Diagnostics And Epidemiology of *Phytophthora infestans*, The Cause of Late Blight of Potato

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

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Director of Studies

Declaration

I hereby declare that this work has not been previously accepted in substance for any other award and is not being currently submitted in candidature for any other degree. I further declare that, except where stated, the work presented in this thesis is original and was performed by the author at the Scottish Crop Research Institute.

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Abstract

A specific and sensitive PCR assay for the detection of *Phytophthora infestans*, the cause of late blight of potato, in soil and plant tissues was developed. A *P. infestans* specific primer pair (INF FW2 and INF REV) was designed by comparing the aligned sequences of internal transcribed spacer regions (ITS) of a range of *Phytophthora* species. Following PCR amplification with primers INF FW2 and INF REV, a 613 bp product was generated from DNA of an isolate of *P. infestans*. No product was amplified when DNA from isolates of various other *Phytophthora* species and potato blemish pathogens was tested, showing that the primers were species-specific. These primers can detect as little as 0.5 pg of pure *P. infestans* DNA. In a nested assay, sensitivity was increased by two fold, and as little as 5 fg *P. infestans* DNA was detected. The primer pair can also detect as few as two oospores, two sporangia and four zoospores of *P. infestans*. Using primer pair INF FW2 and INF REV, ten oospores of *P. infestans* could be detected in 0.5 g soil, besides detecting the pathogen in symptomatic and symptomless leaves, stems and tubers. The assay was validated on field soil samples and commercial potato seed stocks.

Studies on the long term survival of sexual (oospores) and asexual (sporangia and mycelium) inoculum of *P. infestans* were undertaken under natural field conditions. PCR detection of oospores was possible up to 24 months (total length of the study) after burial in soil. The sporangia, although detectable up to 12 months, were less efficiently detected at the nine and twelve months sampling dates, presumably representing a degradation of inoculum not adapted to long term survival. In a baiting assay, sporangial inoculum proved non-viable whereas leaf material containing oospores remained viable up to 24 months after burial.

Population studies are dependant on the development of co-dominant markers. Eight single nucleotide polymorphisms (SNPs) were identified in the *P. infestans* genes *2-phosphoglycerate dehydratase*, *transaldolase*, *glutamine synthetase*, *ubiquitin conjugating enzyme* and *ADP/ATP translocase* which represents a rate of ~ 2 SNP per kb. The majority of the SNPs were synonymous (i.e. did not code for a different amino acid). Screening of randomly selected fragments of non-coding DNA from a BAC (Bacterial Artificial Chromosome) library yielded an additional 28 SNPs which

represents a rate of 2 SNP per kb. Allele specific PCR was used to develop SNPs into useful markers. Use of fluorescently labelled primers markedly increased the assay throughput. Segregation analysis revealed that with the exception of 56E14R and glutamine synthetase (linked in the coupling phase), the markers segregated independently. Seventeen genotypes were detected amongst 42 Scottish *P. infestans* isolates. Cluster analysis based on SNP markers grouped isolates into two clades and broadly supported previous studies in which AFLP markers were used.

In the present studies, there was some evidence of cultivar-specific adaptation of *P. infestans* isolates. The isolates caused larger lesions on the cultivars on which they were maintained for seven successive weeks. The effect of cultivar adaptation was however lost upon removal of the selection pressure. The results were validated in an inter-isolate competition experiment. Generally, more sporangia were produced on a cultivar by the isolate adapted to that cultivar than by its non-adapted competitor isolate. The use of the above developed SNP markers allowed tracking of isolates in the inter-isolate competition within a single lesion. The availability of SNP markers will provide a powerful tool for epidemiological studies of *P. infestans* on a field scale.

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List of Abbreviations

~	approximately
@	at the rate of
χ^2	chi-squared
°C	degree Celsius
<	less than
≤	less than or equal to
μg	microgram(s)
μl	microlitre(s)
μm	Micrometre(s)
μM	micromolar
>	more than
≥	more than or equal to
%	per cent
θ	recombination frequency
x	times
A	adenine
ABI	Applied Biosystems
ADP	adenosine diphosphate
AFLP	amplified fragment length polymorphism
Anon	anonymous
ANOVA	analysis of variance
APS	adenosine phosphosulphate
ATP	adenosine-5′- triphosphate
av.	average
BAC	bacterial artificial chromosome
B.C.	before Christ
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
c.	approximately

Ca	calcium
CIP	International Potato Centre, Lima, Peru
cm	Centimetre(s)
CTAB	hexadecyltrimethyl-ammonium bromide
cv.	cultivar
d.f.	degrees of freedom
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide tri-phosphate
ds-DNA	double-stranded DNA
DW	distilled water
E. coli	*Escherichia coli*
e.g.	*exampli gratia* (Latin: for example)
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EST	expressed sequence tag
et al.	*et alia* (Latin: and others)
F1	first filial generation
F-ddNTP	fluorescently labeled di-deoxynucleotide tri-phosphate
Fe	iron
fg	femto gram(s)
Fig.	figure
g	gram(s)
G	guanine
GDP	gross domestic product
GMA	gene mutation analysis
GMP	guanosine monophosphate
Gpi	*glucose-6-phosphate isomerase*
h	hour(s)
ha	hectare
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
i.e.	*id est* (Latin: that is)
IGS	intergenic spacer region

IMI	International Mycological Institute
ITS	internal transcribed spacer region
K	potassium
k	lysine
kb	kilo base
kcal	kilo calorie
KCl	potassium chloride
KD	kilo Dalton
KOAc	potassium acetate
L	litre(s)
LB	Luria-Bertani
LOD	logarithm of odds
LSD	least significant difference
m	metre(s)
M	molar
mg	milligram(s)
Mg	magnesium
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
N	normal
n	number/asparagine
Na	sodium
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information

ng	nanogram(s)
nm	nanometre
ORF	open reading frame
P	phosphorus
p	level of probability
PCR	polymerase chain reaction
pep	*peptidase*
Pers.comm.	personal communication
Pfu	*Pyrococcus friosus* (source of enzyme)
pg	picogram(s)
PGC	*Phytophthora* Genome Consortium
pH	1/log $[H^+]$
pmol	Picomole(s)
PPi	pyrophosphate
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RH	relative humidity
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	Svedberg unit (sedimentation velocity unit)
s	serine
SCRI	Scottish Crop Research Institute
SDS	sodium dodecyl sulphate
Se	standard error
sec	second(s)
SEERAD	Scottish Executive Environment and Rural Affairs Department
SNP	single nucleotide polymorphism
sp.	specie
spp.	species
SSCP	single strand conformational polymorphism
ssDNA	single stranded deoxyribonucleic acid

SSR	simple sequence repeat (microsatellite)
SDW	sterile distilled water
T	thiamine
t	tonnes/ threonine
TAE	tris-acetate-EDTA buffer
Taq	*Thermus aquaticus* (source of polymerase)
TBE	tris-borate-EDTA buffer
TE	tris-EDTA
Tm	melting temperature
Tris	tris (hydroxy methyl) methylamine
Tris-HCL	tris adjusted to the indicated pH with HCl
U	units of enzyme (μmol product min^{-1} at given temperature)
UTR	untranslated region
UV	ultraviolet
V	volts
v/v	volume for volume
w/v	weight for volume

General Introduction

1.1 The Host

1.1.1 Potato: its history and biology

Potato (*Solanum tuberosum* L.) originated in the Andean Mountains of South America (Rich, 1983). It is tetraploid and probably a natural amphiploid hybrid between *S. stenotomum* and *S. sparsipilum* (Hawkes, 1956; Cribb, 1972). According to Scott (1976), 'Inca' Indians cultivated potatoes as far back as 200 BC. Following domestication, it was adopted and spread rapidly and widely from its centre of origin to neighbouring areas throughout the high Andes. By the early 16th century, its cultivation was widespread in areas known today as Colombia, Ecuador, Peru, Bolivia and Chile (Hawkes, 1967). It was unknown to the Europeans until 1532 but was formally recorded by Lopez de Gomara (1552) and Cieza de Leon (1553) (Hawkes, 1992).

The first introduction of potato into Europe was in 1570, when potato was grown in Spain, where it proved successful as a food and was widely adopted in Italy and thereafter in Austria, France and Belgium. The second major introduction took place in the last two decades of the 16th century when it was imported into England (Askew, 2001). It was also recorded across the Atlantic in Londonderry, New Hampshire in 1719 (Smith, 1968). It is widely believed that introductions to Spain and England, where it became the major food source (Glendinning, 1983), were the main stimulus to the spread of potato across the rest of the world (Hawkes, 1990).

The earlier introductions into Europe were thought to be *S. tuberosum* sub sp. *andigena*. Since these potatoes were adapted to short day conditions of the Andes, they would have produced poor yields with late developing tubers under European conditions (Dongyu, 1996). During the next two centuries after their introduction, selections based on yield and earliness were made that gave rise to modern day potatoes, which became known as *S. tuberosum* sub sp. *tuberosum*. The status of

potato rapidly changed from that of a botanical curiosity to a staple food crop by about mid 18th century (Hawkes, 1992; Hawkes, 1994).

Potato is a member of the family Solanaceae, which includes some other commercially important crops such as tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), and tobacco (*Nicotiana tabacum*). There are as many as 235 species in the tuber bearing section of the genus *Solanum* (section *Petota*). Seven of these species are cultivated and are classified into a complex hierarchy consisting of two sub-sections, *Estolonifera* (containing 2 series) and *Patotoe* (containing 19 series) (Hawkes, 1990). Potato is an annual, herbaceous, dicotyledonous plant, as far as its vegetative growth and flowering habits are concerned. However, its capacity to reproduce by tubers makes it a perennial (Gould, 1999). The tubers, borne on below ground stems, or stolons, are the only edible part of the crop and are also the means of commercial propagation (seed tubers) (Renia, 1997). The cultivated potato, as well as its wild relatives, represent a polyploid series ($2x$–$6x$) with the basic chromosome number, x = 12 (Hawkes, 1979). The sub-species *tuberosum* is tetraploid with a world-wide distribution.

Rowe (1993) divided the growth and development of a potato plant into five stages:

Growth Stage I: When a seed tuber is planted, the eyes sprout and the young plant emerges from the soil. The sprout relies on nutrients and water from the mother tuber as a source of energy at this stage as the roots have just started to emerge.

Growth Stage II: During this stage, the sprout produces secondary stems and foliage from the above ground nodes. Likewise, the roots and stolons develop from below ground nodes. Stolon formation begins at the nodes close to the seed tuber. The young shoot soon moves from being a sink to a source of carbohydrates through photosynthesis. Temperature has a marked influence on stolonisation. High temperature stimulates initiation, growth and branching of stolons mainly by delaying tuberisation (Bodlaender *et al.*, 1964; Struik *et al.*, 1989b), thereby producing more stolons and more potential sites per stolon for tuber production (Struik *et al.*, 1989a).

Growth Stage III: This stage is characterised by the initiation of tubers at the tips of stolons, which is controlled by growth regulating hormones. Typically most tubers are initiated over a short period of time ranging from 10-14 days. The appearance of flowers marks the end of this growth stage. High temperature, particularly during the night, has been reported to adversely affect tuber initiation (Struik and Ewing, 1995).

Growth Stage IV: During this phase of plant growth, tuber bulking takes place. This occurs as a result of expansion of tuber cells as tubers become a major sink for water, carbohydrates and other inorganic nutrients.

Growth Stage V: During the final growth stage, there is a marked reduction in photosynthesis, which is the result of senescence and considerable defoliation. The skin of the tubers thickens and fully developed tubers are formed at the end of this stage. Tubers are specialised stems, which are greatly shortened and thickened and have been adapted to perform special functions i.e. the storage of starch for reproduction (Beukema and Van der Zaag, 1990; Gould, 1999). Eyes present on the surface of a tuber represent clusters of modified buds (Anon, 1986). The number of eyes on a tuber varies considerably depending on many factors such as cultivar, size of tuber and growth conditions. The skin of the tuber has many lenticels, which are comparable to stomata on the leaves (Beukema and Van der Zaag, 1990). Tubers have a dormancy period during which sprout growth is suppressed (Van Ittersum, 1992).

Although potatoes grow best under comparatively cool, moist conditions and in lighter soils (Gould, 1999), the crop is grown extensively in the tropics under conditions with greater day time temperatures (Horton and Monares, 1984; Scott, 1988). Tuber expansion depends upon an ample water supply, and a shortage affects tuber yield and quality (Levy, 2000).

1.1.2 Production and uses

Potato is the 4th most important crop world-wide after wheat, rice and maize, is grown in more than 125 countries of the world and is a major food source for the world population (Horton, 1987). On the basis of tonnage, it is ranked as the largest

dicotyledonous crop (Henicke, 2000; Visser, 2000; Askew, 2001). It is grown over an enormous range of latitudes that extend from the polar circle in Finland to the equator in the highlands of Ecuador (Anon, 2000a). There has been an increase in the production of potatoes in developing countries notably India and China in the last few decades (Walker *et al.*, 1999). In fact, China along with Russia and Poland produces approximately 50% of the world production (Gould, 1999; Gacek, 2000). It is expected that the Asian crop will comprise 90% of the potatoes produced in developing countries by the year 2020 (Swaminathan, 2000). In 1999, it was grown on 20 million hectares with an annual production of around 295 million tonnes (Askew, 2001). The statistical data of regions and countries regarding population, share of GDP, rank, area, production and yield of potato is presented in Table 1.1.

The bulk of world potato production (50-60%) is used for direct human consumption, 25% is fed to animals, approximately 10% is used as seed and the rest comprises raw material for industrial products or is waste (Anon, 1995). Until the early 1900's, potatoes were regarded chiefly as a subsistence crop and eaten primarily as boiled tubers for domestic consumption. However, during the 20th century, there has been a tremendous change in the way potatoes are eaten, with processed products taking on added importance (Love, 2000). The main advantages of a processed product are an enhanced shelf life, and a reduction in the amount of time and energy for their preparation. Their promotion through mass media has also contributed to this change (Scott *et al.*, 1992). Processing also has the added benefits of consistent supply all year round and employment opportunities and income for non-growers such as processing and retail industries as well as distributors (Anon, 2000b). Processing therefore has resulted in overall higher potato consumption which has led to increased production and income for the growers. However, many end users have a strict requirement for specific cultivars, many of which are susceptible to diseases.

Potatoes are processed in a variety of ways with canned potatoes, chips and crisps (Clayton and Percival, 2000) being the most popular forms, closely followed by instant mashed potatoes (Webster, 1997). Crisps, which represent 47% of the value of an estimated $ 8 billion world snack food industry, were discovered in 1853 by a chef named George Crum. One of Crum's customers returned a dish of dried

potatoes asking for properly 'thinned French fries'. Crum, obviously enraged by the complaint, made thin peeled slices, fried them until they were crisp and presented them with a touch of salt. The customer was delighted, hence the birth of potato crisps (Gould, 1988).

Potatoes are also processed into forms such as dehydrated flakes, granules and raw starch (Love, 2000). Around 17% of the world's starch originates from potato (Rus, 2000) and increasingly potato protein is being extracted for supplementing animal feed. Potato starch is used in the paper industry for surface coating and the textile industry for finishing sewing thread and cloth. Potato starch has also been used extensively in the food industry in the form of additives to bread and biscuits as well as thickener in soups (Burton, 1989). Village level processing has been reported from several South Asian countries including India, Pakistan and Bangladesh (Sikka, 1988). In China, potatoes are used for making noodles, flour and various types of snack foods (Gitomer, 1987).

Table 1.1 Potato production in major potato growing regions of the world (Source: Askew, 2001).

Region	Population in 2000 (Millions)	Agricultural share of GDP (%)	Rank relative to other crops	Area (000 ha)	Production (000 t)	Yield (t/ha)
Asia						
China	1225	20	5	3489	47777	14
India	1022	27	3	1116	18627	17
Iran	68	-	2	152	3182	21
Bangladesh	120	30	2	133	1489	11
Africa						
Egypt	62	16	5	130	2656	20
S. Africa	46	5	3	56	1539	28
Algeria	31	12	2	80	1099	14
Malawi	12	36	2	51	379	7
Latin America						
Colombia	40	16	2	170	2770	16
Brazil	175	14	7	182	2701	15
Peru	26	7	1	240	2355	10
Argentina	37	6	4	98	2155	22
North America						
USA	275	-	4	556	21200	38
Europe						
Poland	39	6	1	1390	24295	17
Germany	82	1	3	354	12530	35
Netherlands	16	3	1	183	7834	43
Eurasia						
Russian Federation	147	7	1	3389	38534	11
World	6123	-	4	18381	295118	16

1.1.3 Nutritional value

Potatoes are an excellent source of carbohydrates, proteins, vitamins, minerals and trace elements (Anon, 1998). However, the composition of these nutrients is affected by a number of factors including cultivar type, temperature, moisture and soil nutrients (Gould, 1999). Approximately 80% by weight of potato carbohydrate is starch. On average, cooked potatoes have about 2% protein (Woolfe, 1987; Woolfe, 1992). Potato has been reported to contain all the essential amino acids. Potatoes contain vitamin C and some B vitamins such as thiamine, niacin and vitamin B6 and are also regarded as a moderate source of iron, a good source of phosphorus and magnesium and a rich source of potassium (Anon, 1984).

Potato produces more edible energy and protein per hectare than many cereals and other root and tuber crops (Woolfe, 1987). The quality of the potato protein is also reported to be higher than that of most food crops (Horton and Fano, 1985). Moreover, their role in providing essential amino acids, such as lysine, (deficient in rice) (Woolfe, 1987, Woolfe, 1992), should not be overlooked (Anon, 1995). A comparison of potatoes and other crops in terms of their nutritional value is presented in Table 1.2.

Table 1.2 Comparison of the dietary value of potatoes compared to other food crops per 100 g edible portion.

Source	Food energy	Moisture	Protein	Fat	Ca	P	Fe	Na	K	Thiamine	Riboflavin	Niacin	Ascorbic acid
	Kcal	%	g	g	mg	mg	mg	mg	mg	mg	mg	mg	mg
Potatoes	76	80	2.1	0.1	7	53	0.6	3.0	407	0.09	0.04	1.5	16
Maize	51	87	1.2	0.1	1	10	0.1	205	11	0.02	0.01	0.2	0
Rice	109	73	2.0	0.1	10	28	0.2	374	28	0.02	0.01	0.4	0
Wheat	269	36	8.7	3.2	70	87	0.7	507	85	0.09	0.08	1.2	trace

Source: Wheatley *et al.* (1995)

1.1.4 Potato production and future global trends

The world population is increasing at an alarming rate of about 3% per year (Jordens, 2000). It is projected to grow to 7.5 billion by 2020, up from some 5.7 billion in 1995 (Anon, 1999a). The growth rate in developing countries is expected to account for over 97% of the increase in world's population by 2020 (Pinstrup-Anderson *et al.*, 1999). Further, an increasing proportion of the population will be living in cities, thus not producing their own food. The developing world's urban population is expected to reach 3.4 billion (Anon, 1996) and there will be increasing pressures on the existing farming community and land to produce the necessary food. Thus every effort needs to be made to increase world food production.

Being a rich source of nutrients, potatoes are an important component of the diet in many developing countries where the area under potato has steadily increased. According to the CGIAR (Consultative Group on International Agricultural Research) during 1995-1997, world potato production has grown by 4.5% chiefly as a result of improved economies of Asia, Africa and Latin America (Anon, 1998). A recent report (Swaminathan, 2000) estimates that by 2020, approximately 6 billion people will use roots and tubers in their diet which shows that these crops will be important in providing food, employment and income (Anon, 2000b). With continual population growth, tremendous pressure on the available agricultural land, rising incomes and modest consumption of potato, particularly in the developing countries, potato will continue to be an important food crop.

1.1.5 Potato diseases

Despite their enormous importance, and the fact that we have stepped into the 3rd millennium, potatoes are still one of the most difficult crops to grow to acceptable standards of quality (Rhodes, 2000) and one of the limiting factors is their susceptibility to a number of diseases.

Potato diseases might be caused by viruses (potato leaf roll virus, potato virus X, potato virus Y), bacteria (soft rot, common scab, brown rot), fungi (late and early blight, dry rot, gangrene, grey mould) or nematodes (potato cyst nematode). Insects

also affect the potato crop, either as result of direct feeding (Colorado potato beetle) or acting as virus vectors (thrips, aphids). Abiotic factors including mineral deficiencies, unfavourable environmental conditions and toxic substances can also cause disease referred to as 'physiological disorders' (Rich, 1983). Diseases may affect crop yield and quality at any stage of production (Stevenson *et al.*, 2001). Certain tuber diseases, such as soft rot and late blight, break down tubers in storage under conditions of high temperature and humidity. Soft rot Erwinias colonise the tubers as secondary pathogens and spread to uninfected tubers as the rot progresses, resulting in huge losses.

Control of diseases is central to successful commercial potato production. Some diseases can be controlled by application of pesticides. However, concerns have been raised about the excessive use of such chemicals which not only pollute the environment, but may also pose a risk to consumers in the form of residues present on harvested produce that can accumulate through the food chain. In addition, frequent use of the same chemical increases the risk of insensitivity build up in plant pathogen populations. A recent example is the reduced efficiency of phenylamide class of fungicides against the late blight pathogen, *Phytophthora infestans*. Other methods of disease control include use of resistant cultivars, disease quarantine measures to exclude the pathogen from potato growing areas and a variety of cultural methods (Section 1.9).

Since the European epidemic of 1845, late blight, caused by *Phytophthora infestans* (Mont) de Bary, has been recognised as one of the most important diseases of potato world-wide (Stevenson, 1993; Fry and Goodwin, 1997a) and is often a limiting factor to successful potato production (Anon, 1997; Stevenson, 1993; Reust, 2000). The devastation caused by late blight has been well documented (Large, 1940; Woodham-Smith, 1962) and its threat cannot be underestimated.

1.2 The pathogen

1.2.1 History

A mysterious disease of potato was first reported in the U.S. in 1843 which later appeared in Europe and was reported in The Netherlands and Belgium in 1845

(Bourke, 1991). The disease spread rapidly across Europe to Ireland where it caused almost total destruction of the potato crop. Much of the rural population of Ireland relied on potato as a sole source of food and the results of this mysterious disease were thus devastating and resulted in the 'Irish potato famine' (Nelson, 1995). It was unfortunate that the most widely grown cultivar in Ireland, Lumper, was also highly susceptible to blight (Salaman *et al.*, 1985). An estimated 1.5 million Irish died and a similar number migrated to North America. Late blight is thus one of the most significant plant diseases in recorded history (Large, 1940; Woodham-Smith, 1962). Over the following 20 years, the cause of the disease was debated widely. Theories proposed were; a fungus, excessive moisture, genetic deterioration in the cultivated potato or 'an airborne poisonous miasma'. Most people blamed it on the weather and considered the visible sporulation to be saprophytic fungal growth on dead plant tissue (Bourke, 1991). The Reverend M. J. Berkeley was the principal proponent of the 'fungal theory' (Berkeley, 1845; Bourke, 1991). The 'fungal theory' was severely criticised by many of Berkeley's contemporaries and remained controversial for some time. Montagne in 1845 described the pathogen as *Botrytis infestans* Mont. In 1861, Anton de Bary renamed it *Phytophthora infestans* and conclusively demonstrated it as the cause of the disease rather than a symptom of it (de Bary, 1876).

1.2.2 Taxonomy and etiology

Phytophthora infestans (Mont.) de Bary (1876), belongs to the Kingdom Chromista, Phylum Oomycota, Class Oomycetes, Sub-class Peronosporomycetidae, Order Pythiales, Family Pythiaceae and Genus *Phytophthora* (Cavalier-Smith, 1986, Cavalier-Smith, 1987; Dick, 1990; Barr, 1992; Dick, 1995a; Dick, 1995b). The fungus, along with other oomycetes, has several features which are not found in the so called 'true fungi' (Zentmyer, 1983; Griffith *et al.*, 1992). These include a diploid life cycle (Timmer *et al.*, 1970; Sansome and Brasier, 1973; Shaw, 1991), cell walls composed of cellulose and β-glucans rather than chitin (Bartnicki-Garcia, 1969; Bartnicki-Garcia and Wang, 1983), and an inability to synthesize sterols (Hendrix, 1964). It therefore requires an exogenous source of β-hydroxy sterols for sporulation (Ocana, 1967; Hendrix, 1970; Elliott, 1983). Further, carbohydrate is chiefly stored as mycolaminarin, a β-1,3-glucan (Wang and Bartnicki-Garcia, 1974).

On the basis of sporangial and antheridial structure, species within the genus *Phytophthora* (plant destroyer) have been classified into six morphological groups (Stamps *et al.*, 1990). *Phytophthora infestans* belongs to group IV and is characterised by semi-papillate and deciduous sporangia which do not proliferate internally and are borne on sympodial sporangiophores. It is heterothallic with amphigynous antheridia. All species in this group (*P. colocasiae, P. eriugena, P. hibernalis, P. ilicis, P. infestans, P. macrochlamydospora, P. mirabilis* and *P. phaseoli*) are host-specific and generally infect above ground parts of the plant (Drenth and Goodwin, 1998).

Phytophthora infestans has a hyaline, branched and multinucleate (coenocytic) mycelium, that plays an important role in the overwintering of the pathogen in tubers. With the exception of a single report (Patrikeyeva, 1979) neither hyphal swellings nor chlamydospores have been observed. The vegetative mycelium differentiates into branched sporangiophores which emerge from the stomata, at the tips of which lemon shaped sporangia are produced. The sporangia range from 29x19 μm (Waterhouse, 1963) to 36x22 μm (Tucker, 1931). These are produced under moist conditions and at moderate temperatures (10-24°C), are multinucleate, deciduous and semi-papillate. The sporangiophores are of determinate growth, sympodial with a characteristic swelling just below the sporangium. This sporangial phase is responsible for spread of late blight within and between crops during the growing season. Under cool weather conditions, the sporangia produce 3-8 zoospores which are biflagellate. The longer of the two flagella is smooth and posteriorly directed and is called whiplash type (Desjardins *et al.*, 1969; Desjardins *et al.*, 1973). The other is anteriorly directed and is termed the tinsel type (Anderson *et al.*, 1991). Most of the motile power of the zoospores comes from the thrust of the whiplash flagellum, while the tinsel flagellum controls the direction of the movement (Carlile, 1983). The zoospore phase may be particularly important in tuber infection as sporangia are washed from the stem and foliage into the soil during wet weather. Under warmer conditions, the sporangia can germinate directly resulting in infection without a zoosporic phase. This asexual reproduction brings about a very rapid multiplication of inoculum enabling a rapid spread of the disease under cool and wet conditions. Carlisle *et al.* (2002) reported the production of a fresh crop of sporangia within 3-4 days of infection.

The fungus is heterothallic and therefore requires two different mating types, termed A1 and A2, for sexual reproduction (Galindo and Gallegly, 1960; Shaw, 1991; Brasier, 1992; Shaw, 1996). The production of antheridia and oogonia is initiated when thalli of two different mating types come in close proximity (Judelson, 1996; Smart *et al.*, 1998). Heterothallism is regarded as a physiological trait since both antheridia and oogonia can be produced by thalli of either mating type. The development of antheridia and oogonia is hormone-regulated and on agar occurs in a mating zone where vegetative growth and asexual sporulation are limited. In contrast to true fungi, meiosis is gametangial with a diploid somatic phase (Shaw, 1983). After initial contact of antheridium and oogonium, the haploid nuclei fuse, forming a diploid nucleus in the oogonium. This is followed by the formation of lipid bodies and vacuoles in the cytoplasm of the oogonium, which later migrate to the periphery. A thick wall develops around the oogonium and an oospore is formed. Oospores formed *in planta* (Frinking *et al.*, 1987; Pittis and Shattock, 1994; Drenth *et al.*, 1995) are aplerotic and 24-35 μm (av. 30 μm) in diameter (Erwin and Ribeiro, 1996). Oospores are resistant structures, which can survive for long periods between successive potato crops under adverse environmental conditions (Drenth *et al.*, 1995) and germinate either directly by means of a germ tube with a terminal sporangium, or by direct growth as mycelium. Conditions controlling germination are however poorly understood. Moreover, it results in a sexual recombination and the production of new genotypes and provides a means of long term inoculum survival.

1.3 Disease occurrence and losses

Although potato and tomato are regarded as the most economically important hosts of *P. infestans*, it infects a wide range of plants in the family Solanaceae (Turkensteen, 1978; Vartanian and Endo, 1985; Harrison, 1988; Fry *et al.*, 1993; Fry and Goodwin, 1997b). These include eggplant, hairy nightshade, petunia, bitter sweet and others (Birch and Whisson, 2001; Stevenson *et al.*, 2001). In potato, late blight is a continual threat to production in almost every potato growing region (Duncan, 1999; Stevenson *et al.*, 2001), but is more destructive under cool moist conditions (Agrios, 1997). Although late blight can infect plants at any stage of growth under temperate conditions, it is not usually seen until the crop canopy has closed (Stevenson, 1993). There are, however, indications of the trend towards

earlier infections. Leaf infection results in a loss of photosynthetic area which has a serious effect on tuber yield whereas tuber infection promotes rapid rotting in storage (Kirk *et al.*, 2001). Moreover, such infected seeds pose a threat as a source of infection during the next growing season. The costs of disease control and yield and post harvest losses combined make late blight one of the most commercially damaging plant diseases world-wide (Fry and Goodwin, 1997a; Fry and Goodwin, 1997b). If left uncontrolled, susceptible cultivars can suffer 100% tuber losses (Henfling, 1987). A global annual loss of 10-15% in potatoes due to late blight has been reported (Flier and Turkensteen, 1999). The value of crop loss and the cost of crop protection required for late blight control combined account for US $5 billion annually (Birch and Whisson, 2001), of which $ 3 billion has been reported from the developing countries (Anon, 1999b). In the U.S., growers in the Columbia Basin of Oregon and Washington spent $ 1.3 million in late blight management in 1995 (Johnson *et al.*, 1997). Losses caused by late blight in various countries are presented in Table 1.3.

Table 1.3 Losses attributed to late blight in various countries of the world.

Country	Yield Loss (%)	Year	Reference
Africa	40-70	-	Sengooba and Hakiza, 1999
Belarus	50-80	-	Anoshenko, 1999
China	20-80	-	Wei *et al.*, 1999
Czech Republic	10	-	Zimnoch-Guzowska, 1999
Ecuador	50	-	Gonzales, 1999
Ireland	8	-	Copeland *et al.*, 1993
Kenya	56-76	1991-1998	Hakiza, 1999
Poland	22	1997	Zimnoch-Guzowska, 1999

1.4 Symptoms

On potato, symptoms of late blight are manifested as leaf and stem blight during the growing season (Fry *et al.*, 1993; Fry and Goodwin, 1997a). Late blight symptoms on leaves are characterised by water soaked lesions during cool, moist weather. A white downy fungal growth comprising sporangia and sporangiophores develops on the lower surface of the leaves around the margins of the lesions, which helps distinguish it from *Botrytis* which produces dark brown/grey spores. Late blight can also be distinguished from early blight (*Alternaria solani*) by the presence of lesions which neither have concentric rings nor are restricted by major veins as is typical in early blight lesions (Stevenson *et al.*, 2001). Under conducive conditions, entire leaves of susceptible cultivars are rapidly destroyed by large spreading blight lesions which spread to the petiole and stem. If wet weather prevails, the entire crop may be destroyed within two weeks (Agrios, 1997). In some cases, the first signs of the disease in the field are stem lesions which may form as a result of a deep latent infection in the stem during early sprout growth (Adler *et al.*, 2000).

Tuber infection by *P. infestans* results in a shallow, reddish brown dry rot. Whilst mature lesions in susceptible cultivars are obvious during visual inspection, early infection or restricted lesions on more resistant cultivars may not be so clear and may play a role in the spread of the disease. Storage of infected tubers under cool dry conditions results in drying of the infected tissue and the rot progresses very slowly. However, under warm, moist conditions, rotting occurs quickly as the tuber is frequently invaded by secondary rot microorganisms such as soft rotting *Erwinia*, *Fusarium* spp. or *Phytophthora erythroseptica,* (Rich, 1983).

1.5 Disease epidemiology

In tropical areas that produce more than one crop per season, there is a continual green bridge and blight can occur throughout the year. Under temperate conditions, populations peak during the summer but over the winter *P. infestans* survives as mycelium in infected tubers (Fig. 1.1), which may then be used as seed (Van der Zaag, 1956; Hirst and Stedman, 1960) or in discarded refuse piles (Boyd, 1974; Davidse *et al.*, 1989). The role of alternate hosts in late blight epidemiology has not been fully explored. Hairy nightshade and bittersweet have been identified as other hosts of *P. infestans* by several workers (Deahl and Inglis, 1995; Punja *et al.*, 1998). Infected potato tubers, either seed or ground keepers, spread the disease in the following season (Kadish and Cohen, 1992; Lambert and Currier, 1997). The mechanisms are not yet confirmed. The pathogen may move through a soil phase to infect the developing sprout or the mature canopy as the leaves touch the soil surface (Hirst and Stedman, 1960; Boyd, 1980; Doster *et al.*, 1989). Conversely, the pathogen may move directly from the tuber to the sprouts (Peterson, 1947; Robertson, 1991) and remaining dormant or latent until the crop emerges and then produce a sporulating stem lesion. In practice it seems likely that both processes play a role.

Upon reaching the aerial parts of the plant, the pathogen produces abundant sporangia borne on sporangiophores that emerge from the stomata of the stem and the leaves. The deciduous sporangia are released during the day and spread by a combination of wind and rain splash (Fitt and Shaw, 1989). They land on the surface of potato leaves or stems and infect under cool wet conditions (Cox and Large, 1960). Late blight is favoured by cool (16-21°C), wet weather and infection occurs only if the foliage is wet (Rotem *et al.*, 1971; Sedegui *et al.*, 2000). Such weather conditions facilitate sporangial production, dissemination, infection and subsequent disease development. Dry conditions or temperatures above 30°C will halt a late blight epidemic but do not kill *P. infestans* and the return of cool moist weather allows the fungus to become active again (Agrios, 1997). Survival during the dissemination phase is affected principally by exposure to UV light (Mizubuti *et al.*, 2000). At temperatures of 12-15°C, each sporangium produces 3-8 zoospores, which germinate after encystment. Direct germination by means of a germ tube has been

reported at higher temperatures (Agrios, 1997). A period of two days during which the temperature does not fall below 10°C and R.H. is 90% or higher for at least 11 h on each day, is regarded as favourable for infection and is termed a 'Smith Period'. Upon germination, the sporangia, or cysts, form a germ tube (Clarke, 1983; Coffey and Gees, 1991), the tip of which forms an appressorium, which in turn produces a penetration peg that enters the epidermal cell. In a susceptible cultivar, the primary hyphae rapidly move from the epidermal cells and invade the mesophyll. Once inside the host, mycelium can grow profusely producing intracellular haustoria within the mesophyll cells (Gees and Hohl, 1988). After a brief biotrophic phase, plant cells begin to die and necrotic spreading lesions are formed. A few days after infection, sporangia are produced on sporangiophores emerging from the stomata of lower leaf surface. As many as 10^6 sporangia/lesion have been reported (Harrison and Lowe, 1989) and it is this ability to produce prolific sporulation and a short cycle time that makes the pathogen so devastating. Late blight is thus a polycyclic disease since many asexual generations are completed in one growing season (Van der Plank, 1963).

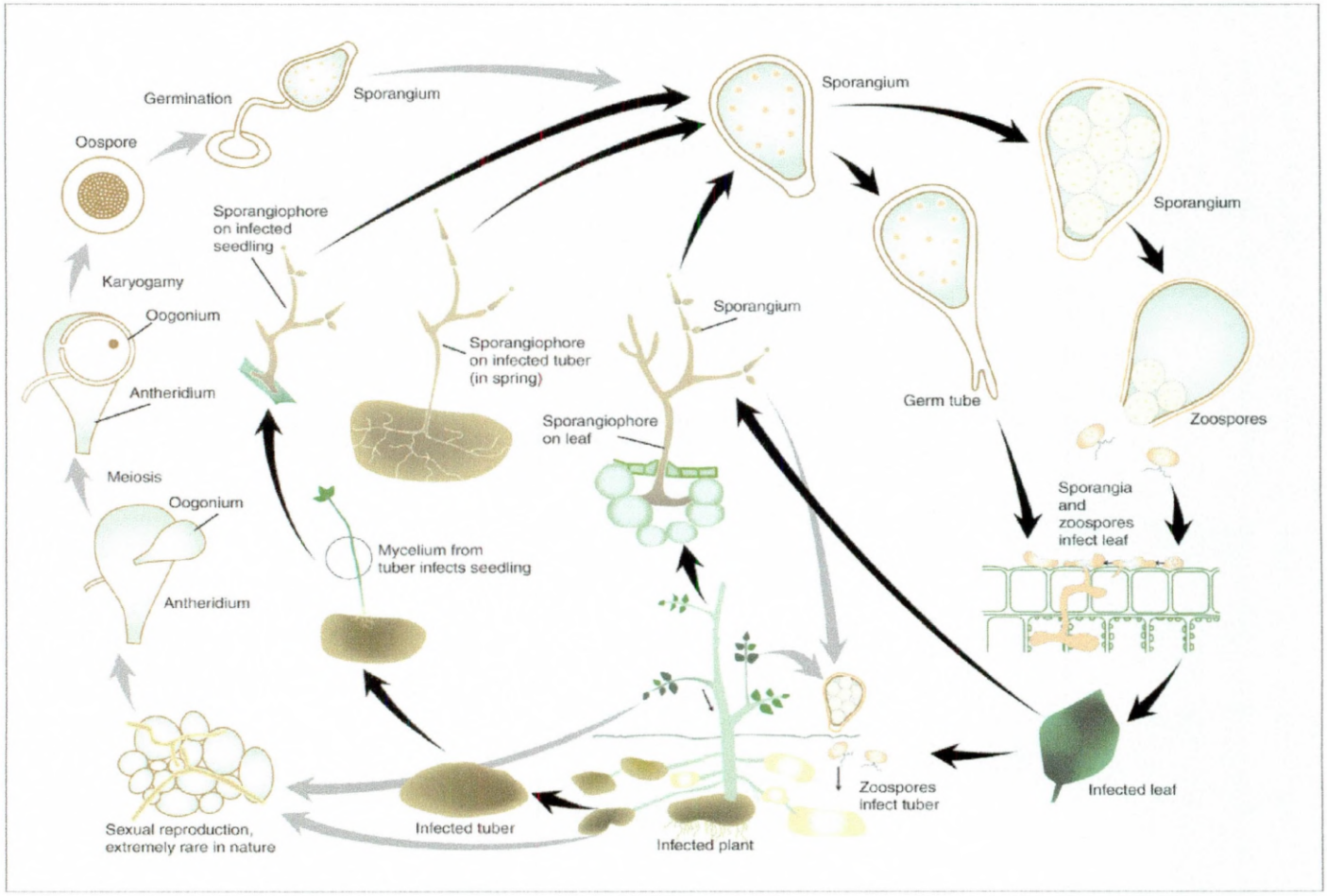

Fig. 1.1 Disease cycle of *Phytophthora infestans*, the cause of late blight of potato. Redrawn from Agrios (1997).

Infection of developing tubers by *P. infestans* occurs shortly after establishment of the disease on the foliage when sporangia are washed down from leaves into the soil (Zan, 1962; Hirst *et al.*, 1965; Lacey, 1966, Lacey, 1967). Consequently, tubers near the soil surface are more vulnerable to disease (Erwin and Ribeiro, 1996). The zoospores infect tubers either through the lenticels, eyes or wounds (Adams, 1975; Walmsley-Woodward and Lewis, 1977). In tubers, the mycelium is mostly intercellular with intracellular haustoria. Infection appears to occur when the soil temperature is at 18°C or below (Sato, 1979). Infection of tubers can also occur at harvest when they come in contact with diseased plants or contaminated soil. It has also been shown that infection can spread from diseased to healthy tubers during tuber washing and grading (Fairclough *et al.*, 1997) or during storage and handling of seed (Lambert *et al.*, 1998). Most of the tubers rot in the ground or later in storage. Tuber infection is influenced by a number of factors, which include soil temperature (Sato, 1979), soil moisture (Lacey, 1967; Lapwood, 1977), soil composition (Andrivon, 1994b), competing microorganisms (Lacey, 1965), and a number of management practices such as use of resistant cultivars (Lapwood, 1977) and haulm destruction (Murphy and Mckay, 1925).

In locations where both A1 and A2 mating types of *P. infestans* co-exist, oospores are produced (Shattock *et al.*, 1990; Daggett *et al.*, 1993) and can serve as survival structures in the soil for months or possibly years (Chang and Ko, 1991; Drenth *et al.*, 1995; Medina and Platt, 1999). Mayton *et al.* (2000) showed that the viability of oospores decreased slightly over a period of 18 months when stored in water at 4°C, in soil at 18°C, or under natural field conditions, and that germination %age appeared to increase after storage in soil when assessed using a potato leaf bioassay. Drenth *et al.* (1995) showed that soil taken from fields where blighted potatoes had grown ten months previously still contained viable *P. infestans* inoculum. Furthermore, RFLP-based DNA fingerprinting with the RG57 probe (Goodwin *et al.*, 1992a) verified that some of the infections in this bioassay were caused by recombinant progeny of the parental isolates that had been used to artificially inoculate the previous crop. Oospores have been shown to survive extremely low temperatures, although moderate increases in heat can affect their viability. For example Drenth *et al.* (1995) reported that oospores exposed to temperatures ranging from -80 to 35°C for two days remained viable but those in soil at 40-50°C did not.

Medina and Platt (1999) showed that oospores had more than 50% viability when exposed to temperatures of -20 and -50°C for up to 90 days under laboratory conditions and exposure to 0, 4 and 15°C resulted in average viabilities from 23-36%. The production of oospores is also favoured by low temperature. Cohen *et al.* (1997) observed that oospore production in detached leaves of two tomato and four potato cultivars increased as temperature decreased. Drenth *et al.* (1995) reported a significantly higher number of oospores in leaf discs at 10°C than at 15 or 20°C. Sprinkle irrigation has also been shown to increase oospore production (Cohen *et al.*, 2000).

Oospores can germinate after coming in contact with newly planted tubers, stems or leaves and cause new infections (Schöber and Turkensteen, 1992). Andersson *et al.* (1998) reported that soilborne oospores were possibly responsible for infection of a subsequent potato crop after remaining dormant in the soil for several months. Despite such observations infections originating from oospores have not been proven conclusively and their role in disease epidemiology is largely unknown.

1.6 Patterns of migration of *P. infestans*

It is likely that the late blight epidemic of 1846 was the result of the migration of *P. infestans* to the U.S. and Europe from its centre of origin, Central Mexico (Niederhauser, 1991; Fry *et al.*, 1992; Fry *et al.*, 1993; Goodwin *et al.*, 1994b) or from a secondary South American centre (Tooley *et al.*, 1989; Abad and Abad, 1995; Abad *et al.*, 1995; Andrivon, 1996). Andrivon (1996) suggests that it was a three step migration, where the pathogen moved firstly from Mexico to the South American Andes some centuries ago, resulting in the establishment of US-1 lineage in that region. US-1 represents the 'old' population of *P. infestans,* which is characterised on the basis of mating type (A1), metalaxyl sensitive, isozyme and RG57 fingerprint (Goodwin *et al.*, 1994b). From the Andes the pathogen then migrated first to North America and then to Europe in the 1840s resulting in the spread of a single clonal lineage throughout the world (Drenth *et al.*, 1994; Goodwin *et al.*, 1994b; Goodwin, 1997). It is assumed that these migrations occurred via the movement of infected plant tissue, most likely tubers (Peters *et al.*, 1999a). Ristaino *et al.* (2001) suggested that more than one strain was introduced to Europe. Nevertheless, genetic analysis of

many late 20th century isolates has shown that a single clonal lineage dominated (Fry *et al.*, 1992; Drenth *et al.*, 1994; Goodwin *et al.*, 1994b; Sujkowski *et al.*, 1994; Fry and Goodwin, 1997b). Regardless of the specific route, blight outbreaks were largely the result of a single clonal lineage (US-1 genotype) for 135 years (Goodwin *et al.*, 1994a). In the absence of a sexual cycle variability must, therefore have originated via mutation, mitotic recombination, a parasexual cycle (Fry *et al.*, 1992) or somatic fusions (Pipe *et al.*, 2000).

The situation changed in the mid 1970s when the A2 mating type was discovered in Europe and North America (Spielman *et al.*, 1991; Fry *et al.*, 1992; Fry *et al.*, 1993; Fry and Goodwin, 1997a). Since then, there have been major changes in population structure of *P. infestans* (Spielman *et al.*, 1991; Fry *et al.*, 1992). The clonal population in many areas has now been largely replaced by a sub-set of the diversity seen in Central Mexico (Goodwin *et al.*, 1994a). The cause of this displacement is not fully understood (Lebreton and Andrivon, 1998), but presumably resulted from a difference in fitness and metalaxyl insensitivity between 'old' and 'new' genotypes (Fry *et al.*, 1992; Fry *et al.*, 1993; Kato and Fry, 1995a; Kato and Fry, 1995b; Day and Shattock, 1997). It has been suggested that an accumulation of deleterious mutations or 'Müllers Ratchet' (Goodwin, 1997) may have occurred in the old population over the decades that it was isolated as a clonal population, and this has contributed to its reduced fitness.

It would appear that the 'new' population consisted of both A1 and A2 mating types, thus providing the opportunity for the fungus to undergo sexual recombination. Novel allozyme alleles at the *glucose-6-phosphate isomerase* (*Gpi*) and *peptidase* (*pep*) loci and RFLP patterns were observed (Drenth *et al.*, 1993a). New genotypes were apparently fitter, given the fact that there was an increase in their frequency after their introduction and that they were highly aggressive (Fry *et al.*, 1993; Goodwin *et al.*, 1994b; Koh *et al.*, 1994; Platt, 1994; Goodwin *et al.*, 1995b; Day and Shattock, 1997; Fry and Goodwin, 1997b; Kato *et al.*, 1997; Miller and Johnson, 1997).

New genotypes resulted in earlier disease outbreaks and foliar studies indicated that the new strains had a shorter latent period (Carlisle *et al.*, 2002) and increased

sporulation as well as being more aggressive on tubers (Kato and Fry, 1995a; Kato and Fry, 1995b; Miller *et al.*, 1995). Lambert and Courrier (1997) reported that new *P. infestans* genotypes caused a severe tuber rot compared to the previous US-1 genotypes.

Several hypotheses have emerged to explain the occurrence of the A2 mating type of *P. infestans* outside its centre of origin. These include:

a) A2 was always present but undetected (Shaw, 1987)
b) A2 was introduced by migration (Spielman *et al.*, 1991)
c) A2 arose by mutation or mitotic recombination
d) A2 evolved by changes to the A1 mating type as a result of induced selfing or exposure to fungicides (Ko, 1994).

Among these causes, migration seems to be the most probable cause (Goodwin and Drenth, 1997). Studies in the U.S., based on allozyme and DNA fingerprint analysis suggest that the A2 mating type was probably introduced into the U.S. from Northwestern Mexico on infected plant tissue around 1992 (Goodwin *et al.*, 1995b; Goodwin, 1997). Peters *et al.* (2001) showed that g-11 (US-11) could have migrated from the Columbia Basin of Washington and Oregon into British Columbia, as the characteristic multilocus allozyme of the genotype was detected at both locations. Sujkowski *et al.* (1994) showed that migration was responsible for a sharp increase in the frequency of 'new' genotypes in Poland around 1988. Drenth *et al.* (1993a), using the molecular probe RG57, demonstrated that the *P. infestans* population in The Netherlands had fingerprint patterns similar to those in Central and Northern Mexico, therefore, implying migration. The A1 mating type strain (Jp-1) was prevalent in Japan in 1987, and on the basis of mitochondrial type, mating type, allozyme alleles and RG57 fingerprint, the same genotypes were soon detected in Korea (Koh *et al.*, 1994). It was postulated that migration was the main cause of the spread of Jp-1, as exchange of potatoes between the two countries was common. Similarly, allozyme data suggest that A2 mating type in Europe and Japan was the result of migration from Mexico (Spielman *et al.*, 1991). The results of these studies and a comprehensive review of the molecular data (Goodwin and Drenth, 1997)

countered the idea that the A2 originated from within the existing US-1 isolates and therefore Ko's hypothesis (1994) was rejected.

Several mechanisms may have been responsible for the spread of novel genotypes. International trade in potato tubers for ware and seed has been reported as the most likely route (Spielman *et al.*, 1991; Goodwin *et al.*, 1995b; Fry and Goodwin, 1997a; Miller *et al.*, 1997; Goodwin *et al.*, 1998; Peters, 1998). It was suggested (Niederhauser, 1991) that the appearance of the 'new' population might have been due to the migration of the A1 and A2 mating type of *P. infestans* as a result of imports of potato into The Netherlands from Mexico in 1976.

Despite rigorous inspection, it is not possible to eliminate blight in such consignments of tubers. It has also been postulated that infected tomato fruits, seeds and seedlings can spread *P. infestans* to new areas (Goodwin *et al.*, 1994a; Goodwin *et al.*, 1995b; Fry and Goodwin, 1997a). Another proposed mechanism of migration of *P. infestans* is in tropical storm systems. It has been suggested that windborne sporangia of *P. infestans* may be dispersed several hundred kilometres (Stevens, 1933). Similar observations were made by Peters *et al.* (1999a) to explain the spread of blight from Nova Scotia to Prince Edward Island, a distance of more than 100 km. However, much of the evidence for such long distance airborne spread is circumstantial in light of sensitivity of sporangia to UV light (Mizubuti *et al.*, 2000) and may only occur in very specific circumstances. Detailed studies on the spread of, for example, marked strains are needed to validate such claims.

1.7 Implications of sexual reproduction

It is clear that a clonal A1 population has, in many countries, been replaced by one of mixed mating type. Reports from The Netherlands (Drenth *et al.*, 1993a); Poland (Sujkowski *et al.*, 1994); Scandinavia (Brurberg *et al.*, 1999) and Scotland (D. E. L. Cooke, pers. comm.) suggest increased genetic diversity that is likely to be due to sexual recombination. Of course, this increased variation is attributable to sexual recombination which implies that A1 and A2 mating types are forming oospores which are germinating and the 'new' genotypes are establishing themselves on the growing crop. Oospores resulting from the association of A1 and A2 mating types are presumably most frequently formed on leaf tissue (Drenth, 1994). Others have

reported the production of oospores in stem (Frinking *et al.*, 1987; Mosa *et al.*, 1991) and tubers (Grinberger *et al.*, 1989). These oospores are highly resistant structures that can survive in soil for long periods even in the absence of the host, thus bridging the gap between successive potato crops.

The implications of this resting phase of the fungus are potentially serious for agriculture. Should oospores be shown to be responsible for significant amounts of disease, longer crop rotations and greater fungicide inputs would be the inevitable and costly consequences. Although both mating types have been present in many potato-producing areas for over a decade, however, their impact on late blight epidemiology remains to be demonstrated conclusively. Early infections of late blight were observed in a Swedish potato field that had two years previously been shown to contain both A1 and A2 mating types of *P. infestans* (Andersson *et al.*, 1998). Intuitively, oospore contamination would be expected to result in early infections independent of infected seed or contamination from tuber dumps or ground keepers. In practice however, it is difficult to prove the origins of such outbreaks.

Another implication of sexual recombination is the risk of accelerated pathogen evolution as a result of continual allele re-assortment. This evolution could enable isolates to adapt to a wide range of selection pressures. Already, scientists and growers believe that the 'new' genetically variable population is more aggressive (Goodwin, 1997). Further, this increased diversity and aggressiveness could result in more rapid erosion of host resistance or generation of insensitivity to new fungicidal active ingredients. The oospores will also permit the fungus to survive during unfavourable environmental conditions within the season and will start colonising the healthy foliage once the conditions become favourable. Very little is known about the processes of infection by oospores. Drenth *et al.* (1995) speculated that oospores could germinate during periods of rainfall, releasing motile zoospores which may infect the leaves or stem near the soil surface. Splash dispersal and sprinkling irrigation could lead to infection in the upper leaf canopy.

1.8 Co-dominant markers and their role in the study of population biology

It is clear that 'old' *P. infestans* populations in many countries have been displaced by 'new' more aggressive or fit strains which are insensitive to phenylamides (Day and Shattock, 1997; Fry and Goodwin, 1997a; Kato *et al.*, 1997). In combination with sexual reproduction, this migration of 'new' isolates has resulted in greater disease pressure (Day and Shattock, 1997) with an impact on disease management. It is therefore important to be able to track such changes in the population, but existing methods have limitations. Ideally, a panel of markers for such a purpose should be co-dominant, rapid to score, reproducible and resolve at sufficiently fine scale to discriminate closely related strains. Many existing markers fail in one or more of these criteria (Chapter 4). Therefore the development of new molecular markers is considered to be a high priority (Trout *et al.*, 1997).

The development of co-dominant markers will help in studies aimed at reliably detecting genetic variations in populations and enable us to understand population biology of *P. infestans* more precisely. On one hand, such markers will allow us to determine the extent of genetic recombination within a given population and, on the other, they will also provide information on the presence (extent) or absence of gene flow between populations. Such markers can also prove useful in monitoring individual isolates in field and glasshouse which will be helpful in comparison of fitness difference in co-inoculation studies. Moreover, use of DNA from spores washed from the surface of single lesions can immensely increase the throughput of the assay and will be helpful in rapid screening of the field populations. Such markers, once optimised, will therefore provide an easy, one step screen with reproducible results. A rapid identification of strains could help in implementation of timely disease management decisions.

1.9 Disease management

Currently late blight management involves a combination of practices aimed at either preventing disease reaching the crop in the first place or reducing the population size and rate of progression of disease (Carlisle *et al.*, 2001). These strategies include use

of well timed chemical applications, resistant cultivars, sanitary measures and adjustment of cultural practices. Education of growers is of fundamental importance to ensure the most appropriate practices are applied (Elansky *et al.*, 2001).

Fungicides remain the most widely used method to control late blight in many areas (Van Bruggen *et al.*, 1986). Fungicides can be grouped into two broad categories according to their mode of action i.e. protectants and curatives. Protectant fungicides are active on leaf surface to prevent sporangial germination or to kill zoospores prior to infection. Examples of such fungicides include Bordeaux mixture, copper oxychloride, dithiocarbamates and organic tin compounds. However, these fungicides have little curative activity and are only effective when a complete cover of the foliage is maintained particularly during periods of rapid crop growth and when conditions are favourable for late blight development. Curative fungicides on the other hand, are systemic, generally absorbed by the plant and can eradicate existing infections. Metalaxyl is an example of one such fungicide that has been used effectively against late blight (Bradshaw and Vaughan, 1996). However, the use of a combination of systemic and protectant fungicides is advisable to prevent build up of insensitivity to systemic fungicides. Sprays normally commence upon canopy closure but earlier sprays are also used, especially when weather conditions are conducive to disease development. Early sprays should use a combination of curative and protectant fungicides since the systemic component of the mixture provides a wider window of protection at a time when crop is actively growing. However, when crop canopy reaches its maximum size, a shift to protectant fungicides is advisable in order to prevent build up of insensitivity and the fact that only existing foliage has to be protected. The frequency of application is governed by risk periods which can be predicted from the accurate local weather records and the susceptibility of the cultivars. Blight forecast systems help predict the time when there is a greater risk of the disease, thereby enabling growers to use fungicides effectively and economically. Grower education, both in the developed and developing countries, regarding crop and weather monitoring and use of blight forecast systems will help reduce the incidence of the disease (Marshall-Farrar *et al.*, 1998). A maximum interval of 4-5 days between successive sprays is recommended under conditions favourable for disease development.

