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Elucidating the impact of novel A1 and A2 Phytophthora infestans strains on existing Irish blight populations

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Elucidating the impact of novel A1 and A2 *Phytophthora infestans* strains on existing Irish blight populations

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Summary

The over-arching objective of this study was to explore the potential impact of novel A1 and A2 isolates of *Phytophthora infestans* on the existing population in Ireland and the impact of these new strains on potato blight management efforts. Towards this, activities were executed to determine *P. infestans* population structure and genetic diversity and afterwards to investigate the potential for changes in *P. infestans* populations through sexual recombination or host resistance associated adaptive change. Simple sequence repeat (SSR) marker based analysis of the sampled Irish population (in 2009) revealed: (i) moderate genetic diversity (ii) two co-existing clusters delineating with mating type and metalaxyl phenotypes (iii) on-going displacement of existing A1 clonal lineages by new genotypes.

In parallel, higher foliar blight aggressiveness on four potato cultivars was recorded for isolates with new genotypes compared to those isolates of 'traditional' genotypes. SSR analysis of F1 progeny derived from crosses between the new A1 and A2 isolates revealed levels of genetic diversity, typically expected from mating. However, the F1 progeny were recorded to be less fit on a moderately resistant potato cultivar Cara compared to parental isolates.

Following several reiterative host-pathogen interactions, the new A1 and A2 isolates demonstrated the potential to adapt to several of the transgenic potato lines tested, which are equipped with the *'resistance to blight'* (*RB*) gene. Adapted isolates showed amino acid substitutions in the carboxylic terminal of the avirulent *ipiO* protein, which is the complementary target of the *RB* protein.

Separately, efforts to develop a quantitative PCR assay for *P. infestans* were also explored culminating in a technique capable of detecting asexual propagules of *P. infestans* in infected tubers.

In light of the emergence of novel A1 and A2 strains of *P. infestans* in Ireland, this study provides valuable information both to the research community and the Irish

potato sector as a whole. This is timely in light of the high blight pressure continuously recorded throughout this study and in regards to the developing debate on the testing of GM blight resistant potatoes in Ireland.

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Abbreviations

µg:	microgram
µl:	microlitre
bp:	base pair
CAA:	Carboxylic Acid Amide
CTAB:	cetyltrimethylammonium bromide
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphate
EDTA:	Ethylenediaminetratraacetic acid
Expt:	Experiment
fg:	femtogram
g:	gram
***GUS*:**	beta-glucuronidase enzyme from *Escherichia coli*
HCl:	Hydrochoric acid
HWE:	Hardy-Weinberg Equilibrium
LRR:	Leucine Rich Repeat
MCMC:	Monte Carlo Marcov Chain
mg:	milligram
MSA:	Microsatellite analyzer
mtDNA:	Mitochondrial DNA
NBS:	Nucleotide Binding Site
NCBI:	National Centre for Biotechnology Information, USA
ng:	nanogram
***npt*II:**	neomycin phosphotrasferase II
PCA:	Principal Component Analysis
PCR:	Polymerase Chain Reaction
pg:	picogram
QiIs:	Quinone Inside Inhibitors
QoIs:	Quinone Outside Inhibitors
QTLs:	Quantitative Trait Loci
RXLR:	a**r**ginine-any amino acid-**L**eucine-A**r**ginine motif
SSR:	Simple Sequence Repeat

[1]Definitions

Allele: an alternative form of a gene that is located at a specific locus on a specific chromosome

Aneuploid: having a chromosome number that varies from a multiple of the haploid number for the species

Apomixis: reproduction without meiosis or formation of gametes

Apoplast: intercellular space

Balanced lethals: arrangement of alleles of two recessive genes in a stable heterozygous chromosome combination in a breeding organism accompanied by loss of any lethal-bearing homozygous chromosomes

Cellulase: an enzyme produced mainly by fungi and bacteria to catalyze the hydrolysis of cellulose

Chromosome: a structure of DNA present in cells and appearing like a coiled thread containing nucleotide sequences and other regulatory elements

Cytoplasm: protoplasm exterior to the nucleus of the cell

Deletion: loss through mutation of one or more nucleotides from a chromosome

Diploid: having a pair of each chromosome

Durable resistance: resistance in a particular host genotype which does not exert selection pressure on a pathogen population to become highly virulent leading to sudden change of the host genotype from being resistant to susceptible

Effector: molecules produced by pathogens to promote parasitic activity during interaction with hosts

[1] definitions provided by: www.biology-online.org

Fitness: extent to which an organism is adapted to an environment or able to produce an offspring in a particular environment

Haplotype: a set of alleles of closely linked loci on a chromosome that tend to be inherited together

Hardy-Weinberg equilibrium: an assertion that in the absence of forces to change them, allele frequencies remains constant over time in a sexually reproducing population.

Heterozygosity: having different alleles at one or more corresponding chromosomal loci

Heterothallic: producing male and female gametangia in different structures

Linkage disequilibrium: occurs when a genotype at one locus is not independent of a genotype at another locus

Locus: specific location of a gene on a chromosome

Non-mendelian inheritance: referring to patterns of inheritance where traits do not segregate in accordance to Mendel's Laws in which each parent contributes one of two possible alleles for a trait

Microsatellite: repeating DNA sequences of 2-6 nucleotides

Mutation: a change of DNA sequence within a gene or chromosome resulting in a new trait not found in the parental type

Non-disjunction: failure of homologous chromosomes to separate during and after meiosis

Non-synonymous substitution: a DNA base pair change in the coding sequence of a gene that leads to alteration in the amino acid sequence of a protein (also called missense mutation).

Nucleotide: any of various compounds consisting of a nucleoside linked to a phosphate group and forming the basic component of DNA and RNA

Nucleus: a part of the cell containing DNA and RNA and responsible for growth and reproduction

Oospores: thick chitinous walled sexual spores that develop following the union of oogonia and antheridia

pH: measure of the acidity or alkalinity of a solution on a scale of 0 to14, it is numerically equal to 7 for neutral solutions but increases with increasing alkalinity and decreases with increasing acidity.

Segregation: separation of paired alleles during meiosis leading to appearance of members of each allele in different gametes

Stability: a stable potato genotype exhibits an unchanged performance regardless of any variation in the environmental conditions

Synonymous substitution: substitution of one base for another in a gene coding for a protein in which the amino acid produced is not modified by that replacement (also called silent mutation).

Triploid: having three times the haploid number of chromosomes in the cell nucleus

Trisomy: the condition of having one chromosome of the set represented three times in an otherwise diploid organism

Thesis outline

This thesis contains an introductory chapter and four chapters of experimental data which build on existing knowledge of our understanding of *Phytophthora infestans* (Mont.) de Bary, the causal agent of potato late blight. The data provide insights into mechanisms involved in *P. infestans* population changes in Ireland and the implications for blight control based on host resistance and fungicides.

The introductory chapter provides a literature review on the status of *P. infestans* population studies, progress towards attaining durable host resistance against potato late blight and the contribution of recently gained insights into oomycete effectors towards unraveling pathogen adaptation to host resistance. A statement of aims and scope of the project are made against this background.

Chapter two presents results of an assessment of Irish *P. infestans* isolates to determine the population structure and diversity. Diversity investigated included mating type composition and frequency of genotypes with resistance to a phenylamide fungicide, metalxyl. It also contains findings of the comparative assessment of recently emerging *P. infestans* genotypes in Ireland for aggressiveness on foliage and tubers of both susceptible and resistant potato cultivars which are grown in Ireland.

Chapter three consists of results of a study on sexual recombination between compatible mating types of current and novel genotypes of *P. infestans* in Ireland and explores the contribution of sexual reproduction to population change. Particularly, it contains data on mating type frequency and metalaxyl sensitivity for the resulting *F1* segregating progeny as well as their aggressiveness on foliage of a moderately resistant potato cultivar, Cara.

Chapter four explores the potential for asexual populations of *P. infestans* to adapt to potato hosts carrying broad spectrum resistance *R* genes and the capacity of the pathogen to overcome this resistance when allowed to complete multiple asexual

generations on these hosts. Specifically, it is in this chapter that selection for fitness in *P. infestans* based on a gene-for-gene, *RB-ipiO* (*Rpiblb1-avrblb1*) interaction is assessed.

In chapter five a technique based on a quantitative real time polymerase chain reaction (PCR) for detection of *P. infestans* propagules in potato host tissues including symptomless tubers is presented.

Chapter six is a general discussion of the findings from each chapter. The implications of the findings are put into perspective as regards the current and future efforts to control potato late blight in Ireland based on host resistance and fungicides. The chapter also identifies questions raised by this study and lists proposed recommendations to provide answers to those questions.

Chapter One

Introduction and Literature Review

1.1 *Phytophthora infestans:* plant destroyer

Among potato's most persistent diseases is 'late blight' caused by *Phytophthora infestans* (Mont.) de Bary. It is best known for its cause of the epidemic that led to the Irish Potato Famine of the mid-nineteenth century in which more than 1 million people died of starvation and 1.5 million more emigrated (Bourke, 1964). The pathogen was first thought to be a fungus and described by French scientist Dr. Charles Montagne as *Botrytis infestans* (Berkeley, 1948). Eventually, Anton de Bary described the life cycle of the pathogen and conclusively showed that it was the cause of potato late-blight disease (de Bary, 1876). The blight pathogen has a global distribution and mainly affects potatoes (*Solanum tuberosum*) and tomatoes (*Solanum esculentum*) (Knapova and Gisi, 2002). Other hosts include hairy nightshade (*Solanum sarrachoides*), petunia (*Petunia hybrida*) and bittersweet (*Solanum dulcamara*) (Dorrance and Inglis, 1997; Platt, 1999).

1.2 Taxonomy, origin and distribution

P. infestans belongs to the Oomycetes, a group of organisms causing many plant diseases. Oomycetes were often classified as true fungi such as basidiomycetes and ascomycetes because they all have a filamentous pattern of growth. However, sequence studies on ribosomal DNA have shown differences between oomycetes and true fungi and provided evidence for taxonomic affinity of oomycetes to kingdom Chromista that also comprises diatoms, chrysophytes and brown algae (Judelson, 1997; Förster *et al*., 1990; Latijnhouwers *et al*., 2003)

Consensus on the phylogenetic affinity of *P. infestans* notwithstanding, debate persists within the scientific community regarding the geographic origin of the pathogen that sparked disastrous blight epidemics of the 1840s in Europe (Ristaino, 2002). Some scientists suggest a direct introduction into Europe and North America from a source in the Toluca Valley of central Mexico (Niederhauser, 1991; Reddick, 1939). Their assertion is based on high levels of genetic diversity

observed in the local populations in this region and mtDNA evidence (Goodwin *et al*., 1992b; Ristaino *et al*., 2001). Additionally, analysis of global populations using molecular markers showed that samples from the Toluca Valley region comprised the only population in Hardy-Weinberg equilibrium (Tooley *et al*., 1985). An alternative hypothesis suggests a three step introduction consisting of firstly, an introduction from Mexico to South America several centuries ago; secondly, migration from South America to the US in 1841-1842 and; thirdly, migration into Europe from South America, US or both in 1843-1844. To support this claim, historical records are invoked as well as genetics and current population structures (Andrivon, 1996; Tooley *et al*., 1989). Both hypotheses are consistent with there being a single clonal lineage of the pathogen prevailing in Europe and the US during most part of the 20th Century (Goodwin *et al*., 1994). The global distribution of *P. infestans* is widely thought to have arisen from two major migrations from its centre of origin. The first of these migrations resulted in the European blight epidemics of the 1840s while the second is believed to have occurred in the late 1970s (Fry and Goodwin, 1997; Henfling, 1987). This spread has been enhanced by the fact that potato is generally propagated by seed tubers which enable pathogens like *P. infestans* to be easily transmitted within the tubers from one crop generation to the next and over vast geographical distances.

1.3 Epidemiology and reproduction

During epidemics, the spread of *P. infestans* is mainly by way of sporangia formed on the foliage of the infected host and this spread is favoured by optimum temperatures of 18-22^{o}C and relative humidity exceeding 90% (Erwin and Ribeiro, 1996). Sporangia are efficiently dispersed by wind and water (Porter and Johnson, 2004) to the next host on which they germinate and infect (Judelson, 1997a). At warmer temperatures of 18-25^{o}C, sporangial germination is by way of hyphal out-growth (Hill *et al.,* 1998). However, at cooler temperatures of 10-13^{o}C, each sporangium releases 6-8 infective zoospores (Krämer *et al*., 1997) which in turn form germ tubes with which to infect the host (Latijnhouwers *et al*., 2003).

Typically, each sporangium forms a single germtube with which to infect a host (Judelson and Blanco, 2005; Tani and Judelson, 2006). Under favourable conditions of temperature and humidity, a single infection can destroy a field of previously healthy plants within a few weeks (Fry, 2008).

Although asexual reproduction is predominant during epidemics, *P. infestans* is capable of sexual reproduction too. This can punctuate clonal propagation by allowing genetic recombination leading to new genotypes, some with enhanced fitness making the occurrence of severe epidemics more likely (Gavino *et al*., 2000). In Ireland, the occurrence of A2 mating type of *P. infestans* in high frequencies as indicated by recent surveys (Kildea *et al.,* 2010) implies likelihood of sexual reproduction to take place giving rise to some genotypes with greater virulence on potato compared with the existing genotypes. *P. infestans* is heterothallic with two mating types known as A1 and A2. Mating and sexual hybridization leading to production of oospores requires interaction between these two mating types (Galindo and Gallegly, 1960). Male and female mating structures (antheridia and oogonia) are produced in response to mating hormones produced by the opposite mating type and although some genotypes may preferentially form oogonia or antheridia, each mating type is capable of forming both sexual structures (Judelson, 1997a).

Isolation and characterization of a mating hormone α1 (1,11,16-trihydroxy-3,7,11,15-tetramethylhexadecan-4-1) from A1 mating type of *P. nicotianae* (Bajpai *et al*., 2007) may provide an indication about the mating behaviour in *P. infestans*. A recent study involving a mating hormone α2, also from *P. nicotianae,* whose aim was to investigate hormonal cross talk during mating demonstrated *in vitro* induction of oospore production by α2 in single isolates of various *Phytophthora* species including *P. infestans*. In each case, there was a corresponding increase in numbers of oospores produced with every increase in the dose of the α2 hormone (Kothe, 2008; Ojika *et al*., 2011). This study suggests the universal nature of the regulatory function of the mating hormone(s) within *Phytophthora.*

Sexual reproduction occurs when haploid nuclei from oogonia and antheridia fuse to form a diploid zygotic oospore which is typically spherical in shape and thick walled (Erwin and Ribeiro, 1996). These oospores are capable of surviving outside the host and remain infective for long periods (Turkensteen *et al*., 2000).

Sexual reproduction in *P. infestans* results in out crossing as well as selfing (Knapova *et al*., 2002) and apomixis: i.e. offspring genetically identical to one of the parents (Judelson and Yang, 1998). Selfing has been proposed to result from non-disjunction (Fyfe and Shaw, 1992), mating between sexual partners with the same genotype forming alternate mating organs (Judelson, 1997a), or autogamy (both oogonia and antheridia formed by an individual) (Smart *et al*., 1998). The role of selfing in particular remains unclear with studies failing to show evidence that such mating can lead to production of viable oospores in plant tissues (Smart *et al*., 1998). In general, however, the implications of sexual reproduction to blight management continue to be a subject of ongoing inquiry. For instance, the resurgence of *P. infestans* has been associated with sexual recombination which is believed to have given rise to genetic diversity in European populations (Drenth et al., 1994; Shattock et al., 1990) with an increasing frequency of aggressive genotypes (Day and Shattock, 1997; Flier and Turkensteen, 1999) which are thought to have led to the displacement of the established genotypes (Levin et al., 2001; Spielman et al., 1991).

1.4 Mating type A2 migrations and changes in *P. infestans* populations

The first indications of a second migration came in the form of the finding of an isolate of A2 mating type outside Mexico (Hohl and Iselin, 1984). This was followed soon by a report on the presence of A2 mating type strains in the UK in imported ware potatoes from Egypt (Shaw *et al*., 1985). Prior to these two events, a single A1 clonal lineage of *P. infestans,* designated US-1, was spread throughout the world while the A2 mating type was confined to central Mexico (Niederhauser, 1956). Evidence exists that suggests association between sexual reproduction and occurrence of aggressive genotypes of *P. infestans* especially so in Europe where

both A1 and A2 mating types exist (Andersson *et al*., 1998; Day and Shattock, 1997). Perhaps the significance of sexual reproduction in aggressiveness of *P. infestans* is best underscored by the occurrence of “blue 13” (13_A2) and “pink 6” (6_A1) strains of blight both which were first reported in the Netherlands (D.E.L Cooke, personal communication).

1.5 Markers for monitoring population diversity

As part of efforts to provide a basis for sustainable control of late blight, an array of techniques have been employed over the last century to study the characteristics of *P. infestans* and develop an understanding of mechanisms that lead to diversity within and between populations. These techniques have evolved over time from basic phenotypic markers to the specific DNA markers presently in use.

1.5.1 Phenotypic markers

Among the phenotypic traits used to study *P. infestans* are mating type (Gallegly and Galindo, 1958), virulence on differential potato genotypes possessing single genes R1-R11 (Malcolmson and Black, 1966) and resistance to phenylamide fungicides more specifically, metalaxyl (methyl-N-(2-furoyl)-N-(2,6-xylyl)-DL-alaninate) resistance (Dowley and O'sullivan, 1981; Cooke, 1981).

The mating type test is performed by pairing a sample isolate with a known A1 isolate and a known A2 isolate on agar plates and observing for the formation of oospores at the interface between the two isolates when hyphae meet after 7-10 days of incubation. The genetic basis for mating type has been the subject of much investigation with studies indicating that the trait is controlled by a single locus (Judelson *et al*., 1995). In one such study the genetic and physical mapping of the mating type determinants in *P. infestans* showed that a single locus controlled each mating type. But recovered progeny from crosses of *P. infestans* have tended to show a non-Mendelian segregation pattern because only two of the four theoretically expected genotypes of the mating type are recovered. This has led to suggestions that ambiguous and potentially deleterious genotypes (i.e. those

expressing both mating types) get eliminated through balanced lethal genes (Judelson *et al*., 1995). In another model, *P. infestans* is proposed to have a single locus where alternate mating types are homozygous and heterozygous (Shaw and Shattock, 1991). Either system has been difficult to test genetically because not all genotypes are recovered due to balanced lethal genes. Yet crosses following both systems result in ratios of A1 and A2 types ranging from 1:1 to an excess of A1 over A2 types (Judelson *et al*., 1995; Shaw and Shattock, 1991).

The second phenotypic marker for characterization of *P. infestans* is the use of a virulence test on differential potato cultivars based on gene-for-gene recognition (Black *et al*., 1953). In this system, the virulence phenotype of an isolate is determined by inoculation on a set of 11 differential potato genotypes each carrying a different single *R* gene. Induction of host cell death or hypersensitive reaction (HR) indicates recognition (Cooke and Lees, 2004). The suitability of this technique has been questioned due to inaccuracies between and within laboratories, and sensitivity of the test to the physiological age of the plant material (Stewart, 1990). The method presents isolate classification difficulties as well as comparison of results where different sets of *R* differentials or inoculation protocols are used by different laboratories. Furthermore, it is time consuming.

The next phenotypic marker is sensitivity to metalaxyl. Metalaxyl showed extensive curative and protective properties against *P. infestans* when first introduced (Gisi and Cohen, 1996). It acts against *P. infestans* by inhibiting the activity of ribosomal RNA polymerase which in turn reduces mycelia growth and sporulation (Davidse, 1995). A single genetic change at the target site in the pathogen makes it resistant to metalaxyl due to the single mode of action of the fungicide. Indeed, resistance was observed within a few years after release (Dowley and O'Sullivan, 1981). Studies on the genetic basis for metalaxyl resistance have suggested that the trait might be governed by a single allele at a locus showing incomplete dominance (phenotype) (Shattock, 1988) and possibly additional loci with cumulative effect contributing to the overall phenotype (Fabritius *et al*., 1997). The ease with which

resistance to metalaxyl could be tested in the laboratory and the presence of resistant genotypes in the population showed that this marker was useful in *P. infestans* population studies. While many laboratories use the floating leaf disc method (Dowley and O'Sullivan, 1981) a lack of uniformity between laboratories in choice of the protocol used to test for sensitivity to metalaxyl compromises the suitability of this test as a universal marker.

1.5.2 Genotypic markers

Phenotypic markers on their own do not fulfill the criteria of an ideal marker for studying *P. infestans* populations (Cooke and Lees, 2004). Genotypic markers offer greater power of discrimination which is needed to track clones within and between populations that cannot be achieved using phenotypic markers.

Between the phenotypic and genotypic markers lie the proteins which because they are encoded by DNA molecules, are classified as phenotypic characters. Of particular interest to population studies are isozymes. Isozymes refer to the occurrence of multiple molecular forms of an enzyme arising from the differences in the DNA nucleotide sequence that encodes it (Smithies, 1955). Heritability, co-dominance and selection neutrality are assumed in studies involving application of an isozyme system (Shaw, 1965). Multiple isozyme systems for *P. infestans* were tested following the initial one that was successfully used to study a segregating population of *P. infestans* to distinguish selfs from hybrid progeny (Tooley *et al*., 1985). Later, greater diversity was observed with respect to glucose-6-phosphate isomerase (*Gpi*) and peptidase (*Pep*) which are now commonly used and whose use has been enhanced by faster product separation using cellulose acetate electrophoresis (Forbes *et al*., 1998; Goodwin *et al*., 1998). Compared to isozyme techniques, DNA genotyping safeguards against influences such as the environment and growth stage of the organism (e.g. culture conditions) that may influence protein transcription and translation compromising the test.

Genotyping techniques have developed rapidly over the last two decades culminating in two groups. The first of these involves methods that relied on digestion of DNA by restriction endonucleases isolated from bacteria (e.g. *Eco*R1). An example of these is the Restriction Fragment Length Polymorphism (RFLP) technique. This technique is based on the principle that restriction endonucleases recognize short nucleotide sequences and cleave DNA at these sites. Consequently, a nucleotide change at the recognition site would prevent digestion while change at a non-target site could result in a new recognition site. Homologous sequences are cut into identical fragments which may then be separated on a gel where identical fragments exhibit the same electrophoresis mobility and the resulting profile for isolates visualized and scored as presence or absence of bands on the gel. Alternatively, fragments are transferred on a nitrocellulose membrane and hybridized with specific probes that have sequence similarity with the DNA sample (Goodwin *et al*., 1992a). The most successful and most commonly used probe is RG57 developed by cloning *Eco*R1 fragments from *P. infestans* and screening them for use as RFLP probes (Goodwin *et al*., 1992a) and has been widely used to study migration events in *P. infestans* (Cooke *et al*., 2006; Deahl *et al*., 2002; Goodwin *et al*., 1994). This probe reveals variation at multiple loci.

The second group of DNA markers consists of those which are Polymerase Chain Reaction (PCR) based. One of them is the Amplified Fragment Length Polymorphism (AFLP) technique (Vos *et al*., 1995) which combines the restriction digestion with ligation of adapters to restriction specific sites to provide binding sites for primers permitting genome wide amplification of a specific set of restriction fragments. Subsequent re-amplification with radioactive or fluorescently labeled primers enables visualization of the fragments by radiography or automated DNA sequencing machines respectively (Vos *et al*., 1995). This method has been employed in studies on *P infestans* (Cooke *et al*., 2003; Knapova and Gisi, 2002) and its resolving power was vindicated when it was used to detect differences in

isolates with similar RG57 profiles (Purvis *et al*., 2001). However, the applicability of AFLP has been impaired by difficulties involved in interpreting the bands (Cooke and Lees, 2004).

Mitochondrial DNA (mtDNA) is a remarkable source of variation and useful for discerning inheritance patterns in a population because unlike nuclear DNA, it is uni-parentally inherited through oogonium (Whittaker *et al*., 1994). RFLP polymorphisms in the mtDNA were identified (Carter *et al*., 1990; Goodwin, 1991) which initially led to two types I and II. Each type was further resolved to give four mtDNA haplotypes (Ia, IIa, Ib and IIb) and ultimately a nomenclature system was developed (Gavino and Fry, 2002). Making use of the availability of the mtDNA sequence (Paquin *et al*., 1997), a PCR-RFLP method was developed to easily distinguish the four mtDNA haplotypes (Griffith and Shaw, 1998). A suitable marker ought not only to be cost effective, but also be robust, easily amenable to commonly available technology and facilitate high through-put sampling (Cooke and Lees, 2004). MtDNA fingerprinting doesn't fulfill the above requirements of an ideal marker.

Simple Sequence Repeat (SSR) markers, also known as 'microsatellites' posses many of these features. SSRs are highly variable tandemly repeated motifs of one to six bases and widely distributed throughout the genome of eukaryotes (Lagercrantz *et al*., 1993). Slippage during DNA replication results in alteration of the repeat length. It is this feature of the SSRs which makes them suitable for accurate sizing of a PCR amplified repeat and its immediate flanking sequence. Further more, the capability to precisely resolve repeat length variation at each locus sets the SSRs apart from other genotyping markers such as AFLPs, such co-dominance data can be used to determine population genetic structure, kinship, the modes of reproduction (Jarne and Lagoda, 1996; Kosman and Leonard, 2005). The application of microsatellites to study plant pathogens is only recent, with description of the first *P. infestans* SSR markers being realized within the last ten years (Knapova *et al*., 2002). Taking advantage of the many features of the SSR

markers; reproducibility of results, increased throughput achieved by multiplexing PCR reactions and automated analysis of resulting PCR products (facilitated by use of fluorescently labeled primers in repeat length PCR amplification), scientists have since employed these markers to either study *P. infestans* population structure or track genotypes within a population (Knapova and Gisi, 2002; Widmark *et al*., 2011).

1.6 Potato (*Solanum tuberosum* L.)

Cultivated potato belongs to genus *Solanum* in the Solanaceae family. The genus contains an estimated 1500 species (Bohs, 2007). There has been controversy within the scientific community in the last century regarding the taxonomic treatment of cultivated potato with anywhere between one to twenty one species recognized (Hawkes, 1990; Huamán and Spooner, 2002). In one classification system potato landraces are grouped into four species namely *S. tuberosum* (with two cultivar groups: Andigenum and Chilotanum comprising diploids, triploids and tetraploids), *S. ajanhuirii* (diploid), *S. juzepczukzii* (triploid) and *S. curtilobum* (pentaploid) based on the evidence of nuclear simple sequence repeat (nSSR) and plastid DNA deletion markers from landraces and related wild species progenitors (Spooner *et al*., 2007). There are 188 wild tuber bearing species distributed in the Americas from Colorado, USA, Uruguay, Chile and Peru (Spooner and Hijmans, 2001). These species are a source of genetic resources which are utilized in breeding programs for disease resistance, environmental tolerance and other agronomic traits (Ross, 1986).

Potato has a number of ploidy levels, based on a haploid number of 12, ranging from diploid (2n=24) to hexaploid (6n=72), and including triploids, tetraploids, and pentaploids. Most cultivated potato varieties are tetraploid (4n=48); many wild species are diploid but may range up to hexaploid (Huamán and Spooner, 2002).

Potato was first cultivated in the Andes in Southern Peru from where it was introduced to Europe through Spain in the 16th century and later spread to the rest of Europe and across the globe (Salaman, 1985). In 2007 the tuber crop was cultivated in over 100 countries with a yield of 314 million tons (FAO, 2010). The unique adaptability of the potato to diverse agro-ecologies has led to a dramatic increase in production of the crop in developing countries to culminate in its endorsement as one of the world's principal food crops when the UN's Food and Agricultural Organization designated 2008 'The International Year of the Potato'. Global population is projected to reach 9 billion people by 2050 (U. N. Secretariat, 2009) and being thus ear-marked to play a crucial role in meeting global food needs, potato is joining the ranks of traditional food crops in many countries. Under ideal conditions, a potato crop can yield more than 15t/ha dry matter, much higher than the average for cereals (Stol *et al*., 1991). Despite the increasing global prominence of the potato, growers worldwide continue to suffer losses amounting to billions of dollars of wasted resources and lost sales due to potato diseases.

1.7 Potato production in Ireland

How the potato got into the Republic of Ireland can only be surmised, but the general view is that it was introduced into the country in the mid 17th century probably from Spain where it had been introduced from South America by returning colonists (Bourke, 1993). The hundred years following potato introduction were characterized by an increasing dependence on the crop by a section of the Irish society such that widespread crop failure caused by late blight led to the starvation and death of an estimated 1 million people and another 1.5 million fleeing the country (Bourke, 1993).

Acreage of land under potato production in Ireland has been declining in the last 50 years as land owners turned to other farming ventures like wheat tillage and dairy farming (Leonard, 2003). In 2011 potato was cultivated on 11,200 ha producing approximately 33t/ha (IFA, 2011). Contribution to national production varies greatly

between the counties. In 2011 for example, 63% of the crop was produced in four of the twenty four counties (Meath, Dublin, Wexford and Louth) only.

Potato variety composition over the decade has been dominated by only a few established popular cultivars (Rooster, Kerr's Pink, British Queen, Golden Wonder and Record). Other cultivars grown albeit with less than 1% share of the area under production in a typical year are Home Guard, Premiere, Lady Rosettea, Maris Piper and Lady Claire. In 2011 just as in other years in the immediate past, two cultivars Rooster and Kerr's Pink took large share of the land under potato production occupying 55.6% and 10% respectively (IFA, 2011).

Potato is a summer crop in Ireland. But the Irish rainy weather conditions coupled with summer temperatures of between 15-18^{o}C make it particularly conducive for late blight. Therefore potato growers have to rely heavily on fungicide to manage the disease. Survey for fungicide usage on potato crops in Ireland for the period 1995-1998 established that a total of twenty different products were used on potato crops and of these six contained metalaxyl (Dowley *et al*., 2002). Data (from the Department of Agriculture Food and Fisheries, Republic of Ireland) on fungicide usage on potato in Ireland in 2004 indicate 12 spray applications were made to deliver 14.1 kg/ha in single season in order to achieve effective blight control (Ewen Mullins personal communication, 2013). The frequency of fungicide applications has since risen to 15 fungicide spray applications in a single growing season (Dowley *et al*., 2008) implying that the amount of fungicide active ingredient has also risen. There has not been any recent compilation of data on fungicide usage in Ireland by Pesticide Service; this is the most up to date dataset.

1.8 Host resistance and potato blight control

Loss of the potato crop to late blight on a global scale is estimated at 10-15% (Turkensteen and Flier, 2002) and in the European Union (EU) alone, this equates to up to €1b of combined worth of crop produce and costs of controlling late blight

(Haverkort *et al*., 2008). Farmers worldwide rely on the use of fungicides to control potato blight. However, host resistance remains the cheapest and the most environmentally friendly tool to ensure late blight disease management (Mendoza, 1993).

Two types of resistance to late blight have been described and used in potato breeding. One kind, "gene-for-gene" resistance, is conferred by a series of dominant resistance genes that render the host resistant to specific races of the pathogen. Eleven such *R* genes originating from *S. demissum*, a hexaploid native to Mexico, have been identified (Black *et al*., 1953; Malcolmson and Black, 1966). This race specific resistance is a qualitative factor which is independent of the number of genes involved (Henfling, 1987). The rapid erosion of this form of resistance is thought to be influenced by the varying race composition of *P. infestans* in a given agro-ecology resulting from the mutation of avirulent genes to give new genotypes not recognized by the R-genes of the host (Visker, 2005).

The second type of resistance, which is quantitative in nature and race non-specific is thought to be much more durable. This resistance has been reported in several different species of *Solanum* (Black, 1970; Umaerus *et al*., 1981). It has been suggested to be proportional to the number of resistance genes inherited (Latin *et al*., 1981). This quantitative and race non-specific resistance is also referred to as "partial" or "field" resistance. Detection of this resistance has been difficult because it is much smaller in magnitude than *R* gene derived resistance, and results in a slow blighting phenotype. This is accomplished by impeding pathogen development expressed in different stages of the disease cycle (Umaerus and Linhell, 1976).

Unfortunately, several limitations have been identified with polygenic resistance. It is not completely known how many genes have to be accumulated for a cultivar to acquire high levels of field resistance uniform across space and time (Parlevliet, 1983; Visker, 2005). It also tends to be associated with major R-genes and late

maturity (Umaerus *et al*., 1981). Other problems include the interaction of field resistance with biotic and abiotic factors as well as the presence of undetected R-genes in the breeding population that may mask field resistance, and unavailability of virulent races of *P. infestans* to reveal all R-genes (Stewart *et al*., 2003). The residual effects of defeated R-genes when associated with field resistance genes could contribute to the overall level of race non-specific resistance (Ordoñez *et al*., 1998). Hence, the total removal of R-genes from potato breeding populations may reduce the level of resistance by removing beneficial associations (Stewart *et al*., 2003). The robustness and durability of race non-specific resistance can be proven by its extensive deployment across a wide area with disparate agro ecological conditions (Forbes *et al*., 2005). Analysis of quantitative resistance in a mapping population identified quantitative trait loci (QTLs) which mapped a known resistance gene cluster. These regions also harbor a major QTL conferring race non-specific field resistance to *P. infestans* (Gebhardt and Valkonen, 2001).

Most field assessments of resistance are mainly based on phenotypic expression of the host and resistance is quantified with parameters measuring apparent infection rate (r) and the area of infected leaf tissue under disease progress curve (AUDPC), after foliar infection readings made at fixed intervals during an epidemic (Campbell and Madden, 1990). Relative AUDPC (RAUDPC) was proposed as a standardized and more stable measure of resistance because it was observed that AUDPC is affected by several experimental factors other than host resistance (Hansen *et al*., 2005). Resistance to late blight in Europe is classified on a scale of 1-to-9 as first proposed by Malcolmson (1976), where 1 represents the most susceptible cultivars and 9 the most resistant ones. The scale has since been modified by several workers including Hansen *et al.,* (2005). As there are indications that newly emerged strains of *P. infestans* have the capacity to adapt to cultivars irrespective of their levels of resistance (Andrivon *et al*., 2007; Montarry *et al*., 2008), how durable is host resistance to changing populations of *P. infestans* ?

1.9 Durability of potato host resistance to *P. infestans*

Durable resistance has been defined as the resistance that remains effective in a cultivar that is widely grown for a long period of time in an environment favourable for disease (Johnson, 1984). Leach *et al.,* (2001) suggested that the definition of Johnson was ambiguous because “long period” is subjective and instead proposed durable resistance to mean the adequacy of resistance throughout the useful time expected from the variety. Although, the race specific hypersensitive resistance conditioned by many *R* genes was initially effective against late blight, it was rapidly overcome after new virulent phenotypes of *P. infestans* emerged (Wastie, 1991). With time, potato breeders started using germplasm with partial or quantitative resistance whose expression is independent of the R genes and was thought to be polygenic and hence more durable (Hawkes, 1979). For instance, in the Netherlands breeding for quantitative resistance was recommended as a means of ensuring durability based on the argument that Dutch potato cultivars without R-genes had expressed stable resistance to *P. infestans* for more than 30 years (Turkensteen, 1993). Despite its advantage over qualitative resistance, polygenic or general resistance is known to be day-length dependent and correlated with late maturity under long-day conditions (Howard, 1970). Besides, erosion of this resistance has also been reported (Flier *et al*., 2003).

Solanum bulbocastanum, a Mexican diploid is one of the tuber-bearing species with known high levels of race non-specific or broad spectrum resistance to late blight (Niederhauser and Mills, 1953). Initially, this resistance was difficult to transfer into cultivated potato cultivars through crossing and phenotypic selection (Hermsen and Ramanna, 1969). Later however, introgression of *S. bulbocastanum* resistance traits into potato was achieved by ploidy manipulation and through a series of bridge crosses resulting in blight resistant germplasm (Hermsen and Boer, 1971). After four decades of breeding effort in the Netherlands following this breakthrough two blight resistant cultivars, Toluca and Bionica, were developed having circumvented difficulties encountered in reconstituting the original potato

parent in the *S. bulbocastanum* and *S. tuberosum* crossings from the resulting quadruple hybrids. A strategy involving somatic hybridization was employed to overcome sexual incompatibility between potato and *S. bulbocastanum* leading to fertile plants that could be used directly in breeding programmes (Thieme *et al*., 1997). Examples of this application include work by Helgeson *et al*., (1998) who developed somatic hybrids from *S. bulbocastanum* and *S. tuberosum* and obtained first and second generation backcrosses with *S. tuberosum* that displayed late blight resistance not dissimilar to *S. bulbocastanum*.

Recurrent crosses to reconstitute a germplasm which meets the standards of a newly bred cultivar can lead to suppression of recombination which confers resistance to blight (Helgeson *et al*., 1998). In recent years, a lot of progress has been made as regards cloning of late blight resistance genes and delivering them directly into potato as a quicker means of exploiting the durable resistance in wild *Solanum* species. As a result of map-based gene cloning and allele mining strategies over two dozen **R**esistance genes to ***P****hytophthora* ***i****nfestans (Rpi)* had been positioned on a molecular linkage map by 2010 (Bradeen, 2011; Hein *et al*., 2009). All of the *R* genes cloned so far belong to the nucleotide binding site, leucine rich repeat (NBS-LRR) superfamily of disease resistance genes that encodes host defense related cytoplasmic proteins (Meyers *et al*., 2003; Vleeshouwers *et al*., 2011). Some of the cloned genes, like *R*1, may have little use in agricultural application, having been circumvented in the past by changing *P. infestans* populations. Others, like *Rpi-blb1* also known as (*RB*) (Song *et al*., 2003; van der Vossen *et al*., 2003) and *Rpi-blb2* (van der Vossen *et al*., 2003) cloned from *S. bulbocastanum,* on the contrary confer broad-spectrum resistance to *P. infestans*. Wang *et al*., (2008) presented data which showed that putatively functional homologues of *Rpi-blb1* are also present in *S. stoloniferum* (sensu Spooner *et al*., 2004), a species that can be crossed directly with cultivated potato (Jackson and Hanneman, 1999). Consequently, *Rpiblb1* has been held up as giving much promise towards realizing durable resistance to potato blight when

deployed in agriculture. The practical utility of this form of resistance including the discovery of blight strains with the ability to challenge *Rpiblb1* are discussed in chapter four. An understanding of the potato-blight interactions at the molecular level is crucial in efforts to identify and exploit *R* genes for resistance to and in deciding the suitable combination of genes to be stacked in single potato genotypes to promote durability of resistance to blight (Zhu *et al*., 2011).

