DOCTOR OF PHILOSOPHY

Variation in aggressiveness and fungicide sensitivity in sexual and asexual progeny of Phytophthora infestans

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Award date:
2004

Awarding institution:
University of Wales, Bangor

Variation in aggressiveness and fungicide sensitivity in sexual and asexual progeny of *Phytophthora infestans*

A thesis submitted for the degree of

Philosophie Doctor

Of the University of Wales

By

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Abstract

Three matings of *Phytophthora infestans* yielded 190, 170 and 185 single oospore progeny (SOP), for cross #1, 2 and 3, respectively. Each cross had a common UK A2 metalaxyl-sensitive (RF 040) parent. Cross #1 A1 parent was a UK (RF 032) metalaxyl-sensitive isolate. Cross #2 A1 parent was a UK (RF 039) metalaxyl resistant isolate. Cross #3 A1 parent was both metalaxyl and propamocarb resistant and of Californian origin. Ratios of A1 to A2 mating type was 132:40, 88:72, and 121:42, for cross #1, 2 and 3, respectively. Self-fertile SOPs totalled eight, four and 16, for cross #1, 2 and 3, respectively, and 22 SOP (10, 6 and 6, cross #1, 2 and 3, respectively) were of undetermined mating type. The morphology of A2 SOPs were observed to be 'lumpy' and could be divided into three categories of lumpiness. Molecular markers, namely RAPDs were used to prove hybridity of SOPs and confirmed with AFLPs. Only 12% of SOPs were non-pathogenic on the most susceptible potato cv. Bintje. No correlation was observed between mating type and any of the aggressiveness components, but there was correlation between latent period and infection frequency, latent period and average lesion expansion and also between sporulation and lesion expansion. On the most susceptible of seven potato cultivars, cv. Home Guard, in cross #1, 11% of the progeny had a latent period shorter than that of the A1 parent and only one SOP had a latent period shorter than the A2 parent. In cross #2, only 8% SOPs had a shorter latent period than either parent. In cross #3, 8% had a shorter latent period than both parents. On cv. Stirling, the most resistant UK potato cultivar used, between 5.5% and 23% SOPs were non-pathogenic and in cross #1, 2 and 3, 6%, 13% and 28% of the SOPs had shorter latent periods than either parent. There were significant ranges in sensitivity of single oospore progeny to four of the five fungicides tested. Cross #1 (A1 MS x A2 MS) produced SOPs that were mainly intermediate. A resistant x sensitive mating (cross #2 & #3) produced all three phenotypes of metalaxyl sensitivity with the majority being intermediate > resistance > sensitive. UK A1 parental isolates (crosses #2&#3) that were metalaxyl-resistant were also resistant (or intermediate) to the other fungicides tested except on azoxystrobin where all isolates were sensitive. Likewise, the metalaxyl - sensitive A1 parent (cross #1) was also sensitive to all the other fungicides. Although the A2 UK parental isolate was sensitive to metalaxyl, it was resistant to cymoxanil and intermediate to propamocarb hydrochloride and fluazinam. Except for azoxystrobin, all crosses produced sensitive, intermediate and resistant single oospore progeny. Sporangial asexual progeny from the parental isolates and from some SOPs were not different from the parental isolates for fungicide sensitivity. However, some zoosporangial progeny differed from their sporangial parents. One cymoxanil sensitive sporangial progeny of the A2 parent gave rise to five very resistant zoosporangial progeny. These aspects of variation are discussed in the context of dynamcs of potato late blight.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

Acknowledgements

I am sincerely grateful to my supervisor, Dr Richard Shattock for his help, patience, encouragement and discussions throughout the project and during the writing of this thesis.

I am also grateful to Dr David Shaw for his assistance and technical support during my time in G47 and for allowing me to use some of his Hungarian potato cultivars for my study. I would like to thank the group in G47 for all the support and the laughs we shared, namely Dr. N. Hardman, Dr. G. E. Harper, Dr. R. Wattier, Louise, Laura, Jadwiga and Debbie. I am mostly grateful to Dr A. Purvis for all the technical support he accorded me in the plant pathology lab at Bangor and even after he had moved to SCRI. I would like to acknowledge Drs Helen Stuart from SCRI and Jenny Vaughan from NIAB for providing me with some of the seed potato used in the study.

I would like to thank parishioners of Our Lady and St James in Bangor for making our stay in Bangor a very pleasant one, especially Canon Bernard Morgan, Mrs Brunhild Tynan and Mrs Roberta Canning. Many thanks to the staff of Index and Littlewoods for all the jokes and laughs we shared. I am also grateful to the African student community in Bangor and to my friends Radek and Rabia and their sons Mubarak, Thomas and Paul for their friendship.

I would like to thank my sponsors Association of Commonwealth Universities for providing financial support to pursue my doctorate and to BSPP for providing conference travel funds. I would also like to thank my employer, University of Swaziland, for assisting me secure this scholarship and for allowing me to go for my PhD.

Last but not least, I must thank my husband Calvin for his patience while I was away pursuing my degree and my sons Jason and Nigel for being with me and helping in every way during my study, guys I would not have done it without your support!

1.0 INTRODUCTION

1.1 Phytophthora infestans

Phytophthora infestans (Mont.) de Bary, is the causal agent of late blight of potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicon* L. syn. *Lycopersicon esculentum* Mill.) and some other solanaceous plants. In Europe, *P. infestans* was first recorded in 1845 in Belgium, but quickly spread throughout Europe and caused severe damage particularly in Ireland, where there was reliance, particularly among the rural poor, on a single crop (potato) as a food source (reviewed by Smart and Fry, 2001). *P. infestans* is a heterothallic pseudofungus that reproduces sexually by means of two mating types (Smoot *et al.*, 1958) designated A1 and A2 (Gallegly and Galindo, 1958) (Figure 1.1). Interaction between hyphae of opposite mating type results in the formation of gametangia, namely paragynous antheridia and oogonia and after fertilisation results in the production of thick-walled oospores (Smoot *et al.,* 1958; Savage *et al.*, 1968). Upon germination, mycelial segregants are either A1 or A2, but sometimes self-fertile phenotypes occur (Al-Kherb, 1988; Shattock, 1988; Fyfe and Shaw, 1992).

Phytophthora infestans is a bisexual diplontic oomycete, i.e. it produces both oogonia (female gametangia) and antheridia (male gametangia) within the same thallus (Galindo and Gallegly, 1960) upon stimulation by pheromones/hormones (Ko, 1978 & 1988). Each mating type is distinguished by its production of a specific pheromone and its response to the pheromone of the opposite type (Ko, 1988). These chemicals induce the attraction of opposite mating types (A2 to A1) vegetative mycelia, followed by differentiation producing both male and female gametangia, in which pre-zygotic meiosis occurs. Nuclear fusion, following passage of an antheridial nucleus into the oogonium, results in diploid oospore with one viable zygotic nucleus, which develops into diploid vegetative mycelia of the A1 or A2 mating type (Brasier and Sansome, 1975; Shaw, 1983; Brasier, 1992). This comes about following germination, first to produce a germ sporangium and then directly to subsequent hyphal development or indirectly after production and release of zoospores (Erwin and Ribeiro, 1996). Although *P. infestans* can form oospores within the same thallus, the opposite mating type is usually required for hormone stimulation to occur, which results in formation of oospores by gametangia of the opposite mating types. An isolate can act as the male or female donor or both, depending on the relative sexuality of the other isolate (Galindo and Gallegly, 1960; Judelson, 1997a).

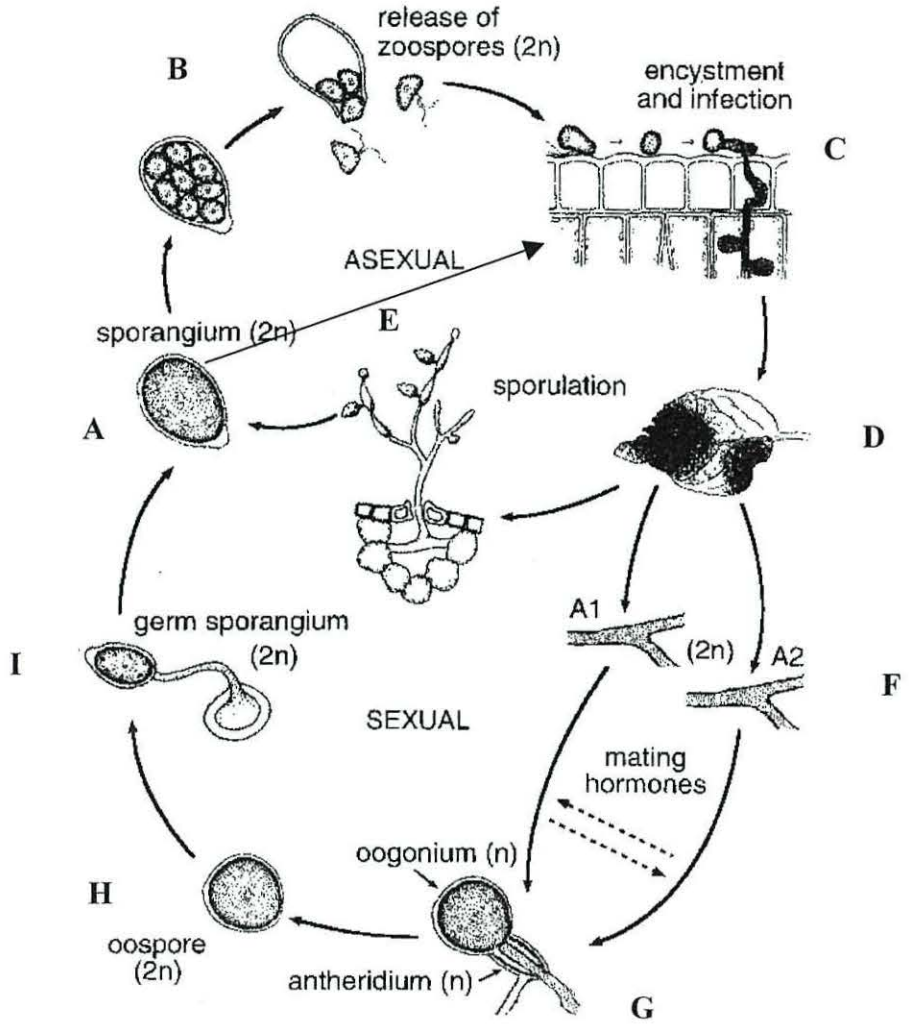


Figure 1.1. Life cycle of *P. infestans* showing the two reproduction cycles; asexual and sexual. A: a diploid multinucleate sporangium may germinate directly to infect a susceptible host or produce uninucleate biflagellate zoospores (B) that encyst (C), germinate and infect a susceptible host. After successful infection (D), sporangiophores bearing sporangia (E) emerge through the stomata at the periphery of the lesion and cause more and more infections. When an A1 and an A2 mycelium grow in close vicinity (F), sexual hormones are released (Ko, 1978 and 1988) which stimulate each isolate to produce sexual gametangia: oogonium (†) and antheridium (°). An amphigynous antheridium is penetrated by the developing oogonium, followed by mitosis, nuclear abortion and meiosis (G). Karyogamy results in a diploid zygotic nucleus followed by development of a thick walled oospore (H), which germinates to produce a germ sporangium (I). Source: Judelson, 1997b.

1.1.1 The discovery and distribution of the A2 mating type

Up until the early 1980s the A2 mating type was thought to be confined to Mexico (Fry *et al.*, 1993). It was detected in Europe for the first time in 1981 (Hohl and Iselin, 1984). Subsequently, the A2 mating type was detected in many countries in Europe: in the UK (Malcolmson, 1985; Shaw *et al.*, 1985; Shaw, 1987; Tantius *et al.*, 1986), Northern Ireland (Cooke *et al.*, 1995), Germany (Rullich and Schober, 1988 as cited by Daggett *et al.*, 1993), Poland (Sujkowski *et al.*, 1994), France (Lebreton and Andrivon, 1998), Sweden (Kadir and Umaerus, 1987), The Netherlands (Frinking *et al.*, 1987; Drenth *et al.*, 1993), and Finland and Norway (Hermansen *et al.*, 2000). The A2 mating type has also been reported from other countries: Japan (Mosa *et al.*, 1989; Therrien *et al.*, 1990; Kato *et al.*, 1998), USA and Canada (Chycoski *et al.*, 1994; Deahl *et al.*, 1990; Dorrance *et al.*, 1999), Egypt (Shaw *et al.*, 1985), China (Fry *et al.*, 1993), and Ecuador (Fry *et al.*, 1993).

Before the A2 mating type was discovered outside Mexico, only the A1 mating type was found around the world: Goodwin *et al.* (1994a) proposed the latter was of a single common clonal lineage, US-1, and believed to have been present for many years, possibly since the 1840s. The US-1 phenotype typically has the dilocus allozyme genotype *Glucose-6-phosphate isomerase* (*Gpi*) 86/100, *Peptidase* (*Pep*) 92/100 (Spielman *et al.*, 1991), was metalaxyl sensitive and contains a 15-band DNA fingerprint (Goodwin *et al.*, 1994a; Goodwin *et al.*, 1995), and a mitochondrial haplotype 1b (Carter *et al.*, 1990).

Although extreme sensitivity to metalaxyl was the ancestral phenotype for the US-1 clonal lineage (Goodwin *et al.*, 1996); there have been reports of US-1, which are resistant to metalaxyl. Koh *et al.* (1994) reported presence of metalaxyl-resistant isolates in the Philippines, where US-1 was the only known lineage. Carter *et al.* (1982) reported presence of metalaxyl resistant isolates in Ireland, which were later shown to have the US-1 genotype (Goodwin *et al.*, 1996). More recently, McLeod *et al.* (2001) reported presence of metalaxyl-resistant isolates from potato in South Africa, even though all isolates were found to be of the US-1 lineage.

The discovery of the A2 mating type in Europe coincided with the appearance of 'new' A1 genotypes. The 'new' genotypes are thought to have originated from an illegal importation of tubers for consumption into Europe from Mexico in 1976 (Niederhauser,

1991; Drenth *et al.*, 1993). The 'new' genotypes appeared to be fitter than those of the 'old' population (US-1 types) in Europe (Spielman *et al.*, 1991; Daggett *et al.*, 1993; Drenth *et al.*, 1994; Day and Shattock, 1997). As a consequence of a separate migration out of Mexico, Goodwin *et al.* (1998) reported an almost total replacement of US-1 within 3 years in the United States by new genotypes US-6, US-7 and US-8, of which the latter two are of the A2 mating type. Similar replacements of US-1 isolates by either new A1 and/or A2 have also been reported in other locations, in the Columbia Basin of Oregon and Washington (Miller *et al.*, 1998), South America (Forbes *et al.*, 1997), Asia (Koh *et al.*, 1994), and in Russia (Maleeva *et al.*, 1999). Day and Shattock (1997) observed that when the 'old' and the 'new' genotype isolates were tested on detached potato leaflets, 'old' genotype isolates were less pathogenic than the 'new' genotype isolates on race non-specific resistant cultivars e.g. Stirling. Such differences in aggressiveness may explain the displacement, in part, of 'old' genotypes by the 'new' genotypes resulting in the dramatic changes in the population dynamics of *P. infestans* worldwide. Although cultural practices differ in various potato producing countries, the intercontinental trade in tubers for planting and consumption have ensured that most countries have witnessed recently these displacement phenomena in late blight populations (Drenth *et al.*, 1994; Koh *et al.*, 1994; Miller *et al.*, 1998).

Although A2 mating type isolates have been reported outside Mexico since the early 1980s, their frequency has not increased in some countries and has not been discovered in others, e.g. in Taiwan (Hartman and Huang, 1995). In some cases, the frequency of A2 has been found not to exceed 5% ((England and Wales) Day and Shattock, 1997; (France) Lebreton *et al.*, 1998; Day *et al.*, 2004)). In other cases, however, high populations of the A2 mating type have been reported ((USA) Marshall and Stevenson, 1995; (Russia) Dolgova *et al.*, 1996; (Finland and Norway) Brurberg *et al.*, 1999; (Finland and Norway) Hermansen *et al.*, 2000)). In Finland about 15% of the tested isolates (200 isolates tested) and about 25% of the tested isolates in Norway (642 isolates tested) were of the A2 mating type (Brurberg *et al.*, 1999). In Russia and countries of the former USSR, percentages of up to 50 of the isolates tested have been reported as A2 mating type and were found to be predominantly resistant to metalaxyl (Dolgova *et al.*, 1996). In some areas in Siberia, only the A2 mating type has been found (Elansky *et al.*, 2001). Repeated sampling has revealed changes in A2 frequency over time. In Israel, Cohen (2002), reported changes in A2 mating type, which fully dominated the population from 1983

until 1991, but was later replaced by A1 mating type, which dominated during 1993 to 2000.

The A2 mating type has been reported on potatoes and on tomatoes (e.g. Shattock *et al.*, 1990; Marshall and Stevenson, 1995). Marshall and Stevenson (1995) reported that from a total of 87 isolates (77 from potato and 10 from tomato) collected from Wisconsin and Northern Illinois (USA), 23 of them were of the A1 mating type (16 from potato and 7 from tomato) and the remaining 64 were Chycoski *et al.*, 1994 of the A2 mating type (61 from potato and 3 from tomato). However, Hammi *et al.* (2001) reported A2 isolates on tomatoes only. When they isolated 108 isolates from both potato and tomato, they discovered that 60% of the isolates were of the A2 mating type and they were all from tomatoes. Population substructuring for various phenotypic traits, not least mating type, may be driven by host specificity, e.g. potato/tomato/indigenous wild species (Ann *et al.*, 1998; Lebreton and Andrivon, 1998; Oryazun *et al.*, 1998; Garry *et al.*, 2000; Wangsomboondee *et al.*, 2002); site characteristics, e.g. commercial/non commercial (Zwankhuizen *et al.*, 1998 & 2000; Groves, 2002; Cooke *et al*, 2003); agricultural practices (e.g. rotation cropping, sanitation practices, fungicides, (Gisi and Cohen, 1996; Peters *et al.*, 1998; Secor and Gudmestad, 1999; Groves and Ristaino, 2000). In all cases, spatial and temporal sampling techniques will affect the accuracy of frequency data of such phenotypes.

1.1.2 Sexual reproduction

Formerly, the absence of the A2 mating type outside Mexico restricted sexual reproduction to Mexico, but with both matings types in European populations of *P. infestans*, there is evidence that sexual reproduction is occuring under field conditions (Drenth *et al.*, 1995). Oospores have been reported in naturally blighted potatoes and tomatoes (Hammi *et al.*, 2001; Strömberg *et al.*, 2001). Strömberg *et al.* (2001) reported oospores formed in naturally infected potato fields planted with seven cultivars, of which six were carrying different levels of race-non-specific resistance and one cultivar with race-specific resistance. Oospores were formed in all but one cultivar that was not attacked by late blight. Drenth *et al.* (1993) reported both a significant increase in virulence and in genetic diversity in Dutch populations. In the former, they reported an increase from 8 races among 148 isolates before 1980, when only 5 virulence factors (i.e. 1, 2, 3, 4, 10) were considered, to 26 races 11 years later when same factors were

considered among 253 isolates (73 races were recognised when 5 more factors were considered, i.e. 5, 6, 7, 8 and 11). In the latter, they reported an increase in RG-57 fingerprint genotype from only one (namely US-1 phenotype) among 5 isolates before 1980 to 134 genotypes among 179 isolates collected after 1980. The authors attributed this sudden change to sexual reproduction after introduction of the 'new' population that comprised of both mating types. Drenth *et al.* (1993) argued that this sudden change could not have been brought about only by the introduction of new isolates. The comparison of allozyme profiles from Mexican and European isolates revealed that in Mexico there were 7 alleles for *Gpi* and 3 for *Pep* (Fry *et al.*, 1993), yet in Europe only 2 alleles for each enzyme were found (Fry *et al.*, 1991; Spielman *et al.*, 1991), of which the *Gpi* allele 90 and the *Pep* 83 allele occur at very low frequencies in Mexico (Goodwin *et al.*, 1993b). Therefore, if an introduction of a large population of diverse genotypes had occurred, more alleles, in combinations similar to those occuring in the Mexican populations would have been detected in Europe.

1.2 Fungicides used to control late blight

Fungicide treatment is still the most effective way of controlling plant diseases all over the world. Latest estimates indicate that approximately $6 billion is spent on all fungicides and about 40% of that is by Europe (Hewitt, 1998). According to Hewitt (1998), the Far East, south-east Asia and Australasia spends $41 million to control late blight on potatoes and tomatoes, South America spends around $39 million, while western Europe spends $103.6 million, and North America spends around $64.3 on diseases of potato and tomato. In 2000, Sender estimated the cost of controlling blight in the United States to be at $155 million annually (Sender, 2000, as cited by Kirk *et al.*, 2001).

Fungicides currently available for the control of late blight (see Table 1.2) are commonly categorised into 3 groups, i) protectant (non systemic); ii) translaminar; and iii) systemic, on the basis of their mobility within the plant.

Table 1.2. List of fungicides used to control potato late blight in the UK

Fungicide	Products	Company	Formulation[a]	Uses	# of treatments
Benalaxyl + mancozeb - systemic and protectant	Galben M Tairel	Sipcam Sipcam	8.65% w/w WP	- apply at blight warning before crop gets infected. - Repeat at 10-21 day interval - When growth ceases use non-systemic fungicides starting not less than 10d after last application	5 per crop
Bordeaux mixture - protectant copper sulphate	Wetcol 3	Ford Smith	30 g/l (copper) SC	- Spray intervals 7-14 days	Not specified
Chlorothalonil - protectant chlorophenyl fungicide	Bravo 500 Flute	Syngenta Sipcam	500 g/l SC 720 g/l SC	-	Varies per crop
Chlorothalonil + mancozeb - multi-site protectant	Adagio Guru	Interfarm Interfarm	201:274 g/l SC 286:194 g/l SC	- start spraying immediately after blight warning - start before disease appears - repeat every 7, 10 or 14 d depending on the blight risk	5 sprays per crop
Chlorothalonil + Propamocarb hydrochloride - contact and systemic fungicide	Merlin	Aventis	375:375 g/l SC	- commence early in the season as soon as there is a risk of infection - use only as protectant, stop use when blight is readily visible	For crop safety limit number of sprays to a maximum of 5 sprays per crop
Copper oxychloride - protectant fungicide	Cuprokylt Cuprokylt FL Headland Inorganic liquid copper	Unicrop Unicrop Headland	50% w/w (Cu) WP 270 g/l (Cu) SC 256 g/l (Cu) SC	- Spray interval 10-14 days	Not specified
Cyazofamid -cyanoimidazolesulfonamide protectant fungicide	Ranman TwinPack	BASF	400 g/l KL	- apply as a protectant before late blight enters the crop - repeat every 7 –10 days	Total of 6 but no more than 3 consecutive sprays

Cymoxanil - a urea fungicide	Sipcam C50	Sipcam	50% w/w WP	- to be used in mixtures with recommended partner containing mancozeb or fluazinam in order to combine systemic and protectant activity - commence spray programme before infection appears - repeat every 7-10 days	4 per crop
Cymoxanil + Famoxadone - preventative and curative fungicide mixture	Tanos	DuPont	25:25% w/w WG	- commence spray programme before infection - repeat every 7-10 days - reduce spray interval if conditions are conducive for late blight	12 per crop
Cymoxanil + Mancozeb - protectant and systemic fungicide	Besiege WSB Curzate M68 Globe Rhythym	DuPont DuPont Sipcam Interfarm	4.5 : 68% w/w WB 4.5 : 68% w/w WP 6 : 70% w/w WP 4.5 : 68% w/w WP	- apply immediately after late blight warning - repeat every 7-14 days	6 per crop
Cymoxanil + Mancozeb + Oxadixyl[b] - systemic and contact protectant fungicide	Ripost Pepite Trustan WDG	Syngenta DuPont	3.2 : 56.8% w/w SG 3.2 : 56.8 % w/w WG	- apply first spray as soon as there is a risk of late blight infection - do not treat crop already showing late blight - repeat every 10-14 days	5 per crop
Dimethomorph + Mancozeb - systemic and protectant fungicide	Invader	BASF	7.5: 66.7% w/w WG	- commence as soon as there is a risk of late blight infection - repeat every 7-14 days	8 per crop

Fentin acetate + maneb - curative and protectant fungicide	Brestan 60SP	Aventis	54 : 16% w/w WB	- commence before infection - repeat every 7-14 days	6 per crop
Fentin hydroxide - curative and protectant organotin fungicide	Barclay Fentin Flow Farmatin 560 Super-Tin 80WP	Barclay Aventis Sumi Agro	480 g/l SC 532 g/l SC 80% w/w WB	- apply on receipt of late blight warning - repeat every 7-14 days	3 per year
Fluazinam - pyridinamine fungicide	Barclay Cobber Shirlan Shirlan	Barclay Zeneca Syngenta	500 g/l SC 500 g/l SC 500 g/l SC	- commence treatment at first late blight risk warning i.e. before late blight enters crop - repeat every 7-14 days	10 per crop
Mancozeb -protective dithiocarbamate	Dithane 945 Manzate 75 WG Micene 80WP	Interfarm/Dow Headland Sipcam	80% w/w WP 75: w/w WG 80% w/w WP	- apply at early late blight warning - repeat every 7-14 days	Varies with crop
Mancozeb + Metalaxyl - systemic and protectant fungicide	Fubol 75 WP	Novartis	67.5 : 7.5% w/w WP	- apply as a protectant fungicide - repeat every 10-21 days	
Mancozeb + Metalaxyl M - systemic and protectant fungicide	Fubol Gold M Fubol Gold M	Syngenta Syngenta	64 : 4% w/w WG 64 : 4% w/w WB	- commence before there is risk of late blight - repeat every 10-14 days	Varies with crop
Mancozeb + Ofurace[b] - systemic and protectant	Patafol	Nufarm UK	67 :5.8% w/w WP	- apply at first late blight warning - repeat every 10 –14 days	5 per crop
Mancozeb _ Oxadixyl[b] - systemic and contact fungicide	Recoil	Syngenta	56 :10% w/w WP	- commence early in the season - use as protectant, potatoes showing blight should not be treated - repeat every 10-14 days	5 per crop including other phenylamides
Mancozeb + propamocarb hydrochloride - systemic and contact	Tattoo	Aventis	301.6 : 248 g/l SC	- commence as soon as there is risk of late blight - repeat every 10-14 days	5 per crop

Mancozeb + zoxamide - protectant fungicide	Electis 75WG Roxam 75 WG Unikat 75 WG	Interfarm Interfarm Candseer	66.7 :8.2 WG " " " " " "	-	
Maneb - protectant fungicide	Maneb 80 Trimanzono X-Spor SC	Rohm & Haas IntraCrop United Phosphorus	80% w/w WP " " " 480 g/l SC	- apply before late blight appears - repeat every 10-14 days	2 or 3 per crop

Source: The UK pesticide guide 2003, pg 109

[a] – formulation abbreviations: KL –Combi pack liquid/liquid
WB – wettable bait
WG- water dispersible granules
WP – wettable powder
SC – suspension concentrate (= flowable concentrate)
SG- suspo-emulsion;

[b] – all approvals for sale and supply of products containing ofurace or oxadixyl will be revoked on July 25, 2003 and farmers have until December 31, 2003 to use, or otherwise dispose off stocks.

1.2.1 Non-systemic

The non-systemic contact fungicides or the protectants act as non-specific poisons by coating the plant's surface and generally killing spores before they can initiate infection (Wain and Carter, 1977). These are, therefore, applied before symptoms are seen (Schwinn and Urech, 1986) and are more suited for mid- or late-season use on potatoes, as at the beginning of the season it is not possible to apply them frequently enough to maintain cover when the plants are growing rapidly without compromising the environment: examples include: mancozeb, chlorothalonil, fluazinam. The latter is subject of further study in this thesis.

1.2.1.1 Fluazinam

Fluazinam is a pyridinamine fungicide (Anonymous, 2003), it belongs to the group called 2,6-dinitroaniline and its mode of action is through uncoupling activity on mitochondrial oxidative phosphorylation (Tomlin, 1994; Hewitt, 1998). This means that these compounds permit electron transport to proceed with the maximum uptake of oxygen, but without the production of ATP necessary for cellular energy (Hewitt, 1998).

1.2.2. Translaminar

This group are eradicants; i.e. they can cure an established infection at the site of application (Wain and Carter, 1977) and as translaminar fungicides, they can move into leaf tissue and across to the other epidermis. They are not, however, translocated within the plant. These are most useful mid-season when they can help to slow down fungal growth immediately after infection. Members of this group include cymoxanil - released into the market in 1977, and dimethomorph - released into the market in 1988 (Albert *et al.*, 1988). Cymoxanil is part of the study described in Chapter 4.

1.2.2.1 Cymoxanil

This fungicide belongs to the group of cyanoacetamide-oximes (Serres and Carraro, 1976, as cited by Schwinn and Urech, 1986). Cymoxanil is more effective against hyphal growth stages than early growth phases (the release of zoospores and their germination) (Hewitt, 1998). It has been found to prevent disease development when applied two to three days after artificial inoculation of field-grown potatoes or tomatoes (Douchet *et al.*, 1977). Its

mode of action is through inhibition of RNA synthesis (Schwinn and Urech, 1986), thus inhibiting nucleic acids and protein biosynthesis (Hewitt, 1998).

1.2.3 Systemic

The third category is the curative or the truly systemic fungicides, which are xylem and apoplastic, but not phloem and symplastic, mobile. These can prevent the development of disease on regions of the plant away from the site of application (Wain and Carter, 1977). They are particularly useful at the beginning of the season as they help protect new growth when the plants are growing rapidly. Examples include propamocarb and the phenylamides (especially metalaxyl). Both of these compounds are subjects of study in this thesis as well as azoxystrobin.

The systemic and the translaminar fungicides tend to be single-site inhibitors and consequently carry a greater risk of selection for resistance (insensitivity) among pathogen strains (Urech and Staub, 1985). To reduce the build-up of fungicide resistant strains, they are normally used as co-formulations with non-systemics such as mancozeb or chlorothalonil (Urech and Staub, 1985). This is based upon the principle that the size of the fungal population actually penetrating the plant and being exposed to the translaminar or systemic fungicides components will have been reduced by initial contact with the protectant component (Schwinn and Margot, 1991).

1.2.3.1 Propamocarb

Propamocarb is thought to have an action related to membrane structure and function (Schwinn and Urech, 1986). In *Pythium ultimum* it caused efflux of cell membrane constituents such as phosphates, carbohydrates and proteins (Hewitt, 1998).

1.2.3.2 Phenylamides

The mode of action of the phenylamides is by inhibition of the ribosomal RNA synthesis via the RNA polymerase I-template complex (Davidse and van der Berg-Valthius, 1989). This results in the thickening of the hyphal walls and eventual death of the fungus (reviewed by

Hewitt, 1998). They are also said to have indirect mode of action, i.e. their effect via plant defence e.g. formation of phytoalexins (Schwinn and Urech, 1986).

This group of fungicides act at specific development stages in the oomycete infection process (Hewitt, 1998). Zoospore release, encystment, germination and initial penetration and 1% haustorium development of the pathogen beyond the formation of the primary haustorium is not controlled (Hewitt, 1998). The widely used phenylamide is metalaxyl. Staub and Young (1980) observed that metalaxyl inhibited formation of sporangium from a germinating chlamydospore of *P. parasitica* var. *nicotianae*, thus preventing build-up of sporangium inoculum thus reducing the impact of secondary cycles of infection. Although it is still being used to control late blight, insensitive phenotypes to the fungicides appeared soon after it was released commercially (Dowley and O'Sullivan, 1981; Pappas, 1985; Deahl *et al.*, 1993). The development of resistance of isolates of *P. infestans* to phenylamides has been used as a phenotypic marker for the pathogen in studies of inheritance (Shattock, 1988; Fabritius *et al.*, 1997; Lee *et al.*, 1999) and population biology (Chycoski and Punja, 1996; Gisi and Cohen, 1996; Day and Shattock, 1997; Peters *et al.*, 2001; Mukalazi *et al.*, 2001; Ghimire *et al.*, 2001; Knapova and Gisi, 2002; Daayf and Platt, 2002; Reis *et al.*, 2003; Cooke *et al.*, 2003; Deahl *et al.*, 2003; Day *et al.*, 2004). Grunwald *et al.* (2001), after sampling *P. infestans* from wild, land-races and modern cultivars of potatoes and testing isolates for metalaxyl sensitivity, concluded that populations of *P. infestans* on wild *Solanum* populations are derived from populations of cultivated potatoes in central highlands of Mexico near Toluca.

1.2.3.3 Azoxystrobin

Azoxystrobin is the most recently developed broad-spectrum fungicide, which is used in most parts of the USA to control mainly early blight (*Alternaria solani* Sorauer). It is also recommended for the control of late blight, but not yet in the UK. It is a synthetic strobilurin, whereby the natural strobilurin (Anke *et al.*, 1977) had its benzene ring replaced by heterocycle. The natural strobilurins were antifungal and antibiotic compounds initially isolated from a wood decaying basidiomycete fungus *Strobilurus tenacellus* (Pers. Ex Fr.) Singer (Anke *et al.*, 1977). Strobilurins were found to inhibit electron transfer in complex III

(bc_1 complex) of the mitochondrial electron transport chain (Becker *et al.*, 1981). This causes inhibition of mitochondrial respiration thus blocking the ATP synthesis i.e. energy production within fungi and oomycetes is stopped (Wiggings and Jager, 1994). Therefore, highly energy-demanding stages of fungal development are particularly sensitive to the effects of strobilurins. The strobilurin-producing fungi are not, however, affected by these secondary metabolites because their cytochromes *b* have altered active sites, rendering them less sensitive to the 2-methoxyacrylates.

Azoxystrobin acts against all major groups of filamentous plant pathogens, namely: ascomycetes e.g. powdery mildews, basidiomycetes e.g. rusts, deuteromycetes e.g. rice blast and oomycetes e.g. downy mildews, and therefore, *P. infestans* (Godwin *et al.*, 1992). The fungicide is systemic when applied as a root drench and also demonstrates translaminar movement. It has also been observed to possess preventative, curative, eradicant and antisporulant activity (Pilling *et al.*, 1996). It has been reported that this fungicide has good persistence effect; when applied on the foliage it can give protection of up to 8 weeks in cereals, thereby maintaining green leaf area until late in the season. The same observation has been made on other analogues. Grossmann and Retzlaff (1997) also reported induction of physiological and developmental alterations in wheat, which were seen to improve yield.

Although azoxystrobin is a relatively new fungicide, insensitivity has already been reported in several fungi: in cereal powdery mildews (Fraaije *et al.* 2002), in grey leaf spot of maize fungus, *Pyricularia grisea* (Vincelli and Dixon, 2002; Kim *et al.*, 2003), in rice blast fungus, *Magnaporthe grisea* (Avila-Adame and Koller, 2003), in *Ustilago maydis* (maize smut) (Ziogas *et al.*, 2002): this insensitivity is because it is a site-specific compound. Mutations in *P. infestans* may produce altered active sites (like the strobilurin-producing fungi) and such mutation (point mutation) has been observed in yeast (*Saccharomyces cerevicae*) to methoxyacrylates (Leroux, 1996). The producers recommend that it be used together with protective fungicides (e.g. mancozeb) as proposed for other systemic fungicides (Brent and Hollomon, 1998).

1.3 Markers used in inheritance studies of *Phytophthora infestans*

There are several markers used to study *P. infestans*. They are divided into three categories: phenotypic, biochemical and molecular markers. Phenotypic markers include: mating type, virulence, sensitivity to antimetabolites, both fungicides and antibiotics e.g. streptomycin and chloramphenicol (Khaki and Shaw, 1974; Shattock and Shaw, 1976; Ann and Ko, 1992). Secondly, biochemical markers based on isozymes e.g. glucose phosphate isomerase, peptidase. These were especially useful because of their co-dominance and the advantage this gives to following patterns of inheritance in diploid organisms. The third group are molecular markers which exploit the much greater levels of which polymorphism associated with regions of DNA both in the mitochondrion and nucleus. These include: mitochondrial DNA (mt DNA), Restriction Fragment Length Polymorphisms (RFLPs) (Goodwin and Fry, 1988; Carter *et al.*, 1990), Random Amplified Polymorphic DNA (RAPDs) (Williams *et al.*, 1990), nuclear RFLPs e.g. RG57 (Goodwin *et al.*, 1992a), Amplified Fragment Length Polymorphisms (AFLPs) (Vos *et al.*, 1995), and microsatellites (Dobrowolski *et al.*, 1998). Some DNA markers are more useful than others for studies of inheritance with diploid organisms and permit heterozygous to be distinguished from homozygous genotypes, e.g. AFLPs, microsatellites (Vos *et al.*, 1995; Dobrowolski *et al.*, 1998). Some of these markers are discussed below.

1.3.1 Mating type

The two mating types, A1 and A2, represent compatibility types that differ in the production of a mating-specific (K) hormone (Ko, 1978 and 1988). Each mating type is bisexual, but self-compatible; responses of each mating type to the hormone of the opposite type are similar, i.e. suppression of aerial mycelium and sporangia, and formation of sexual gamentangia, both antheridia and oogonia (Galindo and Gallegly, 1960). This condition, known as heterothallism, is where different mating types are somatically identical, but carry different alleles at the mating type locus. In *P. infestans* there is some evidence of different morphotypes being distinguished based on mating type, i.e. lumpiness associated with A2 mating type (Shaw *et al.*, 1985; Shattock *et al.*, 1990; Shaw 1991). This aspect is studied in Chapter 2 of the thesis.

Mating type in *P. infestans* is proposed to be determined by a dosage of a dominant allele, with alternate mating types represented by *A/a* and *a/a* (Gallegly, 1970, Shaw, 1991). This model explains the occasional appearance of self-fertile progeny (*Aaa*).

Mating type is also thought to be governed by a single locus (Gallegly, 1970) which displays aberrant segregation, i.e. more A1 than A2 mating type progeny are usually produced, instead of being equal as would be expected for a single locus (Judelson *et al.*, 1995; Judelson, 1996). Because *Phytophthora* species are diploid, presumably each isolate inherits mating type alleles from both parents, therefore, mating type loci potentially operate in a heterozygous state. Judelson *et al.* (1995), using DNA markers, proposed that A1 and A2 mating types are fixed for heterozygosity at the mating type locus, with one allele determining mating type and the second allele being neutral or non-functional. Based on DNA markers flanking the locus, they observed that only two genotypes were recovered among progeny instead of four that would be expected from a diploid. This non-Mendelian pattern of inheritance of the mating type locus was the proposed to be the mechanism for eliminating progeny with ambiguous or sterile genotypes.

1.3.2 Virulence

Resistance genes (R) were obtained from *Solanum demissum* and other species and were bred into the cultivated genotypes of potato (*Solanum tuberosum* L.) (reviewed by Watson, 1970). There are eleven of these genes (R1...R11) (Black *et al.*, 1952). The genetic basis of inheritance of avirulence genes in *P. infestans*, i.e. avirulence being when an isolate fails to produce sporulating lesions on a host plant, suggest a gene-for-gene system (Flor, 1956) having a single avirulence locus with dominant avirulence allelles or recessive virulence alleles determining the reaction of each differential line carrying a single dominant resistance gene (reviewed by Shaw and Shattock, 1991; Al-Kherb *et al.*, 1995).