Metalaxyl, a phenylamide class of fungicides, was used effectively until the appearance of insensitive strains. The selection pressure applied by frequent metalaxyl application has increased the frequency of insensitive isolates in *P. infestans* population. First reported in Germany in 1977 (Daggett *et al.*, 1993), metalaxyl insensitivity has since been reported from many parts of the world including the Middle East (Cohen and Reuveni, 1983), Asia (Therrien *et al.*, 1993; Koh *et al.*, 1994), Europe (Davidse *et al.*, 1981; Dowley and O'Sullivan, 1981), Mexico (Matuszak *et al.*, 1994), South America (Forbes *et al.*, 1997), and the U.S. (Deahl *et al.*, 1995; Miller *et al.*, 1997). Metalaxyl insensitivity has exacerbated the disease control problem (Davidse *et al.*, 1981; Fry *et al.*, 1993; Goodwin *et al.*, 1994c; Matuszak *et al.*, 1994; Davidse, 1995; Chycoski and Punja, 1996; Goodwin *et al.*, 1996; Day and Shattock, 1997; Goodwin *et al.*, 1998; Miller *et al.*, 1998) not only by reducing the efficacy of the fungicide but also because metalaxyl insensitive isolates are more aggressive and produce significantly larger and deeper lesions in potato tubers than metalaxyl sensitive isolates (Grinberger *et al.*, 1995; Kadish and Cohen, 1992).

The use of resistant cultivars provides the most practical, environment-friendly and economical means of controlling late blight. Cultivars with suitable levels of resistance, that are also compatible with marketing needs, are highly desirable, and their use can reduce the number of fungicide applications required for late blight management.

Two types of resistance to late blight can be distinguished. Specific resistance is conferred by single dominant genes (R-genes) and results in a hypersensitive response (HR) (Toxopeus, 1956). By the mid 1950's, four single R-genes (R1-R4) were identified by Black *et al.* (1953) from *S. demissum* and others have subsequently been identified (Malcolmson and Black, 1966; Ross, 1986; Wastie, 1991). Of the 16 distinct R-genes known to exist in *S. demissum* (Niederhauser *et al.*, 1996), 11 (R1-R11) have been introgressed into *S. tuberosum* (El-Kharbotly *et al.*, 1996; Li *et al.*, 1998). A cultivar possessing a particular R-gene will however be resistant to the race of *P. infestans* containing the corresponding avirulence gene. The races thus differ from one another with respect to the R-gene differentials that they can infect. Appearance of a new race with additional virulence genes defeats the

existing R-gene and the cultivar becomes susceptible. Several mechanisms including mutation (Peterson and Mills, 1953; Toxopeus, 1956; Gallegly, 1968), sexual reproduction (Pristou and Gallegly, 1956), parasexuality (Leach and Rich, 1969) or somatic variation (Caten and Jinks, 1968) have been suggested as the possible causes for the appearance of new virulent races. R-gene resistance is therefore effective against specific races of *P. infestans* and is not durable. New potato cultivars have frequently failed soon after their widespread cultivation as a result of the breakdown of resistance by new strains of the pathogen (Wastie, 1991; Andrivon, 1994c; Sujkowski *et al.*, 1994; Platt and Tai, 1998). Complete dependence on cultivars with specific resistance is not therefore prudent, as the appearance of new races of *P. infestans* can often rapidly overcome this form of resistance.

Field resistance, on the other hand, is controlled by many genes (Niederhauser, 1991; Niederhauser, 1993). This kind of resistance, although it does not confer such a high level of resistance, reduces the rate of epidemic development and is effective against a number of races of *P. infestans* (Van der Plank, 1963; Mackenzie *et al.*, 1983). Field resistance, being more durable than specific resistance (Forbes and Jarvis, 1994), is therefore preferable. Many cultivars possessing moderate to high levels of resistance to one or more races of *P. infestans* are available but in many potato production areas are not deployed widely, either because they do not combine resistance and good agronomic characters, or because of the strict requirements of the supermarkets/processors.

Since *P. infestans* overwinters in seed tubers, planting disease-free seed could be a practical means of controlling late blight. However, exclusion of the pathogen is not a trivial task because infected tubers mostly do not have outward disease symptoms and therefore remain undetected. Improved diagnostic tools will however greatly facilitate the detection of cryptic infection in seed stocks and could play a useful role in disease control via eradication. Elimination of inoculum sources such as potato dumps and volunteer plants can also reduce the sources of primary inoculum (Agrios, 1997). Other measures include growing mixtures of potato cultivars or intercropping of potato with other crops such as legumes which reduces the spread of an epidemic either through barrier effects or through the availability of less susceptible tissue for late blight infection (Garrett and Mundt, 2000). Reduced

application of nitrogen hastens tuber maturity and therefore reduces the length of time the crop is exposed to the risk of late blight (Schmitthenner and Canaday, 1983). Similarly, harvest date and soil moisture content have also been implicated in influencing tuber susceptibility (Stewart *et al.*, 1983; Stewart *et al.*, 1993). Use of large potato ridges has been recommended by Rowe and Secor (1993) to offer some tuber protection by filtering *P. infestans* spores out of the soil water suspension. Practices designed to alter the micro-climate, thereby reducing periods of leaf wetness could also affect late blight infection. Such measures include proper spacing to allow air movement and appropriate level of irrigation. A combination of various control strategies is however regarded as the best approach in effective late blight management.

1.10 Host adaptation and its impact on durability of host resistance

Despite the occurrence of gene flow, some degree of pathogenic specialisation exists between *P. infestans* populations on potato and tomato (Lebreton *et al.*, 1999). There are several reports of a clear genetic differentiation of *P. infestans* populations between the two hosts (Fry *et al.*, 1991; Koh *et al.*, 1994; Legard *et al.*, 1995; Forbes *et al.*, 1997; Lebreton and Andrivon, 1998; Oyarzun *et al.*, 1998). Lebreton *et al.* (1999) demonstrated conclusively that *P. infestans* isolates from potato were more aggressive on potato cultivar Bintje than isolates originating from tomato. Differences in aggressiveness could have been due to the lower virulence complexity of tomato isolates (Lebreton and Andrivon, 1998). However, in another study *P. infestans* isolates from potato were less aggressive to tomato compared to a tomato isolate, which was equally aggressive to potato and tomato, thereby suggesting that aggressiveness to tomato was a recently acquired trait (Legard *et al.*, 1995). Oyarzun *et al.* (1998) concluded that adaptation of *P. infestans* to potato and tomato appears to be due to quantitative differences in epidemic components rather than a mutually exclusive trait.

The extent of specialisation of late blight populations within potato cultivars is not known. There have been a few studies on potato genotype specificity with conflicting reports. The presence of putative R-genes in some of the cultivars with partial resistance could be responsible for inconsistent results. It is therefore

important to be able to distinguish the two possible causes of adaptation. Several workers have demonstrated the presence of specificity between *P. infestans* isolates and potato cultivars with partial tuber resistance (Caten, 1974; Bjor and Mulelid, 1991; Peters *et al.*, 1999b). Similar results were reported by Flier (2001) for specificity on foliage. James and Fry (1983), on the other hand, reported limited specificity between isolates and potato cultivars and suggested that it had little practical significance. However, Fry and Doster (1991) have argued that there appears to be no *a priori* reason why such adaptation could not occur. Therefore, if such adaptation exists between *P. infestans* isolates and potato cultivars, then progressive erosion of partial resistance may affect the long term use of such cultivars. Recently, Flier (2001) reported the erosion of partially resistant cultivar Pimpernel under field conditions in The Netherlands as a result of host specificity. There is thus a need to study the extent of host adaptation of 'new' *P. infestans* population on widely grown potato cultivars with rate-reducing resistance.

1.11 Late blight diagnostics and their use in disease management and epidemiology

The potential of *P. infestans* to cause severe epidemics under favourable conditions, the ability of oospores to survive long periods and the appearance of aggressive strains all make it necessary to diagnose infections at the earliest possible stage. Current detection methods to detect infection in tubers include visual examination, isolation of the pathogen and immunodetection. Visual detection is, however, difficult especially in specimens that have latent infections. A recent report suggests that up to 20% tubers in a seed lot can be latently infected with *P. infestans* without showing any outward disease symptoms (Appel *et al.*, 2001). Such tubers often escape detection and thus act as inoculum sources in the next growing season causing disease spread to uninfected areas if used as seed stocks. In such cases, the pathogen often gets well established before the appearance of symptoms, and therefore makes control measures, based on detection by direct observation, less efficient.

Isolation of *P. infestans* is labour intensive and time consuming, and particularly difficult when secondary microorganisms such as soft rotting *Erwinia* are present.

Moreover, such a procedure requires expertise in fungal taxonomy. Although the use of selective media can simplify taxonomic classification, the time required is still not reduced (Davies *et al.*, 1987). Immunodetection methods are rapid, but the existing antibodies for *P. infestans* lack specificity (Harrison *et al.*, 1990). These limitations demonstrate that a new diagnostic tool, that is sufficiently rapid, reliable, specific and sensitive, is needed. PCR detection could prove a powerful tool for detecting latent infections (Chapter 2) and helping in the assessment of seed stock. Healthy seed stocks are a pre-requisite for a healthy crop in the field, and the tool will help researchers and inspectors to make timely recommendations to limit the spread and/or development of blight infection.

Diagnostics can also prove a powerful tool for epidemiological investigations, such as monitoring the pathogen population in irrigation water and field soil, or in air, by trapping airborne inoculum on aerial spore traps, determining longevity of oospores and tracking pathogen movement within the host.

1.12 Aims and objectives of the current project

The development of accurate and reliable diagnostics, their application to epidemiological studies and the development of new molecular markers will aid our understanding of late blight epidemiology. A particular area of importance is the role oospores play in the development of late blight epidemics. The long term aim of such studies is to help to develop improved disease management strategies. The present research project was, therefore, designed with the following objectives:

1) To develop a species-specific and sensitive PCR-based diagnostic test for *P. infestans* and to test its efficacy against a range of propagules in tubers, leaves, stem and soil.

2) To investigate long term survival of oospores and sporangia in soil under field conditions using PCR.

3) To develop novel single nucleotide polymorphic (SNP) markers that can be used to estimate molecular diversity in *P. infestans* and to test the heritability and applicability of these markers. Further, to convert the above developed markers into a high throughput assay for rapid genotyping of the isolates.

4) To examine the response of *P. infestans* isolates to cultivar selection in detached leaf assays and to use the above developed markers in tracking of isolates in competition studies.

Development of a specific and sensitive assay for the detection of *P. infestans* in plant tissue and soil

2.1 Introduction

2.1.1 Late blight and its potential to cause epidemics

Phytophthora infestans differs from many other *Phytophthora* species in that it is a foliar pathogen producing abundant airborne sporangia under cool, moist conditions. These sporangia have the ability to spread over a distance of several hundred kilometres (Aylor, 1986) and are the propagules responsible for the rapid development of late blight epidemics. Under the cool, moist conditions typical of much of the temperate region, the disease may cause total destruction of plants in a field within a couple of weeks (Agrios, 1997).

The influx of the A2 mating type of *P. infestans* from its centre of origin, Central Mexico, to other parts of the world during the 1970s and 1980s resulted in increased severity of late blight, and has once again focused the world's attention on this disease (Fry *et al.*, 1993; Goodwin, 1997). Evidence that sexual reproduction is occurring has been provided through the identification of recombinant genotypes, the co-occurrence of both mating types and the observation of oospores in plant tissues (Drenth *et al.*, 1994; Sujkowski *et al.*, 1994; Chycoski and Punja, 1996; Ristaino, 1998). Soilborne oospores have been reported in many countries (Grinberger *et al.*, 1989; Andersson *et al.*, 1998; Smirnov and Elansky, 1999) and have been shown to cause disease. Strömberg *et al.* (1999) showed that brown discolouration developed on underground stems and tubers of plants grown in soil having 250 and 400 oospores/ml of soil for one month at 15°C and 16 h day length and Drenth *et al.* (1995) demonstrated that oospores could overwinter in natural soil and infect the next crop of potatoes. The implications of sexual reproduction and role of oospore as source of inoculum are discussed in Chapter 1. Similarly, infected seed tubers are thought to be an important source of primary inoculum for late blight epidemics (Davidse *et al.*, 1989; Deahl *et al.*, 1993). Only a few infected tubers are required for survival of the pathogen, as under favourable weather conditions one infected seed

tuber/25 ha of potatoes is enough to initiate an epidemic (Van der Zaag, 1956). The potential of sporangia and zoospores to cause epidemics, the survival of oospores under adverse environmental conditions in the absence of a host and the ability of infected but symptomless tubers to act as a source of inoculum contribute to making late blight one of the most devastating diseases of potato world-wide (Stevenson *et al.*, 2001). Crop losses and crop protection costs that run into billions of dollars are an important reminder that the disease must be controlled for sustainable potato production.

2.1.2 Rationale for blight detection

In view of the devastating effects of late blight, detection of the causal organism (*P. infestans*) at the earliest possible opportunity is warranted. Since *P. infestans* overwinters in infected seed tubers, it is imperative that seed stocks should be disease free. It has been reported that a few infected seed can spread the pathogen throughout an entire storage facility (Secor and Gudmestad, 1999). Post harvest inspection of seed stocks is thus important to minimise such losses. Visual inspection can, however, only reveal established tuber infections, and the contribution of latent infection or even tuber contamination to tuber loss in store and subsequent planting is unknown. A diagnostic test that improves upon visual inspection to give a rapid and sensitive estimate of stock contamination will help to reduce such losses and will be of great benefit to the seed potato industry.

An early detection method for *P. infestans* will only be useful if it has the power to detect the pathogen in pre-symptomatic plant tissue. For example, plant tissues collected while scouting during a spell of dry weather, if positive for *P. infestans*, could serve as a blight warning system. Such warning systems would allow growers to make rational spray decisions on the basis of not only weather conditions but also inoculum pressure. The diagnostic test could also be used as a powerful tool for monitoring the pathogen in the natural environment (e.g. irrigation water and soil). Since the presence of both mating types of *P. infestans* has made oospore production a reality, accurate detection of oospores in soil has become important in order to estimate inoculum load. In addition, new detection methods will prove useful as research tools for epidemiological studies (Chapter 3).

It is therefore anticipated that the development of a rapid and reliable diagnostic test will have many benefits. Early field detection would benefit growers, detection in tubers would help the seed trade to provide a higher standard of product and all benefits to the industry should translate to reduced fungicide application and thus yield both environmental and economic benefits (Askew, 1996).

2.1.3 PCR detection

Various methods of pathogen detection including isolation, visual examination and immunodetection, and their pros and cons, were discussed in Chapter 1. Such traditional methods are not always compatible with the industry requirements for a quick, reliable and economic assay and there is thus a need for alternative diagnostic methods. PCR (Saiki *et al.*, 1988) is one such method, and has been used in many applications (Erlich *et al.*, 1991) including the detection of plant pathogens (Henson and French, 1993; Hadidi *et al.*, 1995; Miller, 1996; Vandermark *et al.*, 2000) and has proved more sensitive than conventional methods. Detection tests based on PCR are sufficiently rapid, producing results in matter of hours. Moreover, such tests are reliable, sensitive, with a theoretical potential of detecting only a few molecules of target DNA, and are specific to the target species. The basis of PCR assays are the specific oligonucleotide primers, which initiate amplification of the target sequence in the species of interest. The design of species-specific primers is thus dependant on the availability of species-specific DNA sequences. The DNA sequence of target and non-target organisms must be compared, and primers designed around the mismatches, maintaining sufficient homology for efficient priming and amplification of the target DNA (Dieffenbach *et al.*, 1993). In addition, the ideal region of choice for PCR primer design should preferentially be present in multiple copies to enhance the sensitivity of the assay.

The DNA that codes for ribosomal RNA (rDNA) in fungi is present as a unit which is repeated in a tandem array. Each unit contains three rRNA genes which can be distinguished as 18S-like rRNA, 5.8S rRNA and 28S-like rRNA. The genes within a unit are separated by internal transcribed spacer (ITS) regions, whereas two units are separated by intergenic spacer regions (IGS) (Edel, 1998). The ITS regions of DNA satisfy the above criteria for the development of successful PCR detection assays and

have proved useful in previous studies (Moukhamedov *et al.*, 1994; Beck and Ligon, 1995; Goodwin *et al.*, 1995a; Sreenivasaprasad *et al.*, 1996). Such regions are an attractive choice for PCR primer design since they are multicopy. In fungal genomes for example, from 60-200 copies have been reported (Bruns *et al.*, 1991; Yao *et al.*, 1992), which will allow sufficient sensitivity for detection even in situations when DNA concentration is very low or where the pathogen DNA comprises a small fraction of the total DNA. In addition, the ITS region is conserved within a species, with a predictable mutation rate and highly conserved flanking sequences (Tooley *et al.*, 1997). The ITS region has been used for the detection and characterisation of a number of fungi including *Magnaporthe* (Bunting *et al.*, 1996), *Phialophora gregata* (Chen *et al.*, 1996), *Ustilago hordei* (Willits and Sherwood, 1999), *Pythium* (Chen, 1992; Levesque *et al.*, 1994), *Peronosclerospora* (Yao *et al.*, 1992), mycorrhizae and rusts (Gardes and Bruns, 1993), *Verticillium* (Nazar *et al.*, 1991), and *Fusarium* (O'Donnell, 1992).

To specifically identify *Phytophthora* spp., a number of molecular approaches have been used in the past. These include random genomic fragments (Goodwin *et al.*, 1989; Goodwin *et al.*, 1990), mitochondrial DNA (Förster *et al.*, 1988; Förster *et al.*, 1990; Carter *et al.*, 1990), rDNA (Lee and Taylor, 1992; Lee *et al.*, 1993; Trout *et al.*, 1997; Liew *et al.*, 1998; Ristaino *et al.*, 1998) and other targets (Ersek *et al.*, 1994; Niepold and Schober-Butin, 1995; Judelson and Messenger-Routh, 1996; Coelho *et al.*, 1997; Lacourt and Duncan, 1997; Schubert *et al.*, 1999). Due to the advantages of rDNA as a target region, the availability of a database of *Phytophthora* ITS sequences (Cooke *et al.*, 2000), and the successful demonstration of ITS-based detection of other *Phytophthora* species (Bonants *et al.*, 1997), this region was selected for the development of the *P. infestans* specific primers. The development of a sensitive, specific PCR assay for the detection of *P. infestans* in plant tissues and soil is described.

2.2 Materials and Methods

2.2.1 Fungal isolates, cultivars and soil.

DNA of various *Phytophthora* spp. was obtained from cultures held in the SCRI culture collection and DNA of various potato blemish pathogens was kindly provided by D. Cullen and A. Lees, SCRI. Potato cultivar Bintje was used for the *in planta* detection of *P. infestans*. Soil used in the experiments was obtained from an SCRI field previously used for barley and raspberry cultivation, sterilised at 121°C for 1 h, air dried on a greenhouse bench for two days and then sieved to remove debris and stones.

2.2.2 DNA extraction

Total genomic DNA was extracted from freeze dried mycelia using the commercially available Phytopure DNA extraction kit (Nucleon Biosciences) according to the manufacturers protocol. Ten mg of freeze dried mycelia were placed in an 1.5 ml Eppendorf tube which was plunged into liquid nitrogen and the mycelia finely ground with Treff homogenisers until a free flowing powder formed. To this, 600 μl of reagent I was added and after mixing thoroughly, 5 μl of RNAse A was added and samples were incubated at 37°C for 30 min. Next, 200 μl of reagent II was added to each sample and the tube was inverted several times until an homogenous mixture formed. The samples were then incubated at 65°C for 10 min with regular manual agitation and placed on ice for 20 min after which 0.5 ml of chloroform (-20°C) was added to each sample. Resin suspension (100 μl) was added to each sample and samples incubated at ambient temperature for 10 min with constant manual agitation. The samples were then centrifuged at 4000 rpm (MSE MicroCentur) for 10 min and the upper aqueous phase containing the DNA was transferred carefully to new Eppendorf tubes. DNA was precipitated by adding 450 μl of cold isopropanol. Tubes were gently inverted and centrifuged at 13000 rpm for 5 min in order to pellet the DNA. Pellets were washed with 70% ethanol and centrifuged again at 13000 rpm for 5 min. The supernatant was discarded and excess ethanol was pipetted out. Pellets, after drying in fume hood for 10 min, were re-suspended in 50 μl of autoclaved HPLC grade water. The integrity of the DNA was

checked by running 5 µl of the extracted DNA on an agarose gel stained with ethidium bromide and viewing on a UV transilluminator. DNA concentration was determined by ultraviolet absorbance at 260 nm and diluted to 10 ng/µl for use in PCR reactions.

2.2.3 Primer selection and PCR conditions

Multiple sequence alignments of internal transcribed spacer regions (ITS) of 47 *Phytophthora* species (listed in Table 2.1) were examined for regions unique to *P. infestans*. Following evaluation of criteria for PCR primer design, such as melting temperature (T_m), self complementarity and G+C content, the *P. infestans* specific primers, INF FW2 (5′-TGGGCGAGCCCTATCAAAA-3′) and INF REV (5′-CGATTCAAATGCCAAGCTAA-3′) were designed. PCR conditions, including primer and $MgCl_2$ concentrations, annealing temperature (52-58°C), time of annealing and extension steps, the number of PCR cycles, and the gel concentration were optimised to maximise the yield of the desired amplification product while minimising levels of non-specific products. PCR was carried out in 10 µl reactions containing 1x PCR buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl], 1 µg of Bovine Serum Albumin (BSA), 100 µM each of dNTPs, 1.5 mM $MgCl_2$, 1 µM of each primer and 0.4 U *Taq* DNA polymerase. PCR was carried out with a programme of 95°C for 2 min (initial denaturation) followed by 30 cycles of 95°C for 20 sec, 54°C for 25 sec, 72°C for 1 min, and a final extension of 72°C for 5 min (MWG Biotech Primus 25/96, Germany). PCR reactions were visualised by electrophoresis in 2.5% (w/v) agarose gels in 1x Tris-Borate-EDTA buffer (90 mM Tris–Borate and 2 mM EDTA) (Maniatis *et al.*, 1982). In some situations, nested PCR was also applied. Nested PCR, with first round primers (DC6 and ITS4) (Table 2.2) based in the 18S and 28S sub units of rDNA is an established method (Bonants *et al.*, 1997; Cooke *et al.*, 2000) for increasing detection sensitivity in situations where the target concentration is low or when inhibitors are present (e.g. soil). In this case a second round of PCR using primers internal to those of the first round is performed (Schesser *et al.*, 1991). An aliquot of the first amplification step, that amplifies DNA from all members of the order Peronosporales (Cooke *et al.*, 2000), was subsequently used in a second round PCR using the *P. infestans* specific nested primers INF FW2 and INF REV.

Table 2.1 Isolates of *Phytophthora* spp. used for multiple sequence alignment for the design of *P. infestans* specific primers. (Source: Cooke *et al.*, 2000).

Species	GenBank No	Host	Origin	Year
Phytophthora arecae	AF266781	*Cocus nucifera*	Indonesia	1991
Phytophthora botryosa	AF266784	*Hevea brasiliensis*	Malaysia	1966
Phytophthora cactorum	AF266772	*Rubus idaeus*	Wales	1985
Phytophthora cajani	AF266765	*Cajanus cajani*	India	-
Phytophthora cambivora	AF266763	*Rubus idaeus*	Scotland	1985
Phytophthora capsicae	AF266787	*Piper nigrum*	India	1989
Phytophthora cinnamomi	AF266764	*Syzygium aromiticum*	Malaysia	-
Phytophthora citricola	AF266784	*Rubus idaeus*	Ireland	1986
Phytophthora citrophthora	AF266785	*Actinidia chinensis*	Chile	1989
Phytophthora clandestina	AJ131989	*Trifolium subterranea*	Australia	1985
Phytophthora colocasiae	AF266786	*Colocasia esculenta*	Malaysia	1995
Phytophthora cryptogea	AF266796	*Lycopersicon esculentum*	New zealand	1951
Phytophthora drechsleri	AF266798	*Beta vulgaris*	USA	1935
Phytophthora erythroseptica	AF266797	*Solanum tuberosum*	USA	-
Phytophthora fragariae var. rubi	AF266761	*Rubus idaeus*	Sweden	-
Phytophthora fragariae var. fragariae.	AF266762	*Fragaria x ananassa*	Scotland	1979
Phytophthora gonapodyides	AF266793	*Salix matsudana*	UK	1972
Phytophthora gonapodyides	AF266791	*Salix matsudana*	UK	1972
Phytophthora heveae	AF266770	*Hevea brasiliensis*	Malaysia	1929
Phytophthora humicola	AF266792	Citrus orchard soil	Taiwan	1981
Phytophthora idaei	AF266773	*Rubus idaeus*	Scotland	1986
Phytophthora ilicis	AJ131990	*Ilex aquilifolium*	UK	-
Phytophthora infestans	AF266779	*Solanum tuberosum*	Netherlands	-
Phytophthora inflata	AF266789	*Syringa*	-	-
Phytophthora iranica	AJ131987	*Solanum melongena*	Iran	1969
Phytophthora katsurae	AF266771	*Cocus nucifera*	Ivory Coast	1993
Phytophthora lateralis	AF266804	*Chamaecyparis lawsoniana*	USA	1942
Phytophthora medicaginis	AF266799	*Medicago sativa*	Australia	1987
Phytophthora megakarya	AF266782	*Theobroma cacao*	Ghana	1990
Phytophthora megasperma	AF266794	*Malus sylvestris*	Australia	1968
Phytophthora megasperma	AF266795	*Asparagus officinalis*	-	-
Phytophthora melonis	AF266767	*Cucumis* sp.	China	1988
Phytophthora mirabilis	AF266777	*Mirabilis jalapa*	Mexico	-
Phytophthora multivesiculata	AF266790	*Cymbidium*	Netherlands	1995
Phytophthora nicotianae	AF266776	-	Australia	-
Phytophthora palmivora	AF266780	*Theobroma cacao*	Papua N Guin	1994
Phytophthora phaseoli	AF266778	*Phaseolus lunatus*	-	-
Phytophthora porri	AF266801	*Brassica chinensis*	Netherlands	1994
Phytophthora primulae	AF266802	*Primula acaulis*	Germany	1997
Phytophthora pseudotsugae	AF266774	*Pseudotsuga menziesii*	USA	-
Phytophthora quercina	AJ131986	*Quercus robur*	Germany	1995
Phytophthora sinensis	AF266768	*Cucumis sativa*	China	-
Phytophthora sojae	AF266769	*Glycine max*	Australia	1994
Phytophthora syringae	AF266803	*Rubus idaeus*	Scotland	1985
Phytophthora tentaculata	AF266775	*Chrysanthemum leucanth*	Germany	1992
Phytophthora trifolii	AF266800	*Trifolium*	-	-
Phytophthora vignae	AF266766	*Vigna sinensis*	Australia	1988

The specificity of the INF FW2 and INF REV primers was tested against DNA from a range of *Phytophthora* spp. and potato blemish pathogens. To ensure that the extracted DNA was of sufficiently high quality, genomic DNA of various *Phytophthora* species including, *Phytophthora erythroseptica*, *P. cactorum*, *P. cryptogea, P. megasperma*, *P. nicotianae*, *P. cambivora, P. gonapodyides*, and *P. citricola*, and that of potato blemish pathogens, including *Helminthosporium solani, Polyscytalum pustulans, Fusarium sulphureum*, and *Rhizoctonia solani* was first amplified with the non-specific primers ITS4 and ITS6 (Table 2.2) which amplify many fungal taxa. The DNA of three other blemish pathogens including *F. coeruleum, Phoma foveata* and *Colletotrichum coccodes* could not be amplified with the above primers so ITS6 was replaced by the very similar primer ITS5 (Table 2.2). To determine specificity of the *P. infestans* primers, PCR was conducted on the DNA of the above mentioned fungal pathogens using the primers INF FW2 and INF REV. To determine the sensitivity of the primers INF FW2 and INF REV, spectrophotometrically quantified DNA was serially diluted with HPLC water over ten orders of magnitude. Sensitivity of detection was then determined with single round and nested PCR.

Table 2.2 Primers used during detection assays of *P. infestans* from soil and plant tissue

Primer ID	Sequence	Length	Tm (°C)	%GC
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	20	55.3	45
ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	22	56.5	40.9
ITS6	5′-GAAGGTGAAGTCGTAACAAGG-3′	21	57.9	48
DC6	5′-GAGGGACTTTTGGGTAATCA-3′	20	55.3	45

2.2.4 Detection of *P. infestans* from plant tissue

2.2.4.1 Inoculum Production

Isolate 95.17.3.2 of *P. infestans* was maintained on Rye A agar, which was prepared by soaking 60 g Rye grains in DW for 36 h. The liquid was poured off in a flask and soaked grains were comminuted, heated for 3 h at 50°C in DW and filtered through muslin cloth. This filtrate was added to the original filtrate and amended with 20 g of sucrose and 15 g of agar. The media was autoclaved after adjusting the volume to one litre. Cultures were passaged twice through the leaves of cv. Bintje in a detached leaf assay, two weeks prior to inoculation (Hodgson and Grainger, 1964; Miller and Johnson, 2000). For inoculation, sporangia were harvested from the surface of the detached leaf in 10 ml of sterile distilled water. Sporangial concentration was adjusted to $2.5x10^4$ sporangia ml^{-1} with a haemocytometer. Potato plants (cv. Bintje) were grown in the glasshouse in 5 L pots filled with a mixture of soil, sand and peat in equal proportions.

2.2.4.2 Inoculation

The adaxial surface of fully expanded leaves of cv. Bintje was inoculated with several 100 μl droplets of a *P. infestans* sporangial suspension prepared as described above. For stem infection, plants of cv. Bintje were inoculated by a sub-epidermal injection of 500 μl of the inoculum with a hypodermic syringe. Inoculated plants were placed in a growth chamber under humid conditions for 24 h to promote infection. Plants were then removed from the chamber back onto glasshouse benches at 16°C until late blight symptoms developed.

Tubers of cv. Bintje were rinsed thoroughly in water for 2 min before inoculation. Using a sterile scalpel, a plug was removed from the surface of the tuber and 100 μl of inoculum ($2.5x10^4$ sporangia ml^{-1}) was placed in the hole. The plug was immediately replaced and sealed with Nescofilm. Inoculated tubers were wrapped in lens cleaning tissue (Whatman) and placed on a mesh nested in wet paper towels in Noble germinators which were incubated at 16°C until symptoms developed.

Tissue samples (c.10 mg) were taken from diseased, as well as healthy areas of infected stem, leaves and tubers. Samples from healthy stem, leaves and tubers served as additional negative controls. Tissues were processed for PCR according to the method of Wang *et al.* (1993) and modified for potato by the method of Ristaino *et al.* (1995) by macerating the samples with Treff homogenisers in 20 µl of 0.5 N NaOH. Samples were centrifuged at 13000 rpm for 5 min and diluted with 100 mM Tris (pH 8.0). One micro litre of this extract was added to a 10 µl PCR reaction mix.

2.2.4.3 Testing tuber washings and peel

In order to determine whether *P. infestans* could be detected from tuber washings, tubers were artificially inoculated as described previously. The outer surface of the tubers was washed with 30 ml of SDW, one week post inoculation. The washings were collected in 30 ml plastic specimen tubes, a 1 ml aliquot removed and centrifuged at 13000 rpm for 5 min prior to DNA extraction using the method of Cullen *et al.* (1999). One microlitre of the extracted DNA was used as a template in a nested PCR reaction. For detection of *P. infestans* in tuber peel, whole tubers were peeled using a hand-held potato peeler. Peel strips (1-2 mm thick) were used for sap expression using a roller press (Meuk, E. Pollähne, Wennigsen, Germany) and sap was collected in 2 ml Eppendorf tubes. DNA was extracted using the bead beating method (Cullen *et al.*, 1999) and 1 µl of the extract was used in 10 µl PCR reactions.

2.2.4.4 Testing commercial samples for latent infection

In two separate studies, batches of commercial seed stocks were screened for latent tuber infection by *P. infestans*.

In the first study, a total of 12, 100-tuber samples of cultivars Shepody, Russet Burbank and Maris Piper were tested. Ninety six tubers were selected from each sample and washed individually under tap water to remove excess soil before two peel strips were taken from each tuber from the rose to the heel ends using a hand-held potato peeler. A Pollähne press was used to express approximately 5 ml of sap from the individual tuber peelings into 10 ml glass vials. After shaking, a 2 ml aliquot was transferred to a 2 ml Eppendorf tube maintained on ice. Sap from each of

the 96 tubers was expressed and transferred to 96 Eppendorf tubes in this way. Between each tuber the Pollähne press was thoroughly washed with water, and between samples with 5% Chlorox followed by 70% ethanol and a rinse with water.

The samples were arranged in a twelve by eight block corresponding to a 96-well microtitre plate, and prior to DNA extraction the sap samples were pooled into eight samples along the columns of the grid. Each of the eight 600 μl samples thus comprised a 50 μl aliquot of sap from each of the 12 individual tubers combined in a single tube. DNA was extracted using the method of Cullen *et al.* (1999). In order to ascertain that the DNA from the expressed sap was amplifiable, amplification was first performed with ITS4 and ITS5 primers that amplify potato DNA. This was followed by a nested PCR, with primers DC6 and ITS4 in the first round and primers INF FW2 and INF REV in the second round.

To verify the results, a single stock was examined in more detail by blind 'spiking' one of the 96 samples with sap from an artificially infected tuber and re-testing each of the 12-tuber and 8-tuber pools. In this way the spiked sample should be identified by the coordinates where the two pools that test positive 'intersect' (Fig. 2.1).

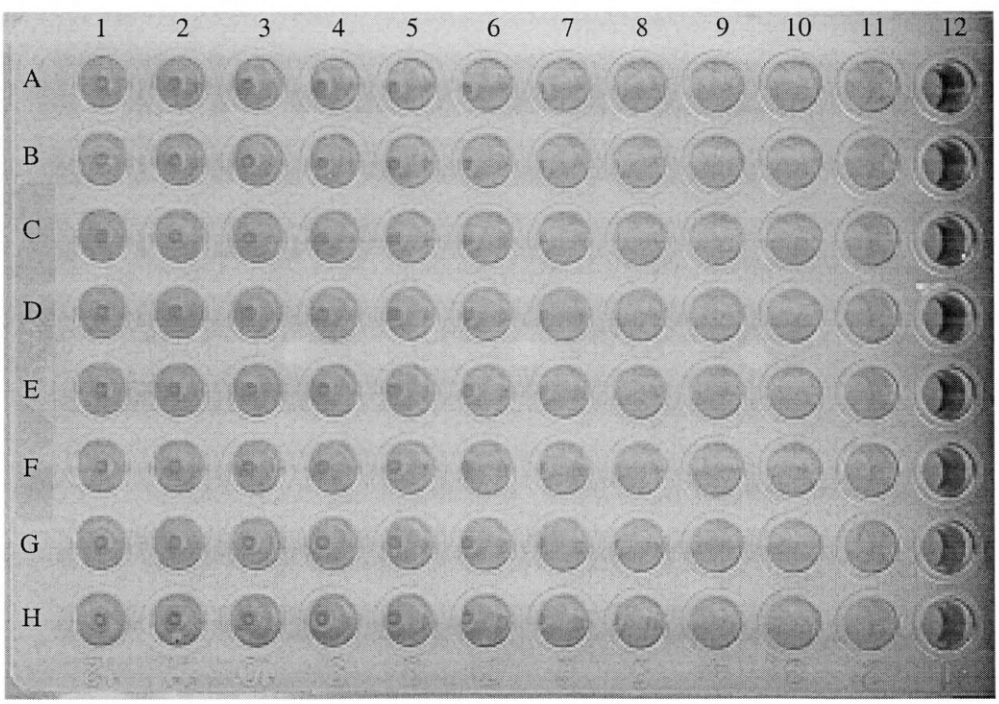

Fig. 2.1 A 96-well plate used to anonymously spike sap from an infected tuber into one of the wells. Pools of 8 and 12 tubers were tested across the plate to detect the spiked sample.

In addition to this, the sap from the single infected tuber was added to pools of increasing depth, i.e. to eight, twelve, sixteen, twenty or twenty four tuber pools and each tested as described previously.

A second consignment of commercial samples consisted of seven samples (100 tubers each) of cultivars King Edward, Shepody, Russet Burbank, and Maris Piper. From each sample, three sub-samples of ten tubers were drawn at random. Sap was obtained from each sub-sample as described previously, DNA was extracted and tested for *P. infestans*. Tuber washings in each case were also tested.

2.2.5 Detection of sporangia and zoospores

Fourteen day old cultures of *P. infestans*, grown on Rye A agar at 16°C, were flooded with SDW and scraped gently with a sterile spatula to dislodge sporangia. The sporangial suspension was filtered through four layers of muslin to remove mycelial fragments (Tooley and Fry, 1985). Between 1 and 15 sporangia were individually selected and added directly to the 10 µl PCR mix. Water from the sporangial suspension containing no sporangia served as an additional negative control. Sensitivity of detection was determined with nested PCR.

For zoospore detection, two week old cultures of *P. infestans*, grown on Rye A agar, were flooded with cold sterile distilled water. The resulting suspension was incubated at 4°C for three hours to promote zoospore release (Melhus, 1915). In order to keep sporangia and mycelia intact, no attempt was made to scrape them off the surface of the culture. Following incubation at 4°C, zoospore concentration was measured using an haemocytometer. A 1 µl aliquot of zoospore suspension (24 zoospores) and a dilution series of a 3 µl zoospore suspension (72 zoospores) were added directly to separate 10 µl reaction mixtures. Nested PCR was performed to determine the sensitivity of the detection assay to zoospores.

2.2.6 Oospore detection from artificially and naturally infested soil

Oospores of *P. infestans* were produced by transferring a 5 mm mycelial plug of each of the two mating types (A1 and A2) onto a Rye A agar plate approximately 25 mm apart and incubating at 16°C for two weeks. Oospores formed where the

colonies met and were harvested by adding 5 ml of chilled SDW to the plates and scraping with a spatula to remove the majority of the mycelium and sporangia. Strips of oospore-bearing agar were homogenised with a glass homogeniser in 25 ml of SDW to release the oospores from the media. The oospores were purified by digesting the remaining mycelia and sporangia with Novozyme @ 7 mg ml^{-1} (Novo Industri, Denmark). Novozyme solution was added to oospore suspension and incubated at 20°C for 24 h. Novozyme was removed by three successive washings of oospore suspension with SDW and low speed centrifugation (3000 rpm for 5 min). The oospores were re-suspended in 25 ml of SDW and stored in the dark at 4°C until further use.

Oospore DNA was extracted according to the method of Edwards *et al.* (1991). Between 1-14 oospores were picked individually by mouth suction with a hand-pulled glass pipette attached to a rubber hose under a binocular microscope and placed into separate sterile Eppendorf tubes along with 400 µl of Edward extraction buffer (200 mM Tris-HCl, 250 mM EDTA and 0.5% SDS). To this, a few grains of sterile sand were added and the mix homogenised with sterile Treff homogenisers and then centrifuged at 13000 rpm for 2 min. The supernatant was carefully transferred to another microfuge tube, precipitated with 400 µl of isopropanol and incubated at ambient temperature for 2 min. Samples were centrifuged at 13000 rpm for 5 min, the supernatant drained off and the pellet was washed with 70% ethanol. After another round of centrifugation at 13000 rpm for 5 min, the ethanol was pipetted off carefully and the pellet was dried for 5 min in a fume hood. The DNA pellet was eventually re-suspended in 50 µl of HPLC water. One micro litre of each DNA sample was amplified in a single round or nested PCR reaction.

Similarly, individual oospores (produced as described previously) were counted and between 9-40 oospores were added to separate 0.5 g soil samples in 2 ml screw-cap vials. Samples with no oospores served as negative controls. DNA was extracted from soil using a bead beating method, followed by purification through Sephadex G75 columns (Cullen *et al.*, 1999). For DNA extraction, 0.1 g of 1 mm diameter sterile glass beads were added to each vial along with 700 µl of extraction buffer (2% CTAB; 1.5 M NaCl, 0.2 M Tris, 25 mM EDTA, pH, 8.0) and subjected to bead beating at 5000 oscillations/min for 1 min in a mini bead-beater (BioSpec Products).

Tubes were then centrifuged at 13000 rpm for 5 min to pellet the soil debris, and approximately 750 μl of supernatant was transferred to a new microfuge tube. To this, 750 μl of chloroform was added and mixed for 1 min until a white emulsion was formed. The tubes were centrifuged again at 13000 rpm for 5 min and the aqueous phase (~750 μl) was transferred to a new microfuge tube and an equal volume (750 μl) of isopropanol added to precipitate the DNA. The Eppendorf tubes were allowed to incubate at room temperature for 20 min after which they were centrifuged at 13000 rpm for 5 min to pellet the DNA. The DNA pellet was washed with 70% ethanol and centrifuged at 13000 rpm for 5 min. DNA pellets were air dried under vacuum for 10 min and re-suspended in 50 μl of HPLC water. The DNA was later purified through Sephadex spin columns (Cullen and Hirsch, 1998).

A Sephadex suspension was prepared by suspending 5 g of Sephadex G75 (Fine grade, Sigma, U.K.) in 200 ml of distilled water and leaving for 25 min. The upper aqueous phase was pipetted off and another 100 ml of SDW was added to the suspension and left to stand for further 20 min. The upper aqueous phase was again pipetted off and the remaining Sephadex was suspended in 200 ml of TE buffer. After thorough mixing, the suspension was autoclaved at 115°C for 15 min.

Sephadex was used to prepare spin columns for DNA purification. Microbio spin columns (Axygen Scientific) were made by adding ~800 μl of Sephadex to the column placed in capless 1.5 ml microtubes using a 1 ml pipette with the end removed. The columns were centrifuged at 2000 rpm for 30 sec and the residual buffer, collected in the lower tube, was discarded. Columns were refilled with more Sephadex and centrifuged again (2000 rpm for 1 min) to remove excess buffer. The DNA sample was added to the column and centrifuged at 2000 rpm for 2 min and clean DNA was collected in a microfuge tube. Single round and nested PCR was performed as described above.

In order to test whether sample size affected the efficiency of detection, DNA extraction from larger soil samples was also tested. For each sample, 10 g of soil was placed in a 30 ml sterile plastic tube with 20 ml of extraction buffer. Samples were vortexed to break up the soil particles. Using a pipette tip with the end removed, 1 ml

of the resulting slurry was transferred to a 2 ml vial with 0.1 g glass beads (1 mm diameter). DNA was extracted as described previously and assayed with nested PCR.

The efficiency of primers was also tested in naturally infested soil. Soil samples (500 g) were obtained from two different gardens in Llandegai, Bangor, Wales, in which potatoes infected with both mating types of *P. infestans* had been cultivated for the past ten years. A total of 35 samples (0.5 g each) were selected at random from each 500 g sample, DNA was extracted and tested using nested PCR as described previously. Larger samples (10 g) were also assayed.

2.3 Results

2.3.1 Primer selection and optimisation of PCR conditions

Multiple sequences of the ITS region of 47 *Phytophthora* spp. were aligned in order to identify a region characteristic to *P. infestans*. Comparisons of ITS1 and ITS2 regions of these sequences revealed differences between *P. infestans* and other *Phytophthora* spp. and those regions showing the greatest dissimilarity were selected (Fig. 2.2) for the design of the putative *P. infestans* species-specific primers (Table 2.3). The primer set INF FW2 and INF REV gave the most consistent amplification of pure *P. infestans* DNA resulting in the production of a 613 bp product following optimisation of PCR conditions including annealing temperature and time, primer extension time, concentration of primers and Mg ion, number of PCR cycles and concentration of the gel. The sequence of the ITS region including the 5.8S rDNA and primer region is shown in Fig. 2.3. The PCR product contains parts of ITS1, ITS2 and whole of the 5.8S sub-unit (Fig. 2.4). Comparison of the primer sequences (Table 2.2) as queries with the NCBI non-redundant nucleotide database by using BLAST 2.2.2 (Altschul *et al.*, 1997) revealed that these aligned with sequences from *P. mirabilis* and *P. phaseoli*. Primer INF FW1 did not match with sequence from any other *Phytophthora* whereas INF FW2 matched with sequence from *P. syringae* and *P. multivesiculata*. Sequence from INF FW3 matched with several *Phytophthora* spp. including *P. nicotianae*, *P. pseudotsugae*, *P. idaei* and *P cactorum*. The reverse primer sequences did not match with any *Phytophthora* spp. apart from *P. mirabilis* and *P. phaseoli*. The primer set INF FW2 and INF REV was selected for further studies.

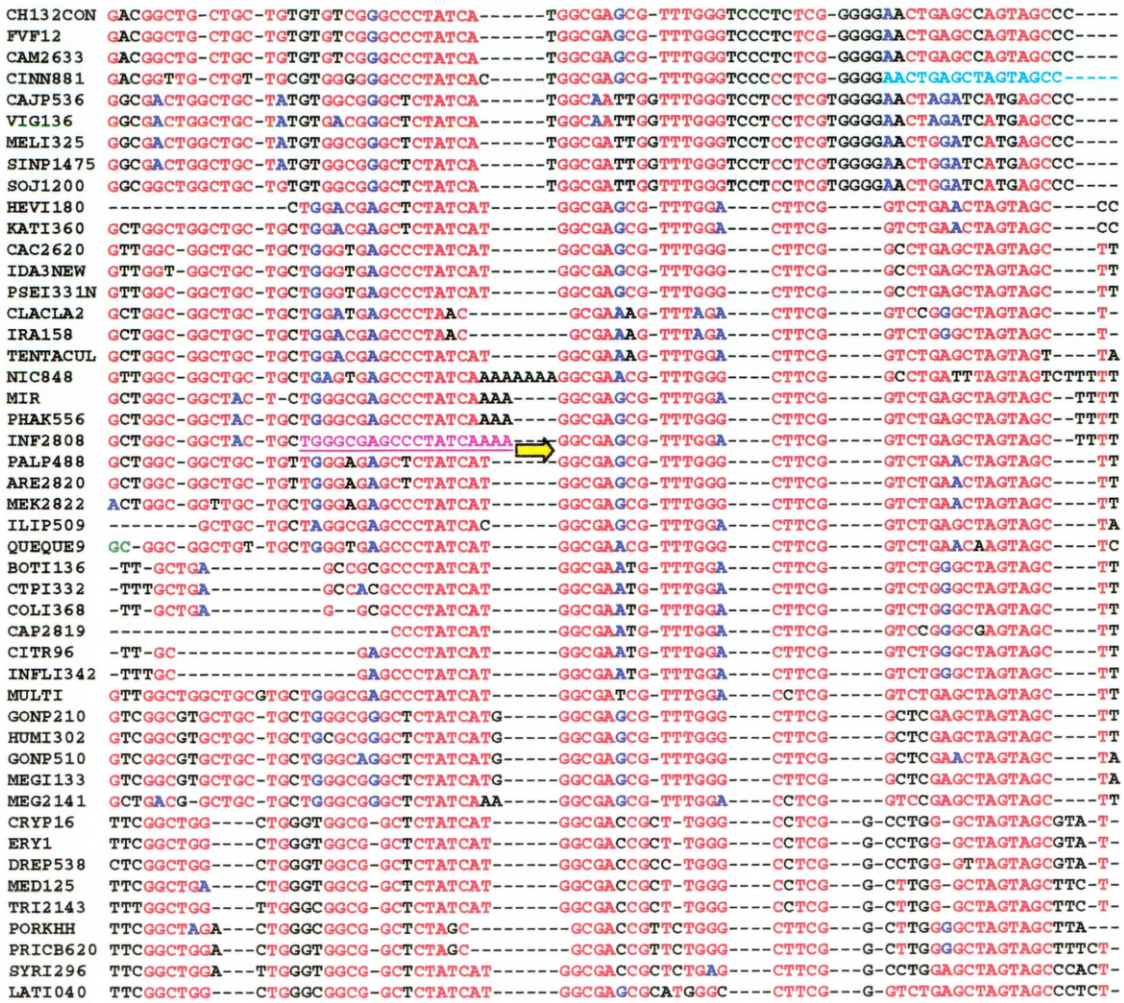

Fig. 2.2 Multiple sequence alignments of ITS regions of 47 *Phytophthora* species. Primer sequences are underlined. The 5′ to 3′ direction of the primers INF FW2 and INF REV is shown by arrows.