1.10 Zig-zag interactions between potato and *P. infestans*

According to the gene-for-gene model of disease resistance, resistance occurs upon recognition of pathogen avirulence (Avr) factors by the corresponding resistance *R* gene product in the host (Dangl *et al*., 1996; Flor, 1942). As a result of this ground breaking finding, it was generally thought that resistance occurs as a result of direct interaction between R and Avr proteins (Keen, 1990). Attempts to prove this, however, failed to demonstrate any direct interaction leading to the proposal of the 'guard' hypothesis which suggests that R proteins guard host proteins which are targeted by the pathogen molecules during infection (Dangl and Jones, 2001; van der Biezen and Jones, 1998). The 'guard' model, suggests that the plant targets or 'guardees' are also cofactors in recognition and further that it is the manipulation of host targets by pathogen molecules rather than their presence that is sensed by the R proteins. The 'guard' hypothesis did not provide clear evidence for virulence targeting of recognition cofactors. This led to yet another model in which these cofactors were thought to have the role of decoys (van der Hoorn and Kamoun, 2008). Alongside research into the gene for gene mode of host resistance, other studies have provided proof for the existence of inducers of plant defense responses that are not pathogen race or cultivar specific (Ebel and Cosio, 1994). The discovery of these host defense inducers merely made efforts to unravel the architecture of host defense machinery more intriguing. Soon a debate ensued about whether there was any relationship between gene-for-gene resistance and elicitor induced defense.

Perhaps a much simpler depiction of the innate immunity in host-pathogen interactions is that provided by Jones & Dangl (2006) in the zig-zag model. This model proposes that the first line of plant defence is formed by pattern recognition receptors (PRRs) which detect conserved molecules (pathogen associated molecular patterns, PAMPS) that are secreted by the pathogens (Nürnberger and Brunner, 2002).

Upon detection of these PAMPs, PRRs activate an innate immune response called PAMP-triggered immunity (PTI). PTI forms the first line of defense that must be overcome by the pathogen in order to establish colonization. Pathogen molecules originally termed as avirulence factors are quite genuinely virulence factors, also called effectors (Boller and Felix, 2009). Successful pathogens secrete these effectors which act either outside or inside host cells to either manipulate or suppress PTI causing effector-triggered susceptibility.

The second line of plant defense is in the form of cytoplasmic R proteins that recognize the presence or activity of pathogen effectors. Majority of these proteins belong to the nucleotide-binding site, leucine-rich-repeat (NBS-LRR) superfamily and have separate recognition and signaling domains where the LRR is the recognition domain while the N-terminal region including the NBS, is the signaling domain (Collier and Moffett, 2009). These proteins function by activating the so called effector triggered immunity (ETI) which is pathogen race or strain specific and is associated with programmed cell death or hypersensitive response (HR) which is an attempt to restrict pathogen spread. ETI represents a selection pressure to which pathogen strains have responded by either abandoning or altering the effectors that are recognized in order to suppress it. In turn, plants develop new receptors that recognize either existing or newly acquired pathogen effectors leading to ETI again in a seemingly endless arms race between host and pathogen.

1.11 The role of *P. infetans* effectors in durability of potato host resistance

Our understanding of the plant-microbe interactions has been aided greatly in recent years by advances in molecular techniques which have made it possible to identify the role of pathogen effectors in the consecutive stages occurring during disease development. During infection, oomycetes effect the colonization of the host tissues by molecular re-organization or manipulation of the host defense system by introducing its effectors which function in the plant host apoplast and cytoplasm (Kamoun, 2006).

But how exactly do oomycetous effectors contribute to pathogenesis? In the early stages of infection, both pathogen and host secrete proteins to control the extra cellular-environment. Therefore, the invading pathogen must overcome the host biochemical barriers. For this purpose, oomycetes synthesize two classes of effectors (cytoplasmic or apoplastic effectors) targeting different sites within the plant host.

Apoplastic effectors are secreted into host plant extracellular spaces while the cytoplasmic effectors are translocated into the plant cells through a mechanism which was previously not clearly understood but has recently received much attention (Kamoun, 2006). Apoplastic effectors interfere with the plant process by inhibition of host enzymes (especially Arginine-Glycine-Aspartic Acid-containing proteins) and/or by mediating toxin-associated host cell death (Kaschani *et al*., 2010; Wang *et al*., 2011). It is still unclear whether oomycetes secrete inhibitors to arm themselves against hydrolytic enzymes produced by the host or to protect their own secretomes from degradation. But it is known that effectors which inhibit toxins that lead to host cell death are necessary for promotion of the biotrophic phase of parasitism (Lee and Rose, 2010).

In the early stages of infection during the biotrophic phase, oomycetes establish an intimate relation with plant cells through formation of haustoria. Besides the mechanical role of penetrating the host cells, haustoria are not only used for

nutrient uptake but have also been implicated as sites of effector production and delivery into the host (Whisson *et al*., 2007).

Although effectors are delivered in the apoplast, those with a host translocation signal get transported into the plant cell. Of these the best known are those that belong to the RXLR effector class. They derive their name from a four amino acid motif (arginine, any amino-acid, leucine, arginine) that was identified among all known oomycete effectors. The RXLR effectors possess an N-terminal domain consisting of a signal peptide motif, an optional amino acid motif known as dEER and a C-terminal effector domain. It is the N-terminus that mediates effector uptake by hosts (Whisson *et al*., 2007; Dou *et al*., 2008).

Experimental data have shown that the RXLR domain enables AVR1b (avirulence protein from *P. sojae*) to bind to phosphatidyl inositol phosphates (PIPs) exposed on the external surface of the host cell plasma membrane which subsequently enter the host cells through vesicle associated endocytosis (Kale *et al*., 2010). However, a more recent study found no binding of PIPs on AVR3a from *P. infestans* and seems to indicate that multiple mechanisms may be involved in oomycete effector translocation (Yaeno *et al*., 2011).

An examination of the recently published genome of *P. infestans* revealed that besides the RXLR effector family, there are large families of the Crinkler (CRN) proteins which are candidate cytoplasmic effectors (Haas *et al*., 2009). CRN effectors possess an N-termini LXLFLAK motif which contains a signal peptide, followed by a conserved DLW-domain and an HVLVXXP motif. The N-terminal LXLFLAK motif was shown to be necessary for translocation of the C-terminal portion of the AVR3a into the plant cell (Schornack *et al*., 2010). *P. infestans* Crinklers target the host nucleus to induce cell death (Schornack *et al*., 2010).

In all plant microbe interactions, disruption of plant metabolism is inevitable once colonization has been established successfully. To this end, pathogens including oomycetes manipulate host metabolic pathways, especially the sugar transport, by

activating the *SWEET* transporter genes (Chen *et al*., 2010; Chen *et al*., 2012). In the course of the infection, biotrophic parasitism switches to necrotrophism possibly by secretion of toxic proteins encoded by the pathogen. Oomycetes secrete necrosis-and ethylene-inducing peptides NEP-1 like (NLP) that trigger cell death in the later stages of the infection.

Being located at genomic sites that enable change through mutation and recombination, effector genes are regularly under selection pressure in an ongoing arms race between the pathogen and host leading to rapid 'birth and death' of effetors. For example 563 RXLR effectors have been predicted for *P. infestans* (Haas *et al*., 2009). Of these, 12 had been isolated and studied by 2010 (Vleeshouwers *et al*., 2011).

The successful isolation of effectors has been accomplished using a variety of techniques including exploitation of the expressed sequence tag (EST) database (Kamoun *et al*., 1999). From this database, the ESTs encoding the secreted proteins are selected and the corresponding genes identified and analyzed for polymorphisms in a set of pathogen isolates with a known race structure (Armstrong *et al*., 2005). *Avr3a,* a gene that triggers *R3a* dependent cell death was identified using this approach, also known as association genetics approach (Armstrong *et al*., 2005).

Also, candidate effectors from prediction models are assayed for *in planta* expression and roles determined. This has been facilitated by the discovery that oomycete *Avr* loci typically carry genes with a conserved RLXR motif. Vleeshouwers *et al*., (2008) used a set of putative effector genes mined from EST databases for transient *in planta* expression and showed that *Avr-blb1* is an ***i**n **p**lanta* **i**nduced (*IpiO*) RXLR effector gene. *IpiO* is a gene family consisting of at least two conserved genes *IpiO1* and *IpiO2* which differ in their protein compositions by only four amino acids (Pieterse *et al*., 1994) and both of which are recognized by the *Rpi-blb1* (*RB*) gene that conditions broad spectrum resistance to *P. infestans* (Vleeshouwers *et al*., 2008).

1.12 Aims

According to reports of blight surveys carried out during the first decade of this new millennium in Ireland, the reappearance of the A2 mating types of *P. infestans* has been accompanied by an increase in the frequency of genotypes with insensitivity to metalaxyl (Griffin *et al*., 2002). Similar changes in *P. infestans* populations have occurred in several North Western European countries that preceded Ireland in reporting the occurrence of A2 mating types (Gisi *et al*., 2011). In these countries, the occurrence of more destructive blight epidemics has led to an increase in the use of fungicides (Cooke *et al*., 2011). The overarching aim of this study was to determine the effect of these shifts in *P. infestans* populations on existing blight control strategies particularly the implications of these changes on the durability of host resistance. To achieve this aim, the study was organized around four specific objectives as follows:

1. To determine genetic diversity within the Irish *P. infestans* population and assess for aggressiveness against commercial potato cultivars
2. To investigate the potential for and implications of sexual recombination between genotypes within the Irish *P. infestans* population.

3. To investigate the potential for genotypes of *P. infestans* to adapt to and overcome transgenic-derived host resistance through mutation.

4. To develop a molecular-based test to quantify levels of late blight infection in infected tissues and soils

Each of the four experimental chapters of this thesis addresses one of the four specific objectives listed above while forming a continuous body of interrelated data that addresses that overall aim of the thesis.

Chapter Two

Pathogenic and genetic variation within the Irish population of *Phytophthora infestans*

2.1 Background

Late blight (*P. infestans*) accounts for an estimated $6 billion worth of annual potato losses in crop yield and costs of control worldwide (Haverkort *et al*., 2008). For Irish potato growers *P. infestans* is ubiquitous, with the disease reaching epidemic proportions in all but four seasons from 1983 – 2007, which equated to a mean loss in total yield over that period exceeding 10 t/ha in the absence of appropriate fungicide control (Dowley *et al*., 2008). The Irish blight population was originally comprised of only the A1 mating type until the arrival of the A2 mating type in the early 1990s (O'Sullivan & Dowley, 1991; Griffin *et al*., 2002). This arrival of the A2 mating type followed a similar event in all countries of North West Europe (Fry *et al*., 1992; Fry *et al*., 1993). Yet, in Ireland the A2 mating type was detected at a frequency not exceeding 5% of sites sampled (Cooke *et al*., 1995; O' Sullivan *et al*., 1995). While fungicides play a crucial role in the integrated control of late blight, the increasing use of fungicides across the primary potato growing regions of North West Europe in the 1990s indicates that newly emerging blight strains have become more destructive and consequently more difficult to control (Cooke *et al*., 2011).

Through 2006 and 2007, a resurgence of A2 mating types was recorded through Northern France, Switzerland, UK, Belgium, Germany, Netherlands, Denmark and Sweden (Gisi *et al*., 2011). Based on the use of a series of SSR markers (Knapova and Gisi, 2002; Lees *et al*., 2006), a majority of isolates possessed allele 154 at locus D13 and were 13_A2 ('blue 13') types (Lees *et al*., 2006). This 13_A2 genotype has since rapidly spread throughout Great Britain with the displacement of the old genotypes of *P. infestans* populations (Lees *et al*., 2009). This has also been recorded in France (Montarry *et al*., 2008). In 2008, over 90% of UK sampled blight populations were represented by just two genotypes with 13_A2 recorded in 80% of samples tested (Cooke *et al*., 2010). Genotype 13_A2 was first detected in Northern Ireland in 2007 (Cooke *et al*., 2009) and in the Republic of Ireland in 2008 (Kildea *et al*., 2010). Of equal concern is the insensitivity of 13_A2 isolates to

phenylamide-based fungicides. Although the occurrence of the A2 mating type and phenylamide resistance has been recorded, previous studies have shown that these two traits did not co-segregate in sexual crosses (Gisi and Cohen, 1996; Gisi *et al*., 2011). Similarly, isolates with the 154 allele did not always show metalaxyl resistance (Gisi *et al*., 2011). Yet, what is evident is that for those 13_A2 isolates that display fungicide insensitivity, there is no apparent lack of fitness that would typically be associated with phenylamide resistance.

Compared to existing A1 genotypes, genotype 13_A2 has shown increased fitness and aggressiveness, especially at lower temperatures (~13^{o}C) (Cooke *et al*., 2010), but *P. infestans* 6_A1 has also increased occurrence in parallel to the dominance of 13_A2. Genotype 6_A1 possesses alleles 152, 160 and 162, at locus Pi02 and alleles 181, 197 at locus Pi89 (Cooke *et al*., 2008). As the potential for oospore formation as a result of co-existing 13_A2 and 6_A1 strains does exist, the primary objective of this chapter was to capitalize on the sampling that was previously conducted in 2009 and:

(i) study the population structure and genetic diversity within a sub-population of the sampled population (Kildea *et al*., 2010) using previously developed microsatellite markers (Knapova and Gisi, 2002; Lees *et al*., 2006) which exhibit a high polymorphism and even dispersion across the *P. infestans* genome

(ii) quantify the metalaxyl sensitivity and aggressiveness of those 13_A2 and 6_A1 isolates on leaf and tuber host tissues, and compare with *P. infestans* isolates of genotypes that previously dominated Irish potato growing regions.

2.2 Materials and methods

2.2.1 *P. infestans* isolates

P. infestans was collected in the form of single-lesion leaf samples, taken from naturally infected commercial potato crops across the major potato production regions of the Republic of Ireland in 2009 (Appendix 2.1). To induce sporulation, harvested leaves were incubated for up to 48 h in 9 cm Petri dishes containing moist filter paper. Pure cultures of 98 isolates were obtained by transferring sporangia from sporulating leaflets onto pea agar media (1.5% agar) (Hollomon, 1965) amended with rifampicin (50 mg/litre) (Appendix 2.2).

2.2.2 Analysis of genotypes

Mycelia were harvested from ten day old pea agar pure cultures of *P. infestans* isolates and DNA extracted using procedures for filamentous fungi described in literature (Raeder and Broda, 1985). Simple sequence repeat (SSR) PCR was performed in a 12.5 µl volume containing 50 ng of standardized (50ng/µl) DNA, 1X Thermal Buffer (Promega), 0.2mM dNTP, 0.25 µM of each forward and reverse primers and 1U of Taq DNA polymerase (Promega). SSR markers used were Pi02, Pi33, Pi70, Pi56, Pi89, G11, Pi04, and Pi16 (Lees *et al*., 2006) along with D13 (Knapova and Gisi, 2002) (marker details in Appendix 2.3) and were synthesized by Applied Biosystems (UK). The forward primers were labeled at the 5' end with FAM, NED, PET, or VIC fluorescent dye to facilitate simultaneous analysis. PCR cycling conditions included an initial cycle of 2 min at 95^{o}C followed by 30 cycles for 20 sec at 95^{o}C, 20 sec at 58^{o}C and 1 min at 72^{o}C, with a final 20 min at 72^{o}C. Into each reaction 0.5µl of the PCR reaction was added to 9.25µl de-ionized formamide containing 0.25 µl of an internal size standard (LIZ-500, Applied Biosystems) and the mixture was denatured for 5 min at 95^{o}C before analysis on an ABI 3130xl Genotyper (Applied Biosystems, USA). SSR loci size data produced from the ABI 3130xl Genotyper was collected by built-in 3130xl Data Collector software v3 before being imported into GeneMapper v.3.7 where

electrophoregrams were visualized and converted into SSR fragment size data and imported copied into an Excel (Microsoft, USA) spread sheet.

2.2.3 Mating type and metalaxyl sensitivity determination

Mating type was determined on un-amended carrot agar medium (Erselius and Shaw, 1982) by plating collected isolates (n = 98 isolates) against known A1 and A2 isolates and recording the presence/absence of oospores after two weeks incubation at 18^{o}C with a 16 h photoperiod (Knapova and Gisi, 2002). To test for metalaxyl sensitivity, stock solution was made by dissolving pure grade metalaxyl (provided by Syngenta) and used to prepare dilutions for the desired product concentrations. Leaf discs (15mm diameter) from 4 week old plants of the blight susceptible cultivar Kerr's Pink were floated on their adaxial surface in 6 cm diameter Petri dishes containing 5 ml of either 0, 5 or 100 µg mL^{-1} metalaxyl. For each metalaxyl concentration, five discs were inoculated with 10 µl sporangial/zoospore suspension of an appropriate isolate and incubated for 7 days under 16h photoperiod. Isolates unable to sporulate on discs floating on 5 or 100 µg mL^{-1} were recorded as sensitive, isolates that sporulated in the presence of 5 µg mL^{-1} but not 100 µg mL^{-1} were characterized as intermediate and isolates were deemed metalaxyl resistant when they sporulated on discs floating in suspensions supplemented with 5 and 100 µg mL^{-1} (Cooke *et al*., 1981).

2.2.4 Aggressiveness of *P. infestans* isolates on detached potato leaflets

The foliar aggressiveness of selected isolates was evaluated on four *S. tuberosum* cultivars selected on the basis of their resistance/susceptibility to late blight according to foliar blight ratings data provided in the British Potato Variety Database (Appendix 2.4) in conjunction with data from a field trial carried out at Teagasc Crops Research Centre, Ireland (Griffin *et al*., 2010). These were cv. Bionica = 8, cv. British Queen = 4, cv. Rooster = 4 and cv. Sarpo Mira = 9, where 1= lowest disease resistance and 9= highest disease resistance. Seed tubers of

each cultivar were acquired from the Teagasc Potato Breeding Programme. Leaf material of uniform size and age for the detached leaflet assay was obtained by growing plants at two week intervals in a glasshouse at Teagasc Crops Research Centre, Carlow, Ireland between April and June 2010. The glasshouse was maintained at 18-23^{o}C with natural lighting and regular watering. Fully expanded leaflets of same age and size were harvested for inoculation from 5-6 week old plants. The glasshouse facility was not only digitalized to allow for remote manipulation of internal temperature, air flow and levels humidity but was also adequately sealed to keep out rain water. All incoming air was filtered and waste air expelled through special vents. As such, natural blight infection was excluded. Additionally, plants in the glasshouse were routinely checked to ensure they were free from blight infection.

From the original collection of 98 *P. infestans* isolates (Appendix 2.5), 30 isolates were selected for a replicated aggressiveness assay from genotypes to which they had been assigned using the SSR data. These included 13 isolates with a 13_A2 genotype, 5 isolates with the traditional Irish 5_A1 genotype, 4 isolates with a 6_A1 genotype and 8 isolates with the older 8_A1 genetic profile (Table 2.1).

Viable inoculum was produced by first inoculating each isolate onto tuber slices (Fig. 2.1) of the susceptible cv. Kerr's Pink. Inoculation was performed under the laminar flow hood by placing a small plug of agar from a seven day culture of a respective isolate onto a tuber slice placed in an inverted 9 cm diameter Petri dish with a sterile and dry Whatman filter paper at the base. Three tuber slices were inoculated for each isolate. The lids were replaced and the Petri dishes were sealed with Parafilm before incubating at 18^{o}C with a 16hr photoperiod. Once colonized after 7 days, the tuber slices inoculated with the same isolate were washed together with 5 ml of sterile distilled water in 50 ml Falcon tubes. Each resulting suspension was standardized with a haemocytometer to a concentration of 2-5 x 10^4 sporangia/ml and incubated at 4^{o}C for 2 hours to release zoospores. A range of spore concentration (2-5 x 10^4 sporangia/ml) rather than a single

concentration was adopted in the preparation of inoculums due to disparities in sporangial production observed among isolates used in this study. For this reason the initial sporangial concentration in the inoculum of each isolate was included as a covariant to parameters of aggressiveness during analyses of variance.

2.2.4.1 Inoculation of detached leaflets of four potato cultivars

Leaves produced according to description in section 2.2.4 were used in this activity. In this experiment which was conducted between May and August 2010, a single leaflet was deemed an experimental unit and 9 individual leaflets per isolate/cultivar interaction were inoculated. A split-plot design was adopted in which isolates were assigned to the main plot while the cultivars were assigned to the sub-plots. The experiment was repeated once and each experiment was deemed a block. For the inoculations, the leaflets were placed abaxially in humid boxes with a layer of dampened paper towel at the base and a transparent cover, before being inoculated in the centre with 20µl of the appropriate sporangial/zoospore suspension. No 'standard' reference isolate was included as control as this was not available at the time of the experiment. Instead a single isolate (DL16-1D) from the 30 in Table 2.1 was selected and utilized as an inter-trial control and was included in each test which comprised three batches of 10 isolates in each experiment. The inter-trial control was thus run six times (3 times in the first experiment and 3 times in the repeat experiment).

Table 2.1: Source, genotypic and phenotypic data of *P. infestans* isolates inoculated onto detached leaflets of four potato cultivars

Isolate (n=30)	County	Mating type	Host	[a]SSR Genotype	[b]Metalaxyl response
CW1-3C	Carlow	A2	Potato	13_A2	R
D5-5B	Dublin	A2	Potato	13_A2	R
DL11-1D	Donegal	A2	Potato	13_A2	R
DL12-5A	Donegal	A2	Potato	13_A2	R
DL7-2-T2	Donegal	A2	Potato	13_A2	R
K1-2	Kildare	A2	Potato	13_A2	R
K1-8	Kildare	A2	Potato	13_A2	R
LO2-2D	Louth	A2	Potato	13_A2	R
LO4-1D	Louth	A2	Potato	13_A2	R
MN1-1B	Monaghan	A2	Potato	13_A2	R
MN1-1D	Monaghan	A2	Potato	13_A2	R
WX7-1B	Wexford	A2	Potato	13_A2	R
WX7-2B	Wexford	A2	Potato	13_A2	R
DL11-1A	Donegal	A1	Potato	5_A1	nd*
DL12-5B	Donegal	A1	Potato	5_A1	S
DL16-1D[c]	Donegal	A1	Potato	5_A1	S
DL5-3B	Donegal	A1	Potato	5_A1	S
DL8-1B	Donegal	A1	Potato	5_A1	S
DL11-5A	Donegal	A1	Potato	6_A1	S
DL12-2B-T2	Donegal	A1	Potato	6_A1	S
DL16-5C	Donegal	A1	Potato	6_A1	S
DL4-3B	Donegal	A1	Potato	6_A1	S
C3-1B	Cork	A1	Potato	8_A1	S
CW1-1C	Carlow	A1	Potato	8_A1	S
DL1-5A	Donegal	A1	Potato	8_A1	R
DL16-3B	Donegal	A1	Potato	8_A1	S
DL7-2A	Donegal	A1	Potato	8_A1	R
KY1-2A	Kerry	A1	Potato	8_A1	S
LO4-2A	Laois	A1	Potato	8_A1	S
MN1-2C	Monaghan	A1	Potato	8_A1	S

[a] SSR nomenclature as follows 13_A2 (marker D13; 136, 154); 6_A1 (marker Pi02; 152, 160, 162); 5_A1 (marker D13; 136, 136); 8_A1 (marker D13; 118, 136) (Knapova and Gisi, 2002). [b]R and S denote resistance and sensitivity to metalaxyl respectively. *nd- not determined. [c]Internal control isolate (DL16-1D)

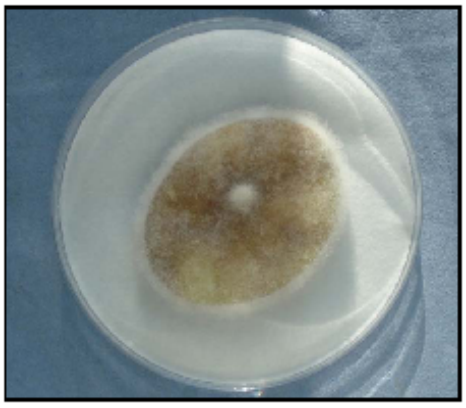


Fig. 2.1: A representative isolate of *P. infestans* growing on a tuber slice of potato cultivar Kerr's Pink at 7 days post inoculation to produce sporangia for leaflet inoculation.

Necessary precautions like surface sterilization of working surfaces with ethanol and use of sterile distilled water for inoculum preparation were taken to avoid unintended introduction blight inoculum other than through appropriate inoculation. Additionally, a set of 18 leaflets in a separate tray was prepared as described above and was included in each experiment. However, instead of zoospore inoculum, each leaflet in the set was inoculated in the centre with a single 20µl droplet of sterile distilled to provide a basis for ascertaining the absence of *P. infestans* propagules in the water used to prepare inoculum for the respective set of isolates in each experiment. Inoculated leaflets were incubated at 18^{o}C for 7 days with a 16 h photoperiod, with disease assessments made every 24 h (Fig. 2.2). Potato is grown as a summer crop in Ireland and reaches flowering and tuber bulking stages when the prevailing temperature is 18^{o}C which together with high humidity provide perfect conditions for blight. The choice of 18^{o}C for the assay simulates these conditions.

Fig. 2.2: Detached leaflets of four potato cultivars (in rows from top to bottom: 1, Bionica; 2, Sarpo Mira; 3, Rooster and 4, British Queen) 6 days following inoculation with *P. infestans*.

Inoculated leaflets were maintained in humid boxes with transparent covers at 18^{o}C with lighting (Fig. 2.2). The remaining zoospore suspension from each isolate was used to determine the infection efficiency of that respective isolate. Again, each leaflet was placed abaxial side up in a 9cm Petri dish with a moist filter paper but each leaflet was then inoculated at 10 individual sites across the leaf surface with 10µl of the appropriate sporangial/zoospore suspension. Leaflets were incubated at 18^{o}C for 3 days with a 16h photoperiod, with 6 leaflets inoculated per cultivar per isolate. Infection efficiency was calculated as a proportion of infected sites with sporulation relative to the total number of inoculated sites per leaflet at the end of the incubation period.

2.2.5 Aggressiveness of *P. infestans* isolates on intact potato tubers

An experiment was carried out in November 2010 and repeated in January 2012 to test for aggressiveness of *P. infestans* isolates on intact potato tubers of four potato cultivars. The four cultivars used (Bionica, Sarpo Mira, British Queen, Rooster) in the assay of isolates for aggressiveness on detached leaflets in section 2.2.4 were again used in the assessment of isolates for aggressiveness on tubers.

For both experiments, tubers were provided by field grown plants. Well sprouted tubers were acquired from the Teagasc Potato Breeding Programme and sown in a well prepared field at Myshall, Count Carlow in May 2010 and May 2011 respectively at the spacing of 30 cm between tubers and 1m between rows. Standard agronomic practices were observed in the maintenance of the crop to maturity including fungicide sprays to keep the crop free from blight. Harvesting was done in November in both years by machine and afterwards tubers were stored at 4^{o}C to await inoculation. Tubers were sorted and washed in water to remove attaching soil prior to artificial inoculation.

2.2.5.1: Inoculation of intact tubers

Ten isolates of *P. infestans* were selected on the basis of their foliar aggressiveness from the study described in section 2.2.4. Inoculum for the selected isolates was prepared by growing them on tuber slices of cultivar Kerr's Pink for 7 days at 18^{o}C. Sporangial suspensions of each culture were obtained by washing the tuber slices in distilled water in 5 litre buckets. Aliquots were taken from each suspension to determine the sporangial density with the aid of a haemocytometer. Afterwards the suspensions were standardized to 15-20 x 10^4 sporangia/ml and incubated at 4^{o}C for 2 hours to induce zoospore release. Inoculum for the selected isolates had varied concentrations because of disparities among isolates in sporangia production as earlier highlighted in section 2.2.4. Prior to inoculation, the tubers were enclosed in net bags with draw back strings at the neck (Edward Kennedy & Co. Ltd, Ireland) and labeled using a water resistant marker on white tyvek tags N-1389596.1B (PWL Creative Labeling Solutions, Ireland). Ten tubers were inoculated in each isolate/cultivar test. Inoculation was performed by submerging enclosed tubers in 3 litres of zoospore suspension of an appropriate isolate for 5 seconds. Inoculated tubers were arranged in plastic boxes which measured 750mm long, 450mm wide and 165mm high (Lin-Pac Ltd, UK) with separate holding compartments. Each box contained all four cultivars treated with the same isolate (Fig. 2.3). There were six technical replicates of each test.

Accordingly, there were six boxes to each isolate. The boxes were enclosed in clear polythene bags to maintain high relative humidity (90-100%) and incubated at ambient store temperature (19±3^{o}C) for three weeks.

In the first experiment, the tubers were inoculated two weeks after harvesting. However, in the repeat experiment, inoculation was completed six weeks after harvesting. This delay in inoculation was due to efforts to complete other activities of this research project on time.

Fig. 2.3: A Lin-Pac plastic box containing potato tubers of four potato cultivars enclosed in net bags (British Queen, Rooster, Sarpo Mira, Bionica) and held in separate compartments after inoculation with a single *P. infestans* isolate. The inoculated tubers (ten per cultivar) were incubated in this box at ambient store temperature (19±3^{o}C) for three weeks to induce tuber blight. To maintain high humidity the plastic box was enclosed in a clear polythene bag.

2.2.6 Data collection

2.2.6.1 Parameters of foliar aggressiveness

The parameters of aggressiveness that were recorded included infection efficiency, latent period (period from inoculation to initial sporulation) in days and area under lesion progress curve (AULPC) calculated as the cumulative totals of sporulating lesions estimated daily for 7 days (Miller *et al*., 1998). A pair of digital calipers

(Work Zone No. DC 0-150) was used to record disease progression with daily lesion areas (mm^2) calculated as per 1/4πab for area of an ellipse with a = breadth of *P. infestans* lesion and b = lesion length (Colon *et al*., 1995). After the seventh day of incubation, leaflets of each isolate/cultivar interaction were washed in sterile 50 ml tubes containing 10ml 10% ethanol. Removed spores were subsequently counted using a haemocytometer, with the sporulation capacity of each isolate/cultivar interaction determined by dividing total spore count by the total lesion area of each isolate on each cultivar. The leaflets that had not sporulated by the 7^{th} day after inoculation were assigned an arbitrary latent period of 8 days to permit statistical comparison as previously proposed (Carlisle *et al*., 2002).

2.2.6.2 Tuber blight assessment

Inoculated tubers were assessed and scored visually for infection using the protocol adopted by the Potato Late blight Network for Europe (EUCABLIGHT). Percentage of tubers with infection was determined for each cultivar/isolate test while percentage of internal infection was recorded for each blighted tuber on a scale of 0%, 5%, 10%, 30%, 50%, 90% or 100% internal lesion size on longitudinally sectioned tubers (Fig. 2.4). Mean tuber lesion size per cultivar/isolate test was calculated from blighted tubers.

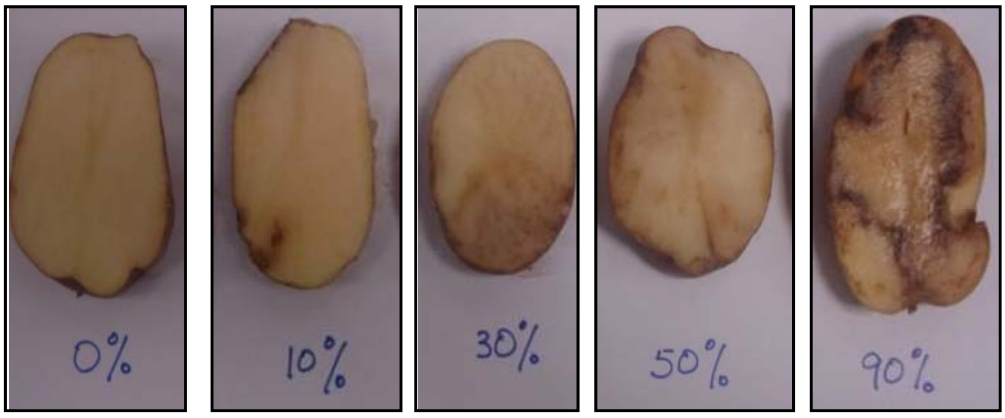


Fig. 2.4: Scale of 0-100% infection adopted during the scoring of tubers for blight disease after inoculation with *P. infestans* isolates and incubation for three weeks.

2.3 Data analysis

2.3.1 Population structure

Population genetic structure was assessed using three complementary techniques. The first was the Bayesian clustering method as implemented in the software Structure software v.2 (Falush *et al*., 2003; Pritchard *et al*., 2000) which provides an iterative algorithm based on distinct marker frequencies for placing individuals into *K* clusters. *K* is a parameter that is chosen in advance but which can be varied in independent Structure software v.2 runs. Structure runs were varied from *K*= 1 to *K*=6 and using the admixture model in the program, ten independent runs were performed for each *K* to assess the consistency of the results. Each run was based on a burn-in period of 50,000 iterations and 100,000 post-burn-in Monte Carlo Markov Chain (MCMC) iterations. To estimate the true *K,* the method of Evanno *et al*., (2005), which is based upon ΔK that evaluates a second order rate of change was used to detect the number of homogenous clusters (*K*). It was assumed that each locus was at Hardy-Weinberg-equilibrium (HWE) and independent of other loci as strongly linked loci lead to spurious clustering (Kaeuffer *et al*., 2007).

In the second method, clustering was performed using principal co-ordinate analysis (PCoA) which reduces multivariate data to clarify relations between variables and reveal major patterns (principal components) within a population. Principal co-ordinates were computed from Nei's pair-wise genetic distance matrices between individuals using the program *GenAlex* 6 (Peakall and Smouse, 2006). PCoA has an advantage over Bayesian algorithm based clustering methods since it does not require assumptions to be made about a population being under the HWE or lacking linkage disequilibrium between the loci (Jombart *et al*., 2009).

For the third method, genetic similarity and cluster analysis of the genotypes was performed based on Jaccard distance coefficient and the unweighted pair group method with arithmetic average (UPGMA) using Free Tree software (Pavlicek *et al*., 1999) after clonal copies were identified and removed. Relationships between genotypes were visualized in a dendrogram constructed in Tree View software.

2.3.2 Genetic diversity

Microsatellite Analyzer software (MSA) V4.05 (Dieringer and Schlotterer, 2003) was used to perform standard population genetic tests including allele frequency, observed and expected heterozygozity (*Ho*, *He*) (Nei, 1973) and Shannon's Index of diversity (I) (Shannon and Weaver, 1949). Wright's *F*-statistics were estimated according to Weir and Cockerham (1978). HWE test and AMOVA procedures were performed in *GenAlEx* following Peakall *et al*., (1995).

2.3.2.1 Heterozygosities (H_o, H_e), Shannon Index of diversity (I) and Wright's *F*- statistics were determined using the formulae

$$H_o = \frac{N_H}{N_g}$$

where N_H is the number of heterozygotes and N_g is the number of genotypes

$$H_e = 1 - \sum_{i=1-k}^{n} p_i^2$$

H_e, expected heterozygosities (also known as gene diversity);

I, Normalized Shannon's Index;

$$I = -\sum \frac{p_i \ln p_i}{\ln N}$$

where P_i is the frequency of the *i*th locus or multilocus genotype and N is the sample size. Shannon ranges from a value of 0 indicates all sample isolates have the same genotype while 1 indicates each isolates has a different genotype.

$$F_{ST} = \frac{\mathrm{var}(P)}{p(1-p)}$$

where variance of allele frequency P is calculated across clusters and $p(1-p)$ is the expected frequency of heterozygosities (Weir and Cockerham, 1984).

2.3.3 Assessment of isolate aggressiveness on foliage and tubers

Statistical analysis of the parameters of aggressiveness saw the AULPC and Percentage of tuber blight incidence and tuber lesion size data subjected to analysis of variance (ANOVA) using GenStat (Payne, 2008). Although, a total of nine leaflets were inoculated in the experiment to assess foliar aggressiveness (5 and 4 leaflets respectively in experiment one and the repeat experiment, or vise versa depending on cultivar), a leaflet was omitted from each isolate/cultivar interaction to leave eight leaflets (4 in each experiment) whose data were used in the analysis (as all water treated leaflets for checking the quality of water did not develop any disease, they were not included in the analysis). Data from the individual leaflets for each parameter of aggressiveness per experiment were pooled and means generated. ANOVA for split-plot was performed in which, the isolate was assigned to the main plot and variety to the sub-plot while the individual experiments were treated as blocks. The initial sporangial concentration in the inoculum was included as a covariate in the analysis of variances to assess its effect on isolate aggressiveness. Where significant differences were detected, means were separated using Fisher's Least Significant Differences (LSD P=0.05). Correlation between parameters of aggressiveness on detached leaflets was determined using Spearman's Rank Correlation. Visual presentation of results in graphs was done in Excel (Microsoft, USA).

Similarly, percentage of tuber blight incidence and tuber lesion size data for each isolate/cultivar interaction were pooled and means generated for each experiment separately. An ANOVA for split-plot was performed in which the isolate was assigned to main plot while the cultivar was assigned to sub-plot and the individual experiments were treated as blocks. Where significant differences were detected, means were separated using Fisher's Least Significant Differences (LSD P=0.05).

2.4 Results

2.4.1 Mating type and metalaxyl sensitivity

Within the sampled population (n = 98), 51% were A1 and 49% were of an A2 mating type. There was a near midway split of the country in terms of the distribution of isolates by mating type, with isolates collected from counties in the eastern half of the country, where commercial potato production is most prominent, being of an A2 mating type while counties in the western half were predominantly of the A1 mating type. Metalaxyl resistance was recorded in 53% of the total population, with resistant and sensitive genotypes present in both the A1- and A2-type populations. Within the A2-type population, 89% of the population were fungicide resistant while 8.3% displayed fungicide sensitivity (Fig. 2.5). In contrast, 64% of the A1-type population were fungicide sensitive and 34% recorded resistance to metalaxyl.