1.3.3 Antibiotics and fungicides

Sensitivity to phenyalamindes, especially metalaxyl, has been widely used as a phenotypic marker particularly in studies of late blight populations (Shattock, 1988; Daggett *et al.*, 1993). Although there are now several molecular markers available to study populations of

P. infestans, *in vitro* metalaxyl sensitivity is regularly used in studies of *P. infestans,* as is mating type (Day and Shattock, 1997; Mahuku *et al.*, 2000; Elansky *et al.*, 2001).

Inheritance studies of resistance to metalaxyl have proposed that one single, incompletely dominant gene is responsible (Shattock, 1988). Other reports suggest one semi-dominant locus combined with the effect of several minor loci resulting in continuous sensitivity segregation in F1 progeny (Shaw 1991; Fabritius *et al.*, 1997). More than one locus may be involved (Judelson and Roberts, 1999) and alternative hypotheses have also been proposed (Lee *et al.*, 1999). Most recently, Knapova *et al.* (2002) crossed metalaxyl sensitive and resistant parents and observed that almost all (31 of 35) of single oospore progeny were intermediate phenotypes. These results support the earliest data (Shattock, 1988), where it was observed that single oospore progeny from a sensitive and a resistant parent were mainly intermediate, and when siblings of intermediate phenotype were crossed, all three phenotypes, sensitive, intermediate and resistant, were recorded among 334 progeny in a ratio of 1:2:1. This and other data involving backcrosses and germination of self-fertile phenotypes suggested that one single, incomplete dominant gene controlled metalaxyl resistance.

These early inheritance studies of fungicide resistance (Shattock, 1988; Chang and Ko, 1993), antibiotic resistance (Whittaker *et al.*, 1996), virulence (Spielman *et al.*, 1990; Goodwin and Fry, 1994; Gisi and Cohen, 1996), relied on biochemical markers e.g. isozymes, i.e. glucose phosphate isomerase and peptidase (Tooley *et al.*, 1985; Shattock *et al.*, 1986b) to track occurrence of hybridisation or selfing of single oospore progeny. The extent of selfing versus hybridisation will disrupt ratios of observed phenotypes and lead to misinterpretation of inheritance patterns.

1.3.4 Isozymes

Isozymes are defined as variant forms of an enzyme that share the same catalytic function and allozymes are isozymes whose variant forms are coded at the same locus (Spielman *et al.*, 1986; Spielman, 1991). They have been used extensively as molecular markers (Tooley *et al.*, 1985; Shattock *et al.*, 1986b). They are single genes and co-dominant chromosomal

markers. Two polymorphic loci, namely glucose phosphate isomerase (*GPI*) and peptidase (*PEP*), which are dimeric, have been used in genetic studies (Tooley *et al.*, 1985). The *Gpi* locus has 7 alleles: 83, 86, 90, 98, 100, 111, and 122; and the *Pep* locus has 3 alleles: 83, 92, and 100. Isozymes have been used for confirmation of diploid Mendelian inheritance and for distinguishing hybrid from selfed progeny (Shattock *et al.*, 1986b). However, they suffer insensitivity as more than one nucleotide change can lead to the same isozyme polymorphism (Purvis, 2000).

1.3.5 RG57 markers

RG57 has been the most widely used DNA probe for population studies of *P. infestans* (Goodwin *et al.*, 1992a; Forbes *et al.*, 1998). The probe is a 1.2 kb genomic fragment derived from a *Phytophthora infestans* genomic library and represents a moderately repetitive nuclear DNA sequence (Goodwin *et al.*, 1992a). It may hybridise to 25 or more *Eco*RI derived different nuclear DNA fragments. Of the hybridising fragments, 13 are known to segregate independently. The fragment patterns are somatically stable and are transmitted to sexual progeny in a Mendelian fashion. Since it is a dominant marker, bands from homozygotes (+/+) are rarely distinguishable from those of heterozygotes (+/-). RG57 fingerprint is therefore a phenotype.

1.3.6 Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA markers (Williams *et al.*, 1990), have been used to investigate hybridity in *Phytophthora cinnamomi* (Linde *et al.*, 2001) and sexual recombination in *P. infestans* (Maufraud *et al.*, 1995). They have also been used to study relatedness of some *Phytophthora* species (Cooke *et al.*, 1996). Sandström *et al* (2001) also used RAPD to study populations of *P. infestans* in Sweden and they then confirmed the results with AFLP and RG57.

1.3.7 AFLPs

This is a DNA fingerprinting technique that detects genomic restriction fragments, which is PCR based. The technique displays presence or absence of restriction fragments rather than length differences (Vos *et al.*, 1995).

Purvis *et al.* (2001) compared AFLP and RFLP (RG57) to study relatedness of isolates of *P. infestans*. They observed that some of the common RG57 genotypes (RF 06 and 40) had closely related AFLP genotypes that differed at no more than two loci. However, other common RG57 genotypes (RF 026 and 39) displayed higher levels of variability for AFLPs. The authors attributed this to a possibility of convergent evolution of some RG57 fingerprints. Flier *et al.* (2003) also compared AFLP and RFLP (RG57) to study genetic variation at DNA level within a sub-sample of 40 isolates. Although they observed a fourfold increase in the number of informative loci from AFLP compared to RG57, there was no significant change in indices used to describe genotype diversity.

1.3.8 Microsatellites

Microsatellites are used as genetic markers in population studies in eukaryotes (Dobrowolski *et al.*, 1998). They are simple sequence repeats (SSRs) and they consist of tandem repeats of very short nucleotide motifs (Queller *et al.*, 1993; Gobbin *et al.*, 2003). Microsatellites arrays are grouped into three classes: (1) perfect repeats, where each repeat follows the next without interruptions, (2) imperfect repeats, where repeats are interrupted by non-repeat bases, and (3) compound repeats, where two or more repeat units are adjacent to each other (Gupta *et al.*, 1996, as cited in Gobbin *et al.*, 2003).

A microsatellite locus is detected by PCR amplification with primers that anneal to the unique flanking sequences of the microsatellite (Moore *et al.*, 1991). Because these flanking sequences and the microsatellite location are often conserved, primers are often transferable among related species (Moore *et al.*, 1991). Microsatellites are known to occur in the chloroplast genome (cpDNA) of plants and they have been developed into useful highly polymorphic genetic markers of pine and soybean populations (Powell *et al.*, 1995a; Powell *et al.*, 1995b). They are codominantly expressed, like isozymes and highly polymorphic (Tautz and Renz, 1984; Cooke *et al.*, 2002; Gobbin *et al.*, 2003). However, Dobrowlski *et al.* (2002), reported non-Mendelian inheritance of microsatellite alleles in progeny of 4 crosses of *P. cinnamomi*.

1.4 Aggressiveness

Phytophthora infestans is a pathogen of potato, tomato and some other species of the Solanaceae (Erwin and Ribeiro, 1996). Simplistically, and in the case of isolates attacking potato, pathogenicity is made up of two components: virulence and aggressiveness. Van der Plank (1968) defined aggressiveness to describe components of pathogenicity such as rate of lesion formation, lesion size, levels of sporulation, when isolates of a pathogen do not interact differentially with varieties of the host. Virulence, however, involves a differential response between isolates of a pathogen and different host genotypes. In the latter case this usually involved different potato genotypes with different R genes (Black, 1952, cited in Fininsa, 1990; Malcolmson and Black, 1966, as in Fininsa, 1990; Al-Kherb *et al.*, 1995) of which eleven have been identified (Malcolmson, 1969). When used to screen potato late blight isolates for virulence such as R genes different genotypic hosts are referred to as differentials. Implicit in the differential response is the gene-for-gene theory as proposed by Flor (1956). Pathogenicity, therefore, includes both aggressiveness and virulence.

In 1960 Knutson and Eide looked at what they called parasitic aggressiveness, which they defined as the ability of a pathogen to increase to epidemic proportions in a host population. They suggested that an aggressive pathogen would be one which has the ability to produce large amounts of viable inoculum (sporulation capacity) and one that can penetrate and infect the host the fastest (inoculation period). Current research on aggressiveness includes the same parameters that were suggested by Knutson and Eide (1960) but they also include latent period and lesion size (Legard and Fry, 1995; Day and Shattock, 1997; Miller *et al.*, 1997; Lee *et al.*, 2002).

Aggressiveness of isolates can be usefully measured to compare performance of host genotypes in breeding programmes and when deployed commercially in different locations and as part of different cropping regimes. Similarly, measurement of infection efficiency, lesion development, etc can be used to assess variation in field isolates of late blight collected over time and space (Dorrance and Inglis, 1997; Flier and Turkensteen, 1999; Lebreton *et al.*, 1999; Dorrance *et al.*, 2001). Equally asexual and sexual progeny can be evaluated for aggressiveness. Whilst the inheritance of virulence has been studied by several different

groups of workers (Spielman *et al.*, 1990; Al-Kherb *et al.*, 1995; Knapova and Gisi, 2002) as well as sometimes including host specialisation for potato and tomato, the inheritance of aggressiveness had not been studied at the inception of the work presented in this thesis.

The discovery of the A2 mating type outside Mexico coincided with the discovery of the 'new' A1 mating type, which was more fit than the old type (Fry *et al.*, 1993; Day and Shattock, 1997). Kato and Fry (1995) reported that 'new' isolates may be more aggressive on potato leaves than 'old' isolates. Day and Shattock (1997) also reported similar findings. They observed that the 'old' genotypes were less pathogenic than the 'new' genotype on the more resistant cultivars Cara and Stirling. Such fitness has been sometimes attributed to these isolates being metalaxyl insensitive, but insensitivity to this fungicide is not always associated with increased fitness among new isolates, i.e. sensitive 'new' isolates are as a class more aggressive than metalaxyl insensitive phenotypes (Day and Shattock, 1997).

Prior to the work described in this thesis and the other studies already cited, characterizing single oospore progeny following matings between A1 and A2 mating type isolates, variation for components of pathogenicity namely, virulence and aggressiveness and growth *in vitro* concentrated on analysis of single sporangial and zoospore isolates (Wallin, 1957; Caten and Jinks, 1968; Caten, 1970, 1971). Such variation as exists is still a contributory factor in late blight population despite the opportunity for sexual recombination/segregation where A1 and A2 mating isolates coexists. Such variation is also described in Chapter 5.

1.5 Aims and objectives

There is limited information on variation among sexual progeny and among asexual progeny of *P. infestans* in aggressiveness and also in sensitivity to currently used fungicides for potato blight control. This thesis describes studies on the inheritance of aggressiveness of sexual progeny from three matings of *P. infestans* isolates from potato. Hybridisation was evaluated using a range of markers, and progeny sets were also evaluated for inheritance of mating type, mycelial morphotypes, and sensitivity to four fungicides. A subset of asexual progeny was also compared for some of these phenotypic characteristics. The questions that were asked were:

1) Is lumpiness a morphological character exclusive to the A2 mating type?

2) Are all sexual progeny, from pathogenic parents pathogenic and are they more or less aggressive than their parents on different potato cultivar with differing polygenic resistance?

3) Is there any difference in growth response to different fungicides among progeny or are they all similar to the parental phenotype?

4) Is there any difference among sexual and asexual progeny in aggressiveness and in sensitivity to fungicides?

2.0 PRODUCTION OF SINGLE OOSPORE PROGENY OF *PHYTOPHTHORA INFESTANS*: INHERITANCE OF MATING TYPE AND MYCELIAL MORPHOTYPES.

2.1 INTRODUCTION

Following the discovery of the A2 mating type and subsequently novel genotypes of A1 mating types due to migrations out of Mexico, first to Europe in the 1970s (Niederhauser, 1991) and then to north America in the 1980s and 1990s (Spielman *et al.*, 1991; Fry *et al.*, 1993; Goodwin and Drenth, 1997), there have been many reports of oospore production both *in vitro* and *in vivo*. Oospores are produced for different studies, mainly for studies of inheritance of important monogenic traits, e.g. mating type, virulence, phenylamide sensitivity, and/or genetic diversity (Smoot *et al.*, 1958; Romero and Erwin, 1969; Shattock *et al.*, 1986a; Shattock 1988; Al Kherb *et al.*, 1995; Hanson and Shattock, 1998; Turkensteen *et al.*, 2000; Knapova and Gisi, 2002; Knapova *et al.*, 2002; Oliva *et al.*, 2002). Oospores have been produced also to study their survival and/or viability (Shaw *et al.*, 1985; Pittis and Shattock, 1994; Drenth *et al.*, 1995; Flier *et al.*, 2001; Hammi *et al.*, 2001), effect of host and fungicides on their production (Cohen *et al.*, 1996; Hanson and Shattock, 1998). In addition, oospores have been reported in nature in naturally blighted potatoes and tomatoes (Hammi *et al.* 2001; Strömberg *et al.*, 2001) and also under experimental treatments. Cohen *et al.* (2000) studied the effect of different sprinkler irrigation regimes on formation of oospores on artificially inoculated potato plants. They observed that an increase in total rain (natural and irrigation), significantly enhanced oospore production in potato leaves, although the number of oospores was still less than those observed under laboratory conditions.

Characterisation of single oospore progeny had been studied as early as the 1960s (Romero and Erwin, 1969), soon after the heterothallic nature of *P. infestans* was discovered in Mexico (Gallegly and Galindo, 1958). The majority of studies, like the first, have been restricted by the small numbers of established single oospore progeny (e.g. 2 and 34 in the study of Smoot *et al.* (1958) and Romero and Erwin (1969), respectively). Exceptions to this were studies by Shattock *et al.* (1986a); Shattock (1988); Smith (1993) and Al-Kherb *et al.* (1995).

This chapter describes the establishment of large numbers of single oospore progeny from 3 matings of one A2 of UK common RG57 fingerprint genotype (RF040)(Appendix 3.0) (Purvis *et al.*, 2001; Shattock, 2002; Day *et al.*, 2004) and three A1s, of which two were UK isolates (RF039 and RF032, common and uncommon RG57 fingerprint genotype, respectively) (Appendix 3.0), and one American isolate (P9463) RF genotype not identified. The genotypes of RG57 have the prefix 'RF' for 'RG57 Fingerprint'. This chapter describes the establishment of three sets of these progeny, their mating type ratios and morphological characteristics. In the latter, some single oospore progeny of the A2 mating type have been observed to be 'lumpy' (Shaw *et al.*, 1985). Lumpy isolates are also observed among field isolates of *P. infestans* of A2 mating type (Shattock *et al.*, 1990) when grown *in vitro* on some nutrient agars but not on others. In this study, the A2 isolate used in the three crosses was a non-lumpy phenotype.

2.2 MATERIALS AND METHODS

2.2.1 Choice and recovery of parent isolates

Parental isolates were recovered from the University of Wales, Bangor late blight collection stored in liquid nitrogen. Cryo-vials containing 3-4 frozen agar plugs, in 15% dimethylsulphoxide (DMSO), of *P. infestans* were thawed under sterile conditions (under laminar airflow). The melted agar plugs in DMSO were then transferred onto a Petri dish containing rye A agar (Appendix 1.1) (Caten and Jinks, 1968) amended with the antibiotics rifamycin (25 :g/mL), ampicillin (25 :g/mL) and nystatin (50 :g/mL) (RAN) (Appendix 1.6). The Petri dishes were then incubated at $^{18^{0}C}$ in the dark at a slanted position to drain excess DMSO.

A total of four parental isolates were selected to be used in the study. Three isolates 96.69, 96.89.43 (Purvis, 2000; Purvis *et al.*, 2001) and P9463 (Bardsley *et al.*, 1998) were A1 mating type. All these were mated with a single A2 isolate 96.70 (Purvis, 2000; Purvis *et al.*, 2001) (Table 2.2.1).

Table 2.2.1. Parental isolates used in this study and their characteristics

Isolate	Origin of isolate	Mating Type	Mt DNA Haplotype	RG 57 Fingerprint	Mycelial morphotype
96.69	Beaumaris (Anglesey, UK)	A1	Ia	RF 039	Fluffy
96.70	Beaumaris (Anglesey, UK)	A2	Ia	RF 040	Fluffy
96.89.43	Pant y ddolen (Wales, UK)	A1	Ia	RF 032	Fluffy
P9463	California (USA)	A1	Ia	NT*	Fluffy

NT - not tested*

2.2.2 Production of oospores

Pairing of an A1 and an A2 isolate was on rye A agar (Appendix 1.1) plates involved placing 1.5 cm long agar strips from edges of actively growing cultures 1 cm apart on rye A agar in Petri dishes sealed with parafilm (BDH Merk. Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leics. LE17 4XN). Mating cultures were incubated at 18 ^{0}C in the dark for a month.

2.2.2.1 Extraction and germination of oospores

After one month, mating of A1 and A2 isolates, oospores were extracted by blending small agar pieces containing mature oospores in 10 mL of sterile deionised water to form a paste using a glass grinder. A membrane filter holder (Swinnex) was used to hold a 20 :m pore nylon filter (Lockertex, LockerWire Weavers Ltd., P.O.Box 161, Warrington, Cheshire, WA1 2SU). The paste was diluted with distilled deionised water to reduce its viscosity and it was collected into a 50 mL syringe. The syringe was then mounted on one end of the filter holder and diluted paste was passed through the filter holder such that the oospores were collected on the membrane. Approximately 100 mL of sterile deionised water were passed through the filter holder to wash-off excess agar from the oospores. The nylon filter was aseptically removed and placed into a universal bottle containing 10 mL of sterile distilled water and vortexed to release the oospores from the filter. A 5:L mixture of RAN antibiotics and 0.01g NovoZym 234 (Novo Biolabs, Nova Enzyme Products Ltd, Farnham, Surrey, UK) (Pittis and Shattock, 1994) were added to the oospore suspension. The mixture was incubated at 18 ^{0}C in the dark for 36 hours after which most hyphal fragments and sporangia

were lysed by the enzyme. Oospores were again collected on a 20:m nylon filter, washed with 100 mL of sterile distilled water, resuspended in 10 mL of sterile distilled water. The oospores were left for 30 minutes to settle at the bottom after which excess water was removed. The spores were transferred and spread onto soft water agar (SWA) (6g of agar per litre) and excess water was evaporated by removing the lid of the Petri dish for a few minutes under sterile conditions (laminar airflow). The Petri dishes were then sealed with Parafilm (BDH Merk. Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leics. LE17 4XN) and incubated at 18 ^{0}C in the light and observed daily for germinating oospores.

2.2.3 Establishment of single oospore progeny

Four days into the incubation of the extracted oospores, about 5% of the oospores were germinating, the percentage germination increased to about 50% after 10 days. Germinating oospores were recognised by their size, morphology and colour. Germinating oospores were about 3 to 4 times bigger than non-germinating ones. They appeared granular and had an orange brown colour while non-germinating ones were smooth and clear or sometimes light brown. The germinating oospores also had an emerging germ-tube.

Germinating oospores were aseptically transferred into Petri dishes containing rye B amended with RAN. This was done by looking through a dissecting microscope and a sterile scalpel was used to cut a chunk of agar on which the germinating oospore was situated to avoid bursting of the emerging fragile germ tube. The transferred germinated oospores were monitored daily and those that continued to grow on the Petri dish were transfered into 24 compartment repliplates (Bibby Sterilin, Staffordshire, UK) containing rye A amended with RAN antibiotics to establish single isolates. Working cultures were then established by inoculating 9 cm Petri dishes containing rye A.

Transferred germinated oospores that were not readily growing were monitored for up to 3 days and were discarded if there was no sign on growth. This process of transferring germinating oospores from the original plates and transferring of transferred growing isolates continued for 3 weeks. The transferring of germinating oospores was then stopped, as it was impossible to identify single isolates as the plates were filling up with germinated isolates

that were not picked in time, however, the establishing of single oospore isolates continued for another 4-5 days.

2.2.4 Maintenance of progeny isolates

Mycelial cultures of single oospore progeny were maintained individually in rye A agar in 9 cm Petri dishes at 18 ^{0}C without illumination. Subcultures were made by inoculating fresh Petri dishes of rye A agar (sometimes amended with RAN to counter contamination) with small mycelial cultures in agar blocks every month.

Medium term storage was either by: (i) suspension of agar plugs in 1.5 mL Eppendorfs containing sterile distilled water, or (ii) suspension of agar plugs in 2 mL tubes containing sterile mineral oil, prepared by heating at 120 ^{0}C for 4 hours. To maintain viable cultures isolates were taken out of the tubes and grown in Petri dishes every 6-8 months and fresh tubes were prepared from the actively growing region of each isolate (as described above).

Long-term storage involved inoculating rye A agar slants in universal bottles. The inoculated slants were incubated at 18 ^{0}C without illumination and when mycelial growth covered the agar surface, sterile light mineral oil was added to a level above the agar. The oil slants were then kept at 18 ^{0}C in lightproof boxes.

2.2.5 Morphological characterisation

The morphological appearance of the single oospore progeny was characterised. The following criteria were used:

(-) = no lumps or non-lumpy, when isolate had a fluffy appearance.

(+) = slightly lumpy, when an isolate had visible but few clusters (lumps) of hyphae, more pronounced on the older part of the colony, i.e. on and around the inoculating plug.

(++) = lumpy, when an isolate had visible lumps throughout the colony but with visible sporangiophores and sporangia.

(+++) = very lumpy, when an isolate had no visible sporangiophores nor sporangia but clusters of lumps that would later coalesce to form a thick mat which became water-soaked.

2.2.6 Mating type characterisation

Rye B agar, which provides a clear see through medium in contrast to the richer, opaque Rye A (Caten and Jinks, 1968), amended with RAN antibiotics at the standard concentration, was used. A central circle of agar of 4 cm diameter was removed from each Petri dish to leave a ring of agar. The antibiotics were used for two purposes, to eliminate bacteria that would interfere with the mating type experiment and also to facilitate recognition of zone of interaction between A1 and A2 mating type mycelia (agar turning pink at the interaction zone while remaining yellow where isolates are growing – see Figure 2.2.1). Mycelial strips of 96.69 and 96.70 (tester isolates), of same length as the agar ring in the Petri plate, were cut from actively growing isolates and placed at the extreme ends of each plate (Figure 2.2.1). Two strips of mycelial cultures of *P. infestans* isolate were placed next to each tester isolate at about 1cm distance, such that the isolates of unknown mating type were tested against each tester isolate, but also against itself (see Figure 2.2.1). In each Petri dish two isolates of unknown mating type were tested on either side of each tester isolate.

The dual cultures were incubated at 18 ^{0}C in the dark. Mating type of the unknown isolates was determined when oospores were produced opposite one or the other tester isolate. Oospores usually formed after about 7 days after incubation.

2.2.7 Scanning electron microscopy of mycelial morphotypes

Three single oospore progeny cultures showing 'lumpy' morphotypes were chosen for SEM studies. The isolates were grown on cellophane overlaid on rye A agar amended with antibiotics (RNA). The isolates formed mycelial lumps on the cellophane as they did on the agar surface but the cellophane could be lifted off the agar and manipulated and cut into small squares to facilitate microscopical examination without disrupting the mycelial architecture. These were fixed in 3% glutaraldehyde in a phosphate buffer (pH 7) for 3 hours at room temperature. The blocks were washed in 6 changes of buffer remaining in the last wash overnight and then dehydrated through an increasing gradient (25, 50, 75, 100%) at 4^{0}C for 30 minutes at each concentration. The blocks were placed in 100% acetone over anhydrous copper sulphate for 30 minutes at 4^{0}C and left to equilibrate to room temperature.

The blocks were critical point dried, mounted on stubs and gold coated using a sputter coater prior to examination using a Hitachi S-520 scanning electron microscope.

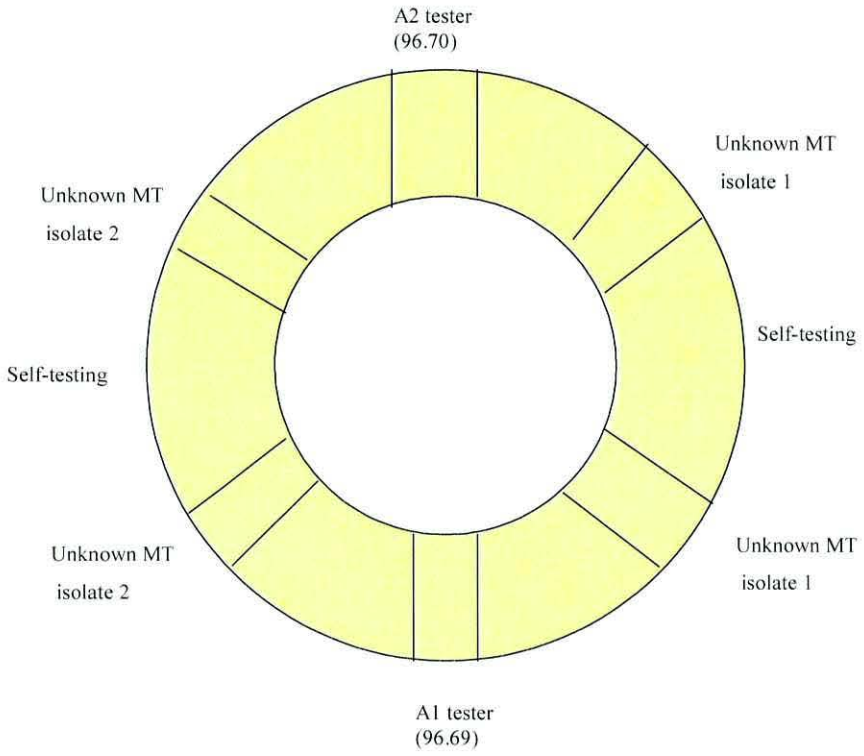


Figure 2.2.1. Diagram showing placement of A1 and A2 mating type tester isolates of *Phytophthora infestans* and tested isolates (two isolates of unknown mating type (MT)) in a 9mm Petri dish. Each isolate of unknown MT was also tested against itself at the region marked 'self testing'.

2.3 RESULTS

2.3.1 Establishment of single oospore progeny

The extracted oospores started germinating 4 days after incubation. Initially, the germination percentage was low, about 5%, however, a week after the starting of germination, the germination percentage had increased to about 50 percent for cross #1 and 2 and over 50% for cross #3. Germinating oospores were recognised by their size as they were 3 or 4 times bigger than non-germinating ones and had a granular appearance with an orange brown colour. Most of the germinating oospores did not give rise to a sporangium rather to multiple germ-tubes. However, few germinating oospores did give rise to a germ sporangium and sometimes 2 or 3 germ sporangia were formed in a chain.

Not all germinated oospores were established as single oospore progeny. At the beginning of the transferring of germinated oospores, care was not taken when lifting them to avoid bursting of the fragile germ-tube. As a result several hundreds of germinated oospores were lost. This problem was rectified by cutting a small block of agar on which the germinating oospore was situated and transferring the block. Keeping the lid of the plate on when looking for germinating oospores also helped reduce dessication and bursting of hyphae. Germinating oospores were first transferred into a Petri dish, containing rye B amended with RAN about 20 to 25 per plate and later transferred into a 24 compartment Repliplate, containing rye A amended with RAN. Oospores that germinated to give rise to one or more sporangia were slow to start growing as the sporangium had to germinate first. These usually took 48 hours or more to start growing while those oospores that germinated to give rise to one a more germ-tube grew more quickly, and sometimes, had to be transferred into Repliplates a few hours after their transfer from the germination plate. Another factor that contributed to failure to capture more germinated oospores, as single oospore isolates was the number of plates that had to be dealt with each day. As more and more oospores germinated, more and more plates had to be transferred and as a result some germinated oospores were not transferred (into Repliplates) in time before 2 or more germinating single oospore cultures grew into each other: such isolates were discarded.

Nonetheless, a total of 545 single oospore progeny (SOP) (cross #1, 190; #2, 170; and #3, 185) were established from the three matings (Table 2.3.1). These were characterised for mating type and morphological characteristics.

2.3.1 Mating type

Across the three crosses, the ratio of A1:A2 mating type of the SOP was approximately 2:1 and the percentage, about 63%, A1, 28%, A2, 5% self fertile and 4% were undetermined. However, the A1:A2 the ratio differed for each cross with cross #1 3:1; cross #2 1:1; and cross #3 3:1 (percentage of A1 to A2, cross #1 69&21, cross #2 52&42, and cross #3 65&23). A total of 22 SOP from all three matings (see Table 2.3.1) had their mating type undetermined. Eight of these progeny when paired with tester isolates (A1 and A2) did not produce any reaction zone, i.e. no sexual gametangia were formed by either isolates (tester and tested) at the interaction zone.

Table 2.3.1 Number of single oospore progeny (SOP) from three matings of *Phytophthora infestans* and their mating types

Parents A1 x A2)	Cross #	A1	A2	Self fertile	Undetermined	Total
96.89.43 x 96.70	1	132 (69)[a]	40 (21)	8	10	190
96.69 x 96.70	2	88 (52)	72 (42)	4	6	170
P9463 x 96.70	3	121 (65)	42 (23)	16	6	185
TOTAL		**341**	**154**	**28**	**22**	**545**

[a] - Number in parenthesis is the percentage of A1 and A2 mating type.

Thirteen isolates when paired with the tester isolates did not grow to the mating zone even when they were placed a few millimetres from the tester isolate. When viewed under the microscope, their advancing hyphae were observed to have burst. This would appear like there was some incompatibility reaction taking place. Most of these isolates were lost and were not used for further studies. One progeny from this group of non-determined isolates, also did not produce any gametangia when paired with the tester but the medium where it was growing was always pink, an indication of oospores (or at least sexual gametangia) formation. This was unique to this isolate as all other isolates only caused the change in colour of the medium when fertilisation was taking place (D.S. Shaw, pers. comm.).

2.3.2 Morphology of SOPs

While all A1 single oospore progeny and 7% (11 of 154) of the A2 single oospore progeny had non-lumpy (-) morphology, the rest (93%) of the A2 progeny exhibited lumpiness of varying degrees. Lumpiness varied from slighty lumpy (+) (17%), lumpy (++) (56%), to being very lumpy (+++) (20%) (Table 2.3.2) and (Figure 2.3.2). There were no visible sporangiophores among the very lumpy progeny; however, sporulation (i.e. number of sporangia per unit area) was not determined. The A2 SOP were lumpy as opposed to their non-lumpy A2 parent, which was a fluffy isolate just like the A1 parents (Figure 2.3.1). Scanning Electron Microscope pictures revealed that the lumpiness was an aggregate of short, stubby incompletely dichotomously branching hyphae (Figure 2.3.3).

Table 2.3.2 Morphological characteristics of A2 mating type single oospore progeny of the three matings of *Phytophthora infestans*.

Mating (A1 x A2)	Non-lumpy (-)	*Slightly lumpy (+)*	Lumpy (++)	Very lumpy (+++)	**Total**
96.89.43 x 96.70	2	12	23	3	**40**
96.69 x 96.70	7	10	35	20	**72**
P9463 x 96.70	2	4	29	7	**42**
Total	**11**	**26**	**87**	**30**	**154**

Figure 2.3.1. *In vitro* cultures of *P. infestans* parental isolate 96.69 (A1) (left) and isolate 96.70 (A2) (right), and an A2 single oospore progeny (centre) showing very lumpy phenotype.

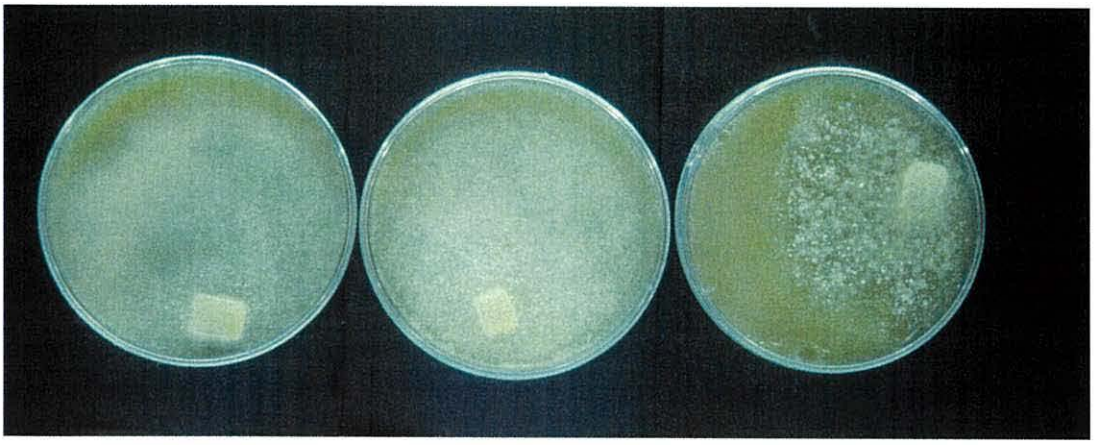


Figure 2.3.2. Three single oospore A2 progeny of *P. infestans* showing the different degrees of lumpiness. Slightly lumpy (+) (left), lumpy (++) (centre), and very lumpy (+++) (right).

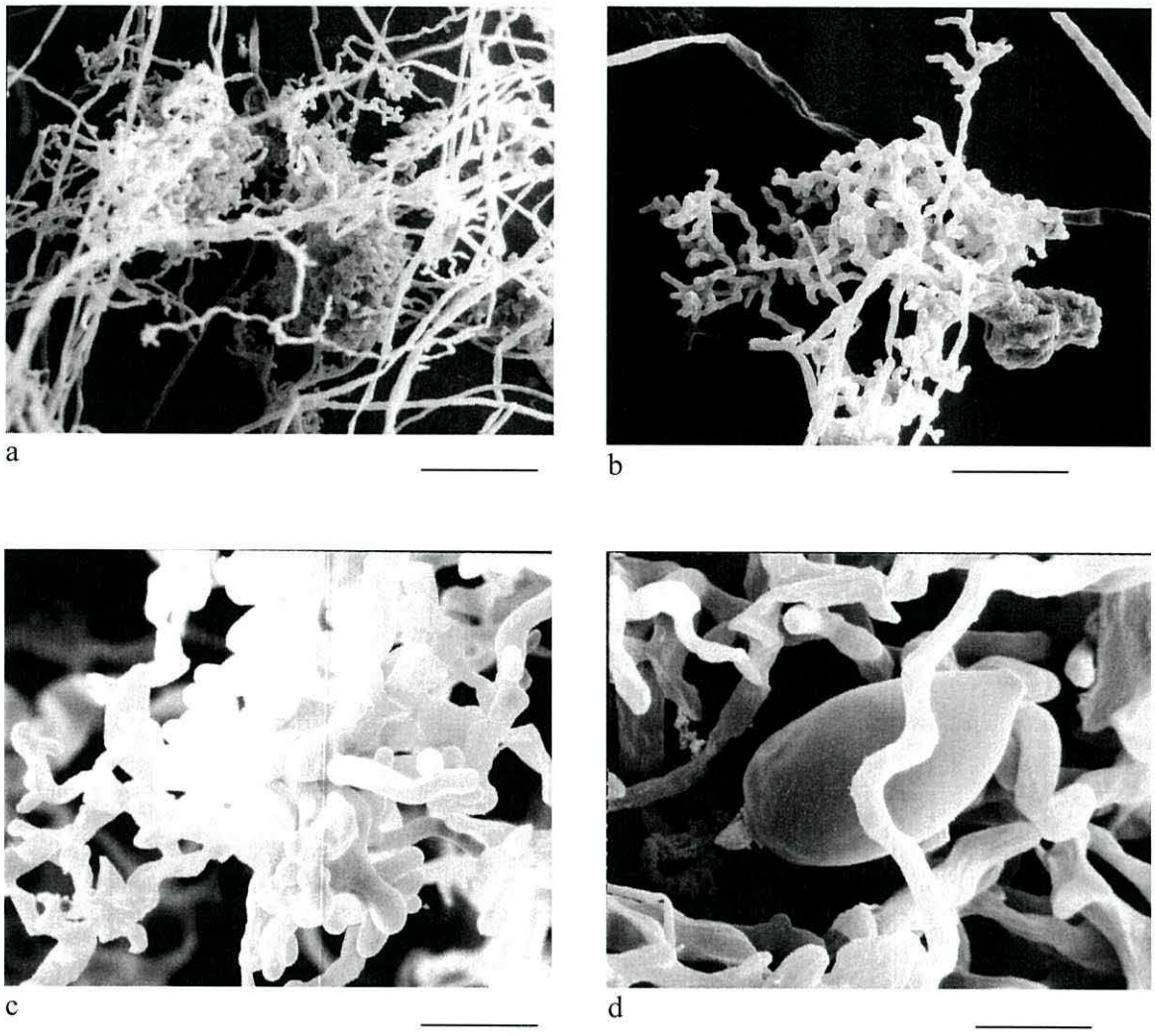


Fig 2.3.3 Scanning electron micrographs showing lumps from an A2 mating type single oospore progeny of *Phytophthora infestans* which was rated as very lumpy (+++).
a - showing the curling hyphae which give rise to the lumps (bar = 50 µm)
b - showing the profuse branching of hyphae (bar = 25 µm)
c - a close-up of the profuse branching of short hyphae (bar = 10µm), and
d - another close-up of the curled short hyphae with a sporangium (bar = 7.5 µm)

2.4 DISCUSSION

In the past, many researchers have not been able to obtain large numbers of single oospore progeny (see Table 2.4.1). In anticipation of such a problem, large pieces of agar were cut from the mating plates to increase the number of oospores to be extracted. However, when blended, the agar posed a problem as it clogged the filter membrane that was used to separate the oospores. As a result of not all the agar being washed off, the oospores were incubated with pieces of agar. The presence of the agar may have proved to be beneficial as it provided nutrients for the oospores. This could have been the reason why there was a high germination percentage and also why the germinating oospores gave rise to multiple germ-tube as opposed to a germ sporangium. Providing nutrients to extracted oospores was found by Ann and Ko (1988) to stimulate germination in oospores of *P. parasitica*. This was also observed by Harrison (1991), who observed that addition of clarified rye A broth to oospores increased the rate and final percentage of germination.

The majority of the germinating oospores were preceded by a swelling followed by production of multiple germ-tubes. This has been reported before (Gallegly, 1968; Romero and Erwin, 1969; Harrison, 1991). Harrison (1991) attributed the swelling of germinating oospores to be a characteristic of germinating oospores in liquid medium. However, in this current study, the oospores were not in a liquid medium yet they were still swollen and also produced multiple germ-tubes, suggesting that the presence of nutrients was responsible for the swelling and production of multiple germ-tubes. Very few of the oospores germinated without a swelling and these gave rise to a germ sporangium.

A total of 545 single oospore progeny were obtained from 3 matings. The number of single oospore progeny per mating were 190, 170 and 185 for crosses #1, #2 and #3, respectively. Only 523 were characterised for mating type and the ratio of A1:A2 was 3:1 for crosses #1 and #3, and 1:1 for cross #2. Although mating type is governed by a single locus (Judelson *et al.*, 1995), aberrant segregation of A1s and A2s is always observed (Table 2.4.1). In 1969, Romero and Erwin reported a ratio of 3:1 (A1: A2) from a total of 34 single oospore progeny. Almost twenty years later, Shattock (1988) reported a ratio of 2:1, and Al-Kherb (1988) reported several different ratios of A1: A2, with the A1 ratio predominantly higher

than the A2 (see Table 2.4.1). In some cases, however, more A2 mating type SOP than A1s have been obtained (Shattock *et al.*, 1986a; Smith, 1993; Al-Kherb *et al.*, 1995). In most cases, when the number of progeny is above a hundred the number of A1 progeny is equal or higher than that of A2 (see Table 2.4). Table 2.4 lists details of mating type of single oospore progeny from 31 matings from 12 studies, although, the list in not exhaustive of available published reports. In this study, in cross #2 there were approximately equal numbers of A1s and A2s and this has been observed also by Shaw *et al.* (1985); Shattock *et al.* (1986a); Turkensteen *et al.* (2000); and Knapova *et al.* (2002). Whether or not the ratio of mating type in sexual progeny is determined by parental phenotype e.g. degree of maleness/femaleness, genetical basis of A1/A2 mating type loci e.g. gene dosage, inhibitor genes, lethal factors, remains to be investigated.