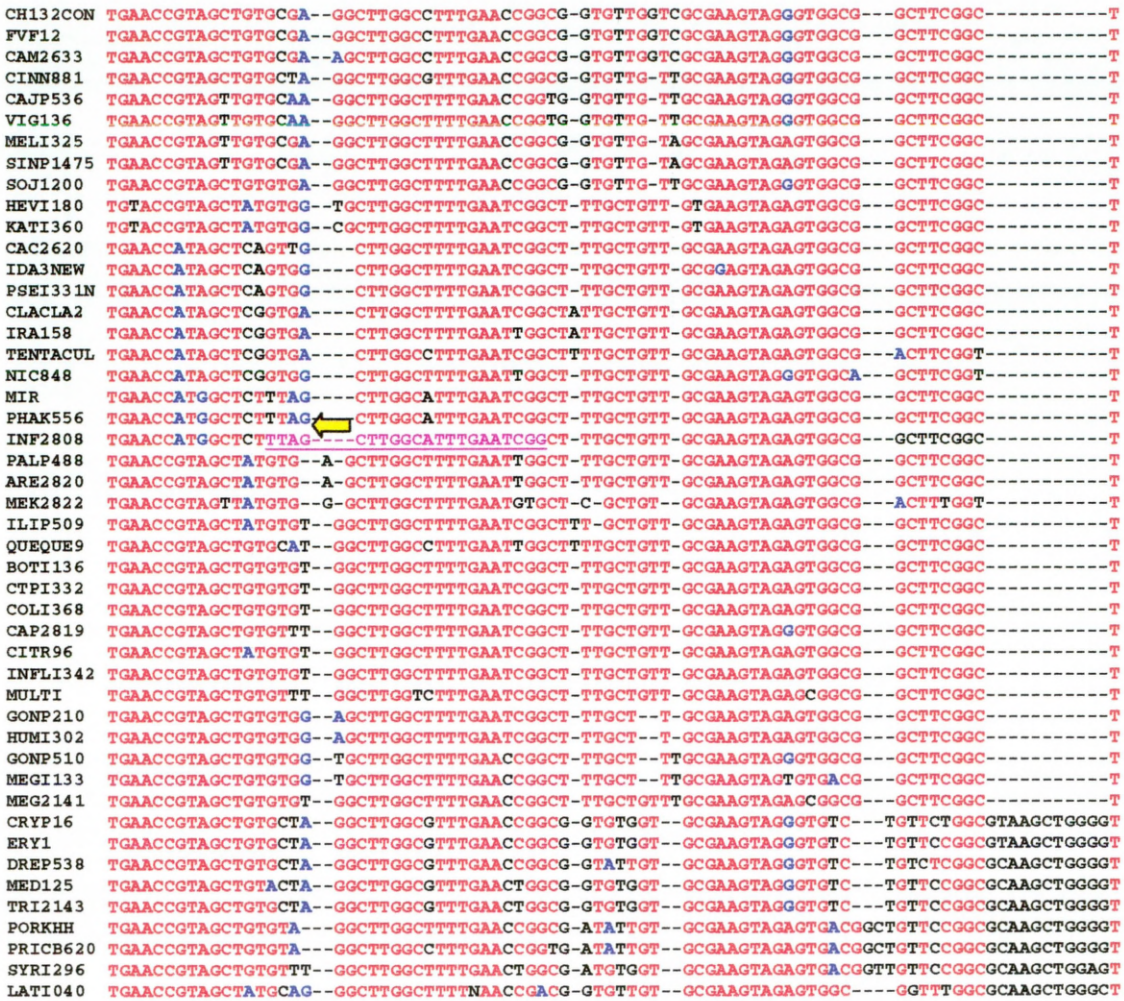

Fig. 2.2 (*Continued*)