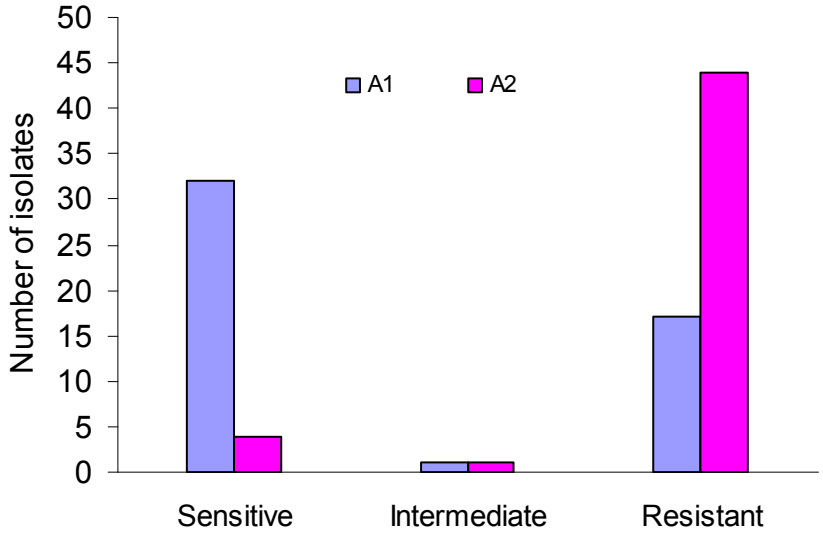


Fig. 2.5: Degree of fungicide sensitivity (scored as sensitive, intermediate or resistant) within each mating type (A1 and A2) population of sampled (n=98) *P. infestans* isolates.

2.4.2 Genotype analysis

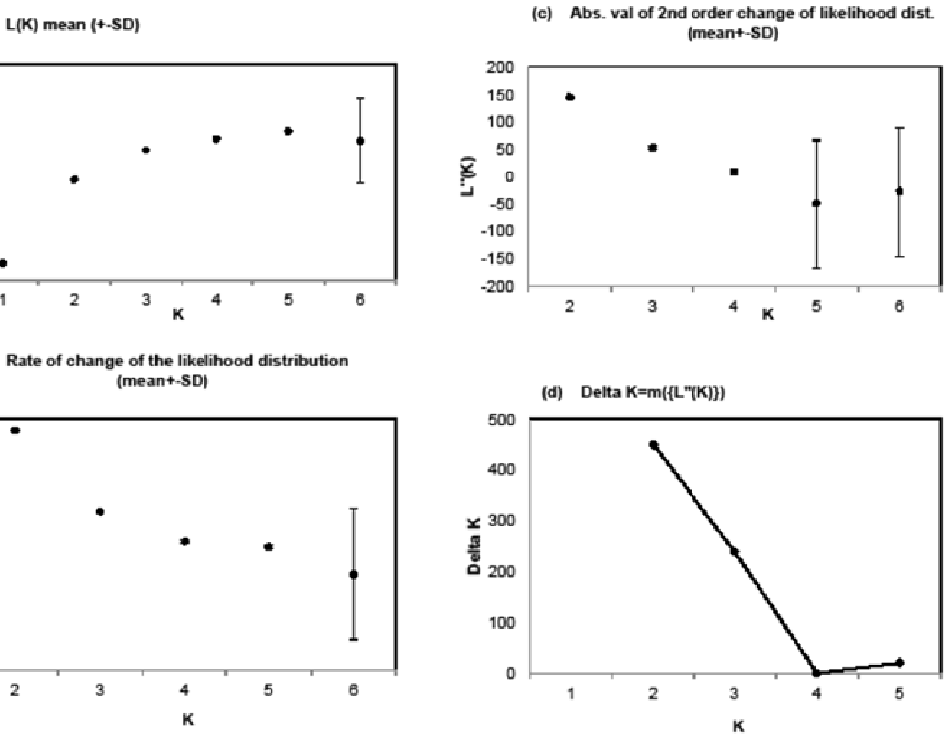
In total, thirty eight genotypes were detected. Genotype-specific alleles were recorded for the 13_A2 (154 at locus G11; 160 and 164 at locus D13) and 6_A1 (189 and 197 at locus Pi89) genotypes. Thirty seven per cent of isolates possessed a 13_A2 genotype with the 6_A1 genotype present in 5.1% of the population. The remaining individuals grouped into either an 8_A1 (32.65%) or 5_A1 (23.5%) genotype (Appendix 2.5). Thirty separate alleles were identified across the sampled *P. infestans* population using SSR markers Pi02, D13, Pi16, G11, Pi56 and Pi89 (Appendix 2.5), with a minimum of 2 (Pi56) to a maximum of 10 alleles (D13) recorded per locus (Table 2.2). Allele 162 (locus Pi02) was most abundant within the population with the rarest alleles being 118 (D13), 138 (D13), 152 (D13), 138 (G11), 150 (G11), 156 (G11), 164 (G11) and 189 (Pi89) (Table 2.2).

Table 2. 2: Allelic frequency at six loci of two clusters of *Phytophthora infestans*

		Cluster 1 (n=51)		Cluster 2 (n=47)	
Locus	Allele size	Allele Count	Allelic frequency	Allele Count	Allelic frequency
Pi02	152	6	0.059	2	0.021
	160	46	0.451	4	0.043
	162	50	0.490	88	0.936
D13	118	1	0.011	30	0.319
	136	53	0.576	59	0.628
	138			1	0.011
	140	7	0.076	2	0.021
	142			2	0.021
	144	6	0.065		
	152	1	0.011		
	154	19	0.207		
	156	2	0.022		
	158	3	0.033		
Pi16	174	3	0.029	2	0.021
	176	45	0.441	41	0.436
	178	54	0.529	51	0.543
G11	138	1	0.010	1	0.012
	150	1	0.010	1	0.012
	154	40	0.417		
	156	1	0.010	17	0.198
	160	29	0.302		
	162			38	0.442
	164	24	0.250	1	0.012
	166			28	0.326
Pi56	174	50	0.490	7	0.074
	176	52	0.510	87	0.926
Pi89	179	90	0.882	46	0.500
	181	6	0.059	46	0.500
	189	1	0.010		
	197	5	0.049		

Model based clustering performed using Structure software v.2 indicated that two was the most appropriate number of clusters obtainable based on the posterior distribution of allele frequencies and maximum membership probability criterion. This was determined by ΔK = 2, (Fig. 2.6d) the upper most level of the structure, which represents the true *K* or number of clusters (Evanno at al., 2005). The overall proportion of the sample in cluster I and II was 0.520 and 0.480 respectively at the mean value of α =0.0431 indicating that most isolates were essentially from one cluster or the other.

Wright's F-statistics showed moderate pair wise genetic differentiation (F_{ST}=0.14) between clusters accompanied by an overall heterozygosity deficit (F_{IS}= -0.472) indicating the absence of or undetectable levels of hybridization and gene flow between the two clusters (Table 2.3). Similarly the mean values of the Shannon Index of diversity (I) showed moderate levels of genetic diversity in both cluster I and II. Using analysis of molecular variance (AMOVA), additional hierarchical partitioning of the *P. infestans* population revealed significant genetic differences (*P*=0.05) between clusters where 55% of the total genetic variation existed within clusters, with 45% recorded between clusters.



: Detection of the number of *P. infestans* clusters *K*. (a) Mean L(K)(±SD) over 10 each *K* value 1-6. (b) Rate of change of the likelihood distribution (mean ±SD). (c) e values of the 2nd order change of likelihood of distribution (mean ±SD). (d) Δ*K* er most level of the structure, here 2, is the true *K* the number of clusters (Evanno 005). There were 50,000 iterations length of burn-in period in and with 100,000 terations. Error bars are standard deviation on mean of 10 replicate runs per *K.*

Table 2.3: Heterozygozity (H_o, H_e), Normalized Shannon's Index of diversity (I) and Wrights indices of genetic differentiation between the two clusters of *P. infestans* (F_{IS} and F_{ST})

	Cluster 1 (n=51)			Cluster 2 (n=47)				
Locus	[a]H_o	[b]H_e	[c]I	*Ho*	*He*	I	F_{IS} (1/2)	[d]F_{ST} (1/2)
Pi02	1.0	0.6	0.9	0.6	0.5	0.3	-0.7	0.2
D13	0.8	0.6	1.3	0.5	0.5	0.9	-0.3	0.1
Pi16	0.9	0.5	0.8	0.5	0.5	0.8	-0.6	0.0
G11	0.9	0.7	1.2	0.7	0.7	1.2	0.01	0.2
Pi56	0.9	0.1	0.7	0.1	0.1	0.7	-0.7	0.2
Pi89	0.1	0.2	0.5	0.5	0.5	0.7	-0.6	0.2
All loci								
Mean	0.8	0.5	0.9	0.5	0.4	0.7	-0.5	0.1
SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.03

Ho and He calculated according to (Nei, 1973)

Multivariate principal co-ordinate analysis (PCoA) provided further support for a two cluster arrangement, with the first two principal co-ordinates accounting for 63.67% of total genetic variability (Fig. 2.7).

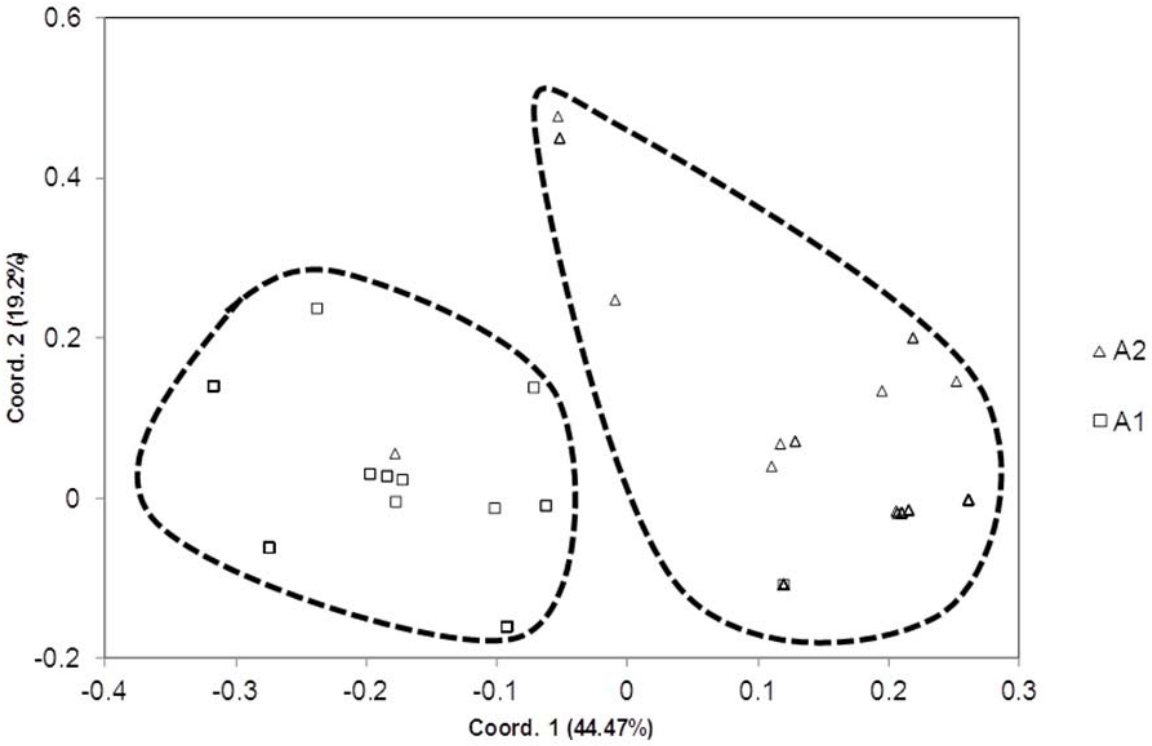


Fig. 2. 7: Two dimensional PCoA performed on the Irish population of *P. infestans* using six microsatellite markers based on Nei's genetic distance (Nei, 1972) and Euclidean distance ($E_i = \sqrt{\sum^{n}_{i=1} (a_i - b_i)^2}$) between two individuals a and b in dimension *i* where n= number of individuals (Sneath and Sokal, 1973). Each point represents a single isolate from each of the thirty eight multilocus genotypes. The co-ordinate 1 (x-axis) accounts for 44.4% (Eigen value of 4.14) of the total variation within the population while the co-ordinate 2 (y-axis) accounts for 19.2% (Eigen value 1.79) of total variation. Both co-ordinates had Eigen values >1 making them inherently highly informative.

Despite the overall difference in locus frequency distributions between the two clusters, there were significant multilocus departures from the Hardy-Weinberg equilibrium (HWE, performed in *GenAlex*, Peakall and Smouse, 2006) for the majority of loci in both clusters indicating the absence of mating. However, some

loci did show no significant *P*-values for deviation from the HWE (D13, Pi 02, Pi 56) (Table 2.4).

Table 2. 4: Chi-Square Tests for Hardy-Weinberg Equilibrium

Cluster	Locus	Df	ChiSq	Prob	Signif
1	Pi02	3	47.7	0.00	***
1	D13	28	41.1	0.05	ns
1	Pi16	3	32.4	0.00	***
1	G11	15	132.0	0.00	***
1	Pi56	1	39.8	0.00	***
1	Pi89	6	102.0	0.00	***
2	Pi02	3	0.2	0.98	ns
2	D13	10	17.5	0.07	ns
2	Pi16	3	22.8	0.00	***
2	G11	15	138.6	0.00	***
2	Pi56	1	0.3	0.58	ns
2	Pi89	1	46.0	0.00	***

ns=not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Of the 38 multilocus genotypes recorded across cluster I and II, cluster I (n = 20 genotypes) largely consisted of metalaxyl resistant phenotypes of an A2 mating type while the majority of genotypes assigned to cluster 2 (n = 18 genotypes) were A1 and metalaxyl sensitive (Fig. 2.8). Twenty four per cent of the total population was made up of genotype G29 (n = 14 isolates; metalaxy resistant A1 mating type) and G5 (n = 10 isolates, metalaxyl resistant A2 mating type). Of interest, G25 and G16 which possess the characteristic 6_A1 genetic fingerprint, claimed greater genetic affinity to the cluster I population that was dominated by the opposite A2 mating type; in contrast to cluster II whose population was more than 94% composed of A1 types.

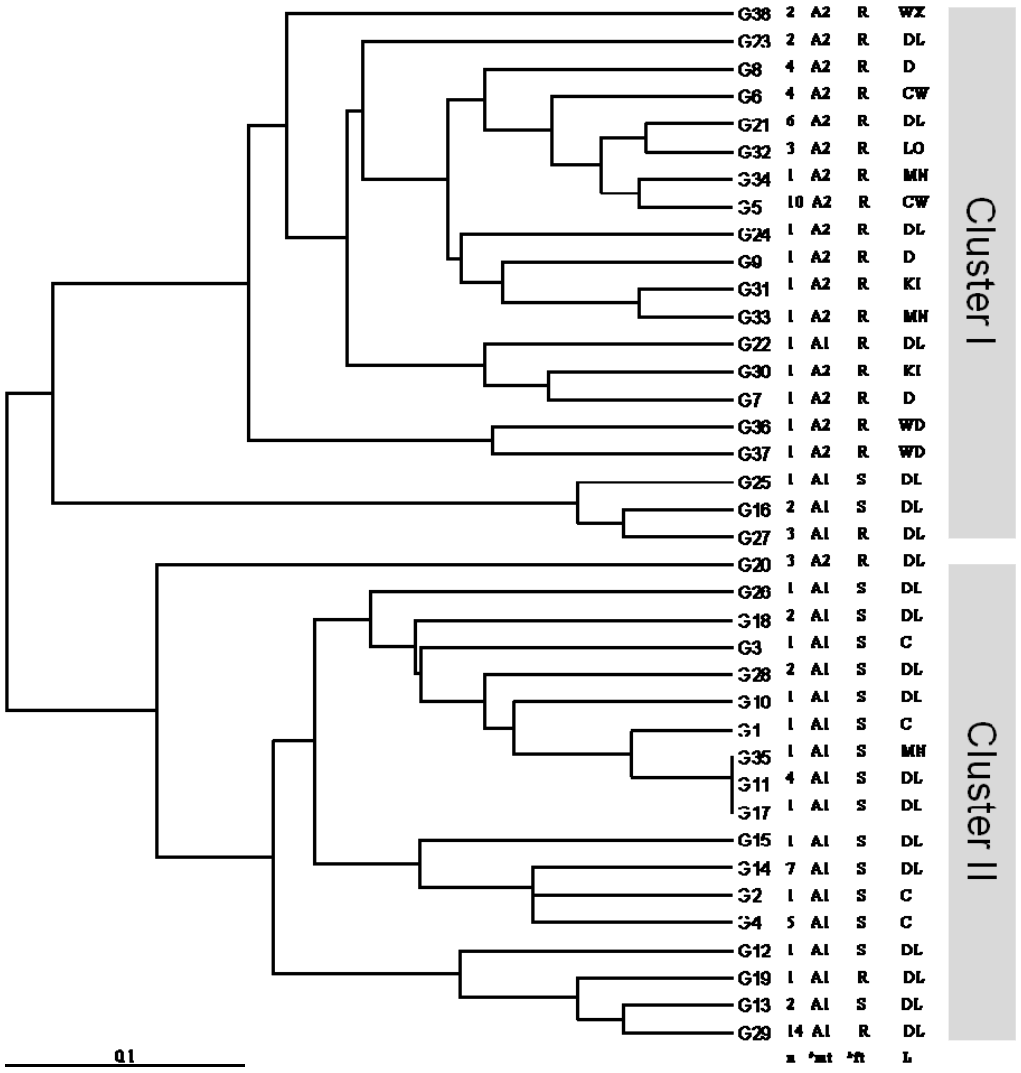


Fig. 2.8: Genetic similarities and cluster analysis of *P. infestans* isolates collected from potato in Wexford (WX), Donegal (DL), Dublin (D), Carlow (CW), Louth (LO), Monaghan (MN), Kildare (KI), Waterford (WD) and Cork (C) in Ireland revealed by un-rooted dendrogram constructed using UPGMA. Genetic distance (0.1 bar) calculated with 1000 repeated re-sampling using Jaccard co-efficient (Pavlicek *et al*., 1999). *Genotypes are arbitrarily numbered and for each genotype information is provided about the location (L) where collected, mating type (mt), metalaxyl sensitivity (ft) and the number of isolates assigned to it (n).

2.4.3 Measurement of parameters of foliar blight aggressiveness

Based on annual crop trials conducted at the Teagasc Oak Park station (2006-2009), the average ranking (data not shown) for the four tested cultivars is British Queen, Rooster, Bionica and Sarpo Mira, with cv. British Queen the most susceptible (mean AUDPC = 2071.2 and cv. Sarpo Mira the most resistant (mean AUDPC = 11.5. These 4 cultivars were employed as host material to gauge the aggressiveness of a sub-group (n = 30) of *P. infestans* isolates representative of the genotypes recorded across both cluster I and II.

Results of each parameter of aggressiveness are presented in separate tables. For each isolate, the scores of each parameter of foliar blight aggressiveness are presented in respective tables 2.5-2.8 for each cultivar on which the isolate was tested (additional ANOVA statistics including separate data on the inter-trial reference isolate DL6-1D are in Appendix 2.6). However, in general terms, highly significant differences ($P<0.001$) were established among isolates and cultivars as well as among the interactions between isolates and cultivars with respect to each of the four parameters of foliar aggressiveness.

Table 2.5 shows the mean infection effeciencies of the isolates on four potato cultivars. Whereas all thirty isolates caused infection on cultivar British Queen, cultivar Sarpo Mira saw six isolates (C3-1B, CW1-3C, DL12-5B, DL5-3B, DL18-1B and KY1-2A) failing to establish blight disease on it. Additionally, isolates had significantly ($P<0.001$) higher infection efficiency on cultivars British Queen and Rooster compared to cultivars Bionica and Sarpo Mira. Consequently, there were significant ($P<0.001$) interactions between isolates and cultivars (Table 2.5). It was established that initial spore concentration in the inoculum, as a covariate, had no significant effect on infection efficiency of the respective isolates. This was also the case with the other three parameters of foliar aggressiveness (AULPC, LP, SI) evaluated.

Table 2.5: Infection efficiency (IE)[a] (%) of thirty isolates of *P. infestans* inoculated onto detached leaflets of four potato cultivars (Bionica, British Queen, Rooster and Sarpo Mira.

Isolate	Bionica	British Queen	Rooster	Sarpo Mira
C3-1B	43.32	91.75	94.17	
CW1-1C	31.74	100.02	72.29	
CW1-3C	85.72	44.32	54.53	86.35
D5-5B	44.16	51.21	28.86	19.72
DL1-5A	51.73	86.21	79.77	16.26
DL11-1A	86.32	100.02	96.06	99.24
DL11-1D	25.49	84.5	85.05	28
DL11-5A	94.78	99.99	100.01	55.89
DL12-2B-T2	29.37	100	93.07	99.98
DL12-5A	41.27	100	99.99	82.56
DL12-5B	45.99	22	67.83	
DL16-1D**	62.51	87.67	97.02	88.66
DL16-3B	11.73	34.17	30.67	5.94
DL16-5C		79.54	71.05	80.09
DL4-3B	37.89	100.01	92.88	100
DL5-3B		83.03		
DL7-2-T2	93.51	97.89	95.51	88.82
DL7-2A	92.11	100	91.73	100.01
DL8-1B	11.01	78.66	20.66	
K1-2	21.01	28.67	19.6	24.2
K1-8	22.46	71.23	32.62	6.22
KY1-2A		19.37	4.39	
LO2-2D	99.99	79.88	92.27	66.59
LO4-1D	92.22	87.48	42.1	61.48
LO4-2A	52.32	100	74.72	37.82
MN1-1B	25.03	99.98	76.14	37.76
MN1-1D	85.93	99.99	95.39	72.28
MN1-2C	97.24	98.65	98.38	99.38
WX7-1B	24.23	96.01	34.94	27.72
WX7-2B	80.01	99.99	98.35	98.27

Isolate (P<0.001)	Covariate* (P=0.98)
Cultivar (P<0.001)	LSD (P=0.05) 9.38
Cultivar/isolate (P<0.001)	

*intial spore concentration in the inoculums used to infect detached leaflets; IE[a] Blanks ( ) in the columns indicate missing values for inoculations which did not produce blight lesions.

**Intertrial control isolate (DL16-1D)

Fig. 2.9 shows the infection efficiencies profiles of individual isolates on each of four cultivars (Bionica, British Queen, Rooster and Sarpo Mira). Regardless of genotype, all isolates infected cv. British Queen. And with the exception of a single 5_A1 isolate (DL12-5B), the same was also the case when cv. Rooster was the host. In contrast, three isolates (DL5-5B, DL16-5C and KY1-2A) were unable to infect cv. Bionica, while 6 isolates (DL12-5B, DL5-3B, DL8-1B, C3-1B, CW13C and KY2-2A) were incapable of initiating disease on cv. Sarpo Mira. On the contrary, 11 (7 A1 and 4 A2) and 10 (4 A1 and 6 A2) isolates achieved greater than 80% infection efficiency on cv. Sarpo Mira and cv. Bionica respectively.

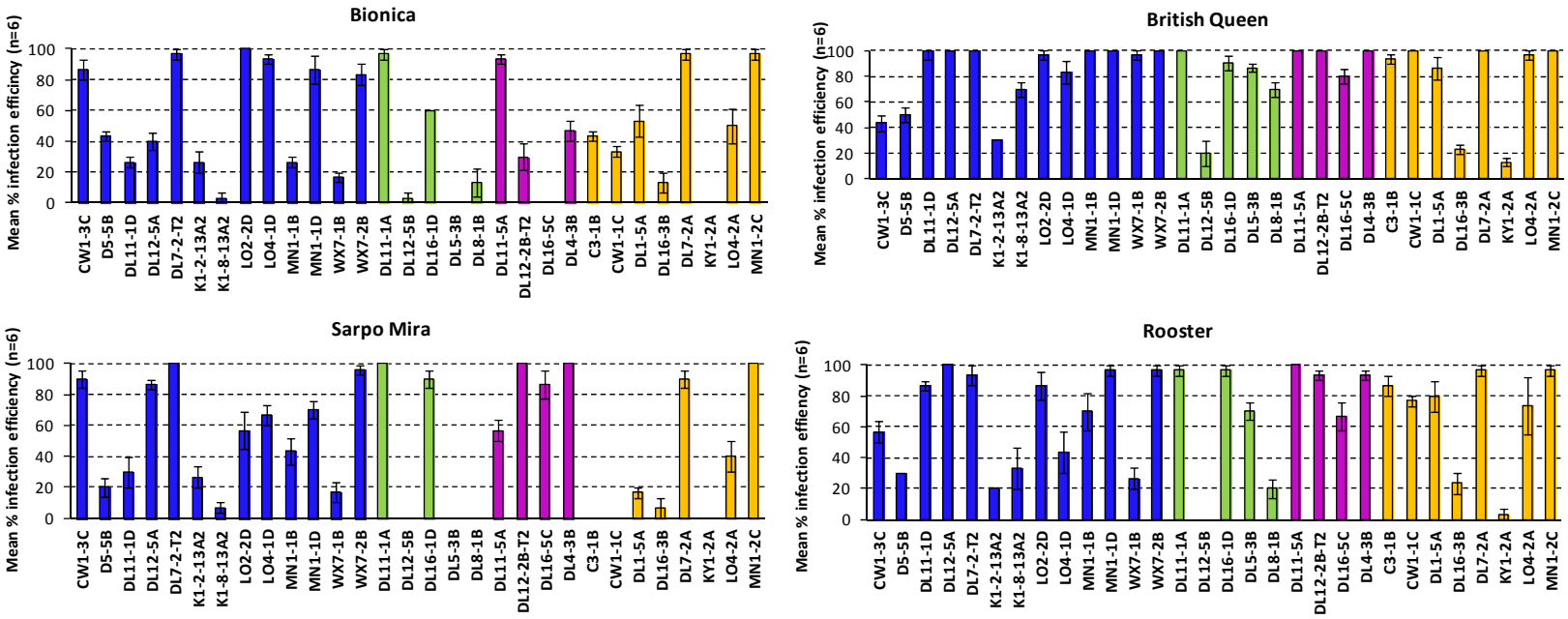


Fig. 2.9: Mean infection efficiency of thirty isolates of *P. infestans* on detached leaflets of four potato cultivars. Error bars represent standard error on mean of six replicates. Isolates are grouped by genotypes and indicated by different colors; blue illustrates a 13_A2 genotype, green a 5_A1 genotype, pink a 6_A1 genotype and yellow an 8_A1 genotype.

Significant differences (*P*<0.001) were observed among isolates (Table 2.6) with respect to the rate of expansion of the lesions they induced on each cultivar in the test. The mean AULPC values ranged from as low as 48 (8_A1 isolate KY1-2A on cv. Sarpo Mira) to the highest value of 4151 (13_A2 isolate MN1-2B on cv. Rooster). Irrespective of *P. infestans* genotype, isolates induced significantly (*P*<0.05) smaller lesions on cv Bionica with only a single (MN1-2C) or three (CW1-3C, WX7-2B, DL2-2-T7) isolates belonging to 8_A1 and 13_A2 genotypes respectively inducing an AULPC > 1000 on this cultivar (Table 2.6). Interestingly, the 8_A1 isolate MN1-2C was the most aggressive isolate of the examined population as indicated by high AULPC values it induced on all the four cultivars. Also, without exception, the isolates in the evaluated population caused significantly larger (P<0.001) AULPC values on cultivars British Queen and Rooster than on cultivars Bionica and Rooster (Table 2.6). When assessed as a covariate, initial sporangial concentration in the inoculums did not significantly affect (P<0.91) the AULPC caused by respective isolates on the potato detached leaflets (Table 2.6).

Table 2.6: Mean AULPC of thirty isolates of *P infestans* inoculated onto detached leaflets of four potato cultivars

Isolate	Bionica	British Queen	Rooster	Sarpo Mira
C3-1B	79	1760	1618	590
CW1-1C	449	2648	2976	1032
CW1-3C	1361	2359	1836	1654
D5-5B	600	1863	1081	1013
DL1-5A	314	2786	3119	1623
DL11-1A	682	2224	1827	1468
DL11-1D	117	2903	2172	110
DL11-5A	94	3077	3276	1632
DL12-2B-T2	440	2801	3710	2708
DL12-5A	255	3074	2493	1494
DL12-5B	71	94	61	59
DL16-1D	119	2779	2796	1102
DL16-3B	139	1224	1940	100
DL16-5C	359	2873	3479	2872
DL4-3B	361	3493	3650	3104
DL5-3B	465	2290	2628	91
DL7-2-T2	1602	2215	2570	2791
DL7-2A	870	2088	1391	1239
DL8-1B	88	1523	1369	408
K1-2	108	144	117	96
K1-8	395	1117	1354	497
KY1-2A	59	473	118	48
LO2-2D	646	2884	2259	1592
LO4-1D	406	1895	1488	1321
LO4-2A	208	1762	1239	186
MN1-1B	686	2331	4151	1274
MN1-1D	748	2207	2143	1524
MN1-2C	2272	3063	2904	3098
WX7-1B	802	2045	2732	1463
WX7-2B	1374	2744	2670	2593
Isolate (*P*<0.001)	Covariate* (P=0.51)			
Cultivar (*P*<0.001)	LSD (P=0.05) 752.3			
Cultivar/isolate (*P*<0.001)				

*intial spore concentration in the inoculums of thirty *P. infestans* isolates used to infect detached leaflets of four potato cultivars, Bionica, British Queen, Sarpo Mira and Rooster.

With regard to latent period (LP), isolates in the tested population differed significantly ($P<0.001$) (Table 2.7). Mean latent periods ranged between 2.83 (lesions induced by DL4-3B on cv Roster) and 8.00 days (in interactions lacking sporulation) (Table 2.7) With the exception of cv. Bionica, genotype 6_A1 isolates took collectively a shorter period than the other genotypes to sporulate on the cultivars examined. Two thirds of the isolates tested formed sporulating lesions on cv Sarpo Mira (Fig. 2.10). Not surprisingly, had shorter latent periods on cvs British Queen and Rooster than on cvs Bionica and Sarpo Mira. Comparatively, isolates sporulated sooner (within 3-4 days post inoculation) on cvs British Queen and Rooster than on cv Bionica and Sarpo Mira (Fig. 2.10). In fact, thirteen isolates from the *P. infestans* population evaluated failed to sporulate on cv Bionica, with only two isolates (13_A2 isolate WX7-2B and 8_A1 isolate MN1-2C) achieving a latent period ≤ 4 days. Significantly, 10 of the 13 isolates with 3_A2 genotype sporulated on cv. Bionica, with a mean latent period of 5.62 days on this cultivar (Fig. 2.10). Notably, the latent periods of the isolates in the tested population seemed to be independent of the initial sporangial concentration in the inoculums used to infect the detached leaflets (Table 2.7; Appendix 2.6). Fig. 2.10 shows the latent period profiles of the isolates.

Table 2. 7: Latent period (LP)[a] (in days) of thirty isolates of *P. infestans* inoculated onto detached leaflets of four potato cultivars

Isolate	Bionica	British Queen	Rooster	Sarpo Mira
C3-1B	8.00	5.21	5.11	6.17
CW1-1C	8.00	4.14	4.26	6.95
CW1-3C	4.25	3.73	3.47	4.06
D5-5B	6.46	4.05	5.37	5.41
DL1-5A	7.67	2.99	3.02	5.44
DL11-1A	7.91	3.24	3.83	4.22
DL11-1D	7.95	3.49	3.56	7.98
DL11-5A	8.00	3.30	3.29	3.49
DL12-2B-T2	7.57	3.36	3.69	3.38
DL12-5A	6.89	2.98	3.06	2.99
DL12-5B	7.94	8.00	7.99	7.94
DL16-1D	8.00	3.72	3.49	4.98
DL16-3B	8.00	6.06	4.44	7.95
DL16-5C	7.47	3.07	3.68	3.31
DL4-3B	7.26	2.82	2.83	3.15
DL5-3B	7.36	4.31	4.17	7.97
DL7-2-T2	4.72	3.00	3.19	3.54
DL7-2A	5.25	3.97	4.35	3.42
DL8-1B	8.00	6.15	5.64	7.76
K1-2-	8.00	7.97	7.91	7.90
K1-8	7.99	6.59	5.75	7.63
KY1-2A	8.00	5.34	7.81	6.89
LO2-2D	5.54	3.37	3.25	3.37
LO4-1D	6.12	4.25	3.73	3.62
LO4-2A	7.99	4.72	4.43	7.61
MN1-1B	6.16	3.75	3.03	4.54
MN1-1D	4.25	3.91	4.09	4.22
MN1-2C	2.92	2.91	3.14	3.00
WX7-1B	7.25	4.16	4.23	6.20
WX7-2B-	4.07	3.21	4.16	3.46
Isolate (P<0.001)	Cultivar/isolate (P<0.001)			
Cultivar (*P*<0.001)	Covariate* (P=0.33)			
LSD (P=0.05) 1.49				

*intial spore concentration in the inoculums used to infect detached leaflets; [a]LP values of 8.0 indicate isolates did not induce sporulating lesion on the respective potato cultivar. An arbitrary value of 8.0 was assigned to interactions lacking sporulating lesions during data collection to permit analysis.

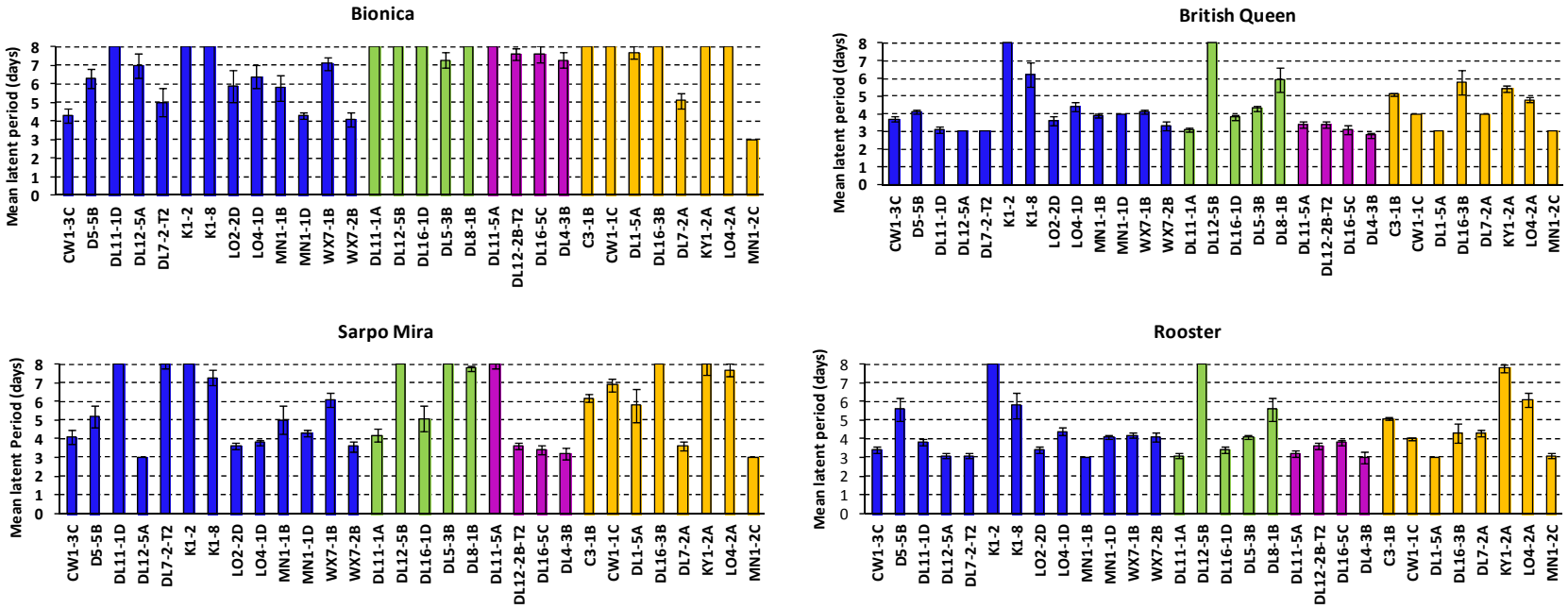


Fig. 2.10: Mean latent period in days for thirty isolates of *P. infestans* on detached leaflets of four potato cultivars. Error bars represent standard error on mean of nine replicates. Isolates are grouped by genotypes and indicated by different colors; blue illustrates a 13_A2 genotype, green a 5_A1 genotype, pink a 6_A1 genotype and yellow an 8_A1 genotype.

There were significant differences (P<0.001) among isolates with respect to sporangia produced per square millimeter of lesion (Table 2.8). With the exception of interactions in which no infections occurred upon inoculation, among the successful infections, sporangia production densities varied from 19.5 (caused by DL7-2A on cv Bionica) to 1000.2 sporangia per square millimeter (caused by DL11-5A on cv Rooster). To a great extent, sporulation densities matched patterns presented by the respective isolates on each potato cultivar with respect to other parameters of foliar blight aggressiveness (IE, LP and AULPC) according to data presented above (Tables 2.5-2.7). Although 6_A1 isolates had a significantly (*P*<0.001) higher sporulation density on cv Bionica than isolates with other genotypes, no isolate produced more than 200 sporangia per mm^2 of lesion (Table 2.8). Fig 2.11 shows the spore production per unit lesion area by 30 *P. infestans* isolates tested on the respective cultivars. Just like the other three parameters of aggressiveness (IE, AULPC, and LP) spore production by isolates on the infected foliage tissue was not significantly (P=0.75) affected by the initial spore concentration in the inoculums (Table 2.8).

Table 2.8: Sporulation density (SI)[a] of thirty isolates of *P. infestans* inoculated onto detached leaflets of four potato cultivars.