Although in this study all three crosses yielded less than 200 single oospore progeny per cross, it is worth reporting that even though the germination rate was not measured, it was observed to have been quite high (≥50%) in all three matings. As a result of the high germination rate, less than half of the germinated oospores were recovered before the Petri dishes were filled with mycelium of germinated oospores. Combinations and placement of isolates have been reported to have a significant effect on oospore formation (Ko and Kunimoto, 1981; Kemmitt, 1993; E.G. Harper personal communication). This means isolates vary in their ability to both detect and produce α pheromones. Kemmitt (1993) observed that pheromone production was influenced by a number of parameters, e.g. age of isolate. He observed that cultures of *P. infestans* older than 12 days were superior α pheromone producers and he suggested 15-day-old cultures to be the best age.

The recent hypothesis relating to the chemical characterization of α hormones is that they are not phospholipids, glycolipids, glycerides or steroids, but more likely neutral lipids with hydroxyl functional group(s) (Chern *et al.*, 1999). Studies by Fabritius *et al.* (2002) interestingly show that certain genes regulated during sexual development in *P. infestans* produce proteins previously shown to elicit plant defence responses. These so called elicitors are known to bind lipid-like molecules (Osman *et al.*, 2001).

Table 2.4.1 Single oospore progeny of *P. infestans* and their A1: A2 ratios established by different researchers.

Date	Country	Number of progeny	*In vitro* or *in vivo*	Ratio of A1:A2	Reference
1956	USA	2	*in vitro*	All A1	Smoot *et al.*, 1958
1969	Mexico	34	*in vitro*	3:1[a]	Romero and Erwin, 1969
1985	Egypt/UK	ND	*in vitro*	1:1	Shaw *et al.*, 1985
1986	USA	361 19 7 6 113 134 55	*in vitro*	1:1 1:3 1:1 2:1 1:1 2:1 1:1	Shattock *et al.*, 1986a
1988	UK	33[b] 111[b] 43[b] 133[c]	*in vitro*	2:1 2:1 2:1 2:1	Shattock, 1988
1988	UK	158 54 60 66 71	*in vitro*	2:1 9:1 1:1 1:1 1:4	Al-Kherb, 1988; Al-Kherb 1995.
1989	Mexico	142 155	*in vitro*	1:1 1:1	Spielman *et al.*, 1989.
1992	UK	56	*in vivo*	1:2	Hanson and Shattock, 1998
1993	UK	327 194 81 14	*in vitro*	1:1 2:1 7:1 1:6	Smith, 1993
1993-94	The Netherlands	13	*in vivo*	1:1	Turkensteen *et al.*, 2000
2000	USA	53	*in vitro*	2:1	Mayton *et al.*, 2000
2002	Switzerland	43	*in vitro*	1:1	Knapova *et al.*, 2002
2002	USA	40 43 52 37	*in vitro*	1:6 1:1 6:1 3:1	Lee *et al.*, 2002
2002	France	23	*in vitro*	8:1	Oliva *et al.*, 2002

[a] only 30 progeny were tested for mating type
[b] The A1 parent was Dutch and the A2 parent is from Egypt
[c] The A1 parent was Dutch and the A2 parent is Mexican
ND – not determined

Of the 523 SOP that were characterised for mating type, 29 were self-fertile (Table 2.3.1). Although self-fertile isolates have a characteristic feature of having appressed hyphae and a shiny waxy appearance (Shattock *et al.*, 1987; Spielman *et al.*, 1990 and Shaw and Shattock, 1991), only 13 of self-fertile progeny showed the typical self-fertile isolate appearance. The remaining 16 appeared fluffy, had sporangia and few oospores that were characteristically light brown in colour. When these isolates were paired with tester isolates, they produced more oospores with both testers. Not all the self-fertile progeny remained self-fertile. Only 7 of them were still self-fertile after 24 months. All these were from the shiny waxy group. The remaining shiny waxy group (6 of them) segregated to become lumpy, a characteristic that was observed to be unique to A2 mating type progeny. Segregation of self-fertile isolates has been reported before (Fyfe and Shaw, 1992; Kemmitt, 1993). Fyfe and Shaw (1992) concluded that self-fertile isolates of *P. infestans* appeared to be intimate mixtures of A1 and A2 hyphae within which rare mating type heterokaryons may occur (Pipe *et al.*, 2000). Most of the shiny waxy group of self-fertile progeny produced very small (in size) oospores compared to those produced by two opposite mating type isolates. Their viability was not tested and efforts to germinate them were unsuccessful. Only one progeny from Cross #3, the Californian and UK cross [P9463 (A1 & Calif.) x 96.70 (A2 & UK)] progeny #70 had normal size oospores, although not very many. Very few oospores were extracted and efforts to germinate them were also not so successful. Only one putative progeny (SF_1) was recovered and it was of the A1 mating type. The other self-fertile progeny (16) that had sporangia with few oospores, when checked after 24 months, were still producing oospores when paired with either tester. It was very difficult to extract their oospores because of the small numbers. All these oospores were tested for pathogenicity and for sensitivity to fungicides (see chapter 4 and 5).

A total of 22 SOP from all three matings did not produce any sexual gametangia when paired with tester isolates. This would mean that these progeny were not able to produce any pheromones, which would have stimulated the tester isolate to produce sexual gametangia, and as a result that the tester isolates neither produced nor received any pheromones.

Characterising mycelial cultures of single oospore progeny has been reported before. Romero and Erwin (1969) described the colony characterisation of 34 putative hybrid single oospore progeny of *P. infestans*. They observed that morphologically, there were remarkable differences in colony type among the SOPs, and none closely resembled either parent. They observed a difference in growth rate in clarified V-8 agar, where some progeny made very little growth on this medium. This phenomenon has been observed in wild type isolates (Shattock *et al.*, 1990), where an A2 isolate (88/24/6) failed to grow on 10% V8 agar and was termed 'V8 shy'. None of the progeny from cross #1, #2 and #3 were grown on V-8 agar and so their V8-shyness (if any) was not determined. Romero and Erwin (1969) also reported differences in colour and texture of mycelium of some progeny to that of parents. Some progeny had white and cottony mycelium as opposed to grey and appressed mycelium of parents. In the study reported here, there were notable differences in the morphology of the progeny compared to their parents. While some progeny had colony morphology similar to their parents, i.e. white fluffy mycelium [non-lumpy (-)], some had smooth appressed mycelium while others had clusters of lumps within their colony (Figure 2.3.1). When mating type of the progeny was established, it was noted that the smooth progeny with appressed mycelium were self-fertile isolates (as discussed above).

Most of the progeny with white fluffy mycelium (non-lumpy) were found to be of the A1 mating type. Few of the fluffy progeny were found to be of A2 mating type. However, when these were viewed under a microscope, some were found to contain lumps that were very small and these were rated as being slightly lumpy (+) (Table 2.3.2). Such observation was also made by Shattock *et al.* (1990), whereby they observed that isolates of the A2 mating type had less obvious lumps when grown on pea agar than in rye A agar, where the lumps were more pronounced. However, Shattock *et al.* (1990) focussed only on characteristics of field isolates *in vitro* and not on sexual progeny from controlled matings.

Lumpiness among the A2 SOP from the 3 matings was divided into 3-levels (Figure 2.3.2): (1) slightly lumpy (+) – these were the SOP which although appeared non-lumpy when observed by naked eye, were found to contain small lumps when viewed under the microscope. The second level was the lumpy (++) – these were SOP that had visible lumps

but still produced visible sporangia. The third level was the very lumpy (+++) – these were SOP that had lumps instead of mycelium and there were no visible sporangia (sporangia were not quantified) and lumps were filled with fluids. When these isolates were grown in liquid medium (Chapter 3), they did not grown horizontally, i.e. to fill the Petri dish, but instead they formed vertical clusters around the mycelial discs used as inoculum. The accumulation of liquids by very lumpy isolates has been reported before (H. Turner, unpublished data), who showed that fluid that accumulated in the network of mycelium did not react with Sudan IV reagent and Steinmetz's reagent. This indicated that it was neither oil nor mucilage and moreover it had the same pH as the condensation in the Petri dish lid, i.e. pH5. Turner (1988) concluded that the fluid was more likely to be condensation than an exudate. This then raises the question why some isolates retain the fluid within their mycelium and some do not and why only certain isolates of the A2 mating type have this ability.

The very lumpy SOP when viewed under the light and scanning electron microscope had short stubby hyphae and even when paired with A1 isolates, the mycelium did not seem to straighten, as would be expected when two different mating types are grown side by side as a result of pheromone induced accelerated growth of A2 towards A1 isolates in paired cultures (Kemmitt, 1993). The morphological characteristics reported previously (Shattock *et al.* 1990) for A2 isolates resembled those illustrated by Shaw *et al.* (1985) who crossed Egyptian A2 isolate (E13a) with a UK A1 isolate (83/3), and observed that some of the single oospore progeny exhibited dichotomously branching hyphae.

Oospores can be produced not only through fertilization of two compatible mating types (Ko, 1978), but also through stimulation by other species of *Phytophthora* e.g. *P. drechsleri* (Skidmore *et* al., 1984; Shattock, 1986a), and through other non related fungi e.g. *Trichoderma* (Brasier, 1975, as cited in Campbell and Duncan, 1985; Brasier, 1978).

Quite a number of researchers have been able to produce and study oospores in the lab (see Table 2.4.1). In most of the studies, two strips of agar containing A1 and another containing A2 were placed side by side, providing a 1:1 ratio of A1 to A2. However, inoculum of A1 and A2 need not be present at equal proportions for oospores to be produced, as the ratio

between A1/A2 sporangia has a minor effect on the number of oospores produced (Cohen *et al.*, 1996). The authors reported extreme proportions of A1/A2 statistically possible for oospore formation to be 1 sporangium of A1 and 19 sporangia of A2 and vice versa, i.e., ratio of 5:95 to 95:5. With such extreme proportions, they were able to obtain between 175 to 232 and 272 and 207 oospores per 8 mm^2 leaf disc on potato and tomato, respectively. Such high numbers of oospore were probably produced through selfing of each isolate, as the few sporangia of the opposite mating type would produce either K^1 (by A1) or K^2 (by A2) sex pheromones (Ko, 1978 & 88) which would stimulate formation of gametangia and then fertilization (Smoot *et al.*, 1958).

In conclusion, the three matings produced large numbers of SOPs contrary to the majority of mating studies previously published. The phenotypic characteristics of the 3 sets of progeny were not atypical of previous reports and like those that did not shed light on reasons of uneven mating type ratios nor the lumpiness of A2 isolates. The data do show that large progeny sets can be obtained but whether this is determined by choice of parents, techniques or chance remains unresolved.

Figure 2.4.1. A photo of tubers with an A2 isolate showing lumpiness as indicated by the arrows. Courtesy of J. E. Pittis (unpublished).

3.0 MOLECULAR MARKERS FOR CONFIRMATION OF HYBRIDITY OF SEXUAL PROGENY OF *PHYTOPHTHORA INFESTANS*

3.1 INTRODUCTION

Asexual reproduction has played a major role in the life history of *P. infestans*, especially outside Mexico where only, until recently, one mating type was found (Fry *et al.*, 1993). Since the discovery of the A2 mating type outside Mexico, sometimes in low proportions (Day and Shattock, 1997; Lebreton *et al.,* 1998), and sometimes is high proportions compared to the A1 mating type (Hermansen *et al.*, 2000; Turkensteen *et al.*, 2000), sexual reproduction has been suspected.

When an A1 and an A2 mating type isolates come in contact, they each produce both sexual gametangia, which later fuse to form hybrid oospores (Judelson, 1997a). Antheridia and oogonia from the same parent may also fuse, resulting in selfing. When the oospores germinate, they give rise to either A1 or A2 and sometimes to self-fertile progeny.

Sexual reproduction brings about new genotypes through recombination of genes of both parents. Before the implication and importance of sexual progeny can be fully understood, there is a need to ascertain the hybridity of the progeny. There are several procedures that can be used to prove hybridity, e.g. allozymes (Tooley *et al.*, 1985), mtDNA (Carter *et al.*, 1990), RG57 fingerprint (Goodwin *et al.*, 1992a), RAPDS (Williams *et al.*, 1990), AFLPs (Vos *et al.*, 1995), etc. Some of the techniques are not sensitive enough e.g. allozymes cannot be used if both parents have the same allozymes, and some are time consuming e.g. AFLPs, making it almost impossible to screen large quantities of progeny. Therefore, there is a need to use simple but conclusive techniques. If one technique is insufficient, more than one technique can be used to confirm the results. Allozyme has been mostly used to prove hybridity of single oospore progeny, however, where the allozyme is similar among the parents, proving hybridity might not be possible.

The probe RG57 is a 1.2 kb genomic fragment derived from a *Phytophthora infestans* genomic library and represents a moderately repetitive nuclear DNA sequences (Goodwin *et*

al., 1992a). It may hybridise to 25 or more *Eco*RI restricted different nuclear DNA fragments. Of the hybridizing fragments 13 are known to segregate independently. The fragment patterns are somatically stable and are transmitted to sexual progeny in a Mendelian fashion. Since it is a dominant marker, bands from homozygotes (+/+) are rarely distinguishable from those of heterozygotes (+/-). RG 57 fingerprint is therefore a phenotype. RG57 probe has been used extensively to investigate genetic diversity of *P. infestans* populations in many countries (Forbes *et al.*, 1998; Day *et al.*, 2004).

Random amplified polymorphic DNA (RAPDs) markers have been used to study genetic structure of fungi. It has been used to study *Pyrenophora teres* (Drechs.) (Peever and Milgroom, 1993); to study population structure of *Beauveria bassiana* (Vuill.) (Couteaudier and Viaud (1997) and of *Phomopsis subordinaria* (Desm.) Trav. (Meijer *et al.*, 1994). RAPDs have also been used to study hybridity in *Phytophthora.* Linde *et al.* (2001) used RAPDs to distinguish hybrids from selfs among single oospore progeny of *Phytophthora cinnamomi.* They have also been used to study genetic variation among asexual progeny of *P. infestans* (Abu-El Samen *et al.*, 2003).

The aim of this chapter was to establish the hybridity of the single oospore progeny from 3 crosses using molecular markers, namely, RAPDS, AFLPs and SNPs.

3.2 MATERIALS AND METHODS

3.2.1 DNA extraction

3.2.1.1 Mycelium for DNA Extraction

Petri dishes (9 cm) containing pea-broth (Appendix 1) and ¼ strength RAN were inoculated with 3 or 4 plugs of hyphae cut from the margins of the colony. Plates were incubated for approximately 10 days at 18 ^{0}C. Mycelium was harvested and the culture medium removed by filtering through No. 1 filter paper 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, about 10 mL of liquid nitrogen was added into each tube. A glass rod was used to break up the mycelium and the tubes were vortexed for 30 seconds until the mycelium was shattered into a fine powder.

3.2.1.2 DNA Extraction Protocol

The following methods were adapted from those of Raeder & Broda (1985). Ground, lyophilised mycelium from two Petri dishes was re-suspended in 3.3 ml sterile DNA extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Samples were homogenised by gentle inversion to give a smooth slurry and incubated at 55 ^{0}C for 30 min., 2.2 mL of liquefied TRIS-buffered phenol (Fisher) was added, followed by 3mL chloroform-isoamyl alcohol (24:1) (BDH). Tubes were then centrifuged at 15,000 rpm (Beckman) for 60 min at 4 ^{0}C. The aqueous layer was removed to a fresh tube and 1mg RNAse A (Sigma) was added to each tube followed by incubation at 37 ^{0}C for 30 min. Chloroform-isoamyl alcohol (24:1) (3.3 mL) was then added and the tubes centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. To the aqueous layer, 0.54 volumes of isopropanol were added and the tubes left for up to 60 min at room temperature or overnight at (minus) -20 ^{0}C. Precipitated DNA was centrifuged at 10,000 rpm for 10 min at 4 ^{0}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.

3.2.2 Randomly Amplified Polymorphic DNAs

Randomly amplified polymorphic DNAs (RAPDs) were generated using the polymerase chain reaction (PCR) with random oligonucleotide primers of 10 nucleotides in length (Williams *et al.*, 1990). PCR was performed in 10 µL reaction volumes in 0.2 mL tube plates (Helena BioSciences) using an Eppendorf Mastercycle gradient. Reaction mixtures contained 1 X buffer, 200 µM dNTPs, 3nM $MgCl_2$, 400 nM primer (Operon Technologies), 0.5U *Taq* DNA polymerase (Promega) and 3ng *P. infestans* DNA. The amplification PCR programme consisted of 1 cycle of 94 ^{0}C for 2 minutes, 35 cycles of 94 ^{0}C for 20 seconds, 45 ^{0}C for 20 seconds, 72 ^{0}C for 20 seconds, and 1 cycle of 72 ^{0}C for 3 minutes and 30 seconds. After PCR, fragments generated by amplification were separated according to size on 1.5% agarose gel run in 0.5 X TBE (45 mmol/L Tris, 45 mmol/L Boric acid, 0.02 mmol/L EDTA pH 8), for 2 hours at 100V. The gel was then stained with ethidium bromide (3 µL/mL), and visualised by illumination with ultra violet light.

A total of 28 primers were screened (Table 3.2.1). Twelve primers were found to be polymorphic; four of which gave reproducible results and were used for the study.

Table 3.2.1. RAPD primers screened to identify *F1* hybrid progeny by the presence of bands exhibited by the parental *Phytophthora infestans* isolates.

Primer	Sequence 5' to 3'	Primer	Sequence 5' to 3'
OPA-04	AATCGGGCTG	OPE-4	GTGACATGCC
OPA-10	GTGATCGCAG	OPE-10	CACCAGGTGA
OPA-11	CAATCGCCGT	OPG-11	TGCCCGTCGT
OPA-17	GACCGCTTGT	OPG-14	GGATGAGACC
OP-A18	AGGTGACCGT	OPG-15	ACTGGGACTC
OPA-20	GTTGCGATCC	OPG-16	AGCGTCCTCC
OPB-08	GTCCACACGG	OPS-14	AAAGGGGTCC
OPB-09	TGGGGGACTC	OPS-20	TCTGGACGGA
OPB-13	TTCCCCCGCT	OPX-12	TCGCCAGCCA
OPB-15	GGAGGGTGTT	OPY-01	GTGGCATCTC
OPB-17	AGGGAACGAG	OPY-05	GGCTGCGACA
OPB-19	ACCCCCGAAG	OPY-07	AGAGCCGTCA
OPB-20	GGACCCTTAC	OPY-11	AGACGATGGG
OPE-03	CCAGATGCAC	OPY-15	AGTCGCCCTT

Source: Operon Technologies, Alameda, CA, USA.

3.2.3 AFLP DNA Fingerprinting

Genomic DNA (0.5 :g) was digested with two restriction enzymes, *Eco*RI (Promega) and *Mse*I (New England Biolabs). *Eco*RI, cuts at sites with the sequence 5'-G˅AATTC-3' and *Mse*I cuts more frequently at the sequence 5'-T˅TAA-3'. Oligonucleotide adaptors and primers were synthesised by GIBCO BRL (Table 3.2.2).

3.2.3.1 Restriction digestion and ligation of adaptors

Genomic DNA (0.5 µg) was mixed with 10 U of *Eco*RI and 8 U of *Mse*I in 30 µL volumes containing 1 x multi-core buffer (Promega) (25 mM Tris-Ac, 100 mM KAc, 10 mM MgAc, 1 mM DTT) and incubated for 3 h at 37 ^{0}C. After digestion for 3 h at 37 ^{0}C, 3 mL of the digest was loaded into a 1% gel and run for 30 minutes to confirm digestion.

The adaptors were prepared from single stranded to double stranded by preparing 50-:M stock of each of the *Mse* forward and reverse adaptors and 5-:M stock of each of the *Eco*RI forward and reverse adaptors. The forward and reverse of each adaptor were mixed at equal volumes, i.e. F+R of *Mse*, F+R of *Eco*RI. These were then annealed, to make double stranded, at 65 ^{0}C for 10 min, 37 ^{0}C for 10 min, 25 ^{0}C for 10 min and then stored at –20 ^{0}C until used.

Into the digest, 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 ^{0}C to allow adaptor ligation.

Table 3.2.2. 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	E19 + GA
	*Mse*I	M40 + AGC; M32 + AAC

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

3.2.3.2 Pre-amplification PCR

Each reaction contained 30 ng of both the *Eco*RI and the *Mse*I universal primers, 0.5 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 22 µL. 3 :L of the digested and ligated DNA was added into the reaction mix. The pre-amplification PCR programme consisted of 30 cycles of 94 ^{0}C for 30 s, 60 ^{0}C for 30 s and 72 ^{0}C for 60 s. The pre-amplified product was checked on 1% agarose gel (TBE) for a smear of digested, amplified genomic DNA. Twenty-five 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.

3.2.3.3 Primer labelling

The *Eco*RI selective primer was 5'end-labelled with $\gamma[^{33}P]$-ATP (Amersham) using T4 polynucleotide kinase (PNK) (GIBCO BRL). Labelling 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), 0.125 U T4 PNK, 0.05 µL $\gamma[^{33}P]$-ATP (10 µCi/:L) (Amersham) and H_2O up to a final volume of 0.5 µL. Labelling was performed at 37 ^{0}C for 1 h and was stopped by heating to 70 ^{0}C for 10 min and it was stored at 4 ^{0}C.

3.2.3.4 AFLP amplification with selective primers

Each PCR reaction contained 15 ng of the *Mse*I selective primer, 200 µM dNTPs, 0.5 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 labelled *Eco*RI primer, 12.5 :L of unlabelled *Eco*R primer, 3 µL of template DNA and H_2O up to a final volume of 15 µL. The selective amplification PCR programme consisted of 1 cycle of 94 ^{0}C for 30 s, 65 ^{0}C for 30 s and 72 ^{0}C for 60 s followed by 11 cycles over which the annealing temperature was decreased by 0.7 ^{0}C per cycle, followed by 23 cycles of 94 ^{0}C for 30 s, 56 ^{0}C for 30 s and 72 ^{0}C for 60 s.

After PCR, samples were immediately mixed with 15 µL of formamide loading buffer (98% formamide, 10mM EDTA, 1% bromophenol blue and 1% xylene cyanol) and denatured at 95 ^{0}C for 5 min, before placing immediately on ice.

3.2.3.5 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, 250 µL of 10% ammonium persulphate (Sigma) and 100 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. 6 µL of each denatured sample was then loaded onto the gel. Electrophoresis was performed at 100-110 W (50 ^{0}C constant temperature) for 3 h. The gel was dried onto Whatman 3 MM paper using a vacuum gel dryer (BIORAD) at 80 ^{0}C and exposed to high-sensitivity Kodak XOMAT-AR film for 96 h. Films were developed manually using Kodak photochemicals.

3.2.4 Ras related gene

Ras involves amplification of template DNA, which is then restricted using two enzymes: *Xba*I and *Nru*I (New England BioLabs). The *Xba*I restricts the 280 bp fragment at 70 bp, if there is the recognition site:

5'...T▼CTAGA...3 '

3'...AGATC▲T...5'

and *Nru*I which further restrict the 210 bp fragment at 104 bp if there is a recognition site:

5'...TCG▼CGA...3'

3'...AGC▲GCT...5'

This restriction results in recognition of 3 alleles: RaRa (70 bp and 210 bp bands), RbRb (70 bp, 34 bp and 176 bp [210 bp restricted]), and RaRb (70 bp, 34 bp, 176 bp and 210 bp).

3.2.4.1 DNA amplification

PCR reaction mixtures (10 µL final volume) contained 1 X buffer, 200 µM dNTPs, 3 nM $MgCl_2$, 400 nM primer (Table 3.2.3), 0.5 units *Taq* DNA polymerase (Promega) and 3 ng of DNA. The amplification PCR programme consisted of 1 cycle of 94 ^{0}C for 2 minutes, 35 cycles of 94 ^{0}C for 45 seconds, 55 ^{0}C for 45 seconds, 72 ^{0}C for 45 seconds, and 1 cycle of 72 ^{0}C for 3 minutes and 30 seconds. After PCR, amplification was tested by running 2 :L of product in 2.5% gel, which was stained with ethidium bromide (3 µL/mL), and visualised by illumination with ultra violet light.

Table 3.2.3. Sequence of the primer used to amplify the genomic DNA for the Ras related gene

Primer	Sequence
Forward	5'-CAT CTC GAC CAT CGG TGT TGA-3'
Reverse	5'-AAC TGC TAG TGA TCG TGC GGA-3'

3.2.4.2 PCR product digestion

The remaining 8 :L of PCR product was mixed with 12 :L of restriction mix to give a total volume of 20 :L. The restriction mix consisted of 1 x Buffer 3, 1 mg mL^{-1}, *Nru*I 2U, *Xba*I 2U and ultra pure sterile water. The mixture of PCR product and restriction mix were sealed and incubated at 37 ^{0}C for 4-5 hours. The restriction product was then evaporated. 10 :L of Ras loading buffer (Appendix 3) was added into the evaporated product. The restricted fragments were separated on 2.5% agarose gel run in 0.5 X TBE (45 mmol/L Tris, 45 mmol/L Boric acid, 0.02 mmol/L EDTA pH 8) for 2-3 hours. The gel was stained with ethidium bromide (3 µL/mL), and visualised by illumination with ultra violet light.

3.3 RESULTS

3.3.1 Randomly Amplified Polymorphic DNA (RAPDs)

A total of 28 primers were screened against the four parental isolates to find primers that were polymorphic and were producing reproducible results. Of the 28 primers, 24 produced DNA products (bands) some of which were polymorphic, some were monomorphic and some were not reproducible between experiments. The remaining 4 primers produced no bands at all or bands that were too faint. Eight primers that produced strong reproducible bands were selected for the study (Table 3.3.1). Five primers: 04 and 10 from the A-kit, 04 from the E-kit, 16 from the G-kit and 05 from the Y-kit produced polymorphic bands that were also reproducible among all three crosses. Three remaining primers: 20 from the A-kit, 01 and 07 from the Y-kit, produced at least one polymorphic band in at least one of the two parents in each cross (Figure 3.3.1).

Table 3.3.1 Details of random decamer oligonucleotide primers used in this study and the number of amplified loci in 4 parental isolates of *P. infestans*

Primer Code	Nucleotide sequence (5' to 3')	Number of amplified loci in parents			
		96.89.43	96.69	P9463	96.70
OPA-04	AATCGGGCTG	5(2)[a]	3(1)	7(2)	5(1)
OPA-10	GTGATCGCAG	9(2)	11(2)	9(2)	12(4)
OPA-20	GTTGCGATCC	3(1)	3(1)	3(3)	2(0)
OPE-04	GTGACATGCC	7(3)	7(1)	8 (3)	6 (4)
OPG-16	AGCGTCCTCC	4(2)	6(1)	8(3)	5(0)
OPY-05	GGCTGCGACA	8(2)	7(1)	12(4)	9 (2)
OPY-01	GTGGCATCTC				
OPY-07	AGAGCCGTCA				

a- value in parenthesis is the number of polymorphic loci

3.3.1.1 Single oospore progeny of cross #1 (96.89.43 x 96.70)

Cross #1, the UK A1 96.89.43 (RF 032) crossed with the UK A2 96.70 (RF 040) had 190 single oospore progeny (132, 40, 8 and 10; A1, A2, selfs and undetermined, respectively) (Chapter 2).

DNA was extracted from 187 SOP, three others did not grow enough to enable large scale DNA extraction. For the progeny of this cross, a total of 4 primers were used to test for hybridity, these were OP- A4, E4, G16 and Y7. The primer OP-G16 was the best primer (Figure 3.3.1).

From the 184 SOP that were tested for their hybridity, 4 contained RAPDs bands similar to the A2 parent when amplified with all the primers. This suggested that they were selfs of the A2 parent.

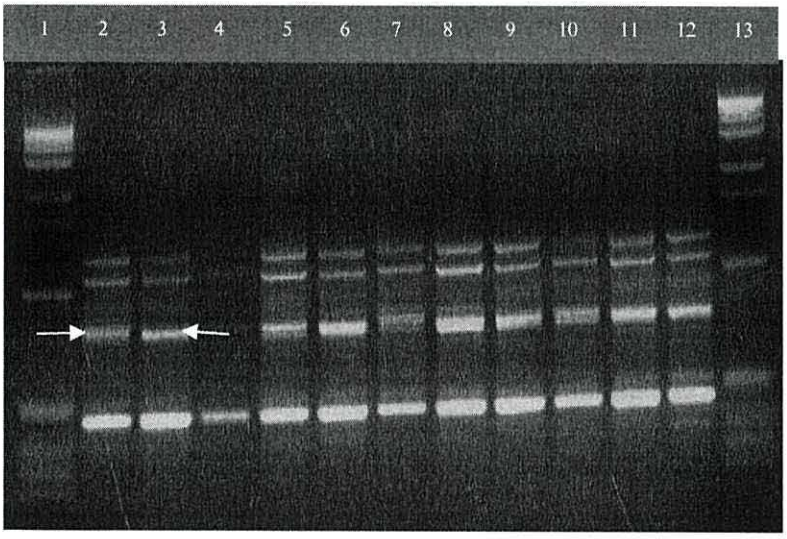
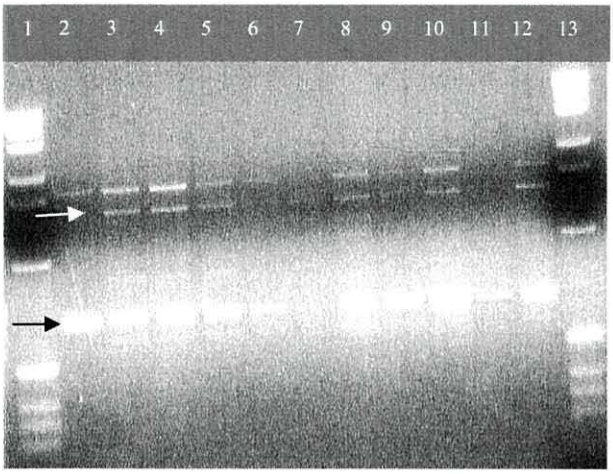


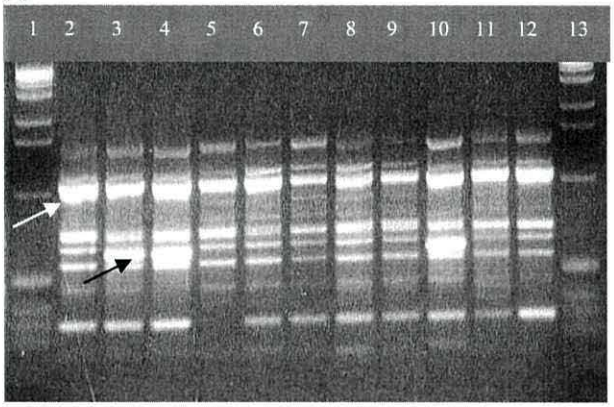
Figure 3.3.1. RAPDs fingerprint of parental isolates 96.89.43 and 96.70, and *F1* hybrids progeny of *P. infestans* using RAPD primers OP-G16. Lanes 1 & 13 are 1kb markers, lane 2 is the A2 parent (96.70), lane 3 is the A1 parent (96.89.43). Lanes 4 to 12 are single oospore progeny 5, 188, 17, 58, 81, 99, 112, 142, and 179. Progeny 5 and 188 (lanes 4 and 5) were confirmed using AFLPs (Section 3.3.2). Polymorphic bands are indicated by arrows

3.3.1.2 Single oospore progeny of cross #2

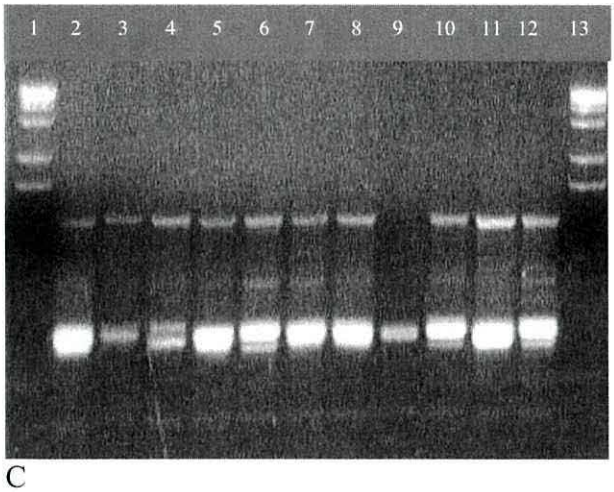
Cross #2, the UK A1 96.69 (RF 039) crossed with the UK A2 96.70 (RF 040) had 170 single oospore progeny (88, 72, 4 and 6; A1, A2, selfs and undetermined, respectively). From a total of 170 single oospore progeny, only 1 isolate did not grow well to have enough mycelium to extract DNA. Six RAPDs primers were used to prove hybridity of the progeny in this cross: OP-A4, A10, A20, E4, G16 and Y5 (Figure 3.3.2 a, b and c). All the progeny were proven to be true hybrids. All but 15 progeny produced RAPDs bands from both parents. The remaining 15 sometimes had bands from the A1 parent in one primer and bands from the A2 parent in another primer. These were also characterised as being true hybrids.



A



B

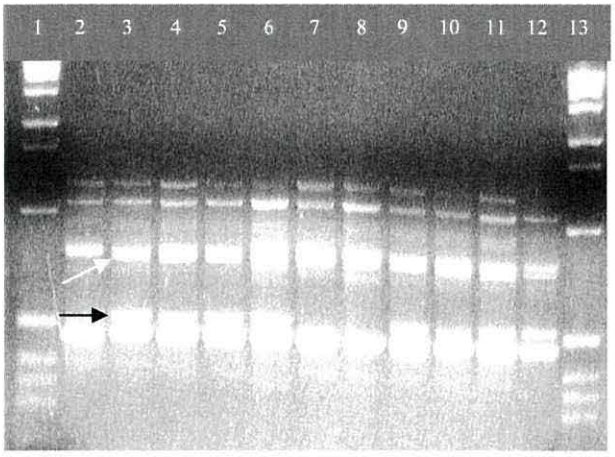


C

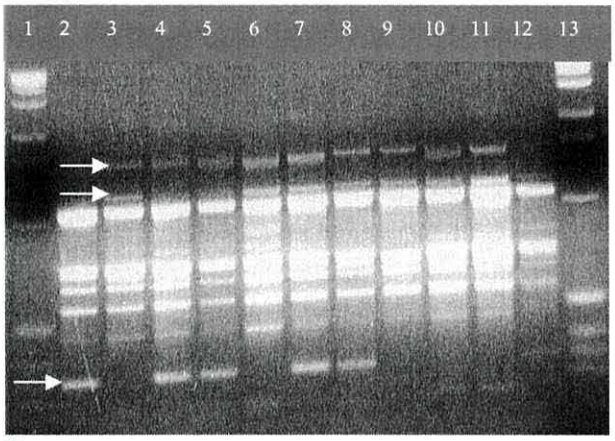
Figure 3.3.2. RAPDs fingerprint of parental isolates [96.70 (A2) and 96.69 (A1)] and 9 *F1* hybrids progeny of the two parents of *P. infestans* using RAPD primers OP-A20, OP-Y05, and OP-A4 for A, B and C, respectively. Lanes 1&13 are 1kb markers, lane 2 is the A2 parent, and lane 3 is the A1 parent. Lanes 4 to 12 are single oospore progeny 40, 103, 88, 169, 65, 92, 108, 132, and 136. Progeny 40 and 103 (lanes 4 and 5) were confirmed using AFLPs (Section 3.3.2).

3.3.1.3 Single oospore progeny of cross #3

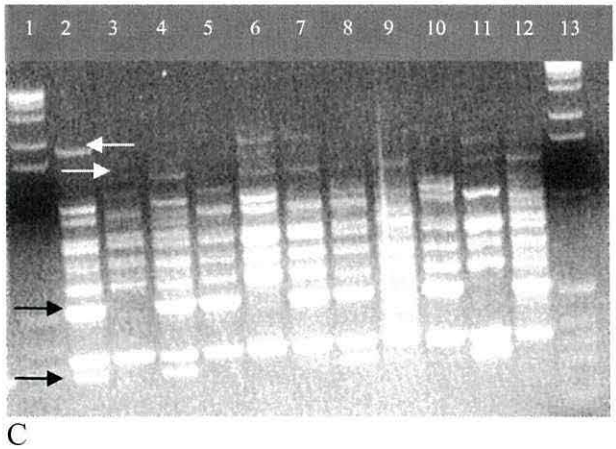
Cross #3, the Californian A1 P9463 (RF undetermined) crossed with the UK A2 96.70 (RF040), had 185 single oospore progeny (121, 42, 16 and 6; A1, A2, selfs and undetermined, respectively). All progeny had their DNA extracted and were tested for hybridity. A total of 6 RAPDs primers were used to test hybridity of progeny in this cross: A4, A10, A20, E4, G16 and Y5 (Figure 3.3.3a, b and c). All progeny were proven to be true hybrids



A



B



C

Figure 3.3.3. RAPDs fingerprint of parental isolates [96.70 (A2) and P9463 (A1)] and 9 *F1* hybrids progeny of the two parents of *P. infestans* using RAPD primers OP-G16, OP-Y05, and OP-A10 for A, B and C, respectively. Lanes 1&13 are 1kb markers, lane 2 is the A2 parent, and lane 3 is the A1 parent. Lanes 4 to 12 are single oospore progeny 71, 72, 12, 44, 89, 103, 148, 153, and 166. Progeny 71 and 72 (lanes 4 and 5) were confirmed using AFLPs (Section 3.3.2).

3.3.2 Amplified Fragment Length Polymorphism (AFLP)

Only 6 single oospore progeny (2 from each cross and indicated in legend to RAPD gels) and the four parents were fingerprinted for AFLP using two combinations of selective primers: i.e. E19 & M40, and E19 and M32. The first primer combination (E19 + M40) yielded more polymorphic bands that were traceable to the progeny (Figure 3.3.4). The second primer combination (E19 +M32) did not yield as many polymorphic bands. The bands were visually scored (Table 3.3.2)

Table 3.3.2 Number of scorable AFLPs bands of four parental isolates and six single oospore progeny of *P. infestans*.