***Phytophthora infestans* IMI66006**

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  1 CCACACCTAA AAACTTTCCA CGTGAACCGT TTCAACCCAA TAGTTGGGGG TCTTACTTGG
 61 CGGCGGCTGC TGGCTTTATT GCTGGCGGCT ACTGCTGGGC GAGCCCTATC AAAAGGCGAG
121 CGTTTGGACT TCGGTCTGAG CTAGTAGCTT TTTTATTTTA AACCCTTTAC TTAATACTGA
181 TTATACTGTG GGGACGAAAG TCTCTGCTTT TAACTAGATA GCAACTTTCA GCAGTGGATG
241 TCTAGGCTCG CACATCGATG AAGAACGCTG CGAACTGCGA TACGTAATGC GAATTGCAGG
301 ATTCAGTGAG TCATCGAAAT TTTGAACGCA TATTGCACTT CCGGGTTAGT CCTGGAAGTA
361 TGCCTGTATC AGTGTCCGTA CAACAAACTT GGCTTTCTCC CTTCCGTGTA GTCGGTGGAG
421 GAGATGCCAG ATGTGAAGTG TCTTGCGGTT GGTTTCCGGA CCGACTGCGA GTCCTTTGAA
481 ATGTACTAAA CTGTACTTCT CTTTGCTCCA AAAGTGGTGG CATTGCTGGT TGTGGACGCT
541 GCTATTGTAG CGAGTTGGCG ACCGGTTTGT CTGCTGCGGC GTTAATGGAG AAATGCTCGA
601 TTCGTGGTAT GGTTGCCTTC GGCTGAACAA TGCGCTTATT GGGTGATTTT CCTGCTGTGG
661 CGTGATGGAC TGGTGAACCA TGGCTCTTTA GCTTGGCATT TGAATCGGCT TTGCTGTTGC
721 GAAGTAGAGT GGCGGCTTCG GCTGCCGAGG GTCGATCCAT TTGGGAAATG TTGTGTACTT
781 CGGTATGCAT CTCAA
```

795 bases

INF FW2	**5′- TGGGCGAGCCCTATCAAAA -3′**
INF REV	**5′- CCGATTCAAATGCCAAGCTAA -3′**
Amplicon	**613 bp**

Fig. 2.3 Sequence of ITS region of *Phytophthora infestans* (IMI 66006). ITS1, 5.8S and ITS2 regions are shown in different colours and primers are underlined.

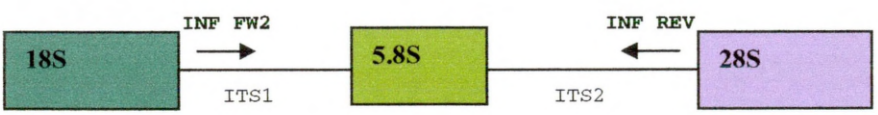


Fig. 2.4 The location of primers INF FW2 and INF REV on the ribosomal DNA gene repeat unit. The 5′ to 3′ direction of the primers is shown by arrows. The product contains ITS1, ITS2 and entire 5.8S sub-unit.

Table 2.3 Primers generated from ITS region of rDNA of *Phytophthora infestans.*

Primer ID	Sequence (5′-3′)	Length	Tm (°C)	%GC
INF FW1	GGACGCTGCTATTGTAGCG	19	58.8	57.9
INF FW2	TGGGCGAGCCCTATCAAAA	19	56.7	52.6
INF FW3	CCAATAGTTGGGGGTCTTACT	21	57.9	47.6
INF REV	CCGATTCAAATGCCAAGCTAA	21	55.9	42.9
INF REV1	CCGATTCAAATGCCAAGCTAAA	22	56.5	40.9
INF REV2	CCGATTCAAATGCCAAGCTAAAG	23	58.9	43.5
INF REV3	CGATTCAAATGCCAAGCTAAA	21	54	38.1
INF REV4	CGATTCAAATGCCAAGCTAAAG	22	56.5	40.9

2.3.2 Specificity and sensitivity of primers.

Primers INF FW2 and INF REV were tested for specificity by their ability to amplify purified DNA of other *Phytophthora* spp. and potato blemish pathogens. To ensure that the DNA was of a sufficiently high quality, total genomic DNA of nine isolates of various *Phytophthora* spp. and eight potato blemish pathogens was amplified using universal primers ITS4 and ITS6 and ITS4 and ITS5. These primer sets amplified a ribosomal DNA fragment of ~900 bp from *Phytophthora* spp. and fragments ranging from ~600 to ~1100 bp from potato blemish pathogens (Fig. 2.5). No PCR products were however amplified from genomic DNA of any of the above species except *P. infestans* for which a predicted band (~600 bp) was consistently amplified with primers INF FW2 and INF REV (Fig. 2.6). This indicates that the primers are species-specific and can be used to differentiate *P. infestans* from other *Phytophthora* spp. and blemish pathogens that could be found associated with the tissues or rhizosphere of a potato plant.

To determine the sensitivity limits of the primer set INF FW2 and INF REV, a 10 fold dilution series of purified total DNA ranging from 500 ng/μl to 0.05 fg/μl was prepared. One micro litre of each dilution was added to 10 μl of PCR reaction mixture containing primers INF FW2 and INF REV. The lowest concentration at which a detectable product amplified was a $1:10^6$ dilution of the DNA, corresponding to 0.5 pg of DNA. No products of any size were seen for dilutions of more than 1: 10^6 of pure *P. infestans* DNA (Fig. 2.7).

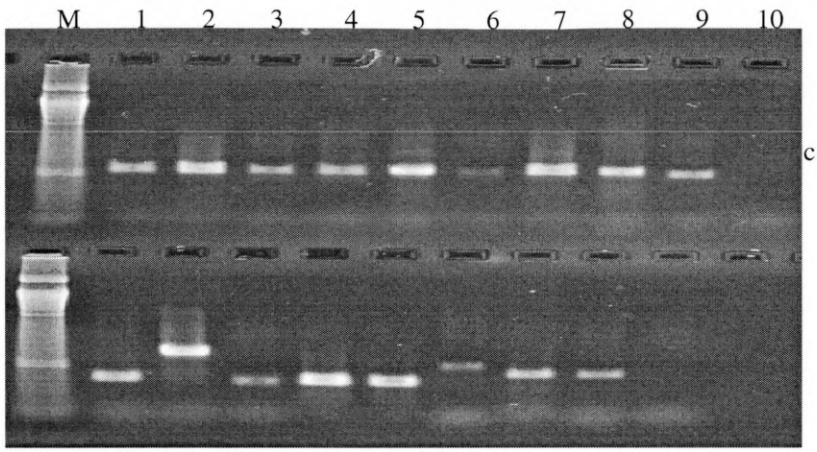


Fig. 2.5 PCR amplification with universal primers ITS4 and ITS6 (ITS5) of DNA from a range of *Phytophthora* spp. (top) and potato blemish pathogens (bottom). Lane 1, *P. infestans*; lane 2, *P. erythroseptica*; lane 3, *P. cactorum*; lane 4, *P. cryptogea*; lane 5, *P. megasperma*; lane 6, *P. nicotianae*; lane 7, *P. cambivora*; lane 8, *P. gonapodyides*; lane 9, *P. citricola* and lane 10, negative control (top); lane 1, *Helminthosporium solani*; lane 2, *Polyscytalum pustulans*; lane 3, *Fusarium sulphureum*; lane 4-5, *Fusarium coeruleum*; lane 6, *Phoma foveata*; lane 7, *Colletotrichum coccodes*; lane 8, *Rhizoctonia solani* and lane 9, negative control. Lane 10 is blank. M represents 100 bp size marker.

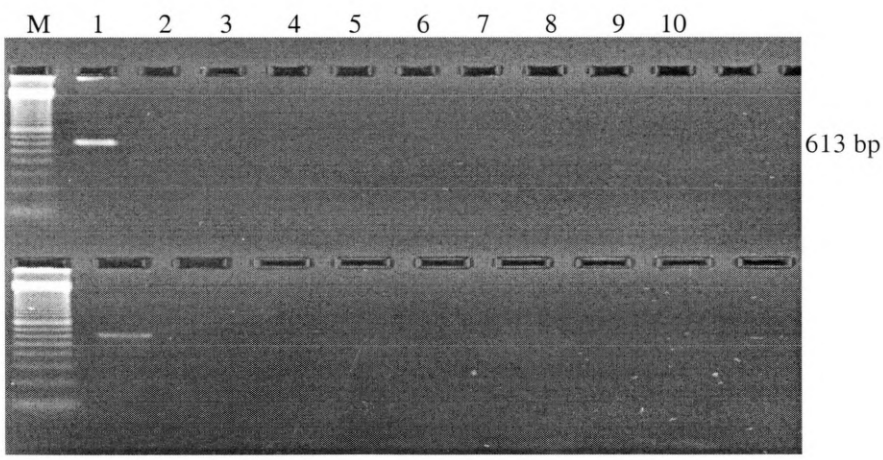


Fig. 2.6 PCR amplification with *Phytophthora infestans* specific primers INF FW2 and INF REV of DNA from a range of *Phytophthora* spp. (top) and potato blemish pathogens (bottom). Lane 1, *P. infestans*; lane 2, *P. erythroseptica*; lane 3, *P. cactorum*; lane 4, *P. cryptogea*; lane 5, *P. megasperma*; lane 6, *P. nicotianae*; lane 7, *P. cambivora*; lane 8, *P. gonapodyides*; lane 9, *P. citricola* and lane 10, negative control (top); lane 1, *P. infestans*; lane 2, *Helminthosporium solani*; lane 3, *Polyscytalum pustulans*; lane 4, *Fusarium sulphureum*; lane 5, *Fusarium coeruleum*; lane 6, *Phoma foveata*; lane 7, *Colletotrichum coccodes*; lane 8, *Rhizoctonia solani* and lane 9, negative control. M represents 100 bp size marker.

Nested PCR increased the sensitivity of detection by two fold ($1:10^8$) over a single round PCR resulting in detectable amplification product from as little as 5 fg of DNA (Fig. 2.8). When a 1:20 dilution of plant DNA was spiked with 1 μl of the dilution series of target DNA (500 ng/μl), amplification of the predicted c.600 bp band occurred at the $1:10^3$ dilution (500 pg) with a single round amplification and $1:10^6$ dilution (0.5 pg) with a nested PCR (Fig. 2.7-2.8).

2.3.3 Detection of *P. infestans* in plant tissue

To test whether the PCR assay could detect *P. infestans* in plant tissue, the late blight susceptible cultivar Bintje was inoculated with *P. infestans* and assayed one week post inoculation. The samples collected from pre-symptomatic and symptomatic areas of an infected leaf did not produce any visible product on ethidium bromide stained gel following a single round PCR amplification with primers INF FW2 and INF REV. Since single round PCR was not sufficiently sensitive to detect infection under these conditions, a nested PCR strategy, using primers ITS4 and DC6 in the first round and INF FW2 and INF REV in the second round, was tested. Total genomic DNA, extracted from plant tissue, was used as a template in the first round of PCR where a larger target sequence (~1300 bp) was amplified with the Peronosporales-specific primers, DC6 and ITS4. An aliquot of the first round product was used as a template in the second round and the predicted band (~600 bp) was then amplified with the nested *P. infestans* specific primers INF FW2 and INF REV. Amplification products produced from DNA extracted from symptomatic and symptomless plant tissue were of the same size (Fig. 2.9a).

Having tested the reproducibility of the PCR assay to detect *P. infestans* in leaves, the assay was used to detect infection in stem and tubers. Nested PCR reactions successfully detected the pathogen in tissues obtained from artificially inoculated stems and tubers (Fig. 2.9b, c). No PCR products were amplified from healthy potato tissue (lane 7, 8). All reactions were repeated at least twice and the results were found to be consistent.

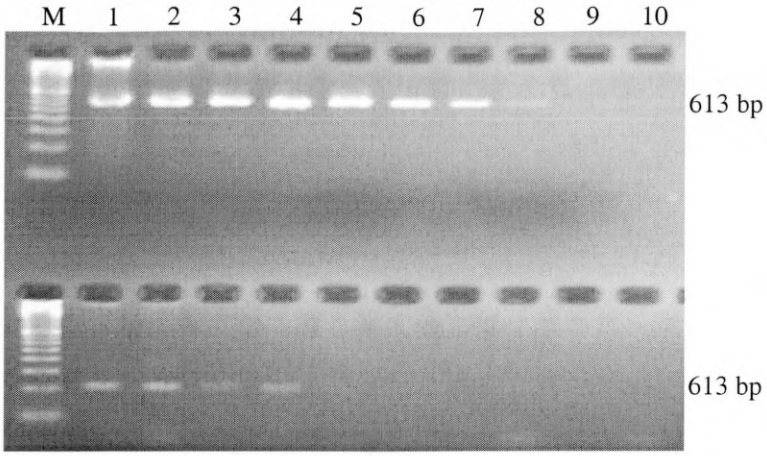


Fig. 2.7 Amplification of dilution series of *P. infestans* DNA (top) and *P. infestans* plus tuber DNA (bottom) tested with primers INF FW2 and INF REV in a single round PCR. Lane 1, undiluted (500 ng/μl); lane 2, 10^1; lane 3, 10^2; lane 4, 10^3; lane 5, 10^4; lane 6, 10^5; lane 7, 10^6; lane 8, 10^7; lane 9, 10^8; lane 10, negative control. M represents 100 bp size marker.

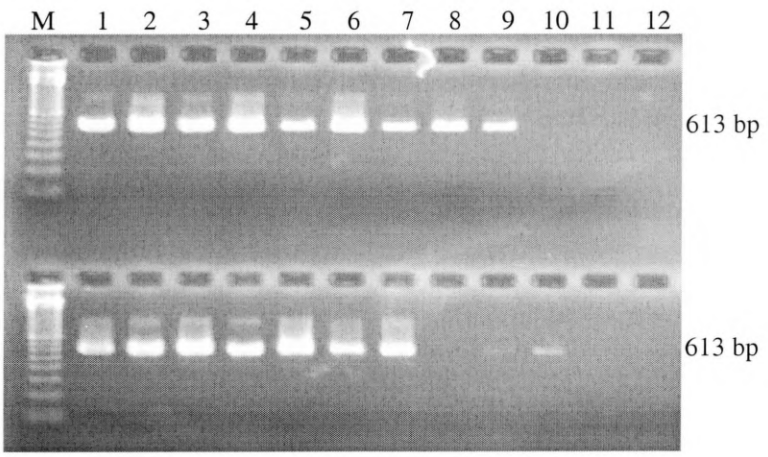


Fig. 2.8 Second round amplification of dilution series of *P. infestans* DNA (top) and *P. infestans* plus tuber DNA (bottom) tested with primers DC6 and ITS4 in the first round and INF FW2 and INF REV in the second round of a nested PCR reaction. Lane 1, undiluted (500 ng/μl); lane 2, 10^1; lane 3, 10^2; lane 4, 10^3; lane 5, 10^4; lane 6, 10^5; lane 7, 10^6; lane 8, 10^7; lane 9, 10^8; lane 10, 10^9; lane 11, 10^{10}; lane 12, negative control. M represents 100 bp size marker.

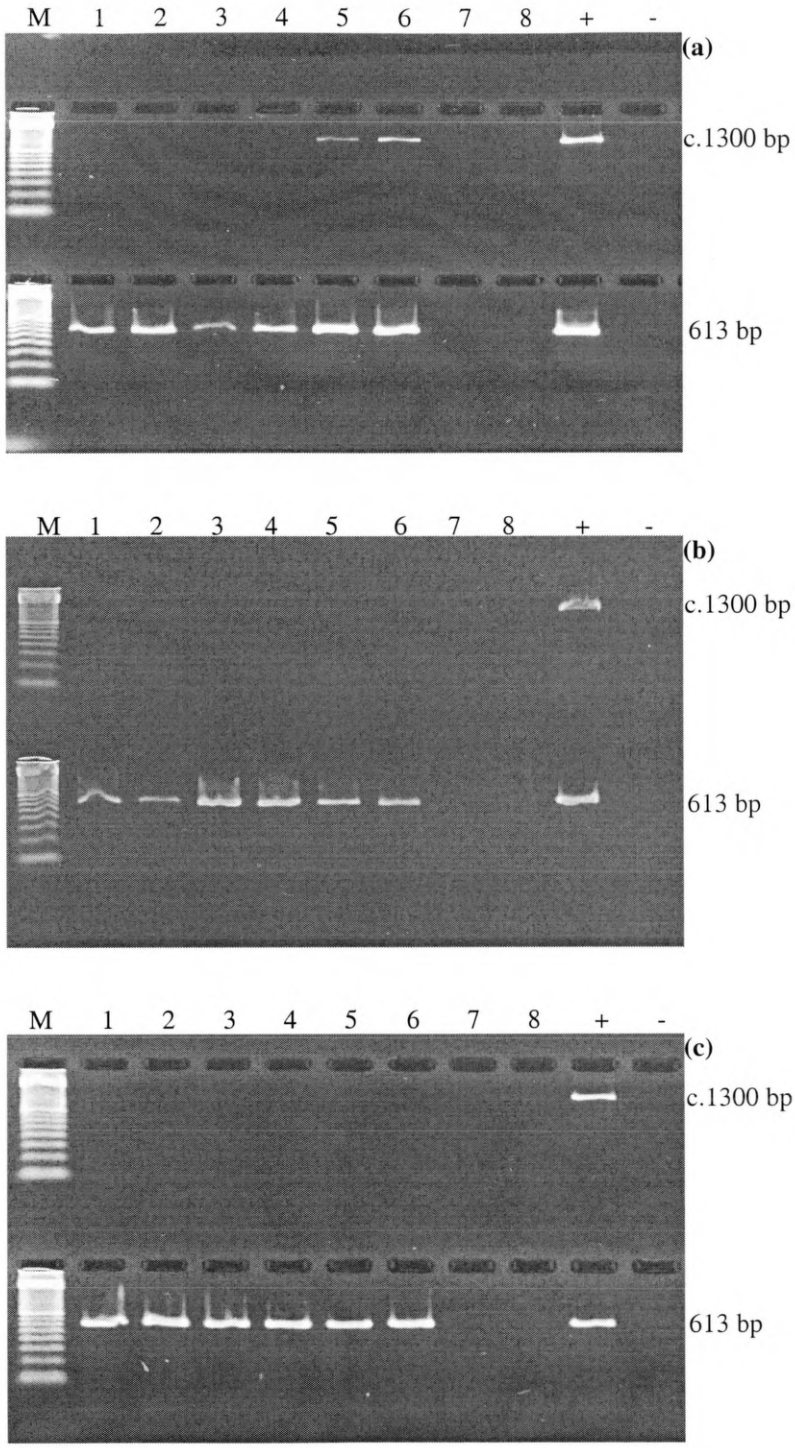


Fig. 2.9 PCR detection of *P. infestans* from potato a) leaves, b) stem, c) tubers after nested PCR with primers DC6 and ITS4 (First Round) (Top) and specific primers INF FW2 and INF REV (Second Round) (bottom). Lanes 1-2 represent samples taken from symptomless areas adjacent to infection; lane 3, sample taken from the centre of a sporulating lesion; lane 4, sample taken from the margin of a sporulating lesion; lane 5, sample taken from the centre of a non-sporulating lesion; lane 6, sample taken from the margin of a non-sporulating lesion; lanes 7-8 represent healthy samples. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

Nested PCR of tuber washings from artificially infected tubers consistently yielded the predicted amplification product. Similarly, sap extracted from peels of these artificially inoculated tubers tested positive.

2.3.4 Detection of *P. infestans* from commercial seed stocks

Primers INF FW2 and INF REV were used successfully to detect latent infection in potato tubers from two different commercial consignments. Before proceeding with the commercial samples, however, sap from an artificially infected tuber was mixed anonymously with the sap of one of the tubers in a batch of 96 tubers held in 12x8 microtitre plate. The infected sample was positively detected in a nested PCR reaction (Fig. 2.10). The results clearly demonstrated that nested PCR with the primer set INF FW2 and INF REV is a reliable technique to specifically detect tuber infection. Since DNA would not amplify due to over dilution as a result of pooling of the sap from tubers, it was necessary to determine the maximum number of tuber sap samples that could be pooled whilst retaining detection sensitivity of the assay. The maximum number of pooled tubers that gave positive PCR signals was found to be 24 (Fig. 2.10) which represents a 4-fold dilution of the infected sap over a pool of 8 tubers.

In each consignment, samples were first amplified with universal primers ITS4 and ITS5. The first consignment consisted of 12 samples (100 tubers each). A sub-sample consisted of DNA pooled from 8 tubers from each sample except 3 samples (A-C) where sap was pooled from 12 tubers. A total of 8 sub-samples were tested in samples A-C whereas 12 sub-samples were tested within each of the samples D-L. The ~700 bp band was produced from all the samples tested (Fig. 2.11). Since the primers can amplify all DNA of fungal and plant origin, this proved that the DNA extracted was of a sufficiently high quality and the absence of PCR signal in subsequent tests with the specific primers should not be interpreted as being due to inhibitors of PCR (i.e. false negatives).

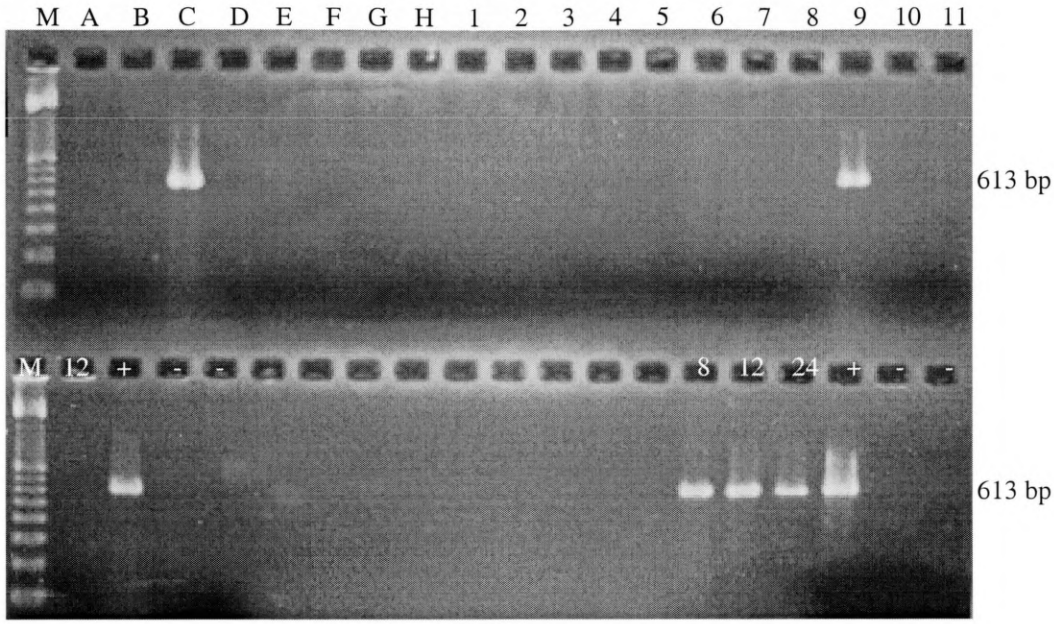


Fig. 2.10 Detection of *P. infestans* DNA from an anonymously inoculated well of a 12x8 microtitre plate with primers DC6 and ITS4 during the first round and INF FW2 and INF REV during the second round of a nested PCR reaction. The numbers above each lane represent the well number across lanes (A-H) and (1- 12) of a microtitre plate. The last six lanes (bottom) show the pooling of sap from various number of tubers and their detection with primers INF FW2 and INF REV after a first round nested PCR with primers DC6 and ITS4. The numbers above each lane represent the number of tubers pooled for DNA extraction and subsequent amplification. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

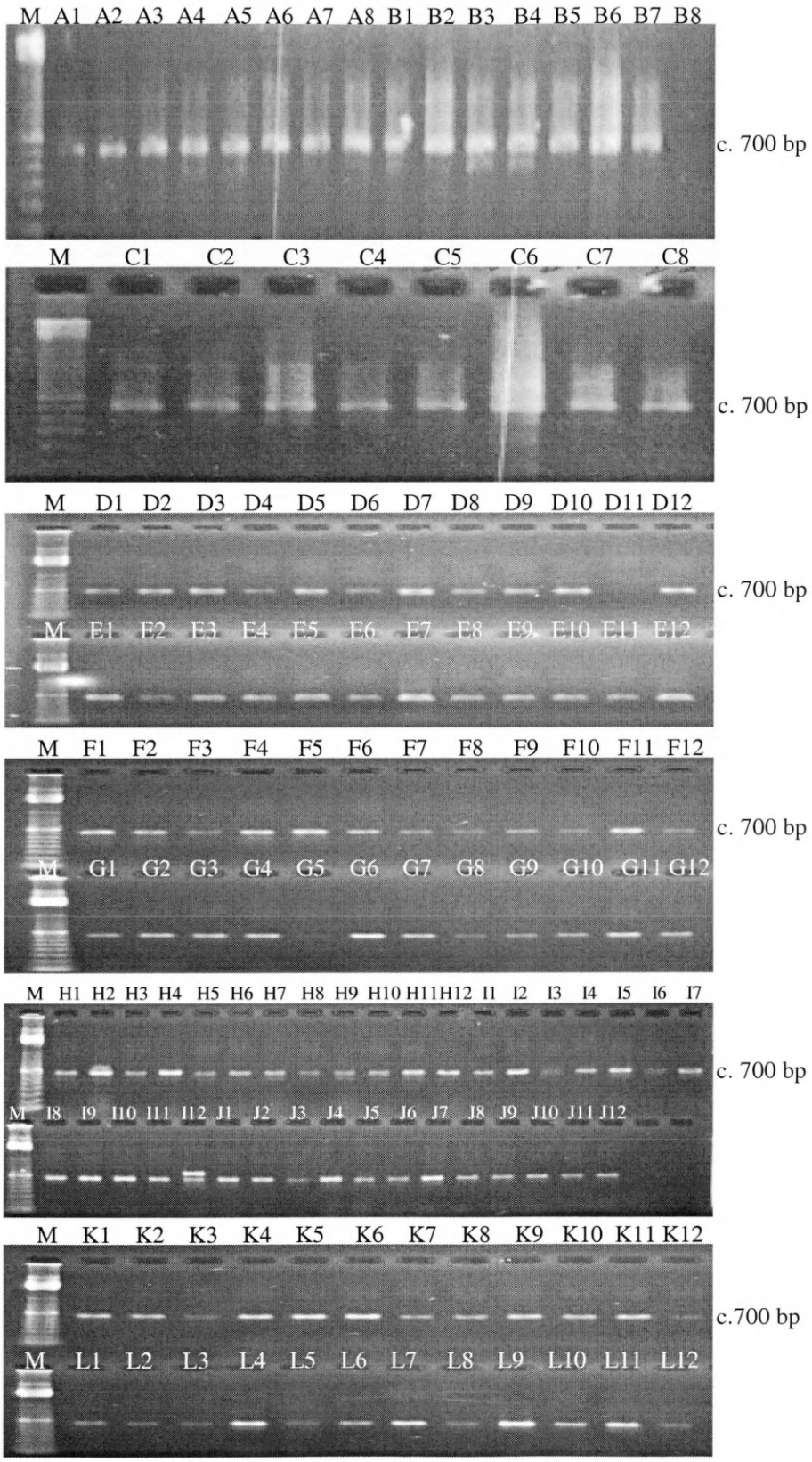


Fig. 2.11 PCR amplification of DNA, extracted from tuber peel extracts, with primers ITS4 and ITS5. DNA was extracted by the rapid NaOH method. The numbers on top of each lane represent the identity of each sample (A-L). Samples A-C were sub-divided into eight sub-samples each whereas samples D-L were sub-divided into 12 sub-samples each. M represents 100 bp size marker.

For samples D, E and L, (Table 2.4; Fig. 2.12), a single PCR fragment of the correct length was observed but positive signals varied in band intensity. The extracts of one sub-sample each from sample D (D10) and sample E (E7) produced intense bands whereas weaker bands were detected from one sub-sample of sample E (E12) and two sub-samples of the sample L (L6 and L7). The results suggest a level of infection of 1.04% in stock D and 2.08% in stocks E and L. The results further showed that two sub-samples considered to be uninfected by visual inspection during peeling, proved positive for the presence of *P. infestans* in the PCR test proving the method to be useful for seed health monitoring. None of the other samples tested in the pooled extracts produced bands after nested PCR. Assays performed on the second commercial consignment (Table 2.5) resulted in positive PCR signals from four (only one of which had a visible lesion) of the 21 sub-samples (Fig. 2.13). The level of infection in stocks B and C was 3% whereas 6% infection was estimated in stock E. None of the tuber washings produced predicted bands after nested PCR.

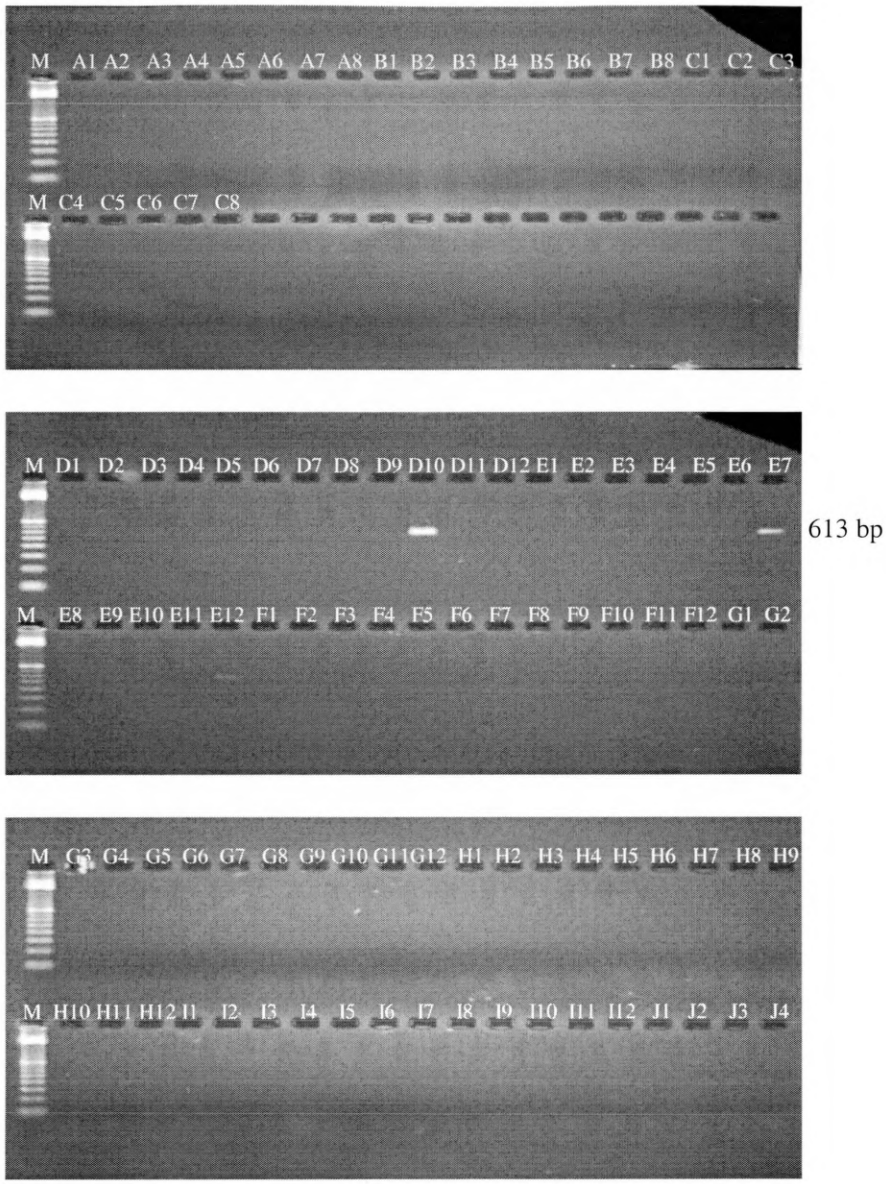


Fig. 2.12 PCR amplification of tuber DNA of samples A1-L12 using *P. infestans* specific primers INF FW2 and INF REV after first round amplification with universal primers DC6 and ITS4. Each tuber sample was divided into twelve sub-samples of eight tubers each except A1-C8 (see text). M represents 100 bp size marker. +/- represent positive and negative PCR controls.

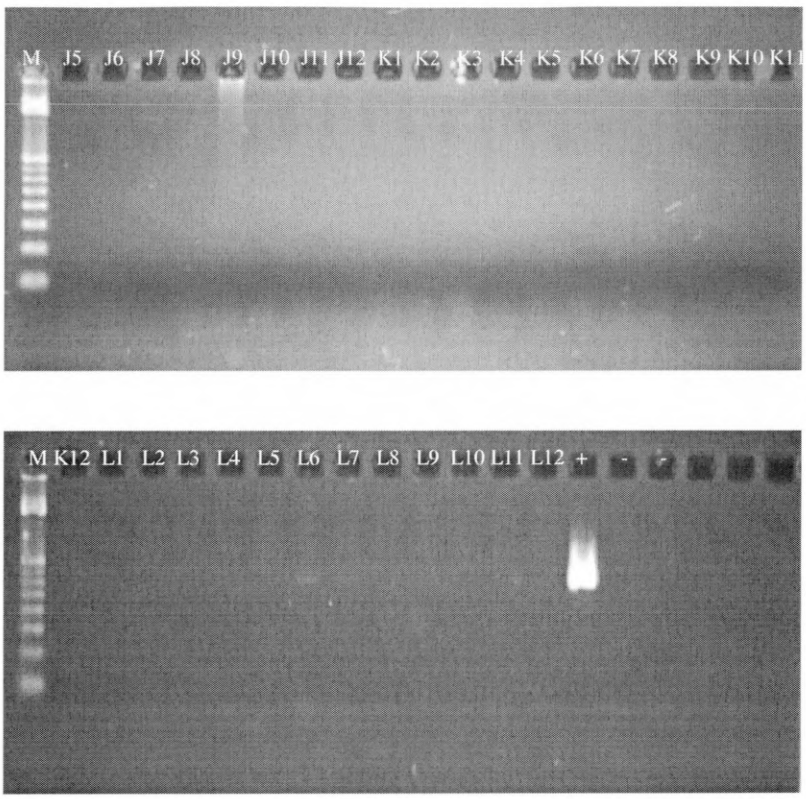


Fig. 2.12 (*Continued*)

Table 2.4 Number of positive and negative samples detected in *P. infestans* detection assays from tubers of various cultivars of 12 commercial seed stocks.

ID Code	Cultivar	Tubers tested	No +ve sub-samples
A	Shepody	96	0/8
B	Shepody	96	0/8
C	Shepody	96	0/8
D	Shepody	96	1/12
E	Shepody	96	2/12
F	Shepody	96	0/12
G	Russet Burbank	96	0/12
H	Shepody	96	0/12
I	Shepody	96	0/12
J	Maris Piper	96	0/12
K	Shepody	96	0/12
L	Shepody	96	2/12

In samples A-C, 8 sub-samples of 12 tubers each were tested
In samples D-L, 12 sub-samples of 8 tubers each were tested

Table 2.5 Number of positive and negative samples detected in *P. infestans* detection assays from commercial seed tubers of various cultivars.

I.D Code	Cultivar	No of sub-samples	Tubers tested	No of +ve sub-samples
A	King Edward	3	30	0/30
B	-	3	30	1/30
C	Shepody	3	30	1/30
D	King Edward	3	30	0/30
E	Russet Burbank	3	30	2/30
F	Maris Piper	3	30	0/30
G	King Edward	3	30	0/30

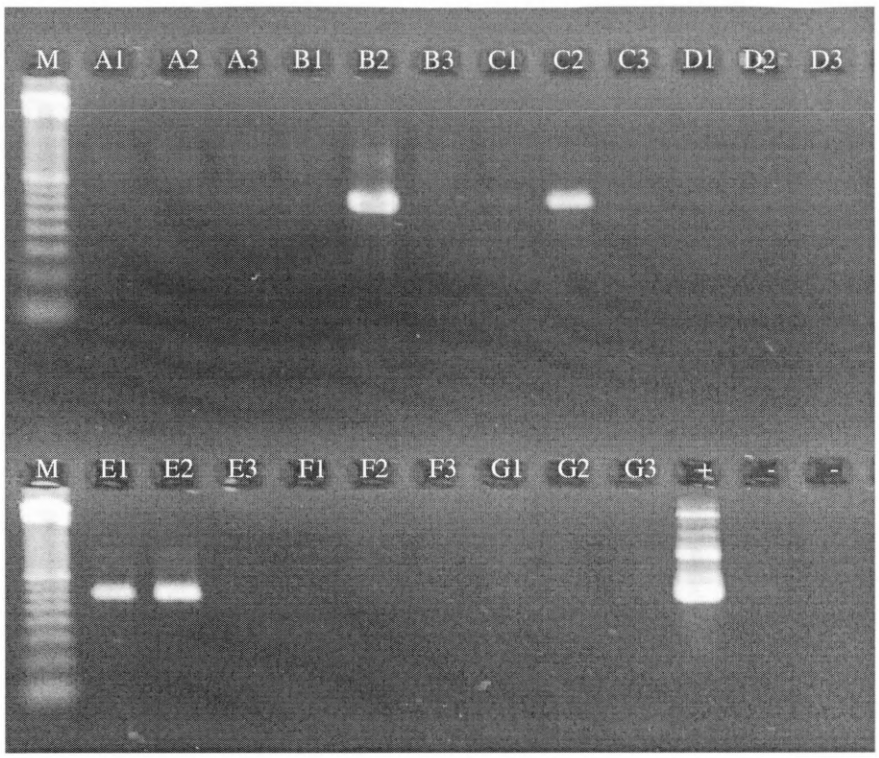


Fig. 2.13 Second round PCR amplification of *P. infestans* using primers INF FW2 and INF REV in seven commercial seed stocks (A-G). Each seed stock was sub-divided into three sub-samples (1-3) of ten tubers. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

2.3.5 Detection of *in vitro* produced sporangia, zoospores and oospores of *P. infestans*

Single round PCR was not sensitive enough to detect representative numbers (number added to each 10 μl reaction) of sporangia, zoospores and oospores of *P. infestans* i.e. no product was visible after agarose gel electrophoresis of the first round PCR products. However, using nested PCR, a visible product was obtained when as few as two sporangia were added directly to the first round PCR reaction. There was no amplification in the negative control containing water from the sporangial suspension (Fig. 2.14). Similarly, an amplification product was obtained in samples containing a 1:20 dilution zoospore suspension (corresponding to approximately four zoospores) (Fig. 2.15) using nested PCR and the intensity of the product detected was proportional to the number of zoospores added to the reaction. The assay also successfully detected oospores produced *in vitro*. The detection sensitivity of the nested PCR assay was two oospores (Fig. 2.16a).

2.3.6 Detection of inoculum of *P. infestans* in soil

To estimate the sensitivity of the detection assay in detecting oospores from soil, 0.5 g soil samples were artificially inoculated with a range (9-40) of oospores. A single round PCR with specific primers INF FW2 and INF REV detected as few as fifteen oospores in 0.5 g of soil, although weaker bands were still visible with as few as twelve oospores added (Fig. 2.16b). The efficiency of detection was improved to 10 oospores/0.5 g of soil using nested PCR (Fig. 2.16c). No PCR product was detected using DNA from nine or less oospores. Also no visible product was seen in larger (10 g) soil samples.

In order to determine the efficiency of the detection assay for monitoring the pathogen in field soil, samples were obtained from two garden sites in Wales. At one of the sites, 5 out of 35 samples (0.5 g) tested positive for *P. infestans* (Fig. 2.17). However, no visible product was detected when seven larger samples (10 g) from this site were tested. No amplification products were obtained in samples collected from the other site.

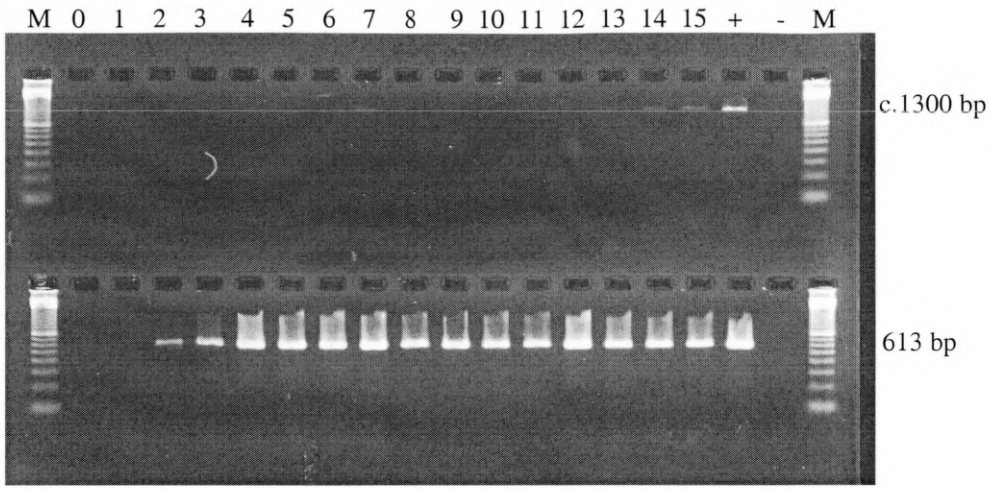


Fig. 2.14 Direct PCR detection of *P. infestans* sporangia after nested PCR with primers DC6 and ITS4 during the first round (Top) and specific primers INF FW2 and INF REV during the second round (Bottom). Sporangia were produced *in vitro* and the numbers tested in each reaction are shown above each lane. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

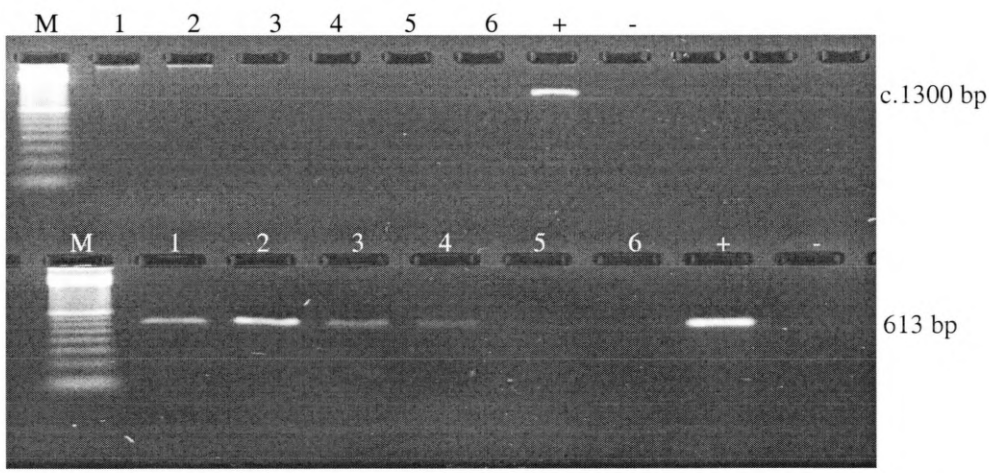


Fig. 2.15 Direct PCR detection of *P. infestans* zoospores, produced *in vitro,* after nested PCR with primers DC6 and ITS4 during the first round (Top) and specific primers INF FW2 and INF REV during the second round (Bottom). Lane 1, twenty four zoospores; lane 2, seventy two zoospores; lane 3, seven zoospores; lane 4, four zoospores; lane 5, two zoospores; lane 6, one zoospore. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

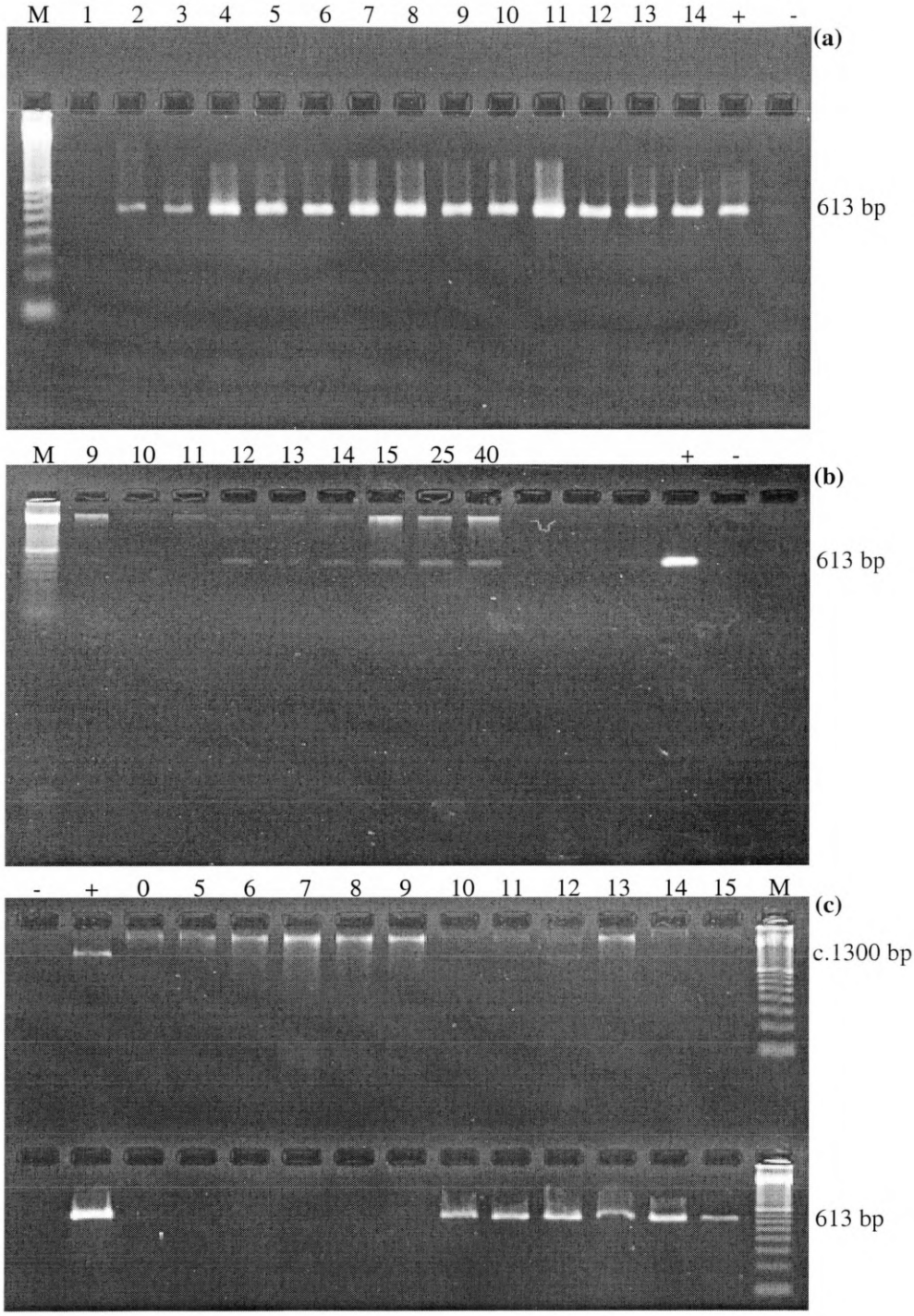


Fig. 2.16 Amplification from oospores of *P. infestans* a) produced *in vitro* and amplified in a nested reaction with primers DC6 and ITS4 in the first round and INF FW2 and INF REV in the second round b) from 0.5 g soil samples with a single round PCR with primers INF FW2 and INF REV c) from 0.5 g of soil with nested PCR. The number of oospores tested in each reaction are shown above each lane. M represents 100 bp size marker. +/- represent positive and negative PCR controls. The three lanes before positive control in b) are blank.

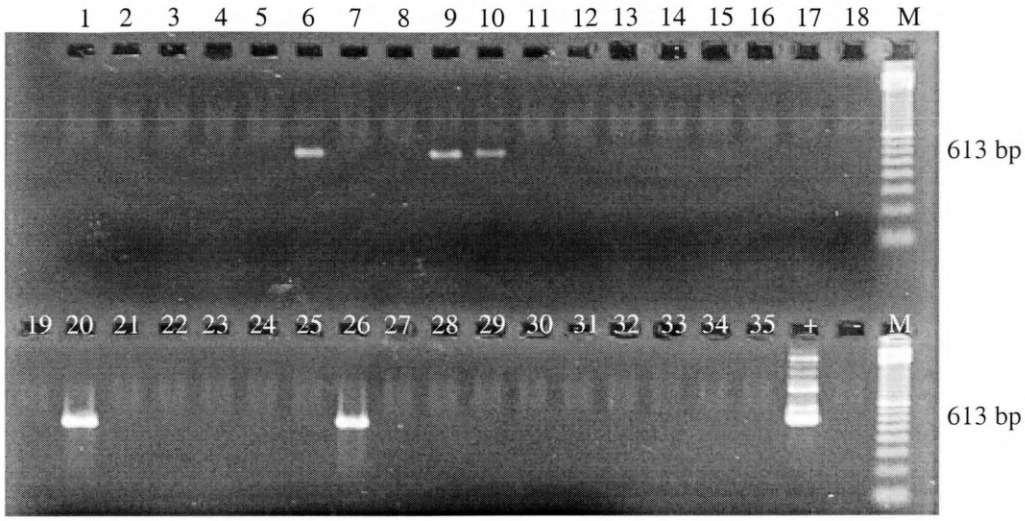


Fig. 2.17 Amplification of a second round PCR using *P. infestans* specific primers (INF FW2 and INF REV) from soil samples (1-35) obtained from a garden site in Wales. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

2.4 Discussion

The frequency and severity of potato late blight epidemics in recent years has increased due to spread of aggressive strains of *P. infestans* (Deahl *et al.*, 1995; Goodwin *et al.*, 1996; Goodwin *et al.*, 1998; Fraser *et al.*, 1999). An accurate and timely diagnosis of the presence of the pathogen is therefore necessary in order to prevent huge losses, reduce production costs and restrict the spread of the disease to uninfected areas. Until now, late blight has been detected by traditional methods such as visual examination of infected plant material, bait tests and use of selective media. However, these procedures are time consuming and expensive when used on a large scale, and are therefore not practical. Similarly, symptom based identification of late blight infected tubers is unreliable and is often confounded by similarity of symptoms with those caused by pink rot. In recent years, PCR has emerged as a powerful tool in plant disease diagnostics (Haff, 1993; Henson and French, 1993). PCR does not require the isolation of pure fungal cultures from infected tissue, and can be used on small samples such as single spores (Lee and Taylor, 1990) or for the detection of obligate parasites. In addition, PCR tests are more sensitive, accurate, robust and rapid, and less labour intensive and more economic than the conventional diagnostic methods (Tooley, 1997). However, the use of stringent operating conditions and strict controls to avoid contamination that could result in false positives, can be regarded as a disadvantage of PCR technology and should not be overlooked (James, 1999).

The use of the PCR assay for identification of *P. infestans* is a realistic approach that eliminates the need to rely on traditional methods and therefore shows potential for plant health monitoring. Molecular detection methods have been used effectively for many plant pathogens in the past (Schots *et al.*, 1994), and one of the objectives of the current study was to develop a PCR-based diagnostic test for *P. infestans* in soil and plant tissue. It is hoped that such a sensitive assay will facilitate disease risk assessments of naturally infected tubers and therefore disease management decisions.

For designing species-specific primers to *P. infestans*, rDNA sequences were selected over other sequences for a variety of reasons. First, rDNA sequences show variation between related taxa (Bachmann *et al.*, 1993; Elder and Turner, 1995) and

are therefore ideal regions of the genome to search for species-specific diagnostic sequences (Goodwin *et al.*, 1989; Hamer *et al.*, 1989; Judelson and Messenger-Routh, 1996). The ITS regions of the rDNA gene repeat unit were targeted since they have been reported to be less conserved than other regions (White *et al.*, 1990; Nazar *et al.*, 1991; O'Donnell, 1992; Xue *et al.*, 1992; Yourno, 1992; Johanson and Jeger, 1993). Secondly, rDNA is found in many copies in the genome. Lee and Taylor (1990) reported that if target sequence is present in multicopies, starting material between 0.1-1.0 ng of the total DNA will suffice for PCR amplification. Genes coding for rDNA in fungi are found on a single chromosome (Peters and Botstein, 1977) in repeated units arranged tandemly with 60-220 copies represented in the haploid genome (Russell *et al.*, 1984; Martin, 1990). Primers designed from such multicopy regions are therefore more sensitive compared with those designed from single copy DNA. Moreover, the availability of the ITS sequence database (Cooke *et al.*, 2000), facilitated the alignment of sequences of a wide range of *Phytophthora* spp. and selection of species-specific primers. Based on sequence variation and evaluation criteria, primers INF FW2 and INF REV were designed to specifically identify *P. infestans* in soil and plant tissue.

Primers INF FW2 and INF REV were designed from the ITS region of greatest dissimilarity in order to obtain maximum specificity of the primers. A guanine/cytosine (G/C) content of 45-55% has been reported to be good for specific binding and yet allows efficient melting during PCR (Sharrocks, 1994). The G/C content of the primers INF FW2 and INF REV was 52.6% and 42.9%, respectively. Other factors, including self-complementarity and annealing of primers to each other (thereby forming large quantities of primer-dimer), were also considered when designing the specific primers. Primer-dimer formation is undesirable since it affects efficient amplification and reduces yield of the desired product and in order to minimise primer-dimer formation, no complementarity between the 3′ ends of the primers should exist. Moreover, the primers should have similar annealing temperatures so that they can efficiently anneal to the target DNA during the annealing step of PCR. PCR conditions including concentration of primers, Mg ion, dNTPs, agarose gel and length time of primer extension were optimised to maximise the yield.

DNA from a range of *Phytophthora* spp. and other pathogens causing potato tuber blemish diseases was amplified with the *P. infestans* specific primers to ensure that there was no cross reaction. Amplification of *P. infestans* DNA only, at an annealing temperature of 54°C confirmed that the primers INF FW2 and INF REV were species-specific. Since DNA from the other fungi was previously amplified with ITS4 and ITS6 primers, the negative results obtained with these organisms were not the result of DNA degradation or the presence of PCR inhibitors (i.e. false negatives). Moreover, since the amplification resulted in strong PCR signals, this suggests that the assay would be useful in detecting low levels *of P. infestans* in plant tissue and soil. Previously, Tooley *et al.* (1997) reported the development of PCR primers that can distinguish *P. infestans*, *P. erythroseptica* and *P. nicotianae* in potato tubers. Since one of the major objectives of the present study was to detect *P. infestans* not only in plant tissue but also in soil, which contains numerous plant pathogens, the specificity of the primers INF FW2 and INF REV was tested against a wide range of *Phytophthora* spp. and other pathogens. This is therefore the first report of detection of *P. infestans* in soil and plant tissue.

The primers developed in current study, did however amplify DNA of *P. mirabilis* and *P. phaseoli*. Although *P. infestans*, *P. mirabilis* and *P. phaseoli* belong to the same taxonomic group (group IV) of the genus *Phytophthora* and evolved from the same common ancestor (Möller *et al.*, 1993; Tooley *et al.*, 1996), *P. phaseoli* is a pathogen of lima beans (Tooley *et al.*, 1997) whereas *P. mirabilis* has been reported from *Mirabilis jalapa* (Four o' clock plant) in Mexico (Galindo and Hohl, 1985). Since neither of these pathogens has been reported to occur in potato (Judelson and Tooley, 2000), the primers developed in this study can be used effectively to detect *P. infestans* in potato. The primers INF FW2 and INF REV amplified a range of *P. infestans* isolates representing Scottish and world-wide diversity, showing that the primers can amplify *P. infestans* DNA across *P. infestans* populations.

PCR conditions including annealing temperature, number of PCR cycles, Mg ion concentration, primer extension time and gel concentration were optimised in a series of experiments. Several factors including annealing temperature and Mg ion concentration have been reported to affect specificity of primers (Bej *et al.*, 1991;

Erlich *et al.*, 1991; Steffan and Atlas, 1991; Arnheim and Erlich, 1992; Bej and Mahbubani, 1992; Riedel *et al.*, 1992).

The assay described allows a detection limit of 0.5 pg of *P. infestans* DNA with single round PCR using 30 cycles of amplification. The assay was therefore more sensitive than those used by Tooley *et al.* (1997) who could detect between 1-10 pg of DNA, and Wangsomboondee and Ristaino (2002), whose assay could detect 10 pg of *P. infestans,* but was less sensitive than that developed by Judelson and Tooley (2000) who reported a detection efficiency of 10 fg of *P. infestans* DNA. The increased sensitivity of the primers developed by Judelson and Tooley could be due to the exceptionally high copy number (14000/nucleus) of the target sequence used for primer design (Judelson and Tooley, 2000). To increase the sensitivity of detection, nested or two-step PCR (Haqqi *et al.*, 1998) was performed by a first round amplification of the target sample DNA using universal primers DC6 and ITS4. A higher sensitivity of nested PCR has been reported by various workers (Tsuhima *et al.*, 1995; Coelho *et al.*, 1997). In addition to the increased sensitivity, increased specificity as a result of the use of nested primers has been reported by a number of workers (Allard *et al.*, 1992; Yourno, 1992; Henson *et al.*, 1993). The universal primers DC6 and ITS4 amplify DNA of all members of the order Peronosporales such as *Phytophthora*, *Pythium* and the downy mildews. A large target sequence (c.1300 bp) was amplified during this step of the PCR. An aliquot of the first round PCR product was then used in the second round with nested specific primers (INF FW2 and INF REV). These primers anneal inside the fragment amplified by the universal primers. A two fold increase in the sensitivity of amplification over single round PCR was achieved using nested PCR and the amount of *P. infestans* DNA detectable was 0.5 fg. Tooley and Therrien (1987) reported that 10-20 nuclei of *P. infestans* roughly contain 10 pg of DNA. Since the detection limits of the present assay are well below this level and the fact that heavily infected tissue will have more DNA, the assay can safely be used to detect *P. infestans* in a variety of substrates. Nested reactions are however more expensive since they need twice the amount of time and reagents. Further, there is an increased risk of contamination due to additional step of DNA handling. Despite this, the high sensitivity of primers coupled with nested PCR will be useful in detecting the target DNA in substrates where the target concentration is low or presence of PCR

inhibitors in the medium such as soil. Furthermore, all amplifications were performed in 10 μl reactions, thereby economising the use of reagents.

The nested PCR assay was used effectively to detect *P. infestans* in plant tissue. The infectivity of these samples was confirmed by isolating the pathogen on selective media. Control amplifications of uninoculated plant tissue were always included to exclude the possibility of false positive results through contamination. DNA used in this assay need not be highly purified in order to obtain amplification and was prepared using a simple NaOH lysis method (Wang *et al.*, 1993). The inclusion of a phenol/chloroform step during the DNA extraction process was therefore eliminated. Moreover, the method does not need many reagents and is less laborious and time consuming than a phenol/chloroform DNA extraction thereby reducing the possibility of contamination during DNA extraction. However, the DNA extracted by this method degrades progressively and is not suited for long term storage (Wangsomboondee and Ristaino, 2002). The amplification of DNA from non-symptomatic plant tissue such as leaves and stems suggests that the assay could be an effective method in early surveys of random leaf samplings and thus could provide a tool for diagnosing plants early in the growing season and therefore having an impact on the time of fungicide application. Moreover, the assay can also be used to monitor the movement of the pathogen within the plant, for example in examining the movement of the pathogen from infected seed into the growing stem. The nested PCR assay was used to screen commercial seed stocks thereby showing that the usefulness of this technique is not just limited to scientific investigations but that it can also be used to screen commercial seed tubers for cryptic infection as well as in epidemiological studies (Chapter 3). Such an assay could provide the basis for determining disease risk and establishment of guidelines for storage of tubers, therefore proving to be an invaluable tool for seed potato industry.

During the expression of sap from tubers for commercial testing, sap was placed on ice for as brief a period as possible, and then stored at -80°C until further use. It is possible that if samples are maintained at room temperature for longer periods of time, inhibitors co-extracted from plants such as humic acid (Kreader, 1996) might accumulate quickly in the sap inhibiting the PCR reaction. Previous reports suggest the effect of plant derived inhibitory compounds on PCR (Steffan and Atlas, 1991;

John, 1992; Tsai and Olson, 1992a; Tsai and Olson, 1992b) that could be a substantial problem with crude DNA extractions. Further, in order to overcome the effect of inhibitors in the extracted DNA, samples were diluted 1:20 fold before PCR.

In the studies described, pooling of sap from up to twenty four tubers allowed detection of *P. infestans* from commercial samples. The potential for further optimisation of sample size for PCR can still be exploited to increase the number of tubers that can be successfully pooled without compromising the sensitivity of the assay. The sampling has practical implications and such a rationalisation will economise time and money spent on the number of DNA extractions and the PCR reactions to be performed.

Tuber washings from commercial consignments were also tested by PCR to confirm whether positive results were due to cryptic infection of the stock or sporangial contamination of the tubers. The negative results obtained from the washings suggest that samples of tuber sap that tested positive were not due to sporangial contamination.

The amplification of DNA with primers ITS4 and ITS5 (White *et al.*, 1990), was used as a positive control to confirm that negative results were not due to DNA extraction failure. However, the occurrence of false negatives still cannot be ruled out due to random sampling error. On the contrary, contamination of PCR samples could result in false positives which can be a serious problem (Yap *et al.*, 1994) in commercial seed testing, and therefore every effort should be made to avoid false positives. In the present study, precautions including use of separate pipettes for loading and preparation of PCR reaction mix and cross linking of PCR master mix prior to dispensing in PCR tubes, were observed to prevent contamination by exogenous DNA, which could act as a highly efficient source of template. The use of commercially prepared PCR mixes or PCR-beads should also be considered. Further, failure of negative controls to amplify in the assay demonstrated that the PCR reagents were not contaminated with *P. infestans* DNA.

Using nested PCR, it was shown that amplification of pure *P. infestans* DNA occurred at a two fold lower concentration when the DNA was diluted in water than when diluted in sap from a healthy tuber. This corresponds to a detection sensitivity of 0.5 pg of *P. infestans* DNA from tubers compared to the detection sensitivity of 5 fg of pure *P. infestans* DNA. The results corroborate previous findings of the presence of PCR inhibitors in plant tissue (Atlas and Bej, 1990; Bej *et al.*, 1991; Bej and Mahbubani, 1992; Atlas and Bej, 1993; Wilson, 1997).

Overall, the level of infection never exceeded 6% in both stocks tested. In most cases, DNA amplification from tubers resulted in quite intense bands. However, rather weak PCR signals were observed in sample L from one of the commercial consignments. This could be because the level of infection was near the limit of detection of the primers for the sample size examined. Moreover, in some samples, which tested positive, no symptoms/signs of infection were visible while extracting sap from the peel. This reaffirms the fact that this assay can be used reliably to detect cryptic infection. The test can be performed in less than four hours including DNA extraction, which makes it a rapid diagnostic assay.

The present assay detected as few as two sporangia and approximately four zoospores *in vitro* using a nested PCR approach. Both these propagules were added directly as spore dilutions to the PCR master mix. This eliminated the need for DNA extraction thereby saving time and effort. Detection of single propagule type (oospores, sporangia and zoospores) has been reported by Wangsomboondee and Ristaino (2002). The reason for differences in the sensitivity limits for zoospores and sporangia could be attributed to the fact that those authors used DNA extracted from these propagules. In the present study, however, no attempt was made to extract DNA from these propagules. Detection of sporangia can be used to detect the pathogen in tuber washings and aerial spore traps and that of zoospores can be used to monitor the pathogen in irrigation water.

Attempts to amplify DNA of oospores directly without DNA extraction failed. The most likely reason for this failure could be the resistant oospore wall, which did not allow the release of oospore DNA. The results are in agreement with those of Wangsomboondee and Ristaino (2002). It was interesting to note that intact oospores

could still be seen in PCR products under a microscope after 30 cycles of amplification. Therefore, in subsequent studies DNA extractions of oospores were used. The assay can detect as few as two oospores extracted from a pure culture of *P. infestans*. Single propagule amplification could be applied in genetical studies under situations of difficult oospore germination, if the sensitivity level of this assay was improved to a single oospore level.

By contrast, the assay could detect no fewer than ten oospores/0.5 g of soil. The lower sensitivity of detection of oospores in soil than from pure culture could be due to the presence of soil inhibitors or small amount of target DNA in total volume of soil tested. One way of resolving this problem is by diluting the DNA or purifying it through spin columns. DNA extracted from soil in the present study was cleaned with Sephadex spin columns to remove inhibitors such as humic acid and phenolic compounds which are found in soil and have been reported to inhibit the action of *Taq* polymerase (Zhou *et al.*, 1996). The DNA extraction method used (Cullen *et al.*, 1999) also has a huge impact on PCR and subsequent amplification efficiency. In the present study, the DNA extraction method was probably not rigorous enough to extract DNA from soil containing less than ten oospores/0.5 g of soil. Wangsomboondee and Ristaino (2002) reported that DNA extraction method can affect quality and quantity of the extracted DNA, which in turn will affect the success of PCR. Nonetheless, the assay consistently detected suitably low number of oospores in soil, which could prove very helpful in identifying this source of inoculum. This will help growers to devise future control strategies particularly in areas where both mating types have been reported. It was interesting that both single round and nested PCR were able to detect oospores from soil, albeit at different levels of sensitivity, yet single round PCR failed to detect DNA of oospores extracted from culture. It is possible that different methods of DNA extraction used could have accounted for such results (Wangsomboondee and Ristaino, 2002). An additional positive control from artificially infested soil was included in the assay to exclude the possibility of DNA extraction failure.

Two soil samples from garden sites, where both mating types had previously been detected, were also evaluated for the presence of soilborne inoculum of *P. infestans*. A total of 35 sub-samples were tested from each sample. Five positive PCR signals

were detected in one of the samples. No PCR signals were detected from the sub-samples taken from the second site, which could be due to random sampling variation. It is possible that the size of the soil sub-samples (500 mg) might have been too small to detect the oospores with a patchy distribution in the field. However, use of larger soil samples has the risk of over dilution of the inoculum. This was evident when no PCR signals were detected in larger samples of artificially infested soil. Several factors still need to be optimised to establish maximum sensitivity and reliability of the assay including development of efficient sampling techniques, sample size, and refinement of the protocol for removal of inhibitors such as humic acid and phenolic compounds (Zhou *et al.*, 1996). Also different DNA extraction methods may be required to extract DNA from different types of soils (Wallenhammar and Arwidsson, 2001) and the possibility of false positives resulting from non-viable oospores cannot be ruled out. However, this can be overcome by performing PCR assay in conjunction with soil baiting assay.

The survival of *P. infestans* inoculum in soil and plant tissue

3.1 Introduction

3.1.1 Oospores: a possible source of inoculum

In the period between 1845 and 1980, the late blight fungus, *Phytophthora infestans*, is believed to have undergone only asexual reproduction outside its centre of origin and diversity (Goodwin *et al.*, 1994b). Despite the dramatic reduction in population size at the end of each growing season (annual bottlenecks) that the A1 mating type of *P. infestans* underwent, it managed to survive in infected seed tubers from one season to the next. *P. infestans* also coped successfully with a range of selection pressures such as host resistance and fungicides in the process (Davidse *et al.*, 1989; Wastie, 1991). In the absence of a sexual stage, the fungus was able to create variability through various means, including migration (Fry *et al.*, 1992: Fry *et al.*, 1993) as well as mutation and heterokaryosis (Le Grand-Pernot, 1986). Although these mechanisms are important, the role of sexual stage, and hence that of oospores, in adaptation and evolution cannot be underestimated (Fry *et al.*, 1989).

The recent world-wide occurrence of both A1 and A2 mating types of *P. infestans* (Spielman *et al.*, 1991; Fry *et al.*, 1993; Fry and Goodwin, 1997a), has increased the possibility of sexual reproduction (Fry *et al.*, 1992; Drenth *et al.*, 1994; Sujkowski *et al.*, 1994; Brown, 1996). The possible role of sexual recombination in influencing the composition and evolution of *P. infestans* has been investigated by various workers. Recent data suggest a high level of variability among *P. infestans* populations in The Netherlands. Different RFLP patterns and distribution of mating types in different areas of The Netherlands suggest the possible role of sexual recombination in influencing late blight epidemics and population structure (Drenth *et al.*, 1993a). Using molecular markers, scientists in Poland (Sujkowski *et al.*, 1994) and the United States (Goodwin *et al.*, 1995b), have detected genotype diversity and gene flow, which further emphasize this point.

Besides generating novel genotypes and hence a genetically more variable population that is more aggressive (Fry and Goodwin, 1997a), sexual recombination will invariably result in the production of oospores which may act as an additional source of inoculum. In most cases, primary inoculum sources consist of infected tubers planted in the spring (Robertson, 1991), volunteer plants surviving the winter in the field (Drenth *et al.*, 1995) and discarded tubers in cull piles (Boyd, 1974). However, production of oospores may create additional opportunities for the fungus to survive and could eliminate the need for a green bridge between successive potato crops. In addition, oospores surviving in the soil could act as primary inoculum sources, resulting in earlier epidemics. Such evidence of soil serving as source of inoculum exists (Andersson *et al.*, 1998) and Zwankhuizen *et al.* (2000) presented genetic evidence suggesting that oospores can be a potential source of inoculum.

Oospores have been reported in tubers (Grinberger *et al.*, 1989), leaves, stems and stolons (Andersson *et al.*, 1998; Strömberg *et al.*, 1999), although they are most abundant in the stems of blighted plants. This could be due to the fact that stems remain intact longer, and thus provide better chance for oospore survival than leaves, on which the time between infection and extended necrosis is insufficient to support oospore formation (Andrivon, 1995; Flier *et al.*, 2001a). Similarly, tubers do not support much oospore production because of their lower free water content (Levin *et al.*, 2001).

It is not clear how oospores in soil infect the foliage but it has been suggested that the infection court may be on the soil surface (Pittis and Shattock, 1994; Drenth *et al.*, 1995). Control of this phase is thus difficult, since currently used protectant fungicides are applied to the top of the plant canopy. Growers are being advised to spray earlier in the season to control the soilborne inoculum, but the benefits of such a practice have yet to be demonstrated. Furthermore, oospores produced in tubers could result in dissemination of both mating types in planting material, a classic case of the sexual cycle conferring both epidemiological and genetic advantage (Duncan *et al.*, 1998).

3.1.2 Factors affecting oospore production

Oospore production is affected by a variety of biotic and environmental factors. The foremost of these is a constant supply of free moisture to blight lesions in the host tissue. It has been shown that oosporogenesis requires that host tissue should be wet for one week or more. Parts of leaves having no direct contact with water (Cohen *et al.*, 1997) or infected leaves incubated at 50-80% RH, fail to produce oospores (Drenth, 1994). Similarly, lower leaves, which are in contact with the soil surface, are not only older, but wetter, compared to those in the mid canopy, and can support more oospore formation (Cohen *et al.*, 2000). Therefore, areas with prolonged rainy periods are at a high risk of oospore production. The question of oospore production under interrupted rainy periods still remains unanswered.

Although temperatures ranging from 8-23°C favour oospore production, the optimum temperature ranges between 8-10°C (Cohen *et al.*, 1997). The anomaly between optimum temperature for oospore production (8-10°C) and disease development (15-20°C) is probably due to the fact that lower temperature delays development of lesions, thereby allowing more time for oospore formation (Drenth, 1994). Compatibility of the *P. infestans* parental isolates is also regarded as crucial in oospore production and viability (Pittis and Shattock, 1994). Information concerning the relative compatibility of isolates exists (Erwin and Ribeiro, 1996), and Medina and Platt (1999) showed that oospore germination and infectivity varied between different mating combinations. Al-Kherb *et al.* (1995) reported that ten out of 31 different matings between A1 and A2 isolates from different countries produced oospores that germinated within six weeks of plating. Oospore germination varied from 32 to 90%. A cross between isolates Ca 65 (California) and E13a (Egypt) produced oospores with the highest germination percentage. Recent work by Flier *et al.* (2001a) suggests that oospore yield and viability are related, since matings yielding low numbers of oospores also showed reduced levels of oospore viability. These observations suggest the presence of a lethal factor or sexual incompatibility, thereby resulting in aborted or non-viable oospores (Erwin and Ribeiro, 1996).

The ratio of A1 and A2 mating type sporangia does not appear to affect oospore formation. Cohen *et al.* (1997) showed that ratios of 1:19 of A1 and A2 sporangia or *vice versa* resulted in oospore production. Thus the low frequencies of A2, currently detected in many countries, e.g. 20% in Europe (Gisi and Cohen, 1996) may be sufficient to allow sexual recombination to occur. The hormonal induction of oospores, reported *in vitro* (Ko, 1978; Ko, 1980), also seems to work *in vivo*, and results in the formation of oospores between discrete A1 and A2 lesions produced on the same leaf in tomato. Light has minimal effect on oospore production as oospores are produced both under light and dark conditions.

3.1.3 Oospore germination and infection of the host

Despite many studies (Ribeiro, 1983), factors affecting oospore germination are poorly understood. Oospore germination in the laboratory is cumbersome, requiring pre-treatment with chemicals, enzymes, digestion by snails (Andrivon, 1995) or other factors (Chang and Ko, 1991). Also there is little information regarding the infection of plants by the oospores in the field. The most likely event is the germination of oospores in response to high rainfall, and the emergence of motile zoospores from the germ sporangium moving to the surface of the soil and infecting leaves and stems which are in contact with the soil surface. The likelihood of infection of leaves in the upper canopy as a result of splash dispersal during rainfall as well as overhead sprinkling irrigation cannot be ruled out (Drenth *et al.*, 1995). Further, it is very rare to have 100% oospore germination in *Phytophthora* spp. and germination rates remain unpredictable ranging between 10-15% (Ribeiro, 1983; Förster *et al.*, 1983). The asynchronous germination pattern of oospores results in a continuous supply of infective propagules, thereby increasing the chances of survival of the fungus (Hord and Ristaino, 1991).

3.1.4 Survival of oospores and other inocula

Although long term survival of mycelium and zoospores of *P. infestans* would not be expected, soils artificially or naturally infested with sporangia remained infective to potato tubers for 15-77 days, depending upon soil type, soil moisture and pH

(Murphy, 1922; Zan, 1962; Lacey, 1965; Bogulavskaya and Filippov, 1977; Sato, 1980; Andrivon, 1994a; Holliday, 1995). Recent data also suggest that aluminium toxicity (Benson, 1993) and soil microflora (Andrivon, 1994b) might affect the survival of sporangia in soil. In contrast, oospores are resistant structures and their survival during adverse environmental conditions makes them a potentially long term source of inoculum (Ribeiro, 1983; Fay and Fry, 1997). Oospores have been shown to survive even when exposed to temperatures between -80 and 25°C for 48 h (Drenth *et al.*, 1995). Recent work has shown that oospores can survive at least one winter in the field under European and American conditions (Drenth *et al.*, 1995; Pittis and Shattock, 1994; Medina and Platt, 1999) but that survival could exceed two years (Perches and Galindo, 1967) in Central Mexico. The presence of oospores in soil will drastically change disease management strategies (Umaerus and Umaerus, 1994). This could include need for longer crop rotation and extended protection of the crop with fungicides from blight epidemics.

Since oospores were previously rarely produced under field conditions outside Central Mexico, their biology, ecology and role in epidemiology remains unexplored (Andrivon, 1995). Although there are presently gaps in our knowledge about the ecology, biology and epidemiology of oospores, their ability to remain in soil may enable *P. infestans* to survive in the absence of the host. Due to a lack of precise data on the actual duration of inoculum survival in soil under natural conditions, an experiment was designed to compare the relative survival time of *in planta* generated sporangia and oospores and those in soil under field conditions. The experiment was established to test viability of these propagules by determining their infectivity every three months for up to two years. In addition, the molecular detection of such sources of inocula was tested by PCR.

3.2 Materials and Methods

3.2.1 Isolates and cultivars

Cultures of *P. infestans* used in this study were obtained from the SCRI culture collection. Isolate 95.19.2.2 was A1 mating type, metalaxyl insensitive, isolated in 1995 from an allotment garden in Dundee. Isolate 97.40.1.3 was an A2 mating type, metalaxyl sensitive, isolated in 1997 from a garden in Dundee. Cultures had been maintained on Rye A slopes at 4°C. Potato cultivar Desirée was used for inoculum production.

3.2.2 Inoculum production and experimental set up

An agar plug (5 mm diameter) of each isolate was transferred from Rye A slopes on to Rye A agar in 9 cm Petri plates. Plates were incubated at 18°C for one week. Cultures were passaged through cv. Bintje in order to activate their virulence by inoculating the leaves with sporangia of each isolate in a detached leaf assay. Leaves were incubated at 18°C under artificial light until blight lesions developed. Sporangia of each isolate were harvested on a weekly basis and used to inoculate a new set of detached leaves. This was repeated for three weeks.

After three weeks, the sporangia were harvested from the leaves in 5 ml of DW and adjusted to a concentration of $2.5x10^4$ sporangia ml^{-1}. The sporangia were incubated at 4°C for 3 h to induce zoospore release for inoculation of leaves for the production of sporangia and oospores.

For the production of sporangia, leaves were detached from 6-7 week old plants of cv. Desirée and placed abaxial side up on moist paper towels in transparent plastic boxes. Leaves were inoculated with zoospore suspension of either isolate 95.19.2.2 (A1) or 97.40.1.3 (A2), by depositing 100 μl of sporangial suspension on either side of the main leaf vein. For oospore production, two methods were adopted. Either the sporangial suspensions of A1 and A2 isolates were mixed in a 1:1 ratio, and 100 μl of the resulting mixture was then used for inoculum, or 50 μl each of the sporangial

suspension of the two parental isolates was deposited on either side of the midrib of a detached leaf. Leaves inoculated with sterile distilled water served as control. Leaves were incubated for 1-2 weeks at 18°C under artificial light. High relative humidity was maintained in the boxes by wetting the paper towels with DW as required throughout the experiment.

The number of oospores or sporangia produced on leaves was determined by removing approximately 1 cm^2 diameter disk of the infected leaf after the propagules were produced abundantly in leaves. The number of each propagule was counted microscopically at x125 magnification.

The experiment was set up as a randomised complete block design with four replications. The experimental layout is presented in Fig. 3.1. Two week old inoculum, produced *in planta,* was used in an overwintering survival study. Inoculated leaves containing either A1 or A2 isolates (therefore, representing asexual inoculum) or both A1 and A2 (therefore representing mixed asexual and sexual inoculum sources) (Fig. 3.2) were exposed to field conditions. Inoculated leaves were placed in individual bags made from 20 μm nylon mesh (Verseidag Vtech Fab, Germany) (Fig. 3.3), chosen in order to prevent escape of oospores or sporangia from the bags. Bags were buried in a 2 m^2 field plot at a depth of 12 cm.

In a parallel study, survival of the two types of inoculum (sporangia and oospores) was studied in soil. Fifty oospores or sporangia of either mating type were added separately to 0.5 g soil in 2 ml plastic screw-top cryo-tubes (Fig. 3.3), which were buried alongside mesh bags in soil.

Samples were buried at the beginning of June 2000 and recovered every three months until June 2002. At each date, the samples were recovered from the field and processed in the laboratory in order to determine the longevity of the propagules by two different methods. Survival of the propagules was investigated by determining their detectability via PCR and viability in a baiting assay.

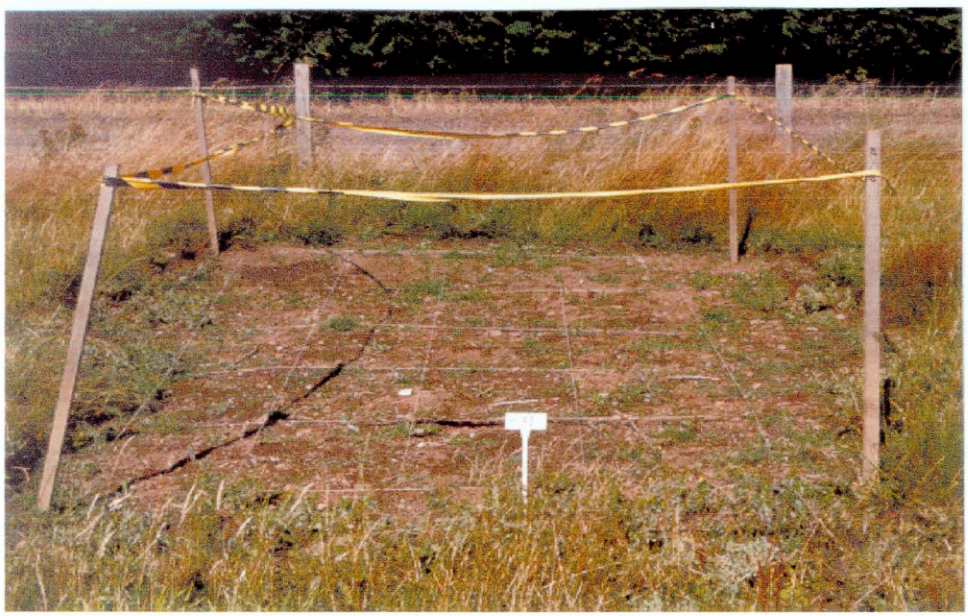


Fig. 3.1 Experimental set up of the inoculum survival study. The inoculum was buried in a 2 m^2 plot in a field at SCRI and recovered every three months and tested with PCR and baiting assay to determine survival and viability of the propagules.

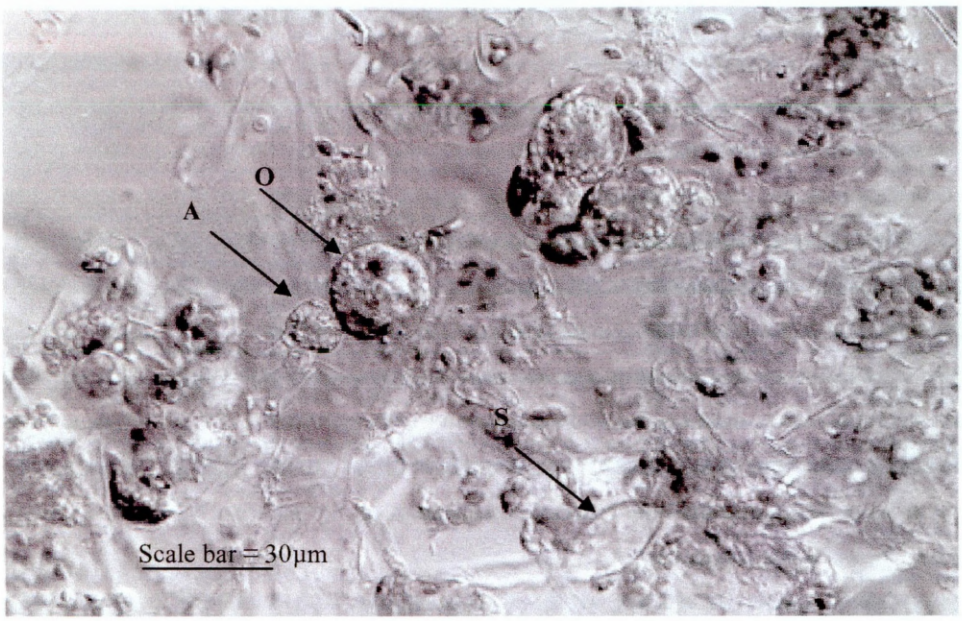


Fig. 3.2 Oospore formation of *P. infestans* in leaves of cv. Desirée incubated at 18°C. The amphygynous antheridia (A) are attached to the oogonia (O). Empty sporangial cases (S) are also visible.

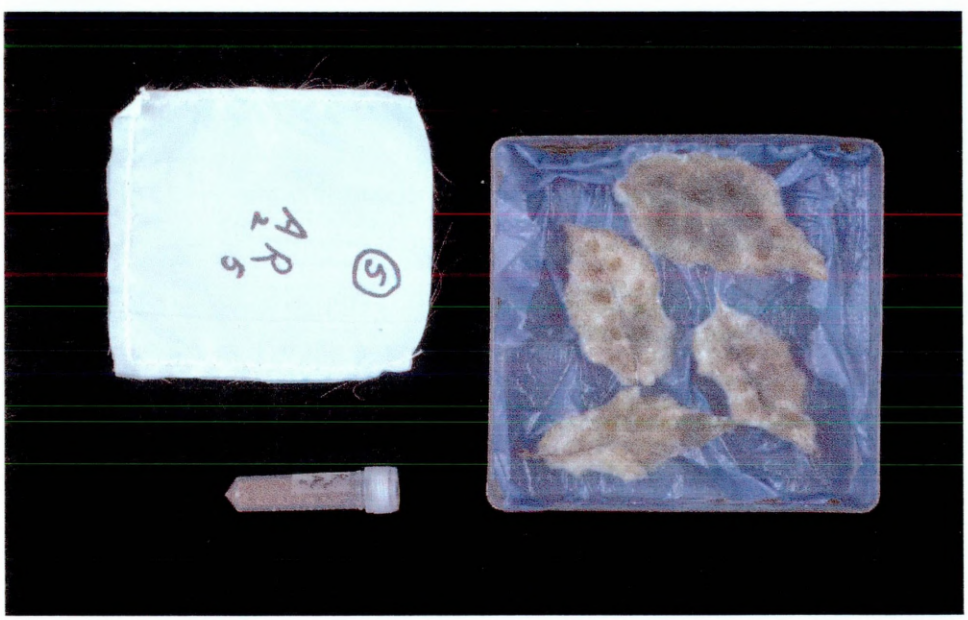


Fig. 3.3 Leaves of cv. Desirée infected with oospores and sporangia of *P. infestans* after incubation at 18°C for 10 days. The nylon bags used had a mesh diameter of 20 µm. Screw cap vials were used to study the inoculum survival in soil.

3.2.3. Determination of inoculum survival with PCR assay

Nested PCR was used to determine whether oospores and/or sporangia were still detectable in the nylon bags following burial in soil for 24 months. At each recovery date, individual bags containing oospores and sporangia were cut open and leaf debris was collected from the mesh bags. Approximately 10 mg of leaf material was used for DNA extraction. Tissue samples were processed for PCR according to the method of Wang *et al.* (1993) as described previously. In order to dilute the effect of tannins and other phenolic compounds that might have accumulated in the leaves over time, the extracted DNA was diluted 1:20 before being used for PCR. The debris from each bag was also examined under the microscope to verify the presence or absence of propagules of *P. infestans* in the leaf tissues. Various sections of leaves were mounted on glass slides and examined at x500 magnification under a microscope.

Spiked soil samples were also assayed for survival of inoculum by subjecting the soil samples to DNA extraction by the method of Cullen and Hirsch (1998) and Cullen *et al.* (1999) (Chapter 2). One micro litre of extracted DNA was used in a nested PCR, as described previously, to give an indication of the period over which different inocula were detectable.

3.2.4 Viability test

PCR results at each sampling date were complemented by determining the viability of the buried propagules. Two separate strategies were adopted to check the viability of the inoculum. Initially, this was done by determining the germination rates of the propagules of *P. infestans* on a range of media, including water agar (0.5, or 7%), Potato dextrose agar, French bean agar, Rye A agar, Rye B agar and potato soil leachate (Table 3.1). Petri dishes containing oospores were incubated at temperatures ranging from 10-22°C from one week to two months. Subsequently, a baiting assay on detached leaves of potato cv. Bintje was used. Leaf material recovered from the mesh bags (source of inoculum) was added separately to a plastic tray containing 50 ml of SDW (Drenth, 1994). Four leaflets of glasshouse grown potato plants were

floated adaxial side up in each tray. The trays were incubated at 16°C under blue light until symptom development (Shattock *et al.*, 1986; French and Tooley, 1987). Blue light was created by covering the fluorescent light bulbs with sheets of blue coloured cellophane. Leaflets were examined for infection by *P. infestans* almost daily until symptom development. Propagules were considered viable if a visible sporulating lesion was observed.

Table 3.1 Media used for oospore germination along with their recipes and pH.

Media	Recipe	PH
Water agar (0.5%)	Agar 1 g/200 ml DW	-
Water agar(7%)	Agar 14 g/200 ml DW	-
Potato dextrose agar	Potato dextrose agar 39 g/L	-
French bean agar	Ground French bean 30 g/L	6.3
	Agar 15 g/L	
Rye A agar	Rye 60 g/L	7.0
	Sucrose 20 g/L	
	Agar 10 g/L	
Rye B agar	Rye 60 g/L	7.0
	Sucrose 20 g/L	
	Agar 10 g/L	
Potato soil leachate	Soil 150 g/1.5 L	-

3.3. Results

3.3.1 Detection of propagules from leaves

Phytophthora infestans propagules (oospores + sporangia) buried in a field were recovered every three months after burial in order to monitor their survival over time. Examination of the debris, recovered from the bags, by PCR and baiting assay revealed that the duration of survival of oospores and sporangia varied over the course of the experiment. Oospores, produced in leaves, were detectable under local soil conditions during the study period, and their survival was longer than that of sporangia of A1 and A2 mating type isolates. There was, however, no difference between the survival of A1 and A2 mating type sporangia. DNA extracted from oospores resulted in PCR amplification of a product of the expected size for up to 24 months.

Both oospores and sporangia were consistently detectable with strong PCR signals during the initial period of the experiment (6 months) (Fig. 3.4-3.5) i.e. the first two samplings, a period during which average soil temperature dropped from 13.3 to 4.1 and 4.7°C in the top 10 and 20 cm layer of the soil respectively, with varying average rainfall (Fig. 3.6-3.7). However, the detection of sporangia by PCR decreased as the exposure time increased. The first sign of degradation of DNA was observed after nine months of burial when relatively weak bands were amplified from three out of four replications of A2 sporangia produced in leaves. No PCR products were detectable in two replications of the treatment containing A1 sporangia produced in leaves (Fig. 3.8a). Twelve months after burial, the oospores still produced strong PCR signals, whereas sporangia (A1 or A2) were detectable in some replications but their overall detectability was very low (Fig. 3.8b). This trend continued with an increase in the exposure time of the propagules to the field conditions. After 15 months of burial, oospores were the only propagules still detectable (Fig. 3.8c). No PCR signals from sporangia (either A1 or A2) were recorded from any replication at this time point. Further, the results show that there was no significant difference in the survival rates of A1 and A2 sporangia.

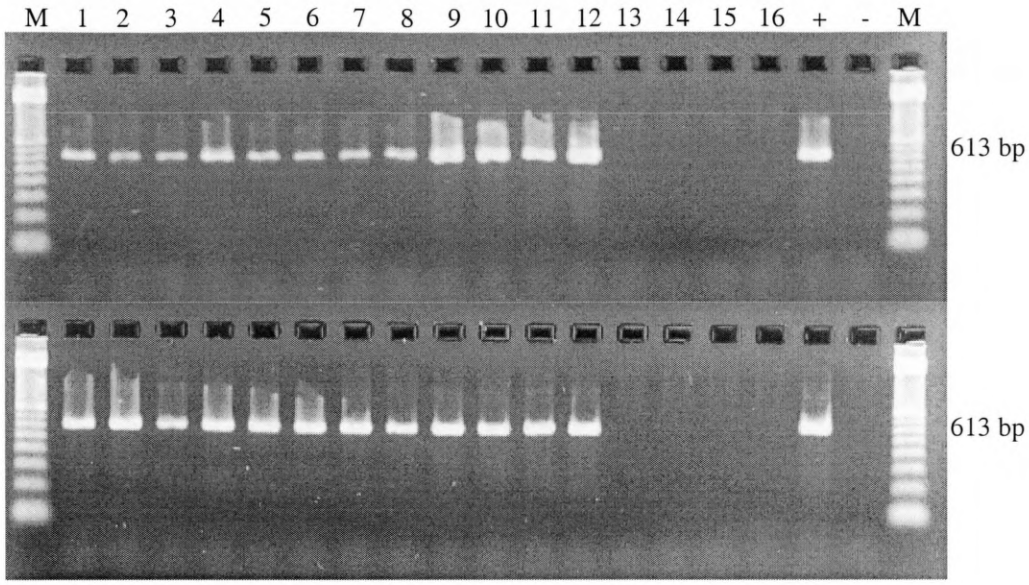


Fig. 3.4 Second round PCR amplification of *P. infestans* DNA from soil (top) and leaves (bottom) inoculated with a single mating type or both mating types and recovered **three months** after burial in the field. Nested PCR was performed with primers DC6 and ITS4 and specific primers INF FW2 and INF REV. Lanes 1-4, sporangia of A1 mating type; lanes 5-8, sporangia of A2 mating type; lanes 9-12, samples containing oospores after infection with both mating types and lanes 13-16, uninoculated controls. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

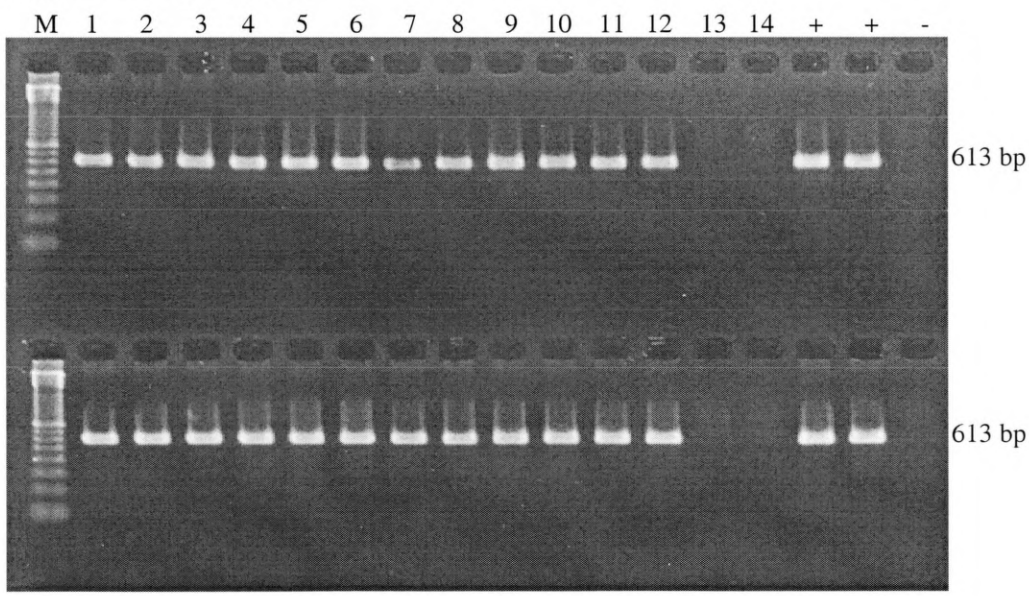


Fig. 3.5 Second round PCR amplification of *P. infestans* DNA from soil (top) and leaves (bottom) inoculated with a single mating type or both mating types and recovered **six months** after burial in the field. Nested PCR was performed with primers DC6 and ITS4 and specific primers INF FW2 and INF REV. Lanes 1-4, sporangia of A1 mating type; lanes 5-8, sporangia of A2 mating type; lanes 9-12, samples containing oospores after infection with both mating types and lanes 13-14, uninoculated controls. M represents 100 bp size marker. +/- represent positive and negative PCR controls.

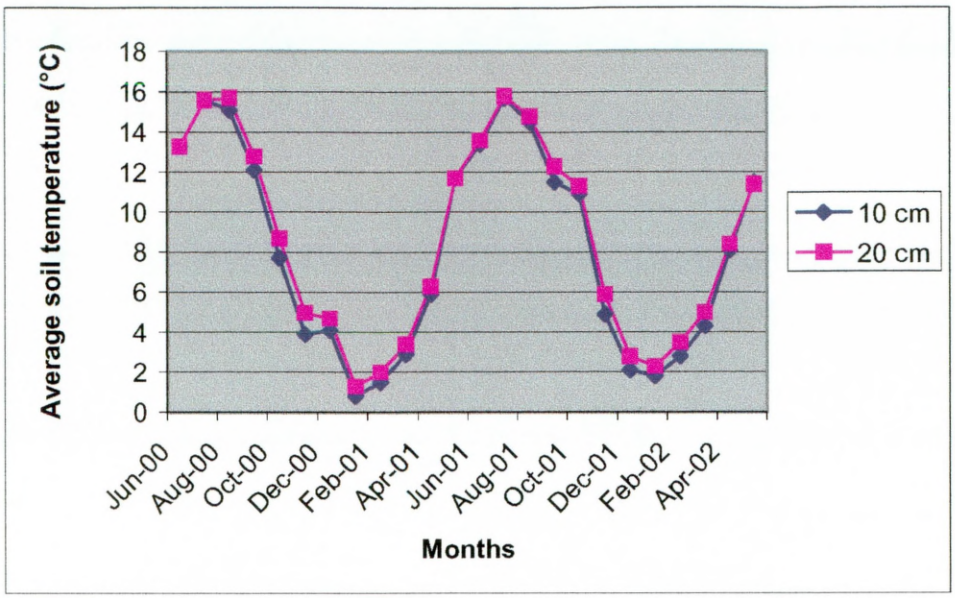


Fig. 3.6 Soil temperature (°C) taken at two different soil depths from the field used for inoculum survival study between June 2000 and May 2002.

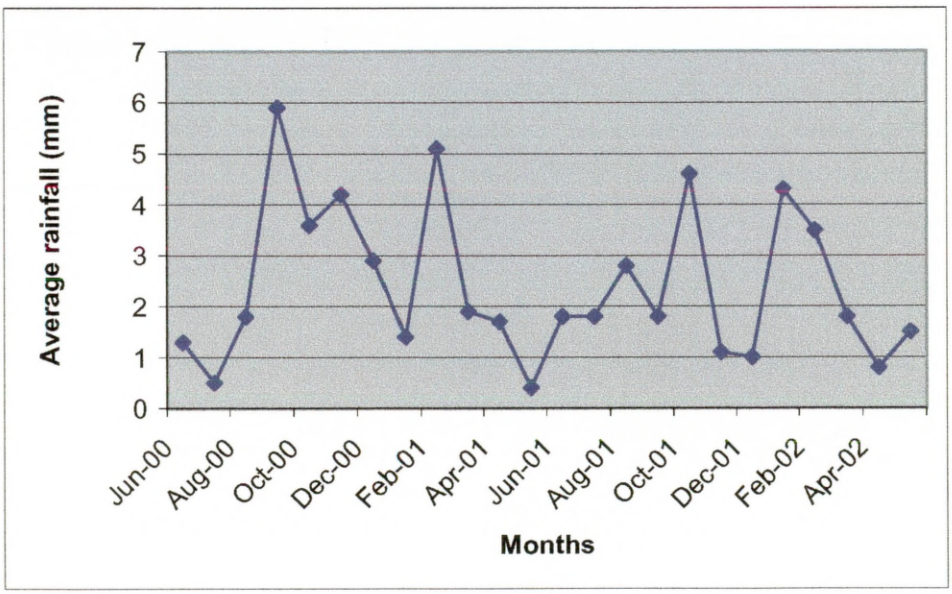


Fig. 3.7 Average monthly rainfall (mm) from June 2000 to May 2002.

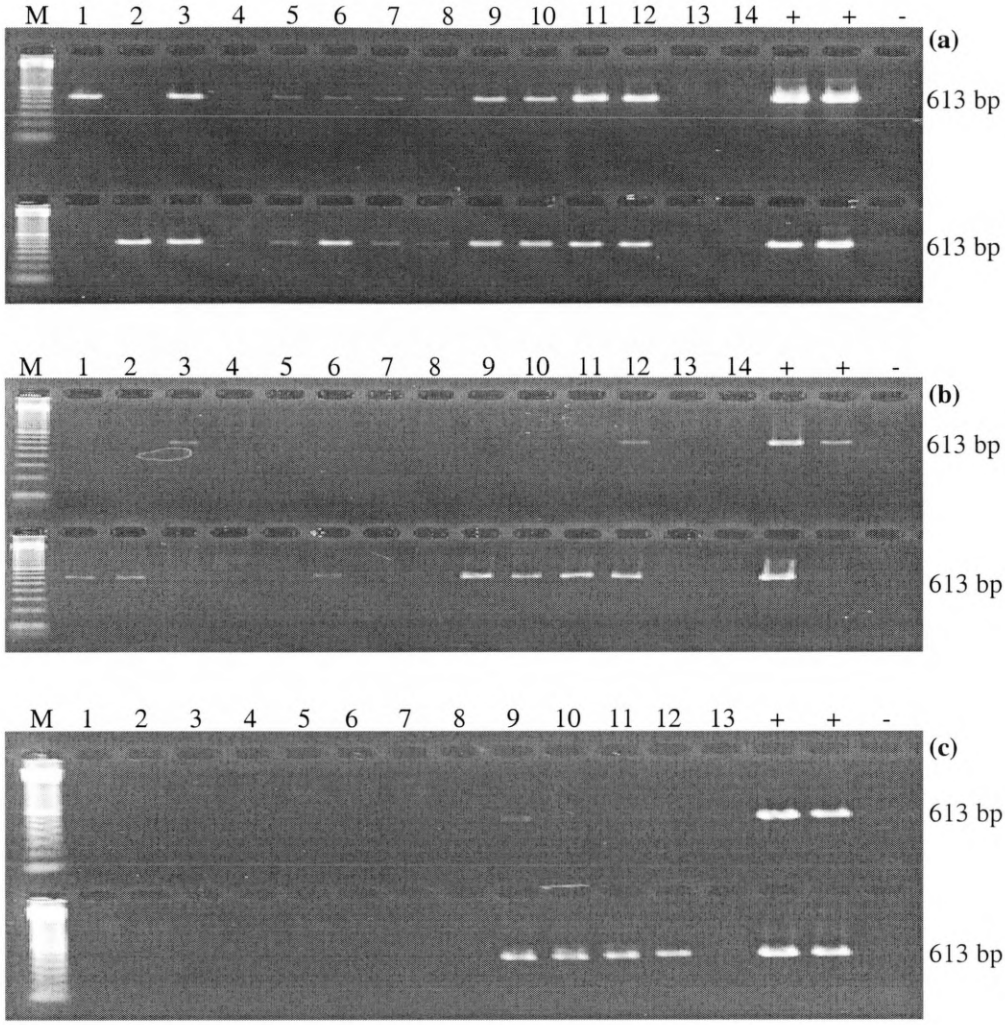


Fig. 3.8 Second round PCR amplification of *P. infestans* DNA from soil (top) and leaves (bottom) inoculated with a single mating type or both mating types and recovered a) **nine months** b) **twelve months** and c) **fifteen months** after burial in the field. Nested PCR was performed with primers DC6 and ITS4 and specific primers INF FW2 and INF REV. Lanes 1-4, sporangia of A1 mating type; lanes 5-8, sporangia of A2 mating type; lanes 9-12, samples containing oospores after infection with both mating types and lane 13, uninoculated control. (Lane 14 in a and b is blank). M represents 100 bp size marker. +/- represent positive and negative PCR controls.

Subsequent samplings revealed that oospore DNA was detectable up to 18 months after burial and produced a strong PCR amplification (Fig. 3.9) after which there was a general decline in the levels of detectability of oospores. After 21 months of burial, oospores could be detected from three out of four replications. However, the product bordered the limit of detection of the assay in the majority of the samples (Fig 3.10a). The last sampling, conducted after 24 months of burial in the field yielded the expected PCR product in 50% of the samples (Fig. 3.10b). A microscopic examination of the recovered debris at each sampling date revealed that oospores could still be seen under the microscope even after 24 months of burial in the field. In contrast, sporangia of neither mating type could be seen at later sampling dates. None of the non-inoculated control leaves showed any amplification products with PCR. The results substantiate the hypothesis that the pathogen is likely to survive as oospores in plant debris.

3.3.2 Detection of propagules from soil