Isolate	Bionica	British Queen	Rooster	Sarpo Mira
C3-1B		408.6	420.7	193.7
CW1-1C		824.9	838.6	169.1
CW1-3C	156.3	686.4	550.3	139.8
D5-5B		384.9	338.8	193.4
DL1-5A	108.1	407.9	1000.2	193.2
DL11-1A		332.6	426.9	
DL11-1D		402.5	307.2	
DL11-5A		396.1	267.1	158.1
DL12-2B-T2	115.2	314.3	261.8	189.1
DL12-5A	111.3	211.4	162	190.8
DL12-5B				
DL16-1D		804.5	639.8	182.4
DL16-3B		493.8	464	
DL16-5C	130.9	390.3	643.4	115.9
DL4-3B	151.6	819.2	410.7	168
DL5-3B		553.4	558.1	
DL7-2-T2	128.6	383.7	592.9	113.5
DL7-2A	47.9	620.3	261.6	131.6
DL8-1B		866.7	217.6	
K1-2				
K1-8		233.2	123.3	
KY1-2A		100.8		
LO2-2D		499.8	639.4	194.6
LO4-1D		724.5	576.6	147.9
LO4-2A		556.8	512	59.9
MN1-1B	124	302	315.3	119.8
MN1-1D	100.1	319.5	277	151.8
MN1-2C	179.9	313.5	308.3	174.2
WX7-1B	200.6	380.5	348.6	135.3
WX7-2B	119.5	298.3	293.8	195.3

Isolate (P<0.001)	Covariate* (P=0.75)
Cultivar (P<0.001)	LSD (P=0.05) 127.3
Cultivar/isolate (P<0.001)	

*intial spore concentration in the inoculums used to infect detached leaflets; SI[a] Blanks ( ) in the columns indicate missing values for inoculations which did not produce sporulating lesions

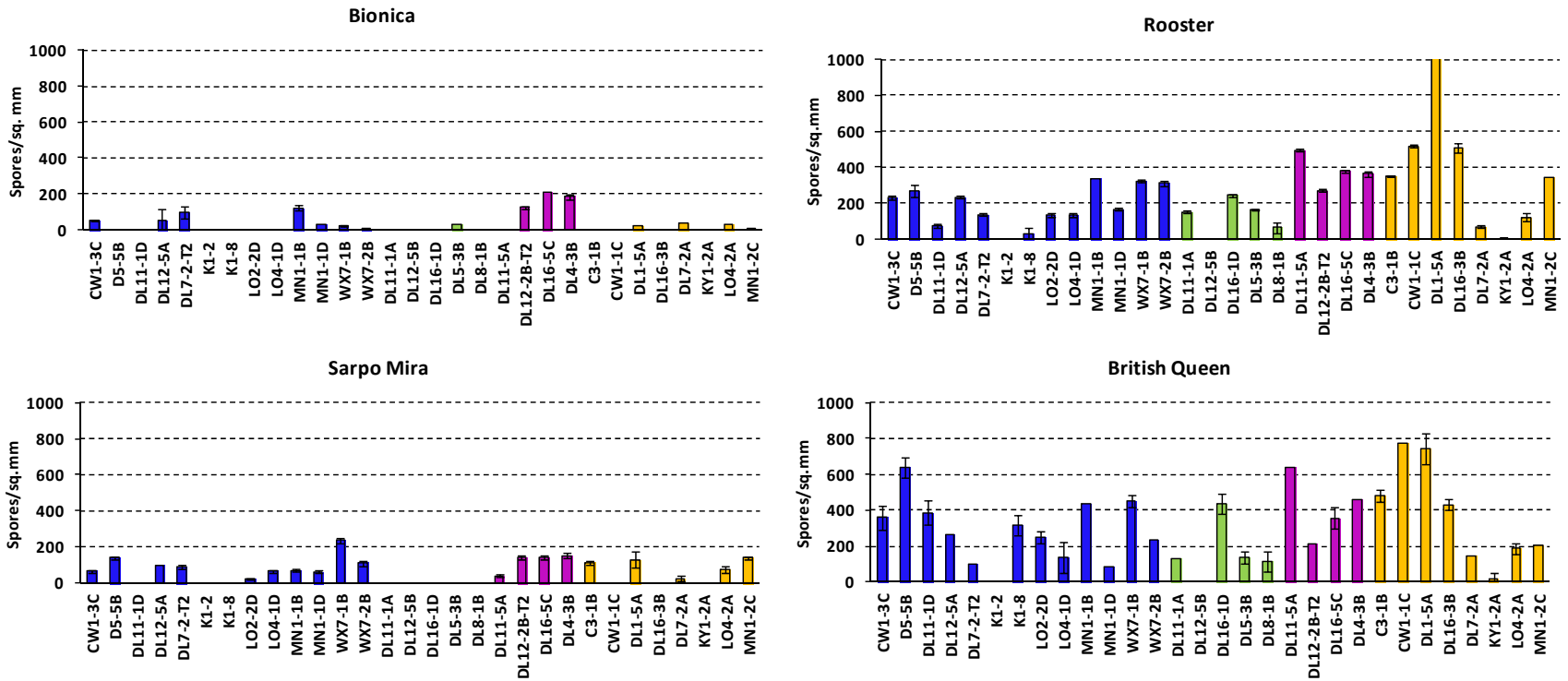


Fig. 2.11: Sporulation density of thirty isolates of *P. infestans* on detached leaflets of four potato cultivars. Error bars are standard error on the mean of nine replicates. Isolates are grouped by genotypes and indicated by different colors; blue illustrates a 13_A2 genotype, green a 5_A1 genotype, pink a 6_A1 genotype and yellow an 8_A1 genotype

Mean AULPC values of individual isolates within a genotype showed that 6_A1 induced lesions that expanded significantly faster ($P<0.001$) than other genotypes on cvs. British Queen, Rooster and Sarpo Mira, while isolates representing the 5_A1 genotype were the least capable of producing rapidly expanding lesions on all four cultivars. Genotype 13_A2 produced the second highest AULPC values after 6_A1 on each of the three cvs British Queen, Rooster and Sarpo Mira. However, genotype 13_A2 had a significantly higher ($P<0.001$) mean AULPC value (707) than the other three genotypes including than 6_A1 (325) on only cv. Bionica (Table 2.9, Appendix 2.6).

Table 2.9: Mean AULPC of four of *P infestans* genotypes (5_A1, 6_A1, 8_A1 and 13_A2) inoculated onto detached leaflets of four potato cultivars.

Genotype	Bionica	British Queen	Rooster	Sarpo Mira
13_A2 (n=13)	707	2149	2090	1355
5_A1 (n=4)	269	1749	1713	650
6_A1 (n=5)	325	3048	3534	2619
8_A1 (n=8)	498	1965	1890	1014
Pi genotype (P<0.001)				
Cultivar (P<0.001)				
Cultivar/Pi genotype (P<0.013)				
Covariate* (P=0.116)				
LSD (P=0.05) 230				

*intial spore concentration in the inoculums used to infect detached leaflets

Correlation analysis between all combinations of the four parameters of aggressiveness returned highly significant associations (P<0.05) (Table 2.10). Latent period correlated negatively with infection efficiency, AULPC and sporulation intensity. Strong positive correlations were observed between all combinations of infection efficiency, AULPC and sporulation intensity. This lends credence to the view that isolates which readily infected host leaflets not only induced faster growing lesions, but also were faster to sporulate and produced more sporangia per unit of lesion area.

Table 2.10: Pearson's coefficients from the correlation of different parameters of aggressiveness of thirty isolates of *P. infestans* on detached leaflets of four potato cultivars

	AULPC	**IE**	**LP**	**SI**
AULPC	1			
IE	0.691**	1		
LP	-0.894**	-0.710**	1	
SI	0.747**	0.414**	-0.654**	1

**P<0.001 (118 df)

The correlation test also demonstrated that each parameter produced a reliable estimate of disease aggressiveness of individual isolates on potato leaflets.

2.4.4 Assessment of isolates for aggressiveness on whole tubers

Every isolate successfully caused disease on each of the four cultivars in both experiments. However, when tuber blight incidence and sizes of internal lesions induced were considered separately, different patterns emerged. Where as no significant differences (P<0.41) were observed among isolates regarding tuber blight incidences (Table 2.11), highly significant differences (P<0.001) were found among isolates with respect to tuber lesion sizes they caused (Table 2.12). Assesment of tuber blight incidence showed that isolate WX7-1B had the highest value (90.00%) on cultivar British Queen while isolate LO2-2D had the lower value (55.8) caused on cultivar Sarpo Mora (Table 2.11). Surprisingly, tuber blight incidences averaged across experiments irrespective of isolate genotype showed no significant differences among cultivars (P<0.16) (Table 2.11).

Table 2.11: Mean tuber blight incidence (%) on four potato cultivars (Bionica, British Queen, Rooster, and Sarpo Mira) inoculated with ten isolates of *P. infestans* based

Isolate	Bionica	British Queen	Rooster	Sarpo Mira
C3-1B	70.8	75.0	72.5	71.7
CW1-3C	64.2	72.5	70.8	72.5
DL11-1A	67.5	85.8	58.3	71.7
DL11-5A	80.0	85.0	68.3	68.3
DL16-5C	80.0	73.3	73.3	67.5
DL4-3B	65.0	60.0	68.3	70.0
DL5-3B	75.0	78.3	61.7	72.5
LO2-2D	62.5	81.7	60.8	55.8
MN1-2C	77.5	81.7	81.7	76.7
WX7-1B	77.5	90.0	77.5	74.2
Isolate (P=0.41)				
Cultivar (P=0.16)				
Cultivar/isolate (P=0.99)				
LSD (P=0.05) 27.37				

The quantity of disease caused on the inoculated tubers as tuber lesion sizes induced following inoculations differed significantly among isolates ($P<0.001$), among cultivars ($P<0.01$) and between experiments ($P<0.001$) (Table 2.12, Appendix 2.6). Isolates produced more disease on each cultivar in the first experiment compared to the second experiment. The discrepancy in the levels of tuber blight between the experiments is attributed to different lengths of storage of the tubers after harvest prior to inoculation with *P. infestans.* In the first experiment, the tubers were inoculated two weeks after harvesting. However, in the repeat experiment, inoculation was completed six weeks after harvesting. This delay in inoculation was an outcome of efforts to complete other activities of this research

project on time. It was presumed that tubers in the second experiment had suberized leading to low levels of tuber blight.

Table 2. 12: Mean tuber lesion size (%) on four potato cultivars (Bionica, British Queen, Rooster, and Sarpo Mira) caused by ten isolates of *P. infestans*

Isolate	**Bionica**	**British Queen**	**Rooster**	**Sarpo Mira**
C3-1B	42.5	56.6	51.1	50.5
CW1-3C	27.7	45.3	30.9	29.4
DL11-1A	34.3	48.4	29.4	39.2
DL11-5A	36.1	56.3	50.8	48.3
DL16-5C	57.9	50.2	53.3	53
DL4-3B	37.8	26.2	29.2	32.3
DL5-3B	38.3	44.1	33.4	31.1
LO2-2D	25.9	39.6	23.8	25.4
MN1-2C	50.0	41.9	40.8	48.1
WX7-1B	45.2	45.8	47.7	46.4
Isolate (P<0.01)				
Cultivar (P<0.001)				
Cultivar/isolate (P=0.75)				
LSD (P=0.05) 18.77				

Although there were differences in disease behavior among isolates with similar genotypes, comparisons of tuber blight incidences induced by isolates on hosts are suggestive of lack of significant differences (P=0.62) among the pathogen genotypes (Table 2.13). Similarly, no significant differences were detected among genotypes in their blight incidences on potato cultivars (P=0.07) nor in the interactions between genotypes and cultivars (P=0.65) (Table 2.13)

Table 2.13: Incidence (%) induced by ten isolates of *P. infestans* inoculated onto whole tubers of four potato cultivars. The ten isolates were grouped according to their SSR genotypes (13_A2, 5_A1, 6_A1 and 8_A1)

Genotype	Bionica	British Queen	Rooster	Sarpo Mira	Mean	SE Mean
13_A2 (n=3)	68.1	81.4	69.7	67.5	74.59	3.03
5_A1 (n=2)	71.2	82.1	60	72.1	76.86	2.32
6_A1 (n=3)	75	72.8	70	68.6	68.64	2.46
8_A1 (n=2)	74.2	78.3	77.1	74.2	77.37	1.58
Pi genotype (P=0.62)						
Cultivar (P=0.07)						
Cultivar/Pi genotype (P=0.65)						
LSD (P=0.05) 16.57						

Presence of significant differences (P<0.01) between pathogen genotypes with respect to the extent of damage to infected tubers internally was noted generally. However, genotype aggressiveness could not be sufficiently discriminated by host cultivars (P=0.32) as no significant interactions (P=0.95) were discerned among pathogen genotypes and potato cultivars (Table 2.14).

Table 2.14: Lesion sizes (%) induced by ten isolates of *P. infestans* inoculated onto whole tubers of four potato cultivars. The ten isolates were grouped according to their SSR genotypes (13_A2, 5_A1, 6_A1 and 8_A1)

Genotype	**Bionica**	**British Queen**	**Rooster**	**Sarpo Mira**	**Mean**	**SE mean**
13_A2 (n=3)	32.9	43.6	34.1	33.8	36.09	2.79
5_A1 (n=2)	36.3	46.2	31.4	35.2	37.27	2.31
6_A1 (n=3)	43.9	44.2	44.4	44.5	42.49	4.42
8_A1 (n=2)	46.3	49.3	46	49.3	47.75	1.81
Pi genotype (P<0.01)						
Cultivar (P=0.32)						
Cultivar/Pi genotype (P=0.95)						
LSD (P=0.05) 16.93						

2.5 Discussion

Even before the occurrence of strains with resistance to metalaxyl in the Irish *P. infestans* population, potato yield losses to late blight were already significant and fungicide based control expensive. Therefore the potential emergence and dominance of national populations with aggressive, fungicide resistant *P. infestans* genotypes represents a major challenge to potato growers. The goal of this study was to firstly ascertain the prevalence of these complex *P. infestans* genotypes in Ireland and thereafter determine their aggressiveness on a series of potato cultivars of economic significance.

Both the recently occurring 6_A1 and 13_A2 genotypes are widespread in potato growing counties and the majority of isolates recovered exhibited a metalaxyl resistant phenotype. The incidence and distribution of fungicide resistant genotypes is determined to a great extent by the agricultural management practices adopted by growers (Gisi *et al*., 2011). Indeed, a trend can be discerned from two decades of blight survey in Ireland during which period, specific blight management practices were in use. For example, although only A1 mating types were recovered in field surveys completed between 1981 and the late 1980s, metalaxyl resistant isolates had already been detected in 1981 following intensive use of metalaxyl in previous years (Dowley *et al*., 2002). Significantly, the subsequent withdrawal of metalaxyl-based fungicides from the market led to a decline in the incidence of metalxyl resistant isolates (Dowley *et al*., 2002). The A2 mating type was isolated from field populations of *P. infestans* in Ireland in the early 1990s up to a frequency of 35% (O'Sullivan and Dowley, 1991), but this was followed by a steady decline in the incidence of the A2 mating type in the Irish populations until only A1 types were detected during national surveys through the early 2000s. However, in 2007 field assessments began to detect A2 genotypes and since then their prominence has steadily increased within the Irish population to the current incidence of 49% as shown for the subpopulation analyzed in this study.

Notably, Irish *P. infestans* population changes and apparent displacement involving only A1 mating type clonal lineages had occurred in Ireland in the 1980s. This had become clear from a study that used isozyme markers to analyze isolates collected between 1988 and 1989. The authors found only four isozyme types including the two dominant ones, Gpi 100/100, Pep 100/100 and Gpi 90/100, Pep 100/100, possessed by strains not previously present in Ireland (Tooley *et al*., 1993). A decade later, mtDNA and RG57 fingerprinting of *P. infestans* isolates showed the presence of IIa and Ia mtDNA haplotypes and 12 different RG-57 genotypes. However, there was a low level of genetic diversity within the population since 80% of isolates analyzed belonged to two clonal lineages IE-1 and IE-5 (Dowley *et al*., 2002; Griffin *et al*., 2002).

In the present study, microsatellite fingerprinting of the Irish blight population confirms the presence of two clusters, which predominantly correspond to each mating type. A moderate level of genetic diversity uncovered by microsatellite fingerprinting is similar to that reported for the French populations of *P. infestans* that were characterized by co-existing clonal lineages (Montarry *et al*., 2010). Additionally, the population deviates from the HWE and shows heterozygosity deficits, both which provide anecdotal evidence for absence of sexual reproduction or detectable gene flow between the two clusters within the *P. infestans* population. Yet, inferences about the mode of reproduction based on HWE can only be indicative because it has been suggested that compliance or non-compliance of allele frequencies to those expected at HWE is not conclusive due to the fact that limited diversity may arise from multiple generations of asexual reproduction during an epidemic (Fry *et al*., 1991). Consequently, results indicate consistency with large scale asexual reproduction but the occurrence of sexual reproduction cannot be ruled out.

Yet, as observed by Anderson and Kohn (1995), the genetic affinity between genotypes recovered from distant geographical locations within Ireland (i.e. Carlow and Donegal) further supports the relative clonality of those genotypes. In fact the

pattern of geographical distribution of genotypes which emerges seems to suggest a westward invasion by genotypes with an A2 mating type and that the observed changes are being driven primarily by a migration into Ireland of multiple genotypes which are establishing and systematically replacing the old population.

The absence of confirmed mating to date in such a moderately diverse population that consists of both mating types of the pathogen is curious. Clearly the occurrence of both mating types in near equal proportion increases the risk that both of these can occur on the same plant in a field making it highly probable for compatible genotypes to mate and produce segregating progenies with the ability to adapt to selection pressure including host resistance and fungicides. It is acknowledged that the absence of recombinants in this research does not indicate the absence of recombination in the field, which could be occurring at a low frequency. This possibility can be inferred from the observation that the two clusters did not completely align with the mating types A1 and A2. An equally plausible hypothesis could be that any recombinants that have formed to date are not able to dominate over the two existing clusters due to a lack of fitness. However, this is speculative for until further studies on field collected leaf samples with multiple lesions or the isolation of viable oospores from field collected soil samples is achieved, will we be in a position to determine the potential for and consequence of sexual hybridization within the Irish blight population.

Parellels can be drawn between this study and similar ones in continental European countries where numerous population studies on *P. infestans* have been performed to determine the impact of A2 mating types on local populations. Diverse patterns have emerged ranging from co-existence between clonal lineages of the two mating types A1 and A2 as in France (Montarry *et al.,* 2010) to on-going mating accompanied by high genetic variability in the Nordic countries (Brurberg *et al.,* 2011). Insight into how the population of *P. infestans* in Ireland changes between years can is provided by the findings of a similar analysis on samples collected in 2011 which suggest a decline in the frequency of A2 mating type and a

corresponding decrease in the frequency of isolates with resistance to metalaxyl (Cooke *et al*., 2011).

A detached leaflet assay to screen representative isolates of each new and existing genotype revealed differences among isolates with respect to their aggressiveness to four potato cultivars, as indicated by infection efficiency, AULPC and sporulation intensity and latent period. By grouping isolates broadly into new (13_A2 and 6_A1) and old (5_A1 and 8_A1) genotypes and comparing the groups for differences in aggressiveness, data showed that the new genotypes were significantly more aggressive relative to the old genotypes. This was despite the variation observed among constituent isolates of each genotype. Available data from this study could not permit the assessment of the genetic basis for differences in aggressiveness among isolates of similar genotypes. The reliability of the detached leaflet test compared to a whole plant assay in investigating aggressiveness of blight populations has been confirmed by previous studies (Lebreton and Andrivon, 1999). Although some studies (Lebreton and Andrivon, 1999; Spielman *et al*., 1992) have preferred some parameters over others in the estimation of isolate aggressiveness, there were statistically significant correlations between parameters of isolate aggressiveness for foliage blight in the present study (Table 2.11).

Consequently, each of the four parameters could be relied upon to provide information about the relative aggressiveness of an isolate for foliage blight. However, on the strict basis of blight epidemiology, the most relevant aspects of aggressiveness would consist of latent period and sporulation density. As *P. infestans* infections are polycyclic, the shorter the latent period an isolate has, the more destructive it becomes. Similarly, production of sporangia ensures spread within and between crops as well as survival of an isolate between succeeding seasons.

The relatively higher aggressiveness of isolates with 13_A2 and 6_A1 genotypes explains the elevated incidence of these new genotypes through the Irish population. Firstly, the shorter latent periods enable aggressive isolates to increase the number of disease cycles in a season. In parallel, faster growing lesions enable the isolates to out compete those isolates that take longer to colonize available host tissues and the high rate of sporulation per lesion area they exhibit enables inoculum dispersal over large cropping areas.

Data from this study support this view (Tables 2.6-2.9; Appendix 2.6). Previous studies have similarly associated relative differences in aggressiveness with shifts in *P. infestans* populations (Goodwin *et al.,* 1995) but what is of particular interest here is the level of isolate variability in regards to foliar aggressiveness, within the four genotypes. While, 7 of the 10 most aggressive isolates were of a 13_A2 genotype, older isolates (e.g. 8_A1 MN1-2C and DL7-2A) exhibited an ability to infect and sporulate on all cultivars including the more resistant cv. Bionica. As revealed from a study by Cooke *et al*., (2010), another feature that makes these new genotypes, particularly 13_A2, more aggressive is the temperature range within which they are infective. These authors showed that isolates with 13_A2 isolates had significantly higher infection efficiency and induced larger lesions when inoculated on detached leaflets and incubated at temperatures of between 4 and 18^{o}C than isolates belonging to traditional genotypes of A1 mating type.

As regards tuber blight infection, the extent of damage caused to infected tubers also revealed variation among genotypes and among isolates including those of the same genotype. For instance, genotype 13_A2 induced smaller lesions on the infected tubers than 8_A1 (Table 2.12). On the contrary, isolates of the pre-existing genotypes 5_A1 and 8_A1 (e.g. C3-1B and MN1-2C) rapidly destroyed the tubers of all cultivars on which infection occurred.

Even cultivars which showed resistance to foliar blight were susceptible to tuber blight indicating the possible expression of resistance in the foliage but not in the tubers within the resistant cultivars. In literature, the relationship between levels of foliar and tuber blight resistance in cultivars is not straight forward, with some reports finding significant correlation and others reporting absence of association between the two forms of resistance. A recent study, for instance, found that markers on potato linkage group V that were strongly associated with foliar resistance were marginally associated with tuber blight resistance in the field and in laboratory assays on tuber slices (Mayton *et al*., 2010). In another study, foliar and tuber resistance were reported to segregate either in combination or independently in mapping populations (Park *et al*., 2005).

Of significance is the role that tuber blight is likely to have on blight epidemics. It is widely known that apart from acting as reservoirs in which *P. infestans* survive harsh conditions and absence of the host foliage, infected tubers are an important pathway for blight dissemination (Andrivon, 1995; Dowley and O'Sullivan, 1991; Lambert *et al*., 1998). Genotypes that infect the tubers but fail to destroy them assure the survival of the genotype during the winter.

The frequency of infected tubers under field conditions is dependent on many above and below ground factors which determine the quantity of *P. infestans* inoculum reaching the new crops. For instance, tuber infection is determined to a great extent by the level of resistance of the cultivars grown (Naerstad *et al*., 2007).

Secondly, the architecture of the above ground parts of the plant and distribution of sporulating lesions on the plant also determine the way water with infective spores translocate down to the tubers (Bain and Möller, 1999; Bain *et al*., 1997; Lapwood, 1977). This means cultivars whose leaf spread encourage water to collect and run down the stems are predisposed to tuber blight compared to cultivars in which water is channeled outwards away from the ridges (Lacey, 1967).

Thirdly, the way tubers are distributed on the potato rhizosphere is crucial to the successful initiation of disease by the zoospores reaching the soil (Arora, 1989). In cultivars that form short stolons, tubers cluster around the main stems at shallow depths and get easily infected by spores washed down from the infected plant canopy. In contrast cultivars that form long stolons, distribute the tubers at greater depths protecting them from most of the inocula (Arora, 1989).

The pathogenic and genetic variation of the isolates detailed in this study has implications for both potato breeding and cultivation in Ireland. Germplasm derived from national breeding programmes must be tested with recently occurring *P. infestans* genotypes. For the grower, a continued reliance on fungicide-based control is clearly unsustainable in the longer term but the issue is compounded by the emergence of more aggressive isolates as shown here.

To achieve more effective control of blight into the future, the introgression of novel sources of blight resistance into existing breeding initiatives must be accelerated. The stacking of resistance genes in varieties through either conventional or biotechnology-based techniques will be key to achieving this so that the development of novel cultivars can occur in tandem with the shifts in genotypes in the Irish population of *P. infestans*.

Chapter Three

Investigating the potential for recombination between recent A1 and A2 strains of *Phytophthora infestans*

3.1 Background

The European populations of *P. infestans* have undergone an especially dramatic change following the arrival on the continent of the A2 mating type in the late 1970s. This was soon followed by the systematic displacement of the 'old' US-1 clonal lineage of A1 mating type that previously dominated the continent (Fry *et al*., 1993). Population studies based on Black's R differential phenotypes as well as DNA fingerprint analyses demonstrated that after 1980, a dramatic increase in genetic diversity occurred in the Dutch *P. infestans* populations, implicating the occurrence of sexual reproduction (Drenth *et al*., 1994). Previously, studies on European populations based on polymorphic allozyme markers had showed that the establishment of new A1 and A2 mating types had been accompanied by a displacement of the A1 mating type that was present prior to 1980 (Fry *et al*., 1992; Spielman *et al*., 1991). These developments coupled with the observation that a very high percentage of isolates identified each year had unique genotypes, strengthened the hypothesis that these changes were driven by sexual recombination.

Consequently, Drenth *et al*., (1995) demonstrated the production of oospores under controlled conditions in potato host tissues at temperatures ranging from 5 to 25^{o}C and their ability to survive temperature extremes of -80^{o}C and 35^{o}C. They also showed that viable oospores could over-winter and act as the inoculum source for subsequent epidemics. In this regard, studies on oospores as primary sources of infection under natural conditions was aided a great deal by the development of new fingerprinting techniques including simple sequence repeat (SSR) markers which have better resolution compared to protein based markers as used in previous population studies.

To a large extent, these new markers have been used to study *P. infestans* populations in order to decipher patterns that would indicate presence of genetic recombination (Montarry *et al*., 2010; Widmark *et al*., 2011) but data obtained from use of these markers have invariably been used to draw inferences about the presence or absence of genetic recombination within a population. The large number of genotypes as revealed by fingerprinting *P.*

infestans in the Nordic countries contrasts with the clonal lineages that define population structures of *P. infestans* in other parts of Europe. In the former the proliferation of *P. infestans* genotypes has been deemed to be evidence for the presence of genetic recombination in the former (Brurberg *et al*., 2011). Inferences about genetic recombination have also been made based on the earlier occurrence of late blight epidemics (e.g. in Finland and Sweden) which are thought to be linked to *P. infestans* surviving the winter fallow via oospores to infect the next crop (Hannukkala *et al*., 2007; Lehtinen and Hannukkala, 2004; Widmark *et al*., 2007). Both approaches have proved valuable tools to aid in drawing conclusions about newly occurring genotypes but fall short of affirming unequivocally the occurrence of sexual recombination in specific instances. Meanwhile the newly occurring genotypes continued to be a threat to potato production in many regions in Europe where they occur (Fry *et al*., 2009), including Ireland and the UK. Although significant effort has already gone into the phenotypic and genotypic characterization of these new populations, there are still few data on their biology particularly with respect to the occurrence of genetic recombination. The question still remains: have the newly occurring aggressive strains, especially of the 13_A2 genotype (blue 13), of *P. infestans* with resistance to phenylamide (metalaxyl) fungicide arisen from genetic recombination?

Field based studies to test the occurrence of genetic recombination are prone to "contamination" of experimental plots with airborne sporangial inoculums from a distant source. On the other hand, laboratory-based tests may be detached from the actual field conditions. In response, the goal of the work reported here was to investigate, under both *in vitro* and *in vivo* conditions, the propensity for genetic recombination between representative isolates of the two recently occurring genotypes (blue 13 and pink 6) of *P. infestans* in Ireland. Once recombination was demonstrated, data from phenotypic and genotypic characterization of the resulting *F*1 recombinants was collated to gain insights into the role of oospores in shifts in population structure and fitness in *P. infestans*.

3.2 Materials and methods

3.2.1. *P. infestans* isolates

Two *P. infestans* isolates were selected to investigate the potential for genetic recombination: isolate CW1-3C_A2 which has a 13_A2 genotype (blue 13) and isolate DL4-3B_A1 which possesses a 6_A1 genotype (pink 6). Both genotypes are recent immigrants to Ireland (Kildea *et al*. unpublished) and isolates from each genotype have displayed heightened levels of aggressiveness compared with isolates of pre-existing Irish *P. infestans* genotypes (see Chapter 2). In addition to their opposing mating types, the two isolates differ in their response to metalaxyl; (susceptible and resistant for DL43B_A1 and CW13C_A2 respectively) and were considered appropriate candidates with which to study a segregating *F*1 population, should mating occur. As there was no record of sexual recombination of these two genotypes in Ireland, preliminary crosses were performed to determine their crossability in *in vitro* tests prior to the investigations proper.

The potential for sexual recombination between these two isolates was determined by pairing them in two ways. Firstly, they were tested under *in vitro* conditions in the laboratory and secondly the potential for *in vivo* recombination was tested by simultaneously inoculating both isolates on a potato crop growing in a polytunnel environment. The polytunnel was located at the Henfaes Research Station of Bangor University in North Wales (53^{o}16' 04.50'' N; 3^{o} 56' 21.81'' W).

3.2.2 *In vitro* pairing of *P. infestans* isolates CW1-3C_A2 and DL4-3B_A1

Ten day old pure cultures of CW1-3C_A2 and DL4-3B_A1 grown on pea agar (Hollomon, 1965) (Appendix 2.2) were used to provide mycelia for performing crosses between the two isolates. Crosses were performed by growing both parents in the same plate of pea agar following published procedures (Lee *et al*., 2002) with modifications. An agar strip (1.0cm x 4.0 cm) with CW1-3C_A2 was placed in parallel and 3cm apart to another similarly sized agar strip of

DL4-3B_A1 on a 9cm diameter plate containing 1.5% pea agar. A total of eight replicate plates were prepared for each cross and this experiment was repeated three times. For each experiment, eight control plates were also included with four containing parallel agar strips from isolate CW1-3C_A2 only and the other four with parallel agar strips from isolate DL4-3B_A1. All cultures were incubated at 18^{o}C for 4 weeks in the dark, after which they were checked microscopically for the presence of oospores within the zone between the two strips in each plate.

3.2.3 *P. infestans* oospore harvesting and pre-germination treatment

Once oospore formation was confirmed microscopically, oospores were harvested from paired culture plates by extracting four equidistant agar plugs (0.5cm diameter) along the interface of the merged colonies. All four plugs from each plate were placed in a 2ml Eppendorf tube containing 1ml of sterile distilled water and were vortexed at 3000 rpm for 3 min to break up agar and release embedded oospores into the liquid suspension. The resulting suspension was passed through a single layer of cheese cloth to remove pieces of agar before being collected in sterile 2ml Eppendorf tubes. Six aliquots of 10µl each from the suspension were then examined microscopically with the aid of a haemocytometer to quantify oospores and adjusted to 1000 oospores/ml using sterile distilled water.

Owing to the thick outer wall of the oospore, previous studies used the cellulase NovoZym 234 (Novo Biolabs, Nova Enzyme Products Ltd, UK) to pre-treat the oospores and digest residual mycelia in the harvested suspension (Knapova *et al*., 2002; Pittis and Shattock, 1994). However, this product was later removed from the market following suspected carcinogenic effects associated with its usage. It is a well established fact that the cell walls of oomycetes consist of (1-3)- β-D-glucans, (1-6)- β-D-glucans, and cellulose (Bartnicki-Garcia, 1968). Accordingly, and as a viable alternative in this study, a *Trichoderma reesei* cellulase ATCC 26921 (1,-4-(1, 3:1, 4)-β-glucan-4 glucanohydrolase) (C2730, Sigma Aldrich) was used to perform the necessary pre-germination treatment of the *P. infestans* oospoore suspension.

Prior to use of the cellulase proper, the digestion conditions were optimised with attention focussed on the concentration of the cellulase and the length of time permitted for digestion. Two cellulase concentrations, 1:1 (50%) and 1:0.5 (33%) (v/v) oospore suspension: cellulase respectively and four incubation times (3, 12, 24, 36 hours) were evaluated to determine the optimal conditions at room temperature to prepare oospores for germination.

3.2.4 Germination of *P. infestans* oospores

After successful mycelial digestion, the suspension was centrifuged at 4000 rpm for 2 min and the supernatant discarded. The remaining oospores were washed three times in 500µl sterile distilled water before the oospores were re-suspended in a final volume of 1000µl. The recovered suspensions were examined microscopically for complete digestion of mycelial residues and adjusted to 1000 oospores/ml with distilled water. To encourage germination of the oospores, 100µl of pre-treated and washed oospore suspension was plated on a 9cm diameter plate containing 0.5% water agar and incubated at 18^{o}C (Tooley *et al*., 1985) in the dark. Six plates were prepared per *P. infestans* genotype pairing. Observations for geminating oospores commenced five days after plating.

To facilitate the handling of a potentially large number of oospore-derived isolates, attempts were made to pick and transfer germinating oospores directly into individual wells of a 96 well plate containing liquid rye or tuber extract amended with rifampicin (50mg/L). This was unsuccessful due to difficulties involved in isolating single germinating oospores and persistent contamination of culture plates. However, these challenges were overcome by adopting a different approach which saw the 0.5% water agar plates being overlaid with rye agar (Caten and Jinks, 1968) (Appendix 2.2) and incubated for 3-4 days at 18^{o}C. Each day thereafter these ‘sandwich’ cultures were observed for the appearance of colonies. Single colonies were transferred onto pea agar plates by careful excision and lifting using a sterile needle and thereafter were incubated at 18^{o}C for 10 days to raise putative F1 cultures.

3.2.5 Obtaining single *P. infestans* zoospores from oospore derived cultures

To ensure the purity of each putative *F*1 culture arising from the sandwich plates, a single zoospore culture was prepared for every successfully transferred colony. This was achieved by flooding a ten day old putative *F*1 culture with sterile distilled water to dislodge sporangia into suspension, which was filtered through a double layer of sterile cheese cloth before incubating at 4^{o}C for two hours. A 10µl aliquot was examined microscopically to confirm zoospore release and adjusted to 2 x 10^4 zoospores/ml. Duplicate plates of 1.5% pea agar were prepared for each isolate and to each 20µl of the zoospore suspension was spread evenly across each plate using a sterile plastic inoculum spreader followed by incubation in the dark at 18^{o}C for 2-3 days to germinate the zoospores. A single germinating zoospore was then excised using a sterile needle and transferred onto plates with pea agar. To prevent loss of isolates through contamination or as a result of flaming the needle, multiple isolations were completed for each *F*1. Recovered individuals were subsequently characterized for mating type and response to metalaxyl as described in chapter two. Then DNA was extracted from each isolate and used for mitochondrial DNA (mtDNA) haplotype and simple sequence repeats (SSR) analysis.

3.2.6 Assessing the potential for sexual recombination in *P. infestans* under polytunnel conditions

Experiments on the potential for sexual recombination between CW1-3C_A2 and DL4-3B_A1 on whole potato plants were conducted in a walk-in polytunnel structure measuring 5m wide, 3m high and 20m long at Henfaes Research Centre, Bangor, UK. Certified seed tubers of Maris Piper, Bionica, Toluca, Sarpo Mira, Cara were planted in the polytunnel on 2nd February 2010.

Prior to planting, tubers were visually examined and those with wounds inflicted during harvesting or transportation were discarded. The risk of external blight inoculum infection caused by airborne sporangial inoculum was minimized by the early planting in the polytunnel which ensured the crop reached maturity

before outdoor potato crops on the farm adjacent to the polytunnel had emerged. The tunnel was rotovated, cleared and marked into eight equivalent plots, four on each side of the pathway which ran midway through the length of the polytunnel (Fig. 3.1). Each plot had 10 rows of 6 potato tubers (n=60). The rows of the susceptible cultivar Maris Piper was alternated with rows of each of the other cultivars in each plot to act as bait for any pre-existing soil borne *P. infestans* propagules.

Optimum soil moisture and humidity in the polytunnel was maintained by misting once a day for 30min with the aid of an automatic programming device HOZELOCK (ACI Model 2705) mounted on the outlet hose which supplied the misting nozzles. Previously, the polytunnel was inoculated with mating populations of *P. infestans* in 2006-2008 during a blight research project supported by the Potato Council Ltd (Cooke *et al*., 2010). As such, the first crop in this study was observed weekly after emergence for primary infection and single lesion samples collected for genotyping prior to inoculation with the mating pair of *P. infestans* isolates. SSR analysis of the collected isolates revealed three genotypes. The plants on which the lesions had been sampled were uprooted, bagged and removed from the polytunnel to minimize the chances of unintended hybridization between any of these three previously tested genotypes with the two genotypes that were later to be introduced in the polytunnel.

The isolates used to inoculate the crop in the polytunnel were CW13C_A2 and DL43B_A1. To produce sufficient inoculum of the two isolates, a rye agar culture (Caten and Jinks, 1968) of each isolate was flooded with sterile water and rubbed gently to dislodge sporangia into suspension. The suspensions were incubated at 4^{o}C to release zoospores. The sporangial/zoospore mixture of each isolate was inoculated onto leaves of potato cultivar Maris Piper placed in plastic trays lined with moist paper towels and afterwards covered with lids before incubating at 18^{o}C for 6 days. Sporulating leaves were pooled together into buckets according to isolate and flooded with water and agitated to free the sporangia into suspension.