Isolate	Mating Type	AFLP bands[a] from 2 primer combos	
		E19-M40[b]	E19-M32
96.70	A2	70 (7)[c] (6)[d] (11)[e]	42 (3)[c] (2)[d] (12)[e]
96.89.43	A1	66 (5)[f]	37 (5)[f]
96.69	A1	56 (7)[g]	41 (8)[g]
P9463	A1	58 (9)[h]	19 (2)[h]
Cross #1 SOP 5	A1	57 (3)[i] (2)[j]	28
Cross #1 SOP 188	A2	64 (4) (5)	39
Cross #2 SOP 40	A2	68 (3) (2)	39
Cross #2 SOP 108	A2	64 (6) (3)	41
Cross #3 SOP 71	A1	71 (4) (9)	36
Cross #3 SOP 72	A2	66 (4) (7)	24

a- only strong bands were scored
b- numbers in parenthesis are polymorphic bands
c- unique bands of the A2 parent which are not found in the A1 parent 96.89.43
d- unique bands of the A2 parent which are not found in the A1 parent 96.69
e- unique bands of the A2 parent which are not found in the A1 parent P9463
f- unique bands not found in the A2 parent
g- unique bands not found in the A2 parent
h- unique bands not found in the A2 parent
i – unique bands inherited by the progeny from its A1 parent
j –unique bands inherited by the progeny from its A2 parent

Figure 3.3.4. AFLP fingerprints of the four parents of *Phytophthora infestans* and six single oospore progeny (two from each cross). First and last lanes are a 1kb marker, the next 10 lanes are all the parents and 6 single oospore progeny: first 4 lanes (after marker) are the parents P9463, 96.89.43, 96.70 and 96.69, respectively. Lane 6 and 7 are progeny from cross #2 (96.69 x 96.70), lane 8 and 9 are progeny from cross #1 (96.89.43 x 96.70) and lane 10 and 11 are progeny from cross #3 (P9463 x 96.70). Lanes 2 to 11 are amplified using the selective primers E19-M40. Lanes 12 to 21 are the same isolates as in lane 2 to 11 but amplified with selective markers E19-M32 (unique inherited bands not outlined). Some of the unique bands of each parent inherited by the progeny are indicated by arrows and are colour coded for each parent. Black (sometimes white) for A2 parent 96.70; green for A1 parent 96.89.43; blue for A1 parent 96.69; and red for A1 parent P9463.

3.3.3 SNP Ras related gene

The Ras related protein PiYpt1 (ypt1) gene in *P. infestans* is involved in vesicle transport (Chen and Roxby, 1996). According to the National Centre for Biotechnology Information (NCBI), it has 2202 base pairs, and has the accession code U30474. Single nucleotide polymorphic (SNP) marker was developed at the University of Wales, Bangor (R. Palmer, unpublished data). The sequence of the primers is in Table 3.2.3. The forward primer occurs at 962 bp within the Ras gene. And the restriction site sequence of *Nru*I is 1062-1066 bp.

The parental isolates were heterozygous (RaRb) for the Ras related gene (Figure 3.3.5). Sub-samples of oospore progeny were screened for inheritance of the Ras related gene (Figure 3.3.6). From a sub-sample of 47 putative oospore progeny from cross #1, 4 were homozygous for the Ra genotype, 28 were heterozygous (RaRb) and 15 were homozygous for the RbRb genotype (Table 3.3.3). From a sub-sample of 28 putative single oospore progeny from cross #2, 2 were homozygous for the Ra, 19 were heterozygous, and 7 were homozygous for the RaRb genotype. And from a sub-sample of 57 putative single oospore progeny, 5 were homozygous for RaRa, 34 were heterozygous, and 18 were homozygous for RbRb genotype. The ratios for RaRa to RaRb to RbRb in each of the three crosses were 1:7:3, 1:10:4, and 1:7:4, for cross #1, 2 and 3, respectively.

Table 3.3.3 The Ras genotype of single oospore progeny of *Phytophthora infestans*

Parents	***Genotype of progeny***			
(A1 x A2)	**RaRa**	**RaRb**	***RbRb***	***Total***
96.89.43 x 96.70	4 (1)[a]	28 (7)	15 (4)	47
96.69 x 96.70	2 (1)	19 (10)	7 (4)	28
P9463 x 96.70	5 (1)	34 (7)	18 (4)	57
Total	11 (1)	81 (8)	40 (4)	132

a- number is parenthesis is the approximate ratio of RaRa to RaRb to RbRb

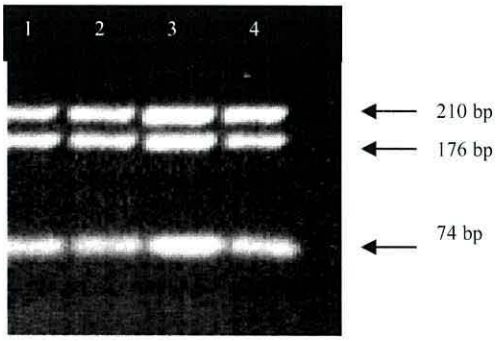


Figure 3.3.5 Ras related gene fingerprint of parental isolates of *P. infestans*. Lane 1 is the UK A2 parent 96.70; lanes 2 and 3 are the UK A1 parents 96.89.43 and 96.69; and lane 4 is the Californian A1 parent P9463.

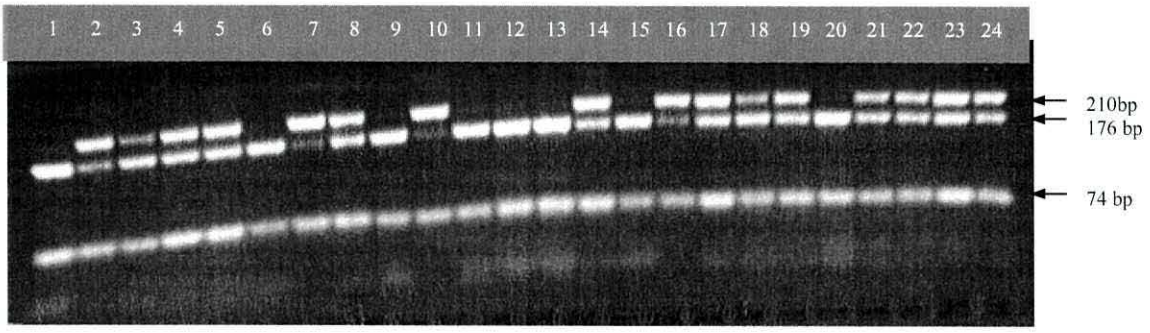


Figure 3.3.6 Ras related gene fingerprint of 24 single oospore progeny of *P. infestans*. The gel shows the 3 different fingerprints: RaRa, lanes 7 and 10; RaRb, lanes 2, 3, 4, 5, 8, 14, 16, 17, 18, 19, 21, 22, 23 and 24; and RbRb, lane 6, 9, 11, 12, 13, 15 and 20.

3.4 DISCUSSION

The aim of this chapter was to prove hybridity of the single oospore progeny of the three crosses of *P. infestans*. Most sets of previously established oospore-derived progeny are hybrids (e.g. Shattock *et al.*, 1986a; Al-Kherb *et al.*, 1995; Judelson, 1997b), although some may arise through self-fertilisation or apogamy (Shaw, 1991). Several molecular markers were investigated to find the best technique to prove hybridity.

In this study, a total of 28 decamer primers were screened to find primers that would be able to show polymorphism between the A1 and A2 parents. Although 24 of these primers did yield bands, only 8 yielded reproducible bands and were used for the study. It is uncommon for a large number of RAPDs primers to be screened and only a small number end up being suitable. Mahuku *et al.* (2000) screened 40 primers when studying genetic diversity of isolates of *P. infestans* in Canada. They only found 6 that exhibited polymorphism and yielded consistent and easily scorable banding patterns. Linde *et al.* (2001) used 55 primers and only found 7 to be able to distinguish hybrids and selfs from parental isolates of *P. cinnamomi*. However, in a study to determine genetic variation among asexual progeny from two isolates of *P. infestans* (Abu-El Samen *et al.*, 2003), over 50 primers (out of 80 screened) produced reproducible polymorphic products from both groups of progeny. The choice of the primers that were screened in this study was based on Mahuku *et al.* (2000), Linde *et al.* (2001), and L. Hill (unpublished work, University of Wales, Bangor).

In cross #1, out of the 184 (of 190) single oospore progeny that were tested for hybridity, all but for 4 were found to be true hybrids. The remaining four had fingerprints of the A2 parent (96.70) and were categorised as selfs of the A2 parent. In cross #2, of the 170 single oospore progeny, 155 progeny were proven to be true hybrids using six primers. The remaining 15 progeny had fingerprints of the A1 parent when tested with primer OP-A20 but had the fingerprint of the A2 parent when tested with primer OP-E4. However, these SOPs were also categorised as true hybrids. In cross #3, all 185 single oospore progeny were found to be true hybrids when tested using 6 primers.

RAPD primers amplify loci that are distributed throughout the genome of the isolate. The decamer primers can bind to any region in the genome that contain a matching sequence and generate polymorphism between two individuals, in this case the two parents. If the sexual progeny inherits that sequence, then RAPD would be useful in proving hybridity of sexual progeny. However, if mutation, be it deletion or substitution, of a base pair occurs especially in the progeny, the primer might not bind to that site and that locus would not be amplified in the progeny. To avoid this limitation, other markers should be used to confirm results of one technique. Clerc *et al.* (1998) found that using RAPDs and AFLP to calculate genetic distances gave similar results.

To confirm the results obtained through using RAPDs, a small selection of the sexual progeny together with their parental isolates, were tested using 2 different combinations of selective markers. The progeny were confirmed to be true hybrids. Based on the number of amplified bands, this technique proved to be by far the best to prove hybridity. It yielded the highest number of polymorphic bands (Table 3.3.2). AFLP markers search for polymorphism in regions of the genome containing restriction sites for the restriction enzymes used in the analysis (i.e. *Eco*RI and *Mse*I sites).

Although this protocol of AFLPs involved radioactive labelling of one of the selective primers, there are new protocols that involve fluorescence labelling of the primers. However, both protocols still require DNA of highest purity. Another disadvantage of the technique is that it is time consuming, which makes it less of a practical technique to use to prove hybridity if dealing with large number of progeny as the case was in this study.

When the parental isolates used in this study were tested for SNPs, all parents were heterozygous (RaRb). If the progeny had inherited the SNP in a Mendelian fashion, the ratio of RaRa to RaRb to RbRb would have been 1:2:1. However, the results showed aberrant rations of 1:7:4, 1:10:4 and 1:7:4, for crosses #1, 2 and 3, respectively (Table 3.3.3)

Another single nucleotide polymorphisms (SNPs) was also attempted on another gene; NiaA gene for proving hybridity. Initially, the results were promising as the 4 parental isolates seemed to have different fingerprint (Figure 3.4.1)

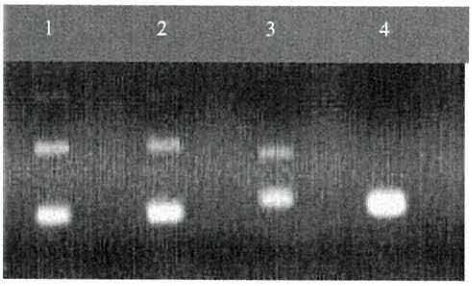


Figure 3.4.1. Microsatellite profile of the 4 parental isolates of *P. infestans* used in this study. Lane 1 is the UK A1 parent 96.69 (referred to as P1), lane 2 is the UK A2 parent, lane 3 is the UK A1 parent 96.89.43, and lane 4 is the Californian A1 parent P9463.

However, when the experiment was repeated with SOPs, the results were inconclusive as the progeny did not seem to have fingerprint similar to the parents. SNP was used however, to prove hybridity in the same lab where this study was conducted (R.M. Wattier, unpublished data, University of Wales, Bangor). However, a PCR product was not separated in an agarose gel, but instead the PCR product was sequenced.

A subset of SOPS from cross #3, i.e. progeny of the Californian A1 parent and UK A2 parent were sent to the Scottish Crop Research Institute (SCRI) where they were used to test inheritance of microsatellite markers from parents to progeny. The PCR product was also sequenced, and consequently all 20 SOPs were found to be true hybrid progeny (H. Shaukat, (SCRI) pers. comm.).

In conclusion, although there is now a wide range of molecular techniques that can be used to test hybridity of sexual progeny of *P. infestans*, there are still technical difficulties that restrict their adoption. Sequencing seems to help where predictions of ratios is impossible,

but the financial implication of sequencing can be a limiting factor if dealing with large numbers of progeny, as was the case in this study.

Taking all the evidence for the various molecular markers described in this chapter, it is appropriate to conclude that the majority of single oospore progeny from each of the crosses were hybrid. This information provides additional insight to patterns of inheritance of mating type and mycelial morphotypes (Chapter 2) and phenotypes among single oospore cultures for aggressiveness (Chapter 4) and sensitivity to various fungicides (Chapter 5).

4.0 AGGRESSIVENESS OF SINGLE OOSPORE PROGENY AND SUBSETS OF SINGLE SPORANGIUM AND ZOOSPORE PROGENY OF *PHYTOPHTHORA INFESTANS* TO SEVEN POTATO CULTIVARS

4.1 INTRODUCTION

Since the discovery of the A2 mating type outside Mexico (Hohl and Iselin, 1984), the centre of origin of *P. infestans* (Fry *et al.*, 1993), sexual reproduction is now believed to occur in many regions, bringing new challenges for controlling late blight (Drenth *et al.*, 1994; Peters *et al.*, 1998; Daayf and Platt, 2000). Sexual reproduction enhances fitness through recombinant genotypes that may be more pathogenic or resistant to fungicides compared with their parents. The discovery of the A2 mating type in Europe coincided with the appearance of 'new' A1 genotypes, thought to have originated from an illegal importation of tubers for consumption into Europe from Mexico in 1976 (Niederhauser, 1991; Drenth *et al.*, 1993). Before the A2 mating type was discovered outside Mexico, only the A1 mating type was found around the world: Goodwin *et al.* (1994) proposed the latter was of a single common clonal lineage, US-1, and believed to have been present for many years, possibly since the 1840s. This view has recently been challenged by examination of herbarium material (Ristaino *et al.*, 2001).

Production of oospores by *P. infestans* might result in oospores being an important source of inoculum because they have the longest survival period for all infective propagules (Erwin and Ribeiro, 1996) and can survive a wide range of temperatures, from freezing temperatures to 40 ^{0}C (Fay and Fry, 1997). Mayton *et al.* (2000) observed that oospores could survive for up to 18 months in different conditions including the soil. However, Pittis and Shattock (1994) observed that although oospores survived up to 10 months in soil, they did not germinate. Sexual recombination might generate new genotypes that are particularly aggressive (Fry and Goodwin, 1997). In Sweden, oospores have been reported as primary inoculum in a field of potatoes (Anderson *et al.*, 1998).

Variations among asexual progeny have been studied as early as 1968. Caten and Jinks (1968) looked at spontaneous variability among single sporangium, single zoospore and

single hyphal tips with regards to rate of growth, sporulation, and colony morphology. After the discovery of the A2 mating type outside Mexico, implications of presence of A2 mating type on fungicide sensitivity and pathogenic characteristics were frequently discussed. Direct studies looked at inheritance of virulence, i.e. how the virulent genes in the pathogen are inherited through sexual progeny. (Spielman *et al.*, 1989; Spielman *et al.*, 1990; Al-Kherb *et al.*, 1995), and how insensitivity to phenylamides was inherited (Shattock, 1988; Judelson and Roberts, 1999). At the inception of this study, however, direct studies the implications of sexual progeny on aggressiveness were relatively few and expression of variation to a range of different fungicides among single oospore progeny were limited.

The aim of this chapter, therefore, was to study the inheritance of aggressiveness of sexual progeny of *P. infestans* with particular reference to latent period, lesion development and sporulation capacity. In addition, and for comparison, a few asexual progeny, single sporangial and single zoosporangial isolates, were established and compared to the sexual progeny. Previously, the question of asexual variation in the *P. infestans* was a dominant issue in late blight research (e.g. Caten and Jinks, 1968; Caten, 1970; Caten, 1971).

4.2 MATERIALS AND METHODS

4.2.1 Pathogenicity testing

The sexual progeny were tested for their ability to cause disease on detached potato leaflets of the susceptible cultivar Bintje (see section 4.2.4 for growing conditions). The leaflets were inoculated with spore suspension obtained from 10 day-old cultures. This was done by harvesting sporangia from Petri dishes, containing rye A agar amended with RAN, by spraying the plate with a fine mist of tap water. The concentration of sporangia was counted, first using a haemocytometer, and later using a quick crude visual counting method (D. S. Shaw, personal communication). Three aliquots of 5 µL of the spore suspension were put onto water agar, on which little grids were marked at the base, to simplify counting. The droplets were left for a few seconds so that the sporangia would settle on the agar. The sporangia in each aliquot were counted under the microscope. The countings were averaged and extrapolated to calculate the concentration of spores, which was adjusted to 2×10^4 spores mL^{-1}. The adjusted spores were used to inoculate the detached leaflets. Each leaflet

was inoculated on the abaxial surface with 15 µL of spore droplets. In cases where there were no visible spores in the Petri dishes, agar discs from actively growing margins of the culture were used. The inoculated detached leaflets were kept in square plastic dishes lined with moistened blue paper. The plates were sealed with masking tape to reduce evaporation and kept at 18 ^{0}C at 16 hours photoperiod. Presence or absence of sporulation and/or necrosis was scored 5-6 days after inoculation. Each isolate was replicated 3 times.

4.2.2 Production of single sporangium and single zoospore progeny

Single sporangium progeny were established from the 3 UK parental isolates and 3 single oospore progeny (one from each cross). Another parental isolate, 96.36.1 (also UK A1), which was used in another study (Earnshaw *et al.*, 2003), and one of its single oospore progeny (A2 parent of the progeny was the same A2 parental isolate used in this thesis), were included. This gave a total of 7 isolates from which the two types of asexual progeny were produced (Table 4.2.1). From each of the 7 isolates, 5 single sporangium were established and from each single sporangium isolate, 5 single zoospore progeny were established (Figure 4.2.1), giving a total of 35 single sporangium progeny and 175 single zoospore progeny.

Table 4.2.1 Isolates of *P. infestans* used to establish asexual progeny

Isolate[a]	Mating type	Nature of isolate	Pathogenicity of isolate
96.70	A2	Parent	Pathogenic
96.69	A1	Parent	Pathogenic
96.69-9	A1	SOP	Pathogenic
96.89.43	A1	Parent	Pathogenic
96.89.83- 80	A2	SOP	Pathogenic
96.36.1	A1	Parent[b]	Pathogenic
96.36.1-19	A1	SOP	Pathogenic

[a] – isolate with a hyphen are single oospore progeny, the isolate number before the hyphen denotes the A1 parent and the number after the hyphen denotes the progeny number.
[b] - the isolate 96.36.1 was used as an A1 parent in another study (Earnshaw *et al.*, 2003)

Single sporangium progeny were established from 7 day old cultures. An inoculating loop with a bubble of sterile water was used to pick a few sporangia. These were spread on soft water agar amended with RAN in a Petri dish. The dish was incubated in an incubator at 18°C for 24 hours. Germinated sporangia were transferred into individual Petri dishes where individual germinated sporangia were allowed to form a single isolate.

To establish single zoospore progeny, initially, establishment of single zoospore progeny from the same sporangium was attempted. A single sporangium from the single sporangium isolate was picked using a sterile inoculating loop with a bubble of sterile water. The sporangium was deposited in a droplet of sterile tap water and chilled at 10°C to release zoospores. This method was unsuccessful and an alternative method was used. A Petri dish with an isolate established from a single sporangium, was flooded with sterile tap water, to harvest sporangia. The harvested sporangia were chilled for about 2 hours to release zoospores. About 50 :L of zoospore suspension was diluted into 1 mL of sterile tap water. The diluted zoospores were spread on a Petri dish containing soft water agar amended with RAN. The Petri dish was sealed with parafilm and incubated at 18°C. After 12 hours and 24 hours, germinated zoospores were transferred into Rye A agar amended with RAN, to establish single zoospore progeny.

4.2.3 Preparation of inoculum

Leaves of cultivar Home Guard or Bintje were used to produce inoculum. The leaves were inoculated with either sporangia or agar discs in cases where the isolates did not produce obvious sporangia. In the case of sporangia inoculation, wedges of agar were used to collect a few sporangia from a culture. The wedges were placed on the leaf and a droplet of water was added onto the wedge. The sporangia were then chilled for about 2 hours at 10 ^{0}C to release zoospores after which the leaves were kept under 16 hour photoperiod of light supplied by 1.2 m 36 W fluorescent tubes at 18 ^{0}C. After 24 hours, the wedge was removed and the droplet was blotted out. Each leaf was kept in a Petri dish lined with moistened blue paper. After 4-5 days sporulation on the leaves was observed. The sporangia were used to inoculate a second batch of leaves. To inoculate subsequent leaves, the leaves were sprayed

with a fine mist of water and the sporulating leaf was dragged over the moistened leaf to inoculate it.

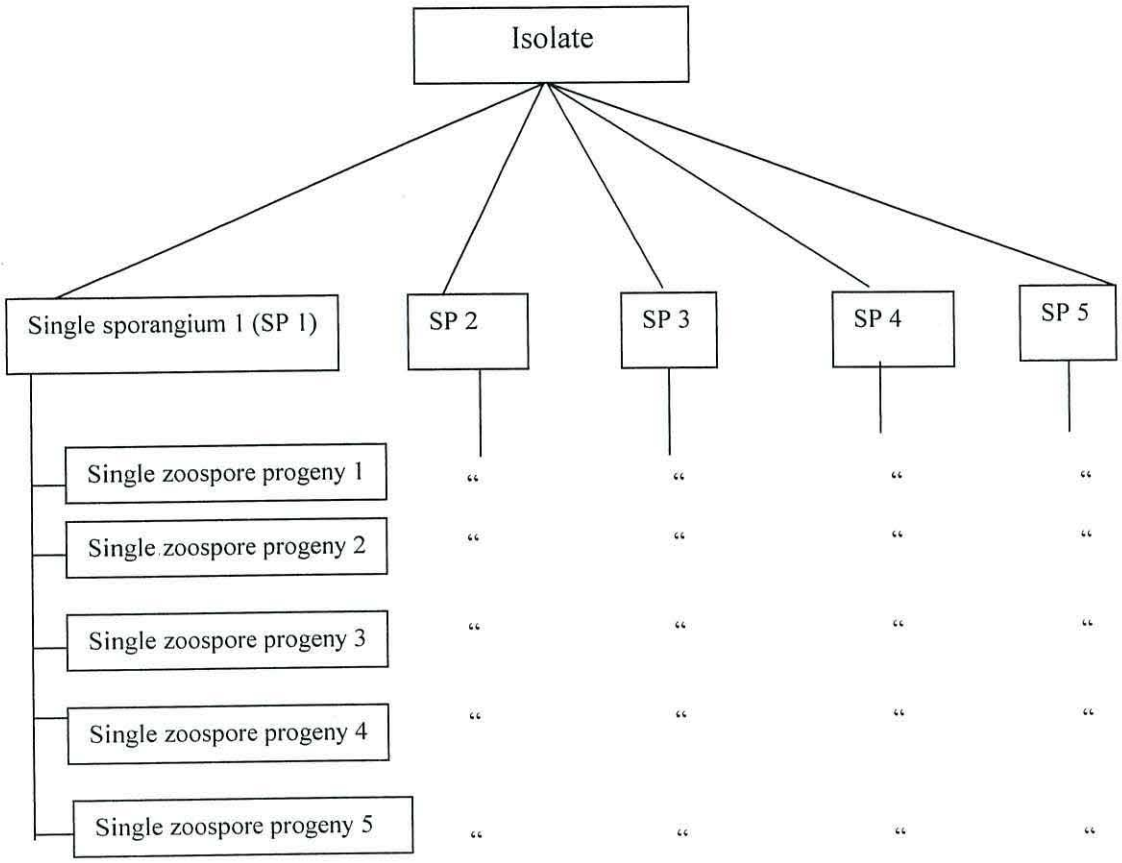


Figure 4.2.1. Diagrammatic scheme for production of single sporangium progeny and single zoospore progeny. Each isolate ended up with 31 isolates: the isolate, 5 single sporangium progeny and 25 single zoospore progeny.

The isolates were cycled through leaves 3-4 times before their sporangia were used as inoculum. After the 3rd or 4th cycle, the sporangia were harvested by spraying a fine jet of water onto each leaf, on both surfaces, held in a beaker. To clean the sporangia of any bacteria, an aliquot of the sporangia of about 1mL was collected at the bottom of the beaker (to get a high concentration of spores), put in an Eppendorf tube and centrifuged at 14 000 rpm for 2 minutes. The supernatant was discarded and the pellet of sporangia was washed with another 1mL of water before it was hydrated with 1 mL of water. The concentration of the harvested sporangia was determined using the crude water agar method as discussed in section 4.2.1. A concentration of 1- 1.2 x 10^4 sporangia per mL was envisaged. Where the concentration was high, the suspension was diluted to the required concentration and where the concentration was low, the suspension was left to settle and extra water was removed.

4.2.4 Determination of aggressiveness

Inoculum was prepared as described in section 4.2.2. Four UK and three Hungarian potato cultivars with increasing race non-specific resistance were used. The UK cultivars were Home Guard, Maris Piper, Stormont Enterprise, and Stirling; the Hungarian cultivars were 84.4.6, Camelon and Mistica (Table 4.2.2). Leaf disks of 26 mm diameter were cut from leaflets of 4-6 weeks old potato plants grown in a green house at 25 ^{0}C. The discs were randomly assigned to isolates. The discs were placed abaxial side up on square Petri plates (48 discs per plate) lined with moistened blue paper (Figure 4.2.1). Each disc was inoculated with 15 :L of spore suspension (adjusted), four discs were inoculated with the same isolate, i.e. 4 replicates. The plates were then chilled for 2-3 hours to release zoospores, after which they were kept at 18 ^{0}C under 16 hour photoperiod of light supplied by 1.2 m 36 W fluorescent tubes. Twenty-four hours after inoculation, the droplets were dried out using a blotting paper.

Figure 4.2.2 Experimental lay out of the potato leaf discs.

Table 4.2.2. Potato cultivars used to assess aggressiveness of single oospore, single sporangium and single zoospore progeny of *P. infestans* with details of blight resistance.

Cultivar[a]	Origin[b]	Resistance to late blight[c]	
		Foliar	Tuber
Bintje	UK	3	3
Home Guard	UK	3	3
Maris Piper	UK	5	3
Stormont Enterprise	UK	6	5
Stirling	UK	8	8
Mistica	Hungary	ND[d]	ND
Camelon	Hungary	ND	ND
84.4.6	Hungary	ND	ND

[a b] –Hungarian cultivars are not commercially available, they are still breeding lines and they were supplied by Dr. D. S. Shaw (Sárvári Research Trust). Jenny Vaughan, National Institute of Agricultural Botany (NIAB), supplied Stormont Enterprise and Stirling was supplied by Helen Stewart (Scottish Crop Research Institute). The rest of the cultivars were purchased as seed potato.
[c] - NIAB 1999, score between 1 and 9 = low to high resistance to late blight; Anon. (1998).
[d] – ND not determined

4.2.4.1 Latent period (LP)

Latent period was measured as the time taken for lesion development and/or sporulation to occur from inoculation. This was expressed in days.

4.2.4.2 Infection frequency (IF)

This assessment was based on the number of inoculated discs with lesion development and/or sporulation (Day and Shattock, 1997; Lebreton *et al.*, 1998). A value of 1 was given to a disc with lesion and/or sporulation and zero for no infection. Therefore, infection frequency was 1.0 if all four inoculated discs were infected or 0.25 if only one out of the four discs was infected.

4.2.4.3 Disease growth

Disease growth was based on the development of a lesion and its expansion during the experimental period. The lesion on each disc was measured from first day of appearance until the whole leaf disc of the most susceptible variety was colonised. This was expressed as growth, in mm, per day.

4.2.4.4 Sporulation capacity (SC)

The number of sporangia produced by each isolate on each leaf discs were calculated. Only one disc from each replication was used. The disc was transferred into universal bottle containing industrial methylated spirit, to fix the sporangia. From each suspension, three 5:L aliquots were taken and counted under the microscope (as described in section 4.2.1). The three readings were averaged and the total spore count was extrapolated, this was then expressed as number of spores per mm of leaf area.

4.2.4.5 Fitness index (FI)

Fitness index was calculated as the product of infection frequency and the mean number of spores per discs (Day and Shattock, 1997), i.e. FI = IF x SC.

4.2.4.6 Composite aggressiveness index (CAI)

The composite aggressiveness index was calculated using all the information gathered on each isolate, i.e. infection efficiency (IE), latent period (LP), average lesion expansion (ALE), and sporulation capacity (SC) (Flier and Turkensteen, 1999), using the formula

$$CAI = IE \times ALE \times SC \times LP^{-1} \text{ or } FI \times ALE \times LP^{-1}$$

Lesion expansion data used was an averaged lesion expansion per day.

4.3 RESULTS

Since the study involved a large number of single oospore progeny, it was not possible to use all isolates at once. The parental isolates were therefore used each time a set of progeny was being evaluated for aggressiveness and so permitted comparison between separate experiments. As a result of this, data for parental isolates involved an average of all the data on them. For initial pathogenicity screening, the progeny were divided into three lots, first and second lot containing 219 single oospore progeny comprising of equal numbers from each cross, i.e. 73 SOP from each cross; and third lot containing 102 SOP (41, 23, 37 for crosses #1, 2 and 3, respectively). Each lot was then tested for pathogenicity together with the 4 parental isolates.

For aggressiveness testing, fewer progeny were used each time, i.e., only 32 SOP (and all 4 parents), giving a total of 36 isolates tested per an experiment. As a result of the small number of SOP used each time, the experiment was done 15 times and the data on the parental isolates is an average of the 15 experiments.

It was not possible to use equal numbers of SOP from each cross per an experiment, therefore, a ratio of 12:12:8 was used per an experiment. The ratio was changed for each experiment so that after 3 experiments, a total of 32 SOP from each cross would have been used. This was repeated 12 times at which point 128 SOP from each cross were tested. The 13^{th} and 14^{th} experiments involved 12 SOP from crosses #1 and 3, and 8 SOP from cross #1. The last experiment (15^{th}) involved 7 SOP from cross # 1, 5 from cross #2 and 13 from cross #3.

4.3.1 Pathogenicity of single oospore progeny

From a total of 540 SOP, from the three crosses, only 64 (12%) were non-pathogenic on the most susceptible potato cultivar Bintje (Table 4.3.1). There were fewer A1 SOP that were non-pathogenic: 5% and 2% for crosses #1 and 3, respectively, compared to A2 SOP: 13% and 9%, for crosses #1 and 3, respectively. However, in cross #2, there was no difference between the two mating types; 8% and 7%, A1 and A2 SOP, respectively. Cross #1, the UK parental isolates, 96.89.43 (rare A1) and 96.70 (common A2) had the highest number of non-

pathogenic progeny compared to the other two crosses. Cross #3, the Californian isolate P9463 (A1) and UK 96.70 (A2), had the least number of non-pathogenic sexual progeny.

In all 3 crosses, the majority of the non-pathogenic progeny were the progeny with undetermined mating type (Chapter 2). Of the 22 SOP with undetermined mating type, 5 were lost before the pathogenicity experiment was carried out; 14 were non-pathogenic and the remaining three caused necrosis but without sporulation. These three SOP were not used for the aggressiveness experiments (Section 4.3.2).

Table 4.3.1 Numbers of non-pathogenic progeny of *P. infestans* from three crosses on leaflets of the susceptible potato cultivar Bintje.

Crosses	**# of non-pathogenic SOP**	Mating type			
		A1	**A2**	**Self**	**Undetermined**
96.89.43 x 96.70	28 (15)[a]	7 (5)	13 (32)	1 (12)	7 (100)
96.69 x 96.70	20 (12)	6 (7)	8 (11)	1 (25)	5 (83)
P9463 x 96.70	16 (9)	3 (2)	9 (21)	2 (12)	2 (50)
Total	**64 (12)**	**16 (5)**	**30 (20)**	**4 (14)**	**14 (64)**

[a] – number in parentheses is a percentage of the mating type

4.3.2 Aggressiveness of sexual progeny

A total of 473 SOP (Table 4.3.2) from all three crosses were found to be pathogenic (Section 4.3.1) and were used for the aggressiveness experiments. The aggressiveness was assessed on seven potato cultivars. There were significance differences ($P<0.05$) in aggressiveness shown by SOPs on the UK and the Hungarian cultivars (Figure 4.3.1).

Table 4.3.2 Numbers of single oospore progeny (SOP) from each cross that were used for the aggressiveness experiment.

Cross	# of pathogenic SOP	# in each mating type		
		A1	**A2**	**Self**
96.89.43 x 96.70	159	125	27	7
96.69 x 96.70	149	82	64	3
P9463 x 96.70	165	118	33	14
Total	**473**	**325**	**124**	**24**

4.3.2.1 Latent period

The latent period of the parental isolates was shorter on the susceptible potato cultivar, i.e. Home Guard and longer on the more resistant cultivars (Table 4.3.3). There was no significant difference ($P<0.05$) among the parental isolates 96.69 (A1 for cross #2), 96.70 (A2) and P9463 (A1 for cross #3) within each potato cultivar where all three isolates had a successful infection. However, parental isolate 96.89.43 (A1 for cross #1) had a latent period significantly ($P<0.05$) longer than the other parental isolates in all potato cultivars.

Table 4.3.3. Mean latent period (in days) of the parental isolates of *P. infestans* on seven potato cultivars of increasing non-race specific resistance

Isolate & Nature	Latent period (days) on different potato cultivars						
	HG	**MP**	**SE**	**ST**	**CA**	**84**	**MI**
96.70	2.5	3.0	3.4	4.2	4.1	4	5.5
96.89.43	3.25	4.25	4.25	4.5	5.3	0	4.6
96.69	2.25	2.75	3.25	5	4.0	4	5.0
P9463	2.3	2.5	3.8	4.8	4.7	0	5.0

N= 60 (15 experiments x 4 reps)

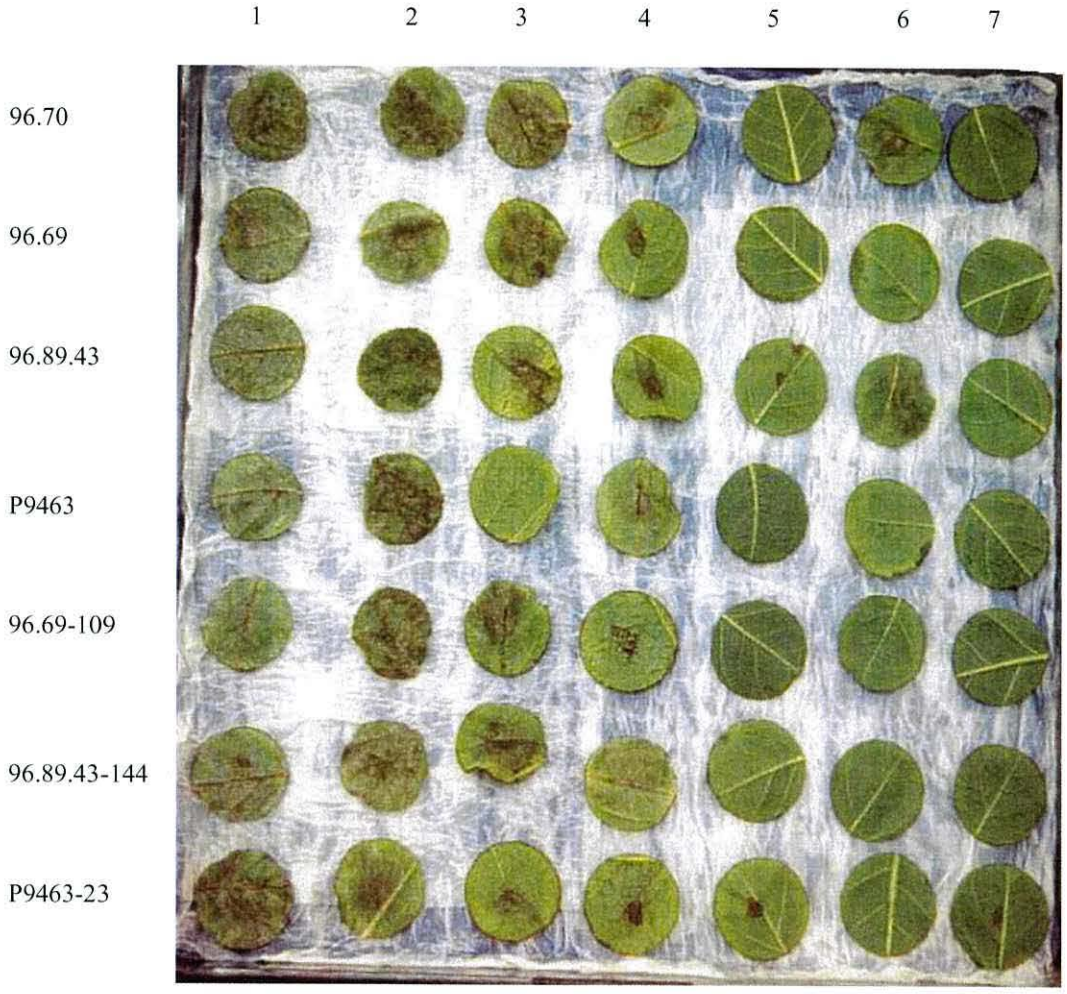


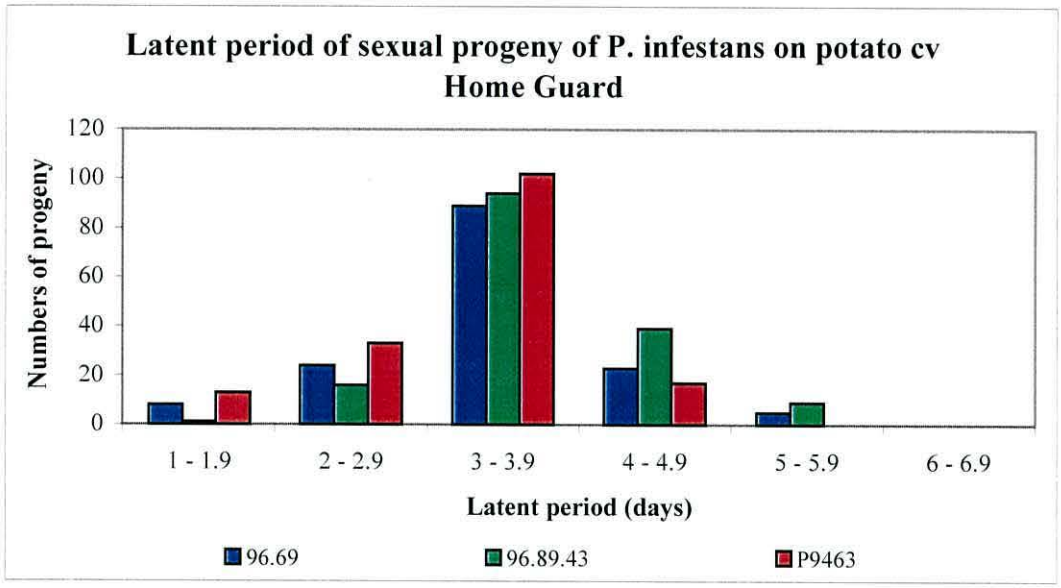
Figure 4.3.1. Potato leaf discs of seven cultivars (4 UK and 3 Hungarian), represented by numbers 1 to 7 (vertical lines), inoculated with the 4 parental isolates and 3 single oospore progeny (one from each cross). The potato cultivars are: the 4 UK cultivars - line 1 Home Guard, line 2 Maris Piper, line 3 Stormont Enterprise, line 4 Stirling, and the 3 Hungarian – line 5 Camelon, line 6 Mistica, and line 7 84.4.6. The photo shows the susceptibility and/or resistance of the potato cultivars. Lane 1 is the most susceptible cultivar and resistance increases towards the right. The parental isolates, and some of their SOPs, are listed on the left hand-side.