PCR amplification of the soil samples spiked with either oospores or sporangia revealed that both types of inoculum were detectable at the first sampling date (three months) (Fig. 3.4). The second sampling was carried out in December 2000, when temperatures fell to 4.1 or 4.7°C in the top 10-12 cm layer of soil, respectively, compared to a temperature of 13.3°C at the start of the experiment in June 2000. However, the sporangia and oospores were still detectable from all replications (Fig. 3.5). In the first post-winter detection assay carried out after nine months (March, 2001), there was a noticeable reduction in the detection of sporangia as depicted by the amount of PCR product, compared to the earlier samples. There was, however, little change in the detectable levels of oospores (Fig. 3.8a). By the 4^{th} sampling after 12 months (June, 2001), there was a substantial reduction in the detection of oospores and sporangia buried in soil. The sporangia and oospores existed at a level below that possible for detection by nested PCR except from one replication each where A1 sporangia and oospores were detected (Fig. 3.8b).

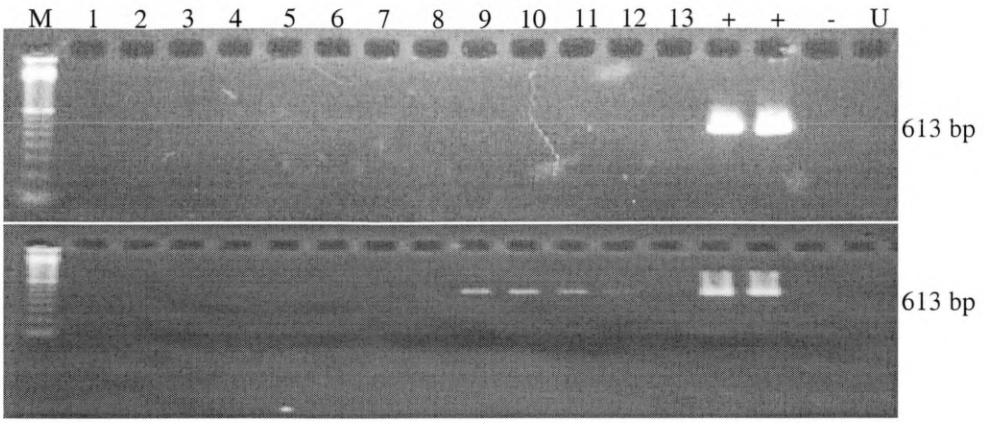


Fig. 3.9 Second round PCR amplification of *P. infestans* DNA from soil (top) and leaves (bottom) with a single mating type or both mating types and recovered **eighteen months** after burial in the field. Nested PCR was performed with primers DC6 and ITS4 and specific primers INF FW2 and INF REV. Lanes 1-4, sporangia of A1 mating type; lanes 5-8, sporangia of A2 mating type; lanes 9-12, samples containing oospores after infection with both mating types. Lane 13 is blank. M represents 100 bp size marker. +/- represent positive and negative PCR controls. The last lane marked 'U' is the uninoculated control.

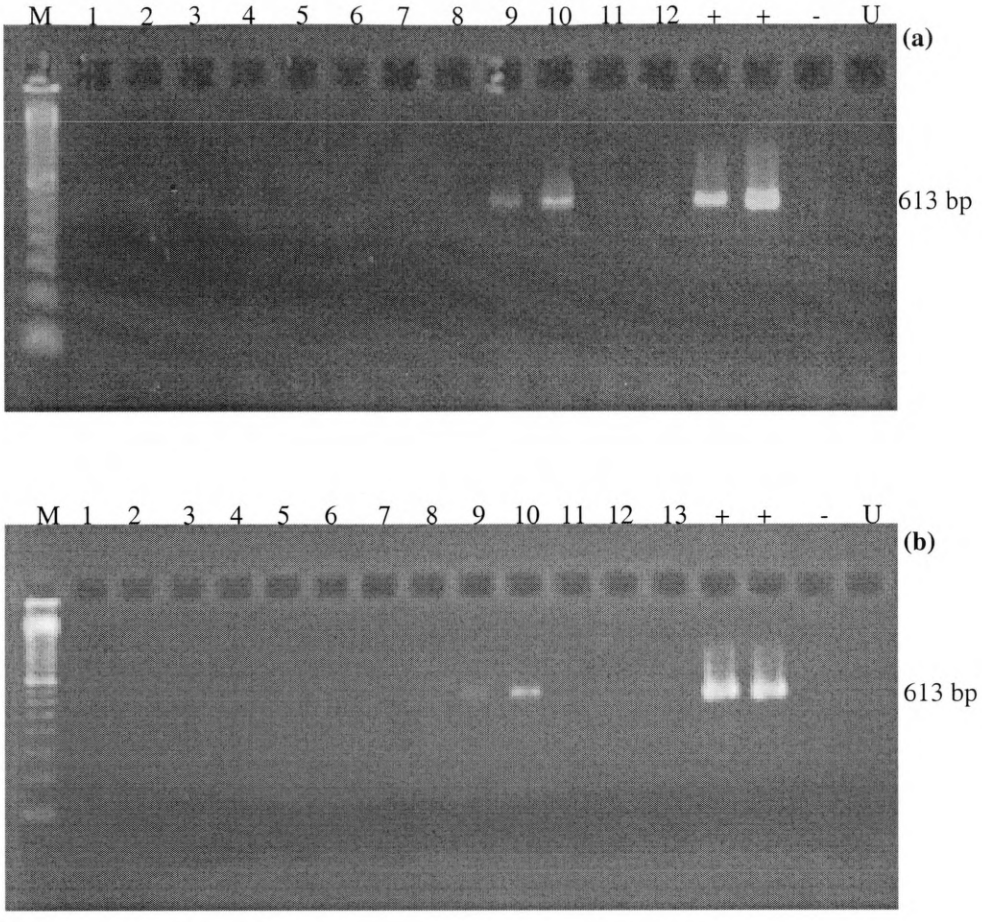


Fig. 3.10 Second round PCR amplification of *P. infestans* DNA from leaves with a single mating type or both mating types and recovered a) **twenty one months** and b) **twenty four months** after burial in the field. Nested PCR was performed with primers DC6 and ITS4 and specific primers INF FW2 and INF REV. Lanes 1-4, sporangia of A1 mating type; lanes 5-8, sporangia of A2 mating type; lanes 9-12, samples containing oospores after infection with both mating types. Lane 13 in b is blank. M represents 100 bp size marker. +/- represent positive and negative PCR controls. The last lane marked 'U' is the uninoculated control.

Fifteen months after burial of inoculum in the field, oospores could still be detected from one out of four replicates with a weak PCR signal. There was no PCR signal from either the A1 or A2 mating type of sporangia. (Fig. 3.8c). Subsequent samplings failed to yield a detectable PCR product from oospores or sporangia (Fig. 3.9). All amplifications performed with soil DNA from sterile autoclaved soil provided negative results. No significant differences were observed in the survival of asexual and sexual spores in soil.

3.3.3 Viability of propagules

Viability of the propagules from the first two samples of leaves (three and six months after burial), was checked by determining their germination rate on a range of media and incubation temperatures and times (Table 3.2). Plating of debris containing oospores on to seven different media demonstrated that oospores failed to germinate on all the media tested. Similarly, the oospores failed to germinate when incubated at a range of different temperatures for varying lengths of time. The debris recovered from bags containing either A1 or A2 sporangia did not show any germination when plated on to Rye A agar plates incubated at room temperature for two weeks.

Table 3.2 Germination of oospores of *Phytophthora infestans* on a range of media, at various incubation temperatures and times.

Media Type	Germination	Incubation Temp (°C)	Germination	Incubation Time	Germination
Water agar (0.5 %)	No	10	No	One Week	No
Water agar (7%)	No	12	No	10 Days	No
Potato dextrose agar	No	14	No	12 Days	No
French bean agar	No	16	No	Two Weeks	No
Rye A agar	No	18	No	Three Weeks	No
Rye B agar	No	20	No	One Month	No
Potato soil leachate	No	22	No	Two Months	No

The lack of success of the germination test in measuring viability of oospores led to the use of baiting test. All debris collected from the bags was tested on potato leaves (cv. Bintje) in a detached leaf assay. Up to 24 months after burial, the oospores of *P. infestans* remained infective and caused blight lesions following two weeks of incubation at 16°C under blue light (Fig. 3.11). In addition to typical blight lesions, sporangia and sporangiophores were recognisable on incubated leaves. The results show that the exposure of oospores for up to 24 months to temperatures ranging from -1.7 to 18.4 °C did not significantly affect their viability.

The sporangia were non-viable throughout the course of this experiment when debris retrieved from bags containing sporangia were used as source of inoculum in the baiting assay, clearly suggesting that such propagules are not suited for long term survival. Similarly, debris collected from bags containing non-inoculated control leaves failed to produce any signs of infection. Although the present data did not allow for a determination of the percentage of viable oospores, the results indicate the potential of the oospores for survival under field conditions and their ability to infect a subsequent potato crop.

Fig. 3.11 Viability of A1 sporangia (top left), A2 sporangia (top right) and oospores (bottom) of *P. infestans* in baiting assay. Leaves of potato cultivar Bintje were used as a bait. The inoculum consisted of leaf debris obtained from bags recovered after twenty four months of burial in the field. Leaves infected with oospores showed typical blight symptoms and abundant sporulation seen here as a whitish growth.

3.4 Discussion

Many studies have linked the increase in disease severity seen in recent years to the displacement of the 'old' population of *P. infestans*, and the appearance of the A2 mating type of the fungus in the late 1970s in areas outside Central Mexico (Drenth *et al.*, 1993b; Drenth *et al.*, 1994; Peters *et al.*, 1999b; Gavino *et al.*, 2000). The co-existence of both mating types of the pathogen could have serious implications for potato production (Hannukkala, 1999). Besides generating recombinant genotypes (Drenth *et al.*, 1995; Hanson and Shattock, 1998a), the presence of A1 and A2 mating types will provide a unique opportunity to the pathogen to survive as thick walled oospores in soil (Shattock *et al.*, 1986; Goodwin and Drenth, 1997; Trout and Ristaino, 1997), thereby eliminating the need for a green bridge between successive potato crops (Pittis and Shattock, 1994; Strömberg *et al.*, 2001). Reports concerning the possible role of oospores, which are regarded as the most persistent structures within the genus *Phytophthora* (Weste, 1983), as a source of inoculum, have emerged in recent years (Pittis and Shattock, 1994; Drenth *et al.*, 1995; Chycoski and Punja, 1996; Andersson *et al.*, 1998; Bagirova and Dyakov, 1998; Schöber-Butin, 1999; Smirnov and Elansky, 1999; Zarzycka and Sobkowiak, 1999; Flier and Turkensteen, 2000; Turkensteen *et al.*, 2000; Zwankhuizen *et al.*, 2000). Changes in the biology of the pathogen are likely to affect its potential to cause severe epidemics, and this must therefore be countered with changes in disease management strategies.

An important aspect of the biology and epidemiology of late blight is the survival potential of oospores under field conditions. Species such as *P. cactorum, P. erythroseptica* and *P. megasperma*, which are homothallic, are known to produce large numbers of oospores that remain viable and infective for many years (Sneh and McIntosh, 1974). Since oospore production in *P. infestans* under field conditions has only recently been observed, information on survival of these propagules is rather limited. Pittis and Shattock (1994) and Drenth *et al.* (1995) showed that oospores were able to survive at least one winter under natural field conditions. Similar results were reported by Medina and Platt (1999), who showed that oospores survived for seven months under gnotobiotic conditions in a potato field. Recently Mayton *et al.*

(2000) reported that oospores could survive for > 18 months under field conditions. In an effort to complement these studies and to provide more comprehensive data on oospore survival, studies were conducted for two years in a field under Scottish weather conditions. Unlike previous studies, molecular techniques alongside a baiting assay were used to determine the survival and viability of oospores.

A moderately resistant potato cultivar, Desirée, was used for oospore production. There are several reports of a positive association between partially resistant cultivars and oospore production (Drenth *et al.*, 1995; Hanson and Shattock, 1998b; Hermansen *et al.*, 2000; Hammi *et al.*, 2001). On the contrary, Turkensteen *et al.* (2000) observed a larger number of oospores in susceptible and resistant cultivars (Bintje and Pimpernel, respectively) than in the moderately resistant cultivar, Nicola. However, Cohen *et al.* (1997) found no significant effect of host genotype on oospore production. Such a relationship between race non-specific resistance and oospore production remains unclear (Strömberg *et al.*, 2001). Since oospore production is promoted by sterols (Elliott *et al.*, 1964; Elliott, 1983), differences in the level of sterol production of different cultivars could be a determining factor in this process (Langcake, 1974). Similarly, the number of oospores produced in leaves in the present study was lower than previously reported. Oospores numbers as high as 20000/cm^2 were reported by Hanson and Shattock (1998b) whereas the number of oospores produced in the present study was 1200/cm^2 of leaf area. The differences could be attributed to the different parental isolates used. Compatibility of *P. infestans* isolates is regarded as crucial in oospore production and viability (Pittis and Shattock, 1994).

The infected leaves were sealed in a 20 μm nylon mesh. The size of the mesh was chosen carefully in order to prevent escape of sporangia and oospores from the bags. At each sampling date when the bags were cut open, it was noticed that leaves containing oospores and sporangia remained substantially intact even after two years of burial in the field. By contrast, breakdown of the control leaves was complete and no material was found in the mesh bags. This was an interesting observation but not easy to explain. It is possible that a metabolite or antibiotic is produced by *P. infestans* upon infection that prevents or reduces the activities of saprophytic

microorganisms. However, this hypotheses remains speculative and further work is needed to substantiate this.

The use of PCR allowed the presence of oospores and sporangia in leaves and soil to be followed over time. First attempts to amplify DNA extracted from leaf debris were not successful. Since the first sampling was done after three months of burial, the accumulation of inhibitors (Kreader, 1996) resulted in PCR inhibition. Therefore, at each sampling date, crude DNA extractions prepared from the recovered debris were diluted 1:20 in an effort to dilute PCR inhibitors but still have enough DNA template left to be amplified by nested PCR. The samples were routinely diluted from this point onward in order to eliminate the effects of inhibitors. Similarly, DNA extractions from soil were purified through Sephadex spin columns. Artificially inoculated controls were always included in the assay at each sampling to make sure that the DNA extraction had worked.

Although PCR was found to be effective in determining the survival of *P. infestans* propagules, the assay fell short of differentiating between viable and non-viable oospores and sporangia. Since a positive PCR signal does not necessarily translate into number of viable spores, the PCR results may overestimate the disease risk. In order to account for this, molecular detection was compared to spore viability studies.

Initial attempts to determine viability via a germination test were unsuccessful. Germination is regarded as the absolute measure of viability (Pittis and Shattock, 1994). However, low (< 10%) and variable germination is common in *Phytophthora* spp. (Förster *et al.*, 1983; Ribeiro, 1983), as the asynchronous germination pattern ensures a continuous supply of inoculum. Since the germination trait is partly genetic (Romero and Erwin, 1969; Duncan, 1985; Pittis and Shattock, 1994; Fay and Fry, 1997), the isolates selected for the cross could have an effect on germination potential of oospores in the present study. Other factors affecting germination include light, nutrition, temperature and maturity (Ribeiro, 1983). Blue light (fluorescent light through double sheets of blue cellophane) has been reported to enhance germination of oospores (Shattock *et al.*, 1986; French and Tooley, 1987). Since oospores are known to germinate with exhaustion of nitrogen (Ribeiro, 1983),

whereas the accumulation of sugars inhibit germination (Banihashemi and Mitchell, 1976), attempts were made to germinate oospores on a range of media. Such attempts were, however, unsuccessful. The results are in conflict with previous studies where low levels (4-25%) of germination were reported (Hammi *et al.*, 2001) on a range of different media. Similarly, Medina and Platt (1999) found no significant effect of media on oospore germination. It is interesting to note however, that while Medina and Platt (1999) observed no germination on potato root extract, a recent study reported 18% germination on the same media (Hammi *et al.*, 2001). Maturity of oospores is an important factor in germination and since oospores are designed for long term survival, it is not unusual to observe delayed germination. The absence of germination during the first two samplings could be attributed to this factor. However, results of the present studies were different from those of Whittaker *et al.* (1994) who reported germination of five day old oospores after four days of incubation.

Because standard techniques used to germinate oospores failed during the first two samplings, alternative methods were sought. Techniques such as MTT and phloxine stain are reported to give false results (Pittis and Shattock, 1994) whereas plasmolysis (Jiang and Erwin, 1990) underestimates the level of viability (Medina and Platt, 1999). A baiting assay (Drenth, 1994) was used to assess the viability of propagules. The test measures viability in terms of infection potential rather than germination. Further, it was a qualitative study measuring the presence or absence of infection potential of propagules.

As expected, there were differences in the survival of oospores and sporangia. Oospores, produced in leaves and buried at a depth of 12 cm in nylon bags, survived up to 24 months (total length of the experiment), although there was a marked decline in their detection after 21 months of burial. Towards the end of the study (24 months), positive PCR signals were seen in only 50% of the samples. Leaf debris could still be seen intact inside those bags from which positive signals were produced. Examination of leaf debris at each sampling revealed that oospores were still intact inside the leaf tissue.

The minimum temperature recorded during the study was -1.7°C at 10 cm soil depth. The results suggest that oospores and plant tissues remain unaffected by temperatures close to zero. Oospores have been reported to survive at temperatures ranging from 0 to -20°C (Fay and Fry, 1997; Medina and Platt, 1999). There were at least three occasions during the course of the study when soil temperature was below zero for a week or more. The results show that the frequent freezing and thawing encountered during the first year of the study did not affect oospore viability, which is a reflection of the potential of their survival under extreme conditions. The results are different from those of Duncan and Cowan (1980) and Umaerus and Umaerus (1994) who reported that freezing and thawing can affect survival. Other factors such as soil type, moisture, pH and microflora and microfauna could alter the survivability of these propagules. Studies on the effect of these factors on survival of oospores are therefore warranted. During baiting tests, using leaf debris retrieved at each sampling date as a source of inoculum, the leaves produced typical blight symptoms with often profuse white sporulation which was easily recognisable under the microscope. Using the baiting assay, oospores were shown to remain viable for at least 24 months (the total length of the study). The results show that even after a decline in the level of oospores, those present were sufficiently viable to cause infection.

Contrary to expectations, the sporangia derived from plant tissue were detectable for up to twelve months after burial. However, results of the viability test showed that sporangia were non-viable. Temperature and relative humidity have been reported as the most important factors determining viability of sporangia (Minogue and Fry, 1981) and it appears that the test conditions were too rigorous for sporangia to maintain their viability over such a long time. The results are in agreement with those of Kuske and Benson (1983) who reported a survival period of two hours on air dried leaves for sporangia of *P. parasitica* (*nicotianae*).

Oospores added to the soil were detectable up to fifteen months after burial. After this period, no PCR signal was seen following amplification of DNA extracted from soil. However, the absence of a positive PCR signal does not necessarily mean that the pathogen was not present. It is possible that the number of oospores present in the soil at the time of sampling was less than the detection threshold of the assay. An indication of poor persistence of sporangia over time in soil was evident during the

first year of the study. Although sporangia were detectable from soil early in the burial period, they declined quickly to undetectable levels after twelve months of burial. These results agree with the previous studies (Murphy, 1922; Zan, 1962; Lacey, 1965; Bogulavskaya and Filippov, 1977; Sato, 1980; Andrivon, 1994a) which mention 15-77 days as the survival period for sporangia in soil.

On the basis of the evidence available from this investigation, viable oospores are capable of long term survival. Harrison (1992) demonstrated that oospores can be produced under a wide range of temperature conditions and suggested that temperatures in the field are conducive for oospore production in leaves in most potato growing areas of the world. It can therefore be concluded that oospores could act as an important source of inoculum.

No attempt was made to compare survival of oospore and sporangia in soil and plant tissue. Obviously, the differences in the number of propagules present in soil and plant tissue was the main reason for differences in the detection of these propagules in these two media. Whereas leaves had initial inoculum densities of 1200 and 2100 oospores and sporangia/cm^2, respectively, in soil, this number was limited to only 50. Therefore, survival differences between the two propagule types was measured in soil and plant tissue independently.

No attempt was made to determine the biotic and abiotic factors that might influence the survival of propagules. Such factors need to be addressed in future research on this aspect of late blight epidemiology and will be helpful in forecasting the potential for disease outbreaks more accurately. The results, nonetheless, have practical significance in light of recent changes in the epidemiology and population biology of *P. infestans*. It is probable that longer crop rotations with non-hosts would be required to combat late blight.

The study demonstrated that PCR has potential as a sensitive and specific detection technique to monitor propagule numbers in the field. Furthermore, the agreement between the PCR results and that of a viability baiting assay supports the use of PCR to detect not only the presence but the viability of soilborne inoculum. It would appear that so long as the oospore is viable, its DNA is protected and available for

detection by PCR and conversely, a loss of viability appears to correspond to a breakdown of amplifiable DNA. This is encouraging evidence for the utility of this assay for oospore detection in soil. Oospores of *P. infestans* can remain viable for up to 24 months, and possibly longer, under Scottish weather conditions and could act as an important source of inoculum. The results confirm that inoculum originating from infected plant debris may pose a threat to newly planted potato crops. Growers could benefit from this information by adopting practices aimed at destruction of leaf debris which will be helpful in reducing the risk of disease epidemics. In addition, sporangia although able to survive up to twelve months after burial, were non-viable and are therefore unlikely to be a significant source of primary inoculum.

Development of novel molecular markers and genotyping of the *Phytophthora infestans* population in Scotland

4.1 Introduction

4.1.1 Molecular markers and their significance

Despite the importance of *Phytophthora infestans*, and the fact that much survey work on mating type and fungicide resistance has been carried out, little is known of the molecular diversity of the pathogen. Surveys have revealed the presence of both clonal and genetically diverse populations, but the mechanisms responsible for creating and maintaining such molecular diversity have not been studied in great detail. Many markers have been used to characterise *P. infestans* populations, each of which has limitations. Three phenotypic markers: mating type, metalaxyl sensitivity (Marshall-Farrar *et al.*, 1998; Sedegui *et al.*, 2000; Elansky *et al.*, 2001) and virulence have been used widely. Mating type clearly has epidemiological significance and is a useful character to score. Metalaxyl sensitivity is not selectively neutral but does provide a good measure of changes in response to control measures. While understanding the basis of *P. infestans* virulence is important, it has had limited utility as a marker for population analysis (Goodwin *et al.*, 1995b; Sujkowski *et al.*, 1996). Isozymes are useful, but there are problems with throughput and subjective scoring of results from different laboratories. Mitochondrial DNA has been used in various studies (Griffiths and Shaw, 1998) but only provides information on a single locus that is uniparently inherited. Although DNA fingerprinting with the moderately repetitive probe RG57 (Goodwin *et al.*, 1992a) and AFLPs, have proved useful, these techniques mostly require the use of radioactivity and large scale extractions of high quality DNA (Rosendahl and Taylor, 1997). Furthermore, an RG57 fingerprint samples relatively few loci, and convergent evolution of identical RG57 patterns from different genetic backgrounds has been reported (Purvis *et al.*, 2001). RAPDs (Punja *et al.*, 1998) are used widely, but a lack of reproducibility makes comparisons between different studies difficult (Devos and Gale, 1992; Ellsworth *et al.*, 1993; Muralidharan and Wakeland, 1993; Penner *et al.*, 1993; Micheli *et al.*, 1994). New markers are required to both validate existing data,

increase throughput, allow a more standardised approach and add detail to the analysis of *P. infestans* populations.

Understanding a pathogen's population biology provides key information on its reproductive biology and disease epidemiology. Reliable and rapid molecular marker technology will also allow fingerprinting and monitoring (tracking) of isolates in both experimental work and field populations. In this manner, critical evaluations of the short (field to field) and long range (country to country) spread of the pathogen will be possible. A knowledge of pathogen population structure will benefit plant resistance screening (Leung *et al.*, 1993) and the formulation of effective disease management strategies (Legard *et al.*, 1995).

Molecular markers have been used in studies on fungal pathogens of crops (Milgroom, 2001) and their use will help distinguish genotypes of a pathogen in sexually reproducing populations. This will also enable us to investigate the establishment of epidemics and understand the role of mutation, recombination (sexual and parasexual), natural selection and genetic drift on *P. infestans* population dynamics.

4.1.2 Dominant and co-dominant markers

Molecular markers can be categorised into dominant and co-dominant types. A dominant marker is frequently reported as the presence or absence of a specific band on a gel. The presence of the band however, may either be due to a homozygous dominant allele or a heterozygote. Therefore when present, it is not possible to discriminate between homozygous dominant or heterozygous state of that locus. Further, it is not clear whether the absence of band (i.e. a null allele) is always due to the same mutation. This lack of ability to discriminate all forms of an allele makes dominant markers less useful. A marker is defined co-dominant, on the other hand, if discrimination between different alleles is possible. Using co-dominant markers, therefore, allows distinction of homozygotes and heterozygotes since a single allele at a particular locus is expressed in homozygotes whereas in heterozygotes both alleles are represented (Duncan *et al.*, 1998). Such discriminatory power makes co-

dominant markers useful for studying gene flow within populations (Powell *et al.*, 1996), as well as linkage analysis (Meksem *et al.*, 1995), and mapping studies (Raeder *et al.*, 1989). Several co-dominant markers have been used in the past, but these have certain limitations. AFLPs have been scored as co-dominant, but scoring relies on band intensities and is not reliable. Isozymes are co-dominant but not only are the range available and polymorphism limited (Carter *et al.*, 1991), but also there are problems with throughput and band nomination (Elansky *et al.*, 2001). RFLPs, although co-dominant (Tooley *et al.*, 1985; Goodwin *et al.*, 1992b), are time consuming to carry out and dependent on a restriction enzyme cleaving the DNA fragment at the polymorphic site. In order to overcome the limitations of the above mentioned markers, novel markers are needed to characterise the rapidly evolving *P. infestans* populations with accuracy and speed.

4.1.3 Single nucleotide polymorphisms (SNP); their pros and cons

Single nucleotide polymorphisms (SNPs) are regarded as useful co-dominant markers because of their abundance, stability and adaptability to automated scoring (Kruglyak, 1999). Pronounced 'snips', SNPs are biallelic co-dominant markers, which are ideal for population studies. SNPs are point mutations i.e. single base pair changes in the genetic code. As a general rule, a frequency of one SNP per kb of sequence of DNA may be expected (Collins *et al.*, 1998; Landegren *et al.*, 1998; Wang *et al.*, 1998; Zhao *et al.*, 1998). SNPs comprise the largest single set of sequence variants in genomes (Kwok *et al.*, 1996; Kruglyak, 1997; Cho *et al.*, 1999). In the human genome, for example, there are thought to be 200,000 SNPs in the coding region, and probably ten fold this number in non-coding regions (Collins *et al.*, 1997). Their abundance and potential for high throughput screening makes them especially attractive. Another advantage of SNPs over methods such as RFLPs is that only small amounts of DNA are required and rapid extraction protocols are therefore applicable. Further, these markers, unlike AFLPs and RFLPs can be scored easily without using radioactive isotopes (Vos *et al.*, 1995) and are highly reproducible. The only drawback of using SNPs is the labour intensive discovery phase, and the fact that they are biallelic. Multiallelic markers such as microsatellites tend to be more appropriate where SNPs show insufficient variability within a given population

(Marth *et al.*, 2001). However, the advantages offered by SNPs outweigh the drawbacks and they are proving useful in population studies. SNPs have been extensively used in medicine, particularly in relating SNPs to genetic diseases and tracking of haplotypes. Cargill *et al.* (1999) used SNP markers to determine the distribution of SNPs among European, African-American, African and Asian human populations. Most SNPs were detected in multiple groups but a statistically significant group of SNPs were confined to single sub-groups. Although the limited sample size prevented the investigators from drawing firm conclusions, patterns of sub-structuring were proposed. Similar results were reported by Halushka *et al.* (1999). SNPs therefore can be used as an attractive approach in comparative population studies (Chakravarti, 1998; Chakravarti, 1999).

The development of SNP markers involves discovery and validation phases as well as the development of appropriate scoring methods (e.g. automation).

4.1.4 SNP discovery

SNP discovery is the labour intensive phase in the development of SNP markers and could be regarded as a limitation in the use of SNP markers. SNP discovery involves PCR amplification of DNA fragments from a panel of individuals from the population of interest. Fragments representing coding regions may be selected from the GenBank database or Expressed Sequence Tag (EST) databases, whereas non-coding regions may be selected at random from libraries of cloned genomic DNA fragments. Whichever is the case, the fragments from the individuals are screened for sequence variation. Alternatively, in cases where multiple sequences of the same region are available (e.g. in an EST database) an *in silico* screen is feasible, in which duplicate sequences are aligned and polymorphic regions identified. Once amplified, the DNA fragments need to be screened for sequence variation through sequencing. A panel of isolates is PCR amplified and the PCR products are purified with commercially available DNA purification kits and sequenced directly (i.e. without cloning). The sequences are edited, aligned and examined for potential polymorphisms. Sequencing, although precise, is a time consuming and expensive method of SNP discovery.

Direct sequencing is a commonly used approach but is likely to involve the frequent and wasteful sequencing of monomorphic PCR products. Scoring all individuals using a DNA folding kinetics assay such as SSCP (single strand conformational polymorphism) first, increases the efficacy of detection as only variable products need to be sequenced. For SSCP analysis, target regions are amplified and double stranded PCR product is melted using formamide and heat treatment before a snap cooling to maintain the strand separation. Electrophoresis e.g. in gene mutation analysis (GMA, Elchrom Scientific, Switzerland) gels are used to visualise the single, most stable conformation of each single stranded DNA fragment. A homozygous PCR product will thus yield two bands, each representing a single strand. In theory, single strand fragments differing by a single base pair will migrate at sufficiently different rates to allow their discrimination by migration distance.

4.1.5 Methods of SNP scoring

As discussed, the identification of SNPs is merely the first step in the development of such markers. The next step is the scoring of SNPs in the population. A potentially bewildering array of high throughput SNP scoring technologies have emerged. Some SNP scoring methods currently being used are described below.

4.1.5.1 Allele specific PCR

This technique is based on specificity of binding and reliable extension during PCR. The 3′ end of an oligonucleotide primer confers most of the specificity and mismatches thus lead to failure of amplification. Primers are designed so that the 3′ nucleotide corresponds to the SNP site. Two identical primers, apart from their 3′ bases, which match either nucleotide occurring at the SNP site, are thus designed. However, studies have shown that a single 3′ mismatch is often insufficient for allele specific amplification (Drenkard *et al.*, 2000), therefore it is beneficial to introduce an additional mismatch in the few bases prior to the SNP base. DNA polymerase extends the mismatched 3′ ends with much less efficiency, whereas the allele specific primer results in normal amplification of the specific allele (Drenkard *et al.*, 2000). Thus to score an allele, two PCR reactions, each with a different primer, are

conducted and run in two lanes of an agarose gel. The appearance of either a band in both lanes or a mutually exclusive band in a single lane indicates hetero or homozygous alleles, respectively.

Although PCR products may be run on an agarose gel, a modification of this procedure is called fluorescent allele specific PCR. In this case the two forward primers (with the alternating SNP bases) are fluorescently labelled, each with a different dye, and the analysis product is run on ABI 377 or 3700 sequence detector. The software automatically calls the SNP genotype according to the presence of one PCR product with the first label (a homozygote), one PCR product with the other label (the other homozygote) or both products (heterozygote).

4.1.5.2 Single strand conformational polymorphism (SSCP)

As well as being an aid to discovery, SSCP analysis can be used for large scale screening of known SNPs. It does however have several disadvantages. The GMA gels are relatively expensive, the gel run times are long and the assay is sensitive to other sequence variations in the same PCR product, so one can not be sure of scoring the same base change in every case.

4.1.5.3 Restriction fragment length polymorphism (RFLP)

Restriction site differences, based at the SNP site, can be used to discriminate alleles. The PCR product is amplified, digested with a specific enzyme and run on an agarose gel. The enzyme is selected to recognise the SNP locus and the presence or absence of digestion will lead to a length polymorphism (Brown, 1999) that forms the basis of the SNP scoring. The method is however, time consuming and success is dependent on the chance availability of a restriction enzyme that cuts the SNP of interest.

4.1.5.4 Pyrosequencing

Pyrosequencing allows rapid analysis of short fragments of DNA sequence. It is a technique described as 'sequencing by synthesis'. PCR products are converted to

single stranded templates with which a sequencing primer is hybridised and incubated with an array of enzymes including DNA polymerase, ATP sulfurylase, luciferase and apyrase and the substrates adenosine 5′ phosphosulphate (APS) and luciferin. In an automated assay, the dNTPs are added one at a time into the reaction mixture. If the first of the four dNTPs added to the reaction does not complement the first base after the 3′ end of the primers, then apyrase degrades this dNTP. This then leads to the addition of the next dNTP. If, however, the added dNTP is complementary, DNA polymerase incorporates it into the template strand. The successful incorporation of dNTP releases equimolar amounts of pyrophosphate (ppi). This ppi is converted to ATP by ATP sulfurylase and the energy from ATP activates luciferase and generates visible light in amounts proportional to the presence of ATP. Sensitive photodetectors interpret this signal as a sequencing pyrogram (Nyren, 1987, Nyren *et al.*, 1993; Nyren, 1994; Ronaghi *et al.*, 1996; Ronaghi *et al.*, 1998; Ronaghi, 2001). The system is fast and accurate though the equipment and running costs are high.

4.1.5.5 Minisequencing

This method relies on extension of the target sequence containing the polymorphic site with a specific SNP primer using fluorescently labelled dideoxynucleotides (F-ddNTP). After amplification of the target, a specific minisequencing primer (SNP primer), which is exactly one base short of the polymorphic site is used to initiate a sequencing reaction. This is followed by the sequential addition of fluorescently labelled ddNTPs. After one incorporation, the reaction is interrupted by the addition of a terminator. The un-incorporated ddNTPs are removed and after denaturation, the product is separated using capillary electrophoresis. Data collection and analysis is performed on automated sequencer using Genescan software. The SNP is scored on the basis of the colour of the peak as homozygous wild type or mutant. The heterozygotes show two different coloured peaks in the electropherogram (Aydin and Bahring, 2001).

4.1.5.6 TaqMan assays

The technique is based on the principle of using two fluorescently labelled oligonucleotide probes specific for wild type and mutant alleles. During PCR, the probe that matches the target sequence specifically hybridises to it, and is readily cleaved by the 5′ nuclease activity of the polymerase. This releases the dye and the resulting increased intensity of that dye can be detected in real time using the ABI 7700 equipment. Detection of a single dye implies homozygosity and both dyes signal heterozygotes.

No matter which method of SNP scoring is used, the primary goal is to develop a series of markers that will streamline the study of *P. infestans* populations. Such markers should be easy to amplify and score, co-dominant, unlinked and ideally work in the presence of plant DNA. The latter will allow fingerprinting directly from plant material without the need to isolate the pathogen. If the procedure is sufficiently sensitive then amplification from sporangia washed from a lesion is another option.

This work focused on the discovery and assay development of selectively neutral, co-dominant SNP markers. Both sequences of known genes, and randomly selected regions of non-coding *P. infestans* DNA were investigated in the discovery phase. The markers were tested against F1 progeny of a *P. infestans* cross, used for the genotyping of Scottish isolates collected during a 1995-97 survey as well as for the epidemiological part of this study (Chapter 5).

4.2 Materials and Methods

4.2.1 Isolates

The *P. infestans* isolates used in this study consisted of a sub-set (n = 42) of a collection of the Scottish population and some isolates representing world-wide diversity (Table 4.1). Isolates were maintained on Rye A agar, and DNA extracted according to Raeder and Broda (1985). Briefly, 750 µl of extraction buffer (200 mM Tris HCL, 250 mM NaCl, 25 mM EDTA and 5% SDS) was added to 30 mg of freeze dried mycelia, which was ground and vortexed. To this, 525 µl of phenol was added and mixed for 2-3 min, followed by addition of 225 µl of chloroform and a further mixing for 2-3 min. The mixture was centrifuged at 13000 rpm for 10 min and the upper clear phase was transferred to a new Eppendorf tube. This was followed by the addition of 1 volume (750 µl) of chloroform, mixing for 2-3 min and centrifugation at 13000 rpm for 5 min. The upper phase was transferred to a new tube and precipitated with 0.54 volume (405 µl) of isopropanol (-20°C). The mixture was centrifuged at 13000 rpm for 10 min, the supernatant discarded and the pellet washed with 70% ethanol, air dried and re-suspended in 50 µl of HPLC water.

4.2.2 SNP discovery in gene sequences

A selection of full length *P. infestans* gene sequences (Table 4.2) (provided by Sophien Kamoun, Ohio State University) were screened for potential polymorphisms. Comparisons of these sequences against *Phytophthora* Genome Consortium (PGC) database of EST (Expressed Sequence Tag) sequences were conducted using BLAST (www.ncgr.org/research/pgc/). Matching clones from this database were aligned using 'multialign', a web based alignment tool (www.toulouse.inra.fr/multialin/results), and checked for polymorphisms. In order to amplify fragments of these genes, primers were designed initially for genes in which polymorphisms had been identified using Genefisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher). In addition, other genes were selected on the basis of the following criteria; single copy i.e. not members of gene families, the length of untranslated regions (UTR), and the quality and length of available sequence. DNA

sequence quality was checked by examining the downloaded chromatograms in Chromas software (www.technelysium.com.au/chromas.html). Approximately 700 bp regions of genes (detailed in Table 4.2) including *2-phosphoglycerate dehydratase*, *ADP/ATP translocase*, *Transaldolase*, *Glutamine synthetase*, *Cyclophilin*, *Phosphoglycerate kinase* and *Ubiquitin conjugating enzyme* were amplified. The primers used are presented in Table 4.3. PCR conditions were optimised to maximise the yield of the desired product, with minimal non-specific product amplification. PCR was based on a standard set of conditions (Section 2.2.3). Fragments of the candidate genes of the eight *P. infestans* isolates were sequenced using Applied Biosystems Big Dye Sequencing Kits and an ABI 373 sequencer. Manual editing and alignment was used to search and validate individual SNPs.

Table 4.1 Isolates of *Phytophthora infestans* used for SNP screening, genotyping and linkage studies.

Isolate No	Source	Origin	Mating type	Metalaxyl resistance	Year isolated
97.40.8.3	SCRI culture collection	Dundee	A1	R	1997
97.41.6.1	SCRI culture collection	Fife	A1	S	1997
97.41.7.3	SCRI culture collection	Fife	A1	S	1997
97.31.2.3	SCRI culture collection	Midlothian	A2	S	1997
97.36.5.2	SCRI culture collection	Lewis	A1	S	1997
97.31.5.3	SCRI culture collection	Midlothian	SF	S	1997
96.21.1.1	SCRI culture collection	Fife	A2	S	1996
97.11.5.1	SCRI culture collection	Rendall	A1	I	1997
97.28.1.2	SCRI culture collection	Meadowbank	A2	I	1997
97.28.1.1	SCRI culture collection	Meadowbank	A2	I	1997
97.31.1.3	SCRI culture collection	Midlothian	A2	S	1997
95.16.3.1	SCRI culture collection	Edinburgh	A1	S	1995
96.10.3.3	SCRI culture collection	Rosyth	A1	S	1996
96.8.4.2	SCRI culture collection	Meadowbank	A1	S	1996
97.39.1.3	SCRI culture collection	Dundee	A2	S	1997
97.31.4.2	SCRI culture collection	Midlothian	A2	S	1997
95.17.3.2	SCRI culture collection	Edinburgh	A1	S	1995
97.28.2.1	SCRI culture collection	Meadowbank	A2	I	1997
95.5.2.2	SCRI culture collection	Aberdeen	A1	S	1995
97.31.3.2	SCRI culture collection	Midlothian	A2	S	1997
96.17.5.3	SCRI culture collection	Ayr	A1	I	1996
97.38.2.2	SCRI culture collection	Dunkeld	A1	R	1997
96.9.5.1	SCRI culture collection	Cononsyth	A1	R	1996
96.13.1.3	SCRI culture collection	Glasgow	A1	R	1996
95.7.3.1	SCRI culture collection	Barsolus	A1	R	1995
95.19.2.2	SCRI culture collection	Dundee	A1	R	1995
96.8.5.2	SCRI culture collection	Meadowbank	A1	S	1996
96.22.3.1	SCRI culture collection	Perthshire	A1	R	1996
97.9.1.1	SCRI culture collection	Orkney	A2	S	1997
97.30.5.1	SCRI culture collection	Aberdeenshire	A1	R	1997
97.40.1.3	SCRI culture collection	Dundee	A2	S	1997
97.40.3.3	SCRI culture collection	Dundee	A2	S	1997
97.40.4.1	SCRI culture collection	Dundee	A2	S	1997
97.40.9.3	SCRI culture collection	Dundee	A2	S	1997
97.41.10.3	SCRI culture collection	Fife	A1	S	1997
95.1.1.6	SCRI culture collection	Perth	A1	R	1995
95.7.10.1	SCRI culture collection	Barsolus	A1	R	1995
96.32.5.3	SCRI culture collection	Barsolus	A1	R	1996
97.9.5.3	SCRI culture collection	Orkney	A2	S	1997

Table 4.1 (*Continued*)

Isolate No	Source	Origin	Mating type	Metalaxyl resistance	Year isolated
96.36.2.2	SCRI culture collection	Lewis	A1	R	1996
97.39.4.3	SCRI culture collection	Dundee	A2	I	1997
97.39.7.2	SCRI culture collection	Dundee	A2	S	1997
61	D. Earnshaw, Univ. of Wales	P9463 x 96.70	SF	R	1999
62	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A2	I	1999
63	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	S	1999
64	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	I	1999
65	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	R	1999
66	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	S	1999
67	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	S	1999
68	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	I	1999
69	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	S	1999
70	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	R	1999
71	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	R	1999
72	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	S	1999
73	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A2	I	1999
74	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	R	1999
75	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	R	1999
76	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A1	S	1999
77	D. Earnshaw, Univ. of Wales	P9463 x 96.70	A2	I	1999
78	D. Earnshaw, Univ. of Wales	P9463 x 96.70	-A1	R	1999
79	D. Earnshaw, Univ. of Wales	P9463 x 96.70	-A1	S	1999
80	D. Earnshaw, Univ. of Wales	P9463 x 96.70	-A1	R	1999
P9463	D. Earnshaw, Univ. of Wales	USA	A1	R	-
96.69	D. Earnshaw, Univ. of Wales	UK	A1	R	1996
96.89.43	D. Earnshaw, Univ. of Wales	UK	A1	S	1996
96.70	D. Earnshaw, Univ. of Wales	UK	A2	S	1996
80029	S. Whisson, SCRI	Netherlands	A1	-	-
88133	S. Whisson, SCRI	Netherlands	A2	-	-
AG4	-	Argentina	A2	R	1998
EC1	Greg Forbes, CIP	Ecuador	A1	S	1998
US467	W. E. Fry, Cornell Univ.	USA	A2	R	1997

Table 4.2 Full length gene sequences of *Phytophthora infestans* used for EST database search.

Gene	Clone I.D	Accession number	Probability
Glutamine synthetase*	MY-01-A-07	SP IP 23712	$1e^{-56}$
K+ channel protein	MY-01-C-01	AC002376	$5e^{-61}$
14-3-3-like protein	MY-01-D-08	SP IQ 39757	$4e^{-65}$
Ubiquitin conjugating enzyme E2-16KD*	MY-03-C-08	SP IP 46595	$4e^{-65}$
ADP/ATP translocase*	MY-03-E-01	AF 025799	$3e^{-57}$
Chaperonin	MY-O3-E-04	SP IP80316	$3e^{-53}$
Proteasome	MY-O3-E-06	SP IP52427	1^{e-54}
Protein phosphatase	MY-O4-B-01	AF 030290	2^{e-64}
Acetylneuraminase lyase	MY-O4-D-03	-	-
Glycerol-3-phosphate dehydrogenase	MY-O4-E-05	SP IP43304	$9e^{-50}$
Inorganic pyrophosphatase	MY-O4-F-06	L32792	$4e^{-60}$
Fructose-1-6-biphosphatase	MY-O4-G-12	SP IP46267	$8e^{-61}$
Argininosuccinate lyase	MY-O5-B-09	M14218	$4e^{-64}$
GMP reductase	MY-O6-D-05	Pir IB32902	$2e^{-59}$
Phosphoglycerate kinase*	MY-O6-E-01	AF042738	$1e^{-77}$
Transketolase	MY-O6-F-01	Pir IS580383	$8e^{-78}$
Serine/threonine protein phosphatase pp2A	MY-O6-G-03	SP IP23696	$1e^{-65}$
Ubiquitin conjugating enzyme E2-17KD	MY-O7-B-10	SP IQ16781	$2e^{-64}$
Electron transfer flavoprotein beta subunit	MY-O7-C-03	SP IP38117	6^{e-69}
NADH dehydrogenase	MY-O7-E-04	Pir IS52386	$1e^{-71}$
2-phosphoglycerate dehydratase*	MY-O7-F-01	SP IQ43130	$8e^{-61}$
Microbial binding protein	MY-O7-F-02	U93506	$3e^{-52}$
Argonante related protein	MY-O7-G-04	U91995	$9e^{-66}$
Homolog of sar 1	MY-O8-B-04	Y08636	$5e^{-59}$
Cyclophilin*	MY-O8-C-01	U22050	$3e^{-99}$
Serine/threonine protein phosphatase ppE1	MY-O8-C-08	SP IP36614	7^{e-79}
6-phosphogluconate dehydrogenase	MY-O8-C-11	AFO61837	$1e^{-64}$
Decarboxylase	MY-10-B-11	-	-
Cystathionine beta synthase	MY-10-D-12	Pir IC42790	-
Thioredoxin peroxidase	MY-10-H-01	SP IP35704	$3e^{-56}$
3-deoxy-D-arabinoheptulosonate-7-phosphate	MY-11-A-03	SP IP00886	$7e^{-51}$
S-adenosyl methionine synthetase	MY-11-B-09	SP IP43281	$5e^{-75}$
Transaldolase*	MY-11-C-10	L19437	$5e^{-71}$
Transcarboxylase	MY-11-D-10	U56964	$2e^{-59}$
Unknown related to human ORF	MY-11-G-02	AC004472	$6e^{-53}$

Genes with an asterisk were the ones examined
E (expect) value represents the number of hits 'expected' by chance during a database search. Small e-value represents a significant match between the query and the subject sequence

Table 4.3 Primers designed from full length gene sequences (Table 4.2) for amplification and sequencing of *Phytophthora infestans* isolates.

Gene	Forward primer	Reverse primer	Product	Tm (°C)
2-phosphoglycerate dehydratase	GTCGATCAAGGCCCGTCA	GGCGATGTGCTGCCACA	403 bp	58
ADP/ATP translocase	CCTCCCCCCTTCAGTCA	GGCAAAGTCAAGCGGGTA	469 bp	58
Transaldolase	ATGGCTGCCTCCCCCAA	AGCCGAAGAGCAGGGTCA	518 bp	58
Glutamine synthetase	GTCCCTCGACGTGTCTGA	TCACGCCGTCCTTCTCGA	386 bp	58
Cyclophilin	GTCGCAAGCAAGACCCAA	GCGTCTGCAATTGGCAGA	637 bp	58
Phosphoglycerate kinase	TCAAGAACGTGGACGTCA	CACCACCCAGAATCGACA	622 bp	58
Ubiquitin conjugating enzyme	CAACAAGGAGCTGCAGGA	GCGTACTTGGCCGTCCA	420 bp	58

4.2.3 SNP discovery in random BAC fragments: sequencing, selection and screening

End-sequencing of randomly selected genomic DNA fragments from an SCRI BAC (Bacterial Artificial Chromosome) library (Whisson *et al.*, 2001) was performed as follows. *Escherichia coli* strains containing *P. infestans* BAC clones were grown overnight in 5-10 ml of LB plus 12.5 μg chloramphenicol. Cells were centrifuged in 2 ml Eppendorf tubes at 1300 rpm for 3-5 min. The supernatant was discarded and the pellets re-suspended in 200 μl of chilled solution I (5 ml 2 M Tris-HCl, pH 8.0; 8 ml 0.5 M EDTA; 3.6 g glucose in 400 ml of water) by vortexing. To this, 400 μl of freshly prepared solution II (2 ml 10% SDS; 400 μl 10 N NaOH in 20 ml of water) was added. Tubes were inverted c.10 times and placed on ice. This was followed by addition of 300 μl of solution III (600 ml 5 M KOAc; 115 ml glacial acetic acid in 100 ml of water) and after inversion of tubes c.10 times, they were placed on ice for 20 min. Tubes were then centrifuged at 13000 rpm for 10 min. The supernatant was transferred to another 2 ml tube and c.900 μl of cold isopropanol was added, mixed by inversion and then centrifuged at 13000 rpm for 10 min. The pellet was washed with cold 70% ethanol and centrifuged for 5 min. The ethanol was pipetted off and tubes were allowed to dry for 30 min. The dried pellet was then re-suspended in 50 μl of water and 0.5 μl of RNAse A and incubated at 37°C for 1 h prior to storage at -20°C.

Sixty one BAC clones were end-sequenced using Applied Biosystems Big Dye Sequencing Kit on ABI 377 sequencer with the universal sequencing primers, T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAG-3′) (New England Biolabs).

The sequences were BLAST searched against protein database at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) to ensure that they did not contain parts of repetitive elements. Based on this criterion, a sub-sample of 46 BAC clones were selected for further analysis. Primers were designed to amplify c.300 bp fragments of these sequences, using PCR conditions described previously (Section 2.2.3) which were then screened by single strand conformational polymorphism (SSCP) analysis using pre-cast GMA gels (Elchrom Scientific, Switzerland).

4.2.3.1 Single strand conformational polymorphism

Three micro litres of PCR product were denatured by adding 7 μl of denaturing solution (99:1 v/v formamide, 1 M NaOH plus a few grains of bromophenol blue as a tracking dye and incubating at 95°C for 5 min followed by snap cooling at 4°C. Electrophoresis was performed at 6 V/cm (72 V) for 15 h at 9°C in 30 mM TAE running buffer in an Elchrom Scientific SEA 2000 electrophoresis apparatus in combination with a refrigerated circulating water bath. Gels were stained for 20 min with SYBR Gold (10 μl/ 100 ml 10 mM TAE) and destained in water for 45 min before photographing under UV light. Based on electrophoretic mobility, the polymorphic isolates were selected and sequenced. Sequences were aligned for manual editing and searched for SNPs.

4.2.4 SNP scoring using allele specific PCR

Allele specific PCR primers were designed by using the web-based primer design programme SNAPER (http://pga.mgh.harvard.edu/cgi-bin/snap3/websnaper3.cgi). A DNA sequence with the polymorphic base marked was submitted along with primer selection criteria including primer length, annealing temperature and product size.

The programme searches for a pair of primers able to discriminate both polymorphic bases in a rapid PCR based assay. The 3′ terminal base is designated to match the SNP. A list of forward and reverse PCR primers was received via e-mail. The forward primer, besides having the alternating SNP base also has an additional variable base to increase its specificity. Two forward (allele specific and alternate) and one reverse primer were selected for each polymorphic gene and BAC clone.

A number (n = 3) of primer pairs for each marker were selected from the list and their specificity tested and PCR conditions optimised. The optimal primer pair will amplify a PCR product for one homozygous allele but not the variant SNP in its homozygous state. Isolates heterozygous at a particular SNP will yield a PCR product with both primer sets. Amplification reactions were performed in a 10 μl volume. Each reaction was prepared with 1x PCR buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl], 1 μg of Bovine Serum Albumin (BSA), 100 μM each of dNTP, 1.5 mM $MgCl_2$, 1 μM of each primer and 0.4 U *Taq* DNA polymerase. Running conditions are described in section 2.2.3.

4.2.5 High throughput SNP analysis

Fluorescent allele-specific PCR was used to increase the throughput of the SNP assays. The method is essentially the same as used in section 4.2.4 except that the forward primer for a specific allele is labelled with a fluorescent dye. When used in combination with a fluorescent detection and electrophoresis device such as ABI 377 or ABI 3700 sequence detector, the presence of both alleles can be differentiated in a single assay well. For each marker, the allele specific and alternate primers were labelled with different fluorescent dyes including FAM, VIC, TET, and NED (Table 4.4) and were used at a concentration of 1 pmol/μl in 10 μl PCR reactions (conditions as described previously). PCR product dilution was optimised using a range of dilutions including 1:2, 1:5, 1:10, 1:50 and 1:100 of PCR product in water. For genotyping, alleles representing seven different loci were amplified from the sub-set of 42 late blight isolates (Section 4.2.1) in a Thermo Fast 96-well non-skirted PCR microplate (AB gene, U.K) and reactions were prepared for running on the ABI 3700 sequence detector (PE Applied Biosystems). The ABI 3700 system, based on a

high throughput multi-capillary eletrophoresis, has many advantages over acrylamide gel based systems. It is a non-gel based, automated robotic assay, with limited sample handling. The laser-based detection system is very sensitive and is ideal for detecting weak products. The separation matrix of the system allows accurate sizing (0.1 bp). Additionally, since each sample runs in a single capillary, no tracking is needed after each run. A mixture of fluorescently labelled PCR products, LIZ size standard (Applied Biosystems) and formamide (HI-DI) in a 0.5:0.5:9 ratio was prepared. After denaturation of the PCR products at 95°C for 2 min in a thermocycler, the samples were snap cooled and run on ABI 3700 sequence detector. After each run, the chromatograms were analysed using Genotyper software, version 2.5 (Applied Biosystems) for genotype assignment. Once optimised, the software will automatically 'call' the SNP according to the presence of one PCR product with the first label (a homozygote), one PCR product with the other label (the other homozygote) or both products (a heterozygote).

Table 4.4 Fluorescently labelled primers, listed according to size, used for automated detection of *P. infestans* isolates using ABI 3700 capillary electrophoresis sequence detector.

Marker	Primer I D	Number of nucleotides	Product (bp)	Fluorescent labels	Dye colour
7I1F	SNP1	20	138	FAM	Blue
	ALT1	20	138	VIC	Green
2-phosphoglycerate	SNP2	20	181	NED	Yellow
dehydratase	ALT2	20	181	PET	Red
Glutamine synthetase	SNP4	20	214	FAM	Blue
	ALT4	19	213	VIC	Green
56G18R	SNP7	20	230	NED	Yellow
	ALT7	19	229	PET	Red
32A2R	SNP2	20	236	FAM	Blue
	ALT2	20	236	VIC	Green
56E14R	SNP1	18	313	FAM	Blue
	ALT3	20	315	VIC	Green
Trnsaldolase	SNP3	20	328	NED	Yellow
	ALT3	21	329	PET	Red

4.2.6 Segregation analysis

Linkage amongst the SNP loci was determined in an F1 progeny from a cross generated by Diana Earnshaw (University of Wales, Bangor) (Table 4.1). In order to examine linkage in an F1 population, parents must be selected with maximal heterozygosity. Four parental isolates, P9463, 96.69, 96.89.43 and 96.70 were evaluated by SSCP analysis (Section 4.2.3.1) and on the basis of this data, P9463 (A1) and 96.70 (A2), were examined. Twenty randomly selected F1 progeny (also provided by Diana Earnshaw) were screened with the allele specific SNP assays described previously, in order to examine segregation ratios. Progeny were scored as homozygous wild type (+/+), homozygous mutants (-/-) or heterozygotes (+/-) for each SNP marker. All tests were repeated to validate the null allele data. The null hypothesis, that each allele segregates independently amongst the progeny was tested using the chi-squared (χ^2) test, and the program 'JoinMap' version 2.0 (Stam, 1993) was used to determine the linkage amongst the markers.

GENSTAT version 4.3 (Rothamsted, U.K) was used to calculate χ^2 values to compare the observed ratios against the expected Mendelian ratios of 1:2:1 and 1:1 depending upon the parental combination. Markers showing a good fit to the expected ratios were then tested in pairwise combinations using a χ^2 test.

Genetic linkage analysis was performed using the software package 'JoinMap'. The LOD (Logarithm of odds ratio) score is the conventional measure of two point linkage, and is defined as the log10 of the ratio of the likelihood, when the loci are linked with their maximum likelihood recombination fraction to the likelihood when the loci are unlinked (Lander *et al.*, 1987). Therefore, in this study two loci were considered linked if they had LOD score of 0.834 or more, which corresponds to a 5% level of probability.

4.2.7 Genotyping of Scottish isolates

In order to test the reliability of the selected markers and assay conditions, a sub-set of 42 isolates from a previous Scottish late blight survey were tested. The isolates were originally collected between 1995 and 1997 from different areas of Scotland

including commercial crops and gardens and allotments. The isolates were selected from across three AFLP clades, defined previously on the basis of scores obtained using 15 AFLP markers. The isolates, maintained in the SCRI culture collection on Rye A agar, were grown in pea broth, and the mycelium was washed and lyophilised as described previously. DNA was extracted according to Raeder and Broda (1985), as described previously.

All nine SNP marker primer sets were used with each of the isolates. PCR products were run on agarose gels and visualised under UV transillumination after electrophoresis, as described previously. The results were later validated with fluorescent allele specific PCR (Section 4.2.5).

Following PCR, the presence and absence of the bands for each SNP marker was scored by eye. A similarity matrix based on simple matching coefficients was created and hierarchical cluster analysis using group averages was performed with the statistical program GENSTAT (Payne, 1997). Genetic relatedness was calculated on the basis of SNP genotypes, and a dendrogram was constructed.

4.3 Results

4.3.1 SNP discovery in full length gene sequences

On the basis of the analysis of *P. infestans* EST sequences, eighteen genes were selected for the first step of SNP discovery. Polymorphism scoring in genes that were members of gene families or in regions of poor sequence quality were rejected. Seven genes were selected and ~ 400-650 bp regions from eight *P. infestans* isolates (Table 4.1) were amplified. Single PCR products of the predicted size (Table 4.3) for these genes were consistently obtained with pure *P. infestans* DNA of every isolate, and each was sequenced in order to verify the nature of polymorphisms.

Manual editing of the sequences obtained involved removing dye blobs and trimming the forward and reverse sequences, reverse complementing the reverse sequence and aligning with the forward sequences and editing the overlapping region for each isolate. The corrected sequences of each isolate were then aligned with each other and SNPs were validated. Five of the seven gene fragments sequenced showed polymorphism in the isolates tested. Sequences of three genes; *2-phosphoglycerate dehydratase*, *Transaldolase* and *Glutamine synthetase* were polymorphic among the Scottish blight isolates tested, whereas *ADP/ATP translocase* and the *Ubiquitin conjugating enzyme* showed polymorphism only among the international isolates (examples shown in Fig. 4.1-4.2). Of the 3.5 kb of genes sequenced, a total of eight SNPs were detected, which represents ~ 2 SNP per kb studied (Table 4.5). In six cases, two different overlapping coloured peaks were found in the electropherogram demonstrating a heterozygous state, whereas the remaining two SNPs were homozygous. The homozygous SNPs comprised one transition (C→T) and one transversion (G→C). Amongst the genes, *Transaldolase* was the most variable having three out of eight SNPs. Amongst the international isolates, isolate 80029 from The Netherlands showed the highest number of polymorphisms (4) whereas no isolate could be singled out for the highest frequency of polymorphisms among the Scottish isolates.

The edited sequences were realigned with the original PGC EST sequences in order to compare them to the original potential SNPs. A web-based Open Reading Frame (ORF) finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to check whether the SNPs in the coding regions were synonymous or non-synonymous (i.e. whether an amino acid was changed). Six of the eight SNPs were synonymous i.e. did not result in a different amino acid, whereas two SNPs did alter the amino acid (Table 4.6). The transversion G→C in the gene *Transaldolase* was non-synonymous, and resulted in amino acid differences between the ORFs of the isolates. Comparison of amino acid sequences showed that isolates with different SNP bases differed from each other by having asparagine (n) instead of lysine (k) at the SNP site (Fig. 4.3a). In *2-phosphoglycerate dehydratase*, the amino acid change was from serine (s) to threonine (t) (Fig. 4.3b).

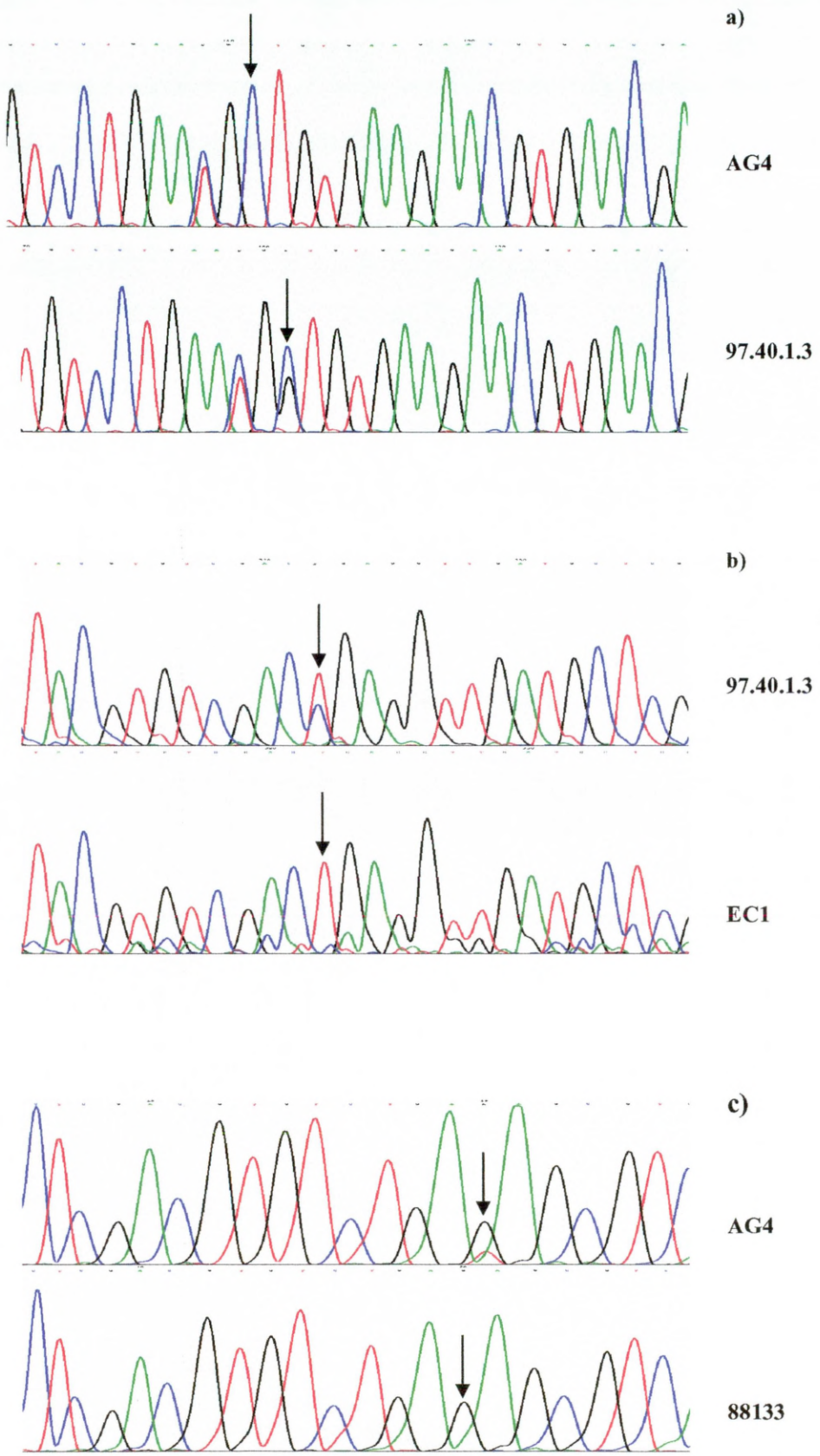


Fig. 4.1 Electropherograms resulting from sequencing of fragments of genes a) *2-phosphoglycerate dehydratase* b) *Transaldolase* (SNP No 322) and c) *Glutamine synthetase*. Polymorphisms between isolates are marked with arrows.

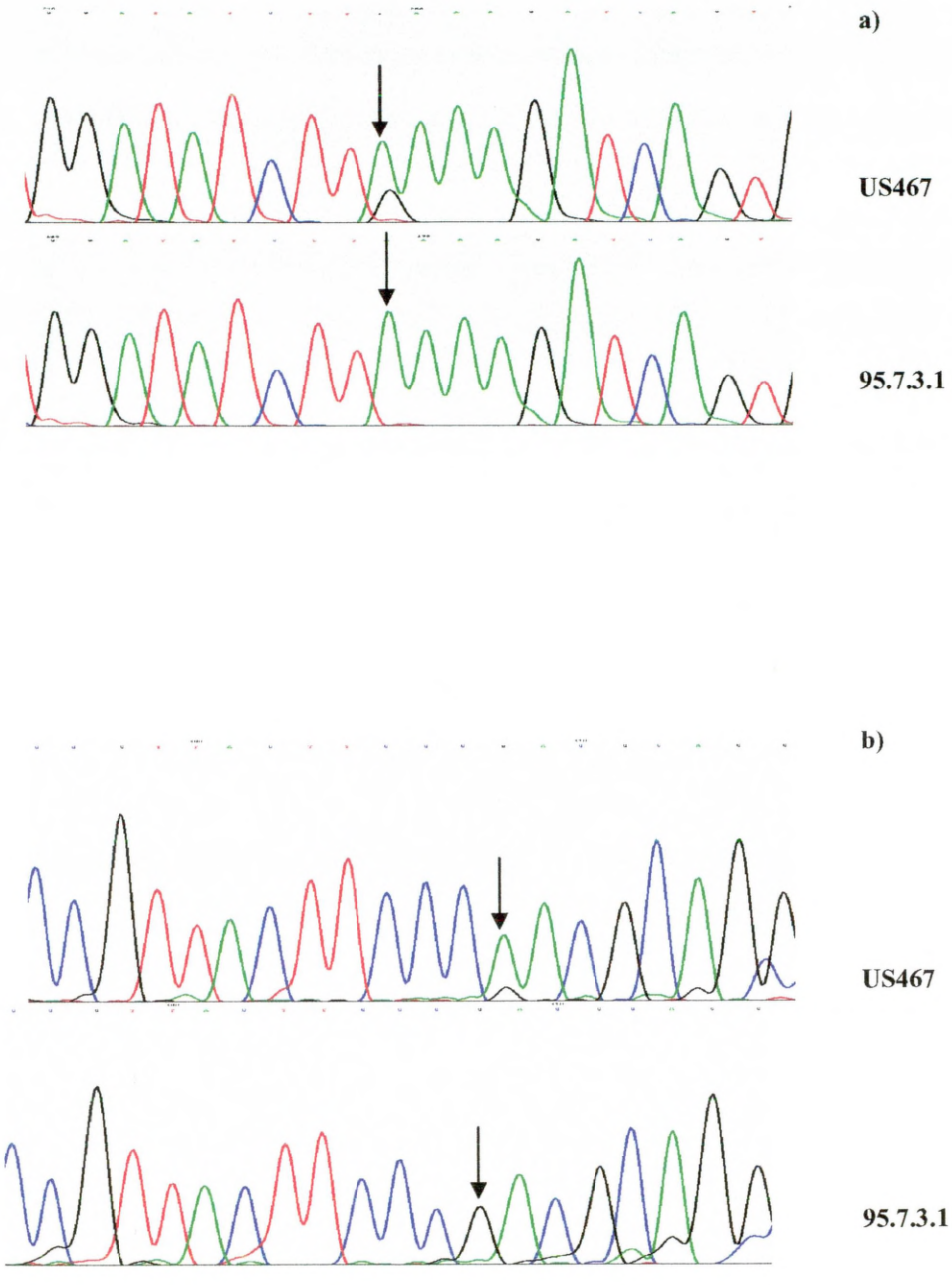


Fig. 4.2 Electropherograms resulting from sequencing of fragments of genes a) *Ubiquitin conjugating enzyme* and b) *ADP/ATP translocase* (SNP No 308). Polymorphisms between isolates are marked with arrows.

Table 4.5 Summary of SNPs detected in seven *Phytophthora infestans* genes.

Isolate	Genes/base numbers									
	Pgd		Aldo		Glu	Ubi	Adp		Cyclo	Pgk
	181	322	433	469	20	249	146	308		
95.7.3.1	**C**	Y=C/T	G	G	K=G/T	A	T	G	-	-
97.36.2.2	S=G/C	**T**	G	G	K=G/T	A	T	G	-	-
97.40.1.3	S=G/C	Y=C/T	G	G	K=G/T	A	T	G	-	-
AG4	**C**	**T**	G	G	K=G/T	A	T	G	-	-
EC1	S=G/C	**T**	G	G	K=G/T	A	T	G	-	-
US467	S=G/C	Y=C/T	G	G	K=G/T	**R=A/G**	T	**R=A/G**	-	-
80029	S=G/C	Y=C/T	**R=A/G**	**C**	**G**	A	**C**	G	-	-
88133	**C**	Y=C/T	G	G	**G**	A	T	G	-	-

SNPs are numbered according to their position in the aligned sequence
Pgd = *2-phosphoglycerate dehydratase*; Aldo = *Transaldolase*; Glu = *Glutamine synthetase*; Ubi = *Ubiquitin conjugating enzyme*; ADP = *ADP/ATP translocase*; Cyclo = *Cyclophilin*; Pgk = *Phosphoglycerate kinase*
PGC Accession numbers are presented in Table 4.2
The bold letter represents the sequence variation as opposed to consensus sequence.

Table 4.6 State and percentage of SNPs and number of heterozygotes in seven *Phytophthora infestans* genes.

Gene	Base number	Synonymous/non-synonymous	% SNP	Number of heterozygotes
2-phosphoglycerate Dehydratase	181	Non-synonymous	12.5	1
Transaldolase	322	Synonymous	37.5	2
	433	Synonymous	-	-
	469	Non-synonymous	-	-
Glutamine Synthetase	20	Synonymous	12.5	1
Ubiquitin conjugating Enzyme	249	Synonymous	12.5	1
ADP/ATP Translocase	146	Synonymous	25	1
	308	Synonymous	-	-
Cyclophilin	-	-	-	-
Phosphoglycerate Kinase	-	-	-	-

% SNPs were calculated as the %age of SNPs for each gene over the total SNPs detected for all gene sequences

a)