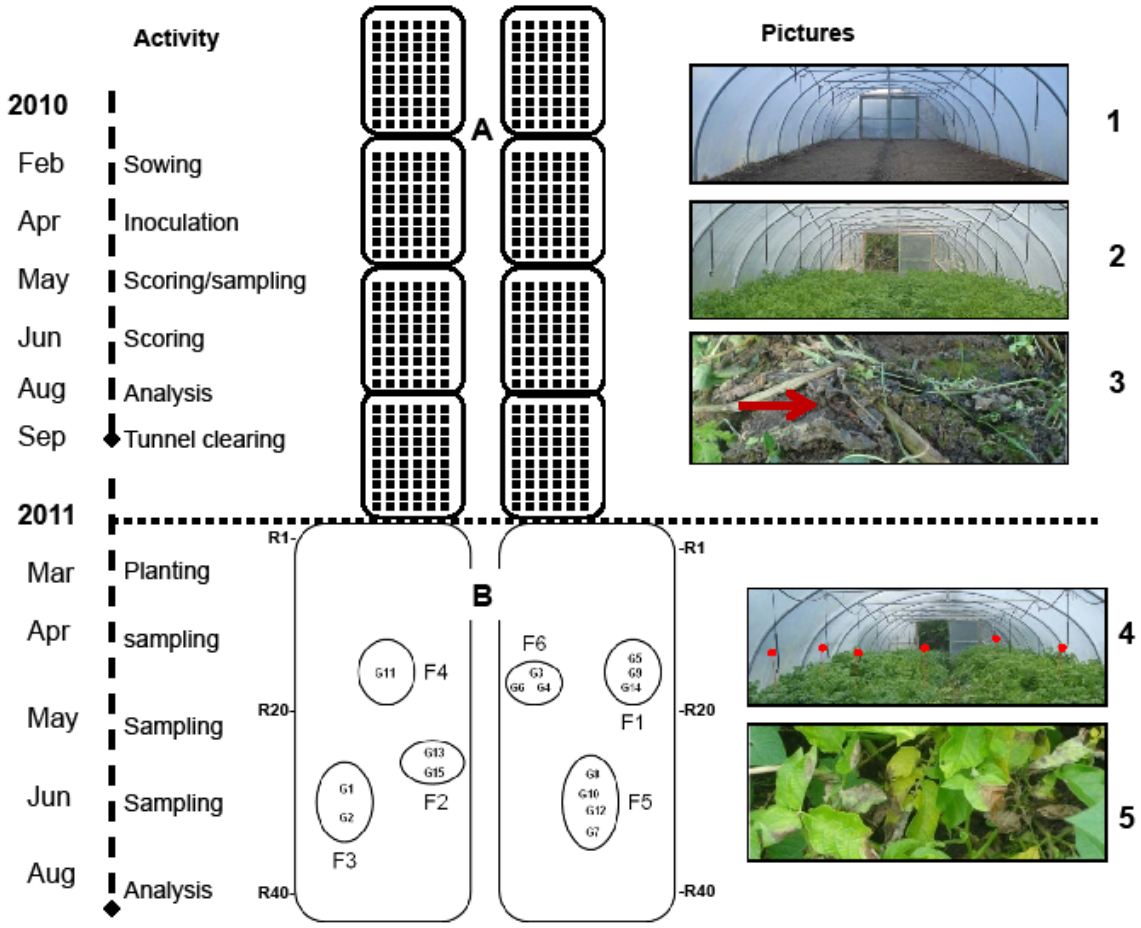


Fig. 3.1 A schematic presentation of activities in the polytunnel. A: Sowing plan in the first season in the polytunnel where eight beds were prepared each having 10 rows of 6 tubers (each dot represents a tuber). In every bed, each of the four cultivars (Sarpo Mira, Bionica, Cara and Toluca) was sown to a single row while cultivar Maris Piper was sown to the six remaining rows resulting in an alternating pattern of cultivars by rows. B: primary foci of soil borne infection F1-F6 caused by *P. infestans* that led to blight outbreak in the second season potato crops of cultivars Cara and Maris Piper in which *P. infestans* genotypes G1-G15 were detected. Pictures 1, Polytunnel prepared for planting (Feb 2010); 2, polytunnel with potato crop (Apr 2010); 3, red arrow indicates rotting leaves of blight infected plants in contact with the soil in the polytunnel (Aug 2010); 4, Red dotted stakes indicating initial blight infection (foci) on lower leaves of potato plants (May 2011); 5, blight lesions on potato leaves in the last phases of the epidemic on the second season's crop (Jun 2011).

The leaves and other debris were removed from each sporangial suspension by filtration. Aliquots were obtained from each volume to quantify the sporangia under the microscope with the aid of a haemocytometer. The suspensions were adjusted to 10,000 sporangia/ml and incubated at 4^{o}C for 2 hours to release zoospores. Inoculation was performed four weeks after tuber planting by applying a sporangial/zoospore suspension of CW1-3C_A2 followed immediately by DL4-3B_A1 using a hand held sprayer to deliver ~70ml of inoculum per plant at a final concentration of 10,000 sporangial/ml.

3.2.7 Foliar blight assessment under polytunnel conditions and isolate genotyping

Foliar blight symptoms were observed 4 days after inoculation (13th Apr 2010) and scoring for disease severity was recorded at, 10, 15, 21 and 27 days post inoculation using a scale of 0-100%; where 0% was no visible infection observed; and 100% indicated that all leaves on the plant as well as stem were infected leading to defoliation or death of the plant.

To ascertain the *P. infestans* genotypes present in the polytunnel during the subsequent epidemic, twenty five leaf samples with single lesions were collected across the polytunnel. Leaves were placed in 9 cm diameter Petri dishes lined with moist Whatmann filter paper and transported to the laboratory in Oak Park where they were incubated for up to 48h at 18^{o}C to induce sporulation. Pure cultures of the isolates were obtained by transferring sporangia from sporulating leaflets onto pea agar media (1.5% agar) amended with rifampicin (50mg/L). Genotypic characterization of isolates was performed using simple sequence repeat (SSR) markers as described in section 3.2.14.

3.2.8. Assessing *P. infestans* oospore production under polytunnel conditions

Forty leaves with multiple, yet independent, blight lesions were collected from each cultivar (n = 5) in the polytunnel. The collected samples were placed in Petri dishes lined with moist tissue paper and incubated at 18^{o}C for 7-10 days to encourage the lesions to merge. Two to three leaf discs of 8mm diameter

were excised from the region of each leaflet where the lesions met and these discs were incubated further by floating them on sterile distilled water for a further 7 days. This was followed by clearing the chlorophyll from the leaf discs by immersion in 5ml of clearing agent (96% ethanol, 100% glacial acetic acid 3:1 v/v) in 50ml Falcon tubes at room temperature for 24 hrs. The cleared leaf discs were blotted dry and mounted on a glass slide in 80% glycerol before observing for the presence/absence of oospores under the microscope. Data were recorded based on the proportion of leaf samples per cultivar possessing oospores. Additionally, the density of oospore production was estimated for each cultivar by obtaining the mean oospore index of 16 leaf discs of the respective cultivar using the scale 0-13 developed by Turkensteen *et al*., (2000) where 0=0 oospores present, 1=1-5 oospores present, 2=6-10 oospores present, 3=11-50 oospores present, 4=51-100 oospores present, 5=101-500 oospores present, 6=501-1000 oospores present, 7=1001-2000 oospores present, 8=2001-3000 oospores present, 9=3001-4000 oospores present, 10=4001-5000 oospores present, 11=5001-10,000 oospores present, 12=10,000-15,000 oospores present, and 13=>15,000 oospores present. Illustrative images of oospores residing *in planta* were taken using a digital camera Zeiss Axio Cam HRm mounted onto a Zeiss-Axioskop 2 Plus microscope and operated by the AxioVision 4.8 computer programme (Carl Zeiss Light Microscopy, Germany).

3.2.9 Assessment of *P. infestans* oospore survival and infectivity over winter under polytunnel conditions

To maximize the potential for oospore entry into the soil, the blighted foliage of the first crop (planted in February 2010) was left to rot down onto the soil (see image 3 Fig. 3.1). By use of a shovel to turn the soil, potatoes were manually removed row by row and placed into buckets and later into pit dumps for destruction. This way, the polytunnel was cleared of all the potato tubers then left fallow through the winter and re-planted with just two cultivars, Maris Piper and Cara on 1st March 2011. Prior to planting, weeds were cleared from the polytunnel and the soil turned via rotovation to incorporate the rotted leaves

from the previous crop. The polytunnel was then prepared into two beds each with 40 ridges on either side of the walkway (Fig. 3.1). Six tubers of cultivars Maris Piper or Cara were planted in alternating ridges of each bed. Wetness in the polytunnel was maintained by misting as in the previous crop. The crop was monitored for the appearance of natural blight infection on low lying leaves on a weekly basis commencing on 19th April 2011.

Due to logistical challenges involved in completing sampling trips between Oak Park, Carlow and Henfaes, Bangor which typically took 3 days per trip, only one sampling exercise could be performed per week. This comprised of travelling to the experimental site on the first day, sampling on the second day and a trip back to Oak Park Carlow on the third day to transfer isolates into pure culture. Staff of Sarvari Research Trust based on site assisted with regular monitoring of the experiment and liaison to plan sampling trips. These challenges imposed an obvious limit on the intensity of the sampling that could be performed but sampling was achieved for a period of nine weeks from first observation of blight lesions in the polytunnel.

During sampling, infected leaves were stored in Petri dishes as before and returned to Oak Park for analysis with pure cultures of recovered isolates used in the determination of mating type, metalaxyl resistance and genotyping using simple sequence repeats (SSR) markers. At the end of the experiment (July 2011) and in order to determine the dominant genotypes (via SSR) in the polytunnel after the observed epidemic, ninety six random leaf samples were collected across the polytunnel once the entire crop was infected with blight. In total, 147 samples were obtained from the 2011 polytunnel crop. These included 51 as potential *F*1 progeny derived from oospore-based inoculum and 96 which were taken at the peak of the epidemic to determine the dominant genotype within the polytunnel (Appendix 3.1). Contaminations during isolation of potential *F*1 led to the loss of some isolates, leaving 38 of the 51 going forward for mating type, metalaxyl response as well as mtDNA haplotyping and SSR profiling.

3.2.10 *P. infestans* oospore baiting from soil samples

In November 2010, soil samples were obtained from the polytunnel in an attempt to quantify the degree of oospore release into the soil from rotting leaves of the first crop. Eight samples (250g) of soil were collected beneath decomposed leaves at eight locations across the polytunnel. The samples were combined to form a composite sample which was transported to the laboratory at the Teagasc Crops Research Centre.

The soil was mixed thoroughly and divided into 2 x 1kg sub-samples. For one sub-sample the soil was separated into four portions of 250g. One was designated a control and autoclaved at 121^{o}C for 15 min, one was frozen at -20^{o}C for 24hrs and thawed at room temperature to destroy any sporangia or hyphae in the sample (Wangsomboondee and Ristaino, 2002). The other two portions were left unmodified. Each portion of soil including the control sample was dispensed in a separate ½ litre plastic pot and planted with 10-15 seeds of tomato cultivar Money Maker and maintained in the glasshouse at 18-20^{o}C under natural lighting and regular watering using sterile water (Fig. 3.2). Germination of seeds occurred 5 days after planting and the seedlings in each pot were monitored for blight infection on the stems for four weeks before the plants were discarded.

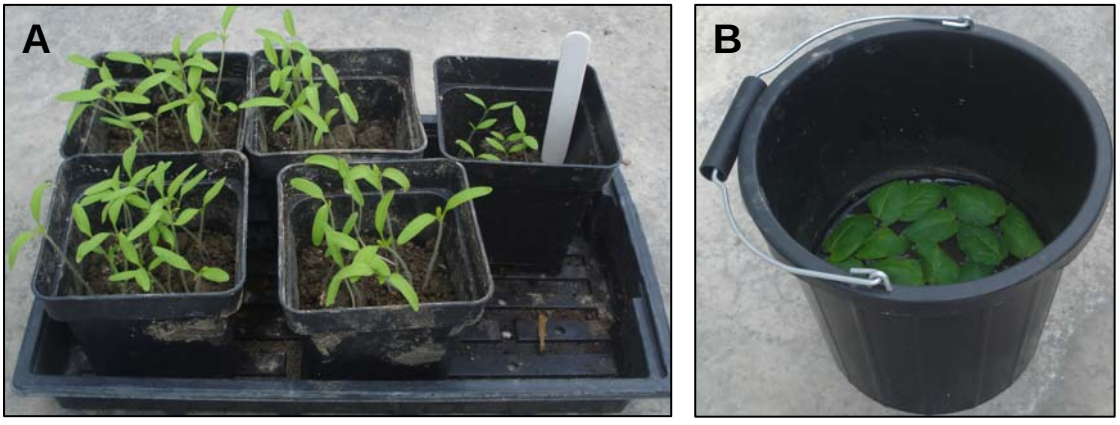


Fig. 3.2: Oospore baiting from polytunnel soil by A: using seedlings of tomato cultivar Money Maker; B: floating leaflets of susceptible potato cultivar Bintje on a water suspension of each polytunnel soil sample (Drenth *et al*., 1995).

The second method to bait oospores was based on a procedure described in the literature (Drenth *et al*., 1995) which also saw four soil sub-samples prepared as described above. However, in this case each sub-sample was placed in a separate 10 litre plastic bucket to which 3 litres of sterile water was added followed by stirring to create a turbid suspension. The samples were left to stand to allow heavier particles to settle at the base of the bucket while lighter ones including infectious propagules remained in suspension. Detached leaflets of potato cultivar Bintje were spread on the entire surface of the suspension with the lower side down. The buckets were placed on the bench in the glasshouse which was maintained at 18-20^{o}C with normal lighting. The leaflets were checked daily for blight lesions. After 7 days the individual leaflets were removed and placed in separate Petri dishes lined with moist Whatman paper, cover lid were placed over. The Petri dishes with leaflets were enclosed in clear polythene bags to maintain humidity and placed incubators maintained at 18^{o}C for a further 7 days and observed for blight symptoms. Meanwhile, the buckets received a new set of leaflets each week to replace those which were collected for further incubation in Petri dishes. Oospore baiting ceased four weeks after setting up the experiment (Fig. 3.2).

3.2.11 *P. infestans* mating type, metalaxyl sensitivity determination

The mating type of isolates collected from *in vitro* crosses and the polytunnel was determined by plating each isolate (n = 83) against known A1 and A2 isolates on un-amended carrot agar (Erselius and Shaw, 1982) and recording the presence/absence of oospores after two weeks incubation at 18^{o}C with a 16 h photoperiod (Knapova and Gisi, 2002). To test for metalaxyl sensitivity, the floating leaf disc procedure (Cooke, 1981) described in section 2.2.3 was performed in which an initial test was completed on each isolate at metalaxyl concentrations of 0, 5 or 100 mg/L (Fig. 3.3). To further elucidate the 'intermediate' phenotype, isolates from both *in vitro* crosses and the polytunnel identified to have intermediate insensitivity to metalaxyl during the initial screening were subjected to further testing against 0 mg/L, 5 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L of metalaxyl. As in the initial metalaxyl test, parental

isolates CW1-3C_A2 and DL4-3B_A1 were retained as reference isolates (Fig. 3.4).

3.2.12 Mitochondrial haplotyping of *P. infestans* F1 progeny derived from *in vitro*, *in vivo* conditions

Mitochondrial DNA (mtDNA) haplotyping of *P. infestans* isolates obtained from the *in vitro* crosses and the polytunnel was completed as per the method of Griffith and Shaw (1998). DNA was extracted from one-week-old mycelium grown on un-amended pea agar using the method of Raeder and Broda (1985). DNA quality and concentration was estimated by comparison with a known quantity of phage *λ* DNA (Promega, Wisconsin, USA) on 1% agarose gels in 0.5× TBE buffer stained with ethidium bromide (0.5 µg/ml) and visualized using a Kodak Imager (Image Station 440 CF, Kodak Digital Science ^{TM}, USA).

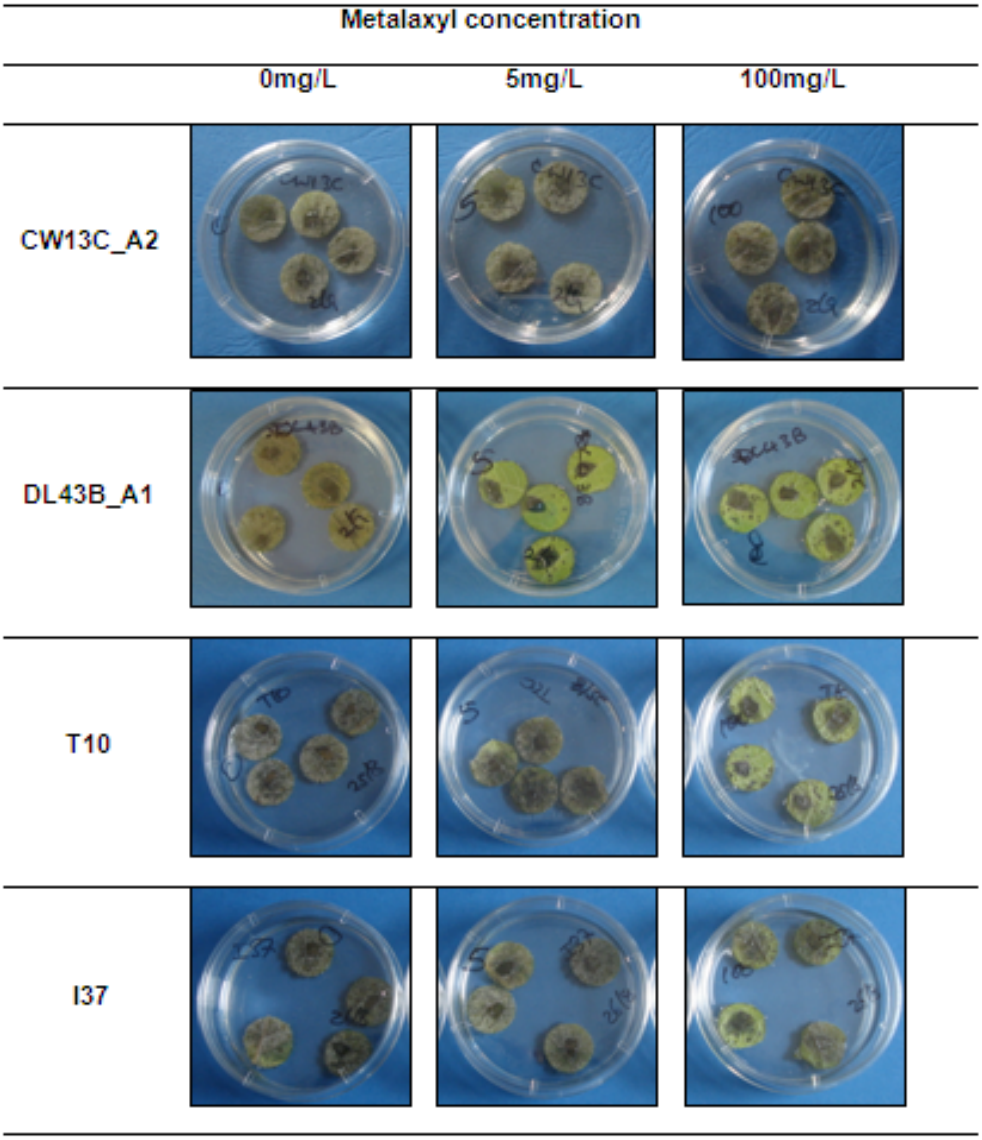


Fig. 3.3: Assessment of the putative *F1* progeny for metalaxyl sensitivity in comparison to resistant and sensitive parental isolates, CW1-3C_A2 and DL4-3B_A1 respectively (first two rows). Sensitive isolates sporulated on leaf discs floated on water with no sporulation on leaf discs floated on 5mg/L or 100mg/L metalaxyl. Isolates with intermediate response to metalaxyl (i.e. isolates T10 and I37; bottom rows) sporulated on leaf discs floated on water and 5mg/L but not 100mg/L metalaxyl and isolates with resistance to metalaxyl sporulated on leaf discs floated on water, as well as 5mg/L and 100mg/L metalaxyl.

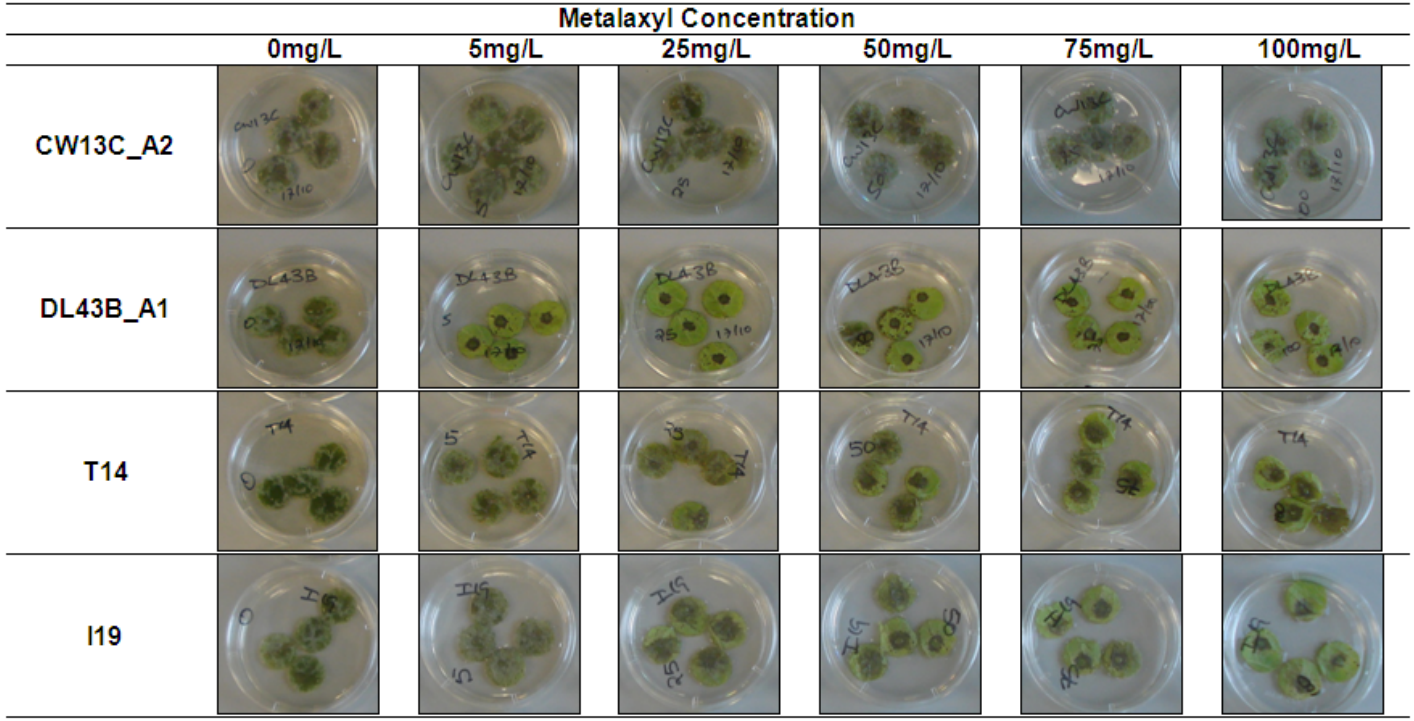


Fig. 3.4: Assessment of the putative *F1* progeny for intermediate metalaxyl sensitivity in comparison to resistant and sensitive parental isolates, CW1-3C_A2 and DL4-3B_A1 respectively (first two rows). Sensitive isolates sporulated on leaf discs floated on water with no sporulation on leaf discs floated on 5mg/L or 100mg/L metalaxyl. Isolates with intermediate response to metalaxyl (i.e. T14, I19; bottom two rows) sporulated on leaf discs floated on water and 5mg/L, 25mg/L and 50mg/L but not 100mg/L metalaxyl and isolates with resistance to metalaxyl sporulated on leaf discs floated on water, as well as 5mg/L and 100mg/L metalaxyl.

Polymorphic regions of mitochondrial DNA were PCR amplified using P2 pair of oligonucleotide primers (forward 5'-TTC CCT TTG TCC ACC GAT-3'; reverse 5'-TTA CGC CGC TTT AGC ACA TAC A-3') with an expected product size of 1,070 bp (Fig. 3.5; Griffith and Shaw 1998). The primers were synthesized by Metabion International AG. PCR were performed in 20ul reaction mixtures comprising of 200 µM dNTP, 0.325 µM primers, 1 × buffer (20mM Tris–HCl (pH 8.4), 50 mM KCl), 1U *Taq* DNA polymerase (New England Biolabs) and 10 ng genomic DNA, with the final volume made up using molecular grade Sigma water. Cycling conditions were 94^{o}C for 90 s followed by 35 cycles of 94^{o}C for 40 s; 60^{o}C for 60 s and 72 ◦C for 90 s.

A 10 µl aliquot of each amplified DNA was digested with 1U of *Msp*I in a 30 µl final volume at 37^{o}C in a water bath for 60 min. Restriction patterns were revealed after electrophoresis of the digested DNA through a 1% agarose gel in a 1×TBE buffer stained with ethidium bromide (0.5 µg/ml) and bands were visualized and the image captured using a Kodak Imager (Image Station 440 CF, Kodak Digital Science^{TM}, USA). A 100 bp DNA ladder (Promega, Wisconsin, USA) was used as a size marker. The DNA haplotype was assigned by determining the molecular weight of the individual restriction fragments of each PCR-RFLP profile (by comparison with the molecular size markers) and referring to the published literature (Griffith and Shaw, 1998).

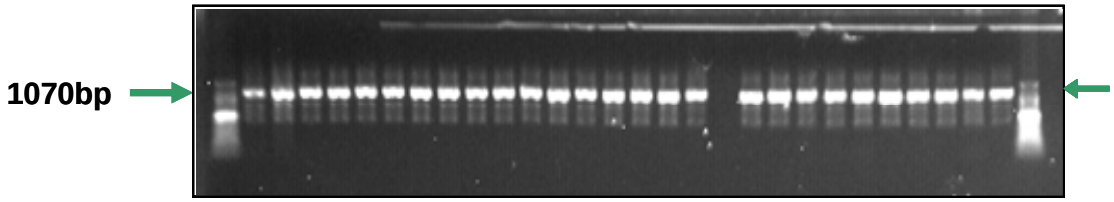


Fig. 3.5: A gel image showing PCR amplicons of the expected size (1,070bp), as indicated by the green arrows, after amplification of *P. infestans* mtDNA with primer pair P2 (Griffith and Shaw, 1998). 100bp ladder is in the far left and far right lanes.

3.2.13 Genotype analysis of *P. infestans* F1 progeny from *in vitro* crosses and polytunnel sampling

Mycelia were harvested from ten day old pure cultures of *P. infestans F*1 isolates from *in vitro* crosses and the polytunnel. DNA was extracted from the samples as per Raeder and Broda (1985) followed by genotypic analysis using a set of 11 SSR markers (used in chapter two) following the procedure described in section 2.2.2. SSR markers used were Pi02, Pi33, Pi70, Pi56, Pi89, G11, Pi04, Pi16 and Pi63 (Lees *et al*., 2006) along with D13 and Pi4B (Knapova and Gisi, 2002) (marker details in Appendix 2.3) and were synthesized by Applied Biosystems (UK). SSR size data obtained were used to determine multilocus genotypes among the tested isolates.

3.2.14 Assessment of *P. infestans* F1 isolates for aggressiveness on detached potato leaflets

Twelve F1 isolates were assessed for aggressiveness on detached leaflets of cultivar Cara in comparison with parental isolates DL4-3B_A1 and CW1-3C_A2 as controls. Due to time constraints, only a small number of isolates was assessed. The isolates were selected to represent the diversity in genotypes identified. The procedure described in section 2.2.4 was used to obtain inoculum of each isolate. Prior to inoculation, six replicated plastic trays lined with moist paper towel were prepared each containing twelve leaflets. Each leaflet was an experimental unit. The experiment was repeated once. In each experiment, inoculation of isolates onto detached leaflets and incubation of inoculated leaflets was performed according to the procedure described in section 2.2.4.1. The parameters of aggressiveness that were recorded were latent period (period from inoculation to initial sporulation) in days and area under lesion progress curve (AULPC) (the cumulative totals of sporulating lesions estimated daily for 7 days according to Miller *et al*., (1998)).

3.3 Data Analysis

3.3.1 Foliar blight assessment and oospore density in leaf tissue

Relative area under disease progress curves (rAUDPCs) was computed for individual cultivars from percent disease severity values using the following equation:

$$RAUDPC = \frac{\sum_{i=0}^{final}(T_i - T_{i-1})(P_{i-1}) + \frac{(T_i - T_{i-1})(P_i - P_{i-1})}{2}}{(T_{final} - T_0)(100)}$$

in which T0 was the day of inoculation, T_i is the ith day after inoculation when an estimation of percentage foliar late blight was made, T_{final} the number of days after inoculation at which time the most susceptible genotype had reached 100% defoliation and P_i was the estimated percentage foliar late blight at T_i. (Stein and Kirk, 2002).

The quantity oospores produced by mating isolates (CW1-3C_A1 and DL4-3B_A1) simultaneously inoculated onto the five potato cultivars (Sarpo Mira, Bionica, Toluca, Cara and Maris Piper) was determined by microscopically visualizing chlorophyll free leaf discs extracted from leaflets of each cultivar. An oospore index was determined for each of the sixteen leaf discs per cultivar.

Oospore index and rAUDPC values were subjected to analysis of variance using the GenStat statistical program and where significant differences were detected, means separated using Fisher's Least significant differences (LSD) test.

3.3.2 Genetic analysis

Multilocus groups were identified by assessing isolates for allelic similarity across all loci. Moreover, standard analyses including number of alleles at each polymorphic locus, polymorphic information content (PIC) at each locus, observed and expected heterozygosities (H_o and H_e) and deviation from the Hardy-Weinberg equilibrium (HWE) were calculated using CREVUS 3.0 software (Kalinowski *et al*., 2007; Marshall *et al*., 1998).

Calculation of observed and expected heterozygosities (H_o and H_e) was based on (Nei, 1973) as described in section 2.3.2.1. PIC was calculated according to formula:

$$PIC = \sum_{i=1}^{n} p_i^2 - 2\sum_{i=1}^{k-1}\sum_{j=i+1}^{k} p_i^2 p_j^2$$

where p_i is the frequency of the *i*th allele (Bolstein *et al*., 1980) as implemented in CERVUS 3.0 software.

3.3.3 Parentage analysis

Confirmation of parentage for progeny from the *in vitro* crosses was performed using the likelihood-based approach as implemented in the CERVUS 3.0 software. In the parental analysis, the CW1-3C_A2 was retained as the maternal parent while the DL4-3B_A1 parent together with a set of randomly selected isolates from the putative *F1* population were treated as candidate parents.

Allele frequencies were calculated based on a complete set of genotypes comprising the progeny and parents. Allele frequency data were used to run simulations for likelihood based parentage analysis. Assuming all loci segregate independently, CERVUS 3.0 assigns parentage to the offspring by calculating the combined likelihood score for each parental pair/offspring combination (Trio LOD). LOD score of zero implies the candidate parents are equally as likely parents as randomly selected individuals. A Positive LOD score implies the candidate parents are the most likely parents than randomly selected individuals. A Negative LOD may result in cases of where candidate parents and progeny share common alleles.

Statistical parentage inference was made from delta, which is the difference between the LOD of the most likely parental pair and the second most likely pair of parents with a minimum value of zero for the latter pair. Delta was calculated at 90% and 80% confidence corresponding to the strict and relaxed confidence levels respectively (Marshall *et al*., 1998).

3.4 Results

3.4.1 *In vitro* oospore production and germination

After four weeks of incubation oospore production was recorded in all of the completed *in vitro* crosses of CW1-3C_A2 and DL4-3B_A1. To determine the optimal conditions for selective digestion of mycelia residues with minimal deleterious effects on the harvested oospores prior to germination, two concentrations of the cellulase *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich) in the digestion mixture (v/v, cellulase to oospore suspension) and four digestion durations were assessed.

Mycelial digestion and the associated effect on oospores varied among the concentrations of cellulase included in the mixture and the digestion duration. The effect ranged from no mycelia digestion and damage to oospores to complete mycelial digestion with severe damage to oospores leading to leakage of oospore contents as illustrated in Fig. 3.6. Generally, digestion of residual mycelia improved with increased digestion duration at both concentrations of the cellulase tested (Table 3.1). However, disintegration of oospore walls resulted when the digestion mixtures at concentrations of 1:1 and 1:0.5 were incubated for 24 or 36 hrs and 36hrs respectively (Table 3.1). Digestion of oospore suspension-cellulase mixture at a concentration of 1:0.5 (v/v) for 24hrs gave complete mycelia digestion (5) with minimal damage to oospores (1.5) (Table 3.1). From these results, the optimum conditions for selective mycelial digestion were determined to be an incubation of a 1:0.5 (v:v) oospore suspension/cellulase mixture for 24hrs at room temperature.

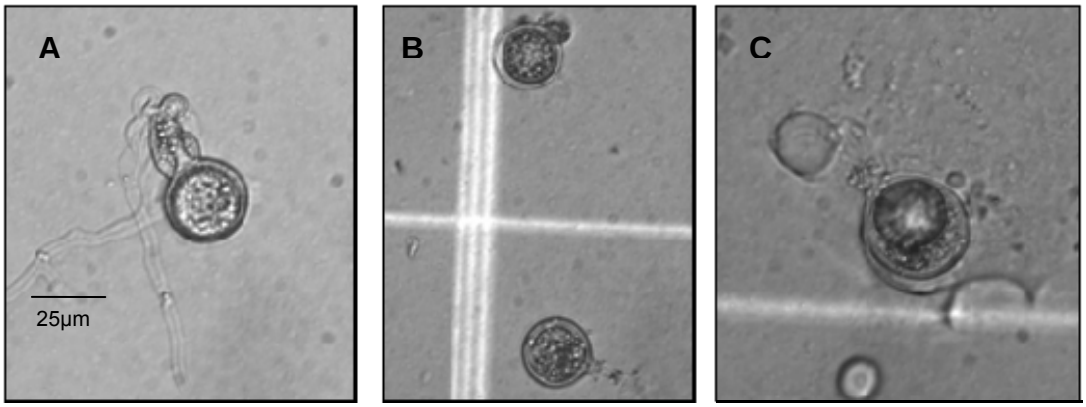


Fig. 3.6: Pre-germinated *P. infestans* oospores. A: oospore with attached undigested mycelia; B: oospores post 24hr mycelia digestion with *Trichoderma reesei* cellulose showing digested mycelia and oogonial walls; C: oospore with ruptured wall and cell leakage after 36hr digestion with *Trichoderma reesei* cellulase.

Table 3.1: Effect of cellulase *Trichoderma reesei* ATCC 26921 concentration and duration of digestion on mycelial disintegration and the integrity of *P. infestans* oospore walls

Suspension: cellulase (V/V)	Digestion duration	Mycelial digestion[a]	SE (±)	Damage to oospores[b]	SE (±)
1:1	3hr	1	0.00	1.00	0.00
	12h	3	0.41	2.75	0.25
	24h	5	0.00	4.00	0.00
	36h	5	0.00	4.75	0.25
1: 0.5	3h	1	0.00	1.00	0.00
	12h	3	0.41	1.00	0.00
	24h	5	0.00	1.50	0.29
	36h	5	0.00	4.50	0.29

[a]Values are means of three replicates based on scoring index of 1-5 where 1 is no evident digestion and 5 is complete digestion of mycelia. [b]Values are means of three replicates based on a scoring index of 1-5 where 1 is no damage to oospores and 5 is severe damage to oospores leading to spillage of contents. The best conditions for pre-germination treatment of oospores are highlighted in grey.

The germination of oospores on 0.5% water agar was observed 4 days after incubation at 18^{o}C for each completed cross of CW1-3C_A2 and DL4-3B_A1. The percentage of germinating oospores was generally low and differed among individual crosses varying from 3.94% to 7.31% with a mean of 5.15% (Table 3.2).

Germinating oospores generally formed germ tubes which extended and divided forming sporangia at the tip of each branch (Fig. 3.7). Attempts to isolate germinating oospores individually by picking using a pin head and transferring them into rye agar (Caten and Jinks, 1968) with 50mg/L rifampicin were unsuccessful due to handling difficulties and persistent contamination. This was subsequently solved by overlaying the water agar plate with rye agar and incubating the 'sandwich' plates for a further 4 -7 days at 18^{o}C to promote growth of germinated oospores.

Germinated oospores emerged from the sandwich plate as minute yet distinct colonies of mycelial growth (Fig. 3.7). Agar plugs with individual colonies were transferred using a sterile needle onto rye agar plates and incubated at 18^{o}C. The number of recovered colonies differed among crosses varying from 2.50 to 3.38 with a mean of 2.98 (Table 3.2). A total of 45 pure cultures were subsequently obtained as a result of single zoospore isolation. The observation of oospore production from these *in vitro* crosses of CW1-3C_A2 and DL4-3B_A1 was presumed to be a positive indicator of sexual compatibility between these two isolates of *P. infestans*.

Table 3.2: Mean number of germinating *P. infestans* oospores and colonies recovered from *in vitro* CW1-3C_A2 x DL4-3B_A1 crosses

CW1-3C_A2 x DL4-3B_A1	Oospore germination[a] per cross (%)	SE (±)[b]	Colonies recovered[c] per cross	SE (±)[d]
Expt 1	3.94	0.28	2.50	0.34
Expt 2	7.31	1.09	3.38	0.13
Expt 3	4.19	0.28	3.06	0.12
[e]Mean/SE	5.15	1.09	2.98	0.26

[a]Mean oospore germination (%) from eight replicate crosses in each experiment (approximately 100 oospores were plated on each replicate plate per cross between isolates CW1-3C_A2 and DL4-3B_A1).

[b]Standard Error on mean oospore germination (%) from eight replicate plates.

[c]Mean of colonies recovered from eight replicate crosses between isolates CW1-3C_A2 and DL4-3B_A1. [d]Standard errors on means of colonies recovered from eight replicate crosses between isolates CW1-3C_A2 and DL4-3B_A1.

[e]Mean values and standard errors on means of oospore germination (%) and number of colonies recovered across experiments.

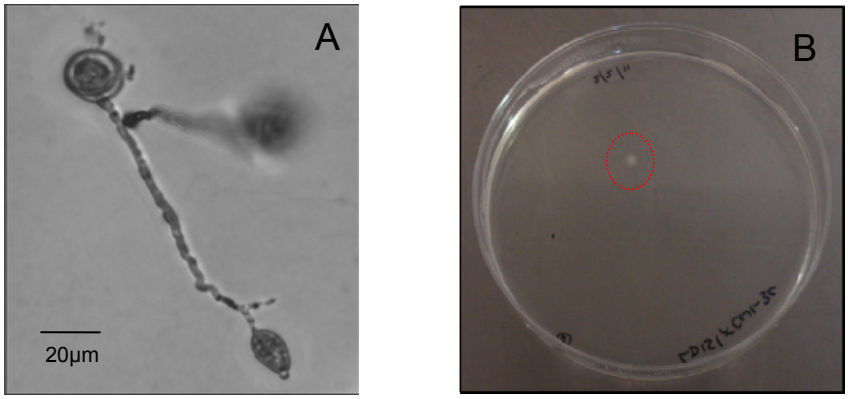


Fig. 3.7: *In vitro* germination of *P. infestans* oospores on 0.5% water agar. A: a germinating oospore with branching hyphae and sporangiospore; B: emerging colony formed by a germinated oospore on a 'sandwich' plate

3.4.2 Phenotypic characterization of *in vitro* isolates

Mating type tests (Fig. 3.8) on 45 progeny derived from *in vitro* crosses between CW1-3C_A2 and DL4-3B_A1 generated 26 (57.8%) A1 and 19 (42%) A2 progeny (Fig. 3.8) indicating an A1:A2 ratio of 1.38: 1.00 and therefore a slight excess of A1 mating types.