The latent period for the sexual progeny on the most susceptible and most resistant of the UK potato cultivars used is presented in Figure 4.3.2.

In the most susceptible potato cultivar Home Guard (HG), in cross #1, the A1 (96.89.43) parent had a latent period one day longer than that of the A2 (96.70) parent. Of the 159 single oospore progeny, 17 (~11%) had a latent period a day shorter than that of the A1 parent, 94 (59%) had latent period similar to that of the A1 parent, and 48 (30%) had a latent period significantly longer than that of the A1 parent. Only one progeny (SOP123, an A2) had a latent period shorter than that of the A2 parent, i.e. 1.8 days; 16 (10%) had a latent period similar to the A2 parent, and 142 (89%) had a latent period significantly ($P< 0.05$) longer than that of the A2 parent. In cross #2, both parental (96.69 & 96.70) isolates had same latent period and only 8 (5%) single oospore progeny had a shorter latent period than the parents. Twenty-four (16%) had a similar latent period to the parents and 117 (~79%) had a longer latent period than that of the parents. In cross #3, again both (P9463 & 96.70) parents had a same latent period, together with 33 (20%) single oospore progeny, only 13 (8%) had a shorter latent period and 119 (72%) had a longer latent period than that of the parents.

In the potato cultivar with intermediate foliar resistance, Maris Piper, all single oospore progeny were pathogenic. In cross #1, both parental isolates had similar latent period, together with 102 SOP (64%), only 7 (4%) had shorter and 50 (31%) had longer latent period than that of the parents. In cross #2, the A1 parent had a shorter latent period than the A2 parent. Only 3 SOP (2%) had a shorter latent period that the A1 parent but 21 (14%) had a latent period shorter than the A2 parent. Eighteen SOP (12%) had a latent period similar to that of the A1 parent and 100 (67%) had a latent period similar to the A2 parent. One hundred and twenty-eight SOP (86%) had a latent period longer than that of the A1 parent while only 28 (~19%) had a latent period longer than the A2 parental isolate. In cross #3, again the parental isolate A1 had a shorter latent period than the A2 parent. Only 9 SOP (5%) had a shorter latent than the A1 parent, while 30 (18%) had a shorter latent period than the A2 parent. Twenty-one SOP (~13%) had a similar one to the A1 parent while 100 (~61%) had a similar latent period to that of the A2 parent. While 135 SOP (82%) had

longer latent period than the A1 parent, only 35 (21%) had a longer latent period than the A2 parent.



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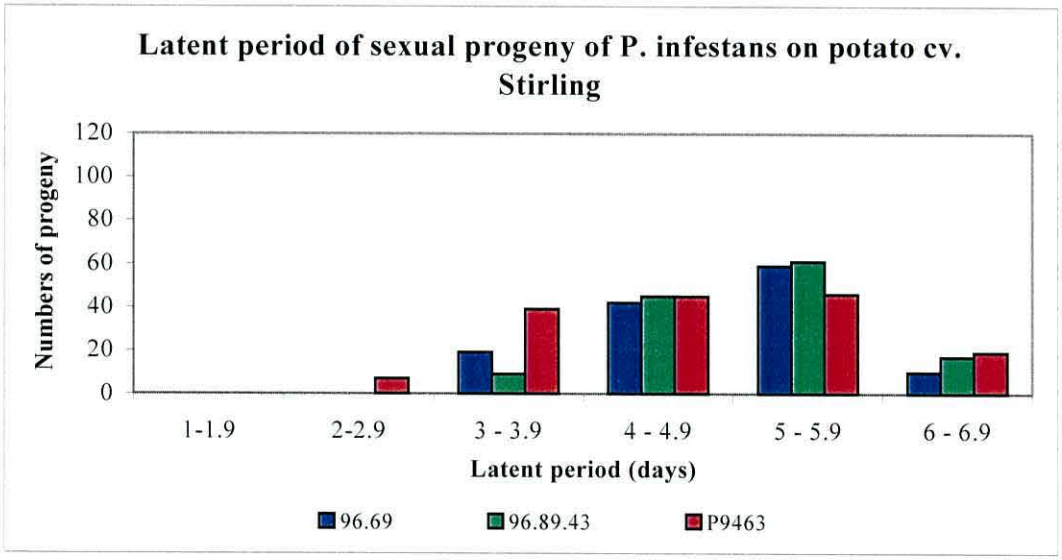


B

Figure 4.3.2. Latent period of sexual progeny of 3 crosses of isolates of *Phytophthora infestans* on two potato cultivars: a susceptible cultivar, Home Guard (A) and a resistant cultivar, Stirling (B). Since the A2 (96.70) parent was common among all crosses, the legend indicates the A1 parent for the SOPs, i.e. 96.69 – A1 parent for cross #2, 96.89.43 – A1 parent for cross #1, and P9463 – A1 parent for cross #3.

In the potato cultivar with the highest race-non-specific resistance, Stirling, not all single oospore progeny tested were pathogenic. The percentage of non-pathogenic progeny of each cross were 22.6, 19.5 and 5.5, for crosses #1, 2, and 3, respectively. In crosses #1 and 3, both parental isolates had a similar latent period. In cross #2, the A1 parent had a longer latent period than the A2 parent. In cross #1, only 9 SOP (~6%) had a shorter latent period than the parents, while in cross #3, 46 (~28%) had a shorter latent period than the parents. About 28% SOP in cross #1, and 27% in cross #3, had latent period similar to both parental isolates. In cross #1, 78 SOP (49%) and 65 (39%) from cross #3, had latent periods longer than the parents. In cross #2, 19 SOP (~13%) had shorter and 69 (46%) had a longer latent period than the A2 parent; while 61 (41%) had a shorter and 10 (~7%) had a longer one than the A1 parent. Forty-two SOP (28%) had a similar latent period to the A2 parent while 59 (~40%) had a similar one to the A1 parent.

Among the parental isolates, Pearson's correlation results showed that there was no significant correlation between latent period and mating type and potato cultivar (see Section 4.3.2.6, Table 4.3.6). However, there was a significant positive correlation between latent period and infection frequency ($r = 0.866$, $P = 0.0001$). There was a positive correlation also between latent period and average lesion expansion per day ($r = 0.583$, $P = 0.0001$). Positive significant correlation ($P < 0.05$) was also observed between latent period and infection frequency of sexual progeny of crosses #2 and 3, i.e. sexual progeny of UK common A1 and A2, and sexual progeny of Californian A1 and UK common A2. However, on cross #1, i.e. UK uncommon A1 and UK common A2; there was a negative significant correlation between latent period and infection frequency ($r = -0.679$, $P = 0.0001$).

4.3.2.2 Infection frequency

On the two most susceptible potato cultivars, Home Guard and Maris Piper, infection frequency of all the parental isolates was 1.0, i.e. all inoculated leaf discs were infected. However, on the cultivars with increased field resistance, i.e. Stormont Enterprise, Stirling and Hungarian cultivars, infection frequency ranged from 0 to 1 (Table 4.3.4). On the most resistant Hungarian cultivar, 84.4.6, infection frequency was significantly reduced ($P<0.01$); UK A1 parental isolates 96.89.43 and Californian A1 parental isolate P9463 were non-

pathogenic. The other two parental isolates, UK A1 and UK A2 96.69 and 96.70, respectively, were pathogenic to a quarter or a third of the inoculated discs.

Table 4.3.4. Average infection frequency of each parental isolate of *P. infestans* on seven potato cultivars of different non-race-specific resistance.

Parental Isolate	Average infection frequency on each potato cultivar						
	HG	MP	SE	St	Ca	Mi	84.4.6
96.89.43	1[a]	1	0.88	0.78	0.75	0.75	0
96.69	1	1	1	0.92	0.82	0.5	0.32
96.70	1	1	1	0.98	0.93	0.94	0.26
P9463	1	1	1	0.96	0.25	0.71	0

N=60 (15 experiments x 4 reps)

[a] – IF = 1 indicates that all leaf discs were infected.

On all three crosses, there was a significant (P = 0.0001) negative correlation between infection frequency and potato cultivars (r = -0.592, -0.623, and –0.704, for crosses #1, 2, and 3, respectively). There was, however, no significant correlation between infection frequency and mating type in all three crosses.

Table 4.3.5 shows the distribution of progeny on each level of infection frequency. Generally, in all three crosses, the majority of sexual progeny were pathogenic in all inoculated leaf discs of potato cultivars Home Guard and Maris Piper. On Home Guard, 73%, 86% and 80% of the SOPs on crosses #1, 2 and 3, respectively, were pathogenic to at least 3 of the 4 inoculated leaf discs. On Maris Piper, a similar trend was observed. However, on the more resistant potato cultivars, the reverse trend was observed: on Stirling, the most resistant UK cultivar used, only 21% and 34% of the SOPs from crosses #1 and 2, respectively were pathogenic to at least 3 of the 4 inoculated leaf discs. On the other hand, for cross #3, it was about half (49%) of the SOPs. The number of SOP non-pathogenic to Stirling were not significantly different among the crosses, they ranged from about 18% and 19% for crosses #2 and 3, respectively, to 23% for cross #1. On the most resistant

Hungarian potato cultivar 84.4.6, the majority of the sexual progeny in all three crosses were either non-pathogenic or had a very low infection frequency.

Table 4.3.5. Average infection frequency (# of infected leaf discs out of total inoculated discs) of sexual progeny of *P. infestans* of three crosses on seven potato cultivars

Potato cultivar[a]	96.89.43 x 96.70						96.69 x 96.70						P9463 x 96.70					
	0[b]	0.25	0.5	0.75	1	TOTAL	0	0.25	0.5	0.75	1	TOTAL	0	0.25	0.5	0.75	1	TOTAL
HG	0[c]	12	31	65	51	**159**	0	4	13	30	102	**149**	0	15	18	23	109	**165**
MP	0	21	23	42	73	**159**	0	11	2	39	97	**149**	0	17	19	40	89	**165**
SE	9	43	23	60	24	**159**	5	17	12	27	88	**149**	0	35	23	51	56	**165**
St	36	57	32	18	16	**159**	29	22	48	19	31	**149**	9	24	51	34	47	**165**
Ca	49	59	21	23	7	**159**	27	13	19	49	41	**149**	31	58	11	30	35	**165**
Mi	56	63	19	17	4	**159**	35	59	21	13	21	**149**	47	21	37	28	32	**165**
84.4	95	40	18	6	0	**159**	76	35	24	9	5	**149**	101	33	17	9	5	**165**

a Potato cultivars: HG – Home Guard, MP – Maris Piper, SE – Stormont Enterprise, St – Stirling, Ca – Camelon, Mi - Mistica, 84.4 – 84.4.6.

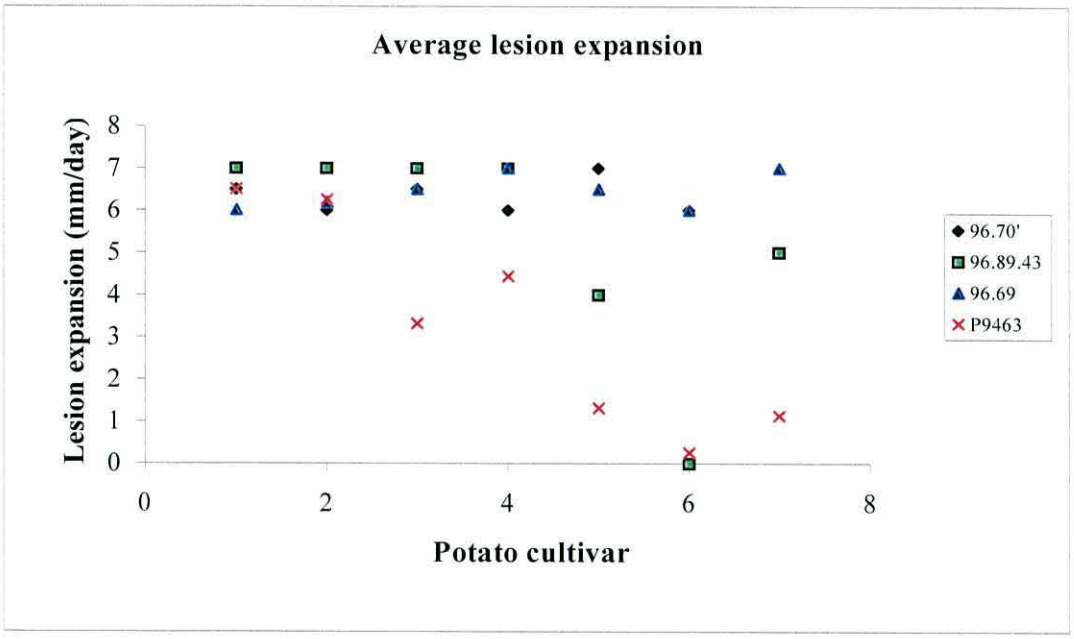
b proportion of infected potato leaf discs: 0 indicates no infection in all four leaf discs; 0.25 indicates only one out four infected; 0.5 indicates two out of four; 0.75 indicates three leaf discs out of four; and 1 indicates all four leaf discs were infected.

c number of single oospore progeny with that infected that many leaf discs in that potato cultivar.

4.3.2.3 Lesion size

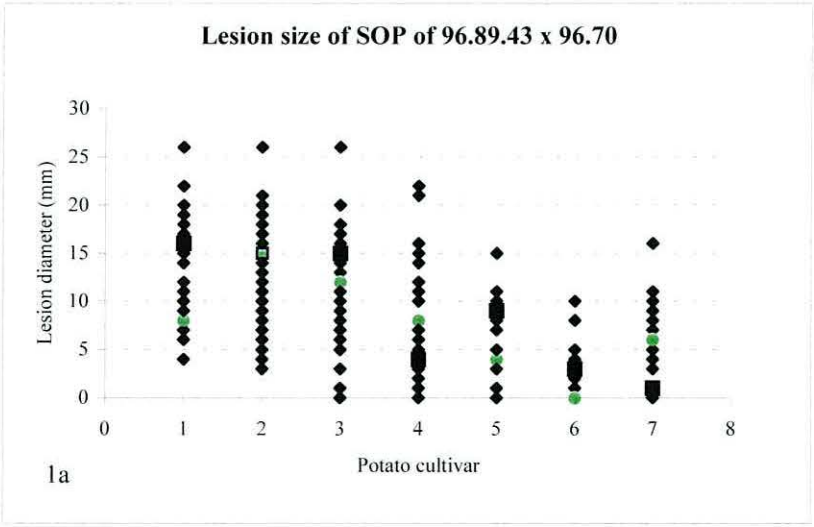
Average lesion expansion of the parental isolates is presented on figure 4.3.3. It was measured as the average of the daily expansion of a lesion on all four leaf discs. For parental isolates, however, this was an average of 15 experiments replicated 4 times and for progeny it was an average of the 4 replications.

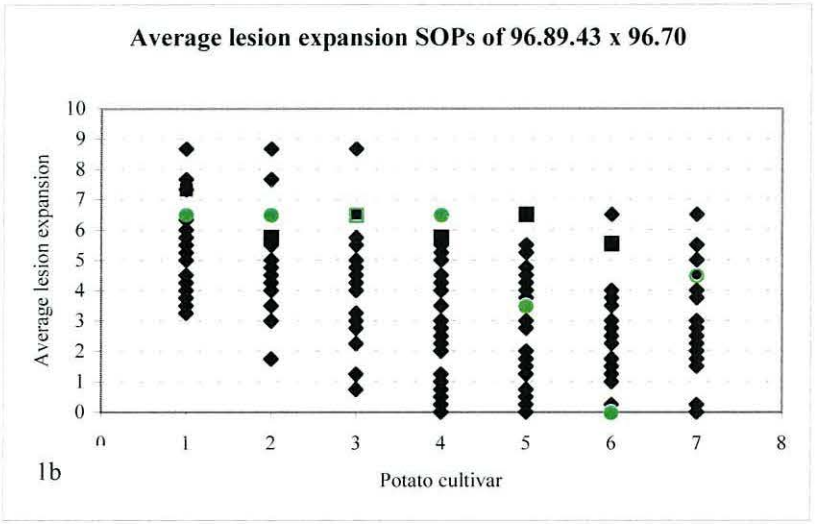
There was no significant difference in average lesion expansion of all UK isolates on all UK potato cultivars. Figure 4.3.4 shows the actual lesion size of the parental isolates and the progeny 5 days after inoculation. Parental isolate 96.89.43 (UK A1 parent with rare RG57 fingerprint) had the highest average lesion expansion (Figure 4.3.3), however, it had the smallest lesion on day 5 compared to the other parents. On the other hand, parental isolate P9463 had the smallest average lesion expansion per day yet it had the large lesions, especially on the resistant cultivars, e.g. Stirling and Mistica ((Figure 4.3.4 (3a)).

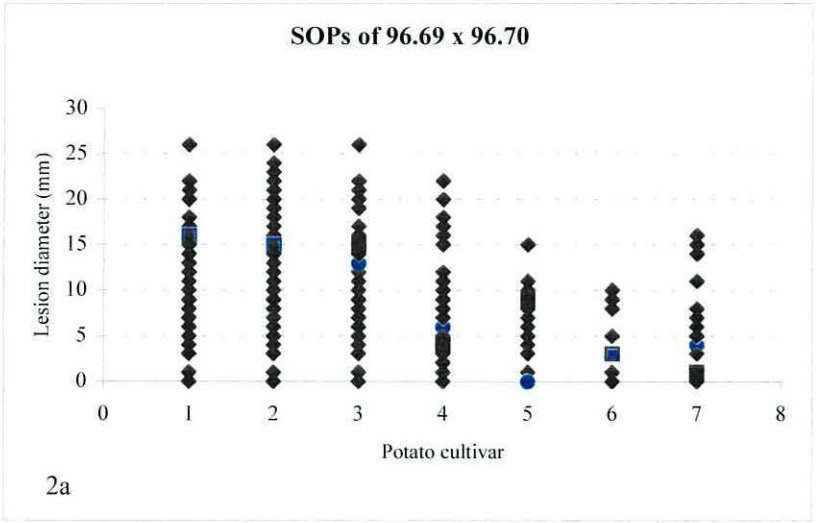


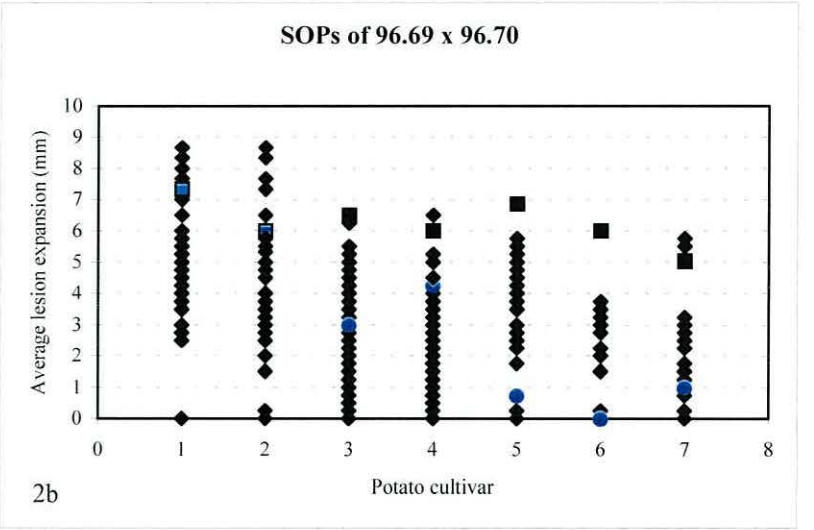
N=60

Figure 4.3.3. Average lesion expansion of the 4 parental isolates of *P. infestans* on seven potato cultivars of different levels of non-race-specific resistance. The potato cultivars were: 1-Home Guard, 2-Maris Piper, 3-Stormont Enterprise, 4-Stirling, 5-Camelon, 6 - 84.4.6, and 7-Mistica.


Lesion size of SOP of 96.89.43 x 96.70
Lesion diameter (mm)
Potato cultivar
1a


Average lesion expansion SOPs of 96.89.43 x 96.70
Average lesion expansion
Potato cultivar
1b


SOPs of 96.69 x 96.70
Lesion diameter (mm)
Potato cultivar
2a


SOPs of 96.69 x 96.70
Average lesion expansion (mm)
Potato cultivar
2b

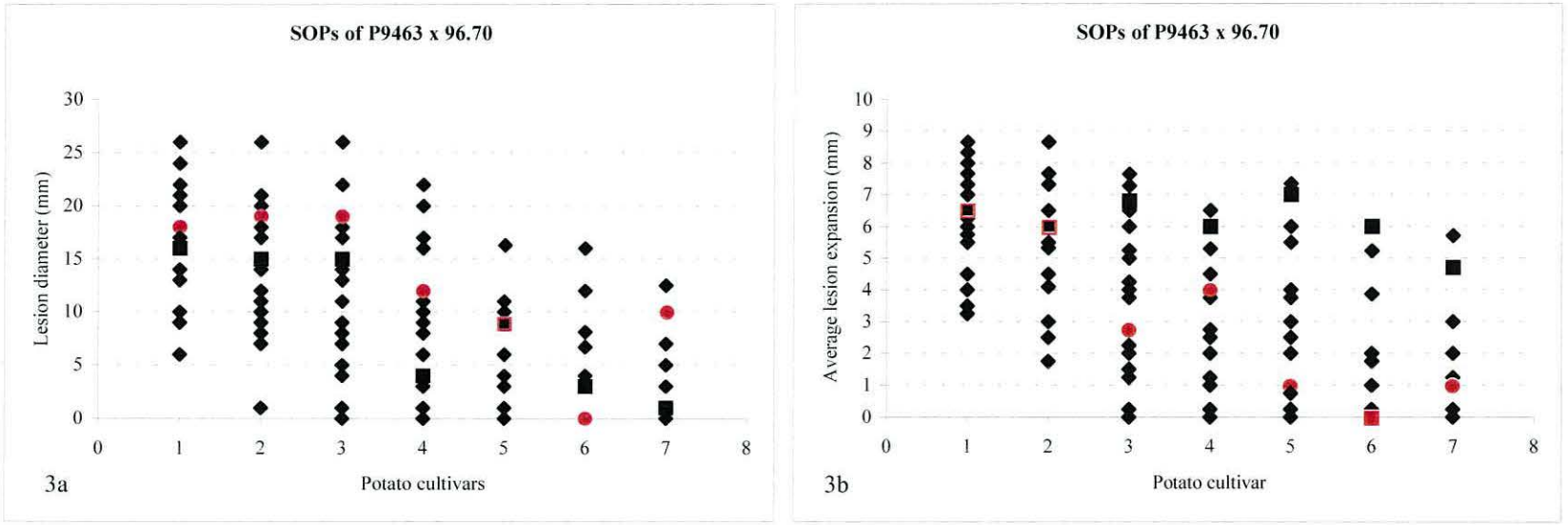


Figure 4.3.4. Lesion diameter (1a, 2a and 3a) and average lesion expansion (1b, 2b and 3b) of single oospore progeny and their parental isolates of *P. infestans* on seven potato cultivars. A1 parents is denoted by a coloured circle, an A2 parent is denoted by a solid black square, and where both parents were the same, they are denoted by a square with the colour of the A1 parent.

4.3.2.4 Sporulation capacity

The number of sporangia among the parental isolates ranged from 51 700 spores cm^{-2} to 62 100 spores cm^{-2} on the susceptible cultivar Home Guard. There was no significant difference among the parents on cultivar Home Guard. There was, however, a significant difference (P <0.05) on sporulation on the rest of the cultivars (Figure 4.3.5).

When the sporulation capacity of the sexual progeny in each cross were ranked and the top ten percent and bottom ten percent sporulating progeny were compared to their parents (Figure 4.3.6), there was no significant ($P < 0.05$) difference between the A1 and A2 parents and the top 10% sporulating progeny on the most susceptible potato cultivar Home Guard on all three crosses.

In cross #1, UK less common A1 and UK common A2 parents, there was no significant difference between the A2 parent and the SOP with the highest sporulating capacity on cultivar Maris Piper. There was however, a significant difference between the A1 parent and the top 10% of sporulating progeny. The average sporulation of 10% of the top sporulating single oospore progeny were not significantly different to the A1 parent in potato cultivars Home Guard, Stormont Enterprise, and Stirling.

In cross #2, the UK common A1 and A2 parents, there was no significant difference among the parents and the top 10% progeny on cultivars Home Guard, Maris Piper and Stormont Enterprise. However, on cultivar Stirling the progeny with the highest sporulation values were significantly higher than both parents. On average they produced 48 900 spores cm^{-2} compared to an average of 18 700 spores cm^{-2} by the A1 parent. Sporulation per unit area was also significantly higher for the top 10% sporulating progeny than either parent in all cultivars.

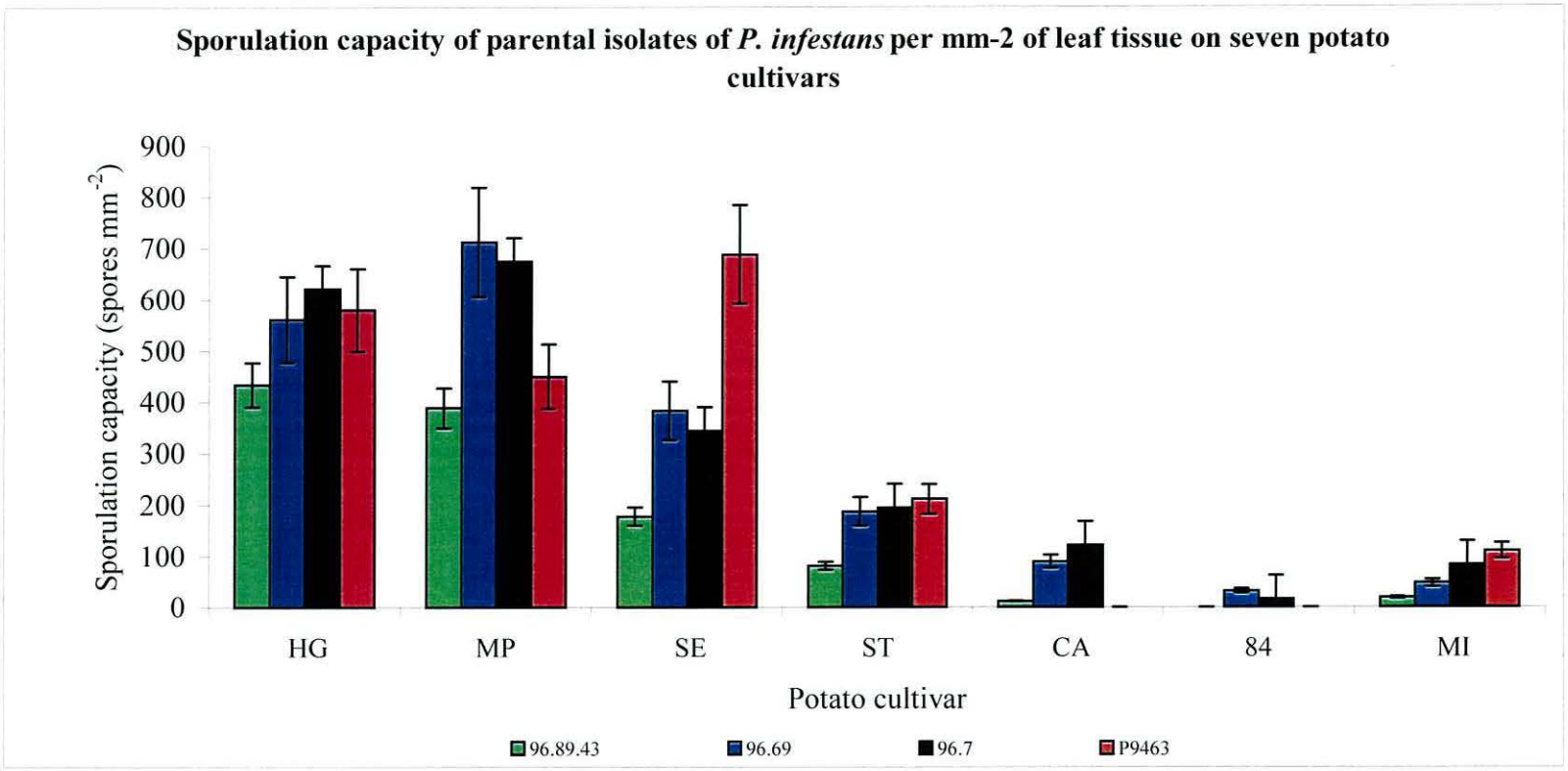
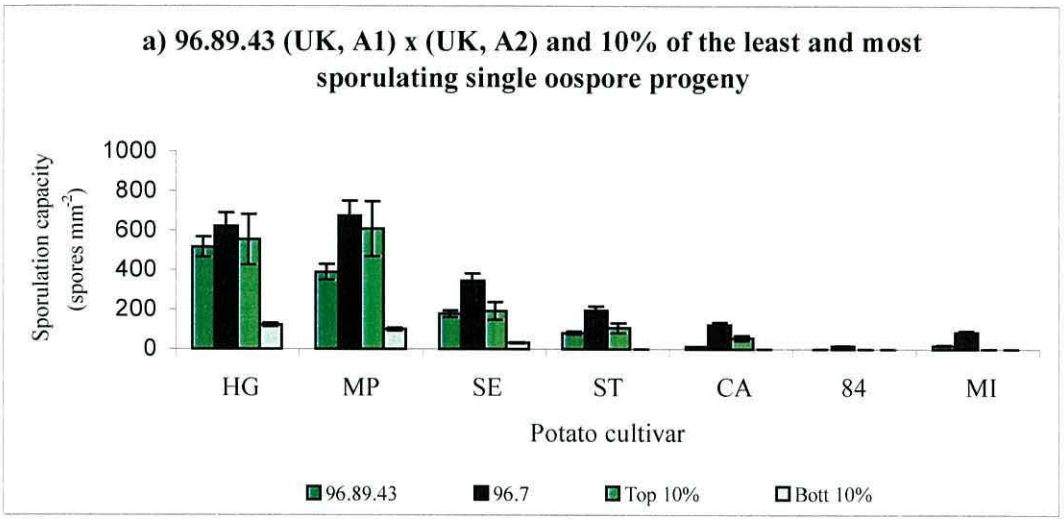
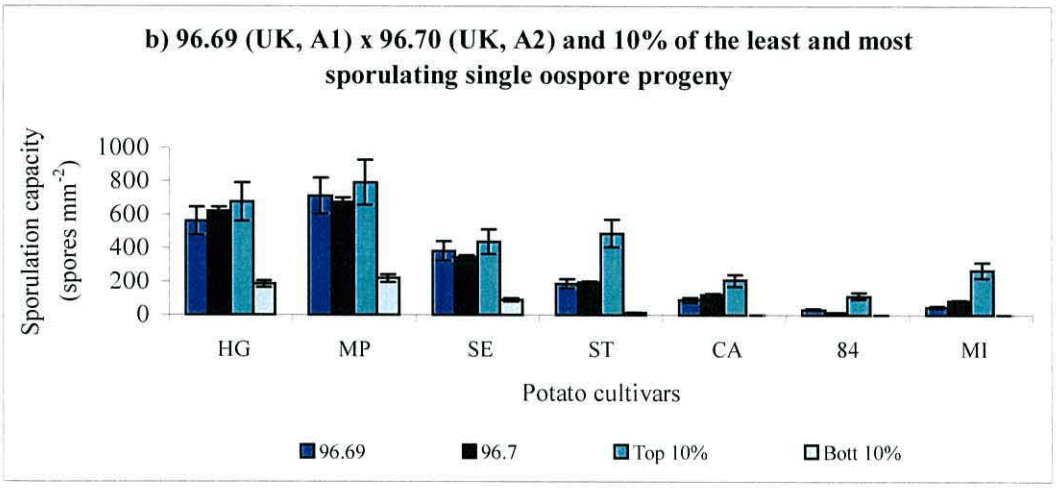


Figure 4.3.5. Sporulation capacity (sporangia mm^{-2}) of the parental isolates of *P. infestans* on leaf discs of seven potato cultivars: HG – Home Guard, MP – Maris Piper, SE – Stormont Enterprise, ST – Stirling, CA – Camelon, 84 – 84.4.6, and MI – Mistica. Bars show SD. *N*=15

In cross #3, Californian A1 and UK common A2 parent, there was no significant difference between the parents and the top 10% sporulating progeny on cultivar Home Guard. There was a significant difference among this group of progeny and both parents on cultivars Stormont Enterprise, Maris Piper and on all Hungarian cultivars. On Maris Piper, there was no significant difference between the A1 parent and the least sporulating progeny, but there was a significant difference between the top sporulating progeny and the A2 parent. While sporulation capacity of the top sporulating progeny on Maris Piper was not significantly different from that on Home Guard, it was the highest sporulation capacity among all seven potato cultivars. On Stormont Enterprise, the A1 parent had sporulation significantly higher than the A2 parent and the top 10% of sporulating progeny.





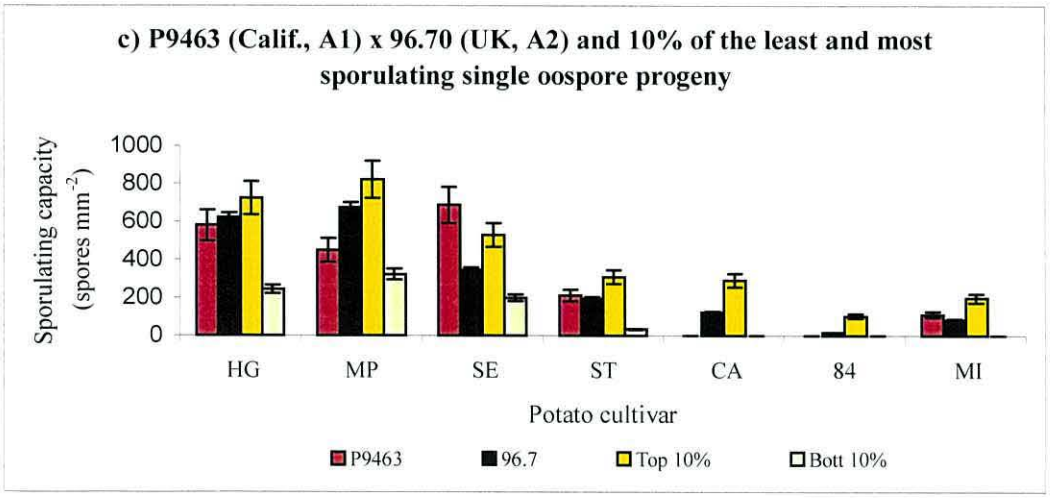


Figure 4.3.6. Sporulation capacity of parental isolates of *P. infestans* and 10% of the most and least sporulating single oospore progeny on leaf discs of seven potato cultivars.

4.3.2.5 Fitness index

Table 4.3.6 Fitness index of parental isolates of *P. infestans* on seven potato cultivars.

Parental Isolate	Fitness Index (Infection frequency x sporulation capacity)						
	HG	MP	SE	St	Ca	Mi	84.4.6
96.89.43	517	389	157	63	9	14	0
96.69	561	712	383	179	73	24	10
96.70	621	675	344	191	104	78	4
P9463	580	450	688	203	0	78	0

There was no significant difference ($P < 0.05$) on fitness index among the parental isolates on the potato cultivar Home Guard and 84.4.6. There was also no significant difference between the UK parental isolates 96.69 and 96.70 (A1 and A2). The Californian A1 isolate, P9463, had a zero fitness index (due to no sporulation) on potato cultivar Camelon although there was disease development after inoculation.

4.3.2.6 Composite aggressiveness

The relationships between the various measures of aggressiveness were explored by means of Pearson rank correlation. Among the parental isolates, there was no correlation between mating type and latent period, mating type and average lesion development, mating type and infection frequency, and potato cultivar and latent period (Table 4.3.7). However, there was a significant correlation between: latent period and infection frequency, and infection frequency and average lesion development. There was a significant negative correlation between: potato cultivar and average lesion development, potato cultivar and infection frequency, and latent period and average lesion development.

As in the case of the parental isolates, there was no correlation between mating type and any of the aggressiveness parameters among the sexual progeny. There was, however, a significant negative correlation between cultivar and average lesion development in all crosses.

Table 4.3.7 Correlation results of parental isolates *P. infestans* on aggressiveness components

Correlated parameters	Correlation coefficient (r)	*P* value
Mating type & latent period	0.156ns	0.102
Mating type & average lesion dev.	0.163ns	0.085
Mating type & infection frequency	0.171ns	0.072
Cultivar & latent period	-0.155ns	0.102
Cultivar & average lesion development	-0.609**	0.0001
Cultivar & infection frequency	-0.508**	0.0001
Latent period & average lesion dev.	-0.583**	0.0001
Latent period & infection frequency	0.866**	0.0001
Infection frequency & aver. lesion dev.	0.763**	0.0001

** - highly significant $P < 0.01$
ns – not significant at $P > 0.05$

There was a significant ($P<0.01$) difference among progeny, cultivars, and the interaction (progeny x cultivar).

4.3.3 Aggressiveness of asexual progeny

A total of 175 single zoospore progeny (SZP) and 35 single sporangium progeny (SSP) together with the parental isolates and their single oospore progeny (Table 4.2.1) were used in this study, giving a total of 217 isolates. During the establishment of single zoospore progeny, it was observed that there were differences in zoospore release. Sporangia from single oospore progeny #6 (not used in the study) from cross #1; the common UK A1 and A2; produced abnormally small zoospores. Their germination was very low (1-2%) and they produced very thin germ-tubes, resulting in only three single zoospore lines being established zoospores isolates were grown as pure cultures, they did not grow normally, but instead formed clusters without radial growth. In contrast, the cultures of SSP cultures established

from the same isolate, grew normally. Two of the three SZP were found to be non-pathogenic and they were all not used for the study.

Composite aggressiveness of the asexual progeny was calculated as described for the sexual progeny (section 4.3.2.6). Among single sporangial progeny (SSP) of each isolate, there was no significant different ($P = 0.05$) in all components of aggressiveness. There was also no significant difference between the SSP and the isolates they were derived from. There was however, a significant difference among the single zoosporangial progeny (SZP). There was also a significant difference between the SSP and SZP of each isolate. While the SSP did not differ significantly from the parental isolates, the SZP were either less aggressive, same or more than their parental SSP. The difference was more pronounced among SZP derived from sporangia of sexual progeny. Of the single zoosporangial progeny derived from SOP 96.89.43-80, an aggressive isolate, 19 were less aggressive and only 6 had the similar aggressiveness as their SSP parents. The percentage of SZP less aggressive than their SSP parental isolates ranged from 16% to 76%.

4.4. DISCUSSION

The aim of this chapter was to assess the pathogenicity and aggressiveness of single oospore progeny and compare them to those of the parents and the two types of asexual progeny, i.e. single sporangia and single zoospore progeny. The results indicate that only a small number of sexual progeny (15%, 12% and 9%, for cross #1, 2,and 3, respectively) were non-pathogenic on the most susceptible cultivars Bintje and Home Guard.