```
           1                                                          50
ALDOC      LDQLKQFTTV VADTGDFEQI NKYKPQDATT NPSLLFKAAQ MEQYSALVDD
ALDOG      LDQLKQFTTV VADTGDFEQI NKYKPQDATT NPSLLFKAAQ MEQYSALVDD
Consensus  LDQLKQFTTV VADTGDFEQI NKYKPQDATT NPSLLFKAAQ MEQYSALVDD

           51                                                        100
ALDOC      AVSYGKGLSA DLSEKERLGY VIDKLSVNFG LEILKVVPGY VSTEVDARLS
ALDOG      AVSYGKGLSA DLSEKERLGY VIDKLSVNFG LEILKVVPGY VSTEVDARLS
Consensus  AVSYGKGLSA DLSEKERLGY VIDKLSVNFG LEILKVVPGY VSTEVDARLS

           101                                                       150
ALDOC      FDTESTIARA HRIIDLYEKA GIKKDRILIK IASTWEGIQA CNHLQKEGIS
ALDOG      FDTESTIARA HRIIDLYEKA GIKKDRILIK IASTWEGIQA CKHLQKEGIS
Consensus  FDTESTIARA HRIIDLYEKA GIKKDRILIK IASTWEGIQA CNHLQKEGIS

           151
ALDOC      CNMT
ALDOG      CNMT
Consensus  CNMT
```

b)

```
           1                                                          50
PHOSPHOG   LPQRLLLGSG TGTAQSKRHT QNRVGAQLLL APAPLVLRAV QLLHHEAVDL
PHOSPSOC   LPQRLLLGSG TGTAQSKRHT QNRVGAQLLL APAPLVLRAV QLLHHEAVDL
Consensus  LPQRLLLGSG TGTAQSKRHT QNRVGAQLLL APAPLVLRAV QLLHHEAVDL

           51                                                        100
PHOSPHOG   GLLRHVLADK SRSDDLVHVL HSXQDTLAHV VLAAVTHLKG LKFARGGSAR
PHOSPSOC   GLLRHVLADK SRSDDLVHVL HTXQDTLAHV VLAAVTHLKG LKFARGGSAR
Consensus  GLLRHVLADK SRSDDLVHVL HSXQDTLAHV VLAAVTHLKG LKFARGGSAR