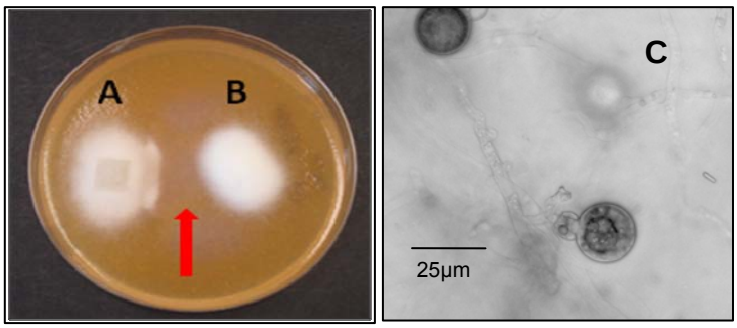


Fig. 3. 8: Mating type test was performed by pairing of each sample isolate with A1 and A2 tester isolates in two separate plates on carrot agar. **A**: tester isolate with known mating type; **B**: sample isolate of unknown mating type. Arrow shows the zone of interaction between paired isolates where their mycelia met; **C**: interaction between opposite mating types leads to production of oospores along the interface.

The frequency of metalaxyl resistant isolates in the *in vitro* progeny was higher among the A2 (22.2%) compared to the A1 (2.2%) mating types. Conversely, all metalaxyl sensitive isolates (26.7%) were of the A1 mating type. The remaining 48.9% (22 isolates) had intermediate metalaxyl responses. Of these intermediates, 59.1% (13 isolates) were A1 mating types and 40.9% were A2 mating types (9 isolates). Accordingly, the distribution of isolates according to metalaxyl phenotypes showed a 1:2:1 ratio for resistant: intermediate: sensitive (Fig. 3.9).

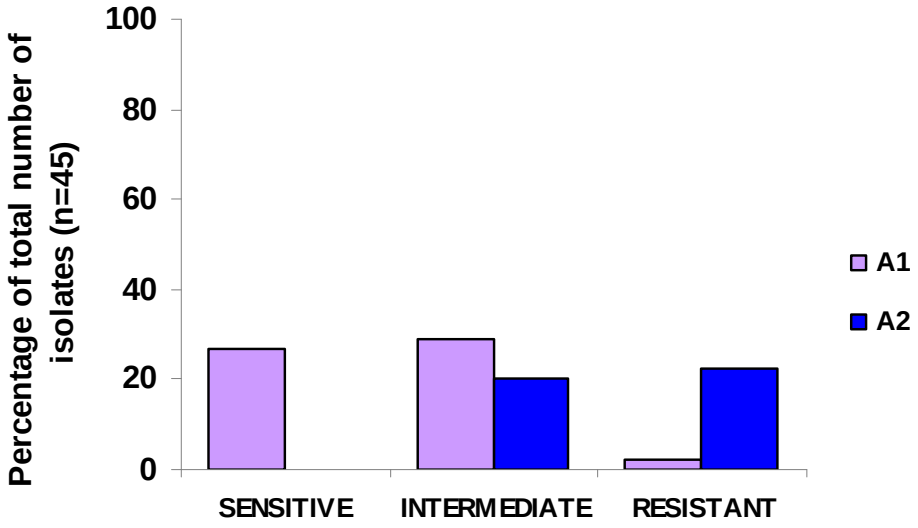


Fig. 3. 9: Percentage of F1 progeny derived from *in vitro* crosses between CW1-3C_A2 and DL4-3B_A1 isolates with sensitive, intermediate or resistant phenotypes relative to their respective A1, A2 mating types.

A more intensive screening of the putative recombinants (n=22) which initially displayed intermediate insensitivity to metalaxyl revealed insights into their limits of tolerance to metalaxyl. The tolerance ranged from 5mg/L to 75mg/L and a larger majority of the isolates (n=14), irrespective of mating type, could withstand metalaxyl up to 5mg/L in suspension only (Fig. 3.10). The frequency of isolates with tolerance to higher dosages of metalaxyl declined until none with tolerance to 100mg/L was detected as expected (Fig. 3.10). Four isolates

(1 of A1 and 3 of A2 mating types) tolerated 25mg/L. A similar number (4) of isolates with similar representation by mating type was had a tolerance limit of 50mg/L. However, only two isolates both of A2 mating type tolerated metalaxyl at 75mg/L (Fig. 3.10).

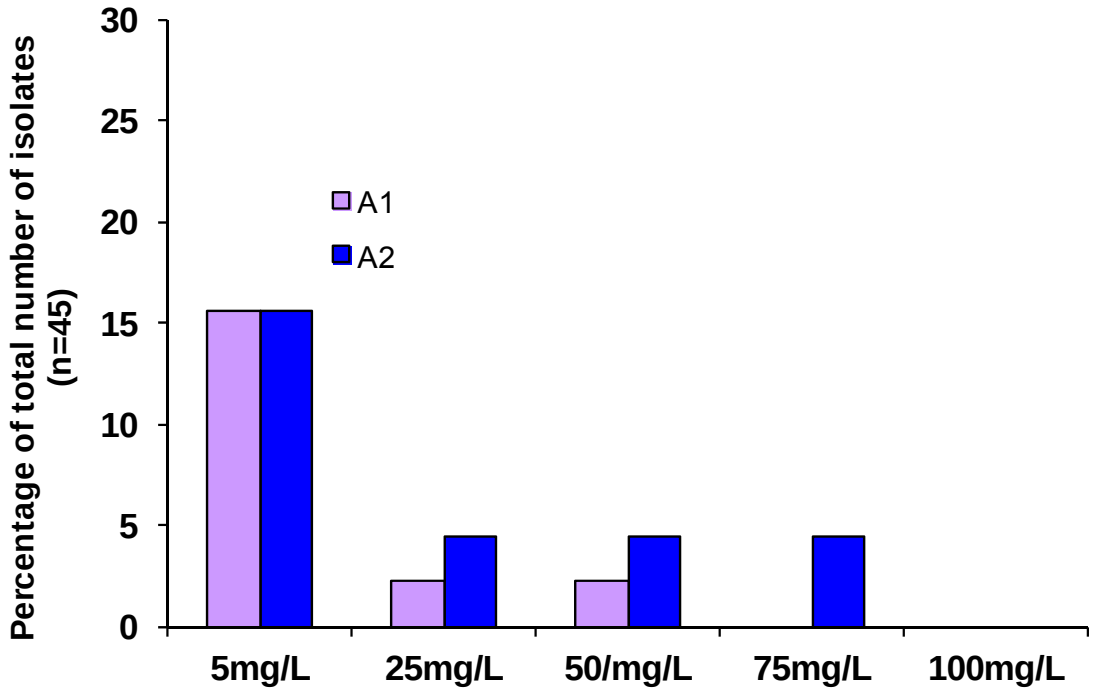


Fig. 3.10: Distribution of the degree of metalaxyl fungicide resistance for those *in vitro F*1 recombinants previously classified as 'intermediate' (Fig. 3.9).

3.4.3 SSR genotyping of *in vitro* isolates of *P. infestans*

Each of the 45 *in vitro F*1 progeny had a unique SSR profile based on 11 markers (Table 3.3). At least one out of two alleles at each of the loci except Pi89 was common to both parental isolates. However, a majority of the progeny showed recombination at each locus. The exception was locus Pi 33 which had a single allele that was common to both parents leading as expected to a homozygous state in all progeny at this marker. Variation in the *F*1 isolates was observed at loci Pi04, Pi4B Pi02 and Pi 89 as shown by their high PIC values, 0.60, 0.55, 0.54 and 0.53 respectively (Table 3.4).

With the least PIC value (0.12) among the eight loci included in the standard molecular analyses (loci Pi33, and Pi 70 were omitted because they were not

sufficiently polymorphic and their inclusion could have skewed the analysis) the *in vitro F*1 progeny displayed the least variation at locus Pi16.

With respect to locus D13, out of 45 *in vitro* isolates analyzed, 14 had a 136, 154 allelic profile which is diagnostic for the 13_A2 genotype ('blue 13' phenotype).

Table 3.3: Mating types, metalaxyl phenotypes and allele sizes (bp) of putative *in vitro* F1 isolates of *P. infestans* from mating of isolates CW13C_A2 and DL43B_A1 as revealed by 11 SSR markers. The last column to the extreme right shows the number of isolates (frequency) per SSR genotype detected, 0= allele absence.

SSR Loci			Pi02	Pi02	Pi02	Pi04	Pi04	Pi4B	Pi4B	D13	D13	Pi16	Pi16	G11	G11	Pi56	Pi56	Pi63	Pi63	Pi70	Pi70	Pi33	Pi33	Pi89	Pi89	Pi89	Freq
Parent	Mating type	Metalaxyl																									
DL43B_A1	A1	S	152	160	162	166	170	213	217	118	136	178	178	160		176	176	151	157	192	195	203	203	181	197	0	
CW13C_A2	A2	R	160	162	0	166	170	205	213	136	154	176	178	154	160	176	176	151	157	192	192	203	203	179	179	0	
*F*1 isolate																											
I45	A1	I	152	160	0	166	170	213	213	136	136	178	178	154	160	176	176	151	157	192	195	203	203	179	181	0	1
I9	A1	I	152	160	162	160	170	213	217	136	136	178	178	154	160	176	176	151	157	192	195	203	203	179	181	0	1
I73	A1	S	152	152	0	170		213	217	136	136	178	178	160	160	176	176	151	157	192	195	203	203	179	181	0	1
I76	A1	S	152	160	162	160	166	205	213	136	136	176	178	160	160	174	176	157	157	192	192	203	203	179	181	0	1
I20	A1	S	152	160	162	166	170	213	217	0	0	178	178	160	160	176	176	151	157	192	195	203	203	181	197	0	2
I72	A1	I	152	160	0	170		205	213	136	136	178	178	154	160	174	176	151	157	192	195	203	203	179	181	0	1
IM4	A1	I	152	160	162	160	166	213	217	0	0	178	178	154	160	174	176	151	157	192	195	203	203	179	181	0	1
IM5	A1	I	152	160	162	160	166	205	213	136	154	178	178	154	160	176	176	151	157	192	195	203	203	179	181	0	1
I23	A1	I	152	160	0	170		205	213	136	136	178	178	154	154	0	176	151	157	192	192	203	203	179	181	0	1
I69	A1	R	152	160	162	166	170	205	213	136	154	178	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I22	A1	I	152	160	0	166	170	205	213	136	136	178	178	154	160	174	176	157	157	192	192	203	203	179	179	0	1
I67	A1	I	152	160	162	166	170	213	217	0	0	178	178	160	160	176	176	151	157	192	195	203	203	181	181	0	1
I49	A1	I	152	162	0	160	166	213	217	0	0	178	178	160	160	176	176	151	157	192	195	203	203	181	181	0	1
I1	A2	R	160	162	0	160	166	205	213	136	154	178	178	154	160	176	176	151	157	192	192	203	203	181	181	0	1
I63	A2	R	160	162	0	166	170	205	213	136	154	178	178	154	160	176	176	151	157	192	192	203	203	181	181	0	1
I71	A2	I	160	162	0	160	166	205	213	136	154	178	178	154	160	174	176	151	157	192	192	203	203	181	181	0	1
I50	A1	I	160	162	0	160	166	205	213	136	136	176	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I29	A2	I	160	162	0	160	166	205	213	136	154	0	178	154	160	176	176	151	157	195	195	203	203	179	179	0	1
I37	A2	I	160	162	0	166	170	205	213	136	136	178	178	154	160	174	176	151	157	192	192	203	203	179	179	0	1
I28	A2	R	160	162	0	160	166	205	213	136	154	178	178	154	160	174	176	151	157	192	192	203	203	179	179	0	1
I13	A2	I	160	162	0	166	170	205	213	136	136	178	178	154	160	174	176	151	151	192	192	203	203	179	179	0	1
I8	A2	R	160	160	0	160	166	205	213	136	154	178	178	154	160	174	176	151	157	195	195	203	203	179	179	0	1
I18	A2	I	160	162	0	166	170	205	213	136	136	176	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I3	A2	R	160	162	0	166	170	205	213	136	154	178	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I36	A2	R	160	162	0	166	170	205	213	136	136	178	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I39	A2	R	160	162	0	166	170	205	213	136	136	178	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I41	A1	I	160	162	0	166	170	205	213	0	0	0	0	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I44	A1	I	160	160	0	166	170	205	213	136	136	176	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I56	A2	R	160	162	0	166	170	205	213	136	154	178	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I57	A2	R	160	162	0	166	170	205	213	136	154	178	178	0	0	176	176	151	157	192	192	203	203	179	179	0	1
IM3	A2	I	160	162	0	160	166	205	213	136	136	176	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
I70	A2	I	160	162	0	166	170	205	213	136	154	178	178	154	154	176	176	157	157	192	195	203	203	179	181	0	1
I21	A1	S	160	160	0	160	166	205	213	154	154	178	178	154	160	174	176	151	157	192	195	203	203	179	181	0	1
I4	A1	S	160	162	0	166	170	205	213	136	136	178	178	160	160	176	176	151	157	192	195	203	203	179	197	0	1
I51	A1	S	160	162	0	160	166	213	217	136	136	178	178	160	160	176	176	151	157	192	195	203	203	179	197	0	1
I48	A1	S	160	162	0	166	170	205	217	136	136	178	178	154	160	174	176	151	157	192	192	203	203	179	197	0	1
I35	A1	S	160	162	0	166	170	213	217	0	0	178	178	160	160	176	176	151	157	192	195	203	203	181	197		1
I62	A1	S	160	162	0	160	166	213	213	0	0	178	178	160	160	176	176	151	157	192	195	203	203	181	197	0	1
I74	A1	S	160	160	0	166	170	205	205	154	0	178	178	160	160	176	176	157	157	192	192	203	203	181	181	0	1
I15	A1	S	160	160	0	166	170	205	213	0	0	178	178	160	160	176	176	0	0	192	195	203	203	181	181	0	1
I66	A1	S	160	162	0	168	0	213	213	136	136	178	178	154	160	176	176	157	157	192	192	203	203	179	181	197	1
I53	A1	S	160	160	0	168	0	213	213	136	136	178	178	160	160	0	0	151	157	192	195	203	203	179	181	197	1
I6	A2	I	162	162	0	166	170	205	213	136	136	178	178	160	160	176	176	151	157	192	195	203	203	179	179	0	1
I24	A2	R	162	162	0	166	170	205	213	136	154	178	178	154	160	176	176	151	157	192	192	203	203	179	179	0	1
IM1	A2	I	162	162	0	160	166	205	213	136	154	178	178	154	154	176	176	157	157	192	195	203	203	179	181	0	1

The other common profile at this locus was a 136,136 allele combination observed in 21 individuals. Allele 118, present in the parental isolate DL43B_A1, was not detected in any of the progeny whereas 154, 154 allele combination was detected in one progeny (Table 3.3).

Twenty six *in vitro F*1 progeny had a 154/160 allele combination at locus G11 which is also diagnostic of blue 13. The over representation of this allele combination in the progeny was presumed to be due to the presence in the DL4-3B_A1 isolate of the 160 allele at this locus. This allele was shared with CW1-3C_A2 which had a 154/160 allele combination at this locus (Table 3.3).

Only two (Pi04 and Pi4B) loci showed significant deviation (p<0.001*)* from the Hardy-Weinberg equilibrium as estimated by the Chi-Square distribution of allele frequency in the software CERVUS. The remaining markers showed either no significant deviation or the test could not be performed because the number of individuals carrying the locus was below the threshold needed for a successful run of the software. However a deduction for a non-clonal population can be premised on the observation that none of the eight loci analyzed showed heterozygozity deficits (Table 3.4).

The SSR profiles of seven (I35, I20, I18, I44, IM3, I69) of the 45 *in vitro* isolates revealed an allele contribution from just one of the two parents in the mating pair, a clear deviation from the pattern of allele inheritance expected from gene recombination (Table 3.3). The seven isolates showed affinity to either CW1-3C_A2 or DL4-3B_A1 indicating that each was capable of selfing. Additionally, aberration in inheritance was noted in some of the progenies that showed allele contribution from both parents at some of the loci while at other loci both alleles were inherited from just a single parent (i.e. I29 at Pi70; I70 at Pi63; I21 at D13) (Table 3.3). The latter form of non-Mendelian inheritance was presumed to result from non-disjunction at the affected loci during meiosis (Shaw and Shattock, 1991).

Parentage analysis data showed positive LOD values for six progeny parent pair combinations with 95% delta confidence confirming that the progeny were

recombinants of the CW1-3C and DL4-3B parental pair (Table 3.5). Similarity between the parental isolates and progeny at some loci due to low marker polymorphism, as was the case in this study, compromised the resolution power of CERVUS 3.0 software in parentage analysis. Furthermore, most population analysis softwares including CERVUS 3.0 software have been developed for handling data from diploid organisms as a consequence of this, triallelic individuals had to be excluded from the analysis.

Table 3. 4: Allele frequency, expected and observed heterozygozities (H_o, H_e), polymorphic information content (PIC) at each SSR locus and Chi-square test for Hardy-Weinberg equilibrium in the *in vitro* F1 population from the *in vitro* sexual cross between CW1-3C_A2 and DL4-3B_A1 isolates of *P. infestans*

		[a]Statistics for HWE test				
Locus	**K**	**N**	H_o	H_e	**PIC**	**Chi**	**[b]P-value**
Pi02	3	53	0.75	0.62	0.54	1.14	n.s
Pi04	6	52	1	0.67	0.60	32.10	***
Pi4B	3	53	0.91	0.63	0.55	12.46	***
D13	3	44	0.41	0.42	0.35	n.d	n.d
Pi16	2	51	0.14	0.13	0.12	n.d	n.d
Pi89	3	52	0.71	0.68	0.53	2.47	n.s
G11	3	52	0.62	0.49	0.51	3.51	n.s
Pi63	2	51	0.86	0.50	0.37	25.12	***
Mean number of allele per locus	3.14						
Mean number of loci	7.0						
Mean expected heterozygozity	0.49						

[a]K, number of alleles at a locus, N, number of individuals typed; Heterozygosities (H_o, H_e), PIC, Polymorphic information content of individual loci. [b]n.d, not done; n.s no significant departure from HWE, ***P<0.001;

Seven triallelic progeny two (I66 and I53) at locus Pi 89 (alleles 179 181 and 197) (Table 3.4;) and five (I9, I76, I20, IM4, IM5) at locus Pi 02 (alleles 152, 160, 162) (Table 3.4) were detected using SSR markers suggesting that one of the *P. infestans* isolates in the mating pair had a chromosome that by chance carried an extra allele at these loci. The occurrence of triallelic progeny in the crosses (Fig. 3.11 & 3.12) indicates the remarkable capacity of *P. infestans* to overcome chromosomal imbalance during sexual reproduction to give rise to viable oospores.

3.4.4 Mitochondrial haplotyping of putative *P. infestans* F1 progeny derived from *in vitro* crosses

Analysis of the mitochondrial DNA of isolates from the *in vitro* crosses between CW1-3C_A2 (Ia) and DL4-3B_A1 (Ib) revealed two mtDNA haplotypes, Ia and Ib (Fig. 3.13). In five of the isolates, PCR reactions repeatedly failed to produce any product. Restriction digestion of amplicons of each of the remaining 40 isolates showed 28 belonged to the mtDNA haplotype Ia while 12 isolates had Ib mtDNA haplotype. All isolates with mtDNA haplotype Ib were of the A1 mating type but mtDNA haplotype Ia possessed isolates of either mating type. Of the 28 isolates with haplotype Ia, 20 were mating type A1 and 8 were mating type A2.

Table 3. 5: Parentage analysis using the software CERVUS of the *F*1 *in vitro* isolates of *P. infestans*

Offspring ID	First Parent	Second Parent	Trio Loci compared	Trio mismatch	Trio LOD score[a]	Trio Delta	Trio confidence[b]
I15	CW1-3C_A2	DL4-3B_A1	5	0	4.39e-01	4.39e-01	*
I20	CW1-3C_A2	DL4-3B_A1	6	0	3.04e+00	3.04e+00	*
I35	CW1-3C_A2	DL4-3B_A1	6	0	1.36e+00	1.36e+00	*
I46	CW1-3C_A2	DL4-3B_A1	5	0	3.04e+00	3.04e+00	*
I67	CW1-3C_A2	DL4-3B_A1	6	0	3.04e+00	3.04e+00	*
I72	CW1-3C_A2	DL4-3B_A1	6	0	3.04e+00	3.04e+00	*

[a]Only values with positive Trio LOD were included in the table; [b]Delta significant at 95% confidence (Marshall *et al*., 1998).

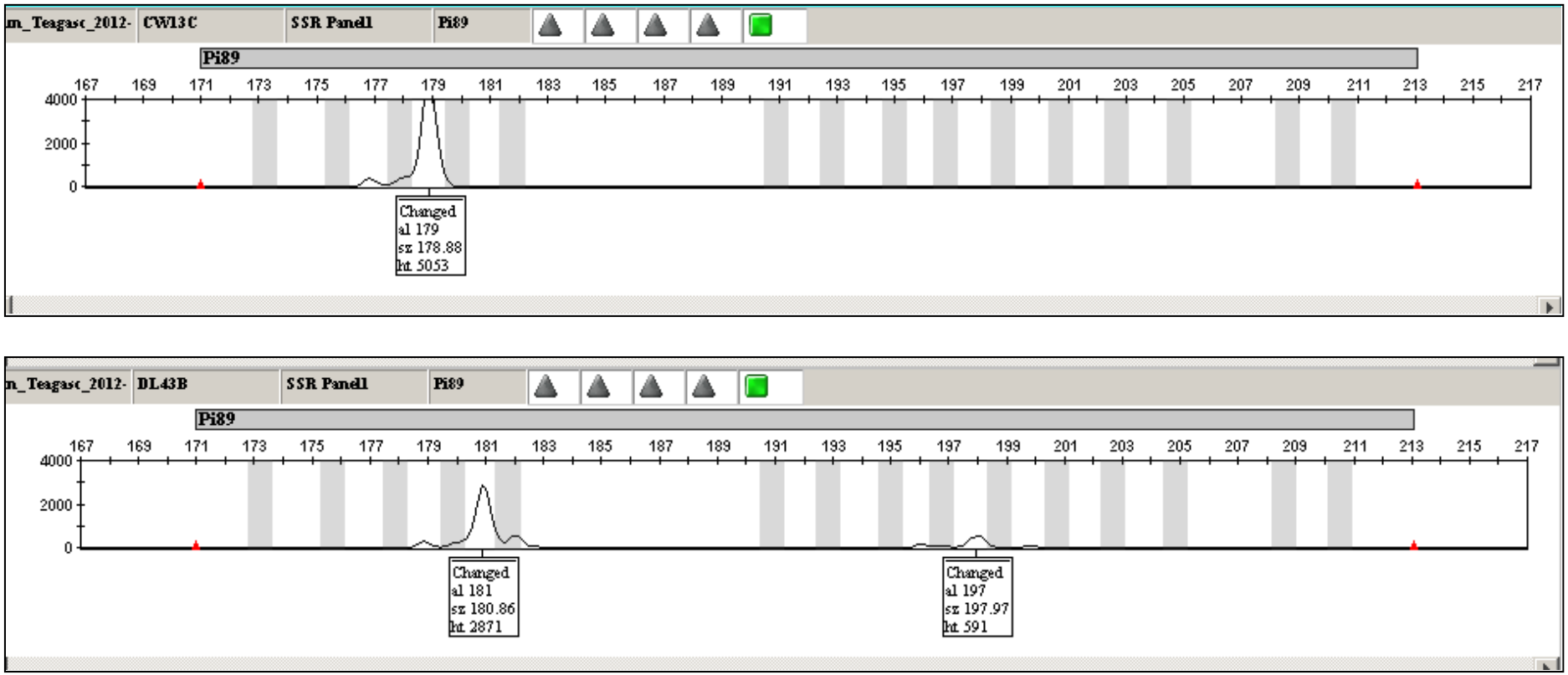


Fig. 3.11: Electropherograms of SSR locus Pi89 as visualized using GeneMapper software showing alleles of CW1-3C_A2 and DL4-3B_A1 parental isolates of *P. infestans*

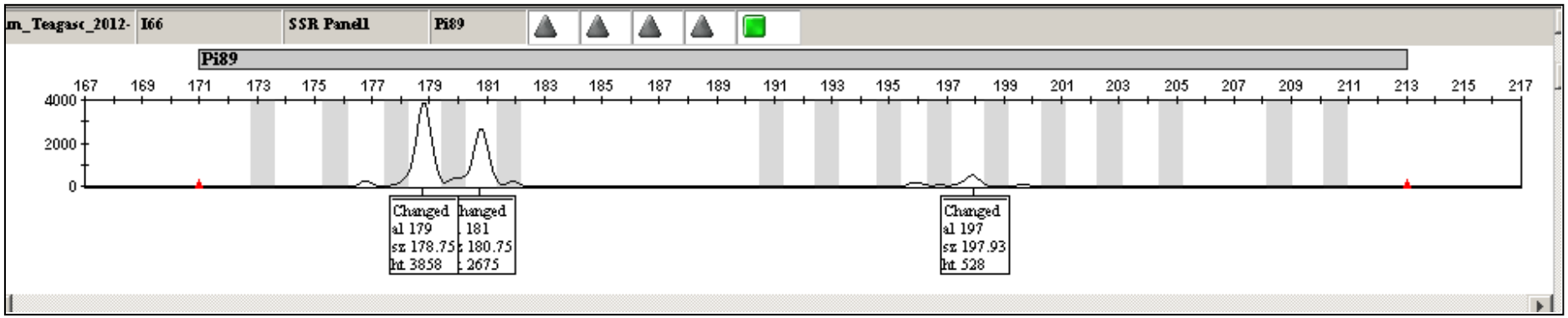


Fig. 3.12: An electropherogram of SSR locus Pi89 as visualized using GeneMapper software showing recombination of alleles in the *F1* triallelic progeny I66 resulting from a cross between CW1-3C_A2 and DL4-3B_A1 isolates of *P. infestans* (Fig. 3.10 above).

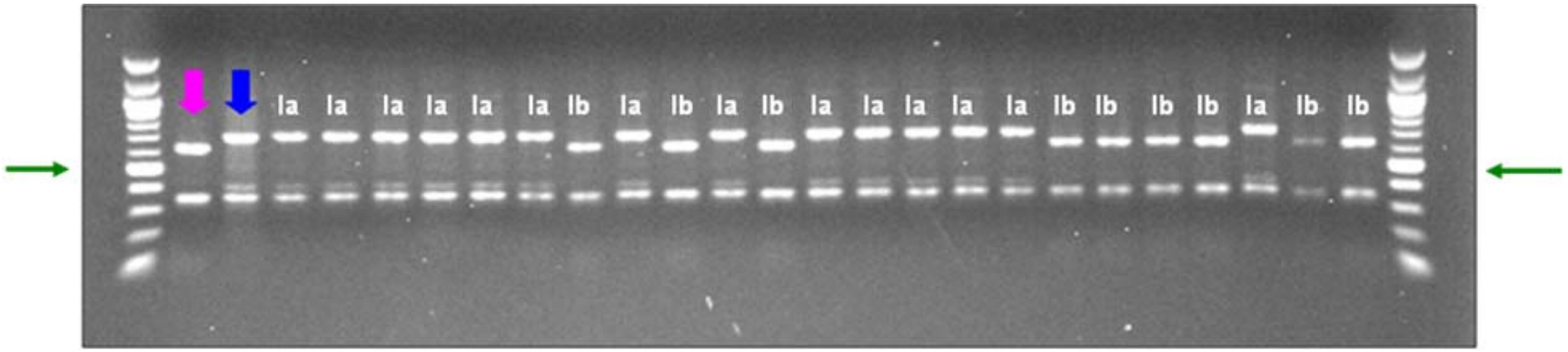


Fig. 3.13: Mitochondrial haplotypes of *F*1 recombinants of *P. infestans* as revealed by *Msp*I restriction of PCR amplicons of the P2 primer pair on mtDNA (Griffith and Shaw, 1998). Green arrows indicate 500bp on the ladder; bold purple and blue arrows indicate parental isolates DL4-3B_A1 and CW1-3C_A2 respectively while lanes 4-26 from left to right are *F*1 progeny showing mtDNA haplotypes Ia or Ib.

3.4.5 Assessment of foliar blight on first cycle crop in the polytunnel

Table 3.6 provides a summary of sampling events for blighted leaflets with single lesions from the two potato crops sown in the polytunnel in 2010 and 2011 for phenotypic and genotypic characterization. Five cultivars grown in the polytunnel in 2010 were compared for response to foliar blight caused by simultaneous inoculation with isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans*.

Overall, there were significant differences ($P<0.001$) among the five cultivars as regards foliar blight progression. Less disease was observed on cultivar Sarpo Mira as indicated by its lowest value of area under disease progresses curve (AUDPC) of (118.4). On the contrary, cultivar Maris Piper had the highest AUDPC value (1222.8) (Table 3.7), followed by Cara (652), Toluca (166.9) and Bionica (123.1).

Notably however, foliar blight progression did not differ significantly on the three resistant cultivars Sarpo Mira, Bionica and Toluca (Table 3.7). Expression of area under lesion progressive curve relative to the length of the epidemic (rAUDPC) largely showed the same trend (Table 3.7). Disease assessment ceased when disease incidence on the most susceptible cultivar Maris Piper reached 100% with complete defoliation. This was four weeks after first observation of blight symptoms on the crop.

Table 3.6: Summary of sampling exercise on the potato crops sown in the polytunnel during the investigation of *P. infestans* oospores biology *in planta* at Henfaes Research Centre, Bangor in 2010 and 2011.

Period	Crop	Phase	Description of sampling	Samples analyzed	SSR genotypes
2010	**1st Crop**	[a]Pre-inoculation	Section 3.2.7	3	Table 3.9
		[b]Post inoculation	Section 3.2.8	12	Table 3.10
2011	**2nd Crop**	[c]Primary infection loci	Section 3.2.10	38	Table 3.13
		[d]Late epidemic	Section 3.2.10	96	Table 3.14

[a]Isolates were collected on the first crop in the polytunnel prior to inoculation with the parental isolates

[b]Single lesion leaf samples collected during blight epidemic on the first crop post inoculation with parental isolates DL4-3B_A1 and CW1-3C_A2 for SSR confirmation of gentoytpes

[c]Single lesion leaf samples collected at primary blight infection loci on the second crop and in subsequent sampling of the polytunnel before the epidemic spread on the entire crop

[d]single lesion leaf samples collected from the crop in later stages of the epidemic

Table 3.7: Area Under Disease Progress Curve (AUDPC) of blight infected foliage of five potato cultivars (Sarpo Mira, Bionica, Toluca, Cara, Maris Piper) sown in the polytunnel and inoculated with a mixture of CW1-3C_A2 and DL4-3B_A1 isolates of *P. infestans*

Cultivar	Mean AUDPC[a]	Mean rAUDPC[b]
Sarpo Mira	118.4a	0.08a
Bionica	123.1a	0.09a
Toluca	166.9a	0.12a
Cara	652.5b	0.47b
Maris Piper	1222.8c	0.87c
Cultivar (P<0.001)		
LSD (P=0.05)	58.31	0.04

[a]AUDPC values followed by the same letter in the same column do not differ significantly at P=0.05; [b]rAUDPC generated according to (Stein and Kirk, 2002); values of rAUDPC followed by same letter not significantly different at P=0.05.

3.4.6 *P. infestans* oospore production in leaves of five potato cultivars grown in polytunnel

To determine oospore production in leaves of plants inoculated with CW1-3C_A2 and DL4-3B_A1, forty leaflets exhibiting multiple blight lesions were assessed from each cultivar. Absence or presence of oospores and their abundance was determined microscopically on leaf discs extracted and cleared of chlorophyll (Fig. 3.14 & 3.15).

The number of lesions per leaflets differed significantly (P<0.001) among cultivars ranging from 2.2 on cultivar Sarpo Mira to (3.5) on cultivars Bionica and Toluca. None of the examined leaf discs from cultivars Bionica and Toluca showed oospore production. Oospore production was observed in a proportion of sampled leaves from each of the remaining three cultivars. Out of forty leaves

in each case, cultivars Sarpo Mira, Maris Piper and Cara had 8, 26 and 32 leaves respectively showing oospore production (Table 3.8).

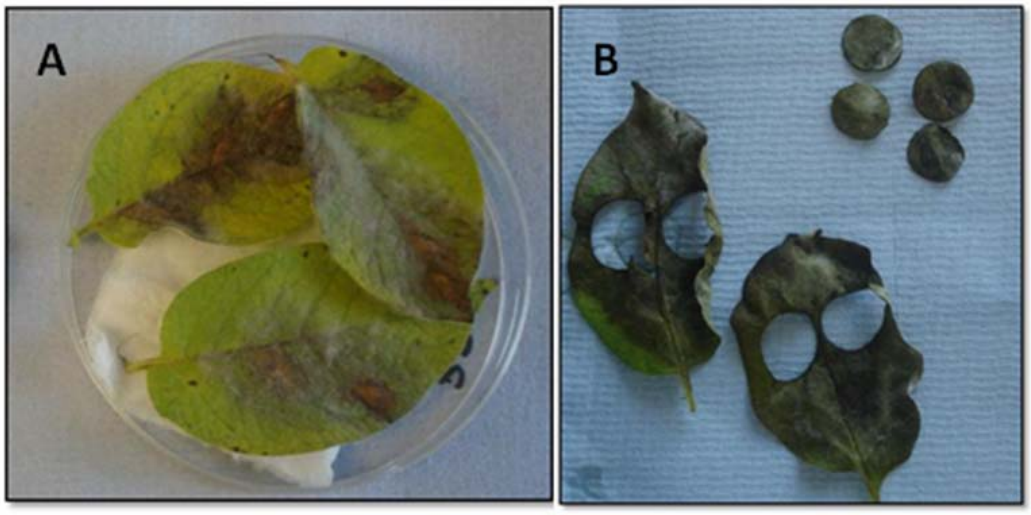


Fig. 3.14: Leaf samples from polytunnel first cycle potato crop inoculated with parental isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans*. **A**: incubation of leaflets with multiple lesions to induce oospore production in a Petri dish with moistened filter paper; **B**: leaf discs obtained from zone of contact between lesions after 7 days incubation.

The quantity of oospores produced in infected leaves as indicated by the oospore index differed significantly (P=0.001) among cultivars. With the exception of cultivars Bionica and Toluca on which no oospore production was detected, the fewest oospores were produced on cultivar Sarpo Mira 1.1. In contrast, large quantities of oospores were produced on cultivars Cara and Maris Piper as indicated by their elevated oospore indices 4.5 and 4.6 respectively (Table 3.8) suggesting an association between host resistance/susceptibility to blight and *in planta* oospore production.

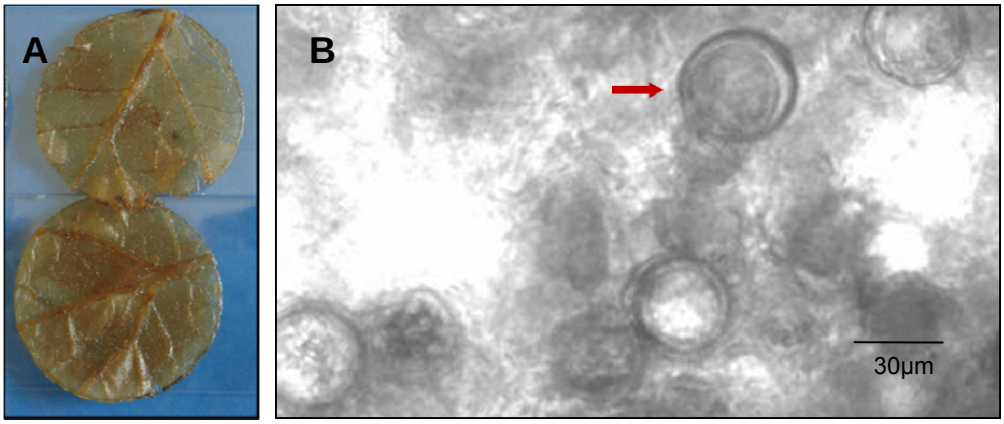


Fig. 3.15: Verification of *P. infestans* oospore production in leaf-samples obtained from the polytunnel after simultaneous inoculation of potato with CW1-3C_A2 and DL4-3B_A1 isolates **A**: leaf discs obtained from junction between lesions on a leaflet and soaked in ethanol-acetic acid to extract chlorophyll; **B**: microscopic observation of oospores in cleared leaf tissues (200X). Arrow points at an oospore.

3.4.7 Oospore baiting from the soil obtained from the polytunnel after 2010 crop

Potato leaflets floated on a polytunnel soil-wash suspension of the un-autoclaved sample began showing necrotic lesions, especially on the leaf edge, after four days of incubation. To confirm whether the lesions were caused by *P. infestans*, the leaflets were incubated in Petri dishes lined with moist paper towels at 18^{o}C and observed daily for sporulation with the aid of a stereo microscope. Additionally, portions of the leaf tissues from the edge of the necrotic lesions were introduced into cut tubers of potato cultivar Kerr's Pink to induce sporulation. Both efforts failed to yield any evidence of *P. infestans* and this was also the case for the baiting approach using germinating seeds of tomato cultivar Money Maker.