Caten (1971) observed some attenuated stock cultures of *P. infestans* could be reversed to restore pathogenicity and aggressiveness by serial transfers on leaves or tubers of host plant. In this study, to avoid use of any attenuated isolates, the parental isolates were maintained on potato leaves of cultivar Bintje and/or Home Guard. Source of single oospore progeny cultures was oil slants (see Section 2.2.4), which had undergone, at most, two transfers after germination. By the time the aggressiveness experiments were done, the progeny had been in oil for at least 12 months. Although the progeny were passed through leaves three times before they were used, this was a real difference compared to the parental isolates that were maintained on potato leaves throughout the experiments.

Aggressiveness of field isolates of *P. infestans* has been studied by many researchers and most recently by Day and Shattock (1997); Miller *et al.* (1998); Peters *et al.* (1999); Flier and Turkensteen (1999); Lebreton *et al.* (1998); Carlisle *et al.* (2002). Most of these authors measured sporulation capacity, lesion size and infection frequency and sometimes latent period, as measures of aggressiveness. There does not seem to be any consistency, however, in the interpretation of the data to reflect aggressiveness of isolates. Aggressiveness can be defined as an overall ability of a pathogen to attack its host plant (Cooke and Deahl, 1998, as cited in Carlisle *et al.*, 2002). This would mean that all components that contribute to the ability of a pathogen to attack are crucial. Some authors do not agree that these components should then be multiplied together. Carlisle *et al.* (2002) argued that multiplying the components would be superfluous to combine them as they all provided similar information about the isolates tested. However, in this study it was decided that all the correlated components should be combined. While distinguishing aggressiveness of isolates can be difficult, especially among wild-type isolates (Day and Shattock, 1997; Carlisle *et al.*, 2002),

it was not difficult with progeny (sexual and asexual) as there were significant differences even on susceptible cultivars.

Although very few sexual progeny in all three crosses were more aggressive than the parents, these few isolates may became economically important if they were to fit the 'red queen' theory (Wuethrich, 1998; Barton and Charlesworth, 1998; as in Mayton *et al*, 2000), i.e. sexual reproduction generating a new genotype that is fit and adapted to the agro-ecological conditions. Whether potato cultivars with race non-specific resistance are indeed stable and not subject to erosion of and lack of durability of their 'horizontal' resistance continues to be debated. For example, Flier *et al.* (2001) indicated some adaptation of *P. infestans* to this type of resistance in Dutch potato cultivars and suggested adaptation was more likely to occur in sexually reproducing populations.

In this study, there was no association found on any component of aggressiveness and mating type among the parents and among the progeny from the different crosses. These results concur with those found by Flier and Turkensteen (1999), whereby they found no association of any aggressiveness component with mating type in the Dutch population of field isolates of *P. infestans*. These authors attributed the lack of association due to presence of sexual reproduction, whereby random assortment during meiosis guarantees that any association between mating type and aggressiveness is broken after a few cycles of sexual reproduction. However, in this case, this reason would not apply as the progeny used was the first generation therefore, breakage of any association would not have occurred.

Tooley *et al.* (1985), when comparing isolates from Mexico and North-eastern USA found that although sexual and asexual populations differed in components of fitness (components used were: infection frequency, lesion area and sporulation capacity), there was no significant difference in overall fitness, i.e. composite fitness, calculated as the compound of the three fitness parameters. These authors, however, did not take into account latent period. Latent period is an important component of aggressiveness as it affects the rate of additional cycles of infection. In this study there was a significant strong negative correlation between latent period and sporulation capacity, i.e. the shorter the latent period, the higher the

sporulation capacity. These results concur with those observed by Miller *et al.* (1998) who studied the aggressiveness of isolates of *P. infestans* collected from naturally infected potato plants in the Columbia Basin of Washington and Oregon on detached leaflets. The workers observed that sporulation was significantly correlated with all measurements of aggressiveness, including latent period. They also observed that the isolates of the lineage US-8 and US-11 were more aggressiveness than isolates of the US-1, US-6 and US-7 lineage. The US-11 lineage is suspected to be sexual progeny of US-6 and US-7 or US-8 (Gavino *et al.*, 2000). Perez *et al.* (2001) after sampling Peruvian populations of *P. infestans* between 1997 and 1999 suggested that PE-3 may have been through recombination between US-1 and EC-1. The short latent period and high sporulation capacity of aggressive isolates means these isolates would colonise plant tissue faster and sporulate more thus increasing the chance of dispersal and dissemination for these isolates.

Among the sporangial progeny tested for aggressiveness, there was no significant difference among them and their parental isolates in all components of aggressiveness measured. However, the zoosporangial progeny were significantly different from their sporangial parents. The percentage of single zoosporangial progeny less aggressive than their sporangial parents ranged from 16% to 76%. Caten and Jinks (1968) observed variation among asexual progeny of wild isolates of *P. infestans* with regards to colony morphology, rate of growth and sporulation capacity. They observed that variation persisted through at least two successive propagations by single zoospores. Attempts to determine the mechanism(s) responsible for the asexual variability were hampered by the lack of information concerning the genetics of the genus. However, the authors suggested five mechanisms: (i) heterokaryosis, (ii) mutation, (iii) parasexuality, (iv) physiological adaptation, and (v) cytoplasmic control. After further analysis of these different mechanisms, the authors concluded that the hypothesis of cytoplasmic control was the most probable mechanism. Their conclusion was in agreement with that of Galindo (1965), who had suggested that the cytoplasm was involved in the determination of mating type in *P. capsici.* Variability among single zoosporangial progeny was observed in this study. Some germinated zoosporangial progeny were not fit to grow after germination and they were of abnormal size. Those that survived to become established cultures were observed to have

abnormal growth. They tended to grow in a lump and aerially instead of growing radially like the normal cultures. These abnormal cultures were non-pathogenic when inoculated onto susceptible potato cultivars, e.g. Bintje. Abnormal zoospore progeny have been observed also by Abu-El Samen *et al.* (2003). These researchers observed that proportions of zoospores that developed into vegetative colonies varied from 2 to 50% depending on the parental isolate. They also observed abnormal small zoospores whose percentage of recovery was very low. In this study, the mating type or DNA fingerprint of these asexual progeny was not determined. However, Abu-El Samen *et al.* (2003) proved that the single zoospores they were working with were true asexual progeny not a result of isolate mix, heterokaryosis, or parasexuality by using the repetitive DNA probe RG57. When conducting virulence studies, the authors observed that there were differences among the single zoospore progeny. While some maintained the same virulence as their parents, some gained virulence to some R-genes, which the parental parents did not possess. Although virulence studies were not conducted in this thesis, differences in aggressiveness were observed among the single zoospore progeny.

Caten (1970) assessed the aggressiveness of four single zoospore cultures, three were of low levels of aggressiveness and one had a high level of aggressiveness, and their single hyphal tip that had been maintained in rye medium for 9 months (before isolation of the single zoospore progeny). It was observed that the single zoospore progeny with low levels of aggressiveness had their aggressiveness increased to almost that of their parental level. This line of research was not pursued in this study.

In conclusion, the study showed that among large samples of single oospore progeny, some are more aggressive than their parents. Although the percentage of such aggressive progeny is relatively small (less than 20%), selection pressure could result is this population dominating. Therefore, the presence of A2 isolates all over the world and the possibility of sexual reproduction, means that there is a danger of new genotypes resulting from matings that might be even more aggressive than the existing populations. The study also showed that there were differences in aggressiveness of asexual progeny, which supports the hypothesis that *P. infestans* has the capability of continuous change in virulence during

asexual reproduction (Abu-El Samen *et al.*, 2003). Although the study showed that differences in aggressiveness of zoosporangial progeny is as different as in oospore progeny, more research has to be done where a large number of sporangial progeny and a large number of zoosporangial progeny can be compared.

5.0 SENSITIVITY OF SINGLE OOSPORE PROGENY OF *PHYTOPHTHORA INFESTANS* TO FOUR COMMERCIAL FUNGICIDES

5.1 INTRODUCTION

Fungicide treatments are, and will remain, essential for controlling diseases. They form a key component of integrated crop management, and their effectiveness must be sustained as much as possible. Controlling epidemic development of *P. infestans* is mainly by the routine application of fungicides (Bradshaw and Vaughn, 1996). Thus if the presence of A2 mating type means possibilities of sexual reproduction, a knowledge of the efficacy of these fungicides to sexual progeny is important.

5.1.1 Propamocarb hydrochloride

Propamocarb hydrochloride, an oxygen analog of prothiocarb, a thiocarbamic acid developed in 1966 by Schering AG and first released in 1974 for control of soilborne oomycetes (Cohen, 1986), is a systemic fungicide first introduced in 1980 (Bardsley *et al.*, 1996). Since 1993 propamocarb hydrochloride, in combination with mancozeb or chlorothalonil, has been introduced for the control of late blight in 1993 (Löchen and Birchmore, 1990). Propamocarb hydrochloride is acropetally translocated in plants (Cohen, 1986). In year 2000, in Scotland, propamocarb hydrochloride was applied onto 2803 ha (Anonymous, 2000), and in England by 2002, 22 914 ha were treated with propamocarb hydrochloride, which accounted for 16.6% of area grown (Anonymous, 2003).

5.1.2 Metalaxyl

Metalaxyl, the widely used phenylamide, is used as a systemic fungicide to control many soilborne plant pathogens of the oomycetous fungi. Although it is still being used to control late blight, insensitive phenotypes to the fungicides appeared soon after it was released commercially (Davidse *et* al., 1981; Dowley and O'Sullivan, 1981; Pappas, 1985; Deahl *et al.*, 1993).

Metalaxyl resistance in *P. infestans* in the United States and Canada is thought to have originated from two sources (Goodwin *et al.*, 1996). First, it is thought to have been

introduced by migration from northwestern Mexico, where by 1989, metalaxyl resistance was almost 100% (Matuszak *et al.*, 1994). The alternative hypothesis is that resistance arose by mutation within United States.

Marshall and Stevenson (1995) reported a large variation, in metalaxyl sensitivity, among 87 isolates that were collected from Wisconsin and Northern Illinois. Isolates in each mating type reacted in as many as three different ways to metalaxyl. The A1 mating type isolates (23 out of 87) were sensitive (17 isolates), intermediate/sensitive (2 isolates) or intermediate (4 isolates). The first two sensitivity reactions were exclusive to the A1 mating type isolates. However, the third sensitivity reaction was shared between A1 and A2 isolates whereby 29 A2 isolates (out of 64) were also intermediate to metalaxyl. The remaining A2 isolates were either insensitive (3 out of 64) or insensitive/intermediate (30 out of 64). Again, the last two sensitivity reactions to metalaxyl were exclusive to the A2.

Similar results have been reported by Peters *et al.* (1998), where populations of *P. infestans* changed from being A1 metalaxyl-sensitive isolates to be dominated by A2 metalaxyl insensitive (MI) within two years in Canada. Mosa *et al.* (1989) reported an increase in A2 in Japan from 61% in 1987, when A2 was first detected, to 90% in 1989. These authors also observed that the A2 mating type isolates were more aggressive than the A1 isolates, causing more epidemics and producing a larger lesion size than A1 isolates. Derie and Inglis (2001) observed that in Western Washington metalaxyl-sensitive isolates were 5% in 1998 and increased to 72% a year later and the population was US-8.

The most common protocol for metalaxyl sensitivity testing is *in vitro* testing, whereby isolates are grown on metalaxyl-amended medium. Sensitivity is expressed as a percentage growth on metalaxyl-amended medium divided by the growth of the same isolate on media without metalaxyl. The relationship between *in vitro* and *in vivo* sensitivity to metalaxyl has been investigated and the results were found to be similar (Shattock *et al.*, 1990; Matuszak *et al.*, 1994). The concentration of metalaxyl used differs with different researchers; some use 10 :gmL^{-1} (Shattock, 1988; Shattock *et al.*, 1990; Sedegui *et al.*, 2000), while some use two concentrations, 5 and 100 :gmL^{-1} (Therrien *et al.*, 1993; Forbes *et al.*, 1996; Mukalazi *et al.*,

2001), or 0.5 and 5 :gmL^{-1} (Fabritius *et al.*, 1997), and other researchers use more. Daayf and Platt (1999) used three different concentrations: 1, 10 and 100 :g/mL^{-1}.

5.1.3 Cymoxanil

Cymoxanil, a cyanoacetamide-oximes (Serres and Carraro, 1976, as cited by Schwinn and Urech, 1986), is more effective against hyphal growth stages than early growth phases (the release of zoospores and their germination) (Hewitt, 1998). It has been found to prevent disease development when applied two to three days after artificial inoculation of field-grown potatoes or tomatoes (Douchet *et al.*, 1977). It is locally systemic with penetrant and translaminar activity. It is preventative and curative by also inhibiting disease development after infection. It has acropetal systemicity. In Scotland, in 2000, 59 657 ha were sprayed with cymoxanil (Anonymous, 2000), and in England by 2002, 120 000 ha were treated with cymoxanil, which is 87% of area grown (Anonymous, 2003).

5.1.4 Fluazinam

Fluazinam is a non-systemic, contact fungicide that belongs to the pyridinamine group (Section 1.2.1.1). Its mode of action is through uncoupling activity on mitochondrial oxidative phosphorylation (Tomlin, 1994; Hewitt, 1998). This means that these compounds permit electron transport to proceed with the maximum uptake of oxygen, but without the production of ATP necessary for cellular energy (Hewitt, 1998). In Scotland, in 2000, 46 146 ha were sprayed with fluazinam, while in England by 2002, 89 840 ha, which is 65.1% of area grown, were sprayed with fluazinam.

5.1.5 Azoxystrobin

Azoxystrobin is a commercially available analogue of strobilurin. Strobilurins were found to inhibit electron transfer in complex III (bc_1 complex) of the mitochondrial electron transport chain by binding to the Q_o site (the outer, quinone oxidizing pocket) of cytochrome bc_1 enzyme complex (Becker *et al.*, 1981). This causes inhibition of mitochondrial respiration thus blocking the ATP synthesis i.e. energy production within fungi and oomycetes is stopped (Wiggings and Jager, 1994). Therefore, highly energy-demanding stages of fungal development are particularly sensitive to the effects of strobilurins

5.1.6 Aim

The aim of this chapter was to assess the inheritance of fungicide sensitivity among the sexual progeny of the three crosses and some asexual sporangial and zoosporangial progeny, originating from parental isolates used in matings and some of their single oospore progeny.

5.2 MATERIALS AND METHODS

5.2.1 Production of inoculum

Inoculum used for *in vivo* experiments was generated by passing each isolate through a susceptible potato cultivar Bintje three times. This was done by harvesting sporangia from 10 days old cultures by spraying the plate with a fine mist of water. The spores were then adjusted to 2 x 10^4 by counting the number of spores in 5 :L on water agar. The counting was done three times per isolate and the average of the three readings was used to extrapolate the concentration of the spores. The adjusted spores were then inoculated onto detached potato leaflets of cultivar Bintje, to restore pathogenicity. These detached leaflets were placed, abaxial surface up, in square Petri dishes lined with moistened blue paper. Each leaflet was inoculated with droplets containing sporangia, about 15 :L of water. The plates were sealed with masking tape to reduce evaporation and kept at 18 ^{0}C at 16 hours photoperiod. Spores were harvested after 6 days by spraying a fine mist of water onto each leaflet held over a glass beaker. The harvested sporangia were again adjusted to 2 x 10^4 and 15 :L of water was used for the actual inoculation.

5.2.2 Metalaxyl sensitivity testing

Testing of metalaxyl sensitivity was done *in vitro*, first, using a method of Goodwin *et al.* (1996), and later using a method of Shattock (1988). In the former, two different concentrations of metalaxyl: 5 :gmL^{-1} and 100 :gmL^{-1}, and in the latter, only one concentration (10 :gmL^{-1}) was used. The fungicide was incorporated into rye B agar medium from a stock solution of 100 mgmL^{-1} made in dimethyl sulfoxide (DMSO). For 5 µgmL^{-1}, 50 µL of stock solution per litre of agar was used, for 10 µgmL^{-1}, 100 µL of stock solution per litre of agar was used, and for 100 µgmL^{-1}, 1 mL of stock solution per litre of agar was used. Control (no fungicide) medium contained 0.1% DMSO (Matuszak *et al.*, 1994; Sujkowski *et al.*, 1995). DMSO solutions with or without fungicide were added into

molten, cooled agar (*ca.* 50 ^{0}C) before the medium was dispensed into Petri dishes. Discs of agar with mycelium (7 mm diameter) were cut from margins of actively growing isolates of 7-10 day old colony growing on rye A agar, using a cork-borer. The agar discs were placed with the mycelium in contact with the test medium. Each Petri dish was inoculated with two agar plugs (2 isolates) at 180 ^{0} from each other. Each isolate was inoculated into three (or 2 in the latter method) Petri dishes: control (no metalaxyl), 5 :gmL^{-1} agar and 100 gmL^{-1} agar and replicated twice. The Petri dishes were then incubated at 18 ^{0}C without illumination. After 7 days the radius of the colonies were recorded. The radius was then corrected for the size of the initial agar disc (7 mm).

5.2.3 Propamocarb hydrochloride sensitivity testing

Two different methods were used, i.e. *in vivo* and *in vitro*.

5.2.3.1 In vivo testing

The *in vivo* testing was done using the floating disk method (Bardsley *et al.* 1996). Leaf disks of 12 mm diameter were cut from potato leaves of the susceptible cultivar Home Guard. The leaf disks were floated abaxial side up in multiwell repliplates (Fisons Scientific Equipment, Loughborough, Leicestershire LE11 0RG, UK) containing 3 ml of fungicide solution or water (control). The different concentrations of the propamocarb hydrochloride used were: 10, 50, 100, 250, and 500 µgmL^{-1} active ingredient (a.i.). Each isolate was tested against all different fungicide concentrations replicated five times (i.e. in each repliplate 5 isolates were inoculated onto leaf disks replicated 5 times). Before inoculation, each isolate was passed through Bintje leaves to restore pathogenicity (see preparation of inoculum section above). Each leaf disk was inoculated with 10 :L of sporangia suspension of 1 x 10^4 sporangia per mL. The repliplates were incubated for 2 hours at 10 ^{0}C to induce zoospore release from sporangia. After zoospore release the plates were then incubated at 18 ^{0}C with illumination for 7 days.

Sporulation was scored using the following method:

0	sporangiophores absent
1	1-4 sporangiophores per disk
2	5-12 sporangiophore per disk
3	moderate sporulation; sporangiophore only visualised under dissecting microscope
4	profuse sporulation visible with the naked eye

After scoring of the sensitivity of the isolates to propamocarb hydrochloride, sporangia from each isolate, in each concentration, were collected and re-inoculated on fresh leaf disks to test their infectivity. The same replication, incubation temperature and period, and scoring method was used.

5.2.3.2 In vitro testing

The *in vitro* testing was performed using the same fungicide concentration as in the *in vivo* testing. The different fungicide concentrations, i.e. 10, 50, 100, 250 and 500 μgmL^{-1} were added into Rye B as discussed in section 5.2.2. Each isolate was replicated twice.

5.2.4 Cymoxanil sensitivity testing

Cymoxanil was tested *in vitro*. Three different concentrations of were used. These were 1, 10 and 100 μgmL^{-1} and the control contained no fungicide. The procedure was similar to the one for metalaxyl (section 5.2.2). Each plate was inoculated with two isolates at either end and each isolate was replicated twice. The Petri dishes were incubated at 18 ^{0}C in the dark and mycelial growth was recorded 6 days after inoculation.

5.2.5 Fluazinam sensitivity testing

The procedure for testing fluazinam sensitivity was similar to the one described above (Cymoxanil). However, only two concentrations were used: 10 and 100 μgmL^{-1}.

5.2.6 Azoxystrobin

The testing of Azoxystrobin was done *in vivo* and *in vitro*. The concentration used were the same for both procedures and they were 1 and 10 μgmL^{-1}.

5.2.6.1 In vivo

For the *in vivo* testing potato cv. Home Guard was used. Two procedures were used: one followed the procedure used for propamocarb hydrochloride; and the other following the procedure by Wong and Wilcox (2000), which involves coating whole leaf discs in the fungicide solution. In the first procedure, leaf discs were floated on different concentrations of azoxystrobin in a repliplate (as discussed in section 5.2.1.1). In the second procedure, the leaf discs were coated with the different concentration of azoxystrobin. This was done by soaking the leaf discs in the different fungicide solutions for 1 hour. The discs were then spread on a square Petri plate, bloated dry and inoculated with 15 :l of adjusted spore suspension.

5.2.6.2 In vitro

In all the *in vitro* testing, the colony radius was measured daily for 7 days. Percentage growth (relative growth) was calculated by subtracting the diameter of the agar plug (7 mm) from the radius of each colony and dividing by the radius of the unamended control colonies after 7 days.

5.3 RESULTS

The parental isolates that were used in this study were chosen mainly because of their metalaxyl sensitivity status (Table 5.3.1).

Although different concentrations of the different fungicides were used for the *in vitro* experiments, only the 10 μgmL^{-1} concentration was used to differentiate sensitivity of isolates. However, for propamocarb hydrochloride, 50 μgmL^{-1} was used as it was the lowest concentration that showed differences among isolates. Isolate were considered sensitive if they had relative growth of less than 10%, intermediate if they had a relative growth greater than 10% but less than 40%, and resistant if their relative growth was over 40 percent. The same categories were used for all *in vitro* testing for all four fungicides.

Table 5.3.1. Sensitivity of the parental isolates of *P. infestans* to five different fungicides *in vitro*.

Isolate	Origin	MT	Met. status[a]	Prop. status[b]	Cymox. status[a]	Fluazin. status[a]	Azoxys. status[a]
96.70	UK	A2	MS (8)[c]	PI (17)	CR (50)	FI (20)	AS (0)
96.69	UK	A1	MR (63)	PR (45)	CR (62)	FR (45)	AS (3)
96.89.43	UK	A1	MS (9)	PS (9)	CS (8)	FS (0)	AS (0)
P9463	California	A1	MR (142)	PR (53)	CR (58)	FS (0)	AS (0)
96.36.1	UK	A1	MR (89)	PR (60)	CR (61)	FI (25)	AS (0)

[a] - 10 μgmL^{-1} or $\mu g/mL^{-1}$ used
[b] - 50 μgmL^{-1} used
[c] – figures in parentheses indicate mean percentage growth in presence of fungicide

UK A1 parental isolate 96.89.43 (RF032) was sensitive to all fungicides tested (Table 5.3.1). The other UK A1 parental isolate 96.69 (RF039) exhibited different levels of insensitivity (>40% growth on fungicide amended agar) for all fungicides tested except azoxystrobin. The A2 UK isolate was sensitive to metalaxyl and to azoxystrobin, it was intermediate in its response to propamocarb hydrochloride and fluazinam, although on the two latter fungicides

it was towards the lower scale, namely 17% and 20% of growth relative to control. The Californian parental isolate P9463 was chosen for its insensitivity to propamocarb hydrochloride (Bardsley *et al.*, 1998). This isolate also exhibited very high level of insensitivity against metalaxyl (Table 5.3.1, Figure 5.3.1 plate a and b). The isolate also exhibited different levels of insensitivity to cymoxanil but it was very sensitive to fluazinam and azoxystrobin. This parental isolate consistently had a higher relative growth on all concentrations of propamocarb hydrochloride, i.e. it grew on all concentrations (Figure 5.3.1 plates f to i). The UK isolate 96.36.1 that was only included in the study for the asexual progeny testing, was resistant to metalaxyl, propamocarb and cymoxanil. For fluazinam it was an intermediate phenotype but it was sensitive to azoxystrobin (Table 5.3.1).

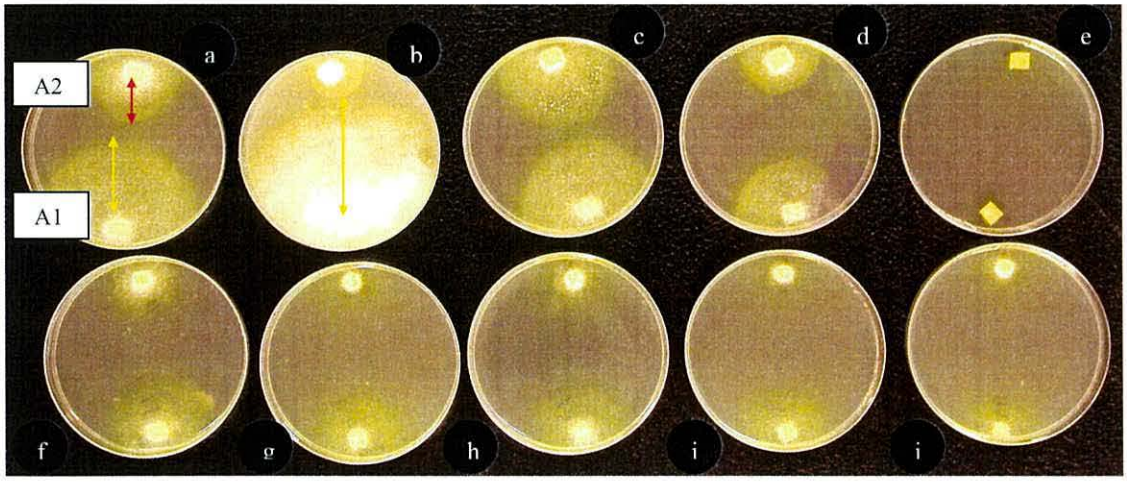


Figure 5.3.1. Two parental isolates, A1 Californian isolate P9463 and A2 UK 96.70, after seven days on Rye B (with RAN) amended with different fungicides at different concentrations. Plate a is the control; b contains 10µgmL^{-1} metalaxyl; plates c, d and e contain 1, 10 and 100 µgmL^{-1} of cymoxanil, respectively; and plates f to j contain 10, 50, 100, 250 and 500 µgmL^{-1} of propamocarb hydrochloride, respectively.

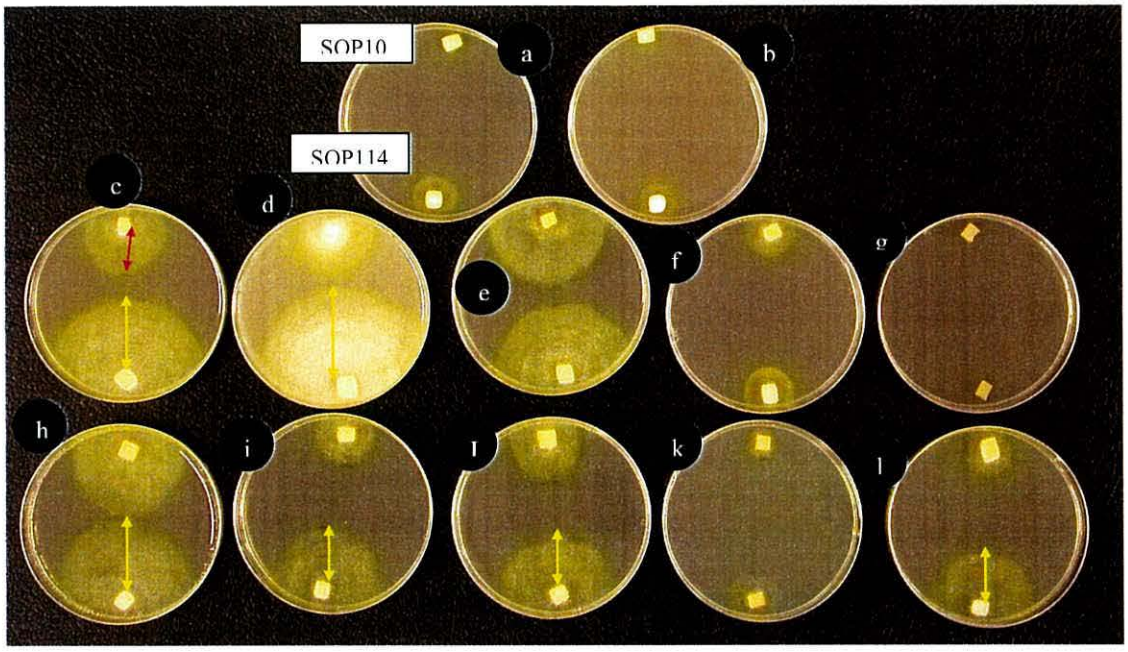


Figure 5.3.2. Two single oospore progeny (SOP) from cross #3 (Californian A1, P9463 and UK A2, 96.70) after seven days on Rye B (with RAN) amended with different fungicides at different concentrations. Petri dishes a and b contain 10 and 100 μgmL^{-1}, respectively of fluazinam. Petri dish c is the control; d contain 10μgmL^{-1} of metalaxyl; plates e, f and g contain 1, 10 and 100 μgmL^{-1}, respectively, of cymoxanil; and plates h to l, contain propamocarb hydrochloride at concentrations, 10, 50, 100, 250 and 500 μgmL^{-1}, respectively.

5.3.1 Sensitivity to metalaxyl

5.3.1.1 Sexual progeny

The ratio of metalaxyl phenotype of single oospore progeny (SOP) of a UK A1 metalaxyl sensitive (MS), RF 032 parental isolate (96.89.43), and UK A2 metalaxyl sensitive, RF 040, parental isolate (96.70), was 31:134:25, for MS: MI: MR, respectively (Figure 5.3.3a). For the SOP that were sensitive, the ratio of A1s to A2s to selfs to undetermined, was 16:7:4:4; for the intermediate the ratio was 100:27:3:4; and for the resistant, it was 15:6:2:2. Of the 25 resistant SOP, none had a colony radius greater than that of the control.

When a UK A1 metalaxyl resistant (MR), RF 039 parental isolate 96.69, was crossed with a UK A2 metalaxyl sensitive (MS), RF 040 parental isolate 96.70, the ratio of metalaxyl phenotype of the sexual progeny was 13:40:117, for MS: MI: MR, respectively (Figure

5.3.3b). Among the sensitive SOP, there were more A1s than A2s (9:2, A1:A2). There were also more A1s among the resistant SOP (64:47, A1:A2). However, on the MI category, there were more A2s than A1s (13:20, A1:A2). Of the 117 resistant SOP, 18 had a colony radius greater than that of the control and two progeny had radius equal to that of the control. There were equal numbers of A1 and A2 among these super resistant progeny (9, 9 and 2, A1, A2 and selfs, respectively).

When a Californian A1 MR (RF not determined), P9463, was crossed with a UK A2 MS (RF 040), 96.70, the ratio of metalaxyl phenotype of sexual progeny was 20:37:128, for MS: MI: MR, respectively (Figure 5.3.3c). In this cross, the ratio of A1 to A2 progeny that were sensitive was almost 1:1 (11:8), for the MI, the ratio was 6:1 (26:4), and for the MR the ratio was almost 3:1 (84:30). Of the 128 resistant progeny, 24 had colony radius either equal to, 9 (6 A1 and 3 A2), or greater than, 15 (10 A1, 4 A2 and 1 self), that of the control.

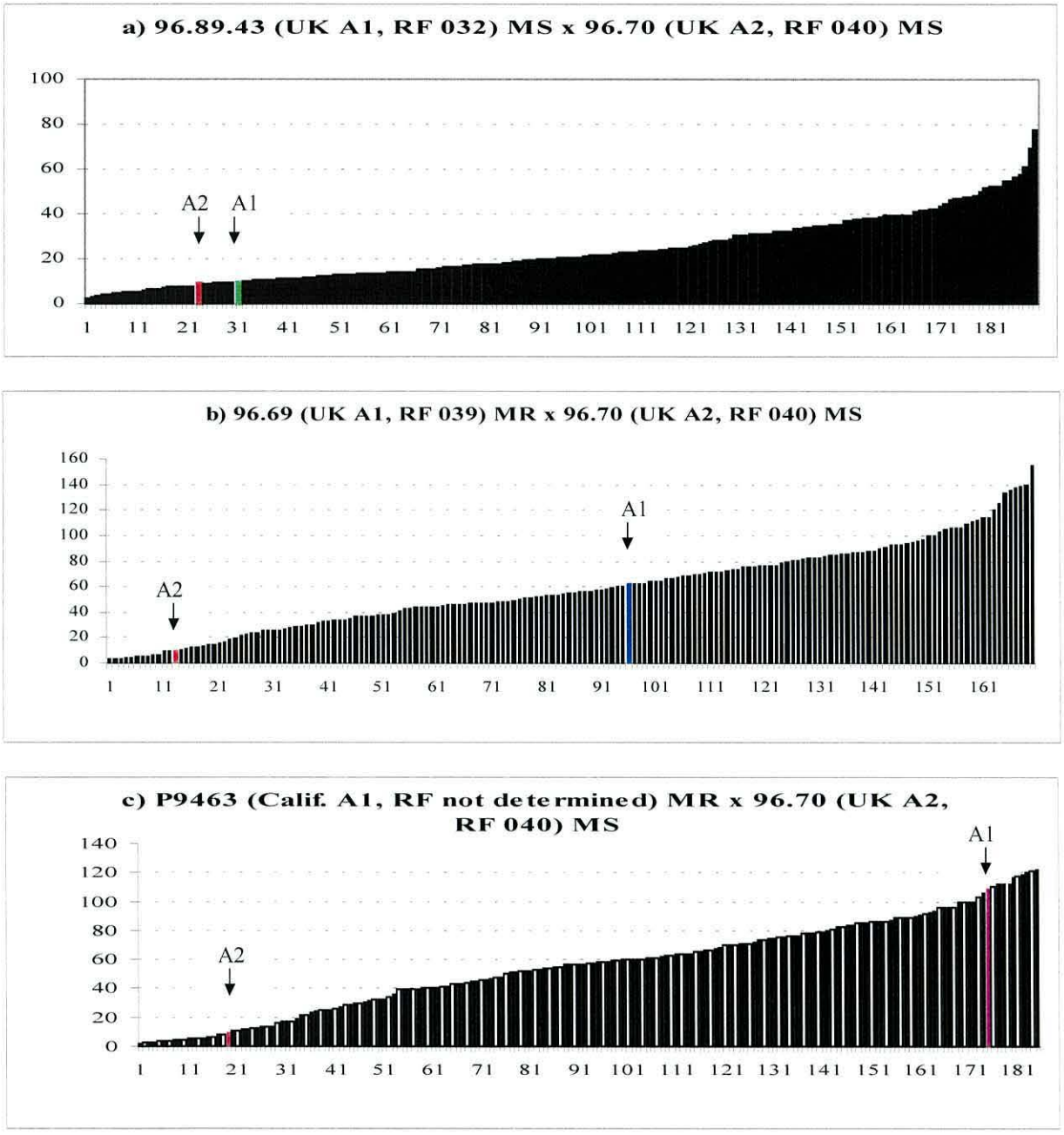


Figure 5.3.3. ***In vitro*** **sensitivity of single oospore progeny and parental isolates of three crosses of** ***Phytophthora infestans*** **to 10 µgmL^{-1} of metalaxyl.**

5.3.1.2 Asexual progeny

The sensitivity reaction of parental isolates, their sporangia (5), their zoosporangia (5 from each sporangium), one SOP from each cross with its sporangia and zoosporangia are presented in Table 5.3.2. Although resistant sporangial and zoosporangial progeny were obtained sometimes even from a sensitive parent, none of these had mycelial radius greater than the control, i.e. negative relative growth, as observed with single oospore progeny.

Table 5.3.2. *In vitro* sensitivity of five sporangial isolates and five zoosporangial isolates from each sporangial isolate of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl.

Isolate and its origin	Met. Status of isolate	Sensitivity of sporangial isolates			Sensitivity of zoosporangial isolates		
		MS	MI	MR	MS	MI	MR
96.70 (UK A2)	MS	5	0	0	14	10	1
96.69 (UK A1)	MR	0	2	3	2	10	13
96.69-9 (SOP A1)	MR	0	2	3	3	17	5
96.89.43 (UK A1)	MS	5	0	0	19	6	0
96.89.43-80 (SOP A1)	MI	2	3	0	11	9	5
96.36.1 (UK A1)[a]	MR	0	2	3	0	3	22
96.36.1 –19 (SOP A1)[b]	MR	0	0	5	0	0	25
TOTAL		**12**	**9**	**14**	**49**	**55**	**71**

[a] – another UK A1 isolate used in a different study (G. E. Harper, unpublished data).
[b] – SOP from a cross of the other UK A1 isolate, 96.36.1, and the A2 used in this study.

All five single sporangial progeny from each of the two UK parental isolates (96.70 and 96.89.43) were metalaxyl sensitive too. Metalaxyl intermediate SOP (80) from the mating between these two metalaxyl sensitive parents had sensitive and intermediate phenotype in their single sporangial progeny but no resistant phenotypes. Metalaxyl resistant parent (96.69) and its resistant SOP (9) both yielded intermediate and resistant but no sensitive sporangial progeny.

Zoospore progeny from the sporangial progeny of parental isolate 96.70 (A2, MS) were mainly sensitive or intermediate, however, one zoosporangial progeny was resistant (Figure 5.3.4b). Those of sporangial progeny of resistant parent, 96.69, were either intermediate or resistant but two of them were sensitive (Figure 5.3.4a). However, those from its resistant SOP (SOP 9) were mainly intermediate, few were either sensitive or resistant, 3 and 5, respectively (Figure 5.3.4c). Zoosporangial progeny of sporangial progeny of sensitive parent, 96.89.43, were mainly sensitive and a few were intermediate but none was resistant. Equivalent progeny from intermediate phenotype SOP (80) were either sensitive or intermediate with five of them being resistant (Table 5.3.1).

Another UK A1 parental isolate that was used in another study (G. E. Harper, unpublished data) was included for the asexual progeny test. This isolate 96.36.1 was metalaxyl resistant and one of its sexual progeny was used for the same assessment. The sporangial progeny of the parental isolate were either MI (2) or MR (3), and all sporangial progeny of the sexual progeny were MR. When zoosporangial progeny derived from the sporangial progeny were assessed, none were sensitive and except for 3 intermediate phenotypes, all zoosporangial progeny from the sporangia of the sexual progeny were resistant.