           101                              131
PHOSPHOG   DSGTVLAELG DEVNLNGGVA TRVKDLTGLD R
PHOSPSOC   DSGTVLAELG DEVNLNGGVA TRVKDLTGL
Consensus  DSGTVLAELG DEVNLNGGVA TRVKDLTGL. .
```

Fig. 4.3 Alignments of amino acid sequences of genes a) *Transaldolase* (No 469) and b) *2-phosphoglycerate dehydratase* with two different bases at the SNP site. In *Transaldolase*, the base change from C to G resulted in an amino acid change from asparagine (N) to lysine (K) at the SNP site. In *2-phosphoglycerate dehydratase*, the nucleotide change from G to C resulted in an amino acid change from serine (S) to threonine (T) at the SNP site. The italic letters G and C represent the allele type in various isolates. Amino acid change in the sequence is highlighted.

4.3.2 SNP discovery in non-coding DNA

In order to yield raw *P. infestans* sequence data from which non-coding regions could be selected for SNP discovery, sixty one BAC clones were end-sequenced. The BAC library was constructed from isolate T30-4 (Van der Lee *et al.*, 1997) and the sequence, because of its clonal origin, contained only a single allele i.e. it was homozygous at each base. The sequences ranged from 200-672 bp in length after manual contig trimming (Table 4.7). The nucleotide sequences were used to search the GenBank sequence database, applying the BLASTX algorithm to match translated sequences in the database (Swissprot). Eleven BAC clones showed sequence homology with small e-values (i.e. a strong match) to repetitive elements (Table 4.7) and were not considered further. Sequences of 50 of the BAC clones showed no significant match in GenBank (Table 4.7). The BAC clone 50P9F had a significant hit with 150 GenBank entries coding for serine/threonine specific protein. The e-value of the match ranged from $1e^{-30}$ - $5e^{-24}$. Sequences of three BAC clones including 56C9F, 7E1R and 72N2R did not show homology to repetitive sequences, but were not evaluated since their opposite end sequences were found to be polymorphic. The sequence of BAC clone 7C1F was too short (209 bp) to merit further analysis.

PCR primers were designed against forty six non-repetitive sequences. PCR products, amplified from a range of *P. infestans* isolates ranging from 134-362 bp were screened for mutations using a single strand conformational polymorphism assay (Section 4.1.5.2). Of the 46 primer sets, 16 did not result in successful amplifications or amplified non-specific PCR products and were not considered in further investigations. The remaining 30 BAC clones gave reproducible amplifications and were retained for further analysis (Table 4.7).

Table 4.7 List of sequenced BAC clones, their size, BLAST results and, if selected for screening, details of primers, melting temperature (Tm) and PCR product size. BAC clones not used and the reason for their rejection is also shown.

BAC clone	Sequence length (bp)	Significant number of hits to GenBank sequences	Forward sequence	Reverse sequence	Product size (bp)	Tm (°C)	Remarks
32A2F	231	3	GCTCTTAAGGGCTCCGTCA	GACTTCTCCGCGACTACGTA	195	59	Monomorphic
7E1F	335	1	CGGAAGTTACCGAGAGAGCA	CAGACTGAAAGGACCCAGCA	308	59	Polymorphic
7I1F	498	-	ATATAACGGCCACCTCGTCA	ATGCTTTTCTACCCCCGCAA	318	59	Polymorphic
50P9F	366	150	CTGCGGTTCGCCTTGCTA	TCGGGCTTTTGCACACCA	306	59	Monomorphic
56I3R	323	3	CGCAAGGTGCTGCGTGA	ATGTGCCAGCTGCAACGA	177	60	Polymorphic
56N16R	625	-	GCTATCTGCTCCCGTGCA	GCTGCGGGTTCAGCAGA	333	60	Monomorphic
63J7R	470	6	ATGAGCGTCGCCATTGGA	CGTCGTGAGCACACTGGA	332	59	Monomorphic
65P12R	580	3	GCTAGCGACGCTGAACGA	CAGTGCCGTCTGCGTGA	316	59	Monomorphic
56C9R	500	6	TCGTCGGTGTCCTCTTCTGA	ATGAGACGCCCAGAAGGAGA	319	65	Polymorphic
72N10R	314	5	GATATCAGAGCGCGGAGC	TTTACACCTCGACGTCCAGA	259	60	Monomorphic
56A5R	542	9	TGTGGCCCTCAAAGCCAA	CAGCTCCGTGAGCTCACA	354	60	Monomorphic
56A9R	595	1	ATAGACGGTCTGATCGTGCA	CGGATAGTCACAAACGACA	312	60	Monomorphic
56D11R	640	-	GTCCGGTTGAGTCGCGAA	TATCCGCTCGGCGTGCTA	307	60	Monomorphic
56E14R	603	10	TGGCAGCGGACAGAGGA	CGCGGATTGACTCCAGCA	301	59	Polymorphic
56F16R	564	1	ACATCGAGCACGGTCGA	CAGCGTAGTGGTGGCGAA	310	59	Monomorphic
56G18R	579	1	GGCACTGGACGACATGGA	ACACGGGAGCCTCTGGAA	351	59	Polymorphic
72N2F	440	3	CTTGGAAGAGCTGCGAGCA	CTTGTGCACGCTGAAATCCA	278	59	Polymorphic
72N6F	303	19	GGGAAACTTCATGCGACA	CGGCCCGTAGATAGTAGACA	265	59	Monomorphic
72N12F	374	7	ATGTCTGTTTCTGCCGACGA	ACGTAGAGACGTCGGCTGA	307	59	Monomorphic
32A2R	331	3	CCAATGGTTGGCCCCTGA	CGACCTTAGTGGGGGAGGA	134	60	Polymorphic
7K1R	517	5	ATGTTTCGTCACGAGGAGCA	AACATCTTCCGCATCGGAA	320	60	Monomorphic
50P9R	415	1	TCGAATTTCTTGGCCGACCA	ATCGGGCTGCCACAGCTA	308	59	Monomorphic
72N4R	493	3	GCTTGCAAACTGCACGCTA	ACACCACTCCTGTACCGGTA	314	59	Monomorphic

Table 4.7 (*Continued*)

BAC Clone	Sequence length (bp)	Significant number of hits to GenBank sequences	Forward sequence	Reverse Sequence	Product size (bp)	Tm (°C)	Remarks
56A9F	488	7	CGAGGACGCTGACAAGGA	TCTCTAGCCGAGCTCGA	330	58	Monomorphic
59B18F	542	1	AGGCTCAGGTGAAGGAGGTA	AACTCTTTCAGCGCTGACGA	324	58	Monomorphic
59O11F	461	-	CTTGGGCAGCTTGTCCGTA	CAGGTCCAACAAAACGCAGA	295	58	Monomorphic
56F16F	547	-	CCGATGCGACACCGGAA	GCGTCGTGCAATGTGTGA	343	59	Monomorphic
38J7R	513	3	AGCGGCAAACCTGAACGA	CACTTCCGGTCGCCGAA	362	60	Monomorphic
56N16F	356	1	TGAAAGGCTGCTCCCTCGTA	CACGCAAACGGCGCCAA	191	59	Monomorphic
56B7F	473	1	GAGAGTGTTCAGCCGCTGA	GTACCCCTGTGTGTTCCACA	359	59	Monomorphic
38J7F	477	1	ACTTTGCGGCCTCGCAA	TCGTGCGCAACTTGGACA	290	52-65	PCR failed
63J7F	392	-	CAGGAAGCCGCCAACGTA	CGTACAATGGGCACTTTCCA	318	52-65	PCR failed
56J20F	479	473	AACCAGATGCCGAAGTCGA	TGGATGCTTTCGAGCGTGA	318	52-65	PCR failed
56I3F	581	2	AGGGCTTTCCATCAGCACA	ATCAAGTGCCGGCTCACA	349	52-65	PCR failed
56H1F	575	1	TAGCTGGAGCGCTGTCCA	CCCGAGCCAGCTTGCAA	318	52-65	PCR failed
72N10F	382	6	CTGCAATCCCGTCGGCAA	CAGGCCAGCAAAGCTCGA	287	58-60	PCR failed
72N4F	395	1	TGGCAACATGGGTCTCCAA	CAGGCCAGCAAAGCTCGA	295	58-60	PCR failed
7KIF	500	3	AGGTCGGTGGTTCAAATCCA	TGGAGAGACCTCGTGGTGA	316	58-60	PCR failed
22B2F	458	12	TAGCGCGGAGGTACGTGA	CGTGCGTGCTCTTCCGTA	296	58-60	PCR failed
32A10F	366	7	CTGCGCAAGCTCGCAGA	TTCCCCCCGGTTGAAGGA	255	58-60	PCR failed
7A1F	386	2	CCATTGATGGGCAGGGACA	TACGTACTGGGGGTGTCTCA	311	58-60	PCR failed
72N12R	200	2	GCTACGCCTTAGCCCGAA	CACGCATCACCACATTCACA	167	58-61	PCR failed
7GIR	470	23	CGGATTCCGTTGGGCGTA	TTTCAGGTCCGAGGCCGTA	310	58-61	PCR failed
7C1R	493	3	GTCTAACGCGTCGGTGA	GTGCGCTCGTTGCGAA	312	58-61	PCR failed
7A1R	275	1	TTCTTCTGCGTCCGTGGA	CGGAAAGGTTGCCCCGAA	228	58-61	PCR failed
56A5F	458	0	GGTTGTAACGGGCCGGAA	GCAGCAGCACACACGGCTA	249	52-65	PCR failed

Table 4.7 (*Continued*)

BAC Clone	Sequence length (bp)	Significant number of hits to GenBank sequences	Forward sequence	Reverse sequence	Product Size (bp)	Tm (°C)	Remarks
65P12F	507	187	-	-	-	-	Repetitive DNA
56D11F	542	719	-	-	-	-	Repetitive DNA
56C9F	294	36	-	-	-	-	Reverse sequence polymorphic
56H1R	672	279	-	-	-	-	Repetitive DNA
56B18R	554	500	-	-	-	-	Repetitive DNA
59O11R	616	19	-	-	-	-	Repetitive DNA
56J20R	521	503	-	-	-	-	Repetitive DNA
50P1R	291	28	-	-	-	-	Repetitive DNA
7I1R	505	20	-	-	-	-	Repetitive DNA
7E1R	380	10	-	-	-	-	Forward sequence polymorphic
50P1F	305	37	-	-	-	-	Repetitive DNA
72N2R	515	10	-	-	-	-	Forward sequence polymorphic
7G1F	550	94	-	-	-	-	Repetitive DNA
22B2R	469	5	-	-	-	-	Repetitive DNA
7C1F	209	1	-	-	-	-	Short product

F and R at the end of BAC clone name represents forward and reverse BAC end- sequences

Amongst the twelve isolates tested, SSCP analysis identified eight out of 30 BAC clones as polymorphic. Typical monomorphic and polymorphic patterns are shown in Fig. 4.4. The SSCP pattern differed from clone to clone, with some yielding simple and others more complex patterns. Within BAC fragment 7I1F (Fig. 4.5a), four different fingerprint patterns were distinguished amongst the 12 *P. infestans* isolates. Within fragments of BAC clones 56I3R, 7E1F and 56E14R (Fig. 4.5b-d), three different fingerprint patterns were observed. Only two fingerprint patterns were clear among BAC clones 56G18R, 72N2F and 32A2R (Fig. 4.6a, c, d). Results of BAC fragment 56C9R were difficult to interpret as isolates formed 3-4 groups which were not sharply defined (Fig. 4.6b). The twenty two BAC clones that were monomorphic across the twelve isolates were not processed further (Table 4.7).

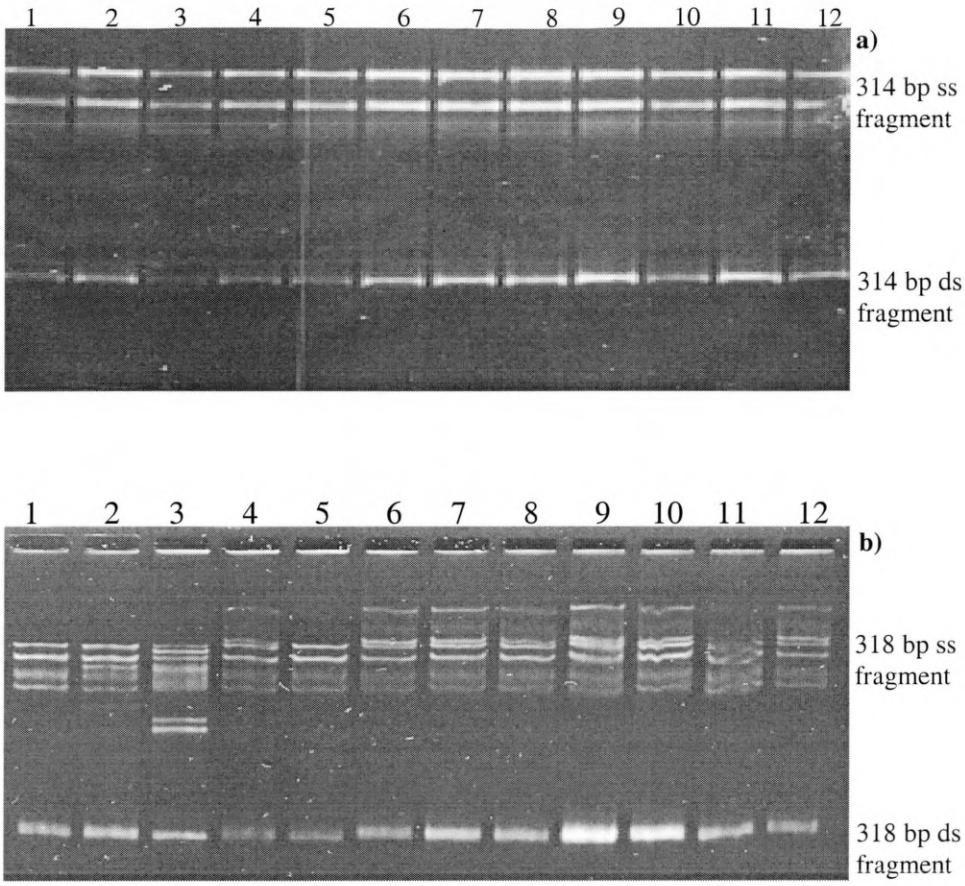


Fig. 4.4 GMA gel analysis of the PCR products from twelve *P. infestans* isolates (Lanes 1-12) amplified with primers designed against BAC clones a) 72N4R and b) 7I1F. These patterns have been selected to demonstrate monomorphic (a) versus polymorphic (b) products revealed by SSCP analysis.

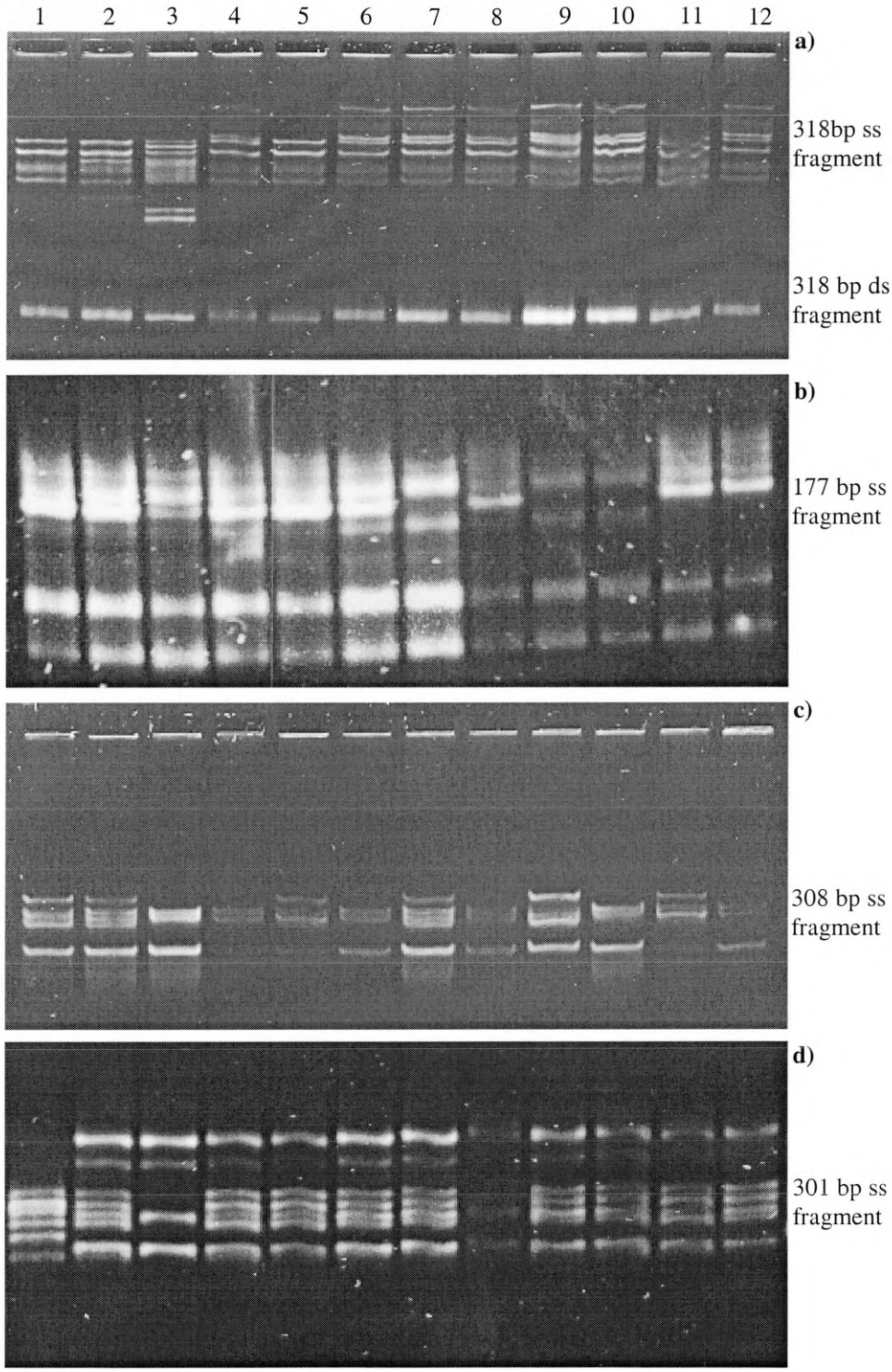


Fig. 4.5 Polymorphisms among twelve isolates (Lane 1-12) of *P. infestans* in various randomly selected BAC clones a) 7I1F; b) 56I3R; c) 7E1F; d) 56E14R revealed by SSCP analysis on GMA gels.

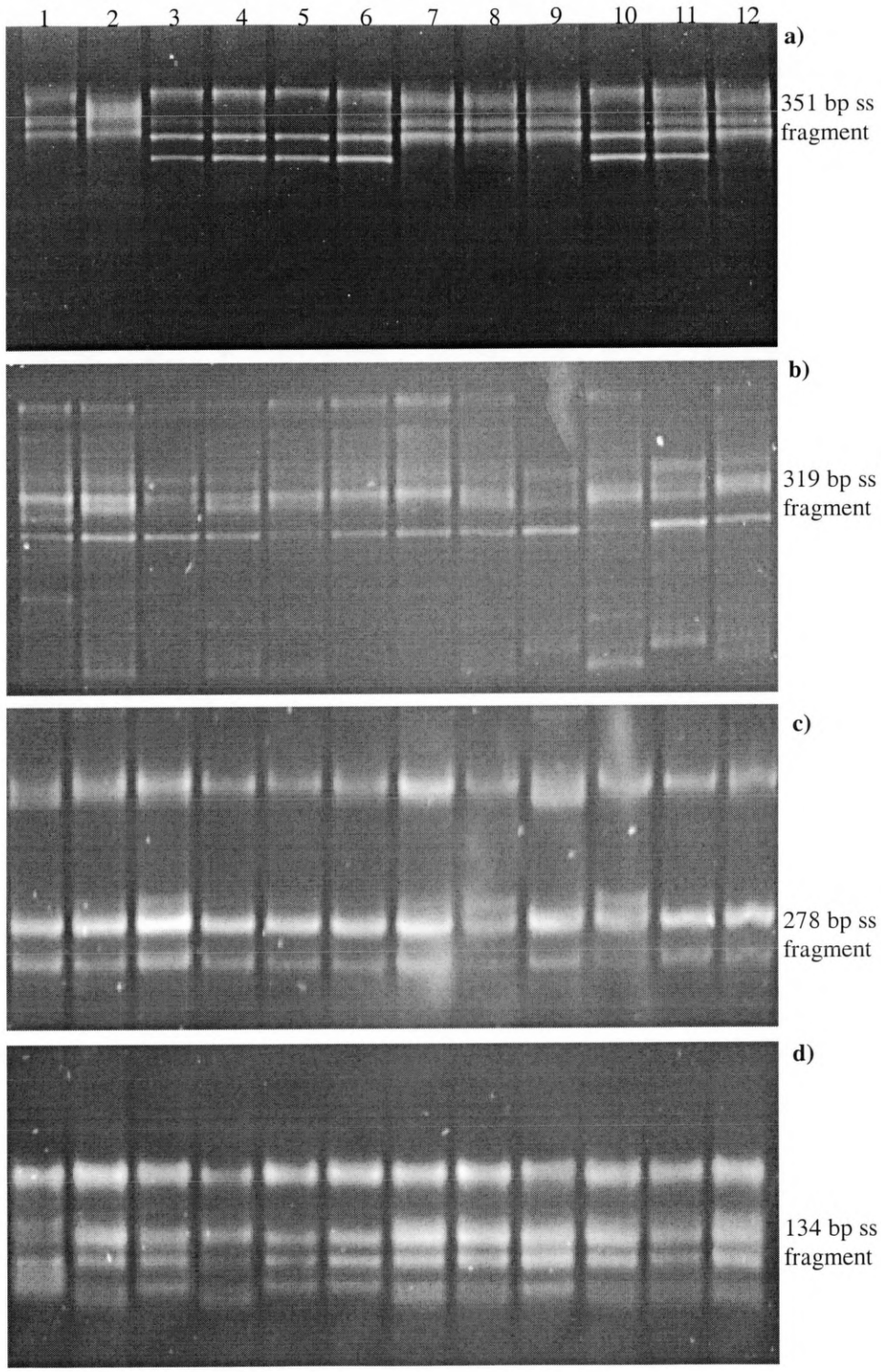


Fig. 4.6 Polymorphism among twelve isolates (Lane 1-12) of *P. infestans* in various randomly selected BAC clones a) 56G18R b) 56C9R; c) 72N2F; d) 32A2R revealed by SSCP analysis on GMA gels.

The PCR products of isolates representing individual pattern on the GMA gels were sequenced to determine the nature of the base change and its position in the sequence. Sequencing always confirmed the SSCP results whenever SSCP patterns showed clear differences amongst the isolates. Although BAC clones 56C9R and 72N2F were considered polymorphic (Fig. 4.6) amongst the twelve isolates in SSCP, the polymorphism was not particularly clear and indeed subsequent sequencing revealed that these clones were monomorphic. SSCP polymorphisms were confirmed in six of the eight BAC clones by sequencing PCR amplified BAC fragments. A total of 28 polymorphisms were detected in the six polymorphic BAC clones (Table 4.8). This represents 2 SNP per kb within the total 14 kb of BAC clones sequenced. In BAC clone 7I1F, the sequence of the isolate US467 showed a deletion of 9 bp (AGAGCTCAG) at nucleotides 247-255, which was responsible for the unusual SSCP patterns of this isolate (Fig. 4.5a). Of the total 28 polymorphisms, 25 were SNPs, two were insertions and there was one deletion. The SNPs consisted of thirteen transitions and seven transversions. In five cases, two different peaks were found in the electropherogram demonstrating heterozygous state. Within the BAC clones, 56G18R showed the highest number of SNPs (11) in a 351 bp sequence which represents 39% of the total SNPs detected stretched over nucleotide numbers 107-299 (Table 4.9).

Table 4.8 Summary of SNPs detected in randomly selected BAC clones of different isolates of *Phytophthora infestans*. Representative isolates were sequenced for each marker. Isolates not sequenced are indicated by gaps. Blank cells represent absence of insertion.

	BAC Clones													
	32A2R		7I1F		56E14R								7E1F	
Isolates	53	91	211	247-255	114	119	122	130	156	165	186	217	62	93
AG4	-	-	-	-	S=G/C	**T**	T	**R=A/G**	Ins A	**S=G/C**	**G**	T	-	-
EC1	-	C	G	AGAGCTCAG	-	-	-	-	-	-	-	-	-	-
US467	-	C	G	Del 9 bp	G	A	A	G		C	T	C	-	-
95.16.3.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96.9.5.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96.10.3.3	-	-	-	-	-	-	-	-	-	-	-	-	T	Ins C
96.17.5.3	-	-	-	-	G	A	A	G		C	T	C	-	-
97.11.5.1	-	A	G	AGAGCTCAG	S=G/C	A	T	G		C	T	T	-	-
97.28.1.1	A	-	-	-	-	-	-	-	-	-	-	-	**C**	
97.31.1.3	R=A/G	A	**R=A/G**	AGAGCTCAG	-	-	-	-	-	-	-	-	T	
97.31.2.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.8 (*Continued*)

	BAC Clones													
	56I3R			56G18R										
Isolates	106	116	143	107	135	140	196	209	244	269	270	273	293	299
AG4	-	-	-	T	G	A	G	G	A	G	A	C	T	T
EC1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
US467	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95.16.3.1	**A**	**C**	**C**	-	-	-	-	-	-	-	-	-	-	-
96.9.5.1	G	**T**	G	-	-	-	-	-	-	-	-	-	-	-
96.10.3.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96.17.5.3	-	-	-	**C**	**C**	**G**	**C**	**A**	**G**	**A**	**T**	**G**	**G**	**C**
97.11.5.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97.28.1.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97.31.1.3	G	C	G	T	G	A	G	G	A	G	A	C	T	T
97.31.2.3	-	-	-	T	G	A	G	G	A	G	A	C	T	T

Bold letters represent SNPs compared to consensus sequences
Numbers under each BAC clone represent the base at which SNP was detected after sequence alignment
Ins = insertion; Del = deletion

Table 4.9 Percentage of SNPs and number of heterozygotes detected in various BAC clones.

BAC Clone	% SNP	Number of heterozygotes
32A2R	3.57	1
7I1F	10.71	1
56E14R	28.57	3
56G18R	39.28	0
7E1F	7.14	0
56I3R	10.71	0

% SNPs were calculated as the %age of SNPs for each BAC clone over the total SNPs detected for all BAC clones

4.3.3 PCR assays using allele specific PCR

In order to convert the SNPs identified into PCR assays, allele specific PCR was developed. To distinguish between alleles that differ by a single nucleotide, the region containing the base change (SNP) was PCR amplified (Drenkard *et al.*, 2000). Each primer set used consisted of an allele specific and alternate primer, which contained both the 3′ terminal base change plus a mismatch in the 3 bp prior to the 3′ terminal base. Two PCR reactions were performed for each isolate to discriminate the alleles on the basis of allele specific amplification. To give flexibility, more than one primer set was designed for each marker, and one of these sets was selected during the optimisation of primers. Allele specific primers developed for markers 7E1F and 56I3R did not reliably discriminate the two alleles at the representative loci and were not considered further. A total of 22 primer sets, designed against the nine SNPs, were tested, and the primer sets that best distinguished the alleles was selected (Table 4.10). PCR products were analysed by agarose gel electrophoresis and the presence or absence of bands in the two reactions was scored for each isolate. Isolates with single bands were scored as homozygous and those with two bands as heterozygous for that SNP.

Table 4.10 Allele specific and alternate primers designed for various sequences used in allele specific PCR. Those marked in bold were the ones selected for routine use. Expected product size and melting temperature (Tm) are also shown.

Marker	SNP #	Primer	Forward sequence	Reverse sequence	Size (bp)	Tm (°C)
Pgd	181	SNP1	CTCGTTCACGTTCTTCGCAG	GATCTTTGACTCGCGTGGC	181	59
		ALT1	CTCGTTCACGTTCTTCACCC	"	181	59
		SNP2	**CTCGTTCACGTTCTTCCCAG**	"	**181**	**59**
		ALT2	**CTCGTTCACGTTCTTCACGC**	"	**181**	**59**
Aldo*	322	SNP1	GCTTAGGCGAGCATCAACCTAG	CCCAACAAGAAGCTCCGC	330	59
		ALT1	GCTTAGGCGAGCATCAACCTTA	"	330	59
		SNP2	AGGCGAGCATCAACCACG	"	326	59
		ALT2	TTAGGCGAGCATCAACCTCA	"	328	59
		SNP3	**TTAGGCGAGCATCAACTTCG**	"	**328**	**59**
		ALT3	**CTTAGGCGAGCATCAACTTCA**	"	**329**	**59**
Glu	20	SNP2	TGTCCCTCGACGTGTCTGTG	GGATCCTTGAAGATGGCCAC	214	60
		ALT2	TCCCTCGACGTGTCGGAT	"	212	60
		SNP3	TCCCTCGACGTGTCTGGG	"	212	60
		ALT3	GTCCCTCGACGTGTCTGGT	"	213	60
		SNP4	**TGTCCCTCGACGTGTCTCAG**	"	**214**	**60**
		ALT4	**GTCCCTCGACGTGTCTGCT**	"	**213**	**60**
Ubi*	249	SNP1	CAGGGCTCCACTGATCTTGC	AGAGCTGCAGGATCTGGGTC	263	59
		ALT1	CAGGGCTCCACTGATCTTGT	"	263	59
		SNP2	CAGGGCTCCACTGATCTGTC	"	263	59
		ALT2	CAGGGCTCCACTGATCGTTT	"	263	59
		SNP3	**CAGGGCTCCACTGATCGTTC**	"	**263**	**59**
		ALT5	**CAGGGCTCCACTGATCCTTT**	"	**263**	**59**

Table 4.10 *(Continued)*

Marker	SNP #	Primer	Forward sequence	Reverse sequence	Size (bp)	Tm (°C)
ADP*	308	**SNP1**	**GAAGTTCAAGGCCTGCGAC**	TCGTGGACTGCTTCGTGC	**107**	**60**
		ALT2	**CGAAGTTCAAGGCCTGCTTT**	"	**108**	**60**
		SNP2	AAGTTCAAGGCCTGCGGC	"	106	58
		ALT1	GAAGTTCAAGGCCTGCGAT	"	107	58
		SNP3	GAAGTTCAAGGCCTGCCTC	"	107	58
		ALT7	CGAAGTTCAAGGCCTGTGTT	"	108	58
32A2R	53	SNP1	TGTACAAGGTATCCCGCGG	GAGTCGCTTTTACCCGCC	235	58
		ALT1	TTGTACAAGGTATCCCGGGA	"	236	58
		SNP2	**TTGTACAAGGTATCCCGTGG**	"	**236**	58
		ALT2	**TTGTACAAGGTATCCCGCGA**	"	**236**	58
7I1F	211	**SNP1**	**GCTAGAGCTTGTATGGGCCG**	CTACCCCCGCAAATATCGC	**138**	**60**
		ALT1	**GCTAGAGCTTGTATGGGCCA**	"	**138**	**60**
		SNP2	GCTAGAGCTTGTATGGCCCG	"	138	60
		ALT2	GCTAGAGCTTGTATGGCCCA	"	138	60
56E14R	114	**SNP1**	**GATCCGAATACAGGCCCG**	GCCCGTTCACTAGCTGCC	**313**	**59**
		ALT3	**TGGATCCGAATACAGGCATC**	"	**315**	**59**
		SNP2	GGATCCGAATACAGGCTCG	"	314	59
		ALT2	GATCCGAATACAGGCCCC	"	313	59
56G18R*	209	SNP6	ACAGCGAAGGTTGTGTGACC	CATGGCACTGGACGACATG	230	65
		ALT6	CAGCGAAGGTTGTGTGGCT	"	229	65
		SNP7	**ACAGCGAAGGTTGTGTGTGC**	"	**230**	**65**
		ALT7	**CAGCGAAGGTTGTGTGCCT**	"	**229**	**65**

Pgd = *2-phosphoglycerate dehydratase*; Aldo = *Transaldolase*; Glu = *Glutamine synthetase*; Ubi = *Ubiquitin conjugating enzyme*; ADP = *ADP/ATP translocase*; Cyclo = *Cyclophilin*; Pgk = *Phosphoglycerate kinase* Markers marked with an asterisk were those for which primers were designed in the reverse orientation relative to the original sequence.

All primers shown in 5′ to 3′ orientation.

All the primer sets with single base changes at the 3′ end resulted in efficient amplification of genomic DNA. Amplification was not robust with a change in the annealing temperature. The reactions were therefore optimised and a standard annealing temperature was applied to each allele specific assay.

Allele specific primers designed for the *2-phosphoglycerate dehydratase* gene successfully discriminated alleles of the Scottish and world-wide *P. infestans* isolates, and this complemented previous sequencing results of the same isolates. Results in Fig. 4.7a show that five isolates (EC1, US467, 97.36.2.2, 97.40.1.3 and 80029) were heterozygous (C/G) at *2-phosphoglycerate dehydratase* locus and were amplified with both the primers, whereas the other three isolates (AG4, 95.7.3.1, and 88133) were homozygous (C/C) and were amplified with the primer having a C at the 3′ end.

Primer $Aldo^A$ (alternate allele primer) generated an amplification product for allele 'T' in isolate 97.36.2.2 and no amplification product for the allele 'C'. Isolates 88133, US467, 95.7.3.1 and 97.40.1.3 produced amplification products with both allele specific and alternate primers $Aldo^S$ and $Aldo^A$ (Fig. 4.7b). Genomic DNA of two isolates which were either homozygous with respect to the SNP site, or heterozygous at the locus *Glutamine synthetase*, as previously determined by sequencing, were each amplified with allele specific primers. Primer Glu^S corresponded to the base 'G' and primer Glu^A corresponded to the variant base 'T'. In isolate US467, a PCR amplification product was produced with both primers. However, no product was visible with primer Glu^A when isolate 80029 was tested, thereby indicating that this was a homozygous isolate (G/G) (Fig. 4.7b).

Primer set $32A2R^S$ and $32A2R^A$ was tested against a range of Scottish *P. infestans* isolates. Results (Fig. 4.7c) show that the allele specific primer set complemented the sequencing results by generating two 236 bp bands for the isolates 97.31.2.3, 97.31.5.3 and 97.31.1.3. For isolates 97.28.1.2, 97.28.1.1 and 97.28.2.1 the primer set displayed the presence of PCR product for the allele *A* only thereby demonstrating that the isolates were homozygous (A/A).

A single band of the expected size was observed in amplification reactions for isolates 97.40.1.3 and 97.40.8.3 with the two primer sets for the marker 7I1F, thereby confirming that the isolates were homozygous (G/G) (Fig. 4.7c). The same marker when tested using genomic DNA of the isolates 95.17.3.2 and 97.38.2.2 resulted in the amplification of the product of expected size with both primers suggesting that the isolates were heterozygous (G/A).

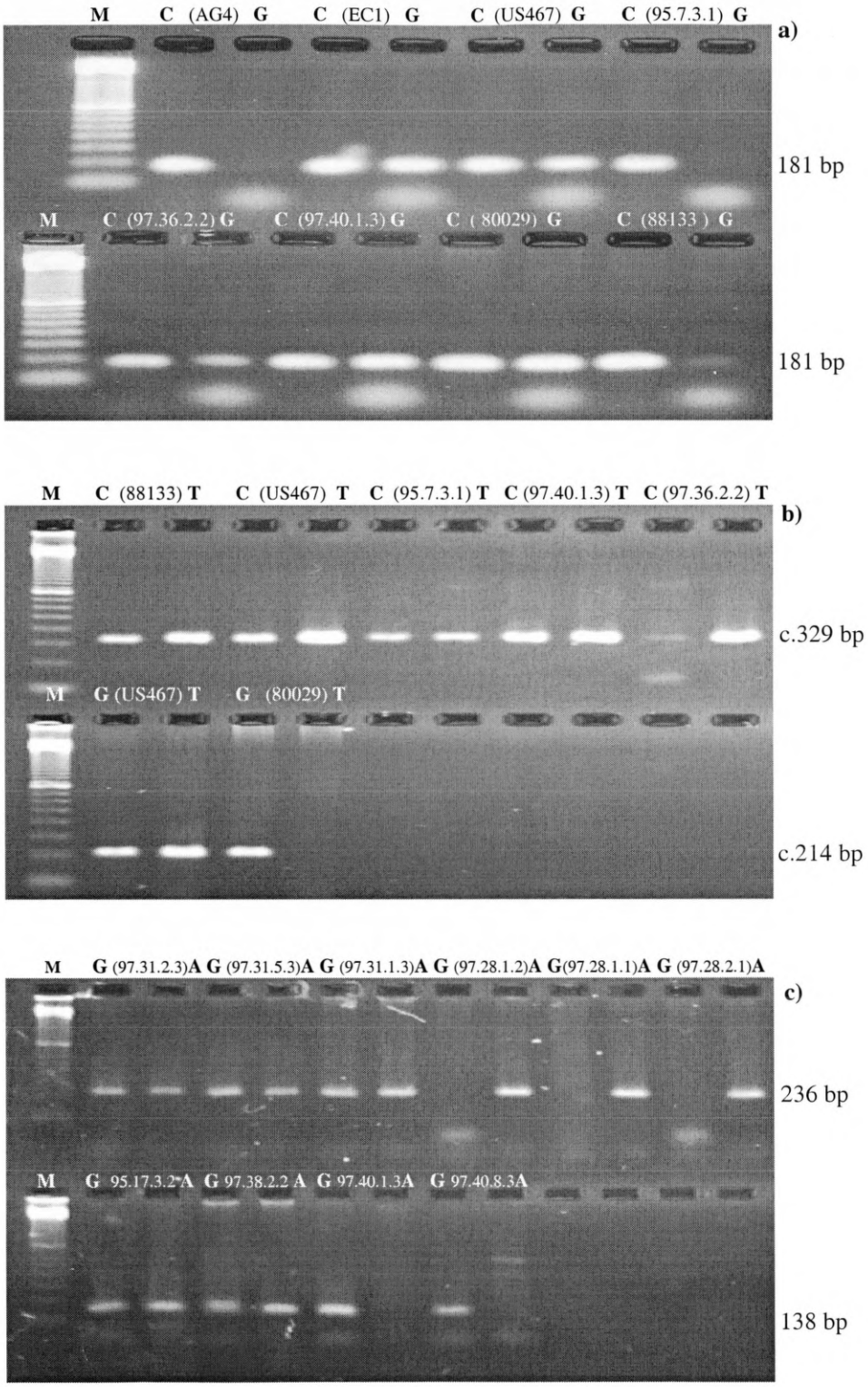


Fig 4.7. Genotyping of Scottish and world-wide isolates of *P. infestans* assessed using an allele specific PCR assay with markers a) *2-phosphoglycerate dehydratase*; b) *Transaldolase* (top) and *Glutamine synthetase* (bottom); c) 32A2R (top) and 7I1F (bottom). Total genomic DNA of isolates was amplified with two sets of primers, with a different SNP base (marked on the top of the lanes) at 3′ ends. The isolates are shown alongside the SNP base combination. The first lane of each gel is a 100 bp size marker (M).

For markers 56E14R and 56G18R, the primer set successfully discriminated the isolates selected on the basis of sequencing results. There was a good correlation between the results obtained via sequencing and allele specific PCR. At locus 56E14R, isolate 97.31.2.3 was heterozygous (G/C) whereas isolate 97.39.1.3 was homozygous (C/C) (Fig. 4.8a). Similarly, at locus 56G18R, isolates 95.17.3.2 and 97.38.2.2 were heterozygous (G/A) whereas isolates 97.31.1.3 and 97.31.2.3 were homozygous for allele *G* (G/G) and isolate 96.17.5.3 was homozygous for allele *A* (A/A) (Fig. 4.8a).

Within the Scottish isolates tested, no polymorphism was detected using the *Ubiquitin conjugating enzyme* and *ADP/ATP translocase* derived SNP markers, but polymorphism was seen amongst the international isolates. Isolate EC1 (from Ecuador) and 97.40.1.3 (from Scotland) were homozygous (A/A) at the *Ubiquitin conjugating enzyme* locus, and isolate US467 (from U.S.) was heterozygous (G/A) at the same locus (Fig. 4.8b). The results of allele specific PCR were again consistent with those of the sequencing. The allelic state of isolates when assessed with the allele specific primers for marker *ADP/ATP translocase*, revealed that isolate US467 (from U.S.) was heterozygous (G/A) and isolates EC1 (from Ecuador), 97.40.1.3 and 95.7.3.1 (from Scotland) were homozygous (G/G) (Fig. 4.8b).

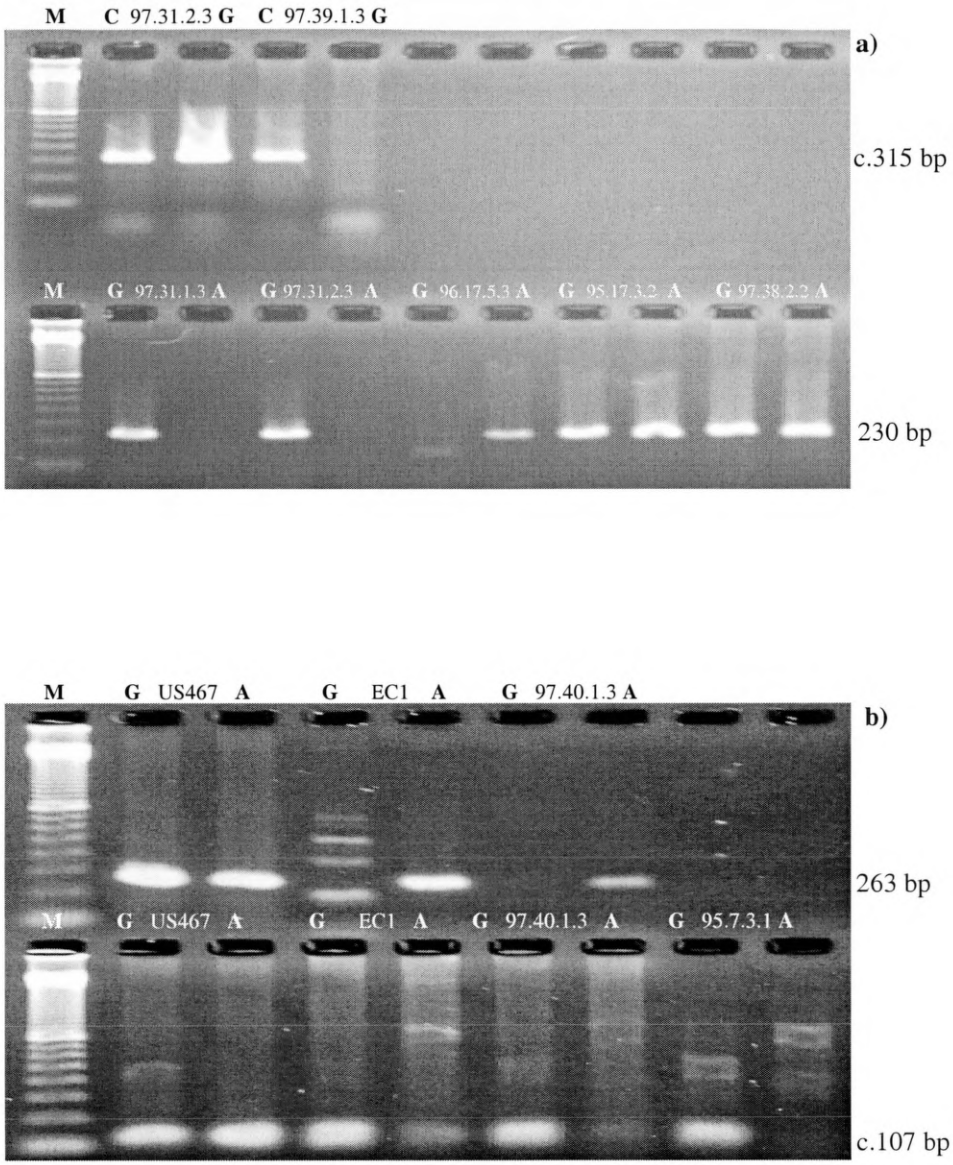


Fig. 4.8 Genotyping of Scottish and world-wide isolates of *P. infestans* assessed using an allele specific PCR assay with markers from a) 56E14R (top) and 56G18R (bottom); b) *Ubiquitin conjugating enzyme* (top) and *ADP/ATP translocase* (bottom). Total genomic DNA of isolates was amplified with two sets of primers, each with different SNP base (marked on the top of the lanes) at 3′ ends. The isolates are shown along with the SNP base combination. The first lane of each gel is a 100 bp size marker (M).

4.3.4 High throughput genotyping assays

Gel-based PCR assays for SNP scoring were converted into high throughput automated assays using fluorescently labelled primers and an ABI 3700 capillary-based sequence detector. Optimisation of the assay was performed by testing a range of PCR product dilutions (1:2-1:100) and a combination of markers in a single well. Dilutions of PCR product (amplified from 50 ng DNA) with water in a range between 1:2 - 1:10, were found suitable for automated detection of SNPs when tested with three markers; *Transaldolase*, 7I1F and 56G18R (Fig. 4.9). In each case, alleles were assigned on the basis of detection of fluorescence from allele specific and alternate primers. Heterozygotes were easily distinguished from homozygotes by the presence of two fluorescent peaks in the electropherogram (Fig. 4.10). Simultaneous analysis of fourteen alleles representing seven different loci in a single well of a 96-well microtitre plate was successful. Despite the limited dye colour combinations used for primer labelling, the differences in PCR product size (138-329 bp) allowed discrimination of different markers in the same well of the microtitre plate.

In order to monitor the quality of genotyping and the allele calling procedure, a sub-set of 42 *P. infestans* isolates (Table 4.1) were used to compare the results of a gel-based genotyping using seven SNP markers with the automated assay. Results showed that there was a good correspondence between the genotypes obtained by fluorescent detection and the conventional gel-based assay. In some cases there were differences in allele scoring between fluorescent and gel-based assays. Comparison of the two genotyping data sets revealed that there was an error rate of ~ 4% in 294 samples. Seven genotypes were scored as heterozygous by the Genotyper software but as homozygous by gel-based assay. Five genotypes were scored as homozygous by the Genotyper, when these were heterozygous according to gel-based assay. Since the gel-based assays were repeated twice, alleles consistent with gel-based assay were considered to be correctly interpreted. The rate of missing genotypes was ~ 2% which were most likely due to pipetting errors.

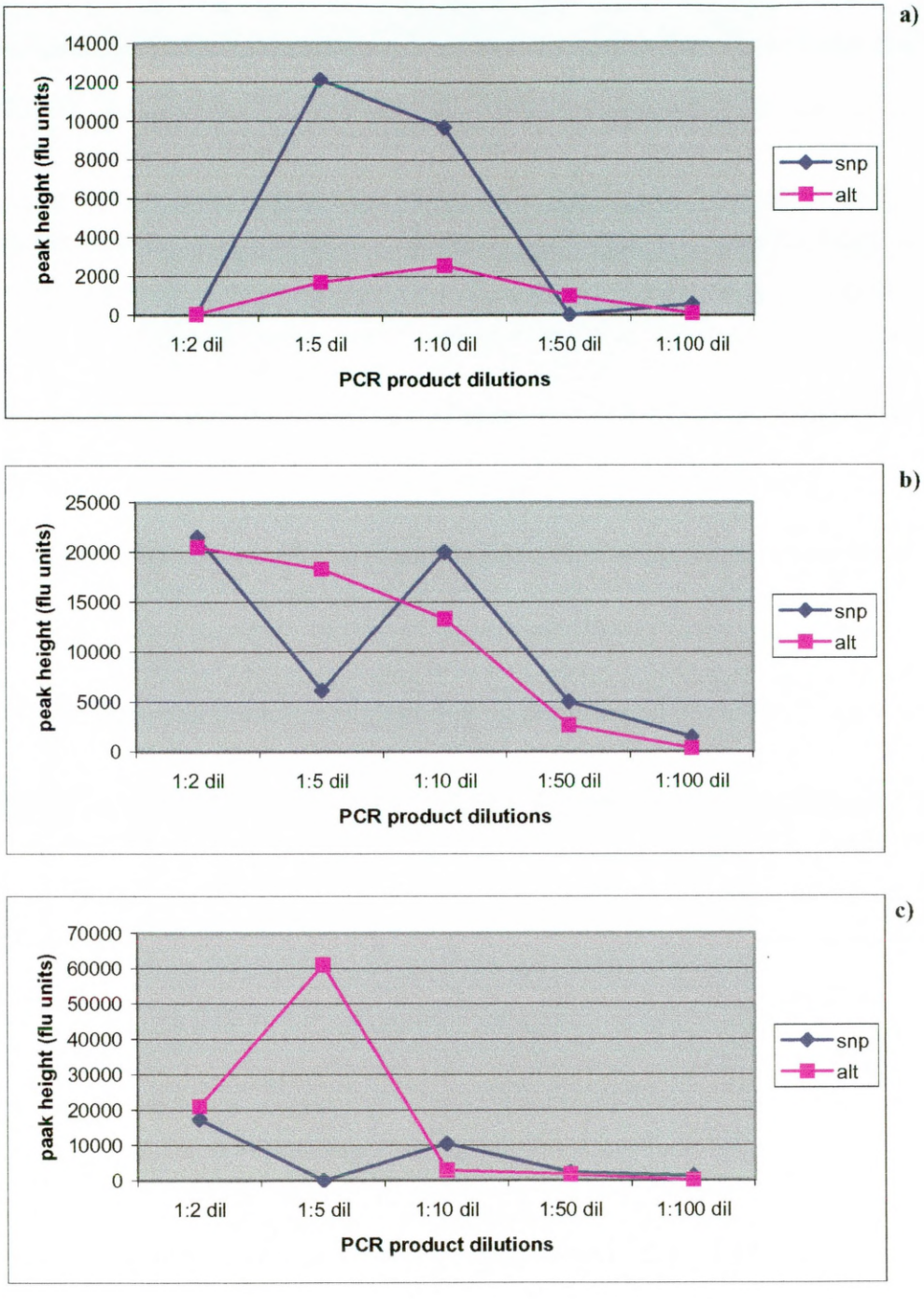


Fig. 4.9 Effect of different dilutions of PCR products on intensity of fluorescence (peak height) for three different markers a) *Transaldolase*; b) 7I1F; c) 56G18R. Forward primers in each case were fluorescently labelled and diluted PCR products were run on the ABI 3700 capillary electrophoresis machine. 'snp' and 'alt' represent the allele specific and alternate primers. Peak height was measured in fluorescence units.

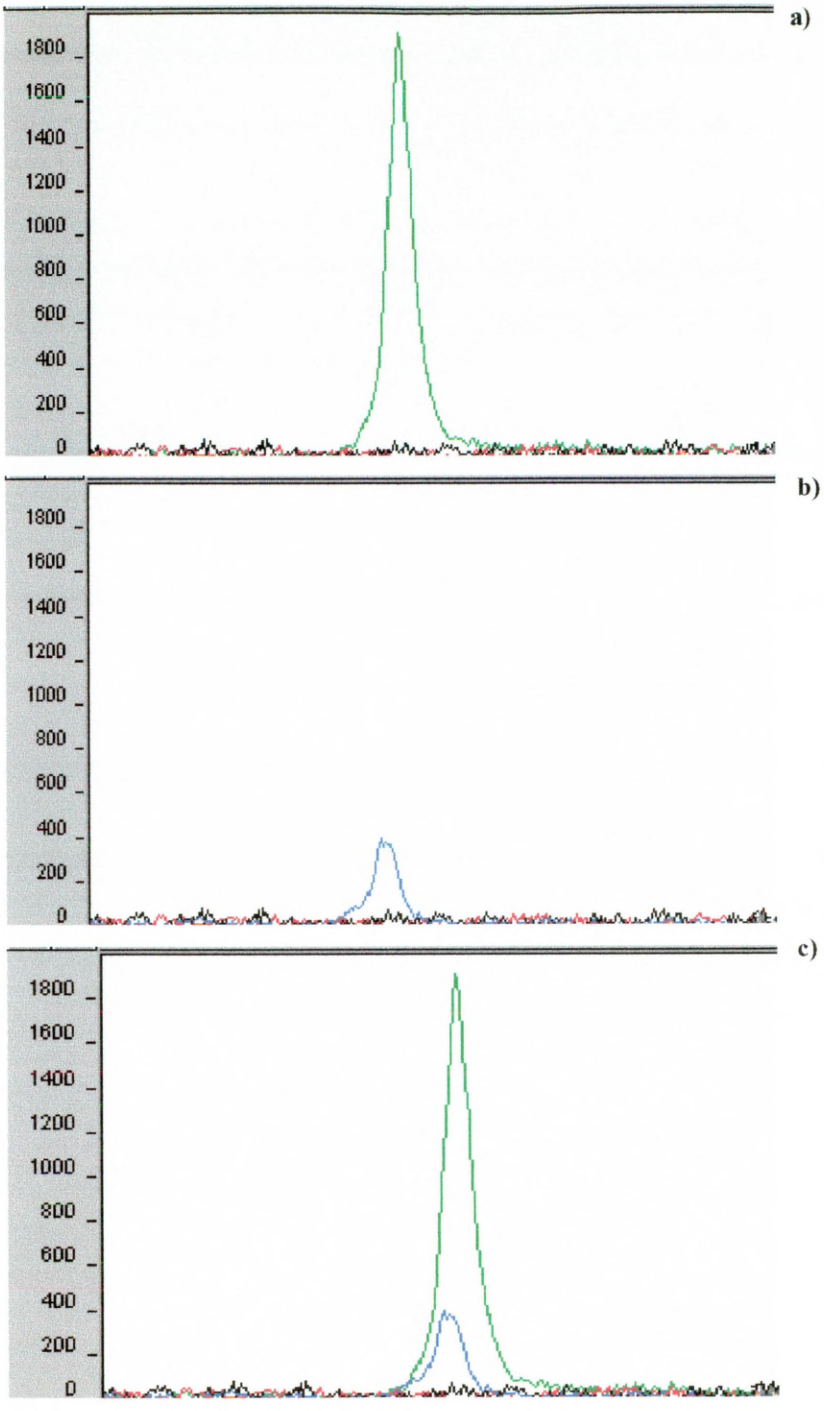


Fig. 4.10 A representative set of electropherograms for the marker *Glutamine synthetase*. The forward primers were labelled with either VIC (green) or FAM (blue) fluorescent dyes. Alleles can be distinguished on the bases of fluorescence where a) homozygous allele *T*; b) homozygous allele *G*; c) heterozygote (T/G). The Y-axis represents the relative fluorescence intensity.

4.3.5 Inheritance of molecular markers

In order to test the inheritance of SNP markers, a progeny of 20 isolates of a cross between two *P. infestans* isolates was evaluated. The progeny genotypes were determined by allele specific PCR for each SNP marker (Fig. 4.11-4.12). Before estimating the recombination frequency, the segregation of each marker was checked with Pearson's chi-squared test. In the case of *Ubiquitin conjugating enzyme*, and *ADP/ATP translocase* loci both parental strains were homozygous and as were all the progeny. Unexpectedly, in the case of *2-phosphoglycerate dehydratase*, where both parents were heterozygous, all the progeny isolates were also heterozygous. Neither of these markers were therefore included in the analysis. Of the remaining markers, 32A2R had a distorted segregation ratio (18 heterozygous and 2 homozygous) and deviated significantly ($p < 0.05$) from the expected 1:2:1 Mendelian ratio. The Pearson's chi-squared value was 12.80 with 1 d.f ($p = 0.001$) (Table 4.11). Similarly, for marker 56G18R, the initial hypothesis was that homozygotes and heterozygotes would occur in a 1:1 ratio. However, the segregation data did not fit this model. Instead, 16 isolates of the progeny were heterozygous and three were homozygous (one was scored as missing value). The segregation ratio, therefore, differed significantly ($p = 0.003$) from the expected 1:1 segregation ratio ($\chi^2 = 8.89$). These two markers were therefore not analysed further. The remaining four loci were inherited in a Mendelian fashion.

Despite the small sample size ($n = 20$), pairwise comparisons of the remaining four loci (*Glutamine synthetase*, 7I1F, *Transaldolase* and 56E14R) revealed all four possible genotypes. The genotypic frequencies expected under the assumption of independent assortment were similar to those observed. The chi-squared test for fit to a 1:1:1:1 segregation ratio demonstrated that all pairs of markers with the exception of 56E14R and *Glutamine synthetase* were unlinked (Table 4.12). The chi-squared value for this latter marker combination was 4.85 with 1 d.f ($p = 0.028$).

The 'JoinMap' calculations of recombination frequency (θ) and LOD score supported the chi-squared analysis and was suggestive of linkage only between markers 56E14R and *Glutamine synthetase*. These two markers were linked in the

coupling phase with an LOD score of 1.09 which exceeded LOD score of 0.834 that corresponds to a 5% level of probability. No linkage was found amongst the other markers. (Table 4.13).

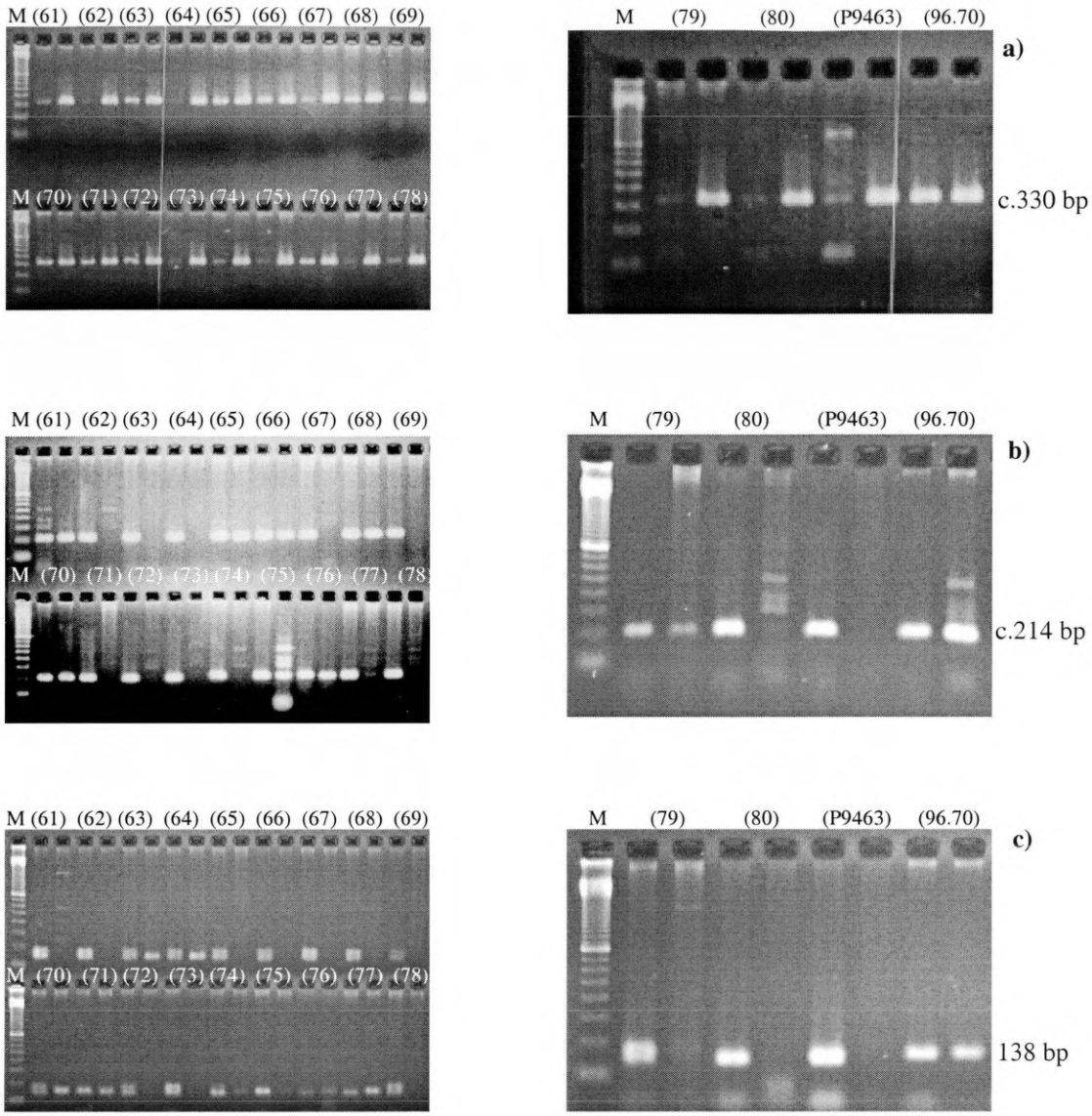


Fig. 4.11 Results of SNP amplification of 20 *P. infestans* progeny (61-80) derived from a cross between isolates P9436 and 96.70, with the allele specific primers for a) *Transaldolase*; b) *Glutamine synthetase*; c) 7I1F. The first lane for each isolate represents amplification with the allele specific primer and the second lane represents the alternate primer. M represents 100 bp size marker.

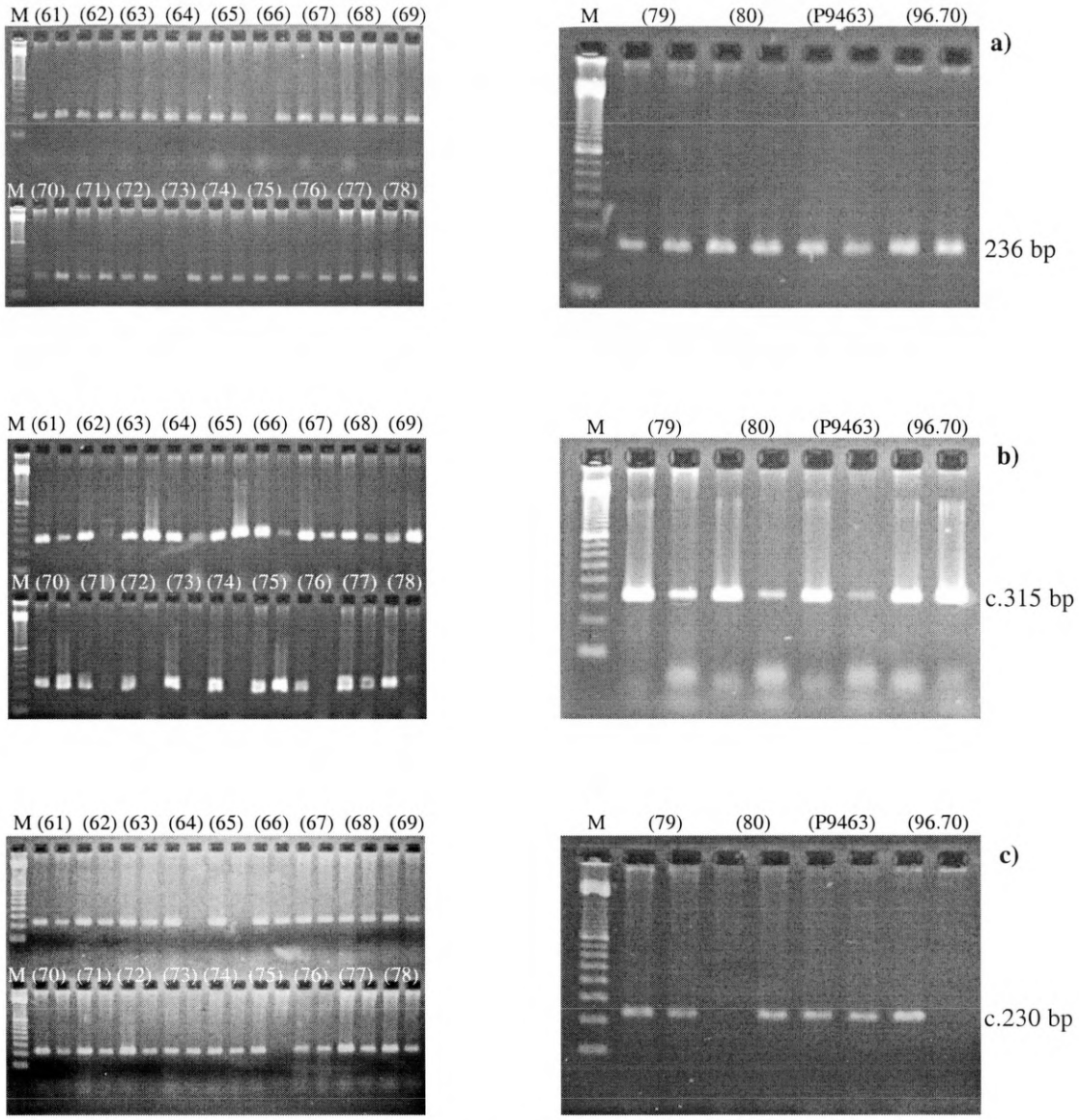


Fig. 4.12 Results of amplification of 20 *P. infestans* progeny (61-80) derived from a cross between isolates P9436 and 96.70, with the allele specific primers for a) 32A2R; b) 56E14R; c) 56G18R. The first lane for each isolate represents amplification with the allele specific primer and the second lane represents the alternate primer. M represents 100 bp size marker.

Table 4.11 Results of a chi-squared test of the segregation ratios of the alleles of each SNP marker amongst 20 progeny of a cross between *P. infestans* isolates P9463 and 96.70.

Marker	Parents	*n*	Genotypic classes observed	χ^2	*P*
	Parent 1 Parent 2				
2-phosphoglycerate dehydratase	Aa x Aa	20	1	-	-
Transaldolase	AA x Aa	20	2	0.80	0.371
Glutamine synthetase	AA x Aa	20	2	0.80	0.371
ADP/ATP translocase	AA x AA	20	1	-	-
Ubiquitin conjugating enzyme	AA x AA	20	1	-	-
56G18R	Aa x AA	20	2	8.89	0.003
56E14R	AA x Aa	20	2	0.20	0.655
7I1F	AA x Aa	20	2	3.20	0.074
32A2R	Aa x Aa	20	2	12.80	<0.001

Table 4.12 Results of a chi-squared test for association between pairs of SNP markers amongst 20 progeny of a cross between *P. infestans* isolates P9463 and 96.70.

Marker	Parents	*n*	Genotypic classes observed	χ^2	*P*
	Parent 1 Parent 2				
Glutamine synthetase x 56E14R	AABB x AaBb	20	4	4.85	**0.028**
Glutamine synthetase x Transaldolase	AABB x AaBb	20	4	2.81	0.094
Glutamine synthetase x 7I1F	AABB x AaBb	20	4	0.16	0.690
56E14R x Transaldolase	AABB x AaBb	20	4	0.13	0.714
56E14R x 7I1F	AABB x AaBb	20	4	0.47	0.492
7I1F x Transaldolase	AABB x AaBb	20	4	2.54	0.111

Table 4.13 Results of a 'JOINMAP' calculations to test for linkage between pairs of SNP markers amongst 20 progeny of a cross between *P. infestans* isolates P9463 and 96.70.

Marker	Parents	Recombination frequency	LOD score	Linkage phase
	Parent 1 Parent 2			
Glutamine synthetase x 56E14R	AABB x AaBb	0.2500	1.0927	Coupling
Glutamine synthetase x Transaldolase	AABB x AaBb	0.3000	0.6166	-
Glutamine synthetase x 7I1F	AABB x AaBb	0.5000	0.0000	-
56E14R x Transaldolase	AABB x AaBb	0.4500	0.0292	-
56E14R x 7I1F	AABB x AaBb	0.4500	0.1041	-
7I1F x Transaldolase	AABB x AaBb	0.3000	0.5495	-

4.3.6 Genotyping of Scottish isolates

A total of 42 Scottish *P. infestans* isolates were analysed with the nine SNP markers developed in Section 4.3.3. Following allele specific PCR, the banding patterns of each isolate were scored on agarose gels. All isolates were monomorphic for the markers *Ubiquitin conjugating enzyme* and *ADP/ATP translocase*. Data for these markers were therefore excluded from the analysis. The allelic data for the remaining seven SNP markers were combined (Table 4.14) and subjected to cluster analysis. Isolate 97.40.1.3 was analysed with only six markers because bands obtained with marker 32A2R were difficult to interpret. Similarly, isolates 96.17.5.3 and 97.40.4.1 were analysed with five markers since data for some of the markers in these isolates could not be scored (Table 4.14). Genetic relatedness was calculated on the basis of SNP genotypes.

The dendrogram of genetic relatedness based on simple matching coefficient using seven SNP markers, showed broad separation of isolates into two clades (A and B) with 25 isolates in clade A and 17 in clade B (Fig. 4.13). Each clade is made up of isolates of both A1 and A2 mating types. The isolates collected over a period of three years consisted of 17 genotypes, 11 of which were unique (Table 4.15). Only two genotypes (genotype 3 and 5) were found in every year of the survey. No relationships between genotypes and mating type, metalaxyl sensitivity, location (garden and allotments and commercial fields) or the year of collection was apparent.

In general the SNP marker combinations of *P. infestans* isolates from the same sample were either identical (e.g. 97.31) or clustered within the same clade (e.g. 97.40). In the latter example both mating types were isolated within the same field.

Table 4.14 Genotypes of *Phytophthora infestans* detected in a sub-set of isolates collected during 1995-1997. Allele specific PCR with seven SNP markers was used to determine the genotype of each isolate. Source and metalaxyl sensitivity of each isolate is also shown. The isolates were clustered into clades A or B on the basis of a simple matching algorithm. X represents isolate/marker combination that could not be scored.

Isolate[1]	Clade	2phosphoglycerate dehydratase	Trans-aldolase	Glutamine synthetase	32A2R	7H1F	56E14R	56G18R	Met sen	Source[2]
97.9.5.3	B	C/G	T	G	G/A	A	G	G/A	S	GA
97.9.1.1	B	C/G	T	G	G/A	A	G/C	G/A	S	GA
96.10.3.3	B	C/G	T	G	G/A	G/A	G/C	G/A	S	GA
97.41.7.3	B	C/G	C/T	G/T	G/A	G/A	G/C	G/A	S	GA
97.41.6.1	B	C/G	T	G/T	G/A	G/A	G/C	G/A	S	GA
97.36.2.2	B	C/G	T	G/T	G/A	G/A	G/C	G/A	R	F
97.41.10.3	B	C/G	T	G/T	G/A	G/A	G/C	G/A	S	GA
96.8.5.2	B	C/G	T	G/T	G/A	G/A	G/C	G/A	S	F
97.38.2.2	B	C/G	T	G/T	G/A	G/A	G/C	G/A	R	F
95.17.3.2	B	C/G	T	G/T	G/A	G/A	G/C	G/A	S	F
96.8.4.2	B	C/G	T	G/T	G/A	G/A	G/C	G/A	S	F
97.36.5.2	B	C/G	T	G/T	G/A	G/A	G/C	G/A	S	F
97.28.2.1	B	C/G	T	G/T	A	G/A	C	G	I	F
97.28.1.1	B	C/G	T	G/T	A	G/A	C	G	I	F
97.28.1.2	B	C/G	T	G/T	A	G/A	C	G	I	F
95.16.3.1	B	C/G	T	G/T	A	G/A	C	G/A	S	F
96.17.5.3	B	C/G	T	G/T	X	G/A	X	A	S	GA
95.7.10.1	A	C	C/T	G/T	G	G	C	G	R	F
95.5.2.2	A	C	C/T	G/T	G/A	G	C	G	S	GA
97.40.4.1	A	C/G	C/T	X	A	X	C	G	S	GA
97.40.8.3	A	C/G	C/T	G/T	G/A	G	C	G	R	GA
97.39.1.3	A	C/G	C/T	G/T	G/A	G/A	C	G	S	GA
96.22.3.1	A	C/G	C/T	G	G/A	G	G/C	G	R	F
97.11.5.1	A	C/G	C/T	G	G/A	G	G/C	G	I	F
96.21.1.1	A	C/G	C/T	G	G/A	G/A	G/C	G	R	GA
97.30.5.1	A	C/G	C/T	T	G/A	G	G/C	G	R	F
97.40.1.3	A	C/G	C/T	T	X	G	G/C	G	S	GA
95.1.1.6	A	C	C/T	G/T	G/A	G	G/C	G	R	F
95.7.3.1	A	C	C/T	G/T	G/A	G	G/C	G	R	F
96.13.1.3	A	C	C/T	G/T	G/A	G	G/C	G	R	GA
96.9.5.1	A	C	C/T	G/T	G/A	G	G/C	G	R	F
96.32.5.3	A	C/G	C/T	G/T	G/A	G	G/C	G	R	F
95.19.2.2	A	C/G	C/T	G/T	G/A	G	G/C	G	R	GA
97.40.9.3	A	C/G	C/T	G/T	G/A	G	G/C	G	S	GA
97.40.3.3	A	C/G	C/T	G/T	G/A	G	G/C	G	S	GA
97.39.7.2	A	C/G	C/T	G/T	G/A	G/A	G/C	G	S	GA
97.39.4.3	A	C/G	C/T	G/T	G/A	G/A	G/C	G	I	GA
97.31.3.2	A	C/G	C/T	G/T	G/A	G/A	G/C	G	S	GA
97.31.4.2	A	C/G	C/T	G/T	G/A	G/A	G/C	G	S	GA
97.31.1.3	A	C/G	C/T	G/T	G/A	G/A	G/C	G	S	GA
97.31.5.3	A	C/G	C/T	G/T	G/A	G/A	G/C	G	S	GA
97.31.2.3	A	C/G	C/T	G/T	G/A	G/A	G/C	G	S	GA

1: The first two components of the isolate name refer to the year of isolation and sample number. 2: source from where the isolate was collected. GA: Garden or Allotment ; F: Field

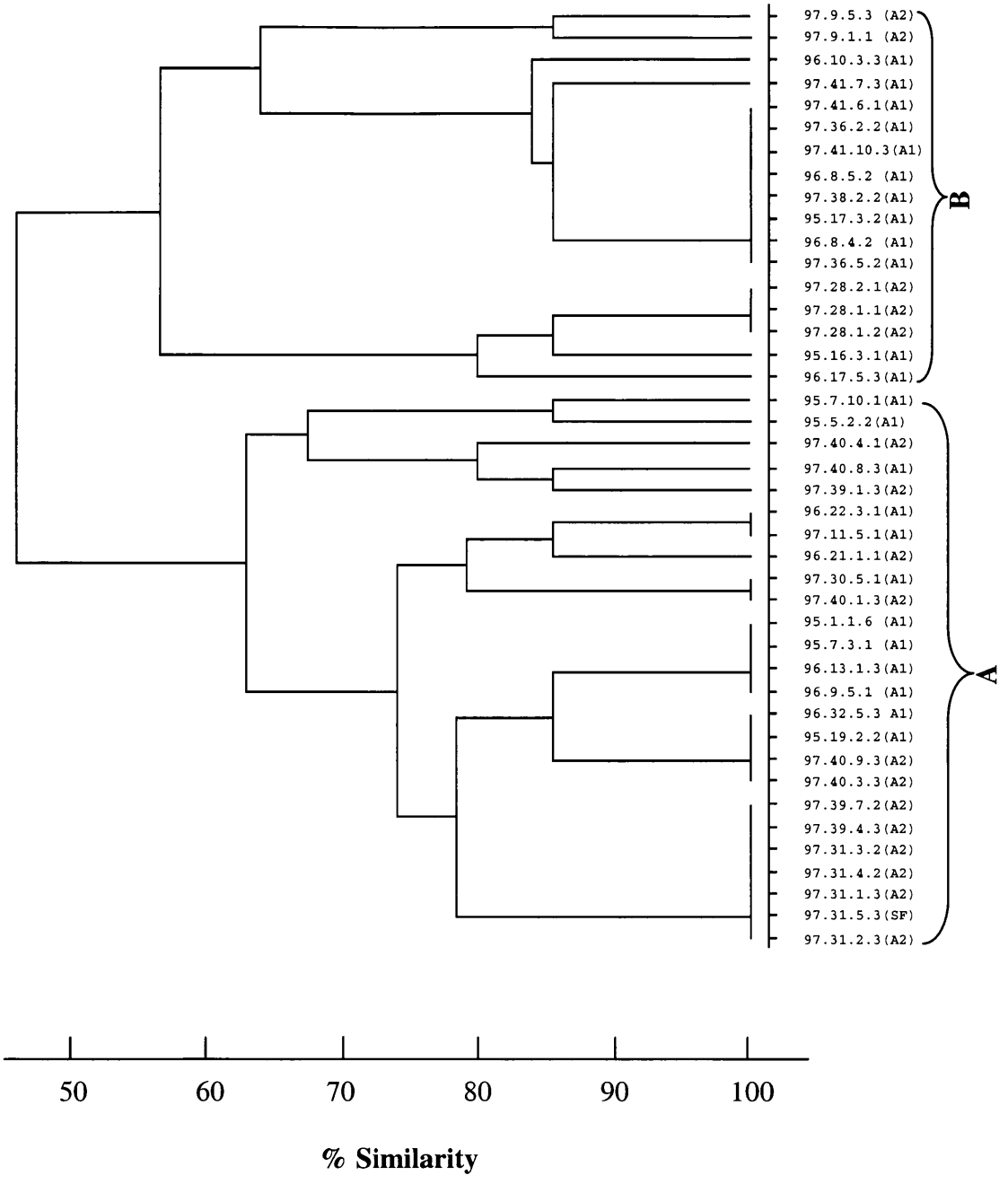


Fig. 4.13 Dendrogram of 42 isolates of *P. infestans* on the basis of seven SNP markers. Simple matching with group averages was used to construct the dendrogram. A and B represent the two clades. Mating type is shown in parentheses after each isolate.

Table 4.15 Number and frequency of occurrence of different SNP genotypes discovered amongst 42 *P. infestans* isolates collected in Scotland between 1995 and 1997.

Genotype	Genotype number	Frequency[1]	Year collected
GT GA GA GC CT CG G	1	7	1997
GT GA GA C CT CG G	2	1	1997
GT G GA GC CT CG G	3	4	1995, 1996, 1997
GT G GA C CT CG G	4	1	1997
GT GA GA GC T CG GA	5	8	1995, 1996, 1997
GT GA A C T CG GA	6	1	1995
G GA GA GC T CG GA	7	1	1996
G A GA GC T CG GA	8	1	1997
G A GA G T CG GA	9	1	1997
GT GA GA GC CT CG GA	10	1	1997
G GA GA GC CT CG G	11	1	1996
G G GA GC CT CG G	12	2	1996, 1997
GT GA A C T CG G	13	3	1997
GT G GA C CT CG G	14	1	1995
GT G GA GC CT C G	15	4	1995, 1996
T G GA GC CT CG G	16	1	1997
GT G G C CT C G	17	1	1995

1: Data for three isolates could not be scored with some markers because of the difficulty in reading the bands

4.4 Discussion

SNP markers have recently emerged as a powerful tool in many studies, occur throughout the genome with much greater frequency than any other markers (Collins *et al.*, 1997; Landegren *et al.*, 1998; Schafer and Hawkins, 1998; Brookes, 1999) and are less prone to mutation than SSRs (Giordano *et al.*, 1999). In addition, several automated detection systems exist for the rapid typing of SNPs (Wang *et al.*, 1998). Dominant markers such as AFLPs can underestimate the variability in a population. The SNP markers are co-dominant, have the ability to distinguish homo and heterozygotes and therefore can be useful in population studies. Further, it is not necessary to extract high quality DNA as is required for RFLP and AFLP. As these markers are neutral, stable (Wang *et al.*, 1998) and dispersed across the genome, they provide an attractive approach to examine the diversity and population biology of *P. infestans*. Such studies are critical for formulating effective management strategies. SNP markers will also prove useful in revealing genetic relationships among isolates.

SNP discovery in the present study involved two approaches; firstly the screening of full length gene sequences, and latterly that of the BAC clones. The initial strategy was to search amongst readily available *P. infestans* gene sequences. A BLAST search of the gene sequences against the PGC EST database, as of May 2000, returned many duplicate sequences. After checking the quality of these sequences in a chromatogram viewer, several candidate SNPs were targeted. Among these candidates, the longest was selected to maximise the probability of locating polymorphic sites (Griffiths and Shaw, 1998). Polymorphisms which appeared to be due to the assembly of ESTs from members of gene families were avoided due to the likelihood of subsequent problems in SNP scoring. The selected loci were amplified from eight different *P. infestans* isolates representing Scottish and world-wide diversity, and visualised on agarose gels to ensure that the primers produced a single amplicon. Sequencing revealed a total of eight potential polymorphisms in the fragments of five of the seven genes. Some polymorphisms including those found in *Phosphoglycerate kinase* and *Cyclophilin* during an EST database search were not confirmed by sequencing. This could be due to sequencing errors in the original EST

clones. Alternatively, the number of isolates tested in each case may not have been sufficient to reveal the polymorphism. For example marker *Glutamine synthetase*, although initially monomorphic amongst the three Scottish *P. infestans* isolates sequenced, was polymorphic when tested against a larger set of Scottish isolates. Screening of a wider panel of isolates may be considered for future studies. The *Ubiquitin conjugating enzyme* and *ADP/ATP translocase* markers could not distinguish amongst the Scottish blight population isolates but showed polymorphism amongst international isolates and can therefore be used to examine diversity on a wider scale.

The second phase of the SNP discovery involved searching randomly selected BAC clones. The reason for using BAC clones was to make a comparison of SNP frequency in coding and non-coding regions. It was noted that in the present studies there was no significant difference between SNP frequency in coding regions and in random genomic sequences from BAC clones. The SNPs occurred at an estimated frequency of 2 SNP per kb.

Another reason for screening BAC clones was to search for neutral markers. Wong *et al.* (2001) reported that mutations in the non-coding regions are expected to be neutral. Since the eukaryotic genomes consist largely of non-coding sequences (Wong *et al.*, 2001), the selection of random sequences was therefore made to identify SNPs in the non-coding regions of the genome. No attempt was made to sequence BAC clones in which a SNP had already been identified in its opposite end. Clearly, such markers would be tightly linked and the objective for population analysis was to select unlinked markers.

All clones yielded sequences of sufficiently high quality to be used reliably for further analysis. These sequences were BLAST searched against the NCBI database. Many sequences showed high homology with repetitive elements known to be found in great abundance in the *P. infestans* genome (Judelson and Randall, 1998). The type of repetitive sequences identified during the database search included previously described retro elements (Kaneko *et al.*, 2000; Sato *et al.*, 2000; Liou *et al.*, 2002). Only one clone (50P9F) matched a gene, that of a serine/threonine specific protein.

Although sequencing proved satisfactory as an initial SNP screening strategy, it is time consuming to score, and relatively expensive. It was thus necessary to streamline the approach for large scale SNP detection amongst the BAC clones. The approach selected was single strand conformational polymorphism (SSCP). The method is less expensive than sequencing and has a high throughput. It also saves the labour involved in purification of PCR products, setting up and loading of sequencing reactions and manual editing of sequences (Maxam and Gilbert, 1977; Sanger *et al.*, 1977). Instead, only those products identified as polymorphic are sequenced. However, like sequencing, the target sequences must be amplified by PCR. The resolution of this technique also has limitations, and the optimum product size is 200-300 bp. Orita *et al.* (1989) and Hayashi (1991) reported 430 bp to be the maximum fragment size distinguishable by this technique. However, Hongyo *et al.* (1993) presented conflicting evidence that fragments as large as 1.35 kb could be resolved by SSCP analysis. Despite this disadvantage, the technique has been successfully used in detecting point mutations (Nigro *et al.*, 1989; Gaidano *et al.*, 1991; Mohabeer *et al.*, 1991; To *et al.*, 1993).

In the present studies, commercially available pre-cast gels designed to resolve fragments of 150-300 bp were used. A mixture of NaOH (Dockhorn-Dworniczak *et al.*, 1991; Yap and McGee, 1992) and formamide was used as a denaturant to prevent the annealing of the two strands of the DNA prior to loading. However, in another study Yap and McGee (1994), added formamide after denaturation and just before sample loading. Further, samples were loaded quickly after heat denaturing to prevent re-annealing of the DNA strands. The running buffer was changed after 2-3 runs in order to retain maximum resolution of the bands. In the present study, ethidium bromide staining was not sensitive enough to reveal SSCP bands and SYBR Gold staining was therefore used. The results are in conflict with Hongyo *et al.* (1993) who reported that ethidium bromide staining was sufficiently sensitive to reveal SSCP bands.

SSCP relies on the maintenance of a continuous low temperature for tightly defined and well separated DNA fragments (Sarkar *et al.*, 1992). Temperature fluctuations affect the migration rates resulting in diffuse bands. In the present studies, the best results were achieved at 9°C. A constant temperature of 10°C has been reported to be

optimal for the best resolution of SSCP bands (Hongyo *et al.*, 1993). The gel running differed according to the product length but ranged from 7 to 15 h. In six out of eight cases, SSCP results were backed up by sequencing. Two clones, 72N2F and 56C9R although apparently polymorphic, were monomorphic when sequenced. This could be due to the presence of PCR artefacts. SSCP not only provided a rapid screen of the BAC clones to identify potential polymorphisms, but also proved cost effective.

In summary, amongst the eight *P. infestans* isolates screened, the SNP discovery phase revealed eight SNPs amongst seven coding and 28 amongst 30 non-coding regions. Amongst genes, *Transaldolase* showed the highest number of SNPs whereas 56G18R had the highest number of SNPs amongst the BAC clones. Two of the seven genes and 24 of the 30 BAC clones were monomorphic amongst the panel of isolates selected. Of these 36 SNPs, nine were eventually developed into SNP markers. Allele specific PCR (Ayres *et al.*, 1997; Hauser *et al.*, 1998; Drenkard *et al.*, 2000), in which two reactions for each SNP allele are run concurrently was used to score the previously discovered SNPs.

The allele specific oligonucleotides (Connor *et al.*, 1983; Orkin *et al.*, 1983) were designed for each marker with a single discriminating base at the 3′ end. A significant problem observed by various workers (Newton *et al.*, 1989; Kwok *et al.*, 1990; Li *et al.*, 1990) with this type of assay was that the mismatch at the 3′ end is extended by DNA polymerase with a varying degree of efficiency. As a result, some primers lack specificity (Kwok *et al.*, 1990; Cha *et al.*, 1992; Kwok *et al.*, 1994b). In order to counter such non-specific amplification in this study, an additional mismatched base was also incorporated within the 3′ terminal four bases (Drenkard *et al.*, 2000). The further destabilisation of the primer-template complex minimises non-specific amplification (Petruska *et al.*, 1988; Newton *et al.*, 1989; Whitcombe *et al.*, 1999; Akada *et al.*, 2001). The reaction also relies on the absence of the 3′ proof reading activity of the enzyme *Taq* DNA polymerase. It is therefore essential that such enzymes as *Pfu*, which possess such a property, should not be used (Newton, 1995). In an effort to eliminate the need to provide different conditions for each locus of interest all the primer pairs were optimised within the range of 58-60°C except 56G18R, which requires an annealing temperature of 65°C. This has practical

implications since it allows the examination of multiple loci simultaneously. Before proceeding with scoring, the DNA concentration of the test isolates was adjusted to 500 ng/μl. This was done in order to avoid mis-scoring resulting from differences in the concentrations of the DNA of various isolates.

The above assay was robust and reproducible and successfully resolved the expected SNP in almost every case. The disadvantage of such a SNP scoring method is however the two parallel reactions that have to be set up for each isolate. This could potentially lead to errors of mis-interpretation of heterozygotes if one of the reactions fails and the other succeeds. The use of commercially prepared PCR master mixes can minimise pipetting steps and thus reduce the chance of error.

Conventional gel-based allele specific PCR can be extremely time consuming since it involves multi step sample processing, which is a disadvantage in high throughput analysis. Automated detection assays are therefore needed to enhance SNP genotyping of isolates. The above developed molecular marker 'toolkit' was refined for high throughput automated analysis. In addition to accurate allele determination, flexibility, and cost are critical for successful assays.

Several high throughput genotyping systems are presently available. These include TaqMan assays (Livak *et al.*, 1995), oligonucleotide ligation assays (Tobe *et al.*, 1996), minisequencing (Chen and Kwok, 1997; Pastinen *et al.*, 1997), molecular beacons (Tyagi *et al.*, 1998), dye-labelled oligonucleotide ligation (Chen *et al.*, 1998), micro-arrays (Hacia *et al.*, 1998; Wang *et al.*, 1998), mass spectrometry (Ross *et al.*, 1998) and the Invader assay (Mein *et al.*, 2000). In the present studies, fluorescent allele specific PCR was used for automated detection. The method was chosen as a continuation of the gel-based allele specific PCR assay already used.

The automated assay proved straightforward, rapid and sensitive for genotyping. The results were easy to interpret and genotypes were assigned in 97% of the cases. The use of 96-well microtitre plates increased the throughput of the assay. Differential fluorescent labelling of primers (Nazarenko *et al.*, 1997) allows the use of allele specific PCR without post-PCR handling and gel electrophoresis. Moreover, the assay allows parallel analysis of several SNPs in the same reaction well by

selecting products of different sizes and a range of fluorescent dyes. This obviates the need for multiplex PCR which is demanding because of the extensive optimisation of the primers and running conditions required for successful amplification (Edwards and Gibbs, 1994). Pooling also reduces the quantity of PCR product required from each sample: only 0.5 μl of the entire PCR product from the pool was sufficient for analysis. This not only saves time but also allows the use of smaller reactions thereby minimizing the cost. In the present study, the most complex genotyping reaction involved combination of 14 primers representing seven loci into a single well. The assays were therefore specifically designed to facilitate such pooling strategy. Pooling of DNA samples has been reported previously for both microsatellites and SNPs (Arnheim *et al.*, 1985; Pacek *et al.*, 1993; Syvänen *et al.*, 1993; Kwok *et al.*, 1994a; Barcellos *et al.*, 1997; Shaw *et al.*, 1998).

Another advantage of this assay is that the software allows automated scoring of the peak heights for each SNP allele, thereby rapidly yielding a table of allele scores. Additionally, the resolution of this system allows fragments that differ by only 0.1 bp to be separated. Such resolution allows scoring of small insertions /deletions such as that observed in the case of marker 7I1F, in which the sequence used for designing allele specific primers had a 9 bp deletion. Although this difference could not be detected on agarose gel, it was apparent in the automated assay, which shows an additional benefit of this scoring system. However, a disadvantage of this approach is the high cost associated with both the use of machine and the specific fluorescent primers required.

In the automated assay, genotypes were independently determined by the software and then compared to the gel-based genotyping results. An error rate of ~ 4% was observed when different results were obtained from gel-based and automated assays. The reason for this anomaly was not clear.

Ideally, markers for population studies should be simply inherited i.e. not linked. In order to test for independent segregation, the inheritance of the markers amongst twenty randomly selected individuals of an F1 progeny of a cross, were examined. As expected, most of the SNP markers, when analysed individually, segregated independently and according to their Mendelian ratios. However, two markers

(32A2R and 56G18R) had distorted segregation ratios. Since isolates were scored twice for each marker, mis-scoring, due to failure of amplification of particular alleles, is unlikely. The reproducible nature of the results therefore eliminates this as a reason for distorted segregation. Further, these markers did segregate in the 42 Scottish *P. infestans* isolates suggesting that the anomaly was either due to small population size (Lu *et al.*, 1998) or related to the particular isolates used for the cross. Further studies with a larger population size would be helpful in determining the reason for such distorted ratios. Pongam *et al.* (1998) reported that inclusion of markers with distorted ratios could result in false linkages. Therefore, markers 32A2R and 56G18R were excluded from the analysis. For markers *2-phosphoglycerate dehydratase*, *ADP/ATP translocase* and *Ubiquitin conjugating enzyme*, the progeny were all identical (i.e. produced a single genotype) and as such were also excluded from the analysis. No skewed segregation was observed for the other markers in the study.

Pairwise combination of the markers showed that all markers, except 56E14R and *Glutamine synthetase*, segregated according to Mendelian ratios and were unlinked. Markers 56E14R and *Glutamine synthetase* had distorted ratios and linkage of the two loci appears to be the most likely explanation for such segregation ratios. This was supported by χ^2 ($p = 0.028$) (Table 4.12) and 'JOINMAP' (Table 4.13) analyses. Analysis of a larger number of progeny from different crosses is needed to confirm these findings. However, these markers can still be used but linkage must be borne in mind while interpreting the results.

SNP markers were used to evaluate a sub-set of Scottish *P. infestans* isolates. Forty two isolates, previously scored by AFLPs were selected from a culture collection of a previous survey (Table 4.1). Each isolate was scored with nine SNP markers. Two markers i.e. *Ubiquitin conjugating enzyme* and *ADP/ATP translocase* were monomorphic and were therefore excluded from the analysis. Seventeen genotypes were identified amongst the 42 isolates examined, of which eleven were unique. Genotype 5 was the most predominant followed closely by genotype 1 (Table 4.15). The cluster analysis and identification of 17 genotypes indicated considerable genetic diversity within the Scottish late blight population.

Based on cluster analysis, the isolates formed two distinct groups, referred to as clades A and B. Some genotypes were detected in all the three years whereas others detected initially were absent in subsequent years which could be due to random sampling variation or seasonal bottlenecks. The lack of sub-structuring of isolates from gardens and allotments and commercial fields suggests gene flow amongst the genotypes. Moreover, clustering of samples did not reflect differences between clades based on metalaxyl sensitivity or mating type.

P. infestans isolates collected from the same site were similar. Within some sites however, isolates were not identical though still clustering within the same clade. The occurrence of both mating types in the same field within a site and the observed genetic variability is suggestive of occasional sexual recombination and re-assortment of alleles. Extending this analysis to the rest of the isolates in the survey would provide detailed information on the genetic structure of Scottish late blight populations.

The present study confirmed results of previous work in which 15 AFLP markers were used for genotyping of a sub-set of Scottish *P. infestans* population (D. E. L. Cooke, pers. comm.). Both marker sets revealed considerable variability amongst the isolates, and showed that clustering of isolates was unrelated to the year of sampling or metalaxyl sensitivity. However, a clear sub-structuring of A2 mating type isolates, evident in the AFLP analysis was not apparent when isolates were genotyped with SNP markers. The small sample size (n = 42) in the present study may explain such differences in the results between the two marker sets.

The studies showed that SNPs are useful markers even though the initial effort involved in their identification is substantial. Such panels of co-dominant markers will be invaluable tools for *P. infestans* mapping studies as well as for studies on its population biology and epidemiology. SNP markers are also essential for determining gene flow between populations and will complement other markers and validate previous studies. The AFLP study on the status of late blight population in Scotland will, for example, benefit from their use. SNP markers can therefore be used as part of a molecular 'toolkit' for *P. infestans* genotyping. The availability of automated assays will allow rapid screening of the population. The markers can also

be used in tracking of individual isolates in competition studies (Chapter 5) as well as monitoring pathogen populations in the field. It will be interesting to use these markers on a wider range of *P. infestans* isolates in future to determine their utility in *P. infestans* population studies on a world-wide scale.

Cultivar-specific aggressiveness and fitness in *Phytophthora infestans* and its characterisation using molecular markers

5.1 Introduction

5.1.1 Aggressiveness and pathogenic fitness

Aggressiveness is regarded as the quantitative component of pathogenicity. It describes relative differences in the amounts of disease caused by various isolates of a pathogen (Lucas, 1998). In practical terms, aggressiveness is the measure of pathogenicity and not only represents the ability to colonise a host but also is a manifestation of the rate of establishment of infection. The criteria used to measure aggressiveness include infection efficiency, lesion size, incubation period, latent period and sporulation capacity (Nelson, 1979; Leonard and Czochor, 1980; Wolfe *et al.*, 1983; Antonivics and Alexander, 1989; Spielman *et al.*, 1992; Leonard, 1997). Reduced aggressiveness has been implicated as a cause of reduced plant pathogen fitness in many studies (Kato and Fry, 1995b; Peters, 1998; Peters *et al.*, 1998). Fitness represents the overall ability of an organism to survive and reproduce (Crow, 1986) and the contribution it makes to the next gene pool (Miller *et al.*, 1998).

There have been several examples of sudden changes of pathogenic fitness resulting in severe disease epidemics. The resurgence of late blight in Europe in the late 1970s was due the world-wide spread of 'new', highly fit isolates of *P. infestans* which were also resistant to the widely used fungicide, metalaxyl. The reasons for the displacement of the 'old' genotypes by the 'new' is still a matter for debate but it is likely that increases in aggressiveness and fitness of the isolates to the cultivars grown were involved (Day and Shattock, 1997). The ability of the 'new' genotypes to infect, colonise and sporulate more efficiently than the 'old' genotypes, points to a more rapid spread of 'new' genotypes within and between potato fields (Miller and Johnson, 2000).

Differences in the aggressiveness and fitness of potato- and tomato-adapted strains of *P. infestans* are well documented (Legard *et al.*, 1995; Legard and Fry, 1996; Marshall and Stevenson, 1996). However, adaptation within potato germplasm itself has not been studied intensively. The rate, mechanisms and significance of such adaptations remain largely unknown. Elucidation of such relationships will provide new insight into the nature of the host-pathogen interaction.

5.1.2 *P. infestans* and host specificity

Host specificity in *P. infestans* has been studied for many years. Although the pathogen attacks as many as 40 species belonging to the family Solanaceae (Turkensteen, 1978), most investigations have focused on the two most economically important hosts, potato and tomato. Earlier reports of tomato late blight make no mention of host specificity (Payen, 1847; Thaxter, 1891; Clinton, 1903; Smith, 1906) and a lack of reliable markers precluded studies on genetic similarity of isolates. However, cross inoculation studies suggested that the *P. infestans* population could be differentiated into tomato and potato specialised forms (Wiltshire, 1913; Giddings and Berg, 1919; Berg, 1926; Small, 1932). Population studies and the characterisation of genotypes of *P. infestans* using molecular and biochemical markers (Tooley *et al.*, 1985; Goodwin *et al.*, 1992a) supports the hypothesis that *P. infestans* populations on two different hosts are to some extent isolated (Lebreton *et al.*, 1996; Lebreton and Andrivon, 1998; Oyarzun *et al.*, 1998).

Such markers have revealed the occurrence of distinct genotypes of *P. infestans* in Brazil (Brommonschenkel, 1988); Northwestern Mexico (Goodwin *et al.* 1992b); The Philippines (Koh *et al.*, 1994); The Netherlands (Fry *et al.*, 1991); France (Lebreton and Andrivon, 1998); Ecuador (Oyarzun *et al.* 1998); and the USA (Legard *et al.* 1995). In a recent study, Vega-Sanchez *et al.* (2000) showed a significant interaction between source of inoculum (potato or tomato) and aggressiveness towards the inoculated host (potato or tomato). Each lineage was more aggressive on the host from which it was isolated but could also cause lesions on the alternative host. In addition, the potato derived isolates had a *glucose-6-phosphate isomerase* (*Gpi*) genotype (*86/100*) which was distinct from those isolated

from tomato (*100/100*). Similarly, Goodwin *et al.* (1992b) found host related differences in the mating type, allozyme and DNA fingerprint patterns between 88 isolates of *P. infestans* isolated from potato and tomato. Several studies have compared the race complexity of isolates from the two hosts, and found that isolates present on tomato generally belong to simpler races than those isolated from potato (Deahl *et al.*, 1993; Lebreton and Andrivon, 1998). Despite this reported host specificity, it is clear that host related differences are merely quantitative and do not affect the fundamental ability of an isolate to cause disease on the other host (Oyarzun *et al.*, 1998).

Ordonez *et al.* (2000) recently provided evidence for the occurrence of a separate population of *P. infestans* on wild *Solanum* spp. (*S. brevifolium* and *S. tetrapetalum*). Differences in mating type, *Gpi* alleles, RG57 banding pattern and mitochondrial DNA haplotypes were seen between populations on wild and cultivated hosts. Although the authors did not speculate on the origin of the new genotypes, they concluded that the two populations were probably isolated by host specificity.

The origin and evolution of host specificity in tomato and potato is unclear. Some workers support the hypothesis that tomato aggressive isolates evolved from potato isolates but this evolution did not however decrease the aggressiveness of isolates towards potato (Legard *et al.*, 1995). Others argue that isolates equally aggressive on both hosts should rapidly replace those that are aggressive on only one host and one would therefore not expect to see differences between the two populations (Turkensteen, 1973).