Table 3.8: *Assessment of oospores in discs (n=16) obtained from leaflets with multiple lesions sampled from five cultivars (Sarpo Mira, Bionica, Toluca, Cara and Maris Peer) of the polytunnel first cycle crop following inoculation with a mixture of isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans*

Cultivar	No. of leaflets with oospores[a]	No. of lesions per leaflet[b]	Oospore index[c]
Sarpo Mira	8	2.2	1.1
Bionica	0	3.5	0.0
Toluca	0	3.5	0.0
Cara	32	2.5	4.5
Maris Piper	26	2.4	4.6
Cultivar		P<0.001	P<0.001
LSD (P=0.05)		0.33	0.24

[a]number of leaflets with oospores (out of maximum 40 leaflets sampled per cultivar); [b]mean number of discrete lesions per leaflet per cultivar; [c]oospore index determined from mean of sixteen leaf discs per cultivar (Index based on Turkensteen *et al.,* 2000)

3.4.8 *P. infestans* oospore survival and infectivity under polytunnel conditions

Blight symptoms in the second crop of the polytunnel in 2011 were first observed on lower leaves of cv. Maris Piper at one primary infection focus seven weeks after planting (21st April 2011). In the course of the next four weeks, before the epidemic spread and covered the entire crop in the polytunnel, a total of six distinct infection foci were identified and marked with a red stake (Fig. 3.16). All six primary foci of blight infection identified in the tunnel started with blight lesions appearing on the lower leaves, which were touching the soil, possibly implicating soil borne propagules of *P. infestans* as the source of infection (Fig. 3.17).

Fig. 3.16: Polytunnel with the second cycle potato crop (cultivars Maris Piper and Cara). The crop was sown following a nine month fallow period (including a winter) after the first crop which was sown in February 2010 and on which the mating pair of *P. infestans* isolates (CW1-3C_A2 and DL4-3B_A1) was inoculated. Red sticks indicate local foci of blight infection on lower leaves.

Fig. 3.17: Lower leaf on a potato plant in the polytunnel with a sporulating blight lesion obtained at one of the natural blight outbreak foci in the 2011 potato crop.

3.4.9 Phenotypic characterization of putative *P. infestans* F1 isolates from the polytunnel

The phenotypic analysis of the polytunnel *F*1 isolates (n=38) collected from the blighted crop of 2011 resulted in obscure patterns of mating type distribution as well as metalaxyl response. For instance, an overwhelming majority of isolates (81.6%) were of the A1 mating type compared to 18.4% of the A2 mating type, indicating a 4:1 ratio in favour of mating type A1 (Fig. 3.18). Metalaxyl resistant isolates with an A1 mating type were not detected among the polytunnel isolates as were metalaxyl sensitive isolates with an A2 mating type (Fig.. 3.18). The seven (18.4%) metalaxyl resistant isolates detected were A2 mating types and all the seven metalaxyl sensitive isolates (18.4%) were of the A1 mating type. With respect to the incidence of isolates with intermediate response to metalaxyl possessing either mating types, there was concurrence in the results from analysis of both *F*1 *in vitro* progeny and polytunnel isolates. Again, the intermediates made up the majority of the polytunnel isolates (62.4%) (Fig. 3.18) but displayed a frequency distribution pattern tending towards a 1:4:1 ratio for resistance: intermediate: sensitive phenotypes for metalaxyl respectively.

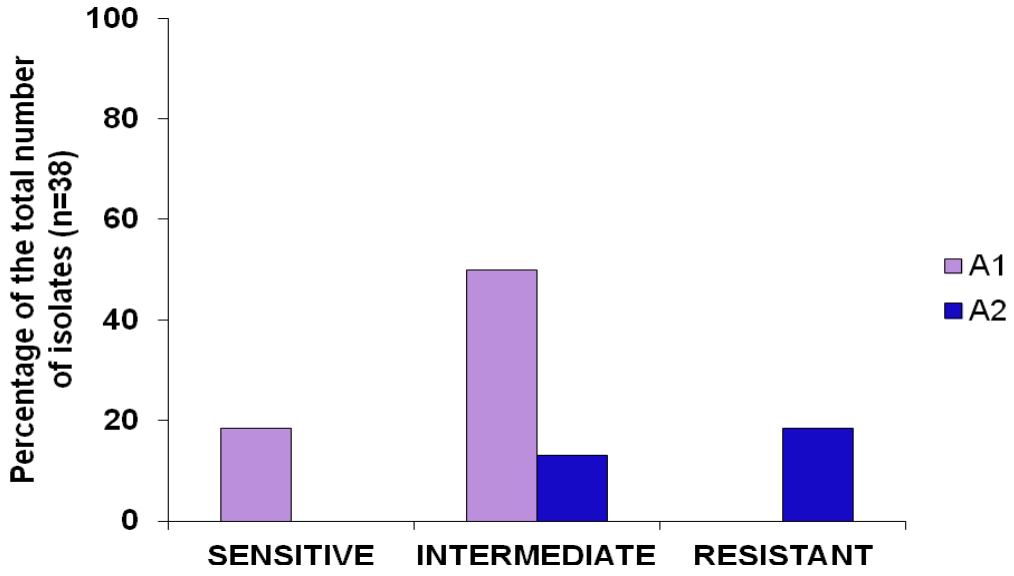


Fig. 3.18: Percentages of mating types with metalaxyl sensitive, intermediate or resistant phenotypes among the isolates from the polytunnel following pairing of CW1-3C_A2 and DL4-3B_A1 isolates of *P. infestans*

Twenty four isolates from the polytunnel which previously showed intermediate resistance to metalaxyl were screened more intensively. The frequency of isolates with intermediate response to metalaxyl declined steadily from a high of 15.8% and 10.5% for mating types A1 and A2 respectively at 5mg/L to only 0% and 2.63% for mating types A1 and A2 respectively at metalaxyl concentration of 100mg/L (Fig. 3.19). The test detected isolates with resistance to metalaxyl at 5mg/L (four A1; three A2), 25mg/L (two A1, one A2), and 50mg/L (two A1; one A2) (Fig. 3.19). Although isolates with intermediate resistance to metalaxyl were detected in each of the *P. infestans* genotypes identified in the polytunnel, isolates with higher tolerance limits of 75mg/L and 100mg/L (e.g. isolate T14; see Fig. 3.4) belonged to multilocus group G11 that later dominated the polytunnel (Table 3.9).

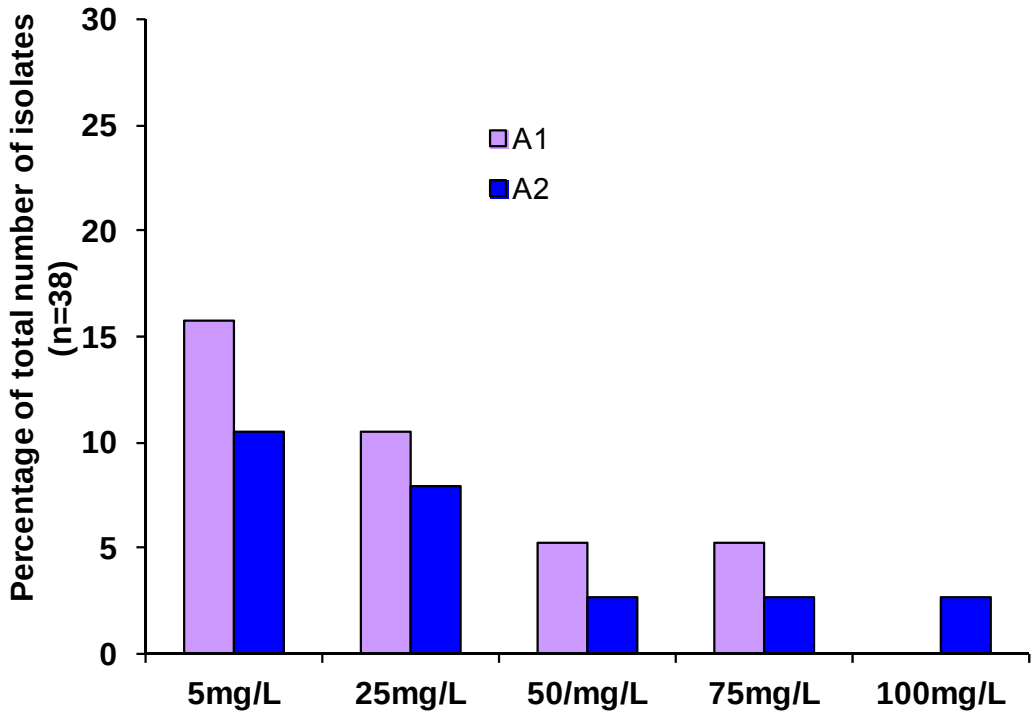


Fig. 3.19: Distribution of the degree of metalaxyl fungicide resistance for those polytunnel *P. infestans F*1 recombinants previously classified as intermediates (Fig. 3.18).

3.4.10 SSR genotyping of polytunnel derived isolates of *P. infestans*

The genotypic profiles of the isolates obtained from the polytunnel prior to the introduction of isolates DL4-3B_A1 and CW1-3C_A2 are shown in Table 3.9. Each of the three isolates was a distinct genotype but all lacked the 136, 154 allele combination at locus D13 which is diagnostic for 'blue 13' genotype and therefore differed from CW1-3C_A2 (blue 13). Also, none of these three isolates had a 181, 197 allele combination at locus Pi 89 nor 152, 160, 162 allele profile at locus Pi02 both which are typical of 'pink 6' the genotype of DL4-3B_A1. Notably, all three isolates had a 179, 179 allele profile at locus Pi89 and one had 154, 160 profile at locus G11 in addition, just like isolate CW1-3C_A2 (Table 3.9).

The genotypic profiles of isolates from blight infected crops of cultivars Cara and Maris Piper (2010) are shown in Table 3.10. SSR data revealed two genotypes which delineated with the SSR profiles of isolates DL4-3B_A1 and CW1-3C_A2 (Table 3.10) and confirmed that both isolates had successfully initiated disease on the potato crops following inoculation and therefore should be directly associated with the subsequent epidemic in the polytunnel.

Analysis of the 38 putative *F*1 isolates recovered from the second polytunnel crop (2011) which had been planted to bait oospores, revealed fifteen discrete multilocus genotypes (Table 3.11). Each of the fifteen genotypes was distinct not only from the two parental isolates (DL4-3B_A1 and CW1-3C_A2) introduced into the polytunnel, but also the three genotypes detected in the polytunnel prior to the inoculation of the first cycle crop (Table 3.9). Thirty of the 38 putative *F*1 isolates showed the 154, 160 allele combination at locus G11 revealing the same pattern of recombination observed among the *in vitro* putative *F*1 progeny. Results indicate allelic recombination occurred at the Pi89 locus which gave rise to a 179, 181 allele combination at this locus in 19 of the 38 putative *F*1 isolates with an allele size of 181bp presumably being contributed from parental isolate DL43B_A1.

Table 3. 9: *P. infestans* genotypes detected by SSR analysis with 10 markers of isolates from the first crop in the polytunnel grown in 2010. Samples were obtained from scouting for blight in polytunnel prior to inoculation of the crop with CW1-3C_A2 and DL4-3B_A1 isolates.

Pre-inoculation genotypes	Pi02		Pi04		Pi4B		D13		Pi16		G11		Pi33		Pi56		Pi63		Pi70		Pi89	
T4-1	160	162	nd**	nd	205	217	118	136	178	178	160	160	203	203	174	176	151	157	192	192	179	179
T4-2	160	162	nd	nd	205	217	126	126	178	178	154	160	203	203	176	176	157	157	192	192	179	179
T4-3	160	162	nd	nd	205	217	118	136	176	178	160	160	203	203	176	176	151	157	192	192	179	179

**nd: not determined

Table 3. 10: SSR profiles of *P. infestans* isolates from the polytunnel sampled 10 days after simultaneous inoculation of the potato crop with isolates DL4-3B_A1 (P1) and CW1-3C_A2 (P2) in 2010.

Isolate	Host	Pi02			Pi04		Pi4B		D13		Pi16		G11		Pi33		Pi56		Pi56		Pi70		Pi89	
DL43B_A1	P1	152	160	162	166	170	213	217	118	136	178	178	160		203	203	176	176	151	157	192	195	181	197
CW13C_A2	P2	160	162	0	166	170	205	213	136	154	176	178	154	160	203	203	176	176	151	157	192	192	179	179
CR1B	Cara	160	162	0	166	170	205	217	118	136	178	178	0	0	203	203	176	176	151	157	192	192	179	179
CR1H	Cara	160	162	0	166	170	205	213	136	154	176	178	154	160	203	203	176	176	151	157	192	192	179	179
CR4B	Cara	160	162	0	166	170	205	213	136	154	176	178	154	160	203	203	176	176	151	157	192	192	179	179
CR8A	Cara	160	162	0	160	170	205	213	136	154	176	178	154	160	203	203	176	176	151	157	192	192	179	179
CR8D	Cara	160	162	0	166	170	205	217	136	154	178	178	154	160	203	203	176	176	151	157	192	192	179	179
MP1C	Maris Piper	160	162	0	166	170	205	213	136	154	176	178	154	160	203	203	176	176	151	157	192	192	179	179
MP2A	Maris Piper	160	162	0	166	170	205	213	136	154	178	178	154	160	203	203	176	176	157	157	192	192	179	179
MP2B	Maris Piper	160	162	0	166	170	205	217	136	154	176	178	160	0	203	203	176	176	151	157	192	192	179	179
CR1F	Cara	152	160	162	166	170	213	217	118	136	178	178	160	0	203	203	176	176	151	157	192	195	181	197
CR8B	Cara	152	160	162	166	170	213	217	118	136	178	178	160	0	203	203	176	176	151	157	192	195	181	197
CR8C	Cara	152	160	162	166	170	213	217	118	136	178	178	160	0	203	203	176	176	151	157	192	195	181	197
CR1G	Cara	152	160	162	166	170	213	217	118	136	178	178	160	0	203	203	176	176	151	157	192	195	181	197

However, allele size 197bp also present in parental isolate DL43B_A1 was not detected in any of the isolates recovered from the polytunnel (Table 3.11).

The same observation was made for locus D13 at which 136/136 allele combination was present in all progeny with no detection of allele size 118bp or 154bp from parental isolates DL4-3B_A1 and CW1-3C_A2 respectively (Table 3.11). Similarly SSR analysis revealed no triallelic isolate among those recovered from the polytunnel (Table 3.11).

A comparison between SSR datasets of the present study and the previous one of 2006-2008 (Cooke *et al*., 2010; Cooke *et al*., 2008) conducted in the same polytunnel showed no genotypes with exactly the same genotype although parental isolates with a typical blue 13 genotype were present in both experiments. In both studies, however, recovered isolates had unique SSR profiles compared with the parental isolates. Similarly, most of the recovered isolates in both experiments had a strong tendency towards inheriting alleles from the blue 13 parent at the G11 locus.

Spatial distribution of the genotypes in the polytunnel revealed that although they were distinct, they essentially clustered at primary infection loci identified during sampling (Fig. 3.1). Most of the genotypes (11 out of 15) were isolated from cultivar Maris Piper (Table 3.11). Only one of the 15 genotypes (G11) comprised A2 isolates with metalaxyl resistance which had been recovered from primary infection focus F4 (Fig. 3.1). SSR analysis of isolates recovered from the polytunnel in the later stages of the epidemic revealed four distinct genotypes (Table 3.12). Of the four detected genotypes, one (1-E1) had dominated the polytunnel to occur at higher frequencies (n=52) in comparison with the other three (Table 3.12). Genotype 1-E1 had similar allele combinations to the CW1-3C_A2 parental isolate at loci Pi02, Pi4B and G11. Compared with the DL4-3B parental isolate, 1-E1 had similar allele combinations at loci D13, Pi16 and Pi70. Additionally just like both parental isolates, genotype 1-E1 had a 166, 170 allele combination at locus Pi04 but carried a 174 allele at locus Pi56 which was absent in either parental isolate. Genotype 1-E1 had a distinct profile 179, 181 at locus Pi89 which suggested contribution of alleles 179 and 181 from

CW1-3C_A2 and DL4-3B_A1 parental isolates respectively. Furthermore genotype (1-E1) also bore similarity with genotype G11 (Table 3.11) differing by a single allele at each of only 2 loci (Pi02 and G11). In general, compared with the *in vitro F*1 population, assessment of the polytunnel isolates for genetic diversity revealed less variation as indicated by the significant deviation from the HWE at four of the nine loci tested (Table 3.13).

Only two loci (Pi02 and Pi89) showed conformity to the HWE expectation of allele frequencies (Table 3.13).This difference in levels of genetic diversity between the *in vitro* and polytunnel isolates can be accounted for by the multiple clonal copies among genotypes recovered from the polytunnel (Table 3.11).

3.4.11 Mitochondrial haplotyping of *P. infestans* F1 progeny recovered from the polytunnel crops

Analysis of the mitochondrial DNA extracted from polytunnel recovered isolates showed each isolate belonged to mtDNA haplotype Ib (Fig. 3.20), with none of the 38 isolates analyzed belonging to mtDNA haplotype Ia.

Table 3.11: SSR genotypes of the mating pair of *P. infestans* isolates DL4-3B_A1 and CW1-3C_A2 and 38 putative F1 isolates sampled from primary infection loci in the second season (2011) polytunnel-crop sown after winter fallow. Genotypes are based on analyses using 11 SSR markers and are arbitrarily and uniquely numbered from G1 to G15 to accommodate the grouping of multiple isolates with similar alleles at every locus typed. The second last column to the extreme right (Freq) indicates the number of isolates identified for each genotype. The last column on the extreme right shows the respective primary infection loci at which the isolates were sampled (Refer to Fig. 3.1)

SSR Loci	Pi02			Pi04		Pi4B		D13		Pi16		G11		Pi33		Pi56		Pi63		Pi70		Pi89		Freq	Primary Focus
Parents																									
DL43B_A1	152	160	162	166	170	213	217	118	136	178	178	160		203	203	176	176	151	157	192	195	181	197		
CW13C_A2	160	162	0	166	170	205	213	136	154	176	178	154	160	203	203	176	176	151	157	192	192	179	179		
Genotypes																									
G1	160	162	0	166	170	205	213	136	136	178	178	154	160	203	203	176	176	151	157	192	195	179	181	4	F3
G2	160	162	0	166	170	205	213	136	136	178	178	154	160	203	203	176	176	151	157	192	195	181	181	8	F3
G3	160	162	0	166	170	205	213	136	136	178	178	154	0	203	203	176	176	151	157	192	195	179	181	2	F6
G4	160	162	0	166	170	205	213	136	136	176	176	160	0	203	203	176	176	151	157	192	195	181	181	2	F6
G5	160	162	0	166	170	205	213	136	136	176	176	154	160	203	203	174	176	151	157	192	195	179	181	2	F1
G6	160	162	0	166	170	205	213	136	136	178	178	154	160	203	203	176	176	157	157	192	195	179	179	3	F6
G7	160	162	0	166	170	205	213	136	136	178	178	160	0	203	203	176	176	151	157	192	195	179	181	1	F5
G8	160	160	0	166	170	205	213	136	136	178	178	154	160	203	203	174	176	151	157	192	195	179	181	1	F5
G9	160	162	0	166	170	205	213	136	136	178	178	154	160	203	203	176	176	157	157	192	195	179	181	1	F1
G10	160	160	0	166	170	205	213	136	136	178	178	160	0	203	203	176	176	151	157	192	195	179	181	1	F5
G11	160	162	0	166	170	205	213	136	136	178	178	154	160	203	203	174	176	151	157	192	195	179	181	7	F4
G12	162	162	0	166	170	205	213	136	136	178	178	154	160	203	203	174	176	151	157	192	195	181	181	2	F5
G13	162	162	0	166	170	205	213	136	136	178	178	160	0	203	203	176	176	151	157	192	195	181	181	2	F2
G14	162	162	0	166	170	213	213	136	136	178	178	154	0	203	203	174	176	151	157	192	195	179	179	1	F1
G15	162	162	0	166	170	205	213	136	136	178	178	154	160	203	203	174	176	151	157	192	195	179	179	1	F2

Table 3.12: SSR genotypes detected in *P. infestans* isolates from the second crop grown in 2011 in the polytunnel. Sampling was done during the last phase of the blight epidemic. The column on the extreme right shows the number of isolates (frequency) with a common SSR profile. Out of the total number of samples (n=96) analyzed, only 63 had complete data sets for each of the eleven SSR markers used (full data sets in appendix 3.1).

Isolate	Pi02		D13		Pi33		Pi04		Pi4B		Pi16		G11		Pi56		Pi63		Pi70		Pi89		Frequency (No.)
1-E1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	192	195	179	181	52
1-H3	160	162	118	136	203	203	166	170	205	213	176	178	154	160	174	176	151	157	192	195	179	181	5
1-H2	160	162	118	136	203	206	166	170	205	213	176	178	154	160	174	176	151	157	192	195	179	181	5
1-D7	160	162	118	136	203	206	170	170	205	213	176	178	154	160	174	176	151	157	192	195	179	181	1

Table 3.13: Allele frequency, expected and observed heterozygozities (H_o, H_e), polymorphic information content (PIC) at each SSR locus and Chi-square test for Hardy-Weinberg equilibrium in the putative *F*1 isolates of *P. infestans* recovered from the polytunnel.

			[a]Statistics for HWE test				
Locus	**K**	**N**	H_o	H_e	**PIC**	**Chi**	[b]**P-value**
Pi02	3	40	0.65	0.50	0.38	1.08	n.s
Pi04	2	42	0.95	0.51	0.37	17.43	***
Pi4B	4	42	0.81	0.55	0.45	10.87	*
D13	3	42	0.05	0.09	0.09	n.d	n.d
Pi16	2	41	0.09	0.29	0.24	n.d	n.d
Pi89	2	41	0.67	0.53	0.44	1.98	n.s
G11	3	42	0.74	0.53	0.41	4.03	*
Pi63	2	42	0.91	0.50	0.37	12.47	***
Mean number of allele per locus	2.71						
Mean number of loci	7.0						
Mean expected heterozygozity	0.42						

[a]K, number of alleles at a locus, Heterozygosities (H_o, H_e), N, number of individuals typed; [b]P-value, n.d, not done, n.s no significant departure from HWE, ***P<0.001;

3.4.12 Assessment of *P. infestans* F1 isolates for aggressiveness on detached leaflets of cultivar Cara

Data on two parameters (latent period and lesion area) of foliar blight aggressiveness caused by *F*1 progeny and parental isolates DL4-3B_A1 and CW1-3C_A2 of *P. infestans* on cultivar Cara are shown in Table 3.14. The test showed that parental isolates were more aggressive on detached leaflets of cultivar Cara than the F1 isolates. Parental isolates had significantly higher values (P<0.001) of AULPC and lower values of latent periods (Table 3.14;

Appendix 3.2). With an AULPC value of 2335.2 and latent period of 3 days, parental isolate DL4-3B_A1 was ranked the most aggressive of the twelve isolates assessed while isolate T4/10 with an AULPC value of 76.6 and latent period of 7.50 days was the least aggressive (Table 3.14).

With the exception of isolates I20 from *in vitro* cross and T10 (genotype G15 in Table 3.11) from the polytunnel, the remaining *F*1 isolates had significantly lower ($P<0.001$) AULPC values (ranging from 76.6 to 572.0) than both parents. Similarly, with regard to latent period isolates I20 and T10 did not differ significantly from both parental isolates. However, the remaining *F*1 isolates had significantly higher ($P<0.001$) latent periods than parental isolates (Table 3.14).

Notably, isolate I20 (mating type A1) from the *in vitro* cross was triallelic at locus Pi02 (allele 152 160 and 162). Additionally, it had a 181, 197 allele combination at locus Pi89 and just like parental isolate DL4-3B_A1 was metalaxyl sensitive (Table 3.4). Taken together, these features give indications that it arose from selfing of the DL4-3B_A1 parental isolate. On the other hand the other *F*1 isolate (T10) with elevated aggressiveness on cultivar Cara was metalaxyl resistant.

Poor foliar aggressiveness of some *F*1 isolates as indicated by their inability to induce expanding and sporulating lesions on leaflets of Cara indicates their loss of virulence on this cultivar as a result of genetic recombination.

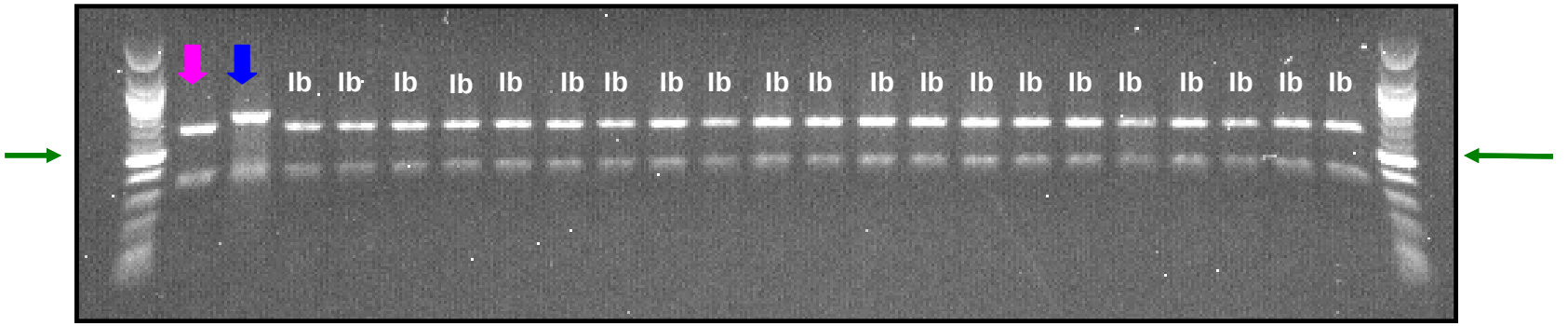


Fig. 3.20: Mitochondrial haplotypes of a subsample of isolates from the polytunnel 'mating' of *P. infestans* as revealed by *Msp*I restriction of PCR amplicons of the P2 primer pair on mtDNA (Griffith and Shaw, 1998). Green arrows indicate 500bp on the ladder; bold purple and blue arrows indicate parental isolates DL4-3B_A1 (Ib) and CW1-3C_A2 (Ia) respectively while lanes 4-26 from left to right are *F*1 progeny showing mtDNA haplotypes Ib.

Table 3.14: Mean Area Under Lesion Progress Curves (AULPC) and Latent Periods (LP) induced by *P. infestans F*1 isolates from the *in vitro* crosses and polytunnel inoculated onto detached leaflets of cultivar Cara

Isolate (n=12)	**[a]Mean AULPC (mm^2)**	**Mean LP (days)**
[b]DL4-3B_A1	2335.2a	3.00a
[c]CW1-3C_A2	1657.5b	3.50a
I20	1785.5b	3.25a
T10	1211.5b	3.83a
I69	572.0c	5.19b
I28	286.3cd	6.75bc
I70	171.4cd	7.67c
I45	106.8d	7.50c
T35	98.0d	7.42bc
I23	80.9d	7.58c
T4/7	79.9d	7.50c
T4/10	76.6d	7.50c
Isolate	(P<0.001)	(P<0.001)
Experiment	(P=0.13)	(P=0.47)
LSD P=0.05	336.5	1.26

[a]Values in the same column followed by the same letter do not differ significantly (P=0.05). [b]DL4-3B_A1: A1 parental isolate; [c]CW1-3C_A2: A2 parental isolate

3.5 Discussion

The objective of this chapter was to determine whether novel isolates that recently appeared in Ireland have the potential to mate and to assess the impact of such a phenomenon. Towards this end, genetic recombination between novel isolates CW1-3C_A2 (metalaxyl resistant) and DL4-3B_A1 (metalaxyl sensitive) was confirmed by crossing these isolates followed by recovering isolates from their mating and systematically assessing them using three techniques: (i) phenotypic screening, (ii) genotypic fingerprinting and (iii) mitochondrial DNA haplotyping.

Phenotypic analysis based on response to metalaxyl detected progenies with intermediate resistance to metalaxyl which composed the majority of isolates recovered from both the *in vitro* crosses as well as the polytunnel. The intermediate phenotypes were presumed to be segregation events because they differed from either resistant or sensitive phenotypes possessed by parental isolates CW1-3C_A2 and DL4-3B_A1 respectively. The genetic based system employed SSR markers to identify progenies that showed allelic recombination based on pre-determined parental SSR profiles as reference and subsequent analysis to confirm levels of genetic relatedness between individual progeny and the parental isolates CW1-3C_A2 and DL4-3B_A1 based on the parentage likelihood method. The last method of discrimination was based on mtDNA haplotype analysis of the putative progeny and confirmed conformity to the uni-parental mtDNA inheritance patterns (Whittaker *et al*., 1994). Overall, all three techniques supported the theory that genetic recombination had occurred between isolates CW1-3C_A2 and DL4-3B_A1 in the present study, effectively endorsing the hypothesis that genetic recombination is possible between opposite mating types of the recently occurring strains of *P. infestans.*

Abundant production of oospores in the host tissue of the colonized crop ensures sufficient numbers reach the soil to generate oosporic infections in the next crop. A fundamental condition to production of out-crossed oospores is the requirement that the mating type A1 and A2 pair of *P. infestans* isolates establish infections

simultaneously on the same host in close proximity to enable interaction between them leading to oosporogenesis. As demonstrated by this study and consistent with earlier findings (Hanson and Shattock, 1998) this condition is more easily fulfilled on blight susceptible cultivars as well as those with intermediate blight resistance than on blight resistant germplasm hence oospore production is more proficient on the former than on the latter. Irish potato production statistics show that susceptible cultivars Kerr's Pink and Rooster jointly accounted for 66.5% of national production in 2011 (IFA, 2011). Besides, it is clear from the results of the present study that germinating oospores caused infection on lower leaves of susceptible Maris Piper more frequently than on moderately resistant Cara, suggesting increased likelihood of oospore mediated blight epidemics when susceptible cultivars are planted in soil containing viable oospores.

Occasionally, both mating types of *P. infestans* may co-exist as strictly clonal lineages even when susceptible potato cultivars are commonly planted (Montarry *et al*., 2010). In the Netherlands for instance, both mating types co-existed for at least 9 years without any sexual reproduction taking place (Fry *et al*., 1991) but mating occurred afterwards (Drenth *et al*., 1994). It is unclear what contributes to this delayed mating, but it is conceivable that the method adopted to control blight has a significant effect. Use of systemic fungicides, for instance, disrupts the synchronous infection of the host tissue by both mating types of *P. infestans.* Typically, farmers in Ireland rely on fungicides including metalaxyl and make an average of 15 sprays in a single growing season to obtain effective control of late blight (Dowley *et al*., 2008). Systemic activity of fungicides significantly reduces the risk of sexual reproduction and oospore production via reduction of intercellular mycelial proliferation leading to inhibition of interaction between opposite mating types (Schwinn and Margot, 1991).

The possibility exists that sexual reproduction is already on-going in the field in Ireland. In this case, the risk of oospore inocula as well as oospore viability as ultimately confirmed by their rates of germination comes into perspective. This

study recorded a 5 % mean germination percentage of oospores recovered from *in vitro* crosses and only fifteen discrete genotypes from the polytunnel crop. The results from the polytunnel crop resonate with findings of other studies that recovered few but unique genotypes from primary infection foci in potato crops grown after short fallow seasons in fields previously cropped with potatoes that were colonized by strains of both mating types *P. infestans* (Evenhuis *et al*., 2007; Widmark *et al*., 2007). Erratic germination of *P. infestans* oospores under both in *vitro* and natural conditions is well documented and available data show germination rates ranging from single digit to over 80% (Drenth *et al*., 1995; Hanson and Shattock, 1998; Pittis and Shattock, 1994; Shattock *et al*., 1985; Singh *et al*., 2004). It is conceivable that the thickened wall of the oospore may account for much of their poor germination rates, but balanced lethals associated with chromosomal translocations which occur during genetic recombination has been invoked to account for the high frequencies of non-viable oospores in *P. infestans* (Knapova *et al*., 2002; Sansome, 1977). Additionally, data from previous studies show that application of metalaxyl at inoculation inhibited oospore production when one or both isolates in the mating pair were sensitive to metalaxyl; and further that oospores were produced irrespective of metalaxyl treatment from mating with resistant parents although there was significant reduction in germination rates in oospores produced on host tissues treated with metalaxyl (Hanson and Shattock, 1998).

Microsatellite analysis of the recovered isolates from both the *in vitro* and polytunnel crops indicated they were out-crossed individuals exhibiting allele segregation including at loci linked to traits of importance to blight control such as sensitivity to fungicides and virulence on existing host germplasm. Variation was also assessed using mtDNA markers which revealed mtDNA haplotypes Ia and Ib among the *in vitro F*1 isolates and Ib among the *F*1 isolates from the polytunnel. Having detected no mtDNA recombinants the findings were taken to affirm the view that progeny assorted with one or the other parental isolate. Over all, the majority

(62.2%) of the *in vitro* isolates possessed mtDNA haplotype Ia therefore mainly as the CW1-3C_A2 parental isolate which acted as the female partner in the mating pair of isolates. On the contrary, all F1 isolates from the polytunnel were of Ib mtDNA haplotype as DL4-3B_A1 parental isolate. A possible reason to account for the mtDNA result of polytunnel isolates is that the sampling failed to capture isolates of Ia mtDNA haplotype. Another possibility is that oospores of this Ia mDNA haplotype failed to germinate and contribute to the genotypes found on the crops in the polytunnel. The latter would imply competitive fitness of haplotype Ib over Ia. Notably, identity of mtDNA haplotypes in historical specimens from the Irish potato famine of the mid-19th century has led to the suggestion that Ia haplotype of *P. infestans* was responsible for that devastating blight epidemic (May and Ristaino, 2004). Other studies have found consistent association between haplotype Ib and the US-1 genotype that predominated Europe (before the 1980s) and other regions of the world (Carter *et al*., 1990; Gavino and Fry, 2002). Surveys reports on the UK *P. infestans* population indicate an increasing prevalence of isolates with a Ib mtDNA haplotype in recent years (Cooke et al., 2010).

Although it is generally thought that mtDNA is uni-parentally inherited from a maternal parent in the mating pair, exceptions to this rule have been reported. Knapova and Gisi (2002) reported a majority of *P. infestans F*1 progeny having mtDNA haplotype similar to the A2 mating type used in the cross while two isolates shared a haplotype with the A1 paternal isolate in the cross. They suggested that the paternal contribution resulted from mtDNA escape into the zygote through the antheridia. It is probable that in the present study parental isolate DL4-3B_A1 also formed oogonia accompanied by alternate formation of antheridia in isolate CW1-3C_A1 in instances where mtDNA was inherited from the DL4-3B-A1 parental isolate. Some isolates of *P. infestans* can preferentially form antheridia or oogonia during sexual reproduction. For instance, an *npt II* and *Gus* labelled Japanese A1 isolate alternately formed antheridia or oogonia under *in vitro* and *in planta* conditions respectively (Gotoh *et al*., 2005). More intriguingly, individual propagules

with an opposite mating type to that of the parental line isolate were detected in cultures of *P. parasitica* (Ko, 1988).

In an attempt to account for the paucity of individuals with recombinant mtDNA in nature, it has been suggested that recombinant mitochondrial genomes produced through mating involving parental *P. infestans* strains with different mtDNA haplotypes are eliminated through selection leaving only offspring with mtDNA haplotypes inherited singly from either parent (Gavino and Fry, 2002; Purvis *et al*., 2001).

As regards phenotypic variation, the present study revealed the occurrence of genotypes with intermediate resistance to metalaxyl at elevated frequencies among isolates from *in vitro* crosses as well as those recovered from the polytunnel. A more intense screening of intermediate individuals on leaf discs floated on a suspension with metalaxyl at concentrations ranging from 0-100mg/L revealed a continuous distribution skewed towards sensitivity. Given that the A1 and A2 parental isolates in the present study were sensitive and resistant to metalaxyl respectively, the result is consistent with the theoretical expectation for a trait under the control of either a single locus with an incomplete dominant effect only or with additional loci having a cumulative effect on the overall phenotype (Fabritius *et al*., 1997; Shattock, 1988).

Observations in the polytunnel on an oospore-initiated blight epidemic in this study, suggest that the prominence of intermediates in an epidemic is short lived because they compete poorly on the host foliage against genotypes with metalaxyl resistant phenotypes which tended to dominate the polytunnel in the later phases of the blight epidemic. This result stands in concurrence with previous findings (Gisi and Cohen, 1996; Kadish and Cohen, 1988). This shift in favour of a single genotype has been shown in other studies to be dependent on the relative fitness of the dominating genotype (Kadish and Cohen, 1989).

All together, the genotypic variation associated with sexual reproduction in *P. infestans* results not just from allele segregation but also from other accompanying genetic events. For example the instability at the mating type locus has been established through analysis of markers linked to this locus which appeared more variable and prone to duplication, translocation or deletion than control loci that were unlinked to the mating type locus (Judelson, 1996). This mobility of loci can become a significant source of variation in *P. infestans* and may be potentially useful for adaptation to a changing environment. Also, phenotypic data exist on the segregation of the mating type from heterokaryons during vegetative growth in *P. infestans* (Pipe *et al*., 2000).

The present study detected the presence of selfed and triallelic individuals as a legacy of sexual reproduction. Selfing in *P. infestans* has been shown to occur (Smart *et al*., 1998) in a variety of circumstances including hormonal stimulation arising from the presence of the opposite mating type of the same or other *Phytophthora* species (Ko, 1988; Skidmore *et al*., 1984), other fungi (Shaw, 1991) as well as treatment with fungicides (Groves and Ristaino, 2000). Employing the use of isozymes to analyze progeny from mating of *P. infestans* isolates, other authors have detected a large number of self fertile progeny and invoked the bisexual nature of *P. infestans* (Shattock *et al*., 1986) to explain the phenomenon.

Triallic individuals are known to occur in sexual populations of *P. infestans* (Carter *et al*., 1999; Shaw and Shattock, 1991; van der Lee *et al*., 2004). This phenomenon arises from chromosomal reorganization and loci translocation during recombination, resulting in multiple copies of loci or regions in the genome of this organism (Judelson *et al*., 1995; van der Lee *et al*., 2004). It is also thought to be an outcome of the absence of disjunction in the parental isolates during meiosis (Carter *et al*., 1999). Also non-Mendelian segregation particularly for the mating type trait where ambiguous genotypes inheriting alleles for both A1 and A2 types are thought to be eliminated through balanced lethals (Judelson, 1997a; Judelson et al., 1995; Shaw and Shattock, 1991). Apparently and quite inexplicably, this

aberration in genetic recombination is unfavourable to the A2 mating type as compared to the A1 mating type and often causes distortion in segregation ratios leading to excesses in A1 mating types (Judelson, 1997b).