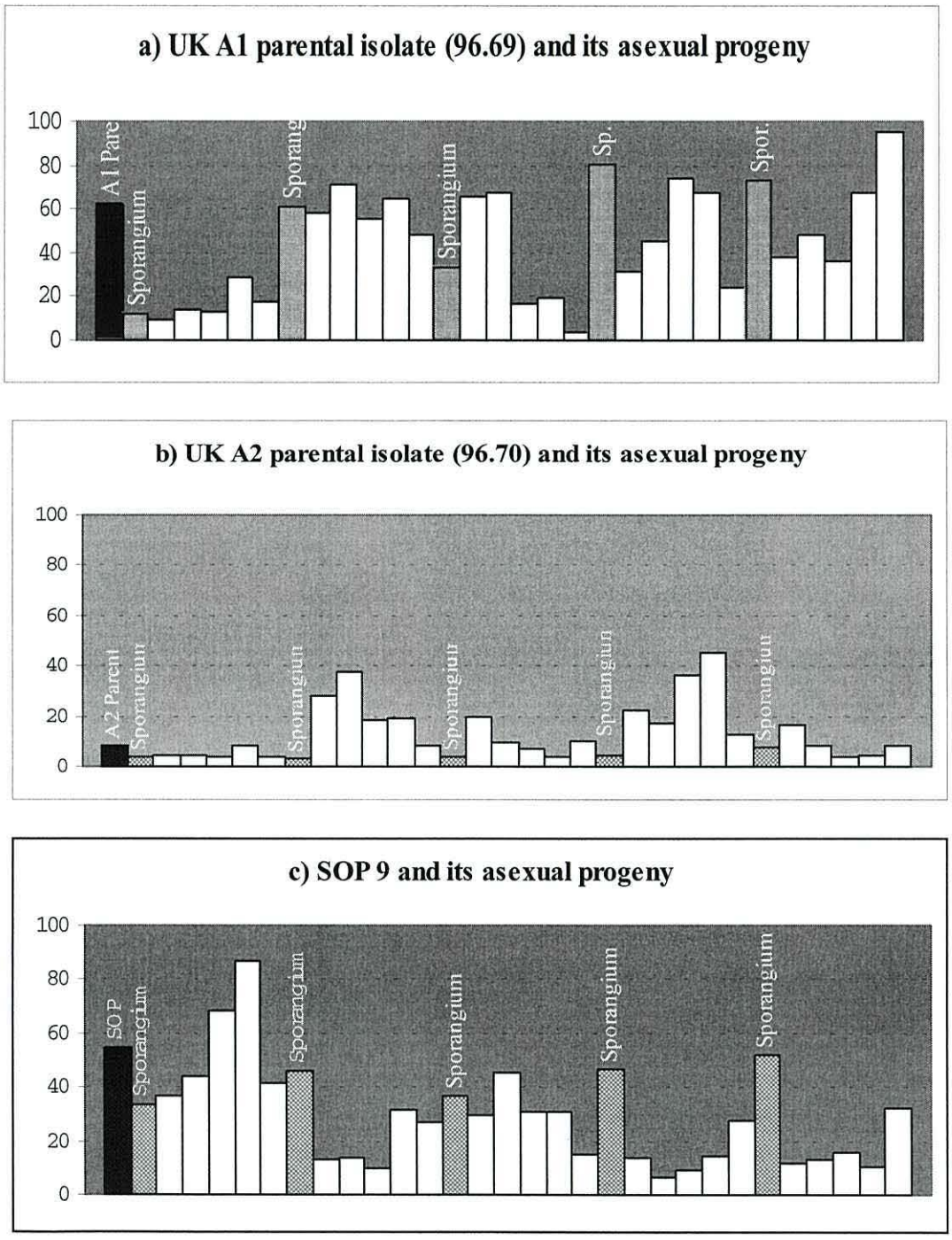


Figure 5.3.4. ***In vitro*** **sensitivity of five sporangial isolates (shaded blocks) and five zoosporangial isolates (blank blocks) from each sporangial isolate of** ***Phytophthora infestans*** **to 10 µgmL^{-1}of metalaxyl. The zoosporangial were derived from the sporangial isolate (on the left of every five sporangial isolates).**

5.3.2. Propamocarb sensitivity

5.3.2.1 In vitro

For propamocarb, five different concentrations were used for the study (10, 50, 100, 250 and 500 μgmL^{-1}) in two methods (*in vivo* and *in vitro*). However, the concentration that was used to differentiate sensitivity of parental isolates and their progeny was 50 μgmL^{-1} for the *in vitro* method.

The UK A1 (96.69) was resistant, whilst among its sexual progeny from cross #2, the ratio of PS: PI: PR was 43: 98: 29 (Appendix 5.2a and 5.2b). No sporangial progeny were found to be sensitive, but were either PI (2) or PR (3). Also, no zoosporangial progeny were found to be sensitive, they were mainly PI (22) and a small number (3) was resistant (Figure 5.3.6a). One sexual progeny that was an intermediate phenotype, had no sensitive sporangial progeny, but instead two were intermediate and the other three were resistant (Figure 5.3.6c). Also, no zoosporangial progeny were sensitive, instead almost equal number were PI (13) and PR (12).

The other UK A1 parental isolate 96.89.43 of cross #1 was sensitive to propamocarb hydrochloride. Single oospore progeny from this isolate (and the UK A2 isolate) had a ratio of 143:35:12 (PS: PI: PR). The intermediate and resistant progeny had relative growth on the low end of the scale (Appendix 5.1a and 5.1b). Sporangial progeny of this isolate were all sensitive and so too were the zoosporangial progeny, except for one zoosporangial progeny that was intermediate. When one sensitive SOP from this isolate (and the UK A2 96.70) had its asexual progeny tested, three sporangial progeny were sensitive and two were intermediate. Zoosporangial progeny derived from the five sporangial progeny were 11:8:6, PS: PI: PR, respectively.

For the Californian A1 parental isolate of cross #3, P9463 (propamocarb insensitive), there was no difference across the range of concentrations used except for the lowest concentration which had mycelial growth similar to the control. The ratio of PM: PI: PR for the sexual progeny was 8:41:136 (Appendix 5.3a and 5.3b). About 60% of the resistant progeny grew to more-or-less same diameter in all concentrations (Figure 5.3.5). No asexual progeny were

assessed for this parental isolates or for any of its sexual progeny (i.e. no sporangia from any sexual progeny was assessed).

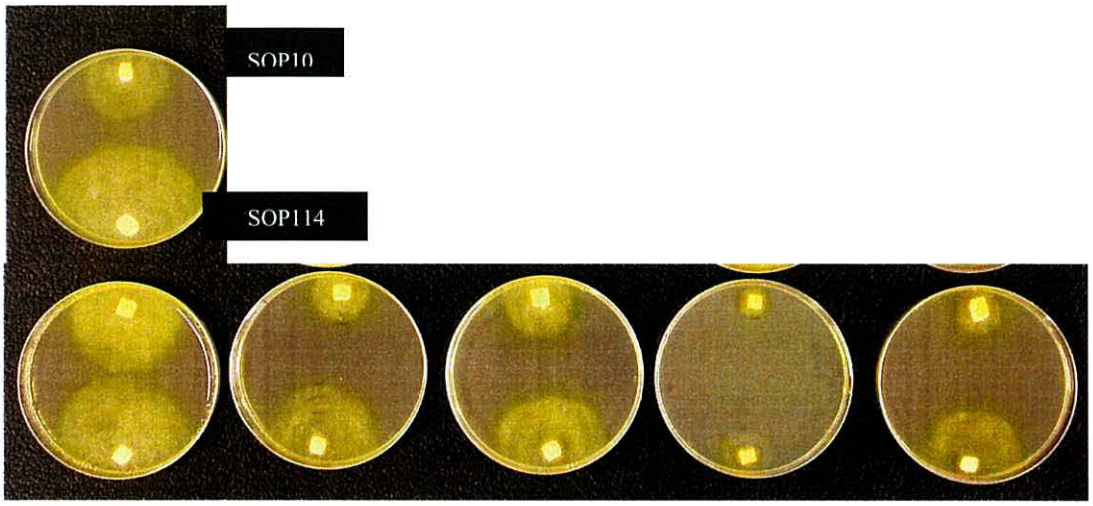


Figure 5.3.5. Single oospore progeny of parental isolate P9463 (Californian A1) and 96.70 (UK A2) showing their growth of control (top Petri dish) and growth on different concentrations of propamocarb hydrochloride (bottom row). The concentrations were 10, 50, 100, 250 and 500 μgmL^{-1}, left to right.

The UK A2, isolate used in all three crosses, was intermediate for propamocarb hydrochloride, however, it was on the lower end of the scale (17% of the control). Sporangial progeny from it were also intermediate (Figure 5.3.6b). The zoosporangial progeny were mainly intermediate (22), only one was sensitive and 2 were at the border between intermediate and resistant (41 and 42% of the control).

The other UK A1 isolate 96.36.1 used in a separate study (G. E. Harper, unpublished data) was resistant, and none of its sporangial progeny were sensitive, only one was intermediate and the rest (4) were resistant. The results were similar for zoosporangial progeny, none were sensitive, 16 were intermediate and 9 were resistant. The sexual progeny (SOP 19) used was resistant to propamocarb hydrochloride, only one of its five sporangial progeny was intermediate and the other four were resistant. However, among the zoosporangial progeny ten were sensitive, 13 were intermediate and only two were resistant.

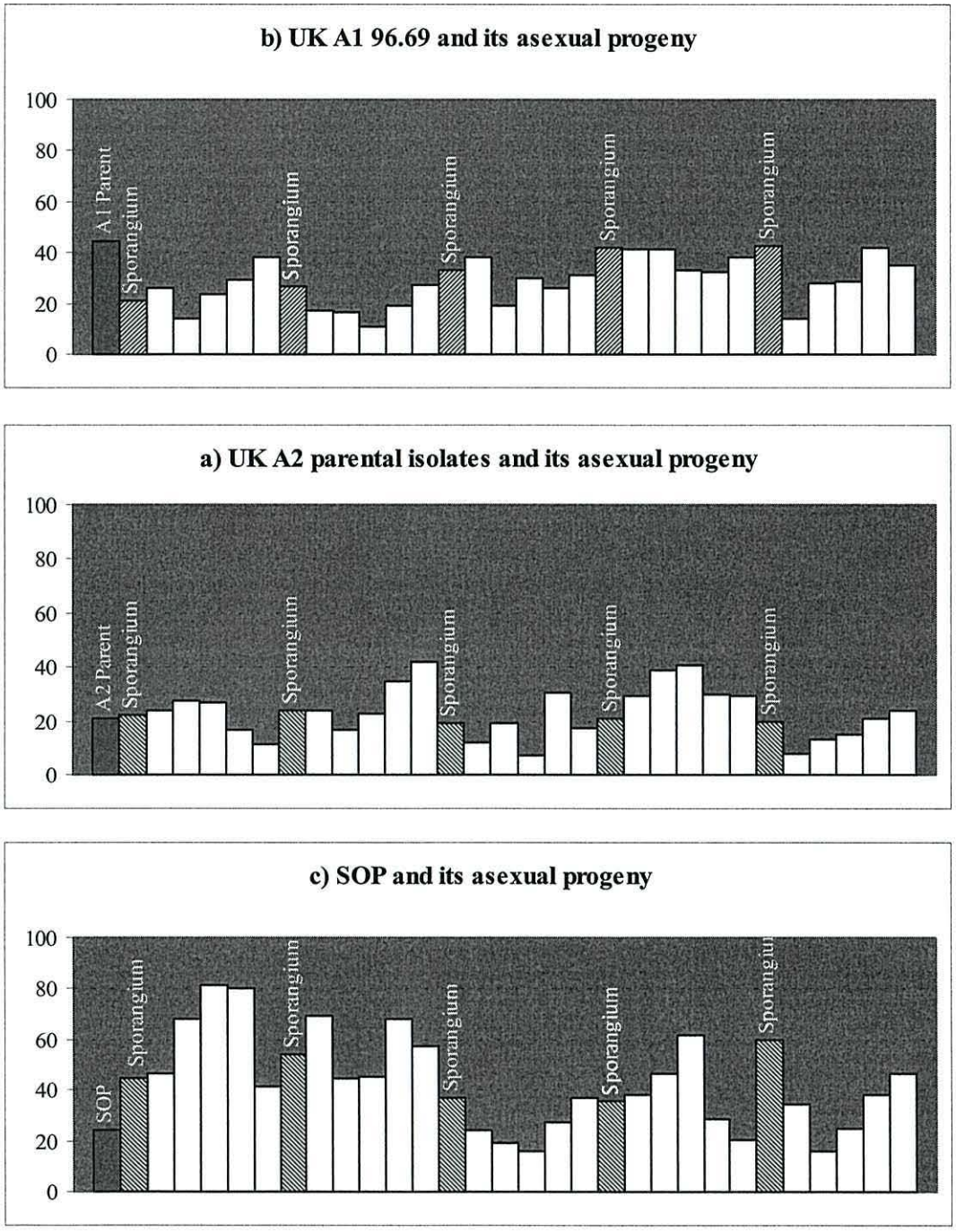


Figure 5.3.6. Propamocarb sensitivity, at 50 µgmL^{-1} of two UK parental isolates of *Phytophthora infestans*, their asexual progeny (sporangia and zoosporangia) and a single oospore progeny and its asexual progeny. Sensitivity of each of five zoosporangial isolates is represented by blank blocks.

5.3.3 Cymoxanil

The ratio of CS: CI: CR among the sexual progeny of cross #1 (96.89.43 x 96.70, both UK) was 161: 24: 5 (Appendix 5.1a & b). Sporangial progeny from the A1 parent were all sensitive but the zoospore derived cultures were 9:14:2, for CS: CI: CR, respectively. Three of the sporangial progeny of the cymoxanil intermediate SOP (80) were sensitive and the other two were intermediate, while none were resistant. When zoosporangial progeny derived from these sporangial progeny were tested, 12 were sensitive and 13 were intermediate, again none were resistant.

Among single oospore progeny of cross #2 (96.69 x 96.70, UK/UK), the ratio of CS: CI: CR was 26:79:65 (Appendix 5.2a & b). Sporangial progeny from UK A1 (96.69) were sensitive (2), intermediate (2), and resistant (1) (Figure 5.3.7a). Zoosporangial progeny derived from them were mainly intermediate (19), only one was resistant and five sensitive. When an intermediate SOP (9) had its sporangial and zoosporangial progeny assessed, the sporangial progeny were either sensitive (3) or intermediate (2) (Figure 5.3.7c). However, the zoosporangial progeny were mainly intermediate (17), only one was resistant and 8 were sensitive.

Sporangial progeny of the A2 isolate (96.70), with relative growth of 50% and therefore classed as a resistant phenotype were either sensitive (2) or intermediate (3). Zoosporangial progeny derived from these sporangial progeny were either sensitive (8), intermediate (10), or resistant (7) (Figure 5.3.7b).

Sporangial progeny from the resistant UK A1 isolate 96.36.1 were mainly resistant (4) and only one was intermediate (none sensitive). There were no sensitive zoosporangial progeny, they were either intermediate (11) or resistant (14). When a resistant SOP had its sporangial and zoosporangial progeny assessed, none were sensitive, instead they were either intermediate or resistant.

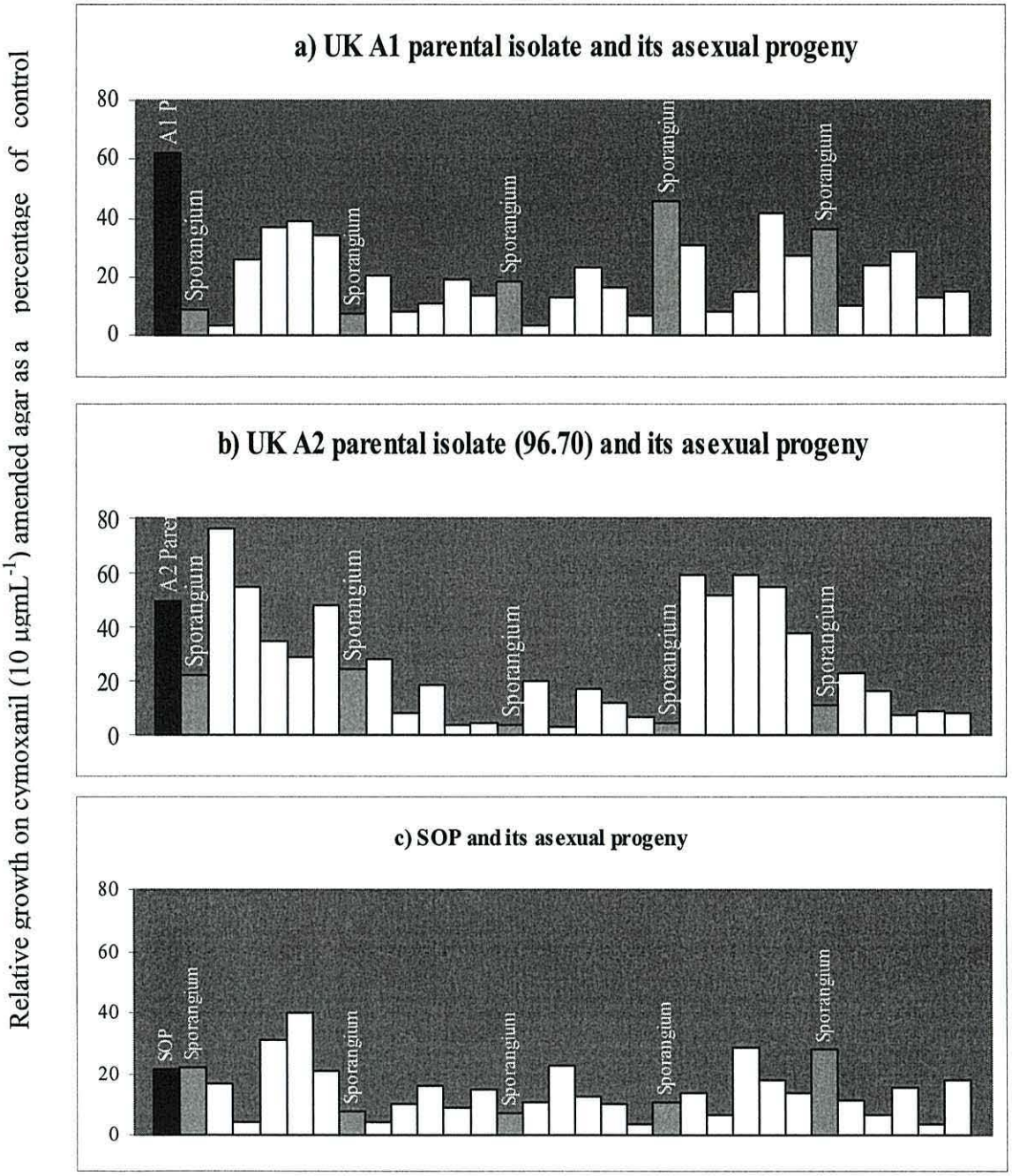


Figure 5.3.7. Cymoxanil sensitivity, at 10 µgmL^{-1} of two UK parental isolates of *Phytophthora infestans*, their asexual progeny (sporangia and zoosporangia) and a single oospore progeny and its asexual progeny. Sensitivity of each of five zoosporangial isolates is represented by blank blocks.

Single oospore progeny from the Californian A1 isolate P9463 (and UK A2, 96.70) had the ratio 17:4:164 for CS: CI: CR, respectively (Appendix 5.3a & b). No asexual progeny were tested for this isolate or for any of its single oospore progeny.

5.3.4 Fluazinam

None of the single oospore progeny of cross #1 (96.89.43 x 96.70), involving sensitive A1 and intermediate A2 parents, were resistant or intermediate to fluazinam (Appendix 5.1a & b). When five sporangial progeny from it were tested, they were sensitive too. Zoosporangial progeny derived from the sensitive sporangial progeny, however, produced two intermediate as well as 22 sensitive progeny.

Among the sexual progeny from cross #2 (96.69 UK (fluazinam resistant) x 96.70 UK (fluazinam intermediate)), the ratio of FS: FI: FR was 91:47:32 (Appendix 5.2a &b). Sporangial progeny of UK A1 (96.69 FR) were either sensitive (2) or intermediate (3) and none were resistant although the parental isolate was resistant (Figure 5.3.8a). Zoosporangial progeny were mainly resistant (11), five were sensitive and four were intermediate. Sporangial progeny from an intermediate SOP (9) were mainly intermediate (4), one was resistant and none was sensitive (Figure 5.3.8c).

Sporangial progeny from A2 (FI) isolate were all intermediate, although the zoosporangial progeny were mainly intermediate (19), only one was sensitive and 5 were resistant (Figure 5.3.8b).

For cross #3, the Californian A1 P9463 (fluazinam sensitive) and UK A2 96.70 (FI), the ratio was 103: 52: 30, FS: FI: FR, respectively (Appendix 5.3a & b). Again, no asexual progeny from either A1 parent or from any of the single oospore progeny was assessed for sensitivity to fluazinam.

Sporangial progeny from UK A1 (96.36.1 FI) were all intermediate. The zoosporangial progeny were mainly sensitive (22), three were intermediate and none were resistant.

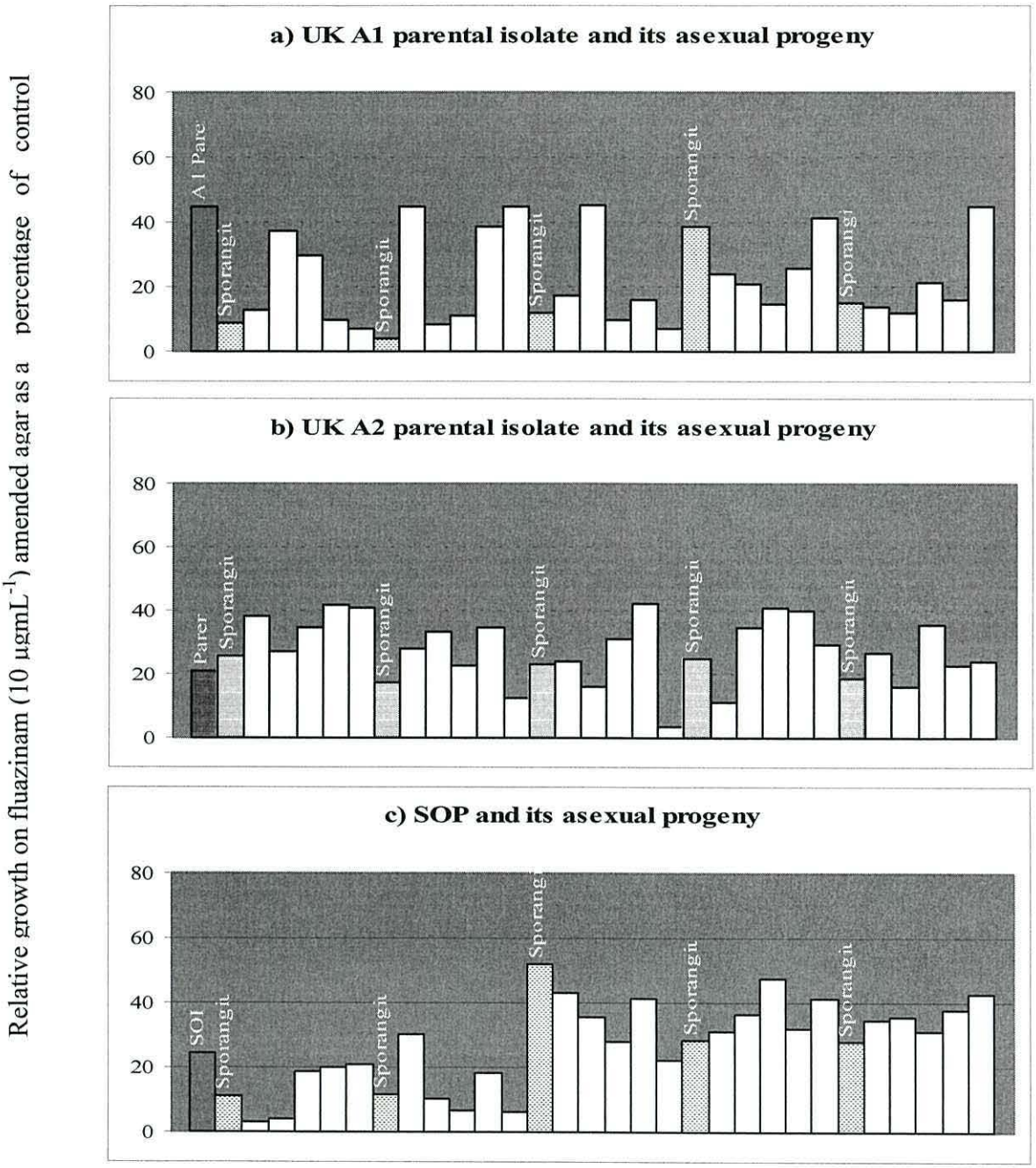


Figure 5.3.8. Fluazinam sensitivity, at 10 µgmL^{-1} of two UK parental isolates of *Phytophthora infestans*, their asexual progeny (sporangia and zoosporangia) and a single oospore progeny and its asexual progeny. Sensitivity of each of five zoosporangial isolates is represented by blank blocks.

5.4 DISCUSSION

Plant pathogen resistance to fungicides can be brought about through: (i) presence of naturally occurring resistant individuals, initially at very low frequency, originating from recurrent mutations conferring resistance, which increase in frequency due to selection pressure e.g. fungicide use, and (ii) also from sexual and asexual recombination (Gisi *et al.*, 2002). The results in this chapter show that resistance can occur among sexual as well as among asexual progeny even when the parental isolates are sensitive.

Shattock (1988) showed that metalaxyl sensitivity is controlled by a single locus exhibiting incomplete dominance. A metalaxyl-resistant and a sensitive parent produced intermediate sexual progeny. However, in this study cross #1 (96.89.43 x 96.70), both sensitive parents, produced mainly intermediate progeny (134 SOPs) and almost equal numbers of sensitive and resistant sexual progeny (31 and 25, sensitive and resistant, respectively). More recent work, using RAPDs markers to characterise resistance in different geographical locations has shown that these Dutch, North American and Mexican isolates map to the same locus (*MEX 1*), while UK isolates map to a different locus (*MEX 2*), however, both loci map to the same linkage group (Fabritius *et al.*, 1997; Judelson and Roberts, 1999). Not all alleles are functionally equivalent, and minor genes interact with *MEX* loci, and therefore sexual progeny from controlled matings express a range of responses to metalaxyl (reviewed in Shattock, 2002).

There was no significant difference in the frequency of metalaxyl resistance among A1 sexual progeny and A2 sexual progeny in all three crosses in this study. However, in natural conditions there has been observation of higher frequency of metalaxyl resistance among A1 than A2 isolates (Hermansen *et al.*, 2000). Gisi and Cohen (1996) did not find any correlation between metalaxyl resistance and the proportion of either mating type, but they stated that the A2 genotypes are primarily metalaxyl-sensitive. However, Peters *et al.* (1998) after sampling for three years (1994, 1995 and 1996) observed a dramatic change from the traditional A1, metalaxyl-sensitive phenotype in 1994, to a complete replacement by isolates of A2 mating type, which were predominantly insensitive to metalaxyl by 1996. Genetic

studies indicate that there was no linkage between the locus for metalaxyl resistance and mating type locus (Lee *et al.*, 1999). Day and Shattock (1997) demonstrated that metalaxyl-resistant isolates in England and Wales were less pathogenic on detached leaves than sensitive isolates. These were isolates occuring under natural conditions (wild-type). The data presented in this chapter indicate that most of the progeny were as aggressive as their parents and yet were more insensitive to fungicides than the parents. From the three crosses (with a total of 540 single oospore progeny), 88% of them were pathogenic on the most susceptible potato cultivar Bintje, and from all three crosses, only 12% of them were sensitive to metalaxyl. Sexual recombination has already been suspected to give rise to metalaxyl resistant lineages. The metalaxyl resistant lineage US-11 in the USA is thought to have risen through sexual recombination between US-6 and US-7 or US-8 (Gavino *et al.*, 2000). The same clonal lineage (US-11) has been isolated in Taiwan, a country where the US-6 or 7 had never been isolated before, indicating an introduction of this lineage from the USA, as opposed to arising from within the country (Deahl *et al.*, 2002).

Although metalaxyl is known to inhibit vegetative growth as well as asexual reproduction in *P. infestans*, metalaxyl stimulated vegetative growth of isolates of *P. infestans* have been observed (Zang *et al.*, 1997). These authors studied five isolates, three A1s and two A2s, isolated from tomato as well as from potato, using two concentrations of metalaxyl, 20 and 100 μgmL^{-1}. While only one isolate (potato A1) grew well on unamended medium (typical of a sensitive isolate), the other four isolates were considered resistant as their colonies grew better, or were retarded by no more than 25% in other cases, compared to cultures grown on medium lacking metalaxyl. In fact, one of these resistant isolates grew well in presence or absence of metalaxyl at both concentrations, and the other two resistant isolates were not only resistant to metalaxyl, but stimulated by the fungicide. Similar results were observed in this study. In cross #2 (UK metalaxyl resistant A1 parent and UK sensitive A2 parent) had two single oospore progeny that were not affected by metalaxyl (1 A1 and 1A2), and 18 SOPs that actually stimulated by presence of metalaxyl (8 A1s, 8A2s and 2 self-fertile). In cross #3, the Californian metalaxyl resistant A1 and UK sensitive A2, there were nine SOPs (6 A1s and 3 A2s) that were not affected by presence of metalaxyl, and there were 15 SOPs (10 A1s, 4 A2s and 1 self-fertile) that were stimulated by presence of metalaxyl.

A study by Mayton *et al.* (2001), where they assessed the role of three fungicides: cymoxanil, propamocarb hydrochloride and chlorothalonil (cymoxanil and propamocarb hydrochloride were mixed with chlorothalonil as a protectant), in the epidemiology of potato late blight observed that while the fungicides could reduce disease development in established epidemics, they could not significantly suppress sporulation from established lesions. In this study the effect of propamocarb hydrochloride on subsequent infection by sporangia produced from propamocarb treated leaves was not tested. However, resistance of both sexual and asexual progeny (sporangial and zoosporangial progeny) was observed to both propamocarb hydrochloride and cymoxanil.

A study by Wong and Wilcox (2000), where they measured the distribution of baseline sensitivities to azoxystrobin among isolates of *Plasmopara viticola* (a causal agent of grapevine downy mildew) showed that the fungicide was active both in the prevention of infection and in restricting the mycelial growth when infection became established, indicating that azoxystrobin can affect multiple stages of the fungal life cycle. The efficacy of the fungicide was observed in this study as it was able to reduce mycelial growth of all isolates tested, parents, sexual and asexual progeny. Although this fungicide seems promising in controlling late blight, its use in controlling other fungal diseases has already been hampered by appearance of resistance to it, e.g. in cereal powdery mildews (Fraaije *et al.*, 2002), in grey leaf spot of maize (Vincelli and Dixon, 2002), in rice blast (Avila-Adame and Koller, 2003) and in maize smut (Ziogas *et al.*, 2002). Other new fungicides which are promising in controlling *P. infestans* include famoxadone, a broad-spectrum fungicide that, at low doses inhibits sporangial differentiation and zoospore release, and at higher concentration can even inhibit mycelial growth and direct germination of sporangia (Andrieu *et al.*, 2001). However, this new fungicide has a similar mode of action as azoxystrobin, i.e. mitochondrial electron transport blocking function (Sternberg *et al.*, 2001). Since resistance to azoxystrobin already exist among other fungi, the future of other fungicide with similar mode of action to strobilurins is not promising.

In conclusion, the results in this study showed that resistance to the most commonly used fungicides against late-blight is possible. Although, there was no resistance to the new

fungicide azoxystrobin observed even among progeny, however, the appearance of resistance to it among other fungal pathogens shows that resistance of *P. infestans* is also possible. Therefore, for sustainable control of late-blight, chemical control should not be the only means. Kirk *et al.* (2001) have shown that the use of cultivars with reduced susceptibility to late blight with reduced fungicide rates and longer application intervals can offer more economic control of this disease.

6.0 GENERAL DISCUSSION

Mexico is generally regarded as the gene centre of *P. infestans* and to date three migrations of the pathogen out of that country have been recorded namely in the 1840s, 1970s and 1980s/90s. Potato production was affected in all cases and later also tomatoes. The latter two migration events introduced not only new A1 mating type isolates but A2 strains too and consequently the opportunity for sexual reproducing populations to develop in late blight affected crops. With sexual reproduction came a new source of perennating inoculum namely the oospore, and such inoculum would release variation through recombination and segregation. Such variation would, it has been assumed, further compromise the efficacy and durability of late blight resistant cultivars and fungicides.

The experimental results presented in this thesis clearly show that this threat is real based on the data from large data sets of three highly fertile matings involving a single A2 mating type parent and two UK and one USA A1 parent. Of course, matings and establishment of sexual progeny from carefully monitored *in vitro* experiments do not necessarily indicate that such sexual activity is widespread in late blight affected crops.

In the UK (each country and epidemiological zone would appear to vary in the structure of their late blight population) the most recent survey of the population (1993-1998) (Day *et al.*, 2004) shows that the A2 isolate occurs at less than 5% of all isolates characterised. This makes the occurrence of sexual mating populations uncommon although where A1 and A2 occur, evidence for sexual reproduction based on neutrally selective markers, e.g. RG 57 fingerprint polymorphism, greater diversity has been postulated as an indication of sexual activity. Diversity alone, however, cannot wholly support the hypothesis that sexual reproduction and gene flow is active. In late blight population biology allelic frequencies need to be determined to address this question and despite substantial progress in studying population dynamics in this pathogen with isozymes, nuclear and mitochondrial polymorphic markers, completely co-dominate nuclear-based molecular markers are needed, such as microsatellites investigated in Chapter 3. In the latter case, RAPDs were used to confirm hybridity of the sexual progeny from a small selection of the oospore progeny of each cross.

Race non-specific rather than race specific (R-gene) resistance was investigated in Chapter 4. Previously, various studies (Spielman *et al.*, 1990; Al-Kherb *et al.*, 1995) indicated that at least some of the virulence/avirulence genes complementary to the potato R-genes were monogenic, behaved in a Mendelian manner of inheritance and conformed to the gene-for-gene theory. More recently, from large progeny sets such as those obtained in Chapter 2, van der Lee *et al.* (2001) have been able to map avirulence genes in *P. infestans* using AFLP markers (used in Chapter 3 to confirm hybridity). These workers used bulked segregant analysis in the same way as Fabritius *et al.* (1997) and Judelson and Roberts (1999) used the technique to begin to map and dissect the genome to identify loci controlling phenylamide resistance and the mating type loci in *P. infestans*, respectively (Judelson *et al.*, 1995). In all these three studies RAPDs were used and their use was too exploited to augment the other molecular markers as different approaches to confirm hybridity or selfing among the single oospore progeny of the three matings (Chapter 3).

More recently, a large library of expressed sequence tags (ESTs) have been and continues to be assembled (Birch and Whisson, 2001). This genomic approach is continuing to expose the late blight pathogen to detailed investigation, which ultimately will expose details of the host-pathogen interaction and offer novel potential targets for disease control. Whilst the large sets of hybrid sexual progeny obtained in this present study provide excellent material for further study of the inheritance of mating type, virulence and fungicide sensitivity, the objective here was to observe the release of variation through sexual reproduction, principally with respect to aggressiveness (non host specific pathogenicity) and sensitivity to currently commercially used fungicides.

Chapter 4 and 5 showed that some single oospore progeny were more aggressive, as shown by the composite aggressiveness value (CAV) of up to 426 on the resistant potato cultivar Stirling, compared to CAV of their parents (98, 250, 273 and 182, for 96.89.43, 96.69, 96.70 and P9463, respectively). In about 47% (44 SOPs) of these sexual progeny with greater than parental CAV, a higher level of insensitivity to three fungicides namely, metalaxyl, propamocarb and cymoxanil was also observed. If such aggressiveness and fungicide insensitive isolates are able to emerge in late blight affected crops then current methods of

control in the UK potato crop will be compromised. Thus far newly introduced classes of fungicides have offered potato growers an effective range of products to control late blight when used in collaboration with decision support strategies such as disease forecasting (Taylor *et al.*, 2003). To date, apart from resistance to metalaxyl the other products have performed well under field conditions, but results presented in Chapter 5 give rise to concern for the longer-term durability of other products. It should be pointed out that even when some isolates have grown on leaf discs treated to high levels of propamocarb hydrochloride (e.g. 500 μgmL^{-1}) sporangia from these have been dysfunctional and lacking in infection potential (Bardsley *et al.*, 1998).

In European agriculture there is a general trend to reduce fungicide input as part of crop protection practices. This will inevitably force growers to adopt higher standards of control such as blight free seed tubers, better sanitation (removing dumps of previously harvested tubers and controlling potato ground keepers in rotational crops), but more importantly, a greater reliance on cultivars with higher levels of resistance to *P. infestans*. Resistance may be mono- or polygenic and the latter was investigated in screening parents and progeny against 7 cultivars with increasing levels of polygenic late blight resistance. Used together higher resistance cultivars e.g. Stirling, can lead to reduced applications of fungicide product for late blight control (Clayton and Shattock, 1995; Kirk *et al.*, 2001). However, this integrated approach to controlling *P. infestans* might be compromised if sexual progeny with higher pathogenicity and higher fungicide sensitivity are selected. This outcome is a distinct possibility and without the public's acceptance of novel forms of resistance, e.g. the RB gene from *Solanum bulbocastanum* (Song *et al.*, 2003) transferred by non-conventional transfer (i.e. genetic engineering), late-blight control will continue to be problematic and could worsen.

Controlling late-blight in various countries has not always remained constant. Zwankhuizen and Zadoks (2002) analysed 47 years of potato late-blight epidemics in The Netherlands and identified three distinct periods 1950s-68, 1969-78 and 1979-96. The first and third periods had similar 'disease intensity' values but the authors suggest that the third period was different when the increased efficacy of fungicides and other disease control improvements

are considered. As such, the severity of late-blight epidemics has increased since 'new' populations became established following the 1976 introduction of exotic strains from Mexico into Europe. The same is true for the USA (Fry and Goodwin, 1997) with the emergence of US-6, 7 and 8 phenotypes, whilst in the UK, Day and Shattock (1997) concluded greater aggressiveness among the 'new' population and this together with other factors, e.g. lack of insensitivity towards phenylamides in the 'old' genotypes, caused the subsequent displacement of the 'old' by the 'new' genotypes.

Clearly, evidence exists indicating altered population dynamics in late blight populations as a result of sexual reproduction. The pathogen, when asexual has, however, previously exhibited variation in pathogenicity towards monogenic (R-gene) and polygenic resistance (reviewed by Shattock, 1977). Similarly, direct evidence for release of variation towards the latter was observed for asexual progeny from both parental cultures and randomly selected single oospore cultures (Chapter 4 and 5). The genetical basis for this variation remains obscure, but with the pathogen being diploid, and evidence for heterokaryosis/heteroplasmosis, then somatic recombination may be just as powerful as sexual recombination in generating variation, together with mutation, in this particular pathogen.

The experiments in this study assessed aggressiveness of sexual and asexual progeny on several potato cultivars with different horizontal resistance. Variation was observed on both types of progeny. Sensitivity of these progeny (sexual and asexual) was evaluated on five fungicides currently used to control late blight. Again, variation was observed. The extent of variation among asexual progeny, especially among single zoospore progeny requires further studies.

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APPENDICES

1.0 Media

1.1 Rye A agar (adapted from Caten & Jinks, 1968)

Organic rye grains	60g
Plant tissue agar	12g
Sucrose	20g

The grains were soaked in filtered water for 36 hours at 18 ^{0}C. The supernatant and the grains were separated. Sucrose was dissolved in the supernatant. The grains were macerated in a blender (Wareing) and filtered through muslin. The filtrate was incubated in a water bath set at 50 ^{0}C for 3 hours. The supernatant was then added to the filtrate and the volume was adjusted to 1L by adding more filtered water. The mixture was then decanted into autoclavable bottles and the agar was added just before autoclaving for 20 minutes at 121 ^{0}C.

1.2 Rye B agar

This medium is prepared in a similar way as Rye A agar (Appendix 1, section 1.1), the only difference is that the grains are not macerated rather they are boiled for an hour. Therefore, there is no incubation involved.

1.3 Soft water agar

Plant tissue agar	6g

The agar was added into 1L of filtered water and autoclaved for 20 minutes at 121 ^{0}C.

