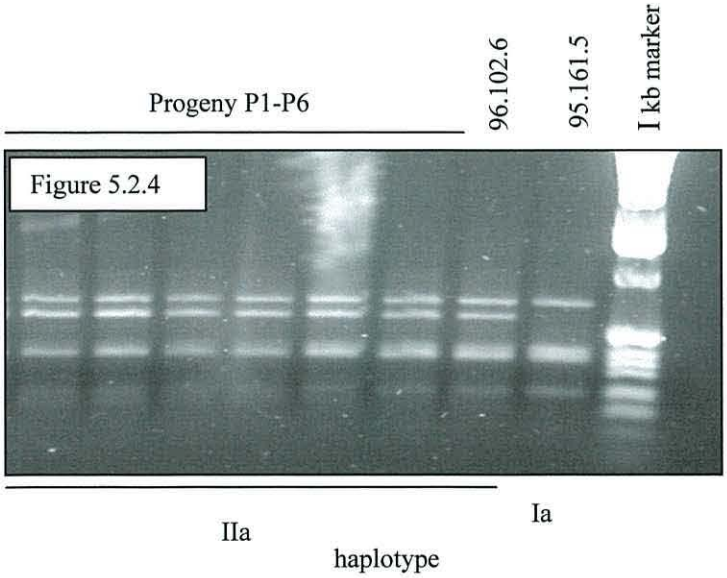


Figure 5.2.3. AFLP fingerprints of progeny from matings A-D. Three polymorphic bands are shown amplified from primers E19-M40.

Figure 5.2.4. mtDNA haplotypes of parents and six progeny from mating F.

5.3 DISCUSSION

5.3.1 Obtaining drug resistant mutants

Many field isolates are resistant to metalaxyl (Day & Shattock, 1997; Day *et al.*, unpublished) when tested at 10 μg ml^{-1}. Subsequent resistance trials in the present study showed that these isolates could grow at concentrations up to 200 μg ml^{-1} (Table 5.2.2) and resistance was stable even when grown on drug-free media. Only one isolate, 95.161.5, was naturally resistant to streptomycin (Table 5.2.3) and this isolate was also naturally resistant to metalaxyl. The other strains resistant to streptomycin were selected as spontaneous resistant sectors on 100 μg ml^{-1} streptomycin amended media: strong selection pressure on the wild-type colonies allowed isolation of such resistant sectors. Wild-type isolates in the present study were able to grow at much higher chloramphenicol concentrations (up to 400 μg ml^{-1}) than observed in previous studies (Shattock & Shaw, 1975; Rooke & Shattock, 1983; Whittaker *et al.*, 1996) (Table 5.3.3). Abnormal morphology of isolates growing on chloramphenicol-amended media, as seen in the present study, has been noticed before (Rooke & Shattock, 1983; El Refai, 1990; Whittaker *et al.*, 1996) and these isolates were not used extensively in further studies. Further resistance tests and mutant isolation revealed that many metalaxyl-resistant, streptomycin-sensitive isolates and four streptomycin-resistant, metalaxyl-sensitive mutants were available for use in further studies (chapter 6) along with three transformed isolates to G418 or hygromycin.

5.3.2 Germination of oospores

Some matings yield oospores that germinate to a high final percentage, whereas others show slow and gradual germination, or no germination at all: these oospores may be aborted or possibly dormant (Shattock, *et al.*, 1986; Al-Kherb *et al.*, 1995). The percentage of oospore germination and establishment of colonies in the present study was low. The percentage germination in all five successful pairings ranged from 0.07%-0.27% (from counting number of germinated oospores per plate) and of these germinated oospores, 4%-16% developed into colonies. Whittaker *et al.* (1996) found that 3%-5% of oospores germinated and of these, 35% were able to grow into colonies. Shattock *et al.* (1986) found that germination of oospores in some crosses

could be between 5-50% and Al-Kherb *et al.* (1995) observed 32-90% germination in a sample of 31 crosses, although they were unable to germinate oospores from many crosses. Variation in genotype (Al-Kherb *et al.*, 1995), place of origin (Harrison, 1991; Al-Kherb *et al.*, 1995) or in ploidy levels (Whittaker *et al.*, 1991b) do not prevent oospore formation and sexual recombination in *P. infestans*. However, isolates from the same or near sites may produce more oospores than those from more distant sites (Harrison, 1991). Of the three pairings (A, B & F) producing most progeny in the present study, two of the parents were isolated from sites 400 km distant (A & B) but the parents in pairing F were both from North Wales. The only pairing (E) in the present study between isolates from different countries (UK x Egyptian) did not reveal any germinated oospores but this Egyptian isolate may have changed genetically since it was first isolated (Harrison, 1991) through intermittent storage in liquid nitrogen.

5.3.3 Identification of hybrid oospores

Most oospore-derived progeny are hybrid (Shattock *et al.*, 1986; El Refai, 1990; Al-Kherb *et al.*, 1995; Whittaker *et al.*, 1996; Judelson, 1997a), although some may arise through self-fertilisation or apogamy (Shaw, 1991). Hybrids can be difficult to identify if appropriate markers are not available or are uninformative. Dominant markers (RG57, RAPD) or co-dominant markers (isozymes, microsatellites) allow parent-specific bands to be identified in the progeny but do not reveal as many loci as AFLPs.

Up to 95% of the legible AFLP bands were monomorphic in parents and progeny, demonstrating that most loci were somatically stable and homozygous. Five (mating F) to 11 (matings A & D) polymorphic parental bands were inherited in some or all of the progeny, as would be expected in a diploid organism depending on the heterozygosity at each locus. Only two progeny (mating B, P5 and mating F, P1) did not reveal bands specific to each parent, possibly the result of selfing (automixis) or non-transmission of loci. Non-transmission and aberrant segregation of DNA markers can occur resulting in hybrids appearing as non-hybrids. Harrison (1991) found a skewed distribution of isozyme genotypes in one cross and suggested that progeny carrying certain isozyme alleles may be less viable. In *P. nicotianae* var. *parasitica*, more than half of the progeny did not inherit a band from both parents at 2 of 5 loci (Förster & Coffey, 1990) possibly a consequence of one of these isolates, a trisomic,

losing one of three homologous chromosomes by mitotic non-disjunction. Although only two progeny from matings A-F did not reveal parent-specific bands from both parents, these progeny may have also been hybrid but may not have inherited one or more of the polymorphic loci revealed with AFLPs. Further crosses with these isolates or further AFLP analysis could determine this.

It is possible that non-transmission resulted in some loci (such as E19-M16 51 and E19-M40 49 in cross A) not being detected in the progeny. Theoretically, a proportion of the progeny should have revealed these bands, depending on the heterozygosity at each locus. In certain regions of the genome, such as the mating type locus (section 1.2.1), aberrant segregation has been observed (Judelson *et al.*, 1995; van der Lee *et al.*, 1997) resulting in a skewed distribution of particular genotypes in the progeny. If these AFLP bands were amplified from such regions, or other regions showing enhanced or reduced fitness or segregation distortion, then aberrant segregation may account for the non-transmission of these bands.

5.3.4 Inheritance of mtDNA and mating type

Sexual crosses should result in equal proportions of A1 and A2 progeny as mating type in *P. infestans* is determined by a single locus (section 1.2.1; Judelson *et al.*, 1995; Judelson, 1996). This has been found in some matings (Shattock *et al.*, 1986; El-Refai, 1990; Chang & Ko, 1990) but others have produced an excess of one mating type (Gallegly, 1970; Khaki & Shaw, 1974; Shattock *et al.*, 1986; Whittaker *et al.*, 1994). The five matings (A-F) all produced populations of oospores of one mating type only. All 17 progeny from crosses A, B, C & D were A1 mating type and co-inherited mtDNA from the A1 parent (Ia from matings A & B and IIa from matings C & D). In mating F, all the progeny were A2 mating type and co-inherited mtDNA (IIa) from the A2 parent. In previous matings, mtDNA and mating type segregated in oospore progeny (Whittaker *et al.*, 1994 & 1996; Carter *et al.*, 1999). The lack of segregation in the present study may be due to the small sample size of progeny; isolating more progeny from further crosses could determine this. *P. infestans* is known to be bisexual (Galindo & Gallegly, 1960) and these data also suggest sexuality is dependent on the pairing. Isolate 96.102.6 acted mainly as the male parent in crosses A & B but as the female in cross F, assuming that mtDNA is inherited uni-parentally (Whittaker *et al.*, 1994) through the oospore.

5.3.5 Inheritance of resistance

In *P. nicotianae* var. *parasitica, P. infestans*, and other Oomycetes, a major component of metalaxyl-resistance may be inherited through a single dominant (Chang & Ko, 1990; Lee *et al.*, 1999) or a single semi-dominant (Crute & Harrison, 1988; Shattock, 1988; El Refai, 1990; Harrison, 1991; Bhat *et al.*, 1993) nuclear locus (section 1.2.2). It is also possible that numerous minor genes (Harrison, 1991; Shaw, 1991; Fabritus *et al.*, 1997) and at least two major, semi-dominant genes are responsible for the inheritance of resistance to metalaxyl (Judelson & Roberts, 1999) and that different genes could be responsible for metalaxyl-resistance in different isolates. With such a small sample size of oospore progeny, it is impossible to distinguish between nuclear and mitochondrial or single and multiple locus-control of metalaxyl-resistance in the isolates used in the present study. However, the major component of metalaxyl-resistance in these isolates is probably located on the nucleus as progeny with resistances intermediate to the parents were obtained.

Mitochondria are inherited uni-parentally (Whittaker *et al.*, 1994) and in some isolates, streptomycin-resistance is inherited through the cytoplasm (Shaw & Elliott, 1968; El-Refai, 1990), probably on the mitochondrion (Whittaker *et al.*, 1996). If this was true for streptomycin-resistant isolates in the present study, none of the progeny from crosses C, D and F should have had the resistant phenotype. The intermediate-resistance of P2 would seem to contradict these findings and could suggest that streptomycin-resistance in 95.161.5, like metalaxyl-resistance, may have had a nuclear component.

5.3.6 Usefulness of particular primers and loci

Data presented in Figure 5.2.2 shows each of the pairings and the polymorphic bands from primer pairs E19-M16 and E19-M40 used to score parents and progeny. Theoretically, AFLP being a random fingerprinting technique, detection of polymorphisms should be similar using primer-pairs with the same number of extension bases, assuming the genome is not biased too heavily in favour of certain bases. A similar number of loci were scored for each primer-pair but the conclusions reached from individual primer-pairs were somewhat different; outcrossing amongst the progeny could be confirmed in only two progeny with E19-M16 but in 17 progeny using E19-M40. Loci detected with E19-M40 may have been more "informative" than those revealed by E19-M16; maybe E19-M40 loci are randomly distributed

throughout the genome whereas many of those detected by E19-M16 are not, as observed for some AFLP markers (Young *et al.*, 1999). In a previous study, van der Lee *et al.* (1997) observed that AFLP markers in all regions of the genome and with all primer-pairs displayed normal segregation with the exception of those near the mating type locus. However, for further studies, E19-M40 may be more useful than E19-M16 for detecting hybrid progeny in *P. infestans*.

5.3.7 Conclusions

Incubating plugs of mycelia on media containing streptomycin allowed isolation of streptomycin-resistant mutants. These complemented the metalaxyl-resistant mutants and produced drug-resistant isolates that could be used for recombination experiments (chapter 6). AFLP was very effective in detecting polymorphisms and hybrid oospore progeny, but primer-pairs E19-M40 were more informative than E19-M16 for detecting recombinants. The percentage germination of oospores was very low, and was probably due, partly, to some isolates being stored in the laboratory for a number of years. Correspondingly, limited data did not allow the inheritance patterns of mtDNA, mating type streptomycin and metalaxyl-resistance to be determined in these isolates.

6 The selection of somatic hybrids of *Phytophthora infestans* using drug resistant markers

6.1 INTRODUCTION

Resistance to antibiotics and other anti-microbial compounds such as metalaxyl often exhibit dominant or semi-dominant inheritance (Shaw & Khaki, 1971; Long & Keen, 1977a; Shattock, 1988; section 1.2.2; chapter 5). Such markers could allow the selection of heterokaryons and somatic recombinants, similar to selection techniques used to identify transformed bacteria (Brown, 1990).

6.1.1 Using drug-resistant markers to identify somatic recombinants

Long & Keen (1977b) paired drug-resistant or auxotrophic mutants of *P. sojae* (*P. megasperma* var. *sojae*) in an attempt to generate a heterokaryon and obtain somatic recombinants. They obtained some growths that had a combination of the parental phenotypes and these could be maintained through single hyphal-tip propagation. The drug-resistant markers subsequently segregated in zoospore progeny, suggesting that the double-drug resistant isolate was a heterokaryon and no recombinant progeny were obtained. Layton & Kuhn (1990) also paired isolates of *P. sojae* (*P. megasperma* f.sp. *glycinea*), resistant to either metalaxyl or fluorotryptophan on soybean leaves, and selected for putative heterokaryons by placing infected plant tissue directly onto agar amended with metalaxyl and fluorotryptophan. Growths on double-drug amended media gave rise to either metalaxyl- or fluorotryptophan-resistant zoospores. However, as these double-drug resistant strains were not isolated through a single sporangium or hyphal-tip, these growths could have been mixtures of the two resistant isolates (and not heterokaryons) that gave rise to zoospores with parental phenotypes.

A1 mutant isolates of *P. infestans* either resistant to chloramphenicol or dependent on streptomycin were co-cultivated on agar or mixed in water (Shattock & Shaw, 1976). Growth from the mixed inoculum was subsequently selected on chloramphenicol + streptomycin-amended agar and a single-zoospore line expressing a combination of the parent phenotypes was isolated. In a similar experiment, mutants of *P. infestans* resistant to acriflavine, blasticidin, oxytetracycline or streptomycin were cultured in pairs and selected on double-drug amended media

(Dyakov & Kulish, 1979, in Shaw, 1983b). Zoospores from these isolates were resistant to both drugs and did not segregate for drug-resistance. The single-zoospore lines with new drug-resistant phenotypes obtained by Shattock & Shaw (1976) and Dyakov & Kulish (1979, in Shaw, 1983b) may have been a result of heterokaryon formation and karyogamy between nuclei of different strains, but this could not be distinguished from mutation, without molecular markers.

El Refai (1990) used isozyme polymorphisms to investigate similar double-drug resistant phenotypes from pairings of A1 isolates resistant to metalaxyl, streptomycin or chloramphenicol. Many single-zoospores from double-drug resistant growths could only grow on one of the drugs, and had parental isozyme genotypes. However, isozyme analysis of double-drug-resistant zoospores revealed that some had a 3-banded isozyme pattern, consistent with the zoospores having determinants from the single-banded, homozygous parents.

More recent studies in *P. infestans* (Judelson & Yang, 1998) have used transformed strains resistant to G418 or hygromycin. After numerous A1 x A1 and A2 x A2 pairings they found that double-drug resistant growths were only obtained when sufficient quantities of zoospores were allowed to swim together; the pairings on leaves or on agar did not reveal double-drug resistant growths. Analysis with unambiguous PCR markers showed that these double-drug growths were probably heterokaryons as single-zoospore lines segregated into the two parental drug-resistant phenotypes. This suggested that nuclear recombination had not occurred in any of these pairings. In a further experiment, matings of A1 x A2 isolates produced over 100 single-oospores that germinated on double-drug amended media. Segregation of RAPD markers in these progeny was not as would be expected in a diploid, outcrossing organism. As parents were + (dominant) and – (null) for all RAPD markers employed, the frequency of the markers in the progeny should have been +:- (1:1) but there was an excess of the + allele (64:4 at one locus) in the progeny. Since most of the progeny had all markers from both parents and since their genotypes did not change through single-zoospore isolation, they were probably polyploid. These experiments suggested that the formation of double-drug resistant hybrids required a mating reaction, as similar polyploid strains were not observed in pairings of vegetative hyphae or zoospores. Although such offspring were somatically stable they have the potential to segregate by chromosomal non-disjunction (section 1.4.1) into aneuploid or recombinant diploid strains.

6.1.2 Using protoplasts to generate somatic hybrids

Lucas *et al.* (1990) had little success in obtaining double-drug resistant cultures when they co-inoculated isogenic strains of *P. capsici* on agar but found that fusing protoplasts of the same strains gave rise to stable, double-drug-resistant colonies, most of which appeared to be recombinants rather than heterokaryons. The behaviour of these protoplast-derived hybrids differed from crosses involving *P. sojae* (*P. megasperma* f. sp. *glycinea*) (Layton & Kuhn, 1988a). They induced protoplasts from isolates resistant to metalaxyl or fluorotryptophan and mixed them before selecting on double-drug amended media. Most of their double-drug resistant colonies were heterokaryons or mixtures as they gave rise to zoospores of parental phenotypes, but 11 double-drug resistant strains also produced large numbers of single-zoospore isolates resistant to both drugs, possibly the result of karyogamy. As the formation of double-drug resistant isolates in the mixtures was much higher than the rate of spontaneous mutation to the drugs, they concluded that the double-drug resistant isolates were true heterokaryons and double-drug-resistant single-zoospore offspring were recombinants but this could not be confirmed without molecular markers.

Protoplasts have also been used to investigate somatic hybridisation in *P. nicotianae* var. *parasitica* (Gu & Ko, 1998). Using metalaxyl- or chloroneb-resistant isolates, microinjection of nuclei of one strain into protoplasts of another resulted in isolates that produced zoospore progeny resistant to both metalaxyl and chloroneb. Some zoospores from these strains were much larger than average and the largest ones were thought to be tetraploid, formed by the fusion of nuclei from both parents. It was suggested that double-drug resistant zoospores with smaller nuclei were recombinant diploids, reduced from the tetraploid state by chromosomal non-disjunction. However, these strains could also have been spontaneous mutants. In *P. infestans*, El Refai (1990) found evidence of recombination fusing protoplasts of a streptomycin-resistant strain with either a chloramphenicol- or a metalaxyl-resistant strain. Some single-zoospore progeny were resistant to both drugs and a small percentage of these had isozyme bands from both parents, suggesting that karyogamy may have occurred. Some of these successful pairings involved two A1 isolates, but others were constructed between an A1 and an A2 isolate.

6.1.3 Aims

The aim of this chapter is to generate and characterise putative somatic hybrids

formed between pairs of drug-resistant isolates of the same mating type. The main approach will be to use strains resistant to one of a number of drugs (chapter 5) and pair these using hyphae, zoospores or protoplasts as inocula. The hybrid or mutational origins of survivors with double-drug-resistant phenotypes will be determined using mtDNA, RFLP and AFLP markers. Similarly, unselected propagations from mixtures of strains on drug-free media were also characterised with these molecular markers.

6.2 RESULTS

6.2.1 Isolates

Isolates were primarily chosen for their different RG57 and AFLP fingerprint patterns and mtDNA haplotype (Table 5.2.1; Table A3.4) and their sensitivity or resistance to metalaxyl, streptomycin, hygromycin, G418, acriflavine or blasticidin (Table 5.2.7). Isolates were selected from a range of populations from the UK and two isolates from Russia (section 5.2.3) and transformed isolates from The Netherlands and the USA (section 5.2.5) were also included.

6.2.2 Strategies for detecting somatic recombination without drug selection

6.2.2.1 *Co-culturing isolates on rye A agar*

Two strips of inoculum (5 x 5 x 40 mm), one from each strain, were placed 4 cm apart on each of three plates of rye A agar. Nine pairings were made in total but three (95.205.11 x 95.205.1; 96.122.1 x 96.5.8; 96.89.41 x 81.9) were not investigated further as there was clear agar, reminiscent of "self-repulsion zones" (Shaw, 1991; Bagirova & Dyakov, 1993) between the two isolates. The remaining six pairings (NS1-6) are described in Table 6.2.1. Two-three weeks after initial contact, the mid-line where the two strains first made contact was cut into 10 mm^2 plugs (40 from each plate) and used to inoculate plates of one-tenth pea-broth agar (appendix 1). Single hyphal-tips (section 2.2.1) were isolated from these plugs and fingerprinted with RG57 (section 2.4.2) and for mtDNA polymorphisms (section 2.4.1). Sixty single hyphal-tips were isolated from the plugs from each pairing and typically, 30-50% of these grew into viable colonies.

Fingerprinting with RG57 revealed polymorphisms amongst parents and single hyphal-tip cultures of each pairing. Of the 25 scored bands, from four (NS3) to six (NS5) were polymorphic (Table 6.2.1; Figure 6.2.3) amongst the parents and progeny. One parent generally revealed all of the polymorphic bands, the exception being pairing NS6 where polymorphic parent-specific bands were present in both parents. All pairings except NS5 gave rise to some hyphal-tip cultures with the same RG57 and mtDNA fingerprint as one of the parental isolates. In total, 62% of the fingerprinted hyphal-tip cultures from NS1-6 were of a parental RG57 and mtDNA fingerprint. All of the hyphal-tip cultures from one pairing (NS1) had the same RG57

and mtDNA fingerprint as one of the parents.

Table 6.2.1. RG57 fingerprints (after Goodwin *et al.*, 1992a) of parents and single hyphal-tip progeny from 6 pairings. Parents (A-H) for each pairing (NS1-6) are indicated, as are the number of progeny (No.) with parental or non-parental genotypes. Only polymorphic bands are shown.

Parents		Mating Type	Mt DNA	No.	RG57 Band								
					2	3	6	7	9	10	16	19	22
95.205.11	(A)	A1	Ia		1	1	1	1	1	1	1	1	1
95.155.22	(B)	A1	IIa		0	0	0	0	1	1	1	0	0
95.162.7	(C)	A1	Ia		1	0	0	0	0	1	1	0	1
95.205.16	(D)	A1	IIa		0	0	1	0	1	1	1	0	0
95.152.3	(E)	A1	Ia		1	0	0	0	0	1	1	0	1
95.154.7	(F)	A2	IIa		1	0	0	0	0	1	0	1	1
96.89.41	(G)	A2	Ia		1	1	1	1	0	1	1	1	1
81.9	(H)	A2	Ia		0	1	1	1	1	1	1	1	1
Hyphal-tip lines													
Pairing NS1 (A x B)													
P1 (parent B type)		A1	IIa	24	0	0	0	0	1	1	1	0	0
Pairing NS2 (A x C)													
P1 (parent C type)		A1	Ia	12	1	0	0	0	0	1	1	0	1
P2		A1	Ia	2	0	0	0	0	1	1	1	0	1
P3		A1	Ia	1	0	0	0	0	0	1	1	0	1
Pairing NS3 (A x D)													
P1 (parent D type)		A1	IIa	14	0	0	1	0	1	1	1	0	0
P2		A1	IIa	1	1	0	1	0	0	1	1	0	0
Pairing NS4 (A x E)													
P1 (parent E type)		A1	Ia	14	1	0	0	0	0	1	1	0	1
P2		A1	Ia	2	1	1	1	1	1	1	1	0	1
Pairing NS5 (F x G)													
P1		A2	IIa	2	1	1	0	1	0	1	1	1	1
P2		A2	IIa	8	1	0	0	0	0	1	1	1	1
P3		A2	IIa	10	0	0	0	0	0	1	0	1	1
P4		A2	IIa	6	0	0	0	0	0	0	0	1	1
Pairing NS6 (F x H)													
P1 (parent F type)		A2	IIa	4	1	0	0	0	0	1	0	1	1
P2		A2	IIa	2	0	0	0	0	0	1	1	1	1
P3		A2	IIa	6	0	0	0	0	0	0	0	1	1

Four pairings (NS2, 3, 4 & 6) gave rise to some hyphal-tip cultures that differed from their parental isolates at between one to three bands. All hyphal-tip cultures from pairing NS5 were of a non-parental genotype.

6.2.2.2 *Mixed zoospores*

Zoospores were induced from 10 day-old colonies as described in section 2.2.3 and the concentration adjusted to 10^4 ml^{-1} of each strain. Five ml of zoospore suspension from 2 (NSZ1) or 5 (NSZ2) parental strains were mixed into fresh 25 ml bottles

(Table 6.2.2) and allowed to swim together for two hours at 10°C. 0.5 ml of the zoospore suspensions were pipetted onto plates of rye A agar and colonies were established during seven days growth at 18°C. Thirty hyphal-tips were isolated (section 2.2.1) from each of NSZ1 and NSZ2 after growth from the mixed zoospore inoculum. Four further zoospore mixtures (95.154.7 x 96.89.41 x 81.9; 96.5.8 x 96.122.1; 95.154.7 x 81.9; 96.89.41 x 81.9) only provided sparse, if any, growth, unlike when these strains were growing singly, and were not investigated further. Table 6.2.2 summarises the fingerprints of the parents and single-hyphal-tip cultures from the zoospore mixtures.

Table 6.2.2. RG57 fingerprints of parents and single hyphal-tip cultures from 2 pairings (after Goodwin *et al.*, 1992a). Parents (A-E) for each pairing (NSZ1-2) are indicated, as are the number (No.) of progeny (P1-P3) with parental or non-parental genotypes. Only polymorphic bands are shown. All isolates and progeny had mtDNA type Ia.

Parents		Mating Type	No.	RG57 Band				
				2	3	6	7	10
96.122.1	(A)	A1		1	1	1	0	1
96.89.41	(B)	A2		1	1	1	1	1
81.9	(C)	A2		0	1	0	0	1
96.5.8	(D)	A1		1	1	1	1	1
95.154.7	(E)	A2		1	0	0	0	1
Hyphal-tip lines								
Pairing NSZ1 (BxE)								
P1		A2	5	0	0	0	0	1
P2 (parent E type)		A2	3	1	0	0	0	1
P3		A2	1	1	1	0	1	1
Pairing NSZ2 (AxBxCxDxE)								
P1		A2	4	0	1	0	1	1
P2		A2	3	0	0	0	0	0
P3		A2	1	0	1	0	1	0

In pairing NSZ1 (Table 6.2.2), four RG57 bands varied amongst the parents and progeny, three of these being present in isolate 96.89.41. The other band (2) was present in both parents but varied amongst the hyphal-tip cultures. Three genotypes were detected in the progeny and one of these was the same as parent E. The most common hyphal-tip genotype was P1 (Table 6.2.2), the same as parent E except that band 2 was not detected. Mixing five isolates (pairing NSZ2) allowed five polymorphic RG57 bands to be investigated in hyphal-tip cultures. Isolates of both mating types were included in this pairing; some oospores were identified but no oospore-germination was observed. None of the fingerprint-patterns amongst the hyphal-tip cultures were the same as any of the parents. Four of the hyphal-tip cultures had a fingerprint pattern similar to isolate 81.9, except that band 7 was also

detected.

6.2.3 Investigating somatic recombination using drug-resistant strains

Seven strategies (A-G; section 6.2.3.1-6.2.3.2) were used to mix strains with different selectable drug-resistance markers and the progeny were investigated for their ability to grow on double-drug amended media (Table 6.2.3). Pairings involving metalaxyl and streptomycin were the most widely available due to the apparent instability of the acriflavine or blasticidin mutants and the general resistance of most wild-type isolates to chloramphenicol (section 5.2.2.3).

6.2.3.1 *Co-culturing drug-resistant strains on rye A agar (A)*

Strips of inoculum of each strain (4 x 5 x 40 mm) were placed 4 cm apart on each of ten plates of rye A agar. Two to three weeks after initial contact, the mid-line where the two strains first made contact was cut into 10 mm^2 plugs (approximately 40 from each plate). These were used to inoculate double-drug amended rye A agar (drugs used depending on the drug resistant phenotypes of the strains employed, as in Table 6.2.3). Plugs were checked every 2 days for growth on double-drug amended media. Any growths were sub-cultured to fresh double-drug amended media.

6.2.3.2 *Pairings (B-G) using zoospores*

Zoospores were harvested from ten-day-old isolates (section 2.2.3) and the concentration of zoospores from each strain was altered to approximately 10^4 ml^{-1}. The following strategies differ slightly in the way the zoospores were treated or handled.

6.2.3.2.1 *Mixing zoospores and pipetting onto double-drug amended media (B)*

The technique was identical to that described earlier (section 6.2.2.2) except that drug-resistant strains were used. Zoospores were allowed to swim together for 2 h after which they were pipetted directly onto appropriate double-drug amended media.

6.2.3.2.2 *Mixing zoospores in liquid pea broth (C)*

Four ml of the zoospore suspensions were co-inoculated into 250 ml flasks (three flasks per pairing) with 200 ml pea-broth (appendix 1) and incubated at 18°C (unshaken) for 7 days. Whole colonies (average 5-15 from each flask) in liquid pea-broth were removed to rye A agar containing double-drug amended media using the

tip of a sterile glass Pasteur pipette. Any growths were transferred to further double-drug amended media.

6.2.3.2.3 *Leaflet infection with drug-resistant strains (D)*

Three detached potato leaflets (cv. Homeguard) were each inoculated with two isolates as described previously (section 2.2.7.2). Sporangia appearing above the surface of the leaves after 3-5 days were removed by adhesion to a 10 x 10 mm block of rye A agar, being careful not to touch the surface of the leaf. These blocks (20 per pairing) were placed onto plates of double-drug amended rye A agar. In a slight modification, one of the leaves from each pairing was cut into 2 cm^2 pieces and placed (eight pieces per plate) abaxial-side down on double-drug amended media.

6.2.3.2.4 *Centrifugation of drug-resistant zoospores (E)*

Five ml of zoospore suspension from each of two isolates was placed into Universal bottles. These mixtures were centrifuged at 1,500 rpm for 2 min after which, 8 ml of the supernatant was removed by pipetting, being careful not to disturb the zoospore pellet. Zoospores were gently re-suspended in 3 ml of one-tenth pea broth and left at 18°C for 2-3 hours. 500 µl of the suspension was inoculated directly onto each of six plates of rye A agar containing double-drug amended media. Any growths were removed to fresh double-drug amended rye A agar.

6.2.3.2.5 *Mixing sporangia from drug-resistant strains (F & G)*

(F). Sporangia were removed from 10-14 day-old cultures (section 2.2.2) to Universal bottles and the concentrations altered to between $10^5 - 10^6$ ml^{-1} (2-3 ml of each strain) depending on the yield. Equal numbers of sporangia from each strain were then mixed and the mixtures were incubated at 10°C for up to two hours to induce zoospore release. Zoospores were then encysted by vortexing for 3-4 s and then incubated at 18°C for 2-3 hours. 0.5 ml of each mixture was then plated onto 10 plates of rye A agar containing double-drug amended media. (G). A modification of this experiment was identical except that 50% polyethylene glycol (4000) (Sigma) was added once zoospores had been released from sporangia.

6.2.3.3 *Identification and characterisation of putative asexual hybrids*

In total, 44 pairings were constructed, a proportion of which were used for each of the seven approaches to investigate somatic recombination as detailed in Table 6.2.3. The

control isolate ("pairing" 1) 95.161.5 resistant to both metalaxyl and streptomycin grew on double-drug (metalaxyl-streptomycin) amended media after all treatments except D (inoculation of leaves). These leaves were heavily contaminated after incubation. Unexpectedly, when 95.161.5 was paired with other isolates (pairings 2-9), double-drug resistant growths were only observed in one pairing (2). Control experiments involving techniques A-G using individual isolates resistant to one drug (metalaxyl, streptomycin or G418) did not produce any growths on double-drug amended media. Further control experiments involved plating plugs or zoospores from the pairings (Table 6.2.3) onto drug-free media as well as media containing either of the two drugs used for selection in that pairing. Isolates could grow on all these control plates (except contaminated plates using technique D), suggesting that parental strains were always viable after experimental treatments.

Methods B, C, E, F & G revealed that zoospores germinated on double-drug amended media, as noticed previously (Shattock & Shaw, 1975), but no aerial hyphae or sporangia were formed in the majority of pairings. Of the seven methods (A-G) employed, only two revealed any growths on double-drug amended media. These techniques (F & G) involved mixing many zoospores and F & G differed only through the addition of polyethylene glycol (section 6.2.3.2.5). Only two (pairings 2 & 30) out of 30 pairings constructed using techniques F & G, yielded growth of aerial hyphae and sporangia on double-drug media. These growths appeared as small, sporulating colonies. They could be sub-cultured once as a mass hyphal transfer to fresh double-drug amended media but the transferred plugs did not grow more than 5-6 mm onto the fresh drug-amended media.

6.2.4 Isolating hybrids from fused protoplasts

Protoplasts were isolated from drug-resistant strains (section 2.2.5) and protoplast fusion was attempted between strains with different drug-resistant markers (section 2.2.6; Table 6.2.4). Protoplast concentrations for all isolates were 10^6-10^7 for each pairing. As 0.5 ml of each protoplast suspension was mixed, 10^6-10^7 protoplasts were involved in each pairing. Seven pairings, all between A1 isolates, were examined for growth on double-drug amended media (Table 6.2.4).

Table 6.2.3. Results of seven strategies (A-G) used to obtain double-drug resistant somatic hybrids. Isolates were paired as indicated (sections 2.2.3.1 & 2.2.3.2). Double-drug markers used to select recombinants is indicated next to the relevant strain used for the pairing.

		Strains and drug-resistant markers employed				Number of double-resistant derivatives obtained using method						
Pair	MT	Strain 1		Strain 2		A	B	C	D	E	F	G
1	A1	95.161.5	SM, MEX	-	-	60	60, 24	12	0	>100	>100	
2		95.161.5	SM, MEX	N18	G418						0	3
3		95.161.5	SM, MEX	97.185.5	MEX	0	0					
4		95.161.5	SM, MEX	96.93.8 Sr	SM	0	0					
5		95.161.5	SM, MEX	5 Aue	AC	0	0					
6		95.161.5	SM, MEX	97.123.1	MEX						0	
7		95.161.5	SM, MEX	98.71.1	MEX						0	
8		95.161.5	SM, MEX	98.57.9	MEX						0	
9		95.161.5	SM, MEX	98.74.7	MEX						0	
10		96.93.22 Sr	SM	97.123.1	MEX	0		0	0	0	0	
11		96.93.22 Sr	SM	98.71.1	MEX						0	
12		96.93.22 Sr	SM	98.64.1	MEX						0	
13		96.93.22 Sr	SM	98.71.1	MEX						0	
14		96.93.22 Sr	SM	98.57.9	MEX						0	
15		96.93.22 Sr	SM	98.74.7	MEX						0	
16		96.93.22 Sr	SM	97.185.5	MEX	0		0	0	0	0	
17		96.93.8 Sr	SM	97.123.1	MEX	0	0, 0	0	0	0	0	
18		96.93.8 Sr	SM	98.88.8	MEX	0, 0	0, 0	0	0	0	0	
19		96.93.8 Sr	SM	98.64.1	MEX	0, 0	0, 0	0	0	0	0	
20		96.93.8 Sr	SM	97.123.1	MEX	0	0				0	
21		96.93.8 Sr	SM	98.74.7	MEX	0, 0	0				0, 0	
22		96.93.8 Sr	SM	98.78.4	MEX	0, 0	0				0	
23		96.93.8 Sr	SM	98.75.13	MEX	0, 0	0				0	
24		96.93.8 Sr	SM	98.57.9	MEX	0, 0	0, 0				0	
25		96.93.8 Sr	SM	98.70.2	MEX	0, 0	0				0	
26		96.93.8 Sr	SM	98.71.1	MEX	0, 0	0				0, 0	
27		96.93.8 Sr	SM	97.185.5	MEX	0, 0	0				0, 0	
28		97.128.1	MEX	96.127.15	CM	0						
29		97.185.5	MEX	96.127.15	CM	0						
30		N18	G418	98.57.9	MEX						5	0
31		N18	G418	97.185.5	MEX						0	0
32		95.162.7	CM	18.2	BL	0	0					
33		95.162.7	CM	5 Aue	AC	0	0					
34		95.162.7	CM	18.2	BL	0	0					
35		96.37.1	CM	18.2	BL	0	0					
36		96.37.1	CM	5 Aue	AC	0	0					
37		18.2	BL	5 Aue	AC	0	0					
38	A2	96.89.41 Sr	SM	E14C2	CM	0						
39	A1/A2	96.89.41 Sr	SM	97.185.5	MEX	0		0		0		
40		96.89.41 Sr	SM	97.123.1	MEX	0		0		0	0	
41		96.89.41 Sr	SM	98.71.1	MEX						0	
42		96.89.41 Sr	SM	98.64.1	MEX						0	
43		96.89.41 Sr	SM	98.57.9	MEX						0	
44		96.89.41 Sr	SM	98.74.7	MEX						0	

Pairs of numbers represent repeated experiments. Blank spaces indicate experiment was not done. MT (mating type). Metalaxyl (MEX) (10 μg ml^{-1}); streptomycin (SM) (50 or 100 μg ml^{-1}); chloramphenicol (CM) (100 μg ml^{-1}); acriflavine (AC) (10 μg ml^{-1}); blasticidin (BL) (140 μg ml^{-1}) or G418 (5 or 10 μg ml^{-1}).

Table 6.2.4. Isolates and marker genes used for protoplast fusion and the number of double-drug resistant colonies observed after growth on double-drug amended media.

Pairing	A1 strains used and drug-resistance markers employed				Colonies on six double-drug plates
	Strain 1		Strain 2		
1	N18	G418	95.161.5	MEX	0
2	N18	G418	97.185.5	MEX	27
3	N18	G418	98.57.9	MEX	C
4	Y1	G418	W1	HYG	0
5	W1	HYG	95.161.5	MEX	15
6	W1	HYG	N18	G418	20
7	Y1	HYG	95.161.5	MEX	0

C (contaminated). Metalaxyl (MEX) (10 μg ml^{-1}), hygromycin (HYG) (10 or 20 μg ml^{-1}), and G418 (5 or 10 μg ml^{-1}).

Numerous colonies were identified on plates from pairings 2, 5 & 6 (Table 6.2.4) and five colonies from each were sub-cultured to fresh double-drug amended media. All sub-cultures from cross 2 were not viable when plated onto fresh double-drug amended media, but could grow on drug-free media and media containing either of the two drugs individually. However, sub-cultures of crosses 5 and 6 were viable on sub-culture to double-drug amended media; cross 5 on metalaxyl-hygromycin and cross 6 on hygromycin-G418. Nuclear genes confer resistance to these compounds so these double-drug resistant growths were putative somatic recombinants. No growths were observed in the control "self" pairing (cross 4) of isogenic isolates W1 (HYG-resistant) or Y1 (G418-resistant), possibly a consequence of lower protoplast yield from Y1. These isolates were controls as they have previously been shown to form heterokaryons (van West *et al.*, 1998).

To investigate double-drug resistant colonies, 20 single hyphal-tips (section 2.2.1) and 20 single sporangia (section 2.2.2) were removed from the fastest growing sub-cultures from crosses 5 & 6 (Table 6.2.4). These propagations were germinated on drug-free rye A medium and were then tested on double-drug amended medium and medium containing either of the drugs individually. All hyphal-tips and single-sporangia from pairings 5 & 6 grew on drug-free medium and media containing the selective drugs individually. However, only 3 hyphal-tips (HT1-3) and 2 single-sporangia (SP1 & 2) from pairing 5 and two hyphal-tips (HT4-5) from pairing 6 grew on double-drug amended media. Fifteen zoospores were isolated from HT1-3 & SP1 (pairing 5) and HT4 (pairing 6). These were tested for ability to grow on double-drug amended media (Table 6.2.5).

Table 6.2.5. The number of germinated single zoospores, from a sample of 15, able to grow on double-drug amended media.

Origin of isolate (pairing) (as Table 6.2.4)	Isolate	Number of single-zoospores forming colonies on double-drug amended media
5	HT1	8
5	HT2	0
5	HT3	0
5	SP1	10
6	HT4	1

HT (hyphal-tip culture), SP (single-sporangial culture). Concentrations of drugs, metalaxyl (MEX), 10 μg ml^{-1}), hygromycin (HYG) 10 μg ml^{-1} and G418 (5 μg ml^{-1}). Pairing 5 MEX-HYG, pairing 6 HYG-G418

All germinated zoospores from HT1, HT4 and SP1 were able to grow on double-drug amended media (Table 6.2.5). The linear growth rates (section 2.2.8.1) of parents, SP1, HT1 and single-zoospore derivatives from these were compared on metalaxyl, hygromycin and metalaxyl-hygromycin amended media (Figure 6.2.1).

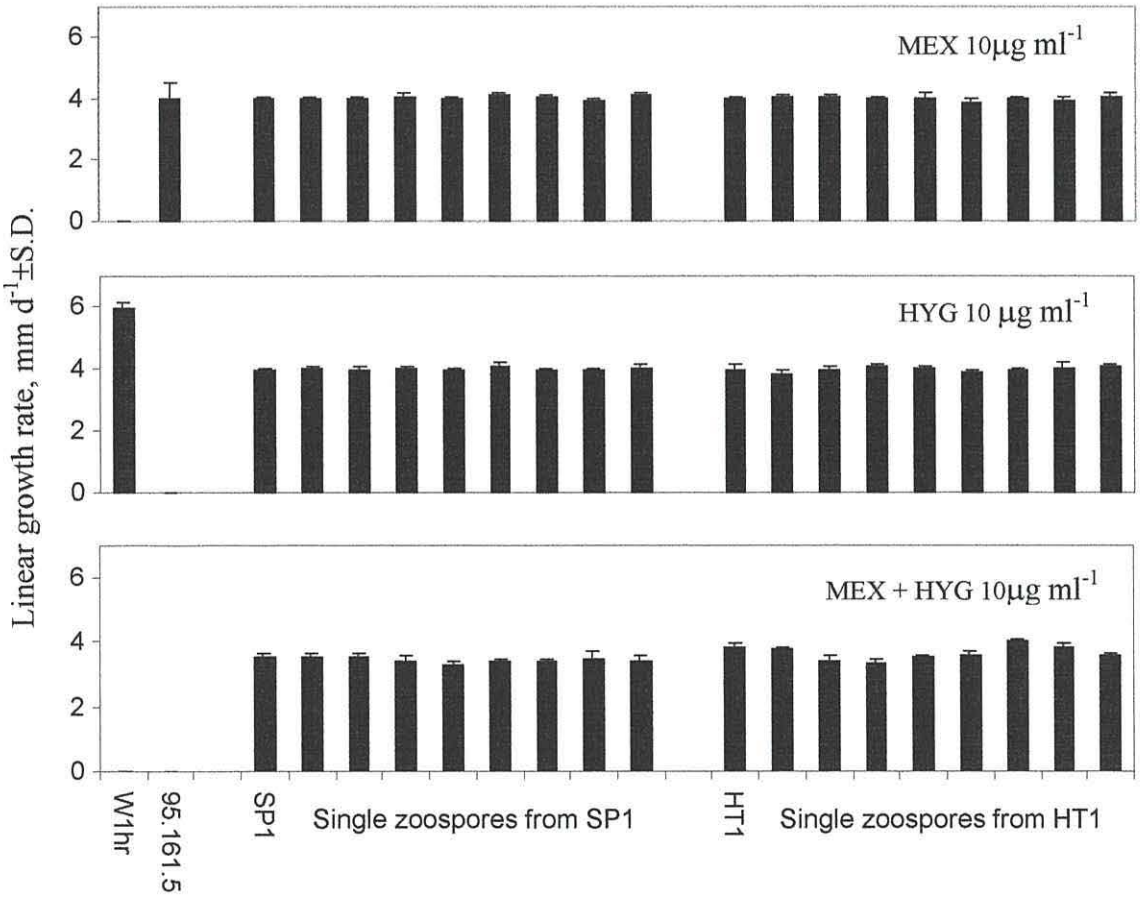


Figure 6.2.1. Linear growth rates of parental isolates, derivatives SP1 and HT1 and single zoospore lines from SP1 and HT1, on single (MEX or HYG) and double (MEX-HYG) drug amended media. Linear growth rates are means of four replicates.

All progeny grew equally as well on metalaxyl, hygromycin and metalaxyl-hygromycin media suggesting the hyphal-tip and sporangial cultures were probably not heterokaryons but could have been tetraploids, aneuploids or mutants resistant to both drugs. Parental isolates grown and treated alone, produced no double-drug colonies suggesting that mutation was not the cause of the observed double-drug resistant phenotypes.

The linear growth rates (section 2.2.8.1) of parents, HT4 and a single-zoospore derivative of HT4 (pairing 6) were compared on G418, hygromycin and G418-hygromycin amended media (Figure 6.2.2).

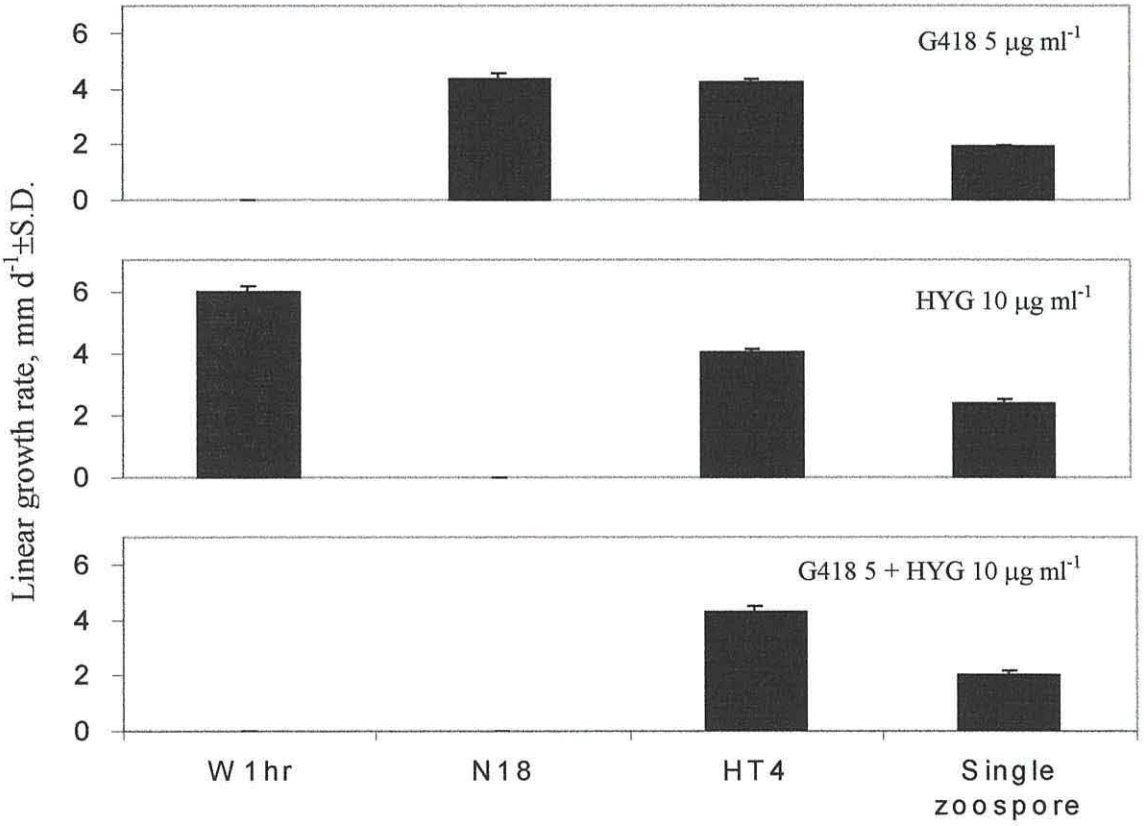


Figure 6.2.2. Linear growth rates of parental isolates, HT4 and a single-zoospore line from HT4 on single (G418 or HYG) and double (G418-HYG) drug-amended media. Linear growth rates are means of four replicates.

Both parental isolates were completely inhibited when grown on either G418 (W1) or hygromycin (N18). Both HT4 and the single-zoopsore derivative were able to grow on G418 or hygromycin individually and on double-drug amended media, although the growth rate of the single-zoospore isolate was consistently less than the

growth rate of HT4 over four replicates (Figure 6.2.2).

6.2.5 Fingerprinting parents and double-drug resistant growths for AFLPs

HT1 and SP1 (pairing 5), HT4 (pairing 6) and the single-zoospore lines derived from these isolates were fingerprinted with two pairs of AFLP primers (section 2.4.3). The two parental isolates in pairing 5 (W1 x 95.161.5), SP1, HT1 and single-zoospore progeny were polymorphic at 14 loci; five of these were specific to W1 and nine were specific to 95.161.5 (Table 6.2.6; Figure 6.2.4).

Table 6.2.6. AFLP fingerprints of hyphal-tip (HT), sporangial (SP) and zoospore (SZ) lines growing on double-drug amended media after protoplast fusion of two parental strains (pairing 5).

	Polymorphic AFLP bands													
	E19-M16								E19-M40					
Isolate	9	10	15	21	38	46	64	66	6	7	17	29	40	34
W1	0	0	0	1	0	1	0	0	0	1	1	0	1	0
95.161.5	1	1	1	0	1	0	1	1	1	0	0	1	0	1
SP1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7 SZ lines	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1 SZ line	1	1	1	1	1	1	0	1	1	1	1	1	1	1
HT1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8 SZ lines	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Bands in red were present in parent W1 only and bands in green were present in 95.161.5 only.
Bands in blue were present in both parents but were polymorphic in the progeny.

Bands specific to both parents were observed in hyphal-tip, sporangial and zoospore progeny. Seven zoospore-derivatives of SP1 had the same fingerprint as SP1, but one zoospore isolate was missing band E19-M16 (66) (Table 6.2.6) that was present in 95.161.5. All single-zoospore lines from HT1 had the same fingerprint pattern as HT1. These data suggest both HT1 and SP1 were not heterokaryons but were probably products of fusion between nuclei from W1 and 95.161.5 as they had drug-resistant phenotypes and AFLP fingerprints that appeared as combination of the two parents.

Table 6.2.7. AFLP fingerprints of hyphal-tip (HT) and zoospore (SZ) lines growing on double-drug amended media after protoplast fusion of two parental strains (mating 6).

	Polymorphic AFLP bands											
	E19-M16		E19-M40									
Isolate	46	66	6	12	16	17	23	24	26	34	39	40
W1	1	1	0	0	1	1	1	0	1	0	1	1
N18	0	0	1	1	0	0	0	1	0	1	0	0
HT4	1	1	1	1	1	1	1	0	1	1	1	1
1 SZ line	1	1	0	0	1	1	0	0	1	1	1	1

Bands in red were present in parent W1 only and bands in green were present in N18 only.

Parental isolates in pairing 6 (W1 x N18), HT4 and the single zoospore line were polymorphic at 12 loci, eight of these were specific to W1 and four were specific to N18 (Table 6.2.7). Bands specific to both W1 were observed in HT4 line and the single-zoospore derivative. Unlike most single-zoospores from mating 5 (Table 6.2.6), the single-zoospore line had a quite different fingerprint to HT4; three AFLP bands observed in HT4 were not detected in the single-zoospore line.

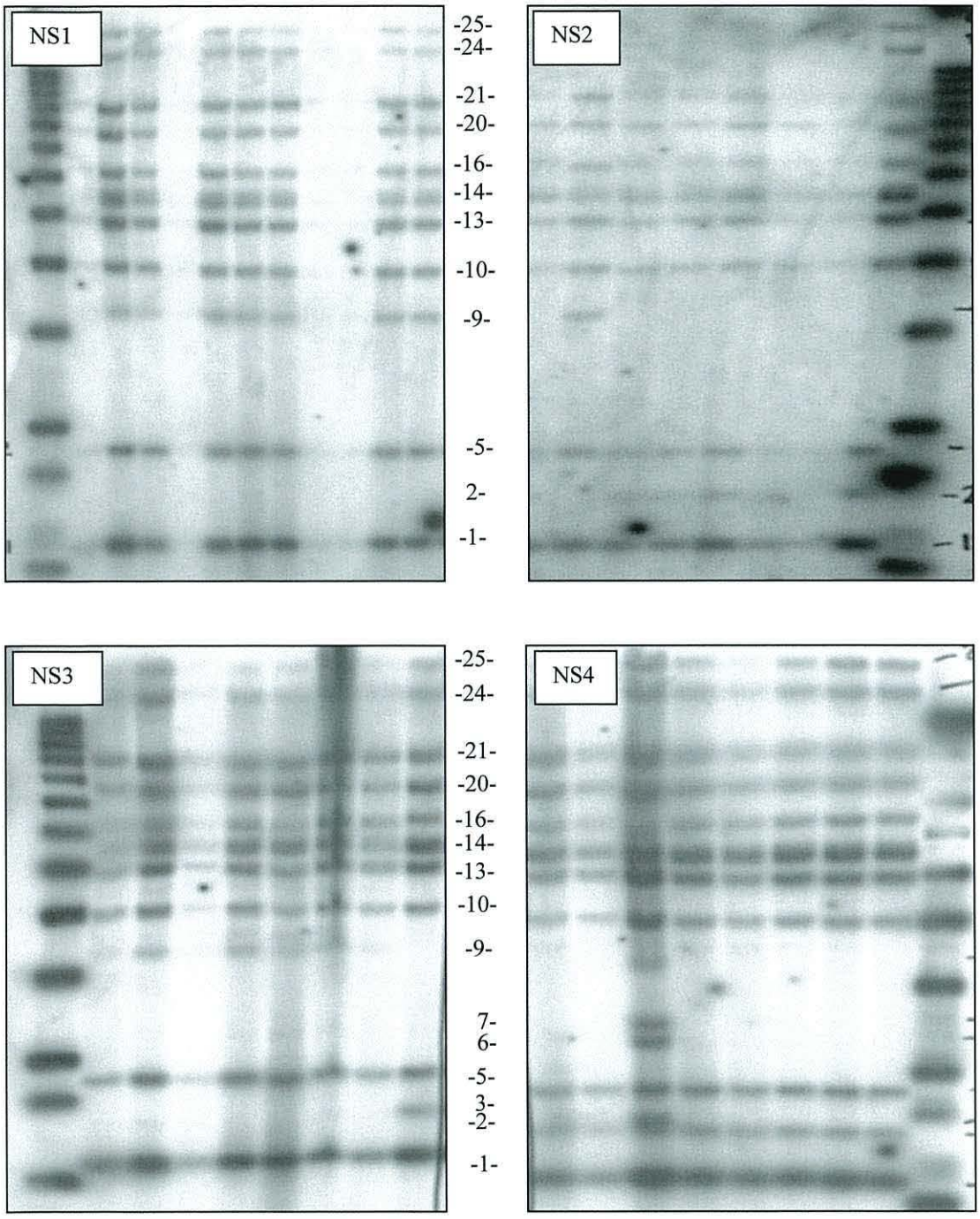


Figure 6.2.3. RG57 fingerprints of hyphal-tip progeny from matings NS1-4 (section 6.2.2.1). RG57 bands labelled after Goodwin *et al.*, (1992a). 1 kb marker is shown on each autoradiograph.

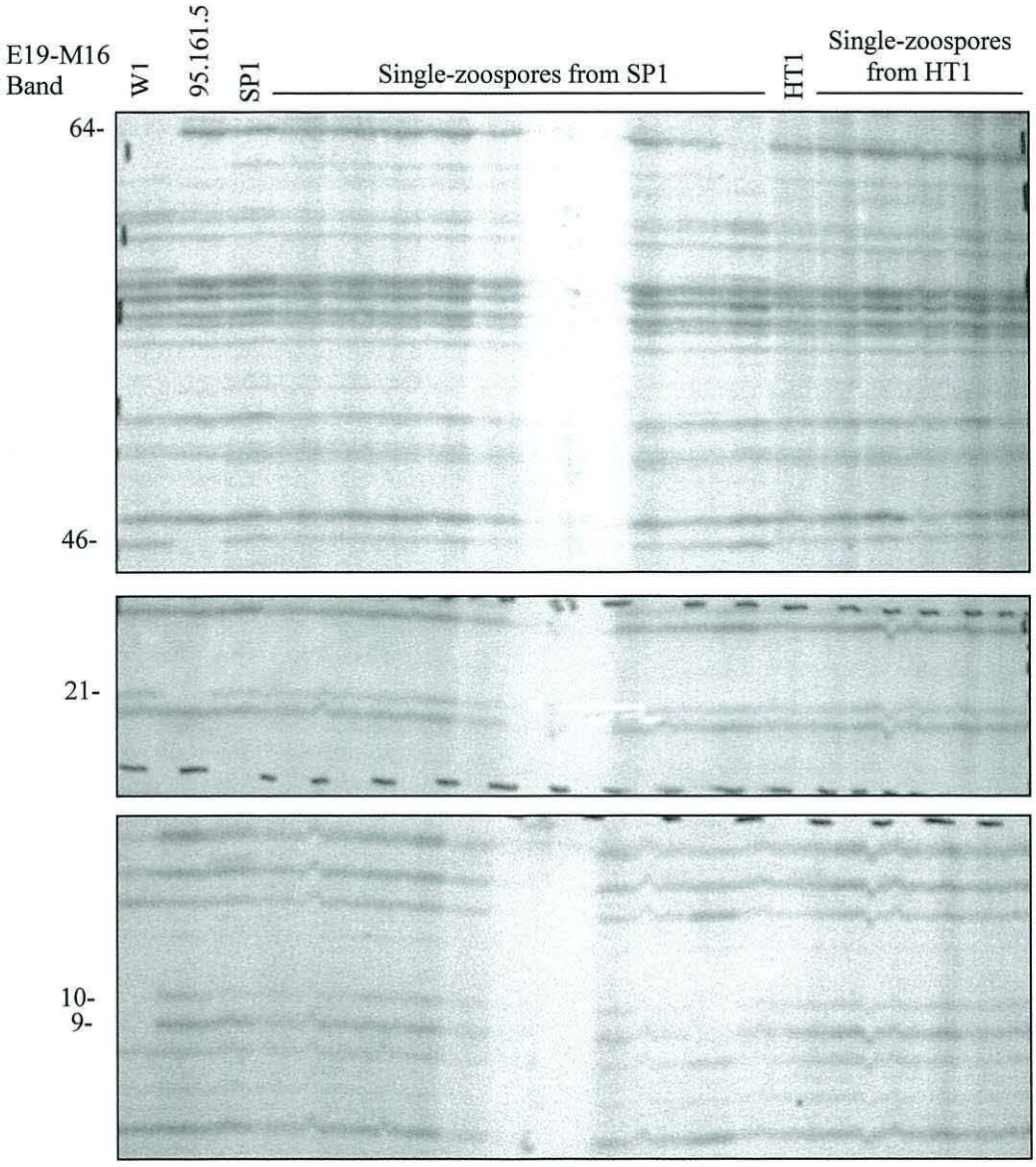


Figure 6.2.4. AFLP fingerprints of single drug-resistant parents and double-drug resistant single-sporangial, hyphal-tip and single-zoospore lines after protoplast fusion (section 6.2.4). Five of the polymorphic bands revealed with E19-M16 are shown.

6.3 DISCUSSION

Previous studies investigating somatic recombination were limited by the fact that stable, neutral, genetic markers were unavailable (Long & Keen, 1977b; Dyakov & Kulish, 1979 [in Shaw, 1983b]; Shattock & Shaw, 1976) and whether or not recombination occurred in these studies is debatable. The present study expands these experiments by pairing drug-resistant isolates of the same mating type using numerous strategies. These strains also had markers for both mtDNA and nuclear DNA to allow somatic recombinants to be positively identified in zoospore cultures. The majority of the experiments described in this chapter employed drug-resistance markers to select for somatic hybrids, though some did not.

6.3.1 Experiments without drug selection

These experiments involved growing two isolates together as parallel inocula or by mixing zoospores on agar. It was thought that this latter technique might have enabled greater contact between the strains. In some crosses, the strains did not collide (section 6.2.2.1) leaving a thin uncolonised zone between them. Also, some pairings where zoospores were mixed (section 6.2.2.2) produced small colonies or no growth at all. In some pairings of *P. infestans*, different isolates of the same mating type repel each other at the juncture of the two mycelia (Gallegly & Galindo, 1958; Bagirova *et al.*, 1998) and this has also been observed when colonies of the same strain are grown together (Galindo & Zentmyer, 1967; Shaw, 1991). These findings may indicate an incompatible reaction between certain isolates, preventing hyphal contact and anastomosis. Media with added charcoal has been used to obtain heterokaryons of *Rhizoctonia solani* (Butler & Bolkan, 1973) and *P. infestans* (Shattock & Shaw, 1976). This media may increase the frequency of heterokaryon formation by adsorbing secreted substances that may be involved in determining incompatibility. Rye A media with 1% charcoal was used for some pairings in the present study, but all isolates had a much reduced growth rate and eventually stopped growing on this medium (data not shown).

6.3.1.1 *Co-culturing and zoospore inoculation of strains without drug-selection*

Forty hyphal-tip cultures with non-parental RG57 genotypes were observed from 109 hyphal-tips isolated from the six pairings NS1-NS6 on agar. The other 68 hyphal-tip

cultures were of the same genotype as one of the parents in the cross. Fourteen of the 18 hyphal-tip cultures obtained from mixing zoospores of different genotypes (NSZ1 & 2) also had non-parental genotypes.

If heterokaryons (and recombinants) were being formed infrequently between some of the paired isolates, observed fingerprint patterns from hyphal-tip cultures should include those with parental genotypes, those with a composite of both parents (e.g. heterokaryon) or novel combinations of markers (after karyogamy). The frequency of these would depend on the frequency and transience of each stage. Hyphal-tip cultures with parental (though always only one parent from each pairing) and novel combinations of RG57 markers were observed in most pairings (Table 6.2.1; Figure 6.2.4) but no fingerprinting evidence supported the existence of heterokaryons amongst the sample of 109 hyphal-tips. The parental genotypes were probably a result of sampling parental hyphae growing into the mid-line (NS1-6) or growing from zoospores (NSZ1-2). Two hypotheses could explain the occurrence of non-parental genotypes in the hyphal-tip progeny. (1) Novel combinations could have arisen by mitotic crossing-over in parental strains where heterozygous loci (+:-) could become homozygous (0:0) at some loci and this could result in the loss of some RG57 bands (Shaw, 1983a & b). Under this hypothesis, hyphal-tip progeny should have similar genotypes to one of the parental isolates with, perhaps, loss or gain of one or two bands depending on where crossing-over occurred. (2) An alternative hypothesis is that somatic recombination may account for the observed novel genotypes. Under this hypothesis, heterokaryon formation and karyogamy, followed by mitotic recombination and/or nondisjunction, would generate recombinant nuclei. At least some recombinants would be expected to reveal combinations of bands specific to both the parental isolates. Unfortunately, as one parent revealed all of the polymorphic bands in most pairings, somatic hybrids could not be distinguished from variants arising through mitotic crossing over in one of the parental isolates.

The RG57 fingerprint has been shown to be somatically stable (Goodwin *et al.*, 1992a) in a total of 63 zoospores from five isolates. All of these zoospores had identical (parental) RG57 fingerprint patterns when they were cultured alone. The observed segregation of RG57 markers in the hyphal-tip cultures could be a property of these isolates growing on their own or it could have been induced by co-cultivation with or without hyphal fusion. Experiments to investigate these mechanisms could yield interesting results in the future.

6.3.2 Selection of somatic recombinants using drug-resistant markers

Only two out of seven techniques (F & G) in Table 6.2.3 produced any evidence of growth on double-drug amended media. Both involved co-culturing A1 isolates and were based on mixing a high density of zoospores and allowing zoospores (or hyphal germ tubes) to fuse. However, growth of both colonies did not continue on transfer to double-drug amended media. These initial growths were probably not a result of mutation as they should have continued to grow on transfer to fresh media and may have been transient heterokaryons that quickly segregated into parental (single-drug resistant) phenotypes. Without further analysis of single hyphal-tips and single-zoospores from these strains, this cannot be confirmed.

The lack of double-drug growths was not expected. Both hyphal fusion (Stephenson *et al.*, 1974) and zoospore fusion (Hiddema & Kole, 1954) have been observed in *Phytphthora* and the use of drug-resistant markers should have allowed detection of somatic hybrids if and when the double-drug resistant phenotypes were generated. Lack of double-drug resistant colonies may indicate that somatic recombination was rare or did not occur amongst these *P. infestans* isolates or may indicate that the conditions employed to detect recombinants were too stringent.

Although double-drug resistant colonies were observed using techniques F & G, drug concentration in double-drug amended media may have been too high to allow the growth of many somatic hybrids. Heterokaryons can be maintained across a wide range of nuclear ratios, such as 20-35:1 (Burnett, 1975) so any heterokaryons may have had reduced gene dosage for one of the resistant gene loci. Previous studies using double-drug amended media to select heterokaryons in *Phytophthora* have used higher, the same or half the concentration of drugs needed to inhibit the sensitive parent and select for double-drug resistance (Shattock & Shaw, 1976; Layton & Kuhn, 1988a & 1990, Ersek *et al.*, 1995). All of these studies produced putative somatic recombinants. Concentrations of drugs used to select for double-drug resistant growths in the present study were generally 5-20% of the highest concentration on which growth of each resistant parent was observed. It is therefore unlikely that the drug-concentrations used were too high to allow double-drug resistant growths, especially as 95.161.5, resistant to both metalaxyl and streptomycin, could grow on metalaxyl-streptomycin double-drug selection plates.

Most of the techniques used to identify double-drug resistant growths (section

6.2.3.2) mixed zoospores for several hours before plating them onto double-drug amended media. In a newly formed recombinant zoospore, expression of both drug-resistance genes may not be induced immediately and it may take several hours or longer for double-drug resistant phenotypes to be expressed in a heterokaryon. Shattock & Shaw (1976) mixed zoospores and plated these directly onto double-drug amended media but did not observe any double-drug resistant growths. However, experiments reported in Judelson & Yang (1998) suggest that double-drug phenotypes can be quickly expressed in a heterokaryon as they applied their selection after 2-3 hours, a similar time-scale to techniques used in the present study. It would be useful to investigate the length of time required before double-drug phenotypes are expressed in a heterokaryon, if compatible strains could be found.

Recently, Judelson & Yang (1998) used transgenic drug-resistant strains and neutral genetic markers to investigate the asexual genetics of *P. infestans*. Using numerous pairings, recombination was only observed when A1 x A2 zoospores were mixed, and not A1 x A1 or A2 x A2. In the present study, double-drug growths were observed between two pairs of A1 isolates but not between two A2 isolates (Table 6.2.3, cross 38) or between A1 and A2 isolates (crosses 39-44). In some true fungi, opposite mating type alleles must be present to override vegetative incompatibility factors (Glass & Kuldau, 1992), whereas in other fungi, the degree of genome similarity at specific loci determines compatibility (section 1.4.2). It is possible that stable recombinants would have been observed if more A1 x A2 pairings were examined. It is also possible that compatibility factors prevented recombination in many pairings. A large number of pairings were made so that some strains should have been compatible but it is possible that compatibility factors are numerous and widespread in *P. infestans*. These mechanisms, if they occur, would have greatly reduced the number of hybrids detected in the present study.

6.3.3 Protoplast fusion

Protoplast fusion can bypass hyphal anastomosis and overcome physical and biochemical barriers to hyphal fusion (section 1.4.2). In some cases, fusion of protoplasts can result in heterokaryons of different species (Ann & Peberdy, 1976).

Seven crosses (Table 6.2.4) involved mixing protoplasts of heterogenic and isogenic strains resistant to different drugs. Of these seven pairings, growth on double-drug amended media was observed in three (pairings 2, 5 & 6). One set of

growths (cross 2) did not continue to grow on sub-culture to double-drug amended media. These growths may have been unstable, transient heterokaryons that quickly segregated, as both parental phenotypes were recovered when plugs were sub-cultured onto media containing each drug separately. Two pairings (5 & 6) (Table 6.2.5) gave rise to double-drug resistant colonies which could grow on sub-culture to fresh double-drug amended media; one of these pairings was the same as that which formed colonies through double-drug zoospore fusion (Table 6.2.3). These double-drug resistant colonies could reflect either a spontaneous mutation to one of the drugs or somatic recombination. Heterokaryons should give rise to single-zoospore lines of one or other (or both) parental types, but mutants resistant to both drugs, or heterokaryons in which karyogamy had occurred, should produce single-zoospore isolates able to grow on double-drug amended media.

The hyphal-tip (HT1 & HT4) and single-sporangial (SP1) lines and their single-zoospore derivatives (Table 6.2.5) all grew well on double-drug amended media (Figures 6.2.1 & 6.2.2). The original double-drug growths from crosses 5 & 6 were therefore not heterokaryons and were most likely to be mutants or the products of karyogamy between nuclei from both parents. Previous studies fusing protoplasts to generate double-drug resistant colonies (Layton & Kuhn, 1988a; Lucas *et al.*, 1990; Gu & Ko, 1998), found that 25% to 83% of zoospore propagations were double-drug resistant. In these studies, some propagations with parental phenotypes were also recovered, so double-drug resistant growths were probably heterokaryons, within which karyogamy occurred.

From both pairings 5 & 6, HT1 & 4 and SP1 revealed AFLP bands from both parents (Table 6.2.6; Figure 6.2.4) and most zoospore lines had the same fingerprint as the isolates (HT1 or SP1) from which they were obtained; no single zoospores had the same fingerprint as either original parent. Mutants would not be expected to reveal combinations parent-specific bands and this would suggest that mutation was not the cause of the observed double-drug resistant phenotypes. For bands from both parents to be present in single-zoospore propagations, karyogamy must have occurred as zoospores of *Phytophthora* species are uni-nucleate with rare exceptions (Castro, 1963; Mortimer *et al.*, 1977; Layton & Kuhn, 1988a; Zheng & Ko, 1996).

Interestingly, not all of the polymorphic markers observed in each parent were detected in the HT or SP progeny and in some zoospore progeny, further bands were not detected. If parental strains were diploid, karyogamy should result in tetraploid

nuclei that may yield aneuploid and diploid segregants, recombinant for parental markers through chromosomal nodisjunction. Such events were confirmed in somatic recombinants of *Magnaporthe grisea* using the multilocus probe MGR586 (Zeigler, 1998) and are presumed to have occurred in tetraploid zoospores of *P. nicotianae* var. *parasitica* (Gu & Ko, 1998), and in some true fungi (Roper, 1966; Burnett, 1975). The absence of certain polymorphic bands in HT, SP and single-zoospore progeny suggests that karyogamy and nondisjunction yielded recombinant aneuploid and/or diploid recombinants. Further analysis with telomeric probes (Pipe & Shaw, 1997) could determine whether recombinants were diploid or aneuploid and also determine the temporal stability of any aneuploid strains.

6.3.4 Conclusions and further work

These data show that heterogenic strains of *P. infestans* from different continents are able to form unstable somatic hybrids following protoplast fusion. The fact that all zoospore propagations obtained from double-drug resistant protoplasts revealed some AFLP bands from both parents, means that karyogamy must have occurred in these isolates. The lack of double-drug growths using techniques other than protoplast fusion suggest that barriers to hyphal or even zoospore fusion exist which might limit somatic hybridisation in the field. Variation in fingerprint patterns observed in propagations from paired but unselected strains could have been a result of somatic recombination but could also be the result of mitotic crossing-over within single strains. This is testable with further experiments employing more markers.

It would be worthwhile attempting some of the methods outlined in Table 6.2.3 again with W1, 95.161.5 and N18 as they have been shown to form recombinant nuclei through protoplast fusion. These experiments may reveal that parental genotype interactions may inhibit or even promote somatic fusions in certain pairs of isolates or that certain techniques are more efficient in producing recombinants.

7 Selection of somatic hybrids with increased virulence to potato leaflets

7.1 INTRODUCTION

7.1.1 Variation in virulence phenotype

Many potato lines and cultivars are available with different combinations of R-genes (section 1.2.3) that confer resistance to some isolates of *P. infestans*. However, *P. infestans* is highly adaptable to host genotypes; even outside Mexico before the spread of the A2 mating type, new physiologic races able to attack previously resistant R-genotypes appeared regularly (Niederhauser, 1961; Malcolmson, 1970).

Theoretically the expected frequency of phenotypes with combined virulences should be a product of the frequency of the individual avirulence-genes in the fungal population (Wolfe *et al.*, 1976) if there is random recombination. Black (1952) suggested that strains with increasing numbers of virulence genes might be selected against in the absence of R-gene selection. Indeed, Shattock (1976) and Malcolmson (1979) inoculated plants with two isolates of different avirulence. New races were found and these revealed increased avirulence compared to the parental isolates. However, *P. infestans* race surveys conducted in North Wales (Shattock *et al.*, 1977) revealed that races of the fungus unselected by host resistance were frequent. In most cases, these complex races reflected the relative frequencies of the individual race characters in the population. This suggested to Shattock *et al.* (1977) that considerable gene flow was occurring in population of *P. infestans*. Before the 1980s, populations of the fungus outside of Mexico were asexual (Smoot *et al.*, 1958; Shaw *et al.*, 1985) and somatic recombination would have been the only possible recombination mechanism in these populations.

7.1.2 Investigating somatic recombination using R-gene selection

Leach & Rich (1969) suggested that two different mechanisms were capable of producing new races of *P. infestans* in the absence of the sexual stage. They paired A1 isolates of different races on lima bean agar (i.e. no selection for recombinants) and used several differential potato lines to test the virulence of single-sporangial and single-zoospore isolates from the pairings. They observed that new virulence, not in

either parent, could be expressed when two races of the fungus were grown together. For instance, pairing race 4 with race 1,3,4 always produced lesions on leaves containing the R2 gene. Secondly, pairing race 2,4 with race 1,3,4 resulted in some single-sporangial isolates of race 1,2,3,4; many single-zoospore derivatives from these single-sporangial isolates also had the recombinant phenotype suggesting that nuclei from the two races had recombined. They concluded that recombination of the two races into one nucleus (through anastomosis and karyogamy) formed a new race with characteristics of both isolates.

Wilde (1961) found that after simultaneous inoculation of r0 potato plants (plants with no race-specific resistance to *P. infestans*) with race 1,2 and race 2,4, new strains of race 1,2,4 could be isolated. Malcolmson (1970) reported similar findings when pairs of A1 isolates of different races were co-inoculated onto potato leaves with no resistance. Sporangia with a new race phenotype were also selected for by inoculating potato plants (R1,2,3,4) that neither parental isolate could infect individually. Pairings of race 4 with race 1,2,3,7 or race 3,4,10 with race 1,2,3,7 resulted in sporangia of race 1,2,3,4,7 or race 1,2,3,4,7,10 respectively. Again, using potato leaves to select recombinants of A1 strains, Denward (1970) reported that mixing race 3 with race 4 gave only race 3,4 but if race 1 was mixed with race 3,4, race 1,2,3,4 could be obtained. Race 2 seemed to appear spontaneously, as reported for some isolates *in vitro* (Leach & Rich, 1969). Race 1 and races 3 and 4 were thought to contain different parts of a race 2 determinant which, when they came together, allowed the expression of the race 2 phenotype. Denward (1970) also produced evidence that certain races could develop spontaneously when single races were grown individually; race 1 became race 1,4 after inoculation on R1, race 3 became race 3,4 on R3 and race 4 became race 3,4 on R4. Denward (1970) thought that the recombination of pathogenicities from the mixed races occurred too frequently to allow any serious suggestion that mutation, not recombination, was the cause, although mutation was proposed to explain the spontaneous appearance of new races in the control experiments.

Unfortunately, no markers other than virulence phenotype could be used at the time to investigate the origin of the new phenotypes (Wilde, 1961; Leach & Rich, 1969; Denward, 1970; Malcolmson, 1970). Virulence markers had been shown to be variable (Mills, 1940; Mills & Peterson, 1952; Graham *et al.*, 1961) as isolates could be "trained" to produce new virulence phenotypes by passage on resistant, young or

senescent leaves, probably a result of mutation (Erwin *et al.*, 1963). Furthermore, the expression of avirulence at some loci may be induced by environmental factors (Denward, 1970) giving a more variable phenotype. Using RG57, Goodwin *et al.* (1995) suggested that extensive variation in virulence phenotype observed in two clonal lineages (US-7 & US-8) of *P. infestans* in the absence of DNA fingerprint variation, was probably a result of mutation and that mutation rates at avirulence loci may be higher than at RG57 loci. Consequently, recombinants identified by virulence phenotype alone may in fact be mutants and not somatic hybrids. Neutral genetic markers such as RFLPs and AFLPs could allow somatic hybrids to be distinguished from mutants and could even allow the extent of any recombination to be determined.

7.1.3 Aims

The aim of this chapter is to mix an isolate of virulence AB with an isolate of virulence CD on r0 leaves. Zoospores can then be used to select for growths on leaves with complimentary resistance to ABCD. AFLP markers should determine the origins of isolates with increased virulence.

7.2 RESULTS

Anouck Busuttil, an undergraduate research student, obtained the results presented in sections 7.3.1 and 7.3.2 as part of a SOCRATES / ERASMUS studentship between March and June, 1999.

7.2.1 Isolates

The two isolates used were donated by Helen Stuart, SCRI, Dundee, and are described in Table 7.2.1.

Table 7.2.1. Origin of isolates used in chapter 7.

Isolate	Code	Race
24.1.1	A	1,3,4,7
34.1.2	B	1,2,4

Both isolates were A1 mating type and had mtDNA haplotype Ia. The AFLP fingerprints of these isolates can be found in appendix Table A3.4.

7.2.2 Virulence of parental isolates and a putative heterokaryon

Isolates A and B were grown separately on rye A agar. After 10 days, sporangia were harvested (section 2.2.7.2), and three detached leaflets of potato (cv Epicure, r0) were inoculated with a sporangial suspension of A, and a further three leaflets with B (concentrations of sporangia not determined). The leaflets were chilled for 1 hour to induce zoospores and incubated (section 2.2.7.2). After four days, spores were collected from A and B by spraying leaves with water into a beaker. The sporangial suspensions were transferred to a Universal bottle where the sporangia could settle for 15 minutes before re-suspending in 5 ml of fresh water. The spore concentration was then adjusted to 10^5 spores ml^{-1} for both A and B. Leaflets of r0 were then inoculated with 10-15 droplets (15 µl each) of isolate A, isolate B or a mixture of A and B (designated M), and were incubated for four days. Sporangia produced on these leaves were collected in a beaker (as above) and the suspensions of A, B. and M adjusted to give three concentrations of spores (Table 7.2.2). Ten 10 µl droplets from each of A, B, and M for each of the three spore concentrations were placed onto r0, R1,2,3 and R1,2,3,4 leaves (Figure 7.2.2). These leaflets were incubated and the percentage of necrosis and sporulation on the three leaflets was recorded visually after four days (Table 7.2.2).

Table 7.2.2. Percentage necrosis and sporulation of parental isolates A and B and the mixture, M, on three differentials at three sporangial concentrations.

		$1.7x10^4$ ml^{-1}			$1.7x10^3$ ml^{-1}			$1.7x10^2$ ml^{-1}		
		r0	R1234	R123	r0	R1234	R123	r0	R1234	R123
A	%N	80	30	15	30	15	0	30	0	0
	%S	80	0	0	5	0	0	0	0	0
B	%N	80	65	50	30	15	15	15	0	0
	%S	80	40	5	5	0	0	0	0	0
M	%N	80	50	30	50	15	0	0	0	0
	%S	80	0	0	50	0	0	0	0	0

N, necrosis; S, sporulation

As expected, both parents A and B and the mixture, M, sporulated well on the r0 leaves at the highest concentration of sporangia. With 10-fold less sporangia, all leaflets produced sporangia but to a lesser extent. At the lowest inoculum concentration, necrosis was observed on the r0 leaves infected with A or B, but there was no sporangium production. At this concentration, M did not induce necrosis or sporulation, even on r0 leaves. On the R1,2,3,4 differential and highest spore concentration, A, B and M all induced a necrotic hypersensitive response but only parent B produced a sporulating lesion. With 10-fold fewer sporangia, necrosis was observed on leaves infected with A, B and M but no sporulating lesions were observed. At the lowest concentration, no necrosis was observed. A similar situation was observed on the R1,2,3 differential although the degree of necrosis and sporulation was always less than on the R1,2,3,4 cultivar. A putative hybrid of A and B isolated from growths of M on R1,2,3,4 leaves (Table 7.2.2) (isolate C) was selected and used in subsequent experiments.

7.2.3 Selection of virulence on R1,2,3,4 leaves

Sporangia of A and B from growths on r0 (Table 7.2.2) and of M from growths on r0 and R1,2,3,4 (i.e. isolate C) were used to re-inoculate the potato differentials, r0. R1,2,3 and R1,2,3,4 (Figure 7.2.2). Each differential line was inoculated with twenty droplets (10 μl) of sporangia (revised concentrations in Table 7.2.3). The leaves were incubated (section 2.2.7.2) and observed after six days.

Once more, symptoms were observed at all three inoculum concentrations on the control (r0) leaves, confirming that the isolates were pathogenic on host material. No necrosis or sporulation was observed on the R1,2,3 or R1,2,3,4 leaves when they were infected with A or B but there was some sporulation of M at the highest

inoculum concentration on R1,2,3,4. However, isolate C produced almost 100% necrosis and sporulation on all three differentials (Table 7.2.3).

Table 7.2.3. Percentage necrosis and sporulation of parental isolates A and B, M and isolate C on three differentials at three sporangial concentrations.

		$1x10^4$ ml^{-1}			$2.5x10^3$ ml^{-1}			$6.25x10^2$ ml^{-1}		
		r0	R1234	R123	r0	R1234	R123	r0	R1234	R123
A	%N	100	0	0	20	0	0	100	0	0
	%S	100	0	0	20	0	0	100	0	0
B	%N	80	0	0	50	0	0	30	0	5
	%S	80	0	0	50	0	0	30	0	0
M	%N	100	20	0	100	0	0	20	0	0
	%S	100	20	0	100	0	0	20	0	0
C	%N	100	100	100	100	100	90	100	100	100
	%S	100	100	100	100	100	90	100	100	100

N – necrosis; S - sporulation

7.2.4 Single-sporangial analysis of isolate C

Isolate C was a putative hybrid since it had the combined virulence of each parent. Six single-sporangial isolates were established from isolate C. The phenotype of three of these single-sporangial lines was determined on differentials, r0, R1, R2 and R3 (Figure 7.2.2) to assess the virulence phenotype. Figures 7.2.1 & 7.2.3 show the percentage sporulation and necrosis of isolates A & B, M, C and single-sporangial lines (SS1-SS3) from C on each differential for parental.

All isolates produced abundant necrosis and sporulation on the control r0 leaves after five days incubation, except one of the single-sporangial isolates (SS1) which did not sporulate well on any of the differential lines. The avirulence of parent A on R2 was confirmed as was the avirulence of parent B on R3 (Figures 7.2.1 & 7.2.3). Disregarding SS1, the mixture M, isolate C and SS2 & 3 derivatives were all virulent on both R2 and R3 to a greater (C, SS2 & 3) or lesser (M) extent and it appeared that the combined virulence could be transmitted through single-sporangia (SS2 & 3) from isolate C.

7.2.5 Single-zoospore and AFLP analysis of putative somatic hybrids

Five single-zoospore lines were established from each six single-sporangial colony. Unfortunately, the single-zoospore lines could not be tested on the r0, R1, R2 or R3 differentials due to time constraints. However, the parental isolates, mixture M, isolate C and the single-sporangia and single-zoospore derivatives were all screened

for AFLPs using primers E19-M16 and E19-M40 (Figure 7.2.2).

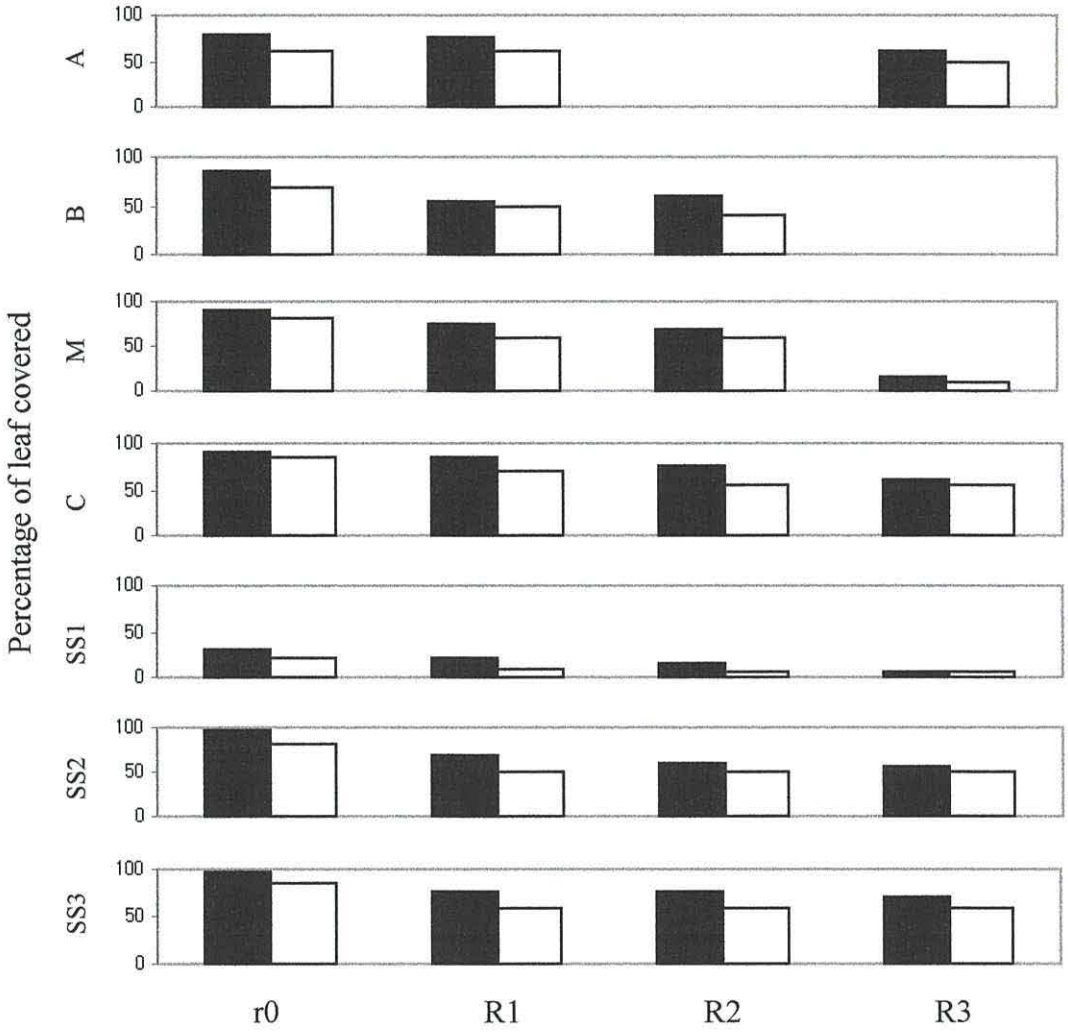


Figure 7.2.1. Percentage necrosis (■) and sporulation (□) of A and B, M, C and three single-sporangial derivatives from isolate C, each of r0-R3. Concentration of sporangia in droplets was 3 x 10^3 ml^{-1} and ten 10 µl droplets were used for inoculation of each of three leaflets.

Three bands unique to parent A and two bands unique to parent B were detected but M and C revealed bands that were unique to parent B only (Table 7.2.4; Figure 7.2.4). The single-sporangial isolates and all but one single-zoospore derivative all had the same fingerprint pattern as parent B with primers E19-M40. One zoospore isolate (SS4 SZ5) revealed band E19-M40 (45) but this band was not detected in the single-sporangial line from which it was derived (SS4). All single-sporangial and single-zoospore isolates had bands E19-M16 (34 & 38), unique to parent B, and none of the single-sporangial or single-zoospore isolates revealed bands

E19-M16 (46 & 53) unique to parent A; they all had the genotype of parent B. Four isolates (M, SS1, SS4 SZ1, SS5 & SS6) did not reveal some bands that were present in both parents (E19-M16 42, 64 & 66) (Table 7.2.4).

Table 7.2.4. AFLP fingerprints of single sporangial (SS) and single zoospore (SZ) derivatives from isolate C with primers E19-M16 and E19-M40. The numbers of each observed zoospore genotype are indicated.

		E19-M40	E19-M16						
	No.	45	34	38	42	46	53	64	66
Parent A		1	0	0	1	1	1	1	1
Parent B		0	1	1	1	0	0	1	1
M		0	1	1	1	0	0	1	0
C		0	1	1	1	0	0	1	1
SS1		0	1	1	1	0	0	0	0
SS1 SZ 1-5	5	0	1	1	1	0	0	1	1
SS2		0	1	1	1	0	0	1	1
SS2 SZ 1-5	5	0	1	1	1	0	0	1	1
SS3		0	1	1	1	0	0	1	1
SS3 SZ	5	0	1	1	1	0	0	1	1
SS4		0	1	1	1	0	0	1	1
SS4 SZ 1	1	0	1	1	0	0	0	0	0
SS4 SZ 2-4	3	0	1	1	1	0	0	1	1
SS4 SZ 5	1	1	1	1	1	0	0	1	1
SS5		0	1	1	0	0	0	0	0
SS6		0	1	1	1	0	0	0	0

Bands in red were only present in parent A, bands in green were only present in isolate B and bands in blue were present in both A and B but were polymorphic amongst the sporangial and zoospore isolates.

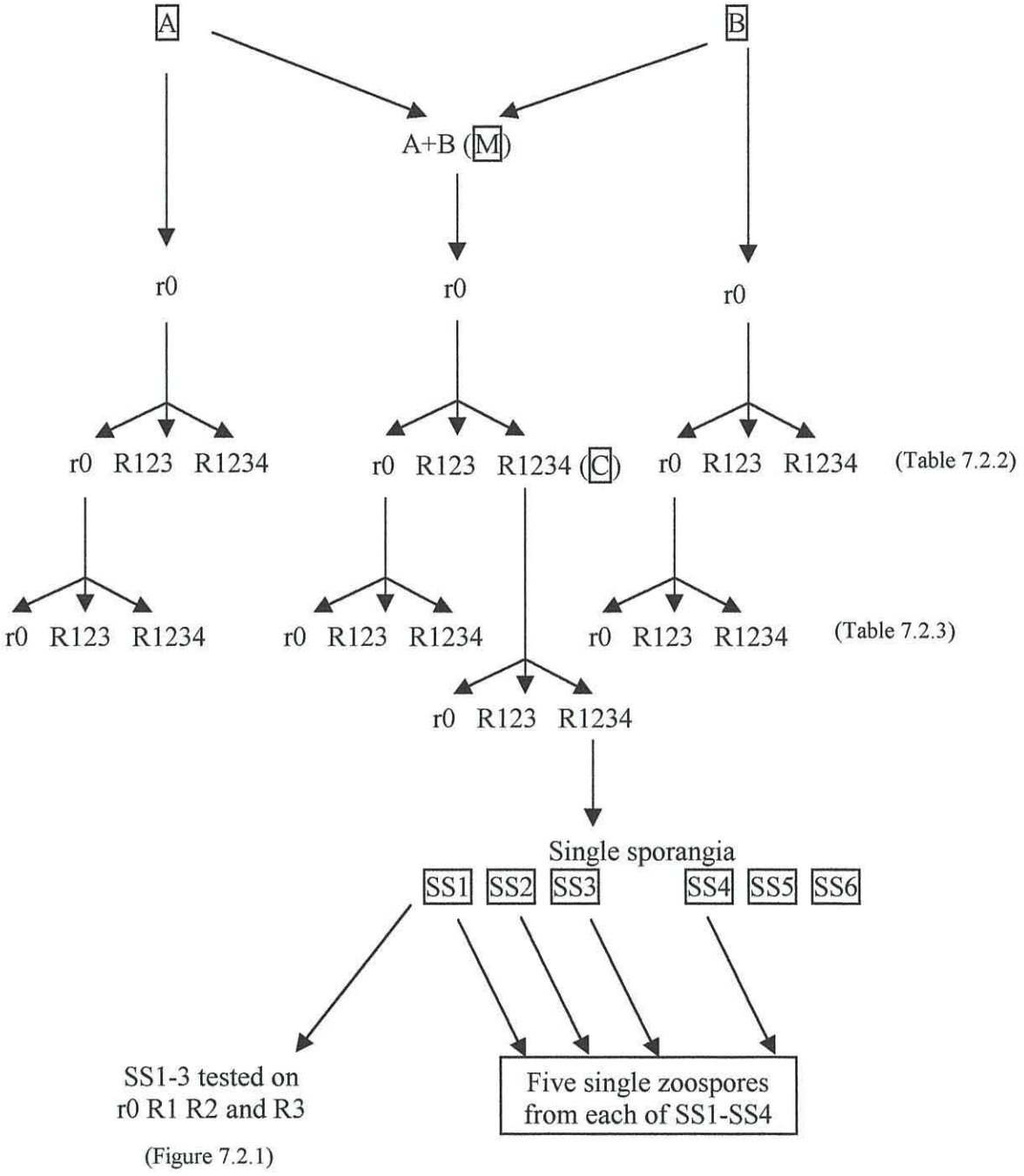


Figure 7.2.2. Flowchart of inoculations for experiments in chapter 7. Appropriate tables presenting results from different inoculations are indicated. Isolates that were fingerprinted for AFLPs (Table 7.2.4) are indicated in boxes.

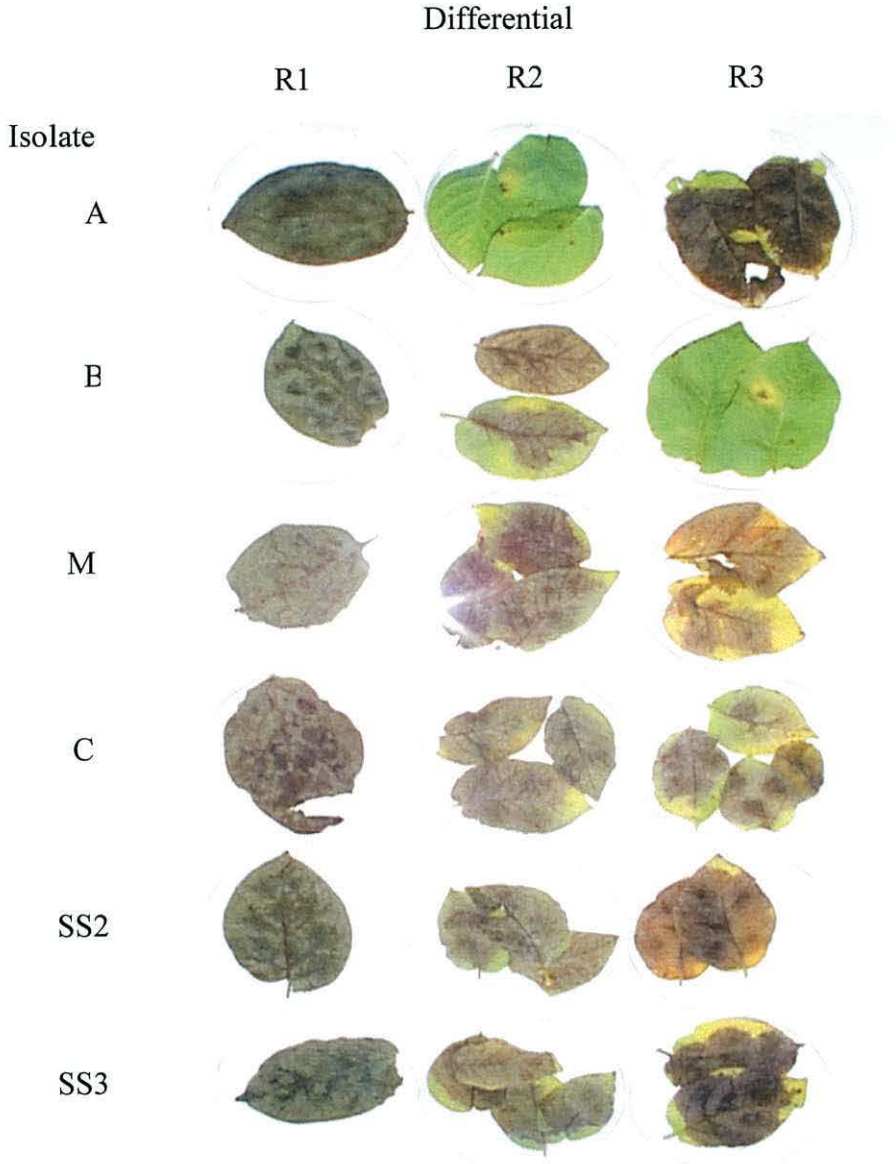


Figure 7.2.3. Necrosis and sporulation on three differential potato lines (R1-R3) of parental isolates A & B, the mixture M, mixture selected on R1234 leaves (C) and two single-sporangial derivatives from isolate C. See Figure 7.2.1.

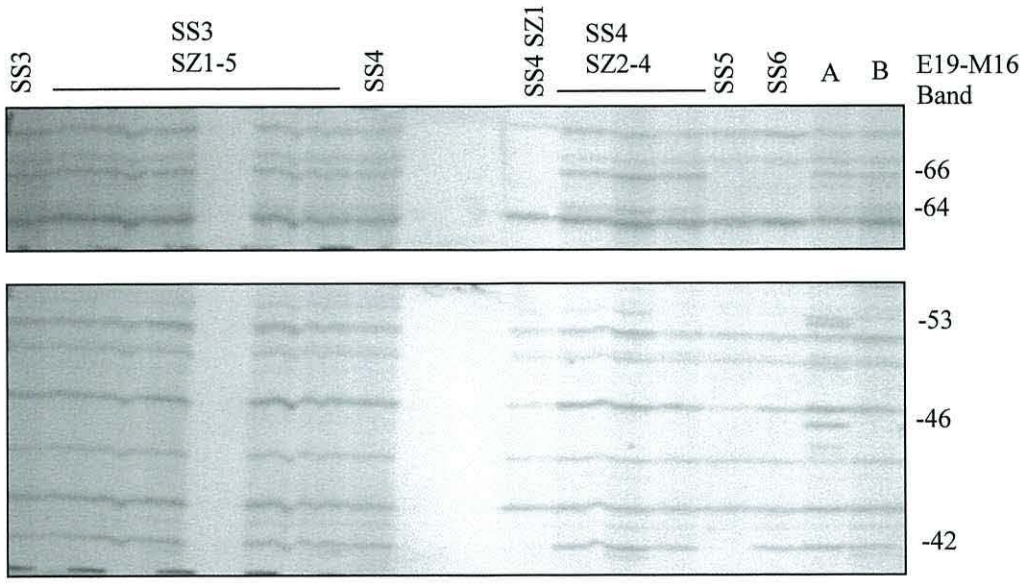


Figure 7.3.4. AFLP variation amongst parents A & B and a sample of single-sporangial and single-zoospore derivatives from isolate C. See section 7.2.5 and Table 7.2.4.

7.3 DISCUSSION

Adaptation through change in virulence phenotype of *P. infestans* has been shown in previous work (Leach & Rich, 1969; Denward, 1970; Malcolmson, 1970). Mixtures of races grown together on a susceptible host (Wilde, 1961; Denward, 1970; Malcolmson, 1970) or on media *in vitro* (Leach & Rich, 1969) seemed to combine genetically to produce new races of the fungus. However, in these studies it was impossible to distinguish mutation from somatic recombination (Mills, 1940; Denward, 1970), as molecular markers were unavailable at the time. One of these experiments was repeated in the present study using both selective (virulence) and neutral (AFLP) markers to establish whether mutation and / or somatic recombination were involved in generating new races.

7.3.1 Infection of potato leaflets with different spore concentrations

Detection of certain virulence factors in *P. infestans* (Sujkowski, 1990) and *P. megasperma* (Eye *et al.*, 1978) is dependent upon inoculum concentration. Fungal isolates are able to infect a normally resistant cultivar if the zoospore (inoculum) concentration is high enough. Conversely, if the spore concentration is too low, then even virulent isolates may be unable to infect susceptible hosts (Eye *et al.*, 1978; Sujkowski, 1990). Accordingly, three spore concentrations were used in each of the experiments to look at susceptible and resistant cultivars (Tables 7.2.2 & 7.2.3). Using the highest concentration of spores (17000 sporangia per 10 µl droplet) (Table 7.2.2), parental isolates A and B were able to elicit necrosis (A & B) and sporulation (B) on the R1,2,3,4 and R1,2,3 differentials. However, at the lowest concentration of spores (1.7 sporangia per 10 µl droplet) both A and B were unable to elicit even a hypersensitive response on either differential. A spore concentration somewhere between these two extremes (Table 7.2.3) inhibited growth of parental isolates on resistant cultivars but allowed selection of the putative recombinant, C.

7.3.2 Pathogenicity of A & B and putative recombinants

Controls of the two parental isolates on r0, R1, R2 and R3 differentials indicated that parent A was race 1,3 whereas parent B was race 1,2 (Figures 7.2.1 & 7.2.3). Neither parent could successfully infect the differentials that possessed both R2 and R3. The co-culturing of A and B (M) and subsequent isolation of C therefore seemed to have

generated a new phenotype appearing, on the basis of the selectable markers available, as a composite of parents A and B (Figure 7.2.3). There are several possible hypotheses to explain the new races.

C may not have been a recombinant but an intimate mixture of A and B hyphae. Theoretically this should not be possible as neither A nor B should have genetic determinants to overcome R2 and R3. However, previous studies have suggested that extracellular factors exchanged between two isolates in close proximity could allow virulence to R2 and R3 to be temporally expressed whilst being associated in a mixture (Leach & Rich, 1969). Alternatively, C could have been a somatic recombinant carrying A and B nuclei in a common cytoplasm, allowing expression of race 2,3 in single culture, assuming virulence was dominant at these loci. It is also possible that one or both parental isolates, when faced with either R2 or R3 resistance genes, may have developed mutations from to race 2 (A) or race 3 (B) allowing new phenotypes to be expressed. Single cultures of *P. infestans* are variable for virulence phenotype (Graham *et al.*, 1961; Denward, 1970) and spontaneous change to virulence on R2 (Black, 1952) and R3 (Graham *et al.*, 1961) has been observed in single culture and during vegetative growth (Reddick & Mills, 1938; Al-Kherb, 1988).

The fact that the three examined single-sporangial isolates established from C produced good growth and sporulation, on R2 and R3 leaves, suggests that these phenotypes were not the result of hyphal mixtures stimulating the expression of race 2 or 3. The possibility that somatic recombination or mutation were the cause of the C phenotype could be investigated with AFLPs.

7.3.3 Molecular analysis of parents and progeny

Only one of the 26 single-sporangial or single-zoospore progeny exhibited bands specific to both parents A and B (Table 7.2.4; Figure 7.2.4) and most (21) of the 26 progeny were identical to parent B for all loci scored. Bands specific to both parents A and B would be expected in some single-sporangial and possibly single-zoospore progeny if C were a somatic recombinant. Assuming the band from isolate A was present in SS4 SZ5 was not a mutation, then it could be argued that isolate C was a somatic recombinant. Nuclei from parent A may have been at a much lower frequency (e.g. 1:35, Burnett, 1975) than those from parent B if isolate C was a heterokaryon but may still have produced the recombinant phenotype. AFLPs

specific to parent A may not have been detected if A nuclei were at a lower frequency than B, so a heterokaryon may appear to have a parent B fingerprint. Perhaps in a heterokaryon with an unequal ratio of A and B nuclei, most sporangia (and resulting zoospores) would be of the common nuclear type (B), as was found. However, infrequent karyogamy between nuclei of A and B could result in both type B and recombinant (or even type A) nuclei migrating from the sporangiophore into the sporangium (Maltese *et al.*, 1995). The presence of the parent A band in SS4 SZ5 may therefore indicate that isolate C was a product of somatic recombination.

An alternative possibility to explain the novel phenotypes and fingerprint patterns is that mitotic crossing-over (Shaw, 1983a & b) or mutation to race 3 occurred in parent B (section 7.3.2) allowing growth on R1,2,3,4, R2 and R3 cultivars. Segregation of avirulence loci in a single rust isolate has been explained by mitotic crossing-over, resulting in the loss of an inhibitor gene pair which suppressed an avirulence allele (Lawrence *et al.*, 1981). Segregation of race 3 has also been observed in *P. infestans* during vegetative growth (Reddick & Mills, 1938; Al-Kherb, 1988). Under either of these hypotheses, the majority of progeny should have a type B genotype but could have new phenotypes. Mitotic crossing-over and / or mutation could result in some heterozygous (+/-) AFLP bands becoming homozygous (-/-) and therefore not detected in some of the progeny, as observed (Table 7.2.4). Further analysis with more AFLPs would be required to determine whether somatic recombination, mutation or mitotic crossing-over had occurred. Without further analysis, mutation to increased virulence and possible mitotic crossing-over are the most likely hypotheses to explain the molecular variation in isolate C and the single-sporangial derivatives.

7.3.4 Inheritance pattern of virulence phenotypes

Avirulence is usually dominant to virulence at most loci, though there are exceptions (section 1.2.3). In heterokaryons constructed with avirulent and virulent nuclei in *P. sojae* (*P. megasperma* f.sp. *glycinea*), Layton & Kuhn, (1988b) found that avirulence was dominant to virulence for race 1 and 3. They suggested that the few heterokaryons showing dominant virulence had undergone karyogamy and may have lost an avirulence gene through sequential loss of chromosomes during diploidisation. If avirulence is generally dominant to virulence in *P. infestans*, mixing race 1,2,4 with 1,3,4,7 would be expected to produce race 1,4 which would be avirulent on R2 and

R3, as observed in putative heterokaryons of *B. lactucae* (Hulbert & Michelmore, 1988). However if the virulence genes were controlled from non-homologous loci in different isolates (Spielman *et al.*, 1990) or did not display such a dominance relationship (Al-Kherb *et al.*, 1995) the results of Leach & Rich (1969), Denward (1970), Malcolmson (1970) and the observations in the present study would be expected. Further investigations are needed to establish the true genetic basis of virulence determination in *P. infestans*, both through sexual and asexual crosses before models regarding heterokaryosis and recombination can be proposed using virulence markers alone.

7.3.5 Conclusions and further work

Novel races of the fungus able to grow on leaves neither parent were able to infect were easy to obtain by co-cultivating the two isolates, A and B, on leaves. Single-sporangia obtained from these putative hybrids also revealed an apparently recombinant phenotype. However, AFLP fingerprints of the parents and progeny suggested that somatic recombination may not have occurred as bands from only one parent were observed in most progeny. Mutation of parent B from race 1,2,4 to race 1,2,3,4 on leaf tissue and possible mitotic crossing-over is the simplest explanation, especially as new races are often observed in single culture. Further investigation with more AFLP markers could identify whether the unexpected parent A band in SS4 SZ5 was a result of recombination or mutation.

Future experiments could be improved using double virulence markers. For instance crossing race AB with CD and selecting on AC may result in recombinants and mutants both resistant to AC. These could be examined on BD, as mutants able to grow on BD would not be expected through selection on AC. Alternatively, isolates with different race and drug resistant markers could be paired on agar. Double-drug resistant growths could be tested for growth on leaves with appropriate R-genes. It would also be useful to cross the two isolates, A and B, with appropriate A2 isolates, to determine the inheritance pattern of virulence markers in these isolates.

8 Summary and conclusions

The study aimed to investigate somatic recombination in *Phytophthora infestans* using several approaches. Initially, the AFLP technique was directly compared to the RFLP technique (RG57) to investigate which would be more appropriate to identify somatic hybrids. Asexual progeny from self-fertile isolates (A1/A2) were analysed for RFLPs and AFLPs to determine whether the self-fertile phenotype was due to a mixture of the two isolates or heterokaryosis and to identify any recombinant asexual progeny. Drug-resistant mutants were isolated and after co-cultivation of strains with different drug-resistant markers, putative recombinants with novel phenotypes were screened for AFLPs. Finally, after mixing two parental strains on detached potato leaflets, sporangia appearing to have the combined race of both parents were investigated to see if they were the result of mutation or recombination.

8.1 Comparing the AFLP and RFLP techniques

Ninety-eight isolates collected from England and Wales were used to investigate whether AFLPs or RFLPs (RG57) would be more useful for detecting somatic hybridisation (chapter 3). In doing this, data revealed by both techniques could be compared through UPGMA analysis. Although the AFLP technique revealed a smaller percentage of polymorphic loci than RG57, almost 3.5 times the number of loci could be screened using a single AFLP primer-pair. Coupled with the fact that there are an almost limitless number of primer combinations, it is possible to screen many more loci using AFLPs when compared to RFLPs. Although there was some variation in AFLP fingerprint pattern amongst replicate DNA extractions or single-zoospore lines from one isolate, other reproducibility experiments did not reveal any variation. The aberrant bands were most likely the result of the initial DNA extraction protocol and mutation (section 3.2.10).

Comparing both RFLP and AFLP data suggested that there was generally a high degree of congruence between the two techniques (Figures 3.2.1 & 3.2.2). UPGMA trees produced from either AFLP or RG57 data revealed that isolates clustered on the basis of their mating type and their mtDNA haplotype. These clusters were further confirmed

using data from six primer-pairs and 57 polymorphic AFLP loci (Figure 3.2.3) where all of the A2 isolates formed a tight cluster and nine of the 11 isolates with mtDNA haplotype IIa also clustered. As neither mating type or mtDNA markers are linked to any RG57 or (presumably) AFLP loci, such a clustering pattern should only be observed if there is no (or little) sexual recombination between isolates with different mating types or mtDNA haplotypes.

There was also evidence for convergence of some RG57 fingerprint patterns. Often, as would be expected, isolates with the same or similar RG57 fingerprint revealed similar AFLP fingerprint patterns. However, some isolates with identical RG57 fingerprints had quite different AFLP fingerprints, suggesting they were not similar to, or clones of that particular lineage (section 3.2.4) and could have had the same RG57 fingerprint as a result of convergence. Further experiments using more AFLP markers (or other RFLP probes) should be conducted to investigate convergence of RG57 fingerprint patterns in populations. This should be compared to theoretical calculations and could be incorporated into further RG57 population data.

8.2 Analysis of self-fertile isolates

Both RG57 and AFLPs were used to investigate asexual progeny from self-fertile isolate, RE-SF, which was synthesised in the laboratory from an A1 and an A2 isolate with known DNA polymorphisms (chapter 4). All progeny revealed the same fingerprint as one of the parental isolates using RG57. However, using AFLPs, polymorphisms could be detected between parents and in the progeny (Table 4.2.2). Some progeny revealed bands from both parents and it is possible that some of these were somatic recombinants. However, only three AFLP bands were polymorphic between the parental isolates and some of the variation observed in the progeny could have been the result of mutation or mitotic crossing-over within a parental strain. Similarly, no evidence of somatic recombination was obtained from investigations of two self-fertile isolates collected from the field, 98.01 and 98.02 (section 4.2.7). Only single genotypes were identified in zoospore progeny and unlike RE-SF, the original parental genotypes were not known. Consequently, it was impossible to determine whether the genotypes of the progeny were parental or recombinant in origin. In light of work presented here and of recent studies

(Judelson & Yang, 1998; Pipe *et al.*, in press), it would be worthwhile investigating more progeny from self-fertile isolates with further molecular markers. AFLP analysis with more primers may reveal further polymorphisms and confirm the occurrence of any somatic hybrids.

8.3 Pairing isolates with the same mating type without selection

Parental and non-parental RG57 fingerprint patterns were observed in hyphal-tip lines after co-culturing A1 isolates with different RG57 fingerprints and different mtDNA haplotypes, on agar and in liquid media (Table 6.2.1). From these cultures, 37% of hyphal-tips had non-parental genotypes and this variation could have been the result of somatic recombinants or mitotic crossing-over within a single strain. Such a proportion of non-parental genotypes would not be expected if they had been generated through mitotic crossing-over, as previous studies suggested that RG57 fingerprints were somatically stable (Goodwin *et al.*, 1992a). However, somatic recombination could not be confirmed in any of these isolates, as bands specific to both parents could not be detected in the hyphal-tip progeny. Similar experiments employing markers such as AFLPs, telomeric probes and single-locus RFLPs would be worth further study in the light of these results.

8.4 Isolating drug-resistant mutants

In order to positively select for somatic recombination, drug-resistance was investigated in a sample of seventeen isolates (chapter 5). Many isolates with stable resistance to metalaxyl were available for use in selection experiments. Resistance to other drugs had to be induced. Incubating plugs of mycelia on drug-amended media allowed four mutant sectors resistant to streptomycin to be isolated (section 5.2.4) and once isolates had acquired resistance, their growth on streptomycin-amended media was stable. It was, however, more difficult to obtain stable, resistant mutants to other drugs (Table 5.2.5). Consequently isolates resistant to streptomycin and metalaxyl, and transformants resistant to G418 and hygromycin were used for subsequent experiments.

Sexual crosses involving streptomycin- or metalaxyl-resistant isolates revealed very low levels (>1%) of oospore germination and establishment (section 5.3.2). Currently, techniques using live water-snails have produced up to 30% germination of *P.*

infestans oospores (D. Earnshaw, personal communication) and may be a more efficient method for establishing germinated oospore progeny in the future. As a consequence of poor germination, few conclusions could be reached regarding the inheritance of drug-resistance or AFLP bands in these crosses.

8.5 Selection of progeny with novel drug-resistant phenotypes

Pairing drug-resistant strains of the same mating type on agar or in liquid media did not produce any evidence of somatic recombination (chapter 6). Lack of recombination (Table 6.2.3) may have been a consequence of the markers (streptomycin and metalaxyl) employed in most of the pairings. If the streptomycin-resistant mutation was mitochondrial and cytoplasmic incompatibility prevented mitochondrial or nuclear exchange, then progeny with novel phenotypes would not be obtained. The fact that certain isolates would or would not collide on agar suggests that incompatibility barriers preventing recombination (Bagirova *et al.*, 1998) may have played a part in the present study. It is possible that cytoplasmic and/or structural barriers may regulate incompatibility and these could be investigated in future studies by injecting cytoplasm of one strain into another (Gu & Ko, 1998) or by forcing isolates together through protoplast fusion.

Fusing protoplasts of drug-resistant strains of the same mating type suggested that somatic recombination had occurred in two pairings (Tables 6.2.6 & 6.2.7). Single hyphal-tip and single-sporangial lines from these pairings had a combination of parental drug-resistant phenotypes also displayed AFLPs specific to both parental strains. Single-zoospores from these growths revealed the same recombinant phenotypes and genotypes suggesting that karyogamy had occurred after protoplast fusion. Most, but not all, parental-specific bands were observed in zoospore progeny, suggesting that these hybrids were not tetraploid and more likely aneuploids or recombinant diploids. These data show that strains of *P. infestans* from different continents are able to form somatic hybrids following protoplast fusion and that recombination had occurred between nuclei of the two parents.

8.6 Selection of somatic hybrids using virulence phenotypes

The final approach to investigate somatic recombination used specific virulence markers

to select for progeny with combined virulence phenotypes of the parents (chapter 7). Single-sporangial progeny with race phenotypes appearing as a combination of the two parents were obtained. However, AFLP fingerprints of the parents and progeny (Table 7.2.4) suggested that that somatic hybridisation had not occurred since bands specific to only one of the parents were observed in most of the progeny. In the absence of further fingerprint data, the best explanation for isolates with novel races was that mutation in one of the parents (B) allowed development of resistance to R3. Indeed, specific virulence markers are variable in single culture (Reddick & Mills, 1938; Graham *et al.*, 1961) and the genetic basis of their inheritance can vary, depending on the locus and the isolate. Virulence markers if used in similar studies in the future should be combined with other selectable markers, such as drug-resistance, to which resistant mutants are less likely to be selected for on leaves.

8.7 Further conclusions

In all experiments pairing isolates on agar or in liquid media, some progeny lost RG57 or AFLP bands that were present in one or both of the parents (Tables 4.2.2, 6.2.1, 6.2.6, 6.2.7 & 7.2.4. Somatic recombination could only be confirmed in one experiment (using protoplasts) and it is possible that mitotic crossing-over within a single strain was the cause of many of the absent bands. Such events may be irregular in single cultures of *P. infestans* but could have been induced through co-culturing isolates with the same mating type. It would be useful to investigate the mechanisms involved in generating this variation. By growing strains singly and in pairs (possibly separated by polycarbonate membranes), before fingerprinting with AFLPs and other markers, variation induced by co-cultivation or vegetative growth could be examined.

The AFLP technique proved to be useful for the study of somatic recombination in *P. infestans*. The technique revealed polymorphic bands in some isolate-pairs where RG57 was monomorphic, and AFLP data allowed conclusions to be drawn, suggesting that mutation (chapter 7) and somatic recombination (chapter 6) were the cause of novel phenotypes. However, as AFLPs are PCR-based, they may not be ideal markers to use alone to investigate somatic recombination. For instance, a sporangium might be a heterokaryon of X and Y but during growth could sector. Subcultures could then vary in

their proportions of X and Y nuclei, all the way from pure X to pure Y. Such variation in nuclear ratio may have allowed detection of a parent A band in SS4 SZ5 (chapter 7; Table 7.2.4) when it was not detected in the parental culture. It is unclear at what dilution bands from one nucleus will not be detected, especially as many bands are being amplified at once in an AFLP reaction. Experiments mixing different proportions of DNA from parents and comparing them to fingerprints of putative hybrids would be worthwhile. It would also be useful in future studies to use AFLPs in conjunction with single-locus (Carter *et al.*, 1999) and telomeric (Pipe & Shaw, 1997) probes. These extra markers are not PCR-based and would be effective in identifying tetraploids and aneuploids through karyogamy.

In AFLP analysis it is always assumed that bands of the same size are homologous (have the same sequence) but this is not always true. For instance, two restriction events may lead to two bands of the same size which might not be allelic (Majer *et al.*, 1996) and variation at these two loci (and evidence for somatic recombination) could consequently go undetected. Furthermore, it was assumed that all of the AFLP bands in the present study were nuclear in origin but if some, especially polymorphic bands, were mitochondrial, some of the conclusions regarding somatic hybridisation would have to be altered. Theoretically, comparing the sizes of the nuclear (Tooley & Therrein, 1987; van der Lee *et al.*, 1997) and mitochondrial genomes (Griffith & Shaw, 1998), 0.02% of AFLP bands should have been mitochondrial in origin. Analysis of the mtDNA sequence from *P. infestans* (Chesnick *et al.*, 1996) would allow the number of AFLP bands amplified with certain primer-pairs to be determined exactly but it is unlikely that mtDNA bands would affect any of the conclusions reached.

The most frequent opportunities for somatic hybrids to form in the field (either through zoospore or hyphal fusion) would be during highest lesion density. Isolates would need to be growing adjacent to each other on diseased tissue (or be associated in a self-fertile) for somatic recombination to occur. Zoospores also have the ability to move and may aggregate in areas favourable for infection (Porter & Shaw, 1978). If zoospores from different strains were attracted to the same aggregate, it is possible that zoospore fusion and somatic hybrids could be formed from isolates growing on different parts of the same plant or on different plants. However, distances moved by zoospores in the soil

are limited. Zoospores of *Pythium* species moved 144 mm h^{-1} and *P. cinnamomi* zoospores failed to infect roots 7.2 cm away (Hickman & Ho, 1966). It seems, therefore, that two isolates would need to be growing close to each other before somatic recombination could occur on the leaf or in zoospore-aggregates.

The stage of the infection cycle and chemical cues from the plant may also stimulate or inhibit somatic recombination (Kuhn, 1991). *P. infestans* displays differential growth physiology on healthy and senescing leaves (Graham *et al.*, 1961) and somatic hybridisation may be induced towards the end of the epiphytotic by cues from senescing leaves. In the present study, the majority of techniques used to identify somatic recombinants paired isolates in liquid media or on agar. Growth on agar may not induce hybrid formation as frequently as on plant tissue as the isolates may receive more nutrients or may be without cues from the plant that may induce recombination. Perhaps inoculation of whole plants with numerous strains of *P. infestans* would reveal more recombinants than experiments employing growth on artificial media. Hybrids would easily be identified from any contaminants in such experiments with molecular markers.

The experiments in the present study evaluated numerous approaches to induce somatic recombination in the laboratory. Variation (mitotic crossing-over) or somatic recombination was observed amongst progeny using particular pairing strategies. The usefulness of AFLPs was demonstrated as bands specific to both parents could be detected in single-zoospore progeny after protoplast fusion between two isolates. However, forcing isolates to form somatic hybrids by selecting double-drug resistant phenotypes and inducing the fusion of membranes may not truly reflect the extent of somatic hybrid formation and parasexual recombination in nature (Caten & Jinks, 1966). Consequently, the true extent and importance of somatic hybridisation in the life cycle of *P. infestans* remains to be determined in further experiments.

Appendices

1 Preparation of media used

1.1 Rye A agar (Caten & Jinks, 1968)

Rye grains	60 g
Lab m agar No. 2	12 g
Sucrose	20 g

Rye grains were soaked in distilled water for 36 h at 18°C. The supernatant was retained and a filtrate prepared from swollen grains by macerating in a blender (Wareing). The filtrate was incubated at 50°C for 3 h and the sucrose was dissolved in the supernatant. The filtrate was collected through muslin, the supernatant added and the volume adjusted to 1 dm^3 by addition of distilled water. Agar was added before autoclaving for 15 min at 1.08 kg cm^{-2}.

1.2 RAN media

Nystatin	1 g
Rifamycin	0.5 g
Ampicillin	0.5 g

The antibiotic cocktail was dissolved in 20 ml DMSO to give a stock solution of 50 mg ml^{-1} nystatin, 25 mg ml^{-1} rifamycin and 25mg ml^{-1} and stored at –20°C. 1 ml was added to 500 ml of rye A agar after autoclaving to give concentrations of 50 μg ml^{-1} rifamycin, 50 μg ml^{-1} ampicillin and 100 μg ml^{-1} nystatin. Dilute RAN contained ¼ strength of the above antibiotics.

1.3 Pea broth and pea broth agar

Frozen peas	300 g
Lab m agar No. 2	12 g

Peas were boiled in 500 ml of distilled water for 10 min. The filtrate was then collected through a sieve and the volume adjusted to 1 dm^3 by addition of more distilled water. Agar was added before autoclaving.

Pea broth was made by omitting the agar and adding 250 µl (¼) RAN per 500 ml broth giving a final concentrations of 12.5 µg ml^{-1} rifamycin, 12.5 µg ml^{-1} ampicillin and 25 µg ml^{-1} nystatin.

One-tenth pea agar contained filtrate from 30 g of peas instead of 300 g in 1 dm^3 and did not contain RAN.

1.4 Rye sucrose mannitol (RSM) medium

RSM used the same quantities of rye and sucrose as for rye A agar, in addition to 1 M mannitol. Rye grains were soaked for 36 h, after which time they were boiled for 20 min. The supernatant was collected through muslin, the sucrose and mannitol were added and the volume adjusted to 1 dm^3. The media was then autoclaved.

1.5 Water agar

Lab m agar No. 2 8 g (0.8%) or 20g (2%)

The agar was mixed with 1 dm^3 of H_2O and autoclaved.

1.6 Metalaxyl medium

1.06 g (94% active ingredient, technical grade, Ciba Geigy) was dissolved in 10 ml DMSO (dimethyl sulfoxide) and made up to 100 ml with sterile distilled water to produce a stock solution of 10 mg ml^{-1}. 500 µl of stock solution when added to 500 ml rye A agar after autoclaving gave media of 10 µg ml^{-1} metalaxyl. Stock was stored at –20°C for up to 1 year.

1.7 Streptomycin medium

5 g streptomycin (Sigma) was dissolved in 50 ml sterile, distilled water to produce a stock solution of 100 mg ml^{-1}. 500 µl of stock solution when added to 500 ml rye a agar after autoclaving, gave media of 100 µg ml^{-1} streptomycin. Stock was stored at –20°C for up to 1 year.

1.8 Chloramphenicol medium

5 g chloramphenicol (Sigma) was dissolved in 50 ml ethanol to produce a stock solution of 100 mg ml^{-1}. 500 µl of stock solution when added to 500 ml rye A agar after

autoclaving, gave media of 100 μg ml^{-1} streptomycin. Stock was stored at –20°C for up to 1 year.

1.9 Hygromycin B and Geneticin (G418)

50 mg (hygromycin) or 150 mg (G418) were dissolved in 1 ml or 3 ml H_2O respectively to give stock solutions of 50 mg ml^{-1}. 10 μl of stock solution was added to 500 ml rye A media after autoclaving to give media of 10 μg ml^{-1}. Stock was stored at 4°C for up to 6 months.

1.10 Acriflavine, blasticidin and phosphorous acid

Stocks of these chemicals were made as described for streptomycin in section1.5.

2 RG57 genotypes

RFLP patterns of RG57 fingerprints (RF-)

Table A2.1 RG57 fingerprint patterns (RF-).

RF fingerprint	Fingerprint pattern
RF-01	100010000100110000111011
RF-02	110010000100110100011011
RF-03	110010000100110100111011
RF-05	100010000100110100011011
RF-06	100010001100110100011011
RF-08	100010000100110100011011
RF-09	100011001100110100011011
RF-11	100010001100110100011011
RF-12	100010001100110100001101 1
RF-13	100010001100110000011101 1
RF-18	101011101100110100011001 1
RF-22	100011001100110100111101 1
RF-24	100011000100110100111001 1
RF-26	111011101100110100111101 1
RF-27	111011101100110110111101 1
RF-28	111011001100110100111101 1
RF-30	111010101100110100111101 1
RF-31	111011101100110100011101 1
RF-32	111011111100110100111101 1
RF-33	111010111100110100111101 1
RF-34	111011100100110100111101 1
RF-35	111011110100110100111101 1
RF-36	111011100100110100110101 1
RF-37	101011101100110100111101 1
RF-39	101011111100110100111101 1
RF-40	111010100100110100111101 1
RF-41	111010000100110100111101 1
RF-42	111010100100110100011101 1
RF-45	101010110100110100111101 1
RF-48	101010001100110100111101 1
RF-49	111010110100110100011101 1
RF-50	101010110100110100011101 1
RF-52	101010110100110100011001 1
RF-63	111010100100110000111101 1
RF-64	111010100100110100011001 1
RF-65	101010100100110100111101 1
RF-67	111010110000110100011101 1

RG57 bands present (1) or absent (0) in each RF- genotype after Goodwin *et al.* (1992a)

3 AFLP bands and genotypes

Approximate size of polymorphic AFLP bands used in the study as determined in section 2.4.3.7. Mobility of fragments is determined by both size and sequence so sizes are approximate.

Table A3.1 Size of all polymorphic AFLP bands (base-pairs)

E19-M16		E19-M40		E19-M14		E54-M16		E11-M16		E54-M14	
Band	Size	Band	Size	Band	Size	Band	Size	Band	Size	Band	Size
1	117	1	119	5	149	2	155	12	145	10	216
2	132	2	128	6	150	3	192	16	156	15	253
9	148	6	138	7	165	6	197	17	160	19	311
10	151	7	140	14	247	8	311	25	205	25	396
13	157	12	149	17	281	9	350	42	285	26	403
15	160	16	154	18	283	11	386			29	525
21	196	17	161	21	377	13	416			30	529
25	207	21	165			14	425				
29	227	23	174			16	428				
34	235	24	177								
38	254	26	187								
42	292	27	193								
45	315	29	198								
46	325	34	232								
47	327	35	235								
51	346	38	260								
52	352	39	262								
53	354	40	277								
64	402	43	303								
66	406	45	330								
68	429	47	336								
73	490	49	351								
		54	431								
		55	439								
		57	444								
		58	523								
		64	598								

TableA3.2. Multilocus genotypes of isolates designated AF1-AF43 in chapter 3 (see Table 2.1)

AF-genotype	E19-M16	E19-M40
1	11111011110101100NNN	11111011000001110101111NNNNN
2	11111011110101100NNN	11111011000001110100111NNNNN
3	11111011110101100NNN	11111010000001110101111NNNNN
4	11111011110101100NNN	11111010000001110100111NNNNN
5	11111011010111100NNN	11111011000001110100111NNNNN
6	11011011110100100NNN	11111010000001110100111NNNNN
7	11011011110101100NNN	11111110000001110101111NNNNN
8	11011011110111100NNN	11111110000011110101111NNNNN
9	11111011110111100NNN	01101011000001110001111NNNNN
10	11011011111111100NNN	11011111000001100101011NNNNN
11	11011011111111010NNN	11011111000001100101011NNNNN
12	11011011111111100NNN	11011111000001100100011NNNNN
13	11011011110111100NNN	11011111000001100100011NNNNN
14	11111011111111100NNN	11011111000001110100011NNNNN
15	11011011110111100NNN	11001110000001100000101NNNNN
16	11011011110111100NNN	11011101000001110010111NNNNN
17	11011011110111100NNN	11011101000001110011111NNNNN
18	11011011110111100NNN	11011100000001110010111NNNNN
19	11011011111111100NNN	11011100000001110011111NNNNN
20	11011011110111100NNN	11011100000001110011111NNNNN
21	11111011110111100NNN	11011100000001110011111NNNNN
22	11011011110111100NNN	11011100000001100010111NNNNN
23	11011011110111100NNN	11011100000001100011111NNNNN
24	11011011110111100NNN	11111101000011110011110NNNNN
25	11111011110111100NNN	11111101000011110011110NNNNN
26	11111011110111100NNN	11111101000011010011110NNNNN
27	11011011110111100NNN	11111101000011110010100NNNNN
28	11111011110111100NNN	11111101000011110010100NNNNN
29	11011011110111100NNN	11111100000011110010100NNNNN
30	11011011110111100NNN	11111100000011110011100NNNNN
31	11011011110111100NNN	11111100000011100011100NNNNN
32	11111011110111100NNN	11111110000000100011100NNNNN
33	11111011111111100NNN	11111110000000100011100NNNNN
34	11011011110111100NNN	11111110000000100011100NNNNN
35	11111011110111100NNN	11111110000000100010100NNNNN
36	11011011110111100NNN	11111110000000100010100NNNNN
37	11011010111011100NNN	11111001000011100100011NNNNN
38	11111011110110100NNN	11111100000011100000001NNNNN
39	11111011111110100NNN	11111000000011100000001NNNNN
40	11111011110110100NNN	11111000000011100000001NNNNN
41	11111011110111100NNN	11001011000001100000001NNNNN
42	11110011110111100NNN	00001011000001100000001NNNNN
43	11111010010111100NNN	11011101000010100001000NNNNN

Presence (1) and absence (0) of a band at a locus. N: Locus unable to be scored
E19-M16 bands: 1, 2, 9, 10, 13, 15, 21, 25, 29, 34, 38, 42, 45, 46, 47, 51, 52, 53, 64, 66, 68.
E19-M40 bands: 1, 2, 6, 7, 12, 16, 17, 21, 23, 24, 26, 27, 29, 34, 35, 38, 39, 40, 43, 45, 47, 49, 54, 55, 57, 58, 64

Table A3.3. AFLP fingerprints of the 40 isolates of RG57 genotype RF-39 at all 17 polymorphic loci. Numbers refer to the numbering of isolates on the tree (Figure 3.2.7).

Isolate	Bangor Code	E19-M16	E19-M40
1	97.43.1	10011001110111111111111	11111100010111010011111011
2	97.248.5	10011001110111111111111	11111100010111010011111011
3	97.247.4	10011001110111111111110	11111100010111010011111011
4	97.250.35	10011001110111111111110	11111100010111010011111011
5	97.282.1	10011001110111111111110	11111100010111010011111011
6	97.250.23	10011001110111111111110	11111100010111010011111011
7	97.46.1	10011001110111111111110	11111100010111010011101011
8	97.146.1	10011001110111111111110	11111100010111010011101011
9	97.147.1	10011001110111111111110	11111100010111010011101011
10	97.241.1	10011001110111111111110	11111100010111010011101011
11	97.250.34	10011001110111111111110	11111100010111010011101011
12	97.250.37	10011001110111111111110	11111100010111010011101011
13	97.303.1	10011001110111111111110	11111100010111010011101011
14	97.351.1	10011001110111111111111	11111100010111010011101011
15	97.250.38	10011001110111111111111	11111100010111010011101011
16	97.250.28	10011001110111111111111	11111100010111010011101011
17	97.250.26	10011001110111111111111	11111100010111010011101011
18	97.250.39	10011001110111111111111	11111100010111010011101011
19	97.250.16	10011001110111111111111	11111100010111010011101011
20	97.205.11	10011001110111111111111	11111100010111010011101011
21	97.353.1	10011001110111111111111	11111100010111010011101011
22	97.250.33	10111001110111111111111	11111100010111010011101011
23	97.250.18	10011001110111111111111	11111100010111010011101011
24	97.48.1	10111001110111111111110	11111100010111010011101011
25	97.250.25	10111001110111111111110	11111100010111010011111011
26	97.52.4	10111001110111111111110	11111100010111010011101111
27	97.267.1	10111001110111111111110	11111100010111010011101111
28	97.82.3	10111001110111111111111	11111100010111010011101111
29	97.81.1	10111001110111111111110	11111100010111010011111111
30	97.361.3	10011001110111111111110	11111100010111010011111011
31	97.325.5	10011001110111111111110	11111100010111010011111011
32	97.347.1	10011001110111111111110	11111100010111010011101011
33	97.356.1	10111001110111111111110	11111100010111010011101011
34	97.250.22	10011001110111111111110	11111100010111010011101011
35	97.250.9	10011001110011111111110	11111100010111010011101011
36	97.250.40	10011001110011111111110	11111100010111010011111011
37	97.249.5	10011001110111111111111	11111100010111010011101010
38	97.283.10	11011001110111111111110	11111100010111010011101011
39	97.295.1	11011001110111111111111	11111100010111010011101010
40	97.312.1	10011001110011110111110	11011111010011100101011011

Presence (1) and absence (0) of a band at a locus.
E19-M16 bands: 1, 2, 9, 10, 13, 15, 21, 25, 29, 34, 38, 42, 45, 46, 47, 51, 52, 53, 64, 66, 68, 73.
E19-M40 bands: 1, 2, 6, 7, 12, 16, 17, 21, 23, 24, 26,, 29, 34, 35, 38, 39, 40, 43, 45, 47, 49 54, 55, 57, 58, 64

Table A3.4. Multilocus genotypes of fingerprinted isolates in chapters 4-7 (see Table 2.3). These isolates could not be scored at all loci at isolates in Table A3.2, so could not be assigned a corresponding AF-number.

Isolate	E19-M16	E19-M40
RE-SF	10111111110111110O1NN	1100101000000110000111110NNN
HT7	10111111110111100O1NN	1100101000000110000011110NNN
98.01	11111000110111111NNNN	1111110001001111011011NNNNN
98.02	11111000110111111NNNN	1111110001001111001111NNNNN
E14C2	11011011111111110NNNN	1111101101000110000101NNNNN
N18	11001011110110110O001	1111100001000111000111NNNN
W1	11001011110111110O011	1101011010110011110111NNNN
Y1	11001011110110110O011	1101011010110111110111NNnN
99.03	11111101010111NNNNNNNN	1111111000001111NNNNNNNNNN
99.02	11111101010111NNNNNNNN	1111111000001111NNNNNNNNNN
12SS5	10111111110111110O1NN	1100101000000110000101110NNN
24.1.1 (A)	11111001100111110O111	1111110001000111010101NNNNN
34.1.2 (B)	11111001111110110O111	1111110001000111010001NNNNN
87.205.4	11111100110111110NNNN	1111100001001111000111NNNNN
95.161.5	11111100111110110111N	1110010000001111000101011NN
96.127.15	11011001111011110NNNN	1101111000000110010011ON1NN
96.102.6	11011111110111110011N	1100111000000110000101011111
96.93.10	1101100111N111110NNNN	1111111101010110010011NNNN
96.93.11	10110011110111110NNNN	1101111000000110010001ON1NN
96.93.14	1101100111N111110NNNN	1111111101010110010011NNNN
97.123.1	11011011110010110O0NN	1110110000001111000100NNNNN
96.89.41 / Sr	11111011110001110ONNN	1111101000000111010011011111
97.185.5	11111101111111110O1NN	1110100000001111000101NN0NN
98.64.1	11111100111111110NNNN	1111100001001111000101NNNNN
98.75.13	11111010111111110NNNN	1111100001001111000101NNNNN
98.70.2	11111100111111110NNNN	1111100001001111000101NNNNN
98.74.7	11111100110111110NNNN	1111100001001111000101NNNNN
98.57.9	11111100111111111NNNN	1111100001001111000101NNNNN
98.88.8	11111100110111110NNNN	1111100001001111000101NNNNN

Presence (1) and absence (0) of a band at a locus. N: Locus unable to be scored.
E19-M16 bands: 1, 2, 9, 10, 13, 15, 21, 25, 29, 34, 38, 42, 45, 46, 47, 51, 52, 53, 64, 66, 68.
E19-M40 bands: 1, 2, 6, 7, 12, 16, 17, 21, 23, 24, 26, 27, 29, 34, 35, 38, 39, 40, 43, 45, 47, 49, 54, 55, 57, 58, 64

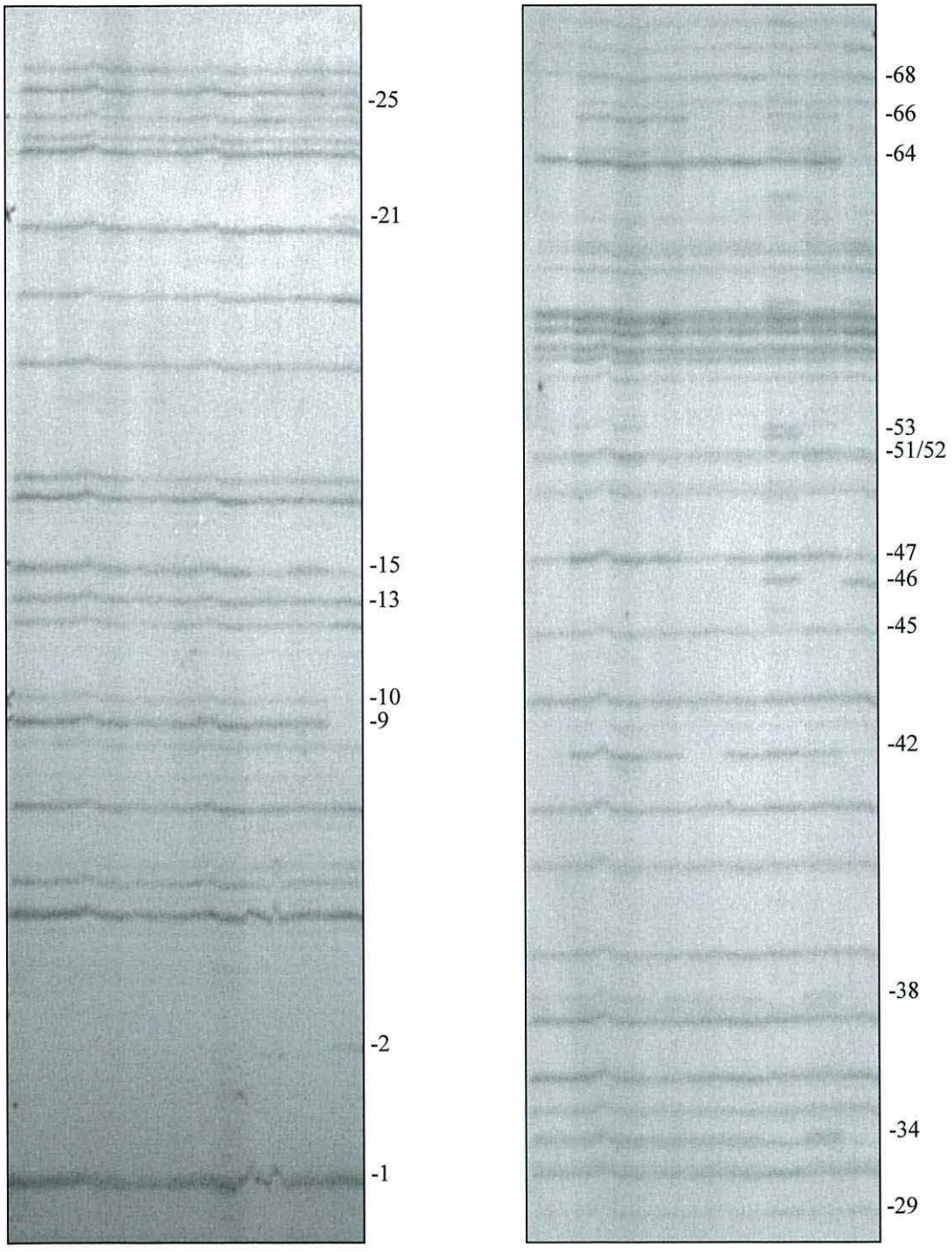


Figure A3.1. Variable AFLP bands generated with primers E19-M16. Band numbers correspond to those in Table A3.1 where approximate size of each band is listed.

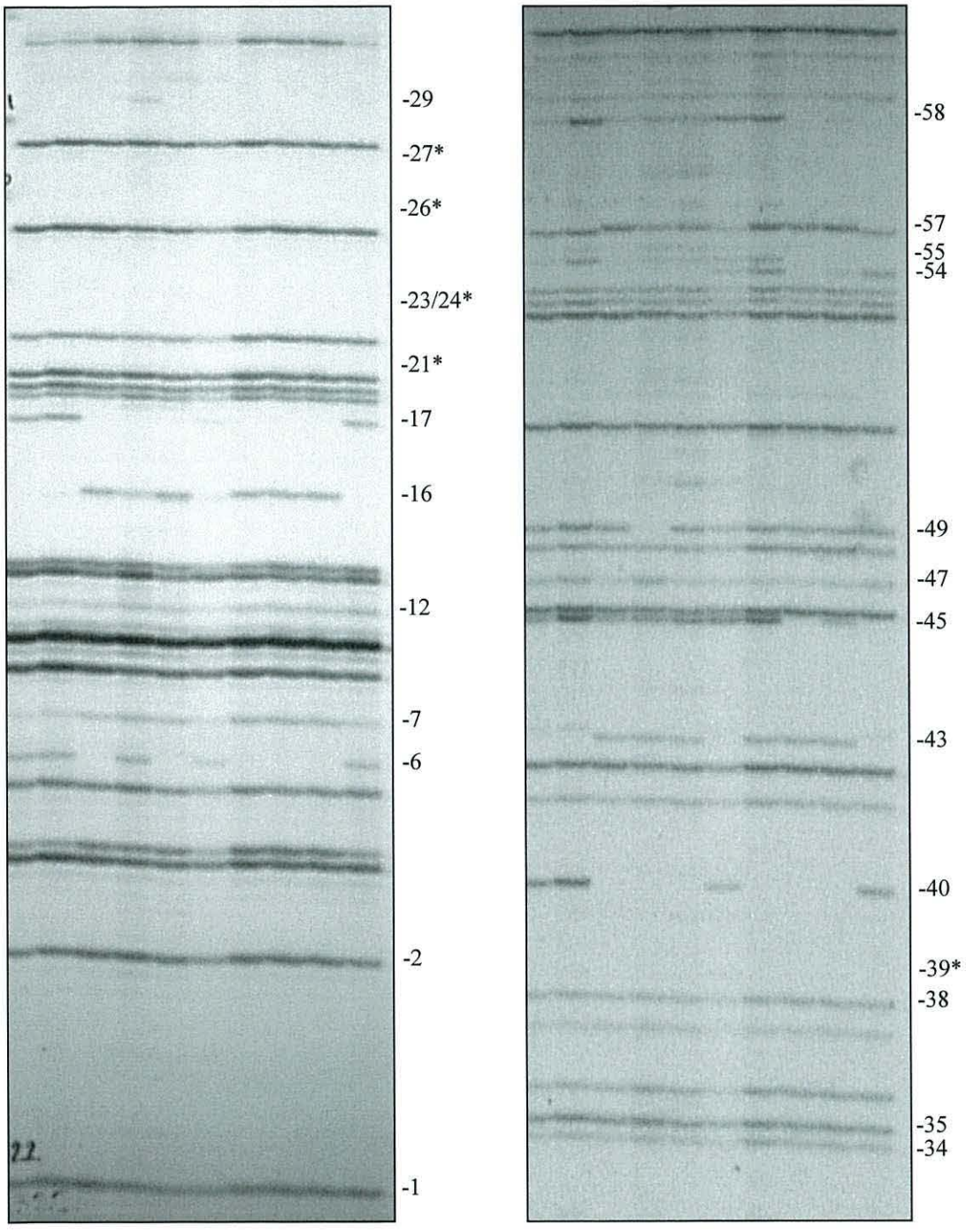


Figure A3.2. Variable AFLP bands generated with primers E19-M40. Band numbers correspond to those in Table A3.1 where approximate size of each band is listed. * Indicates approximate position of band not present in these isolates.

Table A3.5. Further AFLP fingerprints of the 58 isolates (chapter 3) used for initial screening of AFLP primers. These data were combined with data obtained with E19-M16 and E19-M40 (Table A3.2) and used to draw the tree with 58 isolates (Figure 3.2.6) , using 57 AFLP loci.

Isolate	E19-M40	E19-M14	E54-M16	E11-M16	E54-M14
96.105.1	10101	1000001	111110111	01011	1111110
96.110.11	10111	1010001	111000011	01011	1011110
96.110.12	10001	1001101	101100000	00011	0010100
96.148.1	10101	1010001	111100001	01011	1111110
96.127.15	01110	0100110	101100110	11000	1111110
96.104.2	10101	0110100	101100110	11010	1111110
96.110.21	01111	1010011	111110111	01011	1110110
96.154.20	00100	1010001	111000001	01011	1010011
96.126.23	11111	1111100	111000000	00011	0101110
96.154.23	00101	0100100	101110010	11000	1111110
96.154.24	00101	0100100	101100100	11000	1111110
96.110.34	11111	1001100	011100011	01000	1110110
96.110.36	11111	1011100	011000011	00000	1010110
96.126.35	11111	1111101	111110111	01011	0100110
96.110.38	10101	1001100	011000011	01000	1010110
96.110.4	01111	1010011	111110001	01001	1111111
96.126.40	01111	0100100	101100010	11000	1110110
96.126.41	01111	0100100	101110010	11000	1110110
96.110.43	11111	1011100	011110011	01010	1110110
96.126.43	01110	0100100	101100010	11000	1010010
96.110.47	00101	1001100	011010011	01010	1110110
96.110.48	10101	1001100	011100011	01000	1010110
96.110.49	01111	1010011	111110001	01011	1111111
96.101.5	10001	1011101	101000000	00011	0010100
96.51.1	10101	1111100	111000000	00011	0001110
96.127.5	01111	0100100	101110010	11000	1111010
96.52.12	10101	1111100	111000000	00011	0001110
96.110.56	11111	1000001	111100111	01011	1111110
96.102.6	01111	1110100	111111100	00001	0111100
96.130.6	10101	1010001	111010111	01011	1111110
96.102.7	10101	1001101	111000011	01011	1011110
96.54.27	01111	1111100	111100000	00011	0101110
96.127.7	10101	1010001	111110111	01011	1111110
96.54.5	10101	1111100	110000000	00011	0001110
96.96.1	11111	0100110	101110110	11000	1110110
96.95.11	11111	1010001	111110011	01011	1010110
96.91.2	10101	1111100	111000000	00011	0001110
96.95.16	11111	1001100	011110011	01010	1110110
96.95.18	00101	1010001	111000011	01011	1111110
96.95.19	10111	1010001	111000011	01011	1111110
96.93.21	10111	1010001	111000011	01011	1010110
96.93.22	10101	1111100	111000001	00011	0001110
96.91.24	10101	1010001	111000111	01011	1011110
96.91.25	10101	1010001	111000011	01011	1111110
96.93.24	00101	0100100	101000010	11000	1111110
96.90.29	01111	1111100	111100000	00011	0101110
96.95.25	01111	1010001	111100001	01011	1111110
96.89.31	00101	1010001	111000001	01011	1111110
96.90.33	11111	1011101	111110111	01011	1111110
96.89.35	10101	1010001	111000111	01000	1011110
96.89.41	10101	1111100	111000000	00011	0001110
96.89.43	01111	1010001	111110011	01011	1111110
96.89.45	01111	1001011	111110011	01011	1111110
96.95.4	01111	1010001	111100001	01011	1111110
96.93.5	01111	0100100	101000010	11100	1010110
96.93.6	11111	0100100	101000010	11000	1011110
96.95.6	10001	1011101	101110000	00011	0110100
96.93.8	01111	1010011	111110001	01011	1110011

Presence (1) and absence (0) of a band at a locus. E19-M40 bands: 54, 55, 57, 58, 64 (the additional five loci of E19-M40 that could not be scored in all 98 isolates). E19-M14 bands: 5, 6, 7, 14, 17, 18, 2; E54-M16 bands: 2, 3, 6, 8, 9, 11, 13, 14, 16; E11-M16 bands: 12, 16, 17, 25, 42; E54-M14 bands: 10, 15, 19, 25, 26, 29, 30

4 Linear growth rate of isolates in chapter 5.

Table A4.1. Linear growth rates of isolates in chapter 5 on drug-free and drug-amended rye A media. Growth rates are means of four replicates, mm d^{-1}±S.D on each drug concentration (μg ml^{-1}).

Isolate		Metalaxyl			Streptomycin						Chloramphenicol				
	Drug free	10	100	200	0	10	100	200	500	1000	0	10	100	200	400
95.161.5	4.7±0.6	4.0±0.5	3.4±0.2	2.8±0.2	4.7±0.6	4.6±0.4	3.5±0.3	3.3±0.4	3.0±0.4	1.5±0.3	4.7±0.6	3.9±0.2	2.9±0.3	1.5±0.3	1.4±0.3
97.185.5	3.4±0.3	2.8±0.3	3.0±0.4	2.4±0.3	3.4±0.3	0.8±0	0	0	0	0	3.4±0.3	2.9±0.3	2.4±0.2	1.9±0.2	2.0±0.2
97.123.1	4.4±0.2	4.0±0.2	3.5±0.2	2.6±0.2	4.4±0.2	1.8±0.1	0	0	0	0	4.4±0.2	4.9±0.2	3.6±0.2	2.5±0.5	3.1±0.2
98.64.1	3.5±0.1	3.4±0.6	2.3±0.2	1.4±0.2	3.5±0.1	0.9±0.1	0	0	0	0	3.5±0.1	3.7±0.2	2.9±0.3	2.3±0.5	1.9±0.3
98.57.9	3.9±0.5	3.1±0.3	2.8±0.3	2.6±0.4	3.9±0.5	1.4±0.8	0.9±0.1	0	0	0	3.9±0.5	3.4±0.2	2.5±0.3	2.0±0.3	1.8±0.2
98.71.1	3.4±0.1	3.1±0.1	2.6±0.4	1.6±0.2	3.4±0.1	0.6±0.1	0	0	0	0	3.4±0.1	3.0±0.1	2.1±0.1	2.0±0.7	1.9±0.3
98.70.2	3.7±01	3.5±0.1	3.0±0.2	2.2±0.2	3.7±0	0.8±0.1	0	0	0	0	3.7±0	3.7±0	3.0±0.1	2.0±0.3	2.4±0.2
98.74.7	2.6±0.1	2.4±0.1	2.0±0.1	0.7±0.1	2.6±0.1	0.4±0.1	0	0	0	0	2.6±0.1	2.3±0.1	1.3±0.1	1.1±0.4	1.0±0.1
96.104.2	4.4±0.1	1.3±0.1	0.3±0.1	0	4.4±0.1	2.4±0.2	0	0	0	0	4.4±0.1	4.4±0.2	2.0±0.2	0.9±0.3	0
98.75.13	3.4±0.3	3.0±0.2	2.4±0.1	1.5±0.1	3.4±0.3	1.0±0.1	0	0	0	0	3.4±0.3	3.0±0.1	1.8±0.1	1.3±0.5	1.6±0.4
98.88.8	4.0±0.2	3.4±0.4	3.0±0.2	2.2±0.2	4.0±0.2	0.7±0.1	0	0	0	0	4.0±0.2	3.4±0.2	2.5±0.3	1.5±0.1	1.8±0.2
96.93.8	4.1±0.1	0	0	0	4.1±0.1	1.4±0.2	0	0	0	0	4.1±0.1	3.8±0.1	2.7±0.2	C	1.9±0.4
96.93.22	3.7±0.2	0	0	0	3.7±0.2	2.5±0.2	0	0	0	0	3.7±0.2	3.7±0.4	2.9±0.2	2.3±0.2	2.1±0.2
96.89.41	3.8±0.1	0	0	0	3.8±0.1	0.8±0.1	0	0	0	0	3.8±0.1	3.6±0.2	2.3±0.1	0.7±0.1	1.0±0.2
87.205.4	3.1±0.2	3.5±0.1	2.9±0.1	1.3±0.1	3.1±0.2	1.3±0.1	0	0	0	0	3.1±0.2	3.8±0.2	2.6±0.1	0	0
96.127.15	2.5±0.2	1.3±0.3	0.7±0.1	0	2.5±0.2	0.9±0.1	0	0	0	0	2.5±0.2	2.4±0.2	2.8±0.1	2.1±0.2	1.9±0.4
E14C2	4.6±0.1	0	0	0	4.6±0.1	0	0	0	0	0	4.6±0.1	4.9±0.1	4.4±0.1	3.1±0.1	3.4±0.1

Table A4.2. Linear growth rates of transformed isolates from four replicate plates on drug-free and drug-amended media, mm d^{-1}±S.D.

Isolate	Drug concentration (μg ml^{-1})[a]							
		Hygromycin			G418			
	0	5	10	20	5	10	20	50
N18	4.9±0.2	0	0	ND	4.8±0.4	4.8±0.2	4.6±0.1	4.1±0.2
Y1Gr	5.1±0.1	0	0	ND	5.2±0.2	4.8±0.2	4.9±0.4	3.8±0
W1Hr	7.0±0.2	6.1±0.4	7.0±0.2	5.8±0.2	0	0	ND	ND

5 Addresses of suppliers

Address of suppliers of molecular biology reagents, media and hardware

Sigma
Sigma-Aldrich Company Ltd.
Fancy Road
Poole
Dorset
BH12 4QH
England

Promega UK Ltd
Delta House
Chilworth Research Centre
Southampton
SO16 7NS

New England Biolabs, inc.
32 Tozer Road
Beverly, MA 01915

GIBCO BRL
Life Technologies, Inc.
9800 Medical Center Drive
Post Office Box 6482
Rockville, MD 20849

Hoechst Marion Roussel
10236 Marion Park Drive
Kansas City,
MO 64137-1405
USA

BIORAD
Bio-Rad Laboratories Ltd.
Bio-Rad House
Maylands Avenue
Hemel Hempstead
Hertfordshire
HP2 7TD

Eastman Kodak Company
Rochester
New York
14650
USA

Cruachem Ltd.
Glasgow
Scotland
United Kingdom

Novo Biolabs
Nova Enzyme Products Ltd.
Farnham
Surrey
UK

Fisons Ltd
London Road
Holmes Chapel
Cheshire
England

Bibby Sterilin Ltd
Tilling Drive
Stone
Staffordshire
ST15 0SA

Ciba Geigy
Novartis Media Office
Basel
Switzerland

Whatman International ltd
Whatman House
St Leonards Road
20/20 Maidstone
Kent
ME16 0LS

Fisher Scientific Ltd
Whitbrook Way
Stakehill Industrial Park
Middleton
Manchester
M24 2RH

Hybaid Ltd
Action Court
Ashford Road
Ashford
Middlesex
TW15 1XB

Pharmacia Upjohn Ltd
Hatton House
Hunters Road
Weldon North Estate
Corby
Northamptonshire
NN17 5JE

Stratagene
11011
North Torrey Pines Road
La Jolla
CA 92037

References

Akiyama, M. & Nakashima, H. (1996). Molecular cloning of the *acr-2* gene which controls acriflavine sensitivity in *Neurospora crassa*. *Biochimica et Biophysica Acta* **1307,** 187-192.

Al-Kherb, S. M. (1988). The inheritance of host-specific pathogenicity in *Phytophthora infestans*. PhD thesis, University of Wales.

Al-Kherb, S. M., Fininsa, C., Shattock, R. C. & Shaw, D. S. (1995). The inheritance of virulence of *Phytophthora infestans* to potato. *Plant Pathology* **44,** 552-562.

Andrivon, D. (1996). The origin of *Phytophthora infestans* populations present in Europe in the 1840s: a critical review of historical and scientific evidence. *Plant Pathology* **45,** 1027-1035.

Anikina, M. I., Savenkova, L. V. & Dyakov, Y. T. (1997). Self-fertile isolates of *Phytophthora infestans*. (In Russian). *Biologicheskaya* **4,** 414-418.

Ann, P. J. & Peberdy, J. F. (1976). Induced fusion of fungal protoplasts following treatment with polyethylene glycol. *Journal of General Microbiology* **92,** 413-417.

Ann, P. J. & Ko, W. H. (1986). Antibiotic resistant and dependent mutants of cross-inducing species of *Phytophthora*. *Phytopathology* **76,** 1142.

Ann, P. J. & Ko, W. H. (1987). Phenotypic behaviour of progeny from the cross between chloramphenicol-resistant A1 and streptomycin-resistant A2 mating types of *Phytophthora parasitica*. *Phytopathology* **77,** 1711.

Ann, P. J. & Ko, W. H. (1992). Survey of antibiotic-resistance and dependence in *Phytophthora*. *Mycologia* **84,** 82-86.

Bagirova, S. & Dyakov, Y. T. (1993). Reciprocal influence of sexual and asexual compatibility systems in fungi. (In Russian). *Genetics* **29,** 1589.

Bagirova, S. F., Li, A. T. & Dyakov, Y. T. (1998). Mechnaisms of genetic isolation of *Phytophthora infestans* (Mont.) d By. Specific pathogenic forms in sexual and asexual populations. (In Russian). *Mikologiya i Fitopatologiya* **32,** 47-50.

Bailey, A. M. & Coffey, M. D. (1984). A sensitive bioassay for quantification of metalaxyl in soils. *Phytopathology* **74,** 667-669.

Bartnicki-Garcia, S. & Hemmes, D. E. (1974). Some aspects of the form and function of Oomycete spores. In *The Fungal Spore. Form and Function* (ed. D. J. Webber and W. M. Hess), pp. 593-639. Wiley, New York.

Beismann, H., Barker, J. H. A., Karp, A. & Speck, T. (1997). AFLP analysis sheds light on the distribution of two *Salix* species and their hybrid along a natural gradient. *Molecular Ecology* **6,** 989-993.

Bhat, R. G., McBlain, B. A. & Schmitthenner, A. F. (1993). The inheritance of resistance to metalaxyl and to fluorophenylalanine in matings of homothallic *Phytophthora sojae*. *Mycological Research* **97,** 865-870.

Black, W. A. (1952). A genetical basis for the classification of strains of *Phytophthora infestans*. *Proceedings of the Royal Society Edinburgh, B* **65,** 36-51.

Black, W., Mastenbroek, C., Mills, W. R. & Peterson, L. C. (1953). A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. *Euphytica* **2,** 173-179.

Bos, C. J. (1996). Somatic recombination. In *Fungal genetics: Principles and practice* (ed. C. J. Bos), pp. 73-95. Marcel Dekker, New York.

Bourke, A. (1991). Potato blight in Europe in 1845: the scientific controversy. In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 12-24, Cambridge.

Bower, L. A. & Coffey, M. D. (1985). Development of laboratory tolerance to phosphorous acid, fosetyl-Al and metalaxyl in *Phytophthora capsici*. *Canadian Journal of Plant Pathology* **7,** 1-6.

Brasier, C. M. (1992). Evolutionary biology of *Phytophthora* part 1: genetic system, sexuality and the generation of variation. *Annual Review of Phytopathology* **30,** 153-171.

Brasier, C. M., Cooke, D. E. L. & Duncan, J. M. (1999). Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences USA* **96,** 5878-5883.

Brown, T. A. (1990). *Gene cloning, an introduction*. Chapman & Hall, London.

Bruck, R. I., Fry, W. E. & Apple, A. E. (1980). Effect of metalaxyl, an acylalanine fungicide, on developmental stages of *Phytophthora infestans*. *Phytopathology* **70,** 597-601.

Brurberg, M. B., Hannukkala, A. & Hermansen, A. (1999). Genetic variability of *Phytophthora infestans* in Norway and Finland as revealed by mating type and fingerprint probe RG57. *Mycological Research* **103,** 1609-1615.

Burdon, J. J. & Silk, J. (1997). Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology* **87,** 664-669.

Burnett, J. H. (1975). Recombination, segregation and linkage II. Mitotic systems. In *An Introduction to the General Genetics of Fungi* (ed. J. H. Burnett), pp. 73-99. John Wiley & Sons, London.

Butler, E. E. & Bolkan, H. (1973). A medium for heterokaryon formation in *Rhizoctonia solani*. *Phytopathology* **63,** 542-543.

Buxton, E. W. (1962). Parasexual recombination in the banana-wilt *Fusarium*. *Transactions of the British Mycological Society* **45,** 274-279.

Carter, D. A., Archer, S. A., Buck, K. W., Shaw, D. S. & Shattock, R. C. (1990). Restriction fragment length polymorphisms of mitochondrial DNA of *Phytophthora infestans*. *Mycological Research* **94,** 1123-1128.

Carter, D. A., Archer, S. A., Buck, K. W., Shaw, D. S. & Shattock, R. C. (1991). DNA polymorphisms in *Phytophthora infestans*: the UK experience. In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 272-294, Cambridge.

Carter, D. A., Buck, K. W., Archer, S. A., van der Lee, T., Shattock, R. C. & Shaw, D. S. (1999). The detection of nonhybrid, trisomic and triploid offspring in sexual progeny of a mating of *Phytophthora infestans*. *Fungal Genetics and Biology* **26,** 198-208.

Castro, J. (1963). Nuclear condition in zoospores of *Phytophthora infestans*. *Phytopathology* **53,** 24.

Caten, C. E. & Jinks, J. L. (1966). Heterokaryosis: its significance in wild homothallic Ascomycetes and fungi imperfecti. *Transactions of the British Mycological Society* **49,** 81-93.

Caten, C. E. & Jinks, J. L. (1968). Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Journal of Botany* **46,** 329-348.

Caten, C. E. (1971). Single zoospore variation in *Phytophthora infestans* and attenuation of strains in culture. *Transactions of the British Mycological Society* **56,** 1-7.

Chang, T. T. & Ko, W. H. (1990). Resistance to fungicides and antibiotics in *Phytophthora parasitica*: genetic nature and use in hybrid determination. *Phytopathology* **80,** 1414-1421.

Chesnick, J. M., Tuxbury, K., Coleman, A., Burger, G. & Lang, B. F. (1996). Utility of the mitochondrial *nad4l* gene for algal and protistan phylogenetic analysis. *Journal of Phycology* **32,** 452-459.

Clerc, A., Manceau, C. & Nesme, X. (1998). Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmental Microbiology* **64,** 1180-1187.

Clutterbuck, A. J. (1996). Parasexual recombination in fungi. *Journal of Genetics* **75,** 281-286.

Coffey, M. D. & Bower, L. A. (1984). *In vitro* variablility among isolates of eight *Phytophthora* species in response to phosphorous acid. *Phytopathology* **74,** 738-742.

Cohen, Y. & Perl, M. (1973). Stage specificity in streptomycin action against some plant pathogenic Peronosporales. *Phytopathology* **63,** 1172-1180.

Crute, I. R. (1987). Genetic studies with *Bremia lactucae* (lettuce downy mildew). In *Genetics and Plant Pathogenesis* (ed. P. R. Day and G. J. Jellis). Blackwell Scientific Publications, Oxford.

Crute, I. R. & Harrison, J. M. (1988). Studies on the inheritance of resistance to metalaxyl in *Bremia lactucae* and on the stability and fitness of field isolates. *Plant Pathology* **37,** 231-250.

Daggett, S. S. & Gotz, E. (1991). Mating type, metalaxyl sensitivity and allozyme phenotypes of *Phytophthora infestans* from Germany. *Phytophthora Newsletter* **17,** 12-13.

Daly, D. C. (1996). The blight is back. *Natural History* **105,** 31.

Davidse, L. C., Looijen, D., Turkensteen, L. J. & Van der Wal, D. (1981). Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in Dutch potato fields. *Netherlands Journal of Plant Pathology* **87,** 65-68.

Davidse, L. C., van den Berg-Velthuis, G. C. M., Mantel, B. C. & Jespers, A. B. K. (1991). Phenylamides and *Phytophthora*. In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 349-360. University Press, Cambridge.

Day, J. P. & Shattock, R. C. (1997). Aggressiveness and other factors relating to displacement of populations of *Phytophthora infestans* in England and Wales. *European Journal of Plant Pathology* **103,** 379-391.

Debets, A. J. M. (1998). Parasexuality in fungi: mechanisms and significance in wild populations. In *Molecular Variability of Fungal Pathogens* (ed. P. D. Bridge, Y. Couteaudier and J. M. Clarkson), pp 41-52. CAB International, Wallingford.

Denward, T. (1970). Differentiation in *Phytophthora infestans* II. Somatic recombination in vegetative mycelium. *Hereditas* **66,** 35-47.

Drenth, A., Goodwin, S. B., Fry, W. E. & Davidse, L. C. (1993). Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms. *Phytopathology* **83,** 1087-1092.

Drenth, A., Tas, I. C. Q. & Govers, F. (1994). DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in The Netherlands. *European Journal of Plant Pathology* **100,** 97-107.

Drenth, A., Janssen, E. M. & Govers, F. (1995). Formation and survival of oospores of *Phytophthora infestans* under natural conditions. *Plant Pathology* **44,** 86-94.

Duncan, J. M., Cooke, D., Birch, P. & Toth, R. (1998). Molecular variability in sexually reproducing fungal plant pathogens. In *Molecular Variability of Fungal Pathogens* (ed. P. D. Bridge, Y. Couteaudier and J. M. Clarkson), pp 19-39. CAB International, Wallingford.

Dyakov, Y. T. & Kulish, V. B. (1979). Genetic investigation of strains of *Phytophthora infestans* (Mont.) de Bary obtained as a result of paired cultivations of mutant cultures. *Genetika* **15,** 49-56.

El Refai, I. (1990). Somatic hybrids in *Phytophthora infestans*. PhD thesis, University of Tanta, Egypt.

Elliott, C. G. & Glen, B. (1981). Sterol requirement of mating strains of heterothallic *Phytophthoras*. *Journal of General Microbiology* **128,** 859-863.

Ellsworth, D. L., Rittenhouse, D. & Honeycutt, R. L. (1993). Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14,** 214-217.

Ersek, T., English, J. T. & Schoelz, J. E. (1995). Creation of species hybrids of *Phytophthora* with modified host ranges by zoospore fusion. *Phytopathology* **85,** 1343-1347.

Erwin, D. C., Zentmyer, G. A., Galindo, J. & Niederhauser, J. S. (1963). Variation in the genus *Phytophthora*. *Annual Review of Phytopathology* **1,** 375-396.

Erwin, D. C. & Ribeiro, O. K. (1996). *Phytophthora diseases worldwide*. APS Press, St Paul, Minnesota, USA.

Eye, L. L., Sneh, B. & Lockwood, J. L. (1978). Inoculation of soybean seedlings with zoospores of *Phytophthora megasperma* var. *sojae* for pathogenicity race determination. *Phytopathology* **68,** 1769-1773.

Fabritius, A., Shattock, R. C. & Judelson, H. S. (1997). Genetic analysis of metalaxyl insensitivity loci in *Phytophthora infestans* using linked DNA markers. *Phytopathology* **87,** 1034-1040.

Flor, H. (1956). The complimentary genetic systems in flax and flax rust. *Advances in Genetics* **8,** 29-54.

Forbes, G. A., Goodwin, S. B., Drenth, A., Oyarzun, P., Ordonez, M. E. & Fry, W. E. (1998). A global marker database for *Phytophthora infestans*. *Plant Disease* **82,** 811-818.

Förster, H., Coffey, M. D., Elwood, H. & Sogin, M. L. (1990). Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. *Mycologia* **82,** 306-312.

Förster, H. & Coffey, M. D. (1990). Mating behaviour of *Phytophthora parasitica*: evidence for sexual recombination in oospores using DNA restriction fragment length polymorphisms as genetic markers. *Experimental Mycology* **14,** 351-359.

Fry, W. E. & Spielman, L. J. (1991). Population biology. In *Phytophthora infestans, the cause of late blight of potato* (ed. D. S. Ingram and P. H. Williams), pp 171-193. Academic Press, London.

Fry, W. E., Goodwin, S. B., Matuszak, J. M., Spielman, L. J., Milgroom, M. G. & Drenth, A. (1992). Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annual Review of Phytopathology* **30,** 107-129.

Fry, W. E., Goodwin, S. B., Dyer, A. T., Matuszak, J. M., Drenth, A., Tooley, P. W., Sujkowski, L. S., Koh, Y. J., Cohen, B. A., Spielman, L. J., Deahl, K. L., Inglis, D. A. & Sandlan, K. P. (1993). Historical and recent migrations of *Phytophthora infestans*: chronology, pathways and implications. *Plant Disease* **77,** 653-661.

Fry, W. E. & Goodwin, S. B. (1995). Recent migrations of *Phytophthora infestans*. In *Phytophthora infestans150* (ed. L. J. Dowley, E. Bannon, L. R. Cooke, T. Keane and E. O'Sullivan), pp 89-95. Boole Press, Dublin.

Fyfe, A. M. & Shaw, D. S. (1992). An analysis of self-fertility in field isolates of *Phytophthora infestans*. *Mycological Research* **96,** 390-394.

Galindo, J. & Gallegly, M. E. (1960). The nature of sexuality in *Phytophthora infestans*. *Phytopathology* **50,** 123-128.

Galindo, A. J. & Zentmyer, G. A. (1967). Genetical and cytological studies of *Phytophthora* strains pathogenic to pepper plants. *Phytopathology* **57,** 1300-1304.

Gallegly, M. E. & Galindo, J. (1958). Mating types and oospores of *Phytophthora infestans* in nature in Mexico. *Phytopathology* **48,** 274-277.

Gallegly, M. E. (1970). Genetics of *Phytophthora*. *Phytopathology* **60,** 1135-1141.

Garnjobst, L. & Wilson, J. F. (1956). Heterocaryosis and protoplasmic incompatibility in *Nerospora crassa*. *Proceedings of the National Academy of Sciences* **42,** 613-618.

Glass, N. L. & Kuldau, G. A. (1992). Mating type and vegetative incompatibility in filamentous Ascomycetes. *Annual Review of Phytopathology* **30,** 201-224.

Goodwin, S. B. & Fry, W. E. (1988). Mitochondrial polymorphism in *Phytophthora infestans*. *Phytopathology* **78,** 1542.

Goodwin, S. B. (1991). DNA polymorphisms in *Phytophthora infestans*: the Cornell experience. In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 204-219, Cambridge.

Goodwin, S. B. & Fry, W. E. (1991). Global migration of *Phytophthora infestans*. *Phytopathology* **81,** 1191.

Goodwin, S. B., Drenth, A. & Fry, W. E. (1992a). Cloning and genetic analyses of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*. *Current Genetics* **22,** 107-115.

Goodwin, S. B., Spielman, L. J., Matuszak, J. M., Bergeron, S. N. & Fry, W. E. (1992b). Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. *Phytopathology* **82,** 955-961.

Goodwin, S. B., Cohen, B. A., Deahl, K. L. & Fry, W. E. (1994). Migration from northern Mexico as the probable cause of recent genetic changes in populations of *Phytophthora infestans* in the United States and Canada. *Phytopathology* **84,** 553-558.

Goodwin, S. B. & Fry, W. E. (1994). Genetic analyses of interspecific hybrids between *Phytophthora infestans* and *Phytophthora mirabilis*. *Experimental Mycology* **18,** 20-32.

Goodwin, S. B., Sujkowski, L. S. & Fry, W. E. (1995). Rapid evolution of pathogenicity within clonal lineages of the potato late blight disease fungus. *Phytopathology* **85,** 669-676.

Goodwin, S. B. (1997). The population genetics of *Phytophthora*. *Phytopathology* **87,** 462-473.

Goodwin, S. B. & Drenth, A. (1997). Origin of the A2 mating type of *Phytophthora infestans* outside Mexico. *Phytopathology* **87,** 992-99.

Goodwin, S. B., Smart, C. D., Sandrock, R. W., Deahl, K. L., Punja, Z. K. & Fry, W. E. (1998). Genetic change within populations of *Phytophthora infestans* in the United states and Canada during 1994 to 1996: role of migration and recombination. *Phytopathology* **88,** 939-949.

Graham, K. M., Dionne, L. A. & Hodgson, W. A. (1961). Mutability of *Phytophthora infestans* on blight-resistant selections of potato and tomato. *Phytopathology* **51,** 264-265.

Griffith, G. W. & Shaw, D. S. (1998). Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. *Applied and Environmental Microbiology* **64,** 4007-4014.

Gu, Y. H. & Ko, W. H. (1998). Occurrence of a parasexual cycle following the transfer of isolated nuclei into protoplasts of *Phytophthora parasitica*. *Current Genetics* **134,** 120-123.

Gunderson, H., Elwood, H., Ingold, A., Kindle, K. & Sogin, M. L. (1987). Phylogenetic relationships between chlorophytes, chrysophytes and oomycetes. *Proceedings of the National Academy of Sciences, USA* **84,** 5823-5827.

Hammond, S. M. & Lambert, P. A. (1978). *Antibiotics and Microbial Action*. Camelott Press, Southampton.

Harrison, B. J. (1991). The genetics of *Phytophthora infestans*. PhD thesis, University of Wales.

Hawksworth, D. L., Ainsworth, G. C. & James, P. W. (1995). *Ainsworth and Bisby's Dictionary of the Fungi*. CAB International, Wallingford.

Hendrix, J. W. (1970). Sterols in growth and reproduction of fungi. *Annual Review of Phytopathology* **8,** 111-123.

Hickman, C. J. & Ho, H. H. (1966). Behaviour of zoospores in plant pathogenic phycomycetes. *Annual Review of Phytopathology* **4,** 195-220.

Hiddema, J. & Kole, A. P. (1954). Enkle waarnemingen over versmelten van zoosporen bij *Phytophthora infestans* (Mont.) de Bary. *Plantenziekten* **60,** 138-139.

Hohl, H. R. & Iselin, K. (1984). Strains of *Phytophthora infestans* from Switzerland with A2 mating type behaviour. *Transactions of the British Mycological Society* **83,** 529-531.

Hulbert, S. H. & Michelmore, R. W. (1988). DNA restriction fragment length polymorphism and somatic variation in the lettuce downy mildew fungus, *Bremia lactucae*. *Molecular Plant-Microbe Interactions* **1,** 17-24.

Jeffreys, A. J., Wilson, V. & Thein, S. L. (1985). Hypervariable "minisatellite" regions in human DNA. *Nature* **316,** 76-79.

Jinks, J. L., Caten, C. E., Simchen, G. & Croft, J. H. (1966). Heterokaryon incompatibility and variation in wild populations of *Aspergillus nidulans*. *Heredity* **21,** 227-239.

Judelson, H. S. (1991). Transformation of the Oomycete pathogen, *Phytophthora infestans*. *Molecular Plant-Microbe Interactions* **4,** 602-607.

Judelson, H. S., Spielman, L. J. & Shattock, R. C. (1995). Genetic mapping and non-Mendelian segregation of mating type loci in the Oomycete, *Phytophthora infestans*. *Genetics* **141,** 503-512.

Judelson, H. S. (1996). Genetic and physical variability at the mating type locus of the Oomycete, *Phytophthora infestans*. *Genetics* **144,** 1005-1013.

Judelson, H. S. (1997a). The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. *Fungal Genetics and Biology* **22,** 65-76.

Judelson, H. S. (1997b). Expression and inheritance of sexual preference and selfing potential in *Phytophthora infestans*. *Fungal Genetics and Biology* **21,** 188-197.

Judelson, H. S. & Yang, G. (1998). Recombination pathways in *Phytophthora infestans*: polyploidy resulting from aberrant sexual development and zoospore-mediated heterokaryosis. *Mycological Research* **102,** 1245-1253.

Judelson, H. S. & Roberts, S. (1999). Multiple loci determining insensitivity to phenylamide fungicides in *Phytophthora infestans*. *Phytopathology* **89,** 754-760.

Kamoun, S., van der Lee, T., van den Berg-Velthuis, G., de Groot, K. E. & Govers, F. (1998). Loss of production of the elicitor protein INF1 in the clonal lineage US-1 of *Phytophthora infestans*. *Phytopathology* **88,** 1315-1323.

Kavanagh, K. & Whittaker, P. H. (1996). Application of protoplast fusion to the nonconventional yeast. *Enzyme and Microbial Technology* **18,** 45-51.

Kernodle, S. P., Cannon, R. E. & Scandalios, J. G. (1993). Concentration of primer and template qualitatively affects products in random-amplified polymorphic DNA PCR. *Biotechniques* **14,** 362-364.

Khaki, I. A. & Shaw, D. S. (1974). The inheritance of drug resistance and compatibility type in *Phytophthora drechsleri*. *Genetics Research* **23,** 75-86.

Ko, W. H. (1978). Heterothallic *Phytophthora*: evidence for hormonal regulation of sexual reproduction. *Journal of General Microbiology* **107,** 15-18.

Ko, W. H. (1981). Reversible change of mating type in *Phytophthora parasitica*. *Journal of General Microbiology* **125,** 451-454.

Ko, W. H. (1982). Reassessment of the essentiallity of sterols for sexual reproduction in *Phytophthora*. *Phytopathology* **72,** 995.

Ko, W. H. (1988). Hormonal heterothallism and homothallism in *Phytophthora*. *Annual Review of Phytopathology* **26,** 57-73.

Ko, W. H. (1994). An alternative possible origin of the A2 mating type of *Phytophthora infestans* outside Mexico. *Phytopathology* **84,** 1224-1227.

Kohli, Y. & Kohn, L. M. (1998). Random association among alleles in clonal populations of *Sclerotiorum*. *Fungal Genetics and Biology* **23,** 139-149.

Kuhn, D. N. (1991). Parasexuality in *Phytophthora*? In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 242-255. University Press, Cambridge.

Lawrence, G. J., Mayo, G. M. E. & Shepherd, K. W. (1981). Interactions between genes controling pathogenicity in the flax rust fungus. *Phytopathology* **71,** 12-19.

Layton, A. C. & Kuhn, D. N. (1988a). Heterokaryon formation by protoplast fusion of drug-resistant mutants in *Phytophthora megasperma* f.sp. *glycinea*. *Experimental Mycology* **12,** 180-194.

Layton, A. C. & Kuhn, D. N. (1988b). The virulence of interracial heterokaryons of *Phytophthora megasperma* f.sp. *glycinea*. *Phytopathology* **78,** 961-966.

Layton, A. C. & Kuhn, D. N. (1990). In plant formation of heterokaryons of *Phytophthora megasperma* f.sp. *glycinea*. *Phytopathology* **80,** 602-606.

Leach, S. S. & Rich, A. E. (1969). The possible role of parasexuality and cytoplasmic variation in race differentiation in *Phytophthora infestans*. *Phytopathology* **59,** 1360-1365.

Lebreton, L. & Andrivon, D. (1998). French isolates of *Phytophthora infestans* from potato and tomato differ in phenotype and genotype. *European Journal of Plant Pathology* **104,** 583-594.

Lee, T. Y., Mizubuti, E. & Fry, W. E. (1999). Genetics of metalaxyl resistance in *Phytophthora infestans*. *Fungal Genetics and Biology* **26,** 118-130.

Lin, J. J., Kuo, J. & Ma, J. (1996). A PCR-based DNA fingerprinting technique: AFLP for molecular typing of bacteria. *Nucleic Acids Research* **24,** 3649-3650.

Long, M. & Keen, N. T. (1977a). Genetic evidence for diploidy in *Phytophthora megasperma* var. *sojae*. *Phytopathology* **67,** 675-677.

Long, M. & Keen, N. T. (1977b). Evidence for heterokaryosis in *Phytophthora megasperma* var. *sojae*. *Phytopathology* **67,** 670-674.

Lucas, J. A., Greer, G., Oudemans, P. V. & Coffey, M. D. (1990). Fungicide sensitivity in somatic hybrids of *Phytophthora capsici* obtained by protoplast fusion. *Physiological and Molecular Plant Pathology* **36,** 175-187.

Ma, Z. Q. & Lapitan, N. L. V. (1998). A comparison of amplified and restriction fragment length polymorphism in wheat. *Cereal Research Communications* **26,** 7-13.

Majer, D., Mithen, R., Lewis, B. G., Vos, P. & Oliver, R. P. (1996). The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* **100,** 1107-1111.

Malcolmson, J. F. (1970). Vegetative hybridity in *Phytophthora infestans*. *Nature* **225,** 971-972.

Malcolmson, J. F. (1979). Isolation and identification of races of *Phytophthora infestans* from breeders' assessment plots. *Transactions of the British Mycological Society* **73,** 155-156.

Maltese, C. E., Conigliaro, G. & Shaw, D. S. (1995). The development of sporangia of *Phytophthora infestans*. *Mycological Research* **99,** 1175-1181.

Maufrand, R., Archer, S. A., Buck, K. W., Shaw, D. S. & Shattock, R. C. (1995). The use of RAPD markers in genetic studies of *Phytophthora infestans*. In *Phytophthora infestans 150* (ed. L. J. Dowley, E. Bannon, L. R. Cooke, T. Keane and E. O'Sullivan), pp 55-63. Boole Press, Dublin.

Michelmore, R. W. & Sansome, E. R. (1982). Cytological studies of heterothallism and secondary homothallism in *Bremia lactucae*. *Transactions of the British Mycological Society* **79,** 291-297.

Michelmore, R. W. & Ingram, D. S. (1982). Secondary homothallism in *Bremia lactucae*. *Transactions of the British Mycological Society* **78,** 1-9.

Miller, J. S., Hamm, P. B. & Johnson, D. A. (1997). Characterization of the *Phytophthora infestans* population in the Columbia Basin of Oregon and Washington from 1992-1995. *Phytopathology* **87,** 656-660.

Mills, W. R. (1940). Adaptive parasitism of *Phytophthora infestans*. *Phytopathology* **30,** 17.

Mills, W. R. & Peterson, L. C. (1952). The development of races of *Phytophthora infestans* (Mont.) de Bary on potato hybrids. *Phytopathology* **42,** 26.

Mortimer, A. M., Shaw, D. S. & Sansome, E. R. (1977). Genetical studies of secondary homothallism in *Phytophthora drechsleri*. *Archives of Microbiology* **111,** 255-259.

Mosa, A. A., Kato, M., Sato, N., Kobayashi, K. & Ogoshi, A. (1989). Occurrence of the A2 mating type of *Phytophthora infestans* on potato in Japan. *Annals of the Phytopathological Society of Japan* **55,** 615-620.

Niederhauser, J. S. (1956). The blight, the blighter and the blighted. *Transactions of the New York Academy of Science* **19,** 55-63.

Niederhauser, J. S. (1961). Genetic studies of *Phytophthora infestans* and Solanum species in relation to late-blight resistance in the potato. *Recent Advances in Botany* **1,** 491-497.

Niederhauser, J. S. (1991). *Phytophthora infestans*: the Mexican connection. In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 25-45, Cambridge.

Parmeter, J. R., Snyder, W. C. & Reichle, R. E. (1963). Heterokaryosis and variability in plant pathogenic fungi. *Annual Review of Phytopathology* **1,** 51-76.

Pipe, N. D. & Shaw, D. S. (1997). Telomere-associated restriction fragment length polymorphisms in *Phytophthora infestans*. *Molecular Plant Pathology On-Line,* [http://www.bspp.org.uk/mppol/1997/1124pipe].

Pipe, N. D., Azcoitia, V. & Shaw, D. S. (in press). Self-fertility in *Phytophthora infestans*: heterokaryons segregate several phenotypes. *Mycological Research*.

Pittis, J. E. & Shattock, R. C. (1994). Viability, germination and infection potential of oospores of *Phytophthora infestans*. *Plant Pathology* **43,** 387-396.

Poedinok, N. L. & Dyakov, Y. T. (1981). Observation of vegetative incompatibility in *Phytophthora infestans*. (In Russian). *Mikologiya i Fitopatologiya* **15,** 275-279.

Poedinok, N. L., Dolgova, A. V. & Dyakov, Y. T. (1988). Parasexual process in phytopathogenic fungus *Phytophthora infestans* (Mont.) de Bary. (In Russian). *Genetika* **18,** 1423-1428.

Pontecorvo, G. (1956). The parasexual cycle in fungi. *Annual Review of Microbiology* **10,** 393-400.

Porter, J. R. & Shaw, D. S. (1978). Aggregation of *Phytophthora drechsleri* zoospores; pattern analysis suggests a taxis. *Transactions of the British Mycological Society* **71,** 515-518.

Raeder, U. & Broda, P. (1985). Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1,** 17-20.

Reddick, D. & Mills, W. (1938). Building up virulence in *Phytophthora infestans*. *American Potato Journal* **15,** 29-34.

Rholf, F. (1993). Numerical Taxonomy and Multivariate Analysis System. Exeter Software, New York.

Romero, S. & Erwin, D. C. (1969). Variation in pathogenicity among single-oospore cultures of *Phytophthora infestans*. *Phytopathology* **59,** 1310-1317.

Rooke, D. M. & Shattock, R. C. (1983). Effect of Chloramphenicol and Streptomycin on developmental stages of *Phytophthora infestans*. *Journal of General Microbiology* **129,** 3401-3410.

Rooke, D. M. & Shattock , R. C. (1984). Chloramphenicol resistance in an isolate of *Phytophthora infestans*. *Transactions of the British Mycological Society* **82,** 373-376.

Roper, J. A. (1966). Mechanisms of inheritance 3. The parasexual cycle. In *The Fungi*, vol. II, The fungal organism (ed. G. C. Ainsworth and A. S. Sussman), pp 589-617. Academic Press, London.

Sansome, E. (1963). Meiosis in *Pythium debaryanum* Hesse and its significance in the life-history of the biflagellatae. *Transactions of the British Mycological Society* **46,** 63-72.

Sansome, E. (1966). Meiosis in the sex organs of the Oomycetes. *Chromosomes Today* **1,** 77-83.

Sansome, E. & Brasier, C. M. (1973). Diploidy and chromosomal structural hybridity in *Phytophthora infestans*. *Nature* **241,** 344-345.

Sansome, E. (1977). Polyploidy and induced gametangial formation in British isolates of *Phytophthora infestans*. *Journal of General Microbiology* **99,** 311-316.

Sansome, E. (1980). Reciprocal translocation heterozygosity in heterothallic species of *Phytophthora* and its significance. *Transactions of the British Mycological Society* **74,** 175-185.

Savage, E. J., Clayton, C. W., Hunter, J. W., Brenneman, J. A., Laviola, C. & Gallegly, M. E. (1968). Homothallism, heterothallism and interspecific hybridization in the genus *Phytophthora*. *Phytopathology* **58,** 1004-1021.

Schwin, F. J. & Margot, P. (1991). Control with chemicals. In *Phytophthora infestans, the cause of late blight of potato* (ed. D. S. Ingram and P. H. Williams). Academic Press, London.

Shattock, R. C. & Shaw, D. S. (1975). Mutants of *Phytophthora infestans* resistant to and dependent upon antibiotics. *Transactions of the British Mycological Society* **64,** 29-41.

Shattock, R. C. & Shaw, D. S. (1976). Novel phenotypes of *Phytophthora infestans* from mixed culture of antibiotic resistant mutants. *Transactions of the British Mycological Society* **67,** 201-206.

Shattock, R. C. (1976). Variation in *Phytophthora infestans* on potatoes grown in walk-in polyethylene tunnels. *Annals of Applied Biology* **82,** 227-232.

Shattock, R. C., Janssen, B. D., Whitbread, R. & Shaw, D. S. (1977). An interpretation of the frequencies of host-specific phenotypes of *Phytophthora infestans* in North Wales. *Annals of Applied Biology* **86,** 249-260.

Shattock, R. C., Tooley, P. W. & Fry, W. E. (1986). Genetics of *Phytophthora infestans*: determination of recombination, segregation, and selfing by isozyme analysis. *Phytopathology* **76,** 410-413.

Shattock, R. C. (1988). Studies on the inheritance of resistance to metalaxyl in *Phytophthora infestans*. *Plant Pathology* **37,** 4-11.

Shattock, R. C., Shaw, D. S., Fyfe, A. M., Dunn, J. R., Loney, K. H. & Shattock, J. A. (1990). Phenotypes of *Phytophthora infestans* collected in England and Wales from 1985 to 1988: mating type, response to metalaxyl and isoenzyme analysis. *Plant Pathology.* **39,** 242-248.

Shattock, R. C. & Day, J. P. (1996). Migration and displacement; recombinants and relics: 20 years in the life of potato late-blight *(Phytophthora infestans)*. In *Crop Protection Conference - Pests and Diseases*, Brighton.

Shaw, D. S. & Elliott, C. G. (1968). Streptomycin resistance and morphological variation in *Phytophthora cactorum*. *Journal of General Microbiology* **51,** 75-84.

Shaw, D. S. & Khaki, I. A. (1971). Genetical evidence for diploidy in *Phytophthora*. *Genetics Research* **17,** 165-167.

Shaw, D. S. (1983a). The Peronosporales. A fungal geneticist's nightmare. In *Zoosporic Plant Pathogens - A Modern Perspective* (ed. S. T. Buczacki), pp 85-121. Academic Press, London.

Shaw, D. S. (1983b). The cytogenetics and genetics of *Phytophthora*. In *Phytophthora, its Biology, Taxonomy, Ecology and Pathology* (ed. D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao), pp. 81-94. American Phytopathological Society, New York.

Shaw, D. S., Fyfe, A. M., Hibberd, P. G. & Abdel-Sattar, M. A. (1985). Occurrence of the rare A2 mating type of *Phytophthora infestans* on imported Egyptian potatoes and the production of sexual progeny with A1 mating types from the UK. *Plant Pathology.* **34,** 552-556.

Shaw, D. S. (1987). The breeding system of *Phytophthora infestans*: the role of the A2 mating type. In *Genetics and Plant Pathogenesis* (ed. P. R. Day and G. J. Jellis), pp. 161-174. Blackwell Scientific Publications, Oxford.

Shaw, D. S. (1991). Genetics. In *Phytophthora infestans, the cause of late blight of potato* (ed. D. S. Ingram and P. H. Williams), pp 131-170. Academic Press, London.

Sietsma, J. H., Eveleigh, D. E. & Haskins, R. H. (1969). Cell wall composition and protoplast formation of some Oomycete species. *Biochemica et Biophysica Acta* **184,** 306-317.

Smoot, J. J., Gough, F. J., Lamey, H. A., Eichenmuller, J. J. & Gallegly, M. E. (1958). Production and germination of oospores of *Phytophthora infestans*. *Phytopathology* **48,** 165-171.

Sokal, R. R. & Sneath, P. H. A. (1963). *Principles of Numerical Taxonomy*. Freeman, San Francisco.

Spielman, S. J., McMaster, B. J. & Fry, W. E. (1989). Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. *Theoretical and Applied Genetics* **77,** 832-838.

Spielman, L. J., Sweigard, J. A., Shattock, R. C. & Fry, W. E. (1990). The genetics of *Phytophthora infestans*: segregation of allozyme markers in F2 and backcross progeny and the inheritance of virulence against potato resistance genes *R2* and *R4* in F1 progeny. *Experimental Mycology* **14,** 57-69.

Spielman, L. J. (1991). Isozymes and the population genetics of *Phytophthora infestans*. In *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke), pp. 247-257, Cambridge.

Spielman, L. J., Drenth, A., Davidse, L. C., Sujkowski, L. J., Gu, W., Tooley, P. W. & Fry, W. E. (1991). A second world-wide migration and population displacement of *Phytophthora infestans*? *Plant Pathology* **40,** 422-430.

Stephenson, L. W., Erwin, D. C. & Leary, J. V. (1974). Hyphal anastomosis in *Phytophthora capsici*. *Phytopathology* **64,** 149-150.

Sujkowski, L. S. (1990). Effect of some factors on detectability of virulence genes in *Phytophthora infestans* (Mont.) de Bary. *Journal of Phytopathology* **129,** 9-18.

Sujkowski, L. S., Goodwin, S. B., Dyer, A. T. & Fry, W. E. (1994). Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. *Phytopathology* **84,** 201-207.

Sweigard, J. A., Spielman, L. J., Tooley, P. W., Shattock, R. C. & Fry, W. E. (1987). Genetic control of virulence in *Phytophthora infestans*. *Phytopathology* **77,** 122.

Tamura, K., Kimura, K. & Yamaguchi, I. (1995). Blasticidin S deaminase gene (*BSD*): a new selection marker gene for transformation of *Arabidopsis thaliana* and *Nicotiana tabacum*. *Bioscience, Biotechnology and Biochemistry* **59,** 2336-2338.

Tantius, P. H., Fyfe, A. M., Shaw, D. S., Shattock, R. C. (1986). Occurrence of the A2 mating type and self-fertile isolates of *Phytophthora infestans* in England and Wales. *Plant Pathology*. **35,** 578-581.

Tinline, R. H. & MacNeill, B. H. (1969). Parasexuality in plant pathogenic fungi. *Annual Review of Plant Pathology* **7,** 147-169.

Tooley, P. W., Fry, W. E. & Villarreal-Gonzalez, M. J. (1985). Isozyme characteristics of sexual and asexual *Phytophthora infestans* populations. *Journal of Heredity* **76,** 431-435.

Tooley, P. W. & Therrien, C. D. (1987). Cytophotometric determination of the nuclear DNA content of 23 Mexican and 18 non-Mexican isolates of *Phytophthora infestans*. *Experimental Mycology* **11,** 19-26.

Tooley, P. W., Hewings, A. D. & Falkenstein, K. F. (1989). Detection of double-stranded RNA in *Phytophthora infestans*. *Phytopathology* **79,** 470-474.

Tyler, B. M., Förster, H. & Coffey, M. D. (1995). Inheritance of avirulence factors and restriction fragment length polymorphism markers in outcrosses of the Oomycete pathogen *Phytophthora sojae*. *Molecular Plant-Microbe Interactions* **8,** 515-523.

Umaerus, V., Umaerus, M., Erjefalt, L. & Nilsson, B. A. (1983). Control of *Phytophthora* by host resistance: problems and progress. In *Phytophthora, its Biology, Taxonomy, Ecology and Pathology*

(ed. D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao), pp. 315-326. American Phytopathological Society, New York.

van der Lee, T., de Witte, I., Drenth, A., Alfonso, C. & Govers, F. (1997). AFLP linkage map of the Oomycete *Phytophthora infestans*. *Fungal Genetics and Biology* **21,** 278-291.

van West, P., de Jong, A. J., Judelson, H. S., Emons, A. M. C. & Govers, F. (1998). The *ipi*O gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. *Fungal Genetics and Biology* **23,** 126-138.

van West, P., Kamoun, S., van't Klooster, J. W. & Govers, F. (1999). Internuclear gene silencing in *Phytophthora infestans*. *Molecular Cell* **3,** 339-348.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van der Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23,** 4407-4414.

Whittaker, S. L., Shattock, R. C. & Shaw, D. S. (1991a). Variation in DNA content of nuclei of *Phytophthora infestans* as measured by a microflurimetric method using the flourochrome DAPI. *Mycological Research* **95,** 602-610.

Whittaker, S. L., Shattock, R. C. & Shaw, D. S. (1991b). Inheritance of DNA contents in sexual progenies of *Phytophthora infestans*. *Mycological Research* **95,** 1094-1100.

Whittaker, S. L., Assinder, S. J. & Shaw, D. S. (1994). Inheritance of mitochondrial DNA in *Phytophthora infestans*. *Mycological Research* **98,** 569-575.

Whittaker, S. L., Assinder, S. J. & Shaw, D. S. (1996). Inheritance of streptomycin and chloramphenicol resistance in *Phytophthora infestans*: evidence for co-segregation of mitochondrial DNA and streptomycin resistance. *Mycological Research* **100,** 87-92.

Wilde, P. (1961). Ein beitrag zur kenntnis der variabilitat von *Phytophthora infestans* (Mont.) de Bary. *Archive fur Microbiology* **40,** 163-195.

Williams, J. G. K., Kubelik, A. R., Livak, k. J., Rafalski, J. A. & Tingey, S. V. (1990). DNA polymorphisms revealed by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18,** 6531-6535.

Wilson, J. F., Garnjobst, L. & Tatum, E. L. (1961). Heterokaryon incompatibility in *Neurospora crassa* - micro-injection studies. *American Journal of Botany* **48,** 299-305.

Wolfe, M. S., Barrett, J. A., Shattock, R. C., Shaw, D. S. & Whitbread, R. (1976). Phenotype-phenotype analysis: field application of the gene-for-gene hypothesis in host-pathogen relations. *Annals of Applied Biology* **82,** 369-374.

Young, W. P., Schupp, J. M. & Keim, P. (1999). DNA methylation and AFLP marker distribution in the soybean genome. *Theoretical and Applied Genetics* **99,** 785-792.

Zeigler, R. S. (1998). Recombination in *Magnaporthe grisea*. *Annual Review of Phytopathology* **36,** 249-275.

Zheng, X. B. & Ko, W. H. (1996). Continuing variation in successive asexual generations of *Phytophthora cinnamomi* following sexual reproduction. *Canadian Journal of Botany* **74,** 1181-1185.

Zwankhuizen, M. J., van der Lee, T., Govers, F. & Zadoks, J. C. (in press). Population studies on *Phytophthora infestans* in southern Flevoland and adjacent areas (The Netherlands). 1. Genotypes and inoculum sources. *European Journal of Plant Pathology* .