The extent of specialisation of the late blight population within potato cultivars is not known. In the case of the widespread release of R-gene containing cultivars, isolates adapted to overcome such resistance and came to dominate the population (Stevenson *et al.*, 2001). Breeders have since favoured cultivars with more field, or race non-specific resistance, and only a few studies have investigated the adaptation of *P. infestans* to these cultivars. Surveys of the virulence of the blight population have revealed a trend towards increased race diversity (Schöber and Turkensteen, 1992) with increased sexual recombination. Whether increased virulence complexity has an impact on host adaptation is not known.

Adaptation of *P. infestans* to cultivars with rate-reducing resistance has been reported by some workers (Caten, 1974). Day and Shattock (1997) reported that *P. infestans* isolates with mitochondrial DNA haplotype Ia and IIa were more aggressive on cultivars Cara and Stirling than on other cultivars. Similarly, metalaxyl sensitive isolates were more aggressive than metalaxyl insensitive isolates on the same cultivars. James and Fry (1983) on the other hand, reported that adaptation of *P. infestans* isolates to cultivars with rate-reducing resistance is unlikely, since this form of resistance is durable and several mutations in the pathogen are required to adapt to it. Furthermore, changes in growers choice of cultivars from year to year makes it less likely for the pathogen to adapt to a particular cultivar.

The extent to which cultivar-specific adaptation influences disease in the field is largely unknown. How strong is the effect of cultivar adaptation and the possibility of a penalty to aggressiveness where increased aggressiveness on one cultivar decreases aggressiveness on another has also not been studied. Moreover, the question of rate of re-adaptation should also be addressed. The development of co-dominant molecular markers has facilitated monitoring of pathogen populations in competition experiments, and will enable us to answer such questions.

5.1.3 Impact of host adaptation on durability of host resistance and late blight management

In order to reproduce, the pathogen must interact with its host. The widespread cultivation of a particular disease-resistant cultivar over a long period may therefore promote adaptation within the *P. infestans* population and result in overall loss in host resistance. Survival of the pathogen on seed will facilitate adaptation, since the pathogen is continually exposed to cultivar-dependent selection pressure. For example Flier *et al.* (2001b) showed that a differential interaction between cultivars and isolates for tuber infection and colonization could lead to erosion of tuber resistance. However, it is difficult to assess the extent to which such an erosion of resistance could take place. Increased cultivar diversity could be important in preventing adaptation of *P. infestans* to particular potato cultivars and therefore in delaying the erosion of resistance. Unlike with cereals, little attention has been paid

to the strategic deployment of resistant cultivars, primarily because little information is available on how this would influence the pathogen population. The development of co-dominant markers (Chapter 4) and their use in characterisation of individual isolates will be useful in addressing such issues.

Besides having a possible role in broad-scale durability of host resistance, cultivar diversity has also been implicated in reducing late blight epidemics at a field scale (Garrett and Mundt, 1999; Garrett and Mundt, 2000; Garrett *et al.*, 2001). Several mechanisms have been proposed for host diversity effects, which include, a reduction in the proportion of susceptible tissue, and a reduction in the spread of inoculum as a result of resistant plants acting as physical barriers (Chin and Wolfe, 1984; Garrett and Mundt, 1999). These two mechanisms appear to exert mechanical effects related to the way the pathogen spores are spread and to the distribution of resistant and susceptible hosts. Moreover, the infection process may be slowed by the induction of defence responses in susceptible plants by strains of the pathogen that are avirulent on specific host genotype (Lannou *et al.*, 1995). These epidemiological and physiological mechanisms contribute to overall disease suppression.

The present experiments were designed to test the nature and extent of cultivar adaptation by continuous multiplication of an isolate of *P. infestans* on a specific potato cultivar followed by an assessment of its relative aggressiveness on 'host' and 'non-host' cultivars. The relative fitness of two 'trained' isolates was monitored using previously developed SNP markers (Chapter 4) to track each isolate in a co-inoculation experiment with two cultivars.

5.2 Materials and Methods

5.2.1 Isolates and cultivars

Two isolates (95.17.3.2 and 96.13.1.3), that could be differentiated using the SNP marker 56G18R (Chapter 4) were selected from 499 isolates collected during a 1995-97 survey of the Scottish late blight population. The isolates were characterized by mating type, metalaxyl sensitivity, virulence (Table 5.1) and AFLP profiles in previous studies, and represent two different genotypes on the basis of the analysis of 15 AFLP markers (D. E. L. Cooke, unpublished data). Cultures of the isolates were maintained on Rye A agar. Potato cultivars King Edward, Teena, Bintje and Maris Piper were selected for their different levels of resistance to late blight and were grown in the glasshouse under natural light at 15°C in 5 L plastic pots with equal proportions of soil, sand and peat and fertiliser solution containing NPK. The R-gene status and known resistance ratings of the cultivars are presented in Table 5.2.

Table 5.1 Isolates used and their mating types, virulence factor, metalaxyl sensitivity, source and year of collection.

Isolate	Race	Metalaxyl reaction	Host	Cultivar of origin	Source	Year of collection
95.17.3.2 (A!)	1 3 4 7	S	Potato	-	Field	1995
96.13.1.3 (A1)	1 2 3 4 6 7	R	Potato	Ratte	GA	1997

S = sensitive; R = resistant

Table 5.2 Foliar and tuber blight ratings of cultivars used, and the presence of unidentified R-genes.

Cultivar	Foliar blight rating[1]	Tuber blight rating[1]	R- genes[2]
Bintje	2	2	R- gene free
King Edward	3	4	R- gene free
Maris Piper	4	5	R- gene free
Teena	7	8	Unclassified R gene

[1]Ratings according to National Institute for Agriculture Botany (2002). Ratings are based on a scale of 1-9 where 9 is the most resistant.
[2]Based on unpublished data

5.2.2 Isolate 'training'

The experiment comprised eight treatments, consisting of 'training' each of two isolates on four different cultivars as listed in Table 5.2. The 'training' consisted of seven successive generations of leaf infection on a single potato cultivar carried out as detailed below.

Cultures were grown on Rye A agar at room temperature for two weeks prior to inoculation. Plates were washed with 5 ml of SDW and scraped with a glass spreader to produce a sporangial suspension. The concentration of the sporangial suspension was adjusted to $3.5x10^3$ sporangia ml^{-1} prior to incubation at 4°C for 2 h to encourage zoospore release.

Four detached leaves of approximately the same age (of each of the four test cultivars) were inoculated separately (abaxial surface up) with five 100 μl droplets of zoospore suspension of isolates 95.17.3.2 or 96.13.1.3 from herein termed 'a' and 'b', respectively. Thus, a total of eight sub-isolates were formed based on isolate by cultivar combination. These were termed 'aB', 'aK', 'aM', 'aT', 'bB', 'bK', 'bM' and 'bT', to designate the isolate and the cultivar on which they were 'trained'. Each of the isolates sub-cultured on Rye A agar for seven weeks, was also used for comparison and are referred to as 'aR' and 'bR'. Each sub-isolate was passaged continuously through the host plant leaves. The inoculated leaves were incubated on moist paper towels in plastic trays (10 cm^2) at 15°C for one week using a separate

tray for each sub-isolate by cultivar combination. Sporangia from each sub-isolate were harvested on a weekly basis and used to re-infect a new set of detached leaves of the same cultivar in the same way as above. After seven generations the isolates were used immediately in the experiments described below.

5.2.3 Effect of isolate 'training' on aggressiveness

Detached leaflet assays were used to determine the relative aggressiveness of various sub-isolates. Treatments consisted of the five sub-isolates (aB, aK, aM, aT, and aR), each of which was used to infect the cultivars King Edward, Teena, Bintje and Maris Piper. Detached leaflets (five each) of the four cultivars were laid out in plastic boxes (50x25 cm^2) lined with moist tissue paper. Treatments were replicated four times. The boxes were arranged in a randomised complete block design and evenly spaced on the bench of a controlled environment cabinet at 16°C with 16 h of light. A similar experiment was established for sub-isolates bB, bK, bM, bT and bR, separately.

The sporangia harvested from their respective 'training' cultivars (Section 5.2.2) were suspended in SDW and their concentration was adjusted to $3.5x10^3$ sporangia ml^{-1}. The sporangial suspension was incubated at 4°C for 2 h, as described previously, to induce zoospore production. A single drop (10 μl) of the resulting zoospore suspension was applied near to the midrib of each leaflet (abaxial side up). The boxes were then tightly sealed with a thick strip of adhesive tape to maintain leaf wetness, and were incubated as described previously until symptom development.

Four days after inoculation, lesion diameter was measured along two perpendicular lines and the mean lesion diameter was calculated. The data were recorded in EXCEL (Microsoft Office 2000) and analysed using GENSTAT (Payne, 1997). A two way analysis of variance (ANOVA) (Steel and Torrie, 1980) with blocks was performed to test the statistical significance of the interaction between sub-isolates and cultivars. Treatment differences were considered significant where $p \leq 0.05$. The experiment was specifically established to test whether adaptation or 'training' of an

isolate on a cultivar had an effect on that isolate's aggressiveness against that particular cultivar. However, a standard ANOVA tests the 'pool' of possible interactions rather than the specified interactions of interest. The confounding of different interactions is thus likely to yield a statistically non-significant overall interaction. In such an experiment therefore, a sub-division or dissection of the interactions is justified (Snedecor and Cochran, 1989). In this context, the variance of all the interactions has been split into two categories, the interaction of choice (or contrast) and all the remaining interactions (or remainder). This is termed 'comparisons among class means'.

5.2.4 Effect of removal of selection pressure on aggressiveness

A second experiment was performed to test the rate at which any changes in aggressiveness were lost upon the removal of selection pressure. In this case, the previously tested sub-isolates were returned to agar media for six weeks and their aggressiveness was re-tested on the four cultivars. Sub-isolates maintained on their respective 'host' cultivars (King Edward, Teena, Bintje and Maris Piper) throughout the course of the experiment were used as control and are hereafter referred to as 'aH' and 'bH'.

A single lesion isolate of each of the eight sub-isolates was taken from the infected leaves from the above experiment. Leaves were disinfected by immersion in 0.5% NaOCl for 2 min, washed five times with SDW and air dried. Approximately 1 cm sections were cut from the lesion margin and plated on to Rye A agar supplemented with chloramphenicol (34 mg l^{-1}), ampicillin (100 mg l^{-1}), rifamycin (30 μg l^{-1}) and pimaricin (10 μg l^{-1}). Cultures were incubated at room temperature and examined daily for any *P. infestans* growth, which was transferred to fresh Rye A plates. Pure cultures were normally obtained after 1-2 transfers of hyphal tips on media containing antibiotics.

The sub-isolates were maintained on Rye A agar for six weeks and were again used for inoculating a fresh batch of leaves. Sporangial suspensions were prepared for each sub-isolate as described previously (Section 5.2.2) and adjusted to $3.5x10^3$

sporangia ml^{-1}. The cultivars, experimental design and methods were identical to the previous experiment (Section 5.2.3).

5.2.5 Tracking of isolates in glasshouse studies

A detached leaf assay was established to test the relative ability of sub-isolates to cause disease when co-inoculated on to two different cultivars. The two isolates, 95.17.3.2 and 96.13.1.3, as described previously, were used as they could be distinguished by the SNP marker 56G18R. The scale of the experiment necessitated the use of only two cultivars, Teena (resistant) and Bintje (susceptible), selected on the basis of their level of resistance to late blight. A training regime was again established as described above, where each isolate was cultured on both cultivars for seven generations. This was carried out in separate boxes to avoid cross contamination. Treatments consisted of all combinations of four sub-isolate mixtures tested on each of two cultivars with four replicates (Table 5.3). The detached leaflets of the two test cultivars were laid out in plastic boxes (50x25 cm^2) in a split plot design.

Leaflets of approximately the same age were placed on top of moist paper towels (abaxial side up) and were co-inoculated with zoospores from a sporangial suspension ($3.5x10^3$ sporangia ml^{-1}) of the two sub-isolates in each treatment (Table 5.3), mixed in a 50:50 ratio. A single drop (10 μl) of the mixed sporangial suspension was placed near the centre of the leaflet. The boxes were sealed as before and incubated at 16°C in a growth cabinet with 16 h of artificial light.

After five days of incubation, sporangia were collected by immersing diseased leaves in a dish containing 5 ml of SDW. A Pasteur pipette was pulled in a Bunsen flame to create a fine tip and individual sporangia were picked by mouth suction using the modified Pasteur pipette attached to a flexible plastic tube. Single sporangia were transferred to pea broth in individual wells of a 96-well microtitre plate. The pea broth was amended with antibiotics, as described previously, to minimise bacterial contamination. Forty sporangia were selected from each treatment and the total number of sporangia selected from all treatments yielded a total of 1280 colonies.

Each well was examined under a microscope to ensure it contained only a single sporangium. After incubation at room temperature for one week, DNA was extracted (Wang *et al.*, 1993) from the mycelia from each well of the microtitre plate. Mycelium from each well was transferred aseptically to 1.5 ml Eppendorf tubes. The samples were ground in 20 µl of 0.5 N NaOH for approximately 30 sec with a Treff homogeniser and centrifuged at 13000 rpm for 5 min. An aliquot (5 µl) of the supernatant was immediately mixed with 45 µl of 100 mM Tris (pH 8.0) and 1 µl was used as a template for the SNP assay. The scale of this experiment necessitated the extraction of DNA over two consecutive days.

Fluorescent allele specific PCR (Ugozzoli and Wallace, 1991) was used to discriminate the two isolates recovered in each treatment. Individual isolates were characterised on the basis of the previously developed (Section 4.3.3) SNP marker 56G18R. PCRs for each isolate were run as described previously (Section 4.2.4) each using a different fluorescently labelled primer (*56G18R*^{S} and *56G18R*^{A}). Post PCR, the two samples were diluted and mixed in a 50:50 ratio and 0.5 µl run on ABI 3700 sequence detector. Data were analysed using Genescan and Genotyper software. Statistical analysis was performed using Genstat (Payne, 1997).

Table 5.3 Isolates of *P. infestans,* the cultivars used for 'training' and combinations in which they were used to test the interaction between sub-isolates and cultivars in the competition experiments.

Isolate	Trained on	Test cultivar	
		Bintje	Teena
95.17.3.2 (a)		aB x bB	aB x bB
	Bintje (B)	aB x bT	aB x bT
96.13.1.3 (b)	Teena (T)	aT x bB	aT x bB
		aT x bT	aT x bT

5.3 Results

5.3.1 Effect of 'host' cultivar on isolate adaptation

5.3.1.1 Effect of 'host' cultivar on adaptation of isolate 95.17.3.2 (a)

Large differences in aggressiveness were detected amongst the sub-isolates of isolate 95.17.3.2. There was strong statistical support ($p < 0.001$) for aggressiveness differences based on lesion diameter (Table 5.4). The sub-isolates formed two groups based on their overall aggressiveness on different cultivars. Sub-isolates aK and aT produced larger lesions than sub-isolates aB and aM (Table 5.5). The mean lesion size of sub-isolates aK and aT was ≥ 0.59 cm^2 compared to ≤ 0.56 cm^2 for sub-isolates aB and aM. Sub-isolate aR was consistently the least aggressive with a mean lesion diameter of 0.43 cm^2.

Unsurprisingly, marked and statistically significant differences in lesion size were observed between the cultivars ($p = 0.002$) (Table 5.4). The smallest lesions occurred on the cultivars Teena and King Edward whereas the largest lesions were observed on cv. Bintje with a mean lesion diameter exceeding 0.60 cm^2. The lesions on cv. Maris Piper were intermediate in size (Table 5.5).

There was no statistically significant cultivar by sub-isolate interaction when all possible interactions were considered ($p = 0.14$) (Table 5.4), although there was a clear trend towards increased lesion size caused by 'trained' sub-isolates (Table 5.5). The trend was clearly visible when mean lesion diameter of individual sub-isolates on various cultivars were examined (Fig. 5.1a). On three of the four cultivars, the sub-isolate 'trained' on that cultivar produced larger lesions than those caused by sub-isolates 'trained' on other cultivars. On cv. Maris Piper, the 'host' isolate produced lesions slightly smaller than those of the other sub-isolates. Sub-isolate aR was weakly aggressive on all cultivars and not considered in the analysis of the linear contrasts where the variation accounted for in the interactions was partitioned. The contrast that compared the variance between 'host' and 'non-host' interactions

showed a highly significant difference between the comparisons (p = 0.007) (Table 5.4).

There was a general trend towards increased aggressiveness of sub-isolates 'trained' on resistant cultivars to their 'non-hosts' compared to those 'trained' on susceptible cultivars. For example, sub-isolates aT and aM produced larger lesions on cv. King Edward than sub-isolate aB. A similar effect of sub-isolate aggressiveness was observed in all other cultivars.

5.3.1.2 Effect of 'host' cultivar on adaptation of isolate 96.13.1.3(b)

Analysis of variance revealed significant differences in aggressiveness amongst sub-isolates, measured in terms of lesion size ($p < 0.001$) (Table 5.6). On average, sub-isolate bM 'trained' on cv. Maris Piper resulted in larger lesions. The sub-isolate maintained on agar (bR) was consistently the least aggressive with mean lesion diameter of 0.47 cm^2 (Table 5.7).

Again unsurprisingly, a marked (but statistically non-significant) cultivar effect on lesion diameter was observed (Table 5.6). On average, cultivars Bintje and Maris Piper were the most susceptible, and the smallest lesions were formed on cv. Teena (Table 5.7).

The overall interaction between cultivars and sub-isolates was not significant (p = 0.11) (Table 5.6). However, a trend towards larger lesions on the initial 'host' cultivar was clear (Table 5.7). For three of the four cultivars, the sub-isolate 'trained' on that cultivar produced larger lesions on its 'host' cultivar than those 'trained' on a different cultivar (Fig 5.1b). However on cv. Bintje, sub-isolate bK caused larger lesions than the sub-isolate bB 'trained' on cv. Bintje itself. When the sub-isolate bR was removed from the analysis and the sub-isolate by cultivar interaction dissected, as described previously, a highly significant effect of 'training' was observed (p = 0.001) (Table 5.6).

A general trend of increased aggressiveness of sub-isolates 'trained' on resistant cultivars was again apparent amongst the 'non-host' interactions (Fig 5.1b). Sub-

isolate bB, for example was least aggressive for two of its three 'non-host' comparisons.

5.3.1.3 Adaptation patterns of isolate 95.17.3.2 (a) and 96.13.1.3 (b).

A comparison of aggressiveness of the two isolates revealed that the sub-isolates within each isolate produced a similar response on cultivars King Edward and Teena. However, a different response of the two isolates was observed on cultivars Bintje and Maris Piper. In the case of isolate 95.17.3.2 (a), the host adaptation effect was apparent on cv. Bintje but not on cv. Maris Piper (Fig 5.1a). For isolate 96.13.1.3 (b), the order of the two cultivars in terms of host adaptation effect was reversed (Fig. 5.1b)

Table 5.4 Analysis of variance of lesion diameter (cm) caused by isolate **95.17.3.2. (a)**. The cultivars by sub-isolate interaction was further dissected to determine if the 'host' interaction, or Contrast, differed significantly from that of the 'non-host' comparisons, or Remainder.

Source of variation	d.f	SS	MS	F	*p*
Block	3	0.061750	0.020583	3.06	
Sub-isolate	4	0.293750	0.073437	10.92	< 0.001
Cultivar	3	0.112750	0.037583	5.59	0.002
Cultivar x sub-isolate	12	0.123500	0.010292	1.53	0.140
Contrast	1	0.051680	0.051680	7.686	0.0075
Remainder	11	0.071820	0.006529	0.971	0.4827
Residual	57	0.383250	0.006724		
Total	79	0.9750000			

Table 5.5 Mean lesion diameter (cm) produced on different cultivars of potato by four sub-isolates of a *P. infestans* isolate **95.17.3.2 (a)**. Bold values represent the mean lesion diameter of sub-isolates on their respective 'hosts'. Isolates maintained on agar (aR) are included for comparison.

Isolate/Cultivars	King Edward	Teena	Bintje	Maris Piper	Mean
aK	**0.6750**	0.5500	0.6000	0.5875	0.6031
aT	0.5375	**0.5875**	0.6625	0.5875	0.5937
aB	0.4625	0.4875	**0.6875**	0.5750	0.5531
aM	0.5375	0.5125	0.6500	**0.5625**	0.5656
aR	0.4125	0.4250	0.4500	0.4500	0.4344
Mean	0.5250	0.5125	0.6100	0.5525	

5% LSD values = 0.058 (sub-isolate); 0.051 (cultivar); 0.116 (sub-isolate x cultivar)

Table 5.6 Analysis of variance of lesion diameter (cm) caused by isolate **96.13.1.3 (b)**. The cultivar by sub-isolate interaction was further dissected to determine if the 'host' interaction, or Contrast, differed significantly from that of the 'non-host' comparisons, or Remainder.

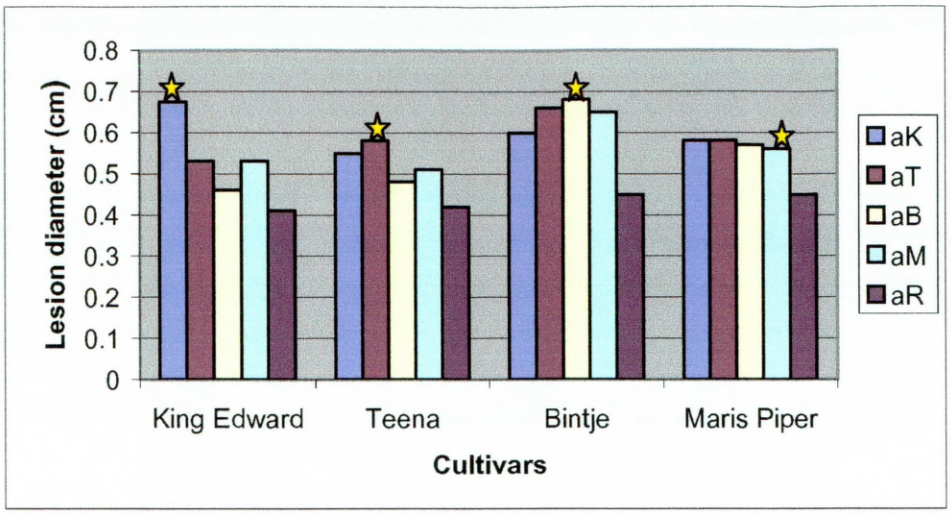
Source of variation	d.f	SS	MS	F	*p*
Block	3	0.103375	0.034458	4.64	
Sub-isolate	4	0.203437	0.050859	6.86	< 0.001
Cultivar	3	0.059625	0.019875	2.68	0.055
Cultivar x sub-isolate	12	0.142562	0.011880	1.60	0.117
Contrast	1	0.085429	0.085429	11.51	0.0012
Remainder	11	0.057133	0.005193	0.70	0.7332
Residual	57	0.422875	0.007419		
Total	79	0.931875			

Table 5.7 Mean lesion diameter (cm) produced on different cultivars of potato by four sub-isolates of a *P. infestans* isolate **96.13.1.3 (b)**. Bold values represent the mean lesion diameter of sub-isolates on their respective 'hosts'. Isolates maintained on agar (bR) are included for comparison.

Isolate/Cultivars	King Edward	Teena	Bintje	Maris Piper	Mean
bK	**0.6750**	0.5250	0.6000	0.6000	0.6000
bT	0.5000	**0.6000**	0.5625	0.5875	0.5625
bB	0.5500	0.4375	**0.5875**	0.5625	0.5344
bM	0.6250	0.4375	0.5875	**0.7000**	0.6125
bR	0.4625	0.4500	0.5375	0.4375	0.4719
Mean	0.5625	0.5100	0.5750	0.5775	

5% LSD values = 0.060 (sub-isolate); 0.054 (cultivar); 0.121 (sub-isolate x cultivar)

a)



b)

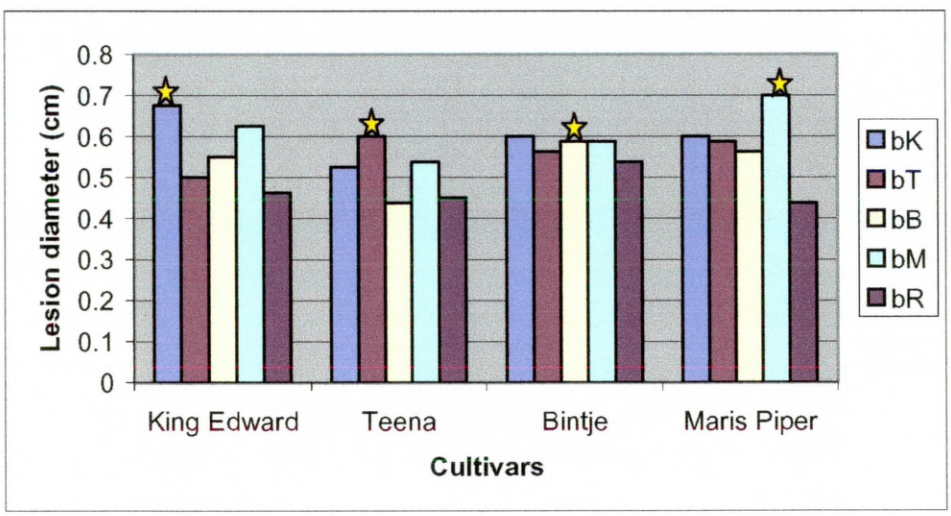


Fig. 5.1 Mean lesion diameter caused by isolate 95.17.3.2 (a) and 96.13.1.3 (b) on their 'host' and 'non-host' cultivars after seven successive generations on each of the four hosts. Isolates maintained on agar (aR and bR) represent the controls. Bars marked with a star represent the 'host' interactions.

Key: K = 'trained' on cv. King Edward; T = 'trained' on cv. Teena; B = 'trained' on cv. Bintje; M = 'trained' on cv. Maris Piper; R = maintained on agar.

5.3.2 Effect of removal of selection pressure on adaptation

The experiment followed on from the experiment described in Section 5.2.3 and tested whether cultivar adaptation was maintained upon the sub-isolate's return to maintenance on agar.

5.3.2.1 Response of isolate 95.17.3.2 (a) to removal of selection pressure

A two-factor ANOVA revealed statistically significant differences ($p < 0.001$) in lesion size caused by different sub-isolates (Table 5.8). The overall mean lesion size caused by sub-isolate aH (the sub-isolates maintained throughout on leaves of their 'host' cultivars) was larger than that caused by those isolates previously 'trained' but then returned to agar culture (Table 5.9). In contrast to the previous experiment, the sub-isolate aK was the least aggressive (Table 5.9). There was an overall reduction in the mean lesion diameter caused by each sub-isolate following culture in agar compared to those not grown on agar.

As expected, cultivar resistance had a marked effect on lesion diameter ($p = 0.011$) (Table 5.8). On average, cv. Bintje was the most susceptible and the smallest lesions were formed on cv. King Edward (Table 5.9). In contrast to the previous experiment, where lesions formed on cv. Bintje were significantly larger than those on all the other cultivars, mean lesion size on cv. Bintje in this experiment was not significantly different from that on cv. Teena (Table 5.9).

Although a significant cultivar by sub-isolate effect ($p = 0.020$) was observed, its contribution to the total variation was very low ($F = 2.26$) (Table 5.8). The F-test for the apportionment of the linear contrast, excluding the data from sub-isolate aH, was non-significant ($p = 0.366$) (Table 5.8). The basis for the significant overall cultivar by sub-isolate interaction is not clear, as no strong interaction was evident (Fig 5.2a). The response of various sub-isolates was variable. The previously observed effects of cultivar 'training' were either absent (cultivars King Edward and Bintje) or less clear (cultivars Teena and Maris Piper) amongst the isolates grown on agar for six weeks (Fig 5.2a).

In no case were sub isolates from agar more aggressive than their counterparts maintained on their respective cultivars.

5.3.2.2 Response of isolate 96.13.1.3 (b) to removal of selection pressure

There were statistically significant differences ($p < 0.001$) in the lesion size caused by different sub-isolates (Table 5.10). As with isolate a, the lesions caused by sub-isolate bH were significantly larger than those of isolates returned to agar medium. There was no significant difference between sub-isolates bK, bB, bM and bT(Table 5.11). In contrast to the previous experiment, sub-isolate bT produced the largest lesions.

Unsurprisingly, cultivar resistance had a significant effect on lesion size ($p < 0.001$) (Table 5.10), with the smallest lesions occurring on cv. Teena and the largest on cv. Maris Piper (Table 5.11). The results are different from those in the previous experiment where the same isolate produced larger lesions on cv. Bintje and cv. Maris Piper. In both experiments however the smallest lesions were produced on cv. Teena.

An examination of the specific interaction between the isolates previously 'trained' on a particular cultivar and the lesion size produced on that cultivar after six weeks on agar, indicated a loss of prior adaptation i.e. a non significant interaction ($p = 0.331$) (Table 5.10, Fig 5.2b). None of the previously 'trained' sub-isolates from agar produced lesions larger than those maintained on plant material.

A comparison of the host adaptation effects between the two isolates revealed similar responses of the sub-isolates in each case. There was a loss of host adaptation effect when sub-isolates were removed from their respective 'host' cultivars.

Table 5.8 Analysis of variance of lesion diameter (cm) caused by isolate **95.17.3.2 (a)**. The cultivar by sub-isolate interaction was further dissected to determine if the prior 'host' interaction, or Contrast, differed significantly from that of the 'non-host' comparisons, or Remainder.

Source of variation	d.f	SS	MS	F	*p*
Block	3	0.0043438	0.0014479	1.47	
Sub-isolate	4	0.0476875	0.0119219	12.07	< 0.001
Cultivar	3	0.0120937	0.0040312	4.08	0.011
Cultivar x sub-isolate	12	0.0268125	0.0022344	2.26	0.020
Contrast	1	0.000826	0.000826	0.83	0.3661
Remainder	11	0.0259865	0.002362	2.39	0.0163
Residual	57	0.0562812	0.0009874		
Total	79	0.1472188			

Table 5.9 Mean lesion diameter (cm) produced on different cultivars of potato by four sub-isolates of a *P. infestans* isolate **95.17.3.2 (a)** after their return to agar culture for six weeks. Bold values represent the mean lesion diameter of sub-isolates on their respective 'hosts'. Isolates maintained on plant material (aH) are included for comparison.

Isolate/Cultivars	King Edward	Teena	Bintje	Maris Piper	Mean
aK	**0.4375**	0.4625	0.5250	0.4500	0.4688
aT	0.4625	**0.5125**	0.4750	0.4750	0.4813
aB	0.4500	0.5000	**0.5000**	0.4375	0.4719
aM	0.5125	0.5000	0.5000	**0.5000**	0.5031
aH	0.5250	0.5250	0.5375	0.5500	0.5344
Mean	0.4775	0.5000	0.5075	0.4825	

5% LSD values = 0.022 (sub-isolate); 0.019 (cultivar); 0.044 (sub-isolate x cultivar)

Table 5.10 Analysis of variance of lesion diameter (cm) caused by isolate **96.13.1.3 (b)**. The cultivar by sub-isolate interaction was further dissected to determine if the prior 'host' interaction, or Contrast, differed significantly from that of the 'non-host' comparisons, or Remainder.

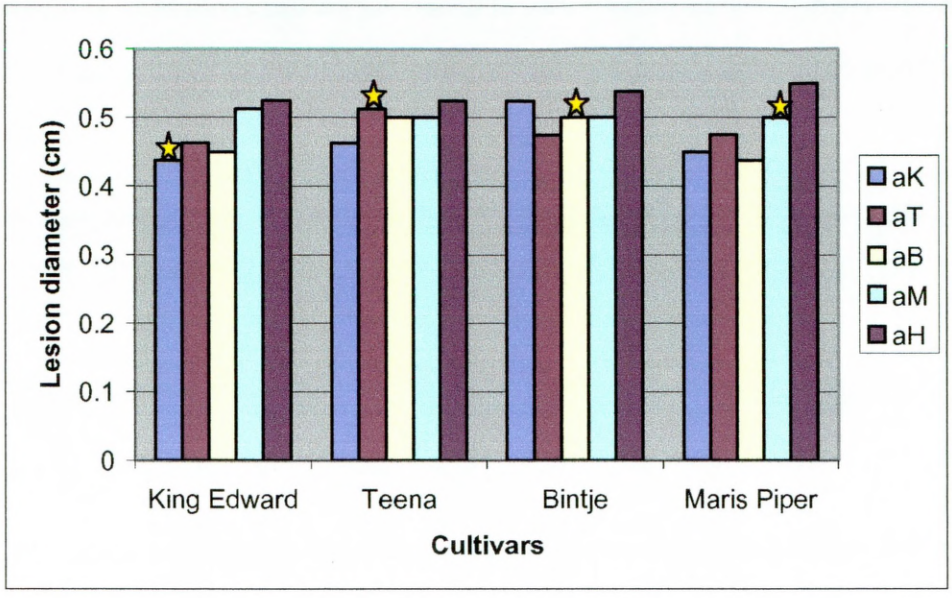
Source of Variation	d.f	SS	MS	F	*p*
Block	3	0.011125	0.003708	1.61	
Sub-isolate	4	0.055437	0.013859	6.01	< 0.001
Cultivar	3	0.042625	0.014208	6.16	< 0.001
Cultivar x sub-isolate	12	0.023313	0.001943	0.84	0.607
Contrast	1	0.002219	0.002219	0.96	0.3313
Remainder	11	0.021094	0.001917	0.83	0.6111
Residual	57	0.131375	0.002305		
Total	79	0.263875			

Table 5.11 Mean lesion diameter (cm) produced on different cultivars of potato by four sub-isolates of a *P. infestans* isolate **96.13.1.3 (b)** after their return to agar culture for six weeks. Bold values represent the mean lesion diameter of sub-isolates on their respective hosts. Isolates maintained on plant material (bH) are included for comparison.

Isolate/Cultivars	King Edward	Teena	Bintje	Maris Piper	Mean
bK	**0.5625**	0.4750	0.5375	0.5000	0.5188
bT	0.5250	**0.5000**	0.5375	0.5750	0.5344
bB	0.5500	0.4750	**0.5000**	0.5250	0.5125
bM	0.5375	0.4875	0.4875	**0.5625**	0.5188
bH	0.6000	0.5500	0.5875	0.6600	0.5844
Mean	0.5500	0.4975	0.5300	0.5525	

5% LSD values = 0.033 (sub- isolate); 0.030(cultivar); 0.067 (sub-isolate x cultivar)

a)



b)

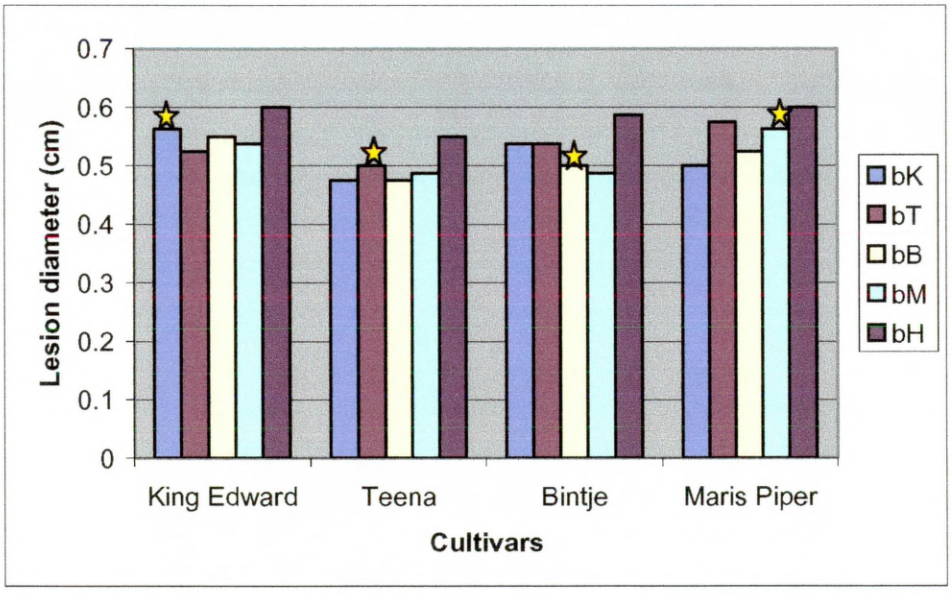


Fig. 5.2 Lesion diameter caused by the host-adapted groups of isolates 95.17.3.2 (a) and 96.13.1.3 (b) on different cultivars after maintenance on agar for six weeks prior to inoculation. Isolates maintained on plant material (aH and bH) are included for comparison. Bars marked with a star represent the response of the previously trained isolate on its host cultivar.

Key: K = 'trained' on cv. King Edward; T = 'trained' on cv. Teena; B = 'trained' on cv. Bintje; M = 'trained' on cv. Maris Piper; H = maintained on respective 'host' cultivar.

5.3.3 Tracking of *P. infestans* isolates

The objective of this co-inoculation experiment, in which sub-isolates were inoculated in a 50:50 ratio, was to examine any interactions between the previously 'trained' sub-isolates and cultivars when competing directly on the same leaf. This was assessed indirectly by examining the number of sporangia produced by each sub-isolate. The sub-isolate combinations were aB with bB, aB with bT, aT with bB and aT with bT with each combination co-inoculated onto the cultivars Teena and Bintje (Table 5.3) in a 2x4 factorial experiment. A total of 40 sporangia were selected at random from each treatment and the numbers from each isolate scored using a previously developed SNP marker (Chapter 4). The data were analysed as the proportion of sporangia of isolate 95.17.3.2 (a) to that of isolate 96.13.1.3 (b) in each possible sub-isolate by cultivar combination.

Variations were found among the number of germinated and non-germinated sporangia in each treatment. Anticipating non-uniform sporangial germination, sporangia were deliberately over-sampled in each treatment. The strategy proved useful in selecting the required number of sporangia from each treatment. Technical difficulties precluded complete genotyping of all sporangia in some of the treatments. Approximately, 14% of the individual sporangia could not be scored due to either failure of DNA extraction or PCR amplification. Allele frequencies were estimated by fluorescent allele specific PCR followed by automated scoring of alleles using the 'allele binning' feature of the Genotyper software. All samples gave fully interpretable genotypes, and allelic configurations were consistently clear, with the exception of a few cases in which signal levels fell below the peak threshold levels.

Analysis of variance showed that there were no statistically significant differences (p = 0.971) between the cultivars in terms of sporangial recovery when measured in terms of proportion of sporangia of isolate 'a' to that of isolate 'b'. (Table 5.12). The overall effect of the different combinations of sub-isolates was also statistically non-significant (p = 0.262). In each case, the treatments necessarily confounded each other, so the lack of clear treatment effects was to be expected. The number of

sporangia of each isolate recovered in the interaction between sub-isolate combination and cultivar was highly significant ($p < 0.001$) (Table 5.12). The most striking differences in the proportion of sporangia of each sub-isolate recovered involved the treatments in which a 'host' sub-isolate competed against a 'non-host' sub-isolate (Table 5.13; Fig. 5.3). For example, among the sub-isolate combinations inoculated on cv. Bintje, the highest proportion (0.66) of sporangia of isolate 'a' (95.17.3.2) was observed from sub-isolate combination aB x bT ('a' trained on cv. Bintje; 'b' trained on cv. Teena). Similarly, among the sub-isolate combinations grown on cv. Teena, the highest proportion (0.70) of isolate 'a' was recovered from treatment combination aT x bB ('a' trained on cv. Teena; 'b' trained on cv. Bintje). When sub-isolates trained on the same cultivar were co-inoculated, similar proportions of sporangia of each sub-isolate were generally recovered. This was particularly clear on cv. Bintje whereas on cv. Teena the proportion was more variable (Fig. 5.3).

Table 5.12 Analysis of variance of sporangial proportion from a 2 by 4 factorial experiment in a split plot design. Figures in parentheses in the d.f column represent the missing values.

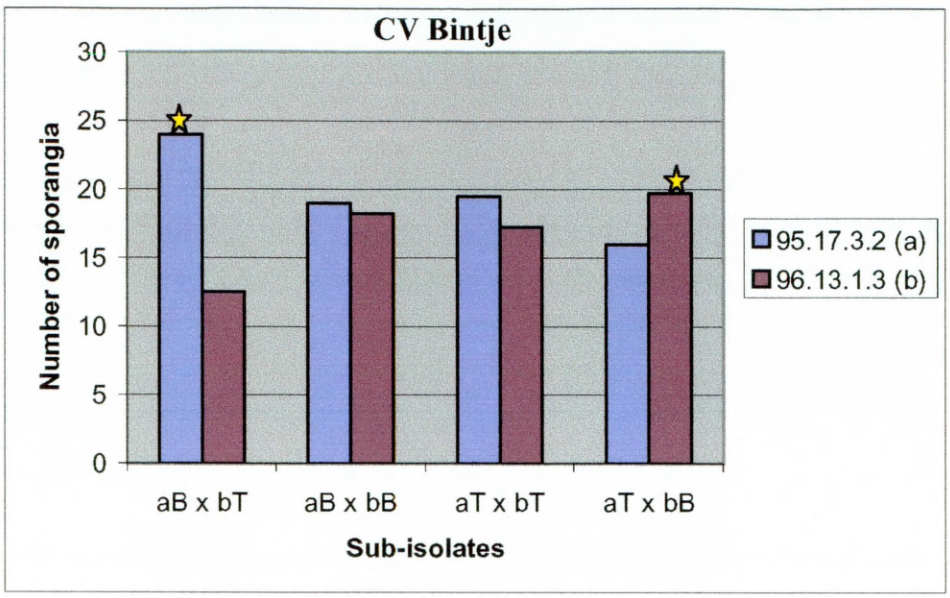
Source of variation	d.f	SS	MS	F	*p*
Replicates	3	0.039095	0.013032	0.55	
Whole Plot					
Cultivar	1	0.000037	0.000037	0.00	0.971
Residual	3	0.070485	0.023495	2.78	
Sub plots					
Sub-isolates	3	0.037022	0.012341	1.46	0.262
Cultivar x sub-isolate	3	0.274596	0.091532	10.85	< 0.001
Residual	16 (2)	0.135036	0.008440		
Total	29 (2)	0.536247			

Table 5.13 Proportion of sporangia of isolate 95.17.3.2 (a) to that of 96.13.1.3 (b) 'trained' on two different cultivars Bintje (B) and Teena (T) and inoculated on their 'host' and 'non-host' cultivars in a competition experiment.

Cultivar/sub-isolate	aB x bT	aB x bB	aT x bT	aT x bB	Mean
Bintje	0.660	0.508	0.534	0.452	0.538
Teena	0.423	0.595	0.438	0.706	0.540
Mean	0.511	0.551	0.486	0.579	

Se = 0.038 (cultivar); 0.032 (sub-isolate); 0.055 (cultivar x sub-isolate)

a)



b)

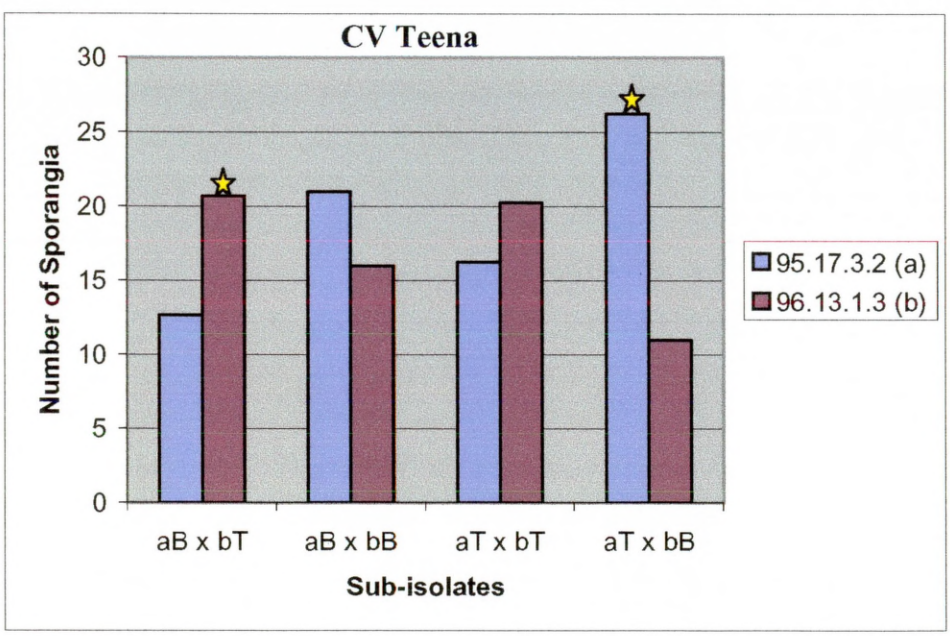


Fig. 5.3 The number of sporangia of each of two isolates recovered from the leaves of cv. Bintje (a) or cv. Teena (b) 5 days after inoculation with a 50:50 mixture of inoculum of each isolate. The isolates were previously 'trained' on either cv Bintje or cv. Teena and all combinations of mixtures of these sub-isolates were compared. The combinations in which the 'host' trained sub-isolate is competing against a 'non-host' trained sub-isolate are marked with a star.

Key: aB and bB= 'trained' on cv. Bintje; aT and bT= 'trained' on cv.Teena

5.4 Discussion

Host adaptation of *P. infestans* has been reported in potato and tomato (Kori, 1972; Legard *et al.*, 1995; Oyarzun *et al.*, 1998; Vega-Sanchez *et al.*, 2000) and has been referred to as quantitative rather than qualitative, since isolates, although more aggressive on their original host, are also pathogenic on the other host (Berg, 1926; Turkensteen, 1973; Vega-Sanchez *et al.*, 2000). The existence of such an adaptation within potato cultivars was central to the hypothesis of this part of the studies. Although cultivar adaptation in other host-pathogen systems has been documented (Johnson and Taylor, 1972; Parlevliet, 1977; Jeger *et al.*, 1981), there are conflicting reports concerning the existence of such a phenomenon in the *P. infestans*-potato system. Jeffrey *et al.* (1962) and Caten (1974) reported better growth of *P. infestans* isolates on tubers of the cultivars from which they were originally isolated than on tubers of other cultivars. The results were refuted by Paxman (1963) and James and Fry (1983) who found no conclusive evidence of adaptation.

In the present studies, there was some evidence to support the hypothesis of host adaptation which is presumably not based on classical R-genes. The experiment was designed to specifically test whether sub-isolates on 'non-hosts' behaved in a different manner to those on their 'host' cultivar and a clear trend towards host adaptation was observed. It was not surprising that the overall interaction was non-significant as this involves the analysis of the interaction data of not only the 'host' but all the 'non-host' interactions. The latter combinations would not be expected to show clear sub-isolate by cultivar interactions and indeed they did not. It was therefore deemed necessary to split the variation accounted for by the sub-isolate by cultivar interaction to determine if the interaction of interest was significant (Snedecor and Cochran, 1989). Such a statistical approach can only be justified in experiments designed to test one specific interaction of the many examined. Such analysis is commonly used in the analysis of clinical trials for example. For both isolates, the contrasts were significant, thereby indicating an effect of host 'training' on isolate aggressiveness. Significant cultivar by sub-isolate interactions were reported by Jinks and Grindle (1963) and Latin *et al.* (1981), who concluded that the *P. infestans* population adapted in response to growth on specific cultivars. Such

significant cultivar by sub-isolate interactions have been variously reported to break down field resistance (Caten, 1974; Latin *et al.*, 1981; Bjor and Mulelid, 1991). Others however believe in the stability of field resistance even if cultivars are attacked by complex races (Niederhauser, 1962; Paxman, 1963; Van der Plank, 1971; James and Fry, 1983; Parker *et al.*, 1992; Colon *et al.*, 1995). Toxopeus (1956) suggested that such erosion may be very limited due to the high number of mutations required by the pathogen to overcome multigene resistance.

It is interesting that such clear interactions were observed in an experiment that was run over seven generations (7 weeks) in a laboratory assay. It is possible that the effects would be even clearer if the isolates are allowed to adapt to a cultivar for a longer period of time. One should caution that lesion size may not be a representative measure of aggressiveness and other parameters such as latent period, infection frequency and sporulation capacity could be included in future studies. The latter two parameters are important since they determine the amount of secondary inoculum produced, thereby translating into secondary infection (Lebreton *et al.*, 1999). Comparisons on whole plants would also be useful although there would be logistical problems of preventing cross-contamination between treatments during the training process.

Efforts were made to select leaves of the same age to confer a similar degree of resistance. Moreover, the experiment was conducted under controlled conditions to analyse the interactions and minimise the effect of confounding factors. Several workers have advocated the validity of detached leaf assays (Lapwood, 1961; Malcolmson, 1969; Umaerus and Lihnell, 1976) and have shown that the results obtained in such assays are in good agreement with those obtained under field conditions (Hodgson, 1962; Singh and Birhman, 1994). Vleeshouwers *et al.* (1999) recently showed that detached leaf assays are a good alternative for field tests in studies on aggressiveness of *P. infestans*. Additionally, detached leaf assays offer many advantages. The comparison of 'indigenous' and 'non-indigenous' isolates can be made easily and the unpredictability of weather conditions can be accounted for in the detached leaf assays. Also it is difficult to compare isolates in the field because of the risk of contamination from airborne inoculum from adjacent fields (Carlisle *et al.*, 2002).

The results of this study were followed up by re-testing the aggressiveness of isolates from the above experiment after maintenance on agar for six weeks. None of the agar isolates produced larger lesions than those continuously maintained on plant tissue. The results are in agreement with others (Gallegly, 1968; Miller *et al.*, 1998; Ordonez *et al.*, 1998) who reported loss of aggressiveness after culturing on artificial media.

The increase in aggressiveness gained through host adaptation was lost to a large extent when isolates were returned to agar culture. This could be interpreted as regression of the isolates to the original state of aggressiveness if the opportunity to infect the same host is denied. The results indicate that adaptation, although being significant, is reversible and presumably does not involve actual change at the DNA level (Van West *et al.*, 1999). The mechanisms for such a process are unknown, but indicate a feedback mechanism between the environment to which isolate is exposed and the expression of its genes. It is hypothesised that a 'trained' isolate is probably able to silence one or more of its non 'housekeeping' genes when infecting a 'host' cultivar in order to bypass the host defences. However, upon removal of the selection pressure, the genes are re-activated thereby indicating this to be a reversible process. In the present study, this was evident from the results of the follow up experiment in which the effect of host adaptation was lost when 'host' cultivars were challenged with previously 'trained' isolates after maintenance on agar culture for six weeks. Detailed molecular studies are however needed to test the validity of this hypothesis.

The results show that in principle, the resistance of cultivars used over long periods, could be eroded as *P. infestans* isolates adapt to them. However, one could argue that many cultivars have been grown for 30 or more years without a breakdown of their partial resistance. Therefore adaptation may be a factor in competition or survival of different strains but not significant enough to fully erode host resistance. On the other hand, Flier (2001) reported erosion of partial resistance of cv. Pimpernel under field conditions in The Netherlands due to host specificity. A possible strategy to counter the effect of host adaptation is therefore the use of cultivar mixtures (Browning and Frey, 1969; Wolfe and Barrett, 1980; Wolfe *et al.*, 1981). However, a concern in deploying cultivar mixtures is the adaptation of a strain of the pathogen against multiple sources of resistance (Groth, 1976; Newton, 1997). Also there could

be difficulties for the growers in practicalities of growing cultivar mixtures since in most cases the end users (e.g. supermarkets) demand single cultivars.

In general, isolates 'trained' on a resistant cultivar were more aggressive on their 'non-host' cultivars than those 'trained' on a susceptible cultivar. It may be speculated that there is a stronger selection pressure for aggressiveness on a resistant cultivar than on a susceptible one. Since agar offers no selection pressure for aggressiveness, there was a consistently low aggressiveness of the isolates maintained on agar than those on plant material.

The isolate tracking experiment showed that PCR assays, coupled with automated detection were successful in determining the proportion of sporangia of each test isolate in a competitive experiment. The assay was cost effective, and the use of 96-well plate and an eight channel multipipette reduced the time for sample preparation. Ideally, individual sporangia would have been amplified directly but the primers were not sensitive for such amplification. Therefore, individual sporangia were added to pea broth amended with antibiotics in microtitre plates to start micro-cultures from which DNA was extracted. Using this high throughput protocol, 1280 isolates were examined within two weeks. However, the assay could be used to screen single lesion isolates directly from leaf lesions, therefore, making it possible to undertake large scale population studies.

This is the first report of its kind on examination of inter-isolate competition within a single lesion. The isolate tracking experiment validated the results of the host adaptation experiment to a large extent thereby suggesting that sporangial count is a reliable measure of aggressiveness. Generally more sporangia were produced on a cultivar by an isolate adapted to that cultivar than by its non-adapted competitor isolate. Moreover, in these comparisons, isolate 'a' generally out-competed isolate 'b' by a greater margin than isolate 'b' out-competing isolate 'a', thereby suggesting that isolate 'a' is the more aggressive isolate.

It can be argued that differences in the latent period of the two isolates could affect the outcome of a competition experiment based on the number of recovered sporangia. However, in the present study, the response of both 'trained' isolates on

their 'host' cultivars was similar suggesting that differences in isolates were due to the effect of host adaptation. Detailed studies examining the effect of latent period of the isolates on their aggressiveness when co-infecting a leaf would be intriguing. The availability of co-dominant SNP markers will be useful in such competition studies between isolates within a single lesion.

General Discussion

In recent years, late blight has become a significant problem in many countries of the world (Erwin and Ribeiro, 1996; Fry and Goodwin, 1997a). Several factors including poor detection of the pathogen, a lack of knowledge of the role of soilborne inoculum and an inability to track isolates in the environment have contributed to increased crop losses. Moreover, a lack of understanding of how *P. infestans* populations evolve under the selection pressures imposed by breeding i.e. how they adapt to new forms of host resistance also needs to be addressed. New information on these aspects is expected to contribute to improved late blight management, and they are therefore addressed in this thesis.

Conventional diagnostic methods used for the identification of *P. infestans* are either not dependable, or are time consuming, and are therefore unrealistic for widespread application. The use of PCR has been shown to be a rapid, sensitive and specific method for pathogen identification and detection (Henson and French, 1993; Hadidi *et al.*, 1995; Miller, 1996; Vandermark *et al.*, 2000). The development of a PCR assay for the detection of *P. infestans* in soil and plant tissue is described. The primers INF FW2 and INF REV were designed from the ITS region of rDNA and were shown to be specific to *P. infestans* when tested against various *Phytophthora* spp. and potato blemish pathogens. The primers did amplify DNA from *P. mirabilis* and *P. phaseoli* in addition to *P. infestans,* but they can still be used effectively, since *P. mirabilis* and *P. phaseoli* do not infect potatoes (Judelson and Tooley, 2000). The sensitivity of the diagnostic assay, as described in Chapter 2, also makes it ideal for amplification of DNA from situations where the target DNA is in low concentrations or is difficult to amplify, such as in soil.

The PCR assay developed in this work enables detection of *P. infestans* in tuber samples that show no outward disease symptoms and is therefore ideal for the detection of latent infection of seed tubers by *P. infestans*. In the present studies, two commercial seed stocks were assayed, and cryptic tuber infection was detected in several samples. It is expected that the commercial application of this rapid and sensitive diagnostic assay will enable indexing of commercial seed stocks according to infection. Such indexing could prevent the use of infected tubers as seed in the

following season thereby preventing the spread of late blight to subsequent crops or, perhaps more importantly, to limit the further spread of new genotypes from one region to another.

The co-occurrence of both mating types of *P. infestans* in many parts of the world (Spielman *et al.*, 1991; Fry *et al.*, 1992; Fry *et al.*, 1993; Fry and Goodwin, 1997a) has led to the production of oospores, with a possible epidemiological impact. It is therefore important to screen field soils to detect soilborne inoculum of *P. infestans* which could initiate late blight epidemics in the absence of other sources of inoculum. The PCR assay described above can be used for such a rapid screen of field soils and has a huge potential for monitoring *P. infestans* soil propagules. The use of molecular techniques to detect oospores in soil has been demonstrated for the first time in this thesis and already scientists at Plant Research International, The Netherlands have applied this assay in a survey of Dutch potato fields. The diagnostic assay could therefore prove useful in potato cropping management decisions regarding selection of suitable fields, cultivars or the adjustment of fungicide spray schedules. A limitation of the use of the PCR assay for evaluating commercial soil samples is its inability to distinguish between live and dead cells of the pathogen. In the present study, although a good correlation was found between PCR results and viability of oospores, as determined by a baiting assay, it is possible that the presence of PCR-amplifiable dead propagules in a sample could produce misleading results. For an interim period, PCR results must therefore be validated by a baiting assay (Drenth, 1994) to determine the infectivity of the propagules detected. Factors such as variable dormancy of oospores (Mayton *et al.*, 2000) however mean that baiting itself is subject to experimental error and perhaps a combination of both methods will provide the best solution. The *P. infestans* specific PCR assay was also used successfully to detect low numbers of zoospores and sporangia, and can therefore be used to verify the presence of *P. infestans* inoculum in irrigation water, tuber washings and aerial spore traps.

The PCR diagnostic assay coupled with the baiting test was an effective tool for epidemiological studies of the survival of oospores and sporangia in the field. The oospore DNA detection under field conditions, using this method, is the first report of its kind. The assay therefore obviates the need to rely on methods such as

plasmolysis and tetrazolium bromide tests (Medina and Platt, 1999), which give inconsistent results concerning the viability of oospores. The present study followed the survival of *P. infestans* inoculum (oospores and sporangia) in the field for two years thereby supplementing previous studies (Pittis and Shattock, 1994; Drenth *et al.*, 1995; Medina and Platt, 1999), which covered shorter periods. Results showed that oospores appear to be an important source of inoculum, capable of long term survival. The intensity and incidence of PCR signals detected from oospores buried in the field over a period of time suggested a trend towards a decline in their number after 21 months. It should be noted however, that a few viable oospores may be enough to initiate a disease epidemic under favourable conditions. The existence of viable oospores even after two years of burial in the field under exposure to natural extremes of temperature and moisture reinforces the point that oospores can be a threat to potato production in areas where both mating types exist. It should be pointed out that the present results can only be interpreted under the set of environmental conditions tested, although these are typical of many potato growing areas in Europe. It is however possible that other climatic and edaphic factors might influence oospore survival differently than that observed in the present studies.

The results of the baiting assay clearly demonstrated that sporangia buried in a field did not infect potato leaves at any of the sampling dates. It can be assumed therefore that survival of sporangia in soil from one crop to another is not a significant factor in late blight epidemiology. The results are in agreement with previous studies (Murphy, 1922; Zan, 1962; Lacey, 1965; Bogulavskaya and Filippov, 1977; Sato, 1980; Andrivon, 1994a).

As mentioned previously, the influx of the A2 mating type of *P. infestans* into many parts of the world has increased the chances of sexual reproduction of the pathogen. This allows the fungus to produce oospores as a potential source of inoculum and can increase the severity of the disease as a result of recombination, leading to the formation of fitter genotypes. Hence, it is important not only to understand the nature of the existing pathogen population but also to keep track of the changes in the population structure of the pathogen over time. An ideal way of detecting such changes is through the use of co-dominant markers. Such markers have an advantage over the existing dominant markers in that they are more rapid and straightforward to

use and have a higher resolving power in detecting heterozygosity in *P. infestans* populations. Their use will make it possible to determine allele frequencies and gene flow between populations. Co-dominant markers can also be used in epidemiological studies including the tracking of individual isolates in co-inoculation studies. In the present studies, SNP markers were developed to reliably characterise *P. infestans* populations.

SNP detection was carried out in coding and non-coding regions of the *P. infestans* genome. Eight SNPs in gene sequences and 28 in non-coding regions were identified, which translates to 2 SNP per kb. A panel of nine SNP markers was eventually developed from the identified SNPs. Segregation analysis revealed that, with the exception of the markers 56E14R and *Glutamine synthetase* (linked in the coupling phase), the markers segregated independently. Scoring of isolates with markers from different loci makes it a multilocus, co-dominant marker system. Two SNPs detected in the genes *ADP/ATP translocase* and *Ubiquitin conjugating enzyme* were monomorphic amongst the Scottish isolates tested, but did discriminate amongst a collection of international isolates.

The automated assays, using fluorescently labelled allele specific, primers (Matsubara *et al.*, 1999) proved a more rapid means of genotyping. The protocol is simple involving only a few operations. PCR is followed by the dilution of PCR products before mixing and running directly on ABI 3700 capillary-based sequence detector. The use of 96-well plates and robotic handling dramatically increases the assay throughput. Processing of the samples takes approximately two hours, multiple plates can be processed in parallel and data are recorded automatically. The final volume of the PCR products used for analysis is only 0.5 μl, which represents a small proportion of the total generated product. Therefore scaling down the original volumes of the reagents not only can save costs but is also useful for samples with a limited amount of starting material. Tests during the course of this study proved reproducible, reliable and sensitive enough to be used in population studies and the use of this technology on spores washed from single lesions could drastically increase throughput of field surveys.

Furthermore, several polymorphic loci could be tested in a single well by pooling of PCR products. Fourteen primers representing seven SNP loci were successfully evaluated simultaneously for the genotyping of the 42 Scottish *P. infestans* isolates. The careful selection of amplicon size and type of fluor label permitted the unambiguous identification of alleles representing all SNP loci. The assay can be modified by adding or substituting additional SNP sites without further optimisation. Of the 294 SNP allele by isolate combinations evaluated in the present study, the assay allowed unambiguous genotyping in 97% of cases. The fluorescence-based assay is therefore robust and efficient for a high throughput analysis of large scale populations.

Genetic diversity was investigated in a sub-population (n = 42) of Scottish *P. infestans* isolates, collected between 1995-97. Seven SNP markers revealed polymorphism within this sub-population and the relationship between the isolates was clearly elucidated. The results were in agreement with earlier studies (D. E. L. Cooke, pers. comm.) where a set of fifteen AFLP markers was used. This study did not attempt to address the issue of population structure of *P. infestans* in Scotland, as only a limited number of isolates were examined. The results are however in agreement with the previous proposed structure of the *P. infestans* population in Scotland with a limited number of dominant clones, between which sexual recombination is ongoing.

The development of SNP markers made the characterisation of individual isolates in competition experiments feasible. The markers thus provide not only a tool for population studies, but also an effective means of answering epidemiological questions. In the present study, the effect of host adaptation on the relative proportion of sporangia produced by two isolates (95.17.3.2 and 96.13.1.3) was evaluated using SNP marker 56G18R in an automated assay. The isolates were 'trained' separately on two cultivars and co-inoculated in a 50:50 ratio on the same cultivars. After rapid analysis of the SNP locus of 1280 single sporangia, the results showed that there was a significant interaction between the origin of the isolates (cultivars on which they were 'trained') and the cultivars in terms of sporangia produced. For example isolate 95.17.3.2 'trained' on cv. Bintje produced significantly more sporangia than isolate 96.13.1.3 'trained' on cv. Teena when they

were co-inoculated on cv. Bintje. The same general trend was observed in separate 'training' experiments in which host adaptation of individual isolates was measured using lesion size as a parameter to measure aggressiveness. The results are in agreement with previous studies of Jeffrey *et al.* (1962) and Caten (1974) who measured aggressiveness on tubers. Although growth of *P. infestans* on tubers and foliage is governed by different factors, and expression of resistance in foliage and tubers is not necessarily related (James and Fry, 1983), the results of the present study clearly demonstrated host adaptation effects when measured as relative sporulation capacity and lesion area. The results show that the adaptation of *P. infestans* to cultivars with rate reducing resistance could affect the durability of existing and future cultivars. Flier (2001) recently reported the erosion of the resistance of the cultivar Pimpernel in The Netherlands as a result of changes. A detailed study involving more components of aggressiveness on a larger set of cultivars and isolates in the field is required to validate these findings. The availability of suitable molecular marker technology, described in this thesis, will help researchers to address this question in large scale studies.

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