Whereas no triallelic or trisomic isolates were observed among isolates recovered from the second cycle crop in the polytunnel, there was disparity in mating type composition with an excess of A1 mating types. In previous late blight experiments in the same tunnel, triallelic loci were detected in large proportions (21%) indicating they appear commonly following sexual recombination (Carter *et al*., 1999; Shaw *et al*., 2009).

The genetic elasticity associated with trisomy has been hypothesized to contribute to fitness and the capacity for adaptation in *P. infestans* (van der Lee *et al*., 2004). However, this hypothesis stands contradicted by a recent study which showed that *F*1 progeny from crosses with at least one triploid parents were less pathogenic than the parental isolates (Hamed and Gisi, 2012). The present study has recorded reduced foliar blight aggressiveness among many F1 progeny and similar levels of aggressiveness in only two F1 isolates in comparison with the two pathogenic parental isolates. These results are in agreement with the findings of other studies on aggressiveness of oospore generated F1 populations on potato (Hamed and Gisi, 2012) and tomato (Klarfeld *et al*., 2009).

As reliance on metalaxyl still remains significant, emergence of novel genotypes with resistance or intermediate response to metalaxyl represent a drawback to blight control programs consisting of use of this active ingredient. In order to pre-empt epidemics caused by oospores acting as primary sources of inoculum adoption of strategies that manipulate conditions necessary for oospore production, germination or epidemic are needed. These strategies include seasonal surveys of local populations of *P. infestans*; promoting the cultivation of resistant cultivars; augmentation of field resistance to blight with preventive fungicides coupled with scouting for oospore production under field conditions.

Chapter Four

Investigating the potential of *Phytophthora infestans* to adapt to host resistance of a transgenic potato cultivar

4.1 Background

P. infestans, like all plant pathogens, has to negotiate intricate defense systems in order to infect its hosts. During the biotrophic phase of *P. infestans* infection, the pathogen forms a structure called the haustorium with which it invaginates the host cell membrane, delivering pathogenicity factors ('effectors') into the host cytoplasm and acquiring nutrients from the targeted cell (Dodds and Rathjen, 2010; Panstruga and Dodds, 2009). Plants may respond to this attack by attempting to restrict colonization, often through effector-triggered immunity which involves a form of programmed cell death popularly known as the 'hypersensitive response'. This is achieved through cellular reorganization involving organelle relocation, cell-wall reinforcement at the pathogen contact site and secretion of anti-microbial molecules such as papain-like cystein proteases (PLCPs) (Bednarek *et al*., 2010). To counteract these plant defenses, effective pathogens such as *P. infestans* secrete extracellular inhibitors of cystein proteases (EPICs) which are thought to bind to and inhibit PLCPs in the apoplast (Kaschani *et al*., 2010; Wang *et al*., 2011).

To facilitate successful colonization, the *P. infestans* genome encodes effectors which operate inside the host cell (Haas *et al*., 2009). The best known *P. infestans* effectors are those containing the RXLR (arginine-any amino acid-leucine-arginine) motif, which represents a conserved sequence determinant of host translocation (Birch *et al*., 2006) and include the *ipiO* gene family which is highly diverse among *P infestans* populations worldwide with class I IPI-O occurring in the majority of isolates (Champouret *et al*., 2009). The antagonistic interactions between *P. infestans* and its potato host impose extreme demands on both organisms. The process is what has come to be commonly referred to as the 'arms race'. In this arms race, the pathogenicity success of *P. infestans* is largely due to its biological lifestyle and extraordinary capacity to rapidly adapt and overcome host resistance (McDonald and Linde, 2002). An examination of the recently published genome of *P. infestans* revealed that besides RXLR-determining effector genes, the genome

also contains large families of Crinkler (CRN) genes, which code for candidate cytoplasmic effectors (Haas *et al*., 2009). CRNs, just like their counterpart RXLR, act within the host cell nucleus to induce cell death (Schornack *et al*., 2010). Consequently, it has been proposed that the well observed pathogenic dynamism of *P. infestans* is borne from the variation enabling plasticity of the gene-poor, repeat rich regions of its genome, which are populated by these RXLR and CRN genes (Haas *et al*., 2009; Schornack *et al*., 2010).

Following the defeat of race non-specific *S. demissum R* genes, focus shifted to broad spectrum or non-race specific forms of resistance (reviewed by Vleeshouwers *et al*., 2011). The wild potato *S. bulbocastanum* has provided a form of resistance mediated by *R* genes including the *RB* gene, also known as *Rpi-blb1* (Helgeson *et al*., 1998; Song *et al*., 2003). The resistance response conferred by the *RB* gene includes the induction of a classical hypersensitive response, callose deposition and up-regulated transcription of pathogenesis-associated defense genes (Bradeen *et al*., 2009; Chen and Halterman, 2011; Kramer *et al*., 2009). However, *RB* only confers strong partial resistance and can permit the growth and sporulation of *P. infestans,* albeit at significantly lower rates compared to the non-*RB* expressing control (Song *et al*., 2003).

RB gene product recognizes the presence of the *ipiO* virulence effector of *P. infestans* and triggers a resistance response when this effector is introduced during invasion by *P. infestans* (Vleeshouwers *et al.,* 2008). The fact that the *ipiO* locus is prone to mutation (Haas *et al*., 2009) suggests that in a scenario where *P. infestans* parasitizes and sporulates on a partially resistant host, each subsequent generation may exhibit increased virulence against the formerly resistant host.

To investigate whether continued parasitism on a host harbouring the *RB* gene would lead to pathogen adaptation and breakdown of resistance, this study experimented with two representative isolates of the recently occurring *P. infestans* genotypes, which were passed ten times through detached leaflets of transgenic

potato plants carrying one, three or five functional copies of the *RB* transgene. Only two isolates were evaluated in this study to keep to the minimum the possible number of isolate-transgenic line interactions that would be occasioned by sequential inoculations over ten vegetative cycles on multiple hosts. At the end of the repetitive passaging, levels of polymorphism within the *ipiO* sequence of each cycled isolate was assessed and compared with the initial isolate.

4.2 Materials and methods

4.2.1 Plant and pathogen preparation

Plant materials used for this study were potato cultivars Maris Peer (MP) and Desiree and the transgenic *RB* potato lines MP 100_4, MP 100_5, MP 100_6, MP 100_9 [cultivar Maris Peer plus 5, 3, 1 and 1 copy of *RB* respectively (Wendt *et al*., 2012)] and DESRB4, cultivar Desiree equipped with a single copy of the *RB* gene (Petti *et al*., 2009). Maris Peer derived transgenic lines were generated via *Ensifer adhaerens* transformation (strain OV14 carrying pCDL04541 and pCAMBIA5105) (Wendt *et al*. 2012) while the Desiree derived transgenic line was generated via *Agrobacterium tumefaciens* mediated transformation (strain AGL1 carrying pCLD04541) (Petti *et al*., 2009).

Plants were propagated from tubers under glasshouse conditions of between 17 and 20^{o}C and a minimum of 16h day length. For the passaging experiments, leaflets (fourth / fifth leaf) below the uppermost fully expanded leaves were collected from glasshouse maintained five to eight week old plant of cultivars Desiree, Maris Peer and the five transgenic lines. The experiment was carried out once between April and August 2011.

P. infestans isolates CW1-3C_A2 (SSR Blue 13 genotype, A2 mating type) and DL4-3B_A1 [SSR genotype 6_A1 (Pink 6), A1 mating type] were sub-cultured on rye A media slants every 4 months.

4.2.2 Passaging of *P. infestans* isolates CW1-3C_A2 and DL3-4B_A1 through detached leaflets of RB-expressing transgenic potato

Prior to commencing, each isolate was firstly passaged through leaflets of a susceptible cultivar, Kerr's Pink, to restore virulence. Viable inoculum was produced 7 days post-inoculation by washing leaflets with sporulating lesions in 5ml of sterile distilled water in 50ml Falcon tubes to dislodge sporangia into suspension. Each resulting suspension was standardized with a haemocytometer to a concentration of 2-5 x 10^4 sporangia/ml and incubated at 4^{o}C for 2 hours to release zoospores.

To conduct the first passage, inoculations were performed using inoculum prepared as described above. A single leaflet was deemed an experimental unit. The leaflets were placed lower surface uppermost in 9cm diameter inverted Petri dishes with a layer of dampened paper towel at the base before being inoculated in the centre with 20µl of the appropriate sporangial/zoospore suspension. Inoculated leaflets were incubated in a growth chamber at 18^{o}C for 7 days with a 16 h photoperiod. Visible lesion area was recorded for each inoculated leaflet as per 1/4πab for area of an ellipse with a = breadth of lesion and b = lesion length (Colon *et al*., 1995). Lesion diameter along the main leaf vein and diameter perpendicular to main leaf vein were deemed length and breadth respectively for consistency. Seven days post inoculation, leaflets of each isolate/cultivar interaction were washed separately in sterile 50 ml tubes containing 2.5 ml of sterile distilled water to obtain inoculum for the next passage. As the same leaves were used for lesion area measurements and for inoculums production for the next cycle, caution was observed to avoid the cross contamination between samples at all stages of the experiment. As there was risk of cross contamination when handling samples with sporulating lesions, working surfaces were sterilized with ethanol between successive samples during measurements for lesion size. Additionally, only cover lids were lifted from only a single sample at a time for assessment.

The same procedure for inoculum preparation and leaflet inoculation was followed for ten consecutive cycles without intervening steps of sub-culturing of isolates on nutrient agar. Pure cultures were obtained upon completion of the tenth cycle by transferring sporangia from sporulating lesions individually onto pea agar without antibiotic. Three plates were prepared for each isolate during transfer of sporangia onto pea agar to reduce the risk of loss of isolates through contamination.

4.2.3 DNA extraction from *P. infestans* after cycling through detached leaflets of potato

Approximately 50mg of *P. infestans* mycelia was harvested from 10 day old pea agar cultures of isolates (recovered from the cycling experiment in section 4.2.2) using sterile scalpels and transferred into 2ml Eppendorf tubes. The mycelia were freeze dried for 24 hrs and pulverized using a mixer mill with sterile glass beads. Extractions were performed using the procedure of Raeder and Broda (1985) in which 750 µl of extraction buffer (0.1M Tris HCl, 0.25M NaCl, 0.025 M EDTA, 0.5% SDS) was added to each ground sample in a 2ml Eppendorf tube and incubated at room temperature for 30min. This was followed by the addition of 750µl of phenol chloroform to each sample, vortexing for 1 min to mix and centrifugation at 13200 rpm for 10 min. The upper phase from each sample was transferred into fresh 1.5ml Eppendorf tubes to which 750µl chloroform added, vortexed for 1 min followed by centrifugation at 13200 rpm for 5 min. The upper phase from each sample was transferred into fresh 1.5 ml Eppendorf tubes to which 450 µl isopropanol was added. The tubes were inverted gently to mix followed by centrifugation at 13200 rpm for 5 min. The supernatant was discarded and the pellet washed with 1000µl 70% ethanol prior to centrifugation at 13200 rpm for 5 min. The supernatant was discarded and the pellet was air dried for 10 min then re-suspended in 50µl HPLC (Sigma Aldrich, Germany) water. RNase A (1µl of 10mg/ml) was added to each sample which was incubated at 37^{o}C for 30 min.

4.2.4 *IpiO* amplification and purification

To determine the level of polymorphism within the *IpiO* gene, the target sequence was firstly PCR amplified under conditions of 0.2mM dNTPs, 1X thermal buffer, 100nM forward primer, 100nM reverse primer, 1 unit Taq polymerase, 50ng/µl genomic DNA and 13.8µl water in a total volume of 20µl. The reactions were completed in a Biometra 3500T Thermo Cycler with an initial cycle of denaturation for 5 min at 95^{o}C followed by 40 cycles of denaturation for 30 sec at 95^{o}C, annealing for 30sec at 58^{o}C and extension for 40 sec at 72^{o}C and a final extension for 5 min at 72^{o}C. The *IpiO* primers used were: 5' ATG GTT TCA TCC AAT CTC and 5' CTA TAC GAT GTC ATA GCA TGA CAC. PCR products were loaded onto 1% (w/v) agarose gel stained with ethidium bromide (1µg/ml) and submerged in 0.5M TBE buffer. A size standard (100bp ladder NEB, UK) was loaded alongside the samples and 70V of current applied for 30 min. Bands were visualized and imaged using a Kodak Imager (Image Station 440 CF, Kodak Digital Science ^{TM}, USA).

Once identified, the requisite bands were excised with a scalpel and transferred into sterile 1.5 ml tubes. DNA was eluted using the Qiaex II Kit (Quiagen, Germany) following the manufacturer's instructions. The resulting supernatant containing the purified amplicons from each sample were transferred into fresh 1.5ml Eppendorf tubes and quantified using a fluorescent Qubit DNA Assay (Invitrogen, USA).

4.2.5 Cloning of *P. infestans IpiO* sequence into *E. coli*

Extracted PCR products were ligated into pGEM-T Easy vector (Promega, Madison, USA) (Fig. 4.1), with a ligation reaction of 10µl volume consisting of 2X Rapid Ligation Buffer (5µl), pGEM-T Easy Vector (50ng), PCR product (42ng), T4 DNA ligase (3 Weiss Units) (Promega, USA) topped up with molecular grade water (Sigma Aldrich, Germany). The ligation reactions were gently vortexed and centrifuged for 30 seconds before incubating overnight at 4^{o}C.

For transformation of the ligated products; vials containing 50µl chemically competent *E coli* cells (Invitrogen, USA), one for each ligation reaction, were removed from -80^{o}C cold storage and thawed on ice for 5 minutes. Transformation of *E. coli* Top 10 chemically competent cells was performed by transferring 2µl of each ligation mixture into individual vials containing 50µl of the chemically competent cells. The tubes were flicked gently to mix and placed on ice for 20 min. Tubes were placed in a water bath at 42^{o}C for 50 sec and immediately put back on ice for 2 min, after which 250µl of SOC media (Invitrogen, USA) was added to each tube followed by incubation at 37^{o}C for 90 min. Each transformation mix was then plated out using a sterile spreader on two Petri dishes containing 2% Luria Bertan (LB) medium (2% LB broth, 1.2% agar) amended with 100µg/ml Ampicillin, 80µg/ml x-Gal (5-bromo-4chloro-3indolyl-β-D-galactopyranoside) and plates were incubated for 24 hrs at 37^{o}C. The appearance of white colonies indicated successful transformation of a circularized plasmid into *E. coli* and these colonies were picked for plasmid DNA extraction.

4.2.6 Mini-prep plasmid DNA extraction using Alkaline lysis method

White colonies of transformed *E. coli* were picked using sterile cocktail sticks and placed into sterile 50ml Falcon tubes containing 5ml of LB broth (Sezonov *et al.,* 2007) amended with 100µg/ml ampicillin and incubated overnight at 37^{o}C with shaking at 220rpm. DNA was extracted from the resulting cultures using a modified alkaline lysis method described by Maniatis *et al.,* (2002). 1.5ml of culture from each sample was transferred into a 2ml Eppendorf tube followed by centrifugation at 13000rpm for 1min and discarding of the supernatant. To increase the DNA yield, another 1.5 ml from the same sample was added to the tube and the step repeated as above. Then, 100µl of solution A (10mM Tris pH 8.0, 1mM EDTA pH 8.0) was added to each tube and vortexed to provide adequate re-suspension of the pellet. The mixture was incubated at room temperature for 5 min. and then 200µl of freshly prepared solution B (0.2N NaOH, 1%SDS) was added to each tube and mixed by inversion followed by incubation for a further 5 minutes. 150µl of

solution C (3M sodium acetate pH 5.2) was added to each tube followed by vortexing and incubation for 20 min at -20^{o}C. The samples were centrifuged at 13000 rpm for 15 min at room temperature. The supernatant from each tube was transferred into fresh sterile 1.5ml Eppendorf tubes and the pellets discarded. 1ml of 100% ethanol was added to each tube and incubated at -20^{o}C for 10 min before the samples were centrifuged at 13000rpm for 15 min at room temperature to pellet the extracted plasmid DNA. Resulting pellets were air dried under the laminar flow hood to vaporize ethanol and then dissolved in 50µl of RNase water (0.1mg/ml).

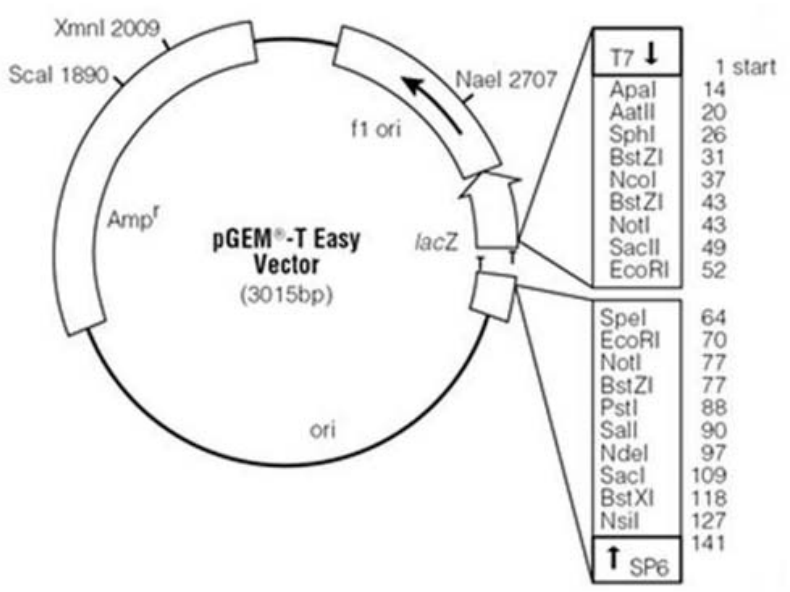


Fig. 4.1: pGEM-T Easy vector (3015 bp). T7 RNA Polymerase transcription initiation site 1; SP6 RNA Polymerase transcription initiation site 141; T7 RNA Polymerase promoter (-17 to +3) 2999-3; SP6 RNA Polymerase promoter (-17 to +3) 139-158; multiple cloning region 10-128; lacZ start codon 180; lac operon sequences 2836-2996, 166-395; lac operator 200-216; beta-lactamase coding region 1337-2197; phage f1 region 2380-2835; binding site of pUC/M13 Forward Sequencing Primer 2956-2972; binding site of pUC/M13 Reverse Sequencing Primer 176-192. (Promega Corporation, Madison, WI).

4.2.7 Post-cycling assessment of *P. infestans* isolates for aggressiveness on detached potato leaflets

Isolates recovered after ten cycles through their respective potato hosts as described in section 4.2.2 were comparatively assessed for aggressiveness on detached leaflets of cultivar Cara. The corresponding un-cycled clonal isolates DL4-3B_A1 and CW1-3C_A2 were employed as controls. In order to select a suitable host for this experiment, insights into disease patterns gained from data in chapter 2 were utilized. Data in chapter 2 showed limited discrimination among isolates on susceptible cultivars and low infection efficiency by isolates on cultivars with high blight resistance ratings. Hence cultivar Cara was selected because it possesses intermediate resistance to *P. infestans* (rating of 5 on a scale of 1-9, where1 is most susceptible and 9 is most resistant) (http://varieties.potato.org.uk).

Inoculum was prepared as per section 2.2.4. Prior to inoculation, six replicated plastic trays lined with moist paper towel were prepared with each containing twelve leaflets. Each leaflet was an experimental unit and the experiment was repeated once. The first experiment was conducted between 8th and 15th June 2012 and a repeat was completed between 15th and 22nd June 2012. In each experiment, the inoculation of isolates onto detached leaflets and incubation of inoculated leaflets was performed according to the procedure described in section 2.2.4.1. The parameters of aggressiveness that were recorded were latent period (period from inoculation to initial sporulation) in days and area under lesion progress curve (AULPC) (the cumulative totals of sporulating lesions estimated daily for 7 days according to Miller *et al*., (1998)).

4.3 Data analysis

4.3.1 Disease Assessment during host-pathogen cycling

Initial inference pertaining to an isolate's cumulative gain or loss of pathogenicity occurring due to continuous exposure to the resistance *RB* gene was made from

phenotypic data on the amount of disease (computed from AULPC) the isolate produced on a host during the 10 cycles of inoculation.

4.3.2 Sequencing and data analysis

Eight colonies containing *ipiO* gene inserts from each isolate were sequenced in one direction using the vector specific primer T7 (5' TAA TAC GAC TCA CTA TAG GG 3') (Fig. 4.1). Sequencing was performed by GATC Ltd. (Germany). Prior to sequence analysis, the vector sequence was removed using BioEdit software (Hall, 1999). Additionally, sequences were visualized against chromatograms in Chromas software (by Conor McCarthy, Griffith University, Australia) and those with ambiguous calls throughout the reads were removed from the analyses. Standard bioinformatics programs were used including: BLAST (Altschul *et al*., 1997), HCV Sequence database (Kuiken *et al*., 2005) to translate sequence into amino acids coding for the *ipi*O effector protein, Emboss-Lite (http://helixweb.nih.gov/emboss_lite/dna.html) to generate the reverse complements of the sequences as required, FaBox (Villesen, 2007) to convert files into appropriate in put formats for MEGA 5. Bioinformatics Multiple sequence alignments of the data were performed in ClustalW (Chenna *et al*., 2003). Sequence analyses for Single Nucleotide Polymorphism (SNPs) and as well as effector protein mutation were performed on recovered isolates after ten passages through the potato host leaflets and the two original isolates CW1-3C_A2 and DL4-3B_A1.

4.3.3 Adaptive selection analysis

For the adaptation analyses, the codon based likelihood approach for nucleotide substitutions (Muse and Gaut, 1994) implemented in MEGA 5 (Tamura *et al*., 2011) was used to determine pair-wise comparisons of sequences at homologous codons for differences between non-synonymous nucleotide substitutions per non-synonymous site (d_N) and synonymous nucleotide substitutions per synonymous site (d_S). The analysis involved 91 nucleotide sequences (325 bases length). There

were a total of 107 positions in the final amino acid sequence dataset. Analyses were conducted in MEGA 5 (Tamura *et al*., 2011).

The numbers of synonymous and non-synonymous substitutions per site were calculated using the formula:

$$d = -3/4 \ln(1-4/3p)$$

where p is either pS or pN, the proportion of synonymous and non-synonymous differences respectively determined as follows:

$$pS = d_S/S$$

where d_S is the number of synonymous differences per codon and S is the average of the total number of synonymous sites for the pair of sequences compared;

Similarly,

$$pN = d_N/N$$

where d_N is the number of non-synonymous differences per codon and N is the average of the total number of non-synonymous sites for the pair of sequences compared (Nei and Gojobori, 1986).

Statistical inference for diversifying selection was based on the relative abundance of non-synonymous substitutions over the synonymous substitutions at a homologous codon between respective pairs of sequences of cycled isolates and the corresponding control isolates. These estimates were produced using the joint Maximum Likelihood (ML) reconstructions under a Muse-Gaut model (Muse and Gaut, 1994) of codon substitution. For estimating ML values, a tree topology was automatically computed. The test statistic $d_N - d_S$ was used for detection of codons that had undergone selection. ML computations of dN and dS were conducted using a HyPhy software package (Kosakovsky Pond *et al*., 2005). A positive value for the test statistic indicated an overabundance of non-synonymous substitutions. In this case, the probability of rejecting the null hypothesis of neutral selection (P-

value) was calculated (Kosakovsky Pond and Frost, 2005; Suzuki and Gojobori, 1999).

i. Null hypothesis; H_o: $d_N=d_S$ strict neutrality

ii. Alternative hypothesis H_A: $d_N \neq d_S$ test of neutrality

    a. $d_N>d_S$ positive selection

    b. $d_N<d_S$ purifying selection

4.3.4 Assessment of genetic distances between isolates, post-selection

Genetic distances between isolates were used to infer the phylogenetic tree (Rzhetsky and Nei, 1992). The phylogenetic tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) at a search level of 0. Analyses were conducted in MEGA5 (Tamura *et al*., 2011).

4.3.5. Post-selection disease assessment

AULPC values from the post-selection assay for aggressiveness on detached leaflets were subjected to analysis of variance using the GenStat statistical program and where significant differences were detected, means were separated using Fisher's least significant differences (LSD) test at P=0.05.

4.4 Results

4.4.1 Disease assessment

Fig. 4.2 shows success or failure of isolates DL4-3B_A1 or CW1-3C_A2 to cause sporulating lesions on respective hosts during repeated inoculations. In the course of the cycling, observations showed that both isolates DL4-3B_A1 and CW1-3C_A2 of *P. infestans* caused disease on detached leaflets of each of the five transgenic potato lines equipped with the non-race specific *RB* blight resistance

gene and the respective non-transgenic comparator cultivars, Desiree and Maris Peer. However, only isolate CW1-3C_A2 produced sporulating lesions consistently on each of the seven potato hosts in each inoculation cycle (Fig. 4.3). In comparison, isolate DL4-3B_A1 failed to produce a sporulating lesion in the fifth cycle of inoculation on transgenic line MP100_4 but like isolate CW1-3C_A2 it also induced sporulating lesions on each of the remaining potato genotypes for all 10 cycles (Fig. 4.3). During cycling, the two isolates displayed similar disease patterns on all transgenic lines with a Maris Peer genetic background except line MP100_4. Comparison of the two isolates on transgenic line MP100_4 for instance showed opposite patterns of decrease and increase in lesion sizes in successive inoculations with DL4-3B_A1 or CW1-3C_A2 respectively (Fig. 4.3). The transgenic line with a Desiree background (DESRB4, containing a single copy insert of *RB*) was also as less discriminating between the isolates as the transgenic lines with Maris Peer background. Additionally, the sizes of disease lesions induced on detached leaflets of DESRB4 by either isolate were comparable to those caused by the particular isolate on the untransformed cultivar Desiree (Fig. 4.3 -).

Deductions about an isolate's cumulative gain or loss of virulence on the basis of AULPC data from the first and last cycle of cycling showed differences between DL4-3B_A1 and CW1-3C_A2 (Table 4.1). For instance, there was a reduction in the amount of disease produced on potato line MP100_4, by isolate CW1-3C_A2 in the 10th cycle relative to the first cycle of inoculation. Isolate DL4-3B_A1 on the other hand was defeated by this host in the fifth cycle of inoculation (Fig. 4.4). *RB* gene dosage effect on host resistance is explored later in this chapter. For the rest of the transformed lines and irrespective of the genetic background, both isolates produced a higher amount of disease in the tenth cycles compared to the first cycles of inoculation. However, isolate DL4-3B_A1 displayed more disease on the tenth cycle compared to CW1-3C_A2 on three MP100_5, MP100_9 and DESRB4 of the four remaining transformed potato lines.

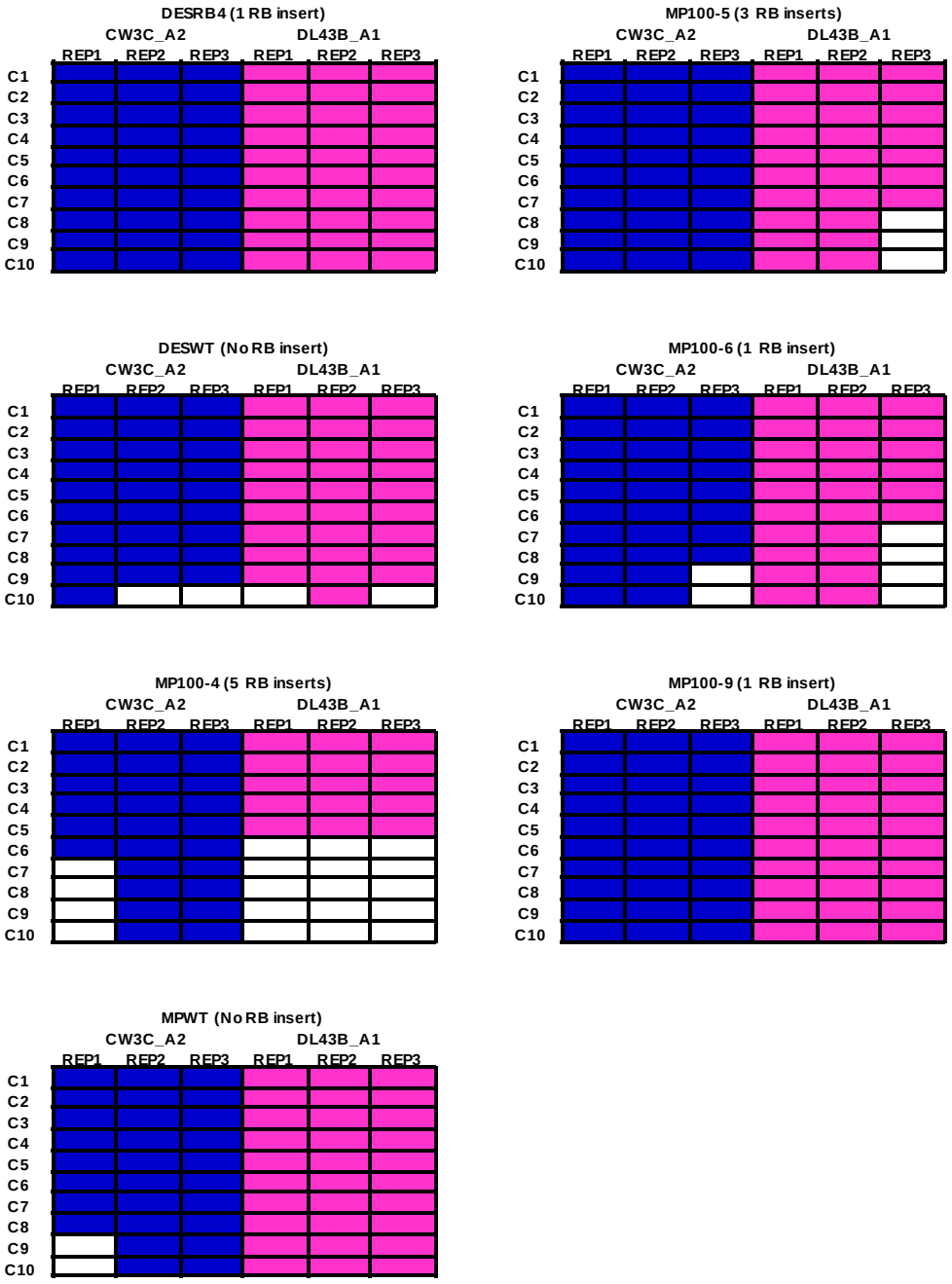


Fig. 4.2 Isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans* cycled ten times through five potato lines equipped with *RB* (non-race specific resistance gene from *S. bulbocastanum*) and two untransformed cultivars (The number of RB gene inserts present is provided in parentheses after the name of the respective potato line). Grids without colour indicate failure of the isolate to sporulate on the leaflets of the respective potato line/cultivar. WT is wild type.

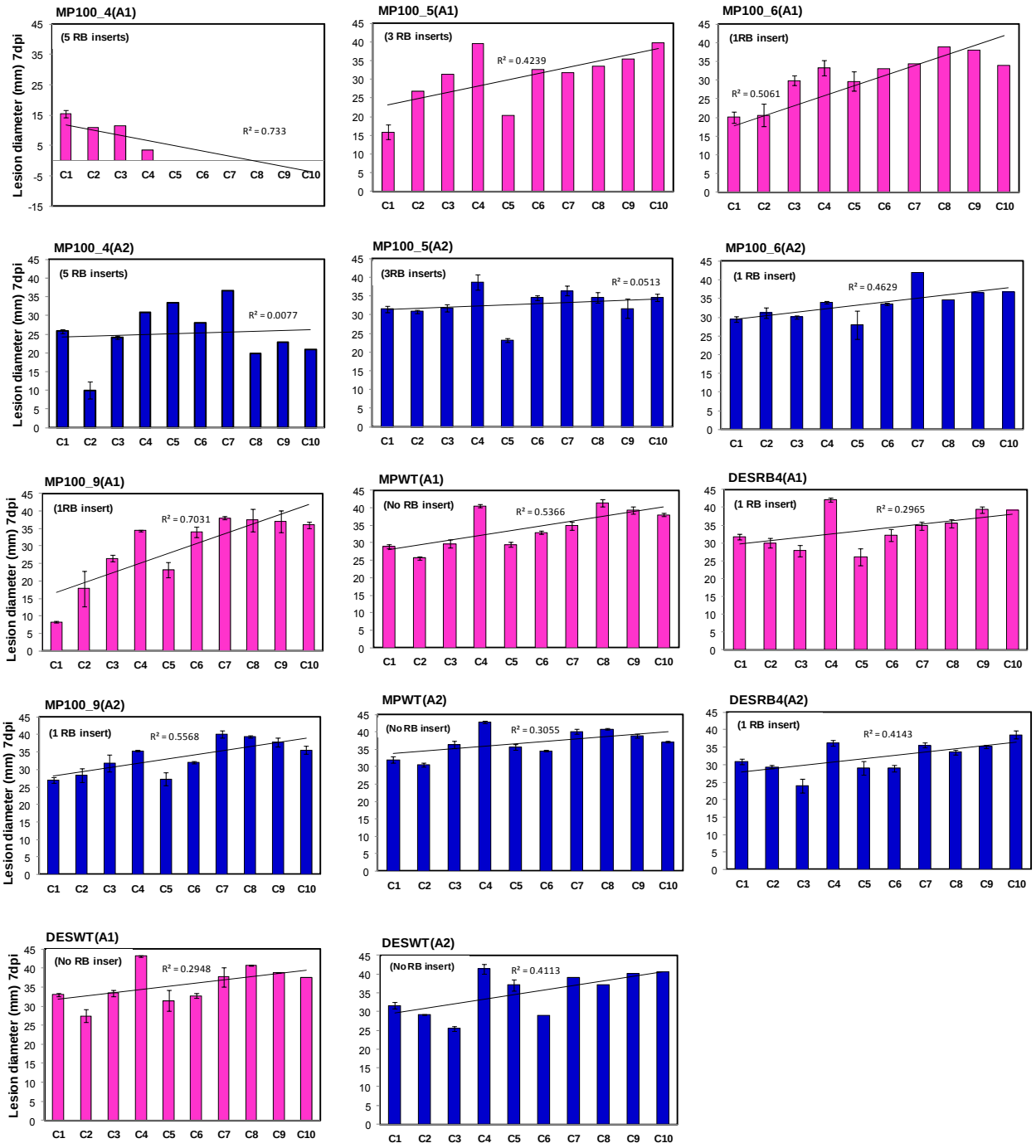


Fig. 4.3: Average lesion diameters (mm) induced by isolates DL4-3B_A1 and CW1-3C_A2 of *P. infestans* during ten cycles of inoculation leaflets of potato lines with or without the *RB* gene. Graphs with Pink bars correspond to interaction between respective potato lines with isolate DL4-3B_A1 while graphs with blue bars correspond to interaction between the respective potato lines with isolate CW1-3C_A2. Inset legend shows number of RB inserts present. For each graph, a trend line including the R^2 value is provided.

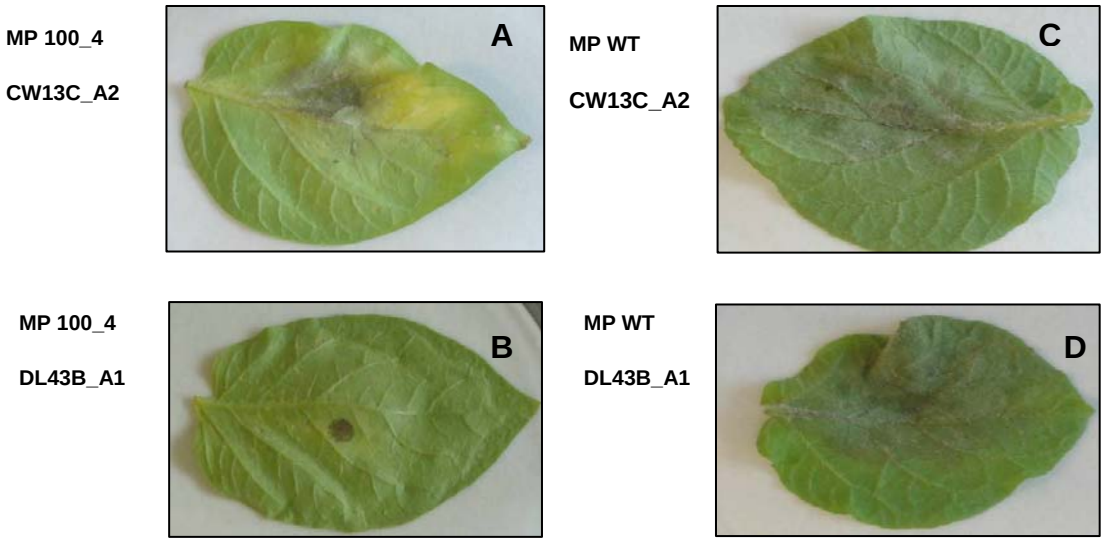


Fig. 4.4: Isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans* inoculated onto potato detached leaves of transformed potato cultivar Maris Peer (equipped with five copy inserts of blight resistance *RB* gene from *S. bulbocastanum*) **A** and **B** respectively or onto untransformed potato cultivar Maris Peer **C** and **D** respectively 6 days post inoculation during the fifth cycle of repeated inoculations (reading not taken for non-sporulating necrotic lesion at site of inoculation on MP100_4 leaflets inoculated with A1 isolate in 5th cycle of inoculation).

4.4.2 Adaptive selection

ClustalW alignment of the amino acid sequences of the *ipi*O effector of the cycled isolates together with those of original isolates DL4-3B_A1 and CW1-3C_A2 showed the occurrence of amino acid substitutions at positions from 72-98 (Fig. 4.5). These substitutions were as follows: leucine with serine at site ^{72}L, isoleucine with arginine at site ^{77}I, glycine with alanine at site ^{89}G, serine with asparagine at site ^{90}S and lysine with asparagine at site ^{98}K. Isolates recovered from cycling of isolate CW1-3C_A2 showed a larger combined number of amino acid substitutions (5) compared to those obtained from cycling of isolate DL4-3B_A1 (2) (Fig. 4.5). In both isolates, these sites are located within the C-terminal domain of the *ipi*O effector.

Table 4. 1: Mean area under lesion progress curves (AULPC) induced by isolates DL4-3B_A1 or CW1-3C_A2 of *P. infestans* inoculated onto detached leaflets of seven potato genotypes with or without the blight resistance *RB* gene at the beginning (C1) and end (C10) of repeated cycles of inoculation