1.4 Pea broth

Frozen peas	300g

The peas were boiled in filtered water for 10 minutes. The filtrate was collected through a sieve and the volume was adjusted to 1L by adding more filtered water. It was then autoclaved for 20 minutes at 121 ^{0}C.

1.5 Clarrified V8 agar

V8 juice	200ml
$CaCO_3$	20g

Plant tissue agar 12g

The juice was mixed with calcium carbonate and centrifuged for 5 minutes at 2000 revs. The clarified supernatant was made up to 1L with filtered water. Agar was then added and the mixure was autoclaved for 20 minutes at 121 ^{0}C.

1.6 RAN

Nystatin	1g
Rifamycin	0.5g
Ampicillin	0.5g
DMSO	20ml

The antibiotics were dissolved in the DMSO to give a stock solution of 50 mg ml^{-1} nystatin, 25 mg ml^{-1} rifamycin and 25 mg ml^{-1} ampicillin. This was then divided into aliquots of 1 ml and kept in the –20 ^{0}C freezer. Each time 1 ml was added to 500 ml of rye A just before pouring to give a concentration of 100 :g ml^{-1} nystatin, 50 :g ml^{-1} rifamycin and 50 :g ml^{-1} ampicillin. Dilute RAN contained 250 :l of RAN per 500 ml.

2.0 Scanning Electron microscope solutions and buffers

2. 1 3% Glutaraldehyde

3 ml phosphate buffer A

5 ml phosphate buffer B

10 ml distilled water

2.2 Phosphate buffer A - dibasic Na_2PO_4

0.2M $NaH_2PO_4.12H_2O$

2.3 Phosphate buffer B – monobasic $NaH_2PO_4.2H_2O$

2.4 0.1M phosphate buffer at pH 6.8

25 ml phosphate buffer A

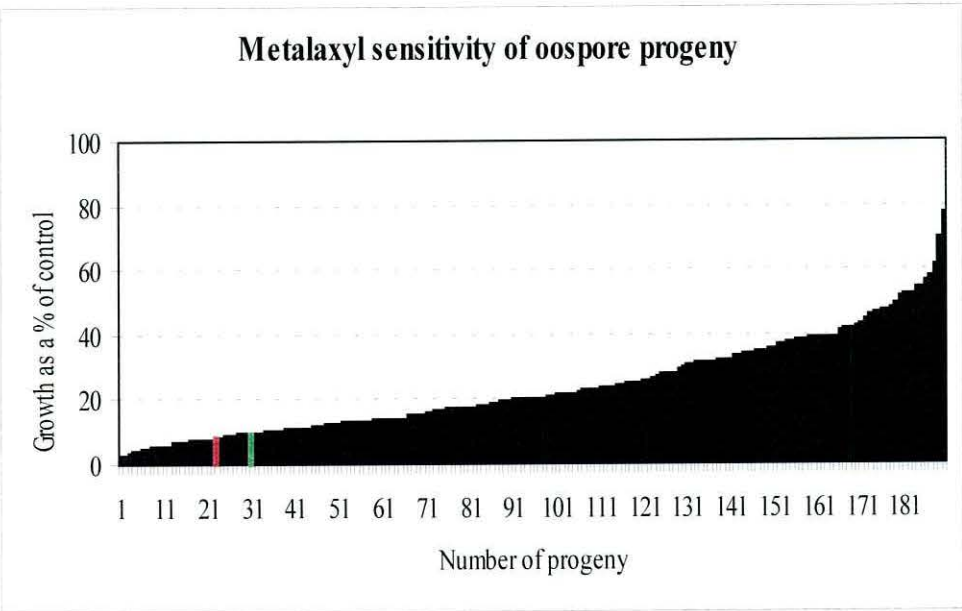
25 ml phosphate buffer B

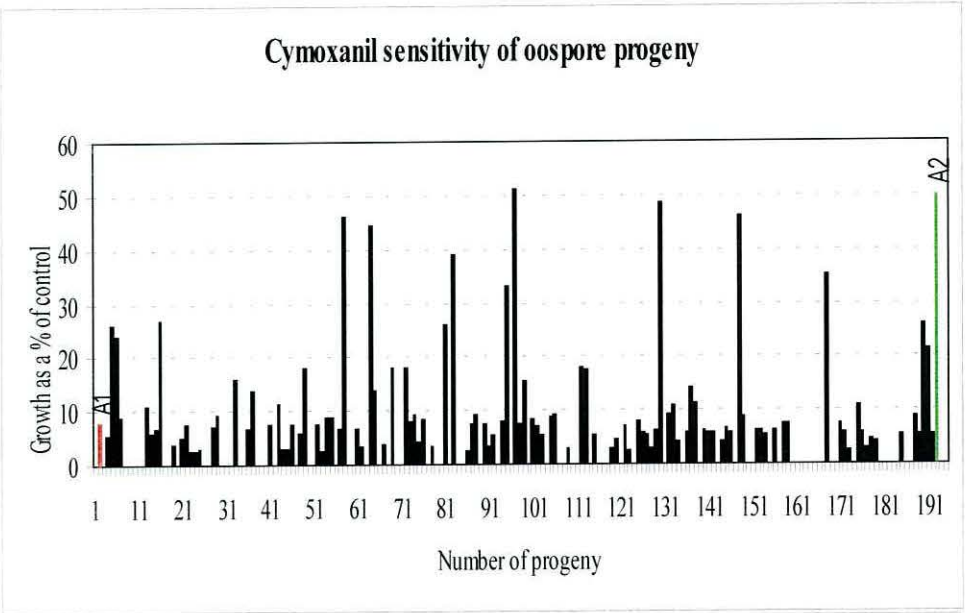
50 ml distilled water

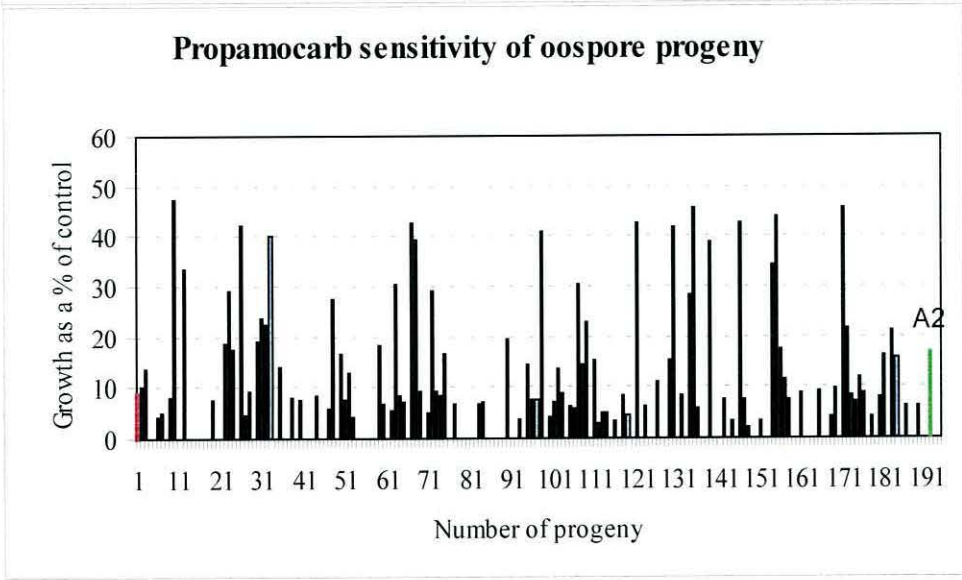
4.0 Aggressiveness data – see enclosed CD

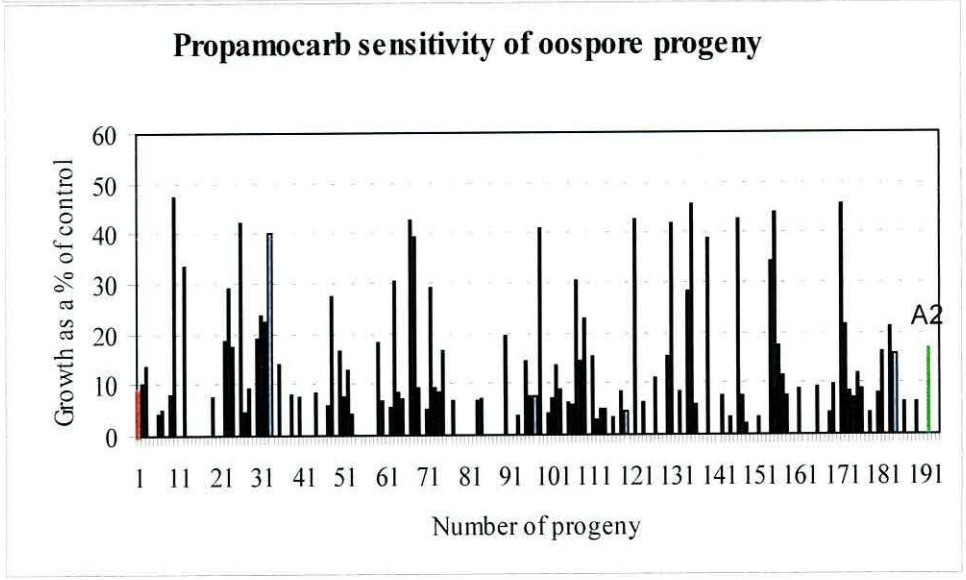
5.0 Fungicide data – see enclosed CD

Appendix 5.1a. *In vitro* sensitivity of single oospore progeny and parental isolates of cross #1 of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl, fluazinam, cymoxanil and 50 μgmL^{-} of propamocarb. Green represent A1 parent and red represent A2 parent

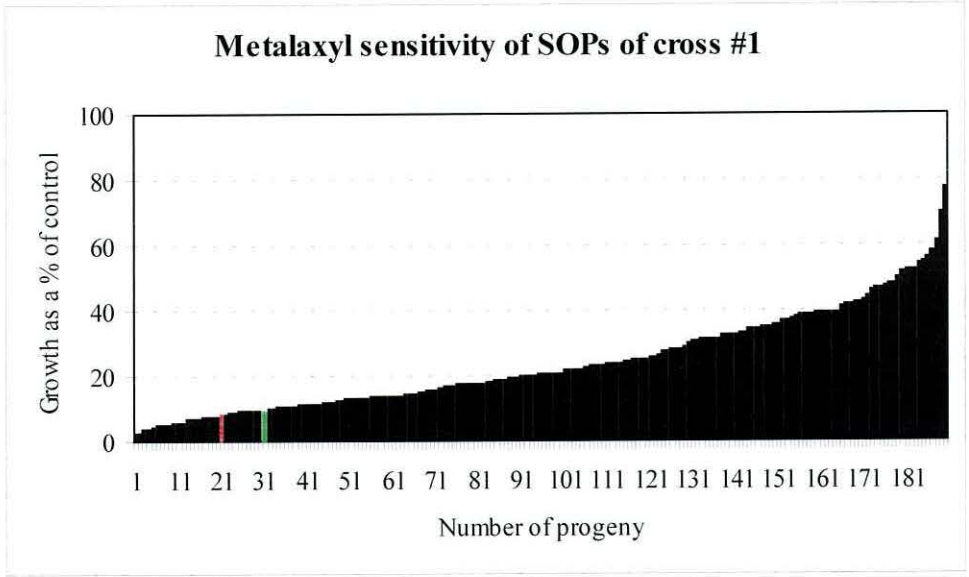


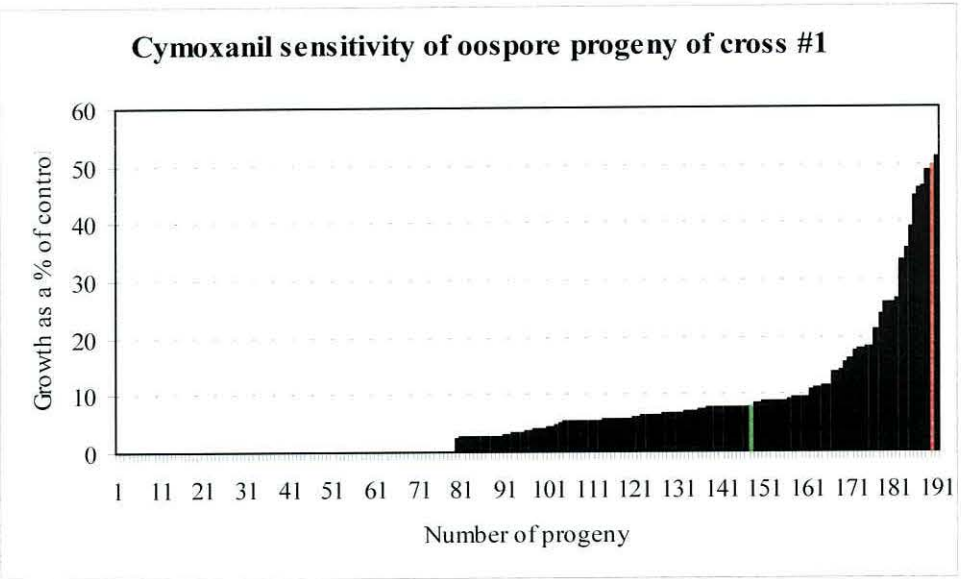


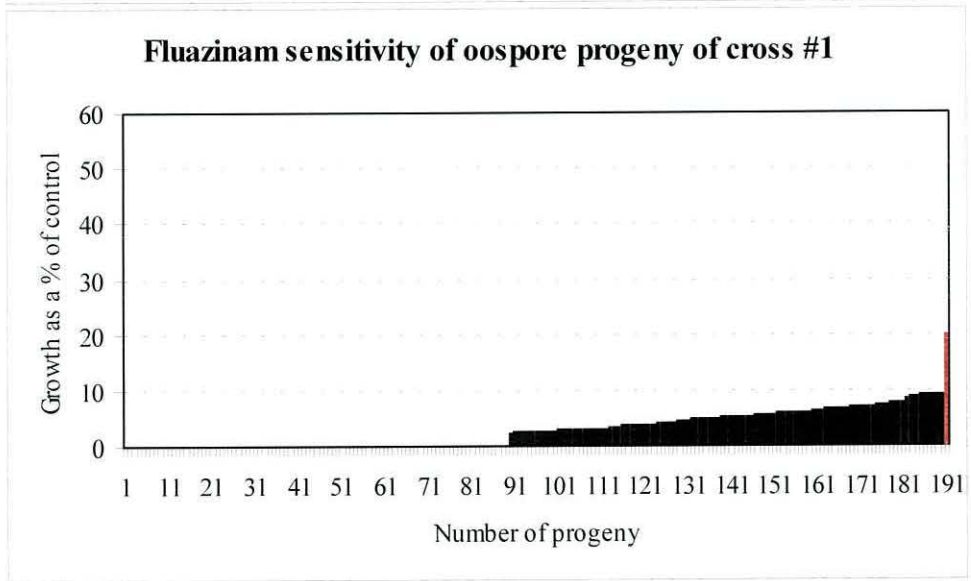


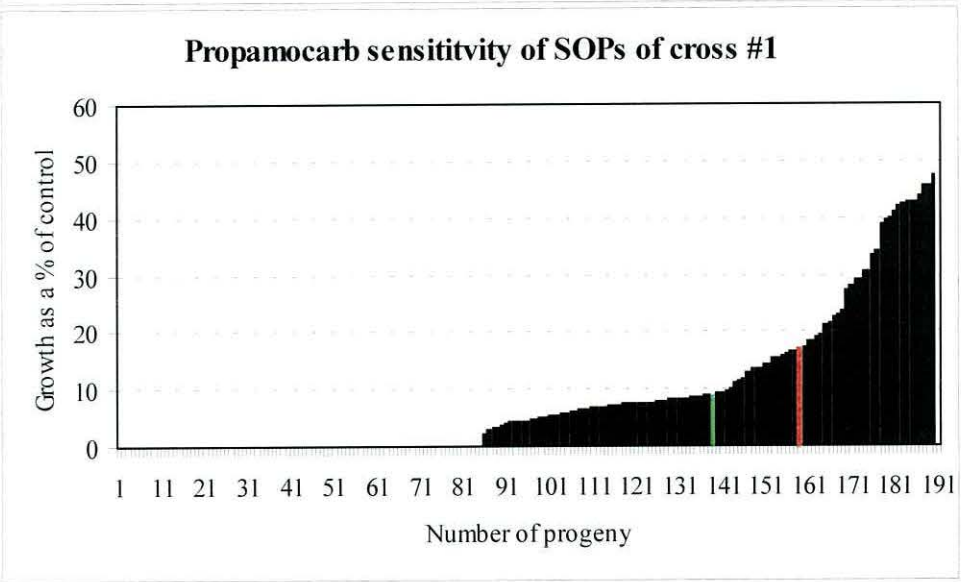


Appendix 5.1b. *In vitro* sensitivity of single oospore progeny and parental isolates of cross #1 of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl, fluazinam, cymoxanil and 50 μgmL^{-} of propamocarb. Green represent A1 parent and red represent A2 parent

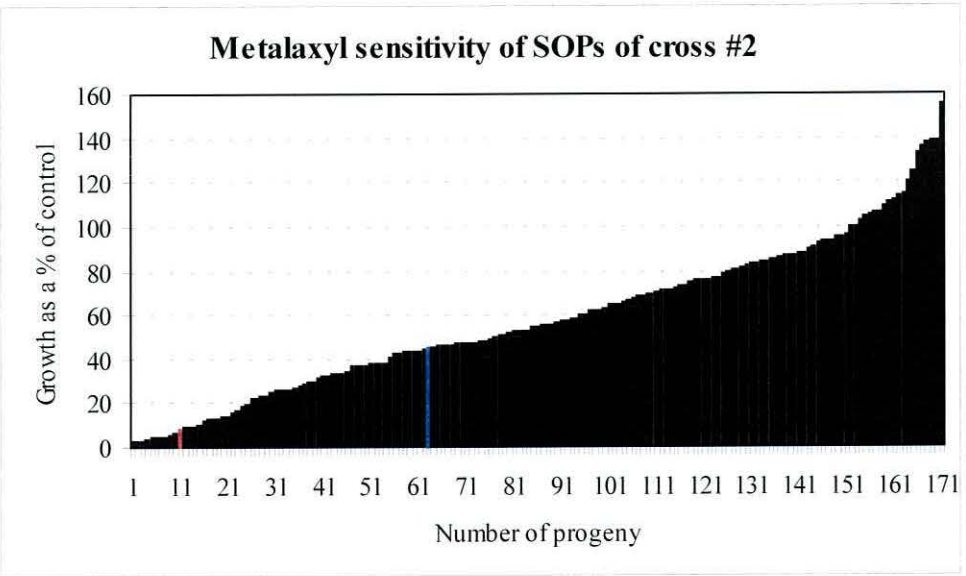


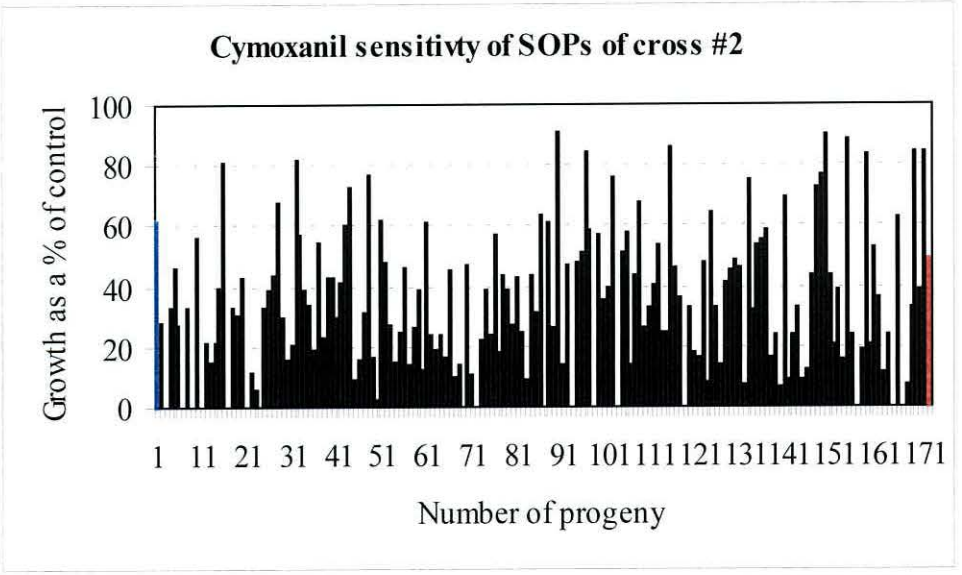


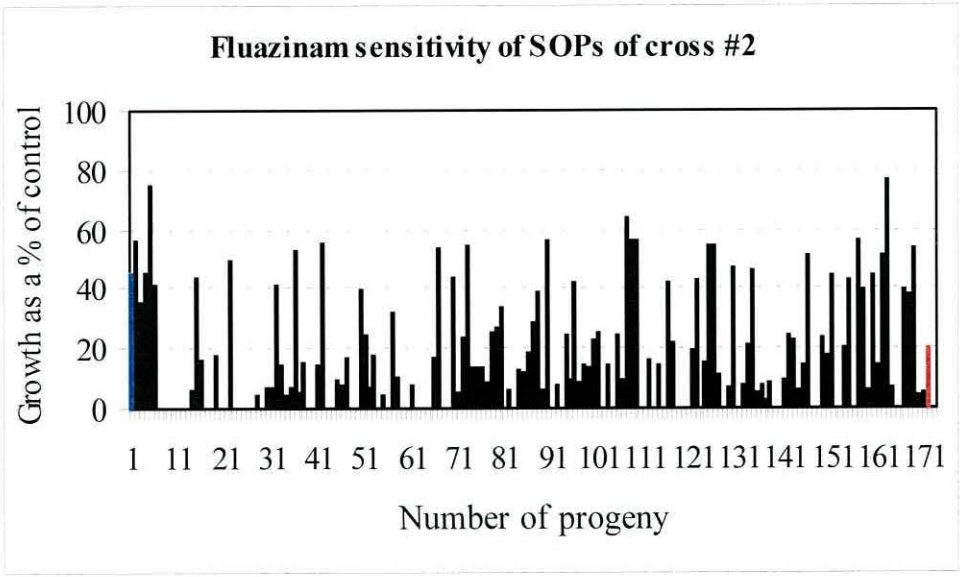


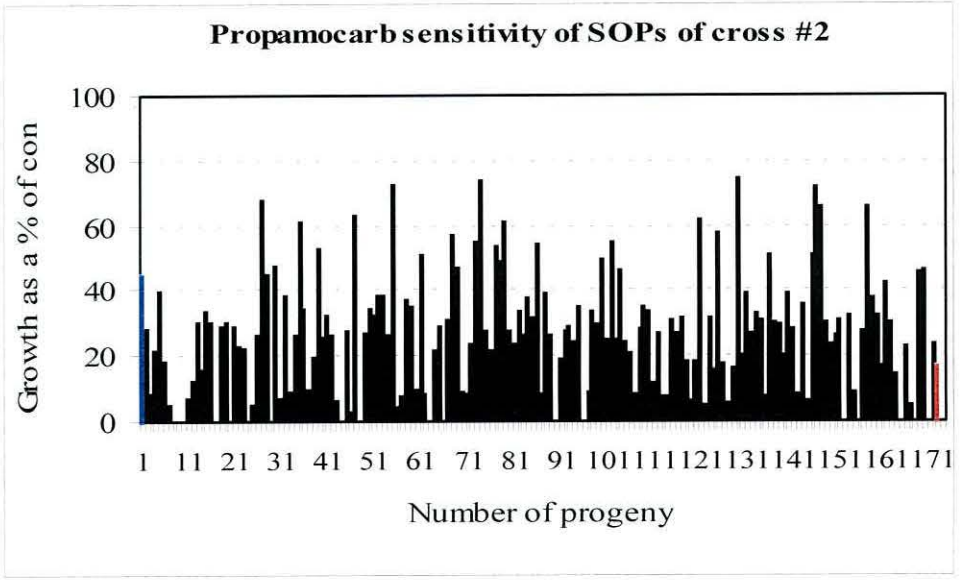


Appendix 5.2a. *In vitro* sensitivity of single oospore progeny and parental isolates of cross #1 of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl, fluazinam, cymoxanil and 50 μgmL^{-} of propamocarb. Blue represent A1 parent and red represent A2 parent

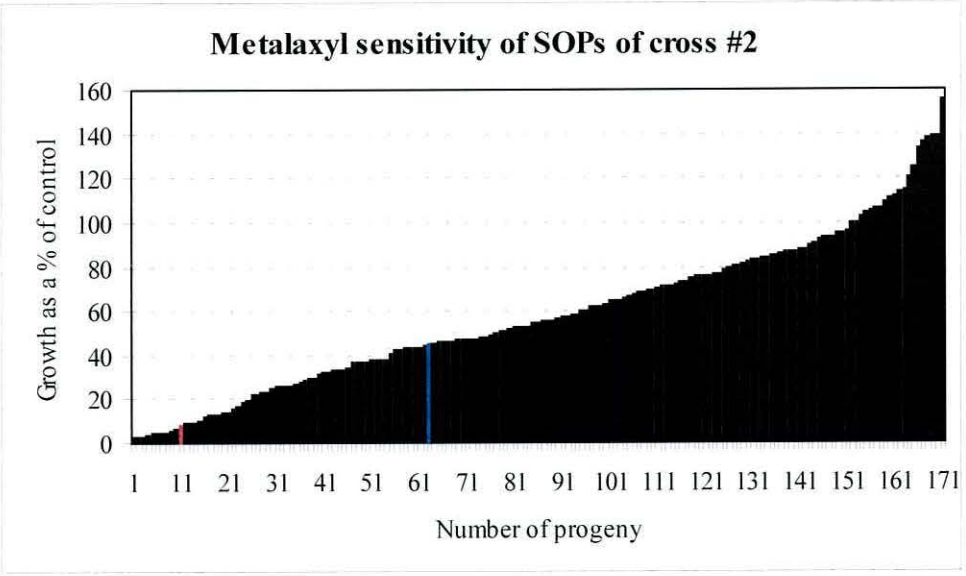


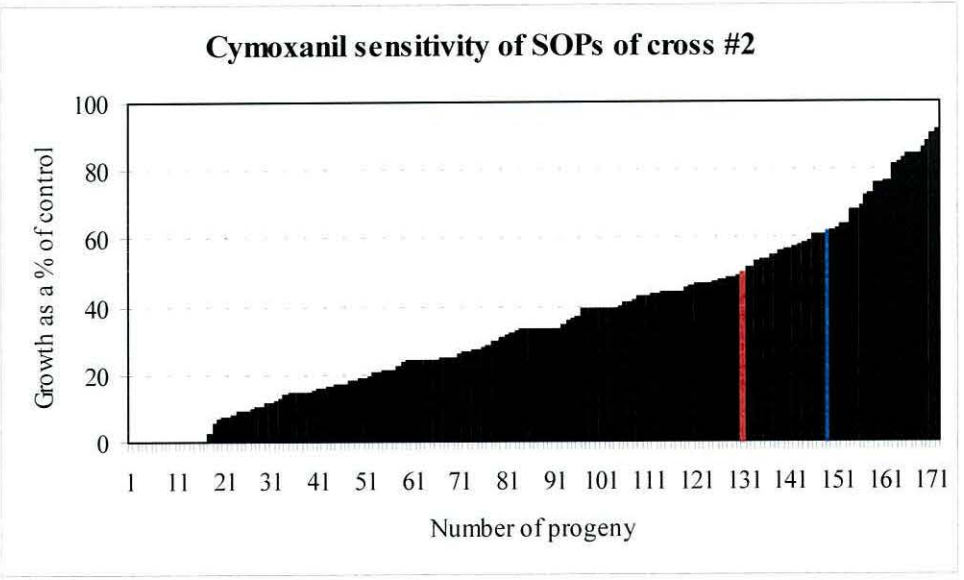


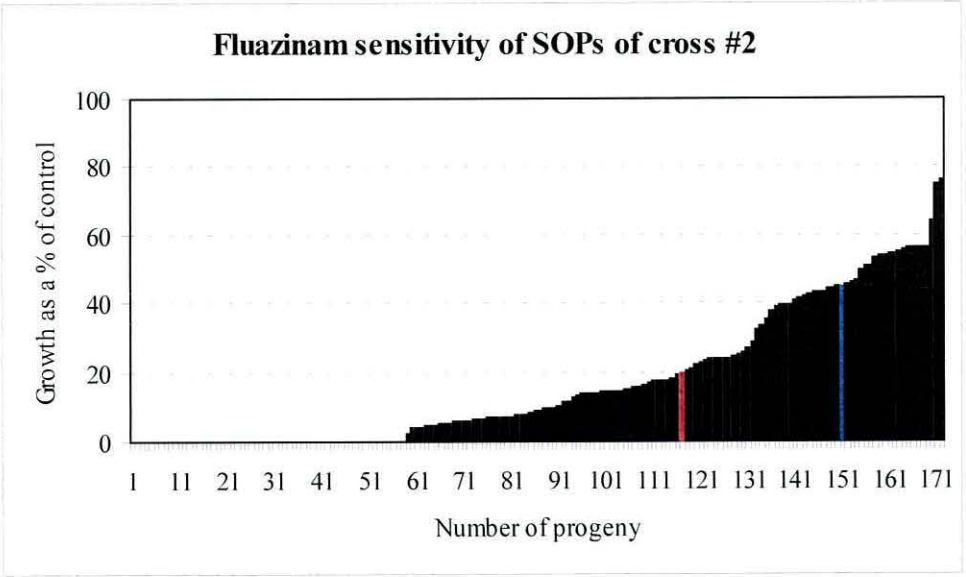


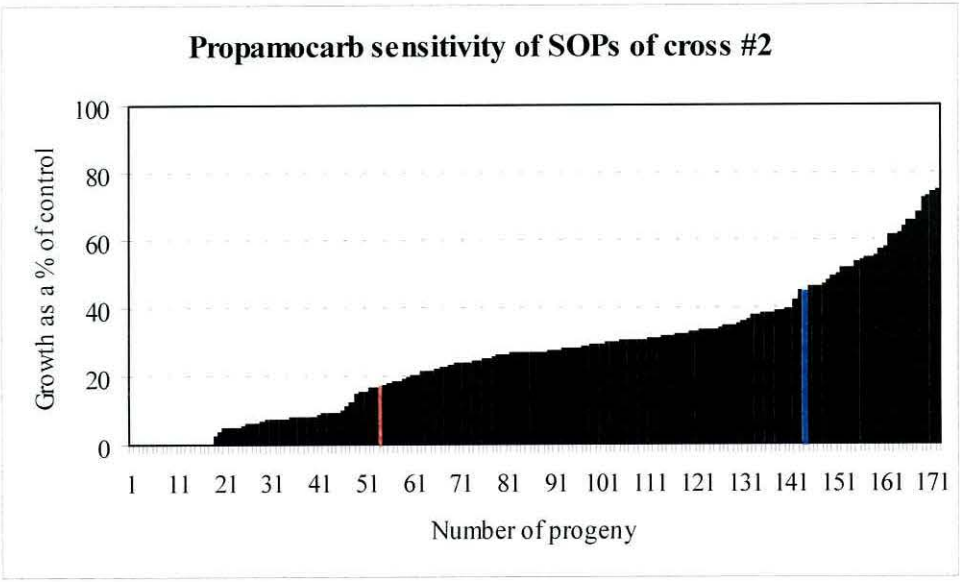


Appendix 5.2b. *In vitro* sensitivity of single oospore progeny and parental isolates of cross #1 of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl, fluazinam, cymoxanil and 50 μgmL^{-} of propamocarb. Blue represent A1 parent and red represent A2 parent

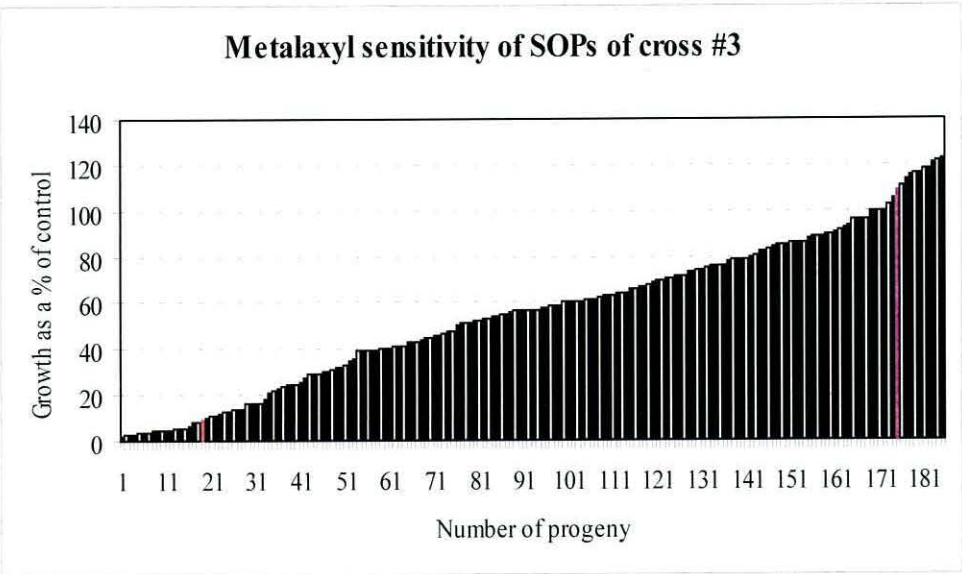


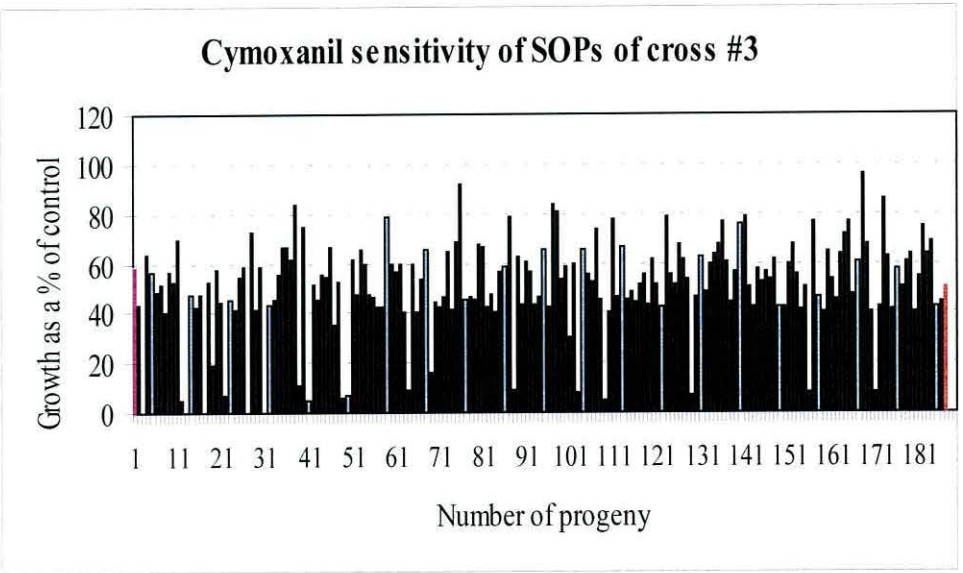


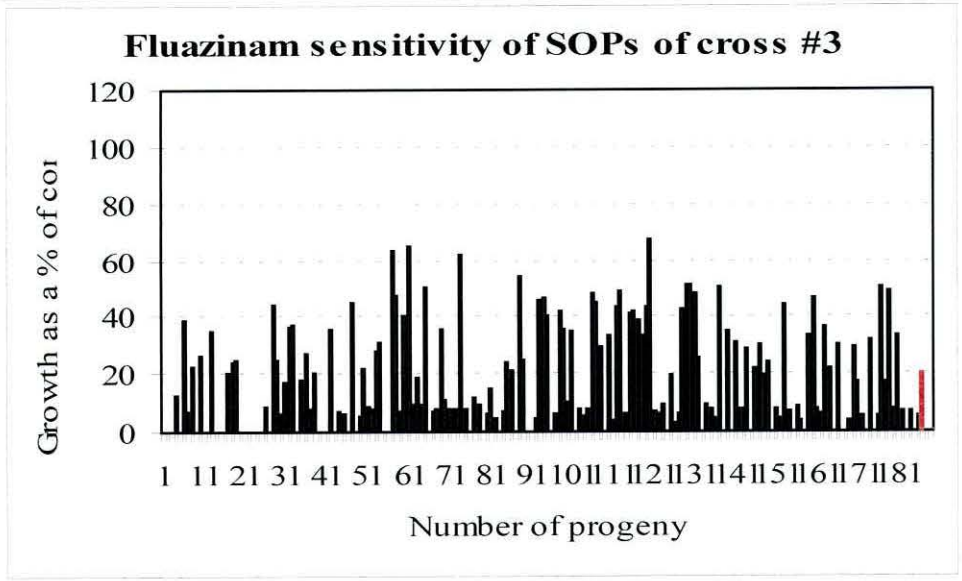


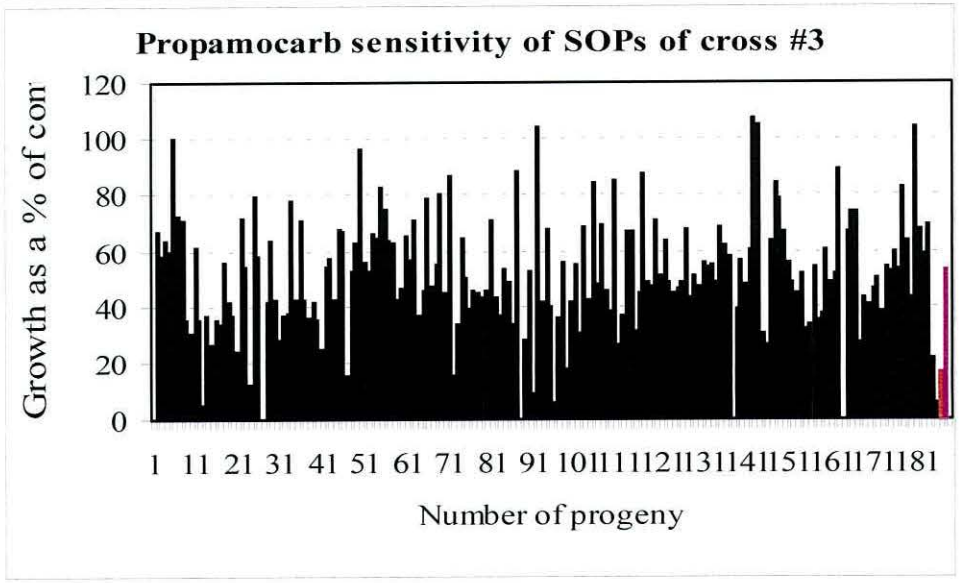


Appendix 5.3a. *In vitro* sensitivity of single oospore progeny and parental isolates of cross #1 of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl, fluazinam, cymoxanil and 50 μgmL^{-1} of propamocarb. Lilac represents A1 parent and red represent A2 parent









Appendix 5.3b. *In vitro* sensitivity of single oospore progeny and parental isolates of cross #1 of *Phytophthora infestans* to 10 μgmL^{-1} of metalaxyl, fluazinam, cymoxanil and 50 μgmL^{-} of propamocarb. Lilac represents A1 parent and red represent A2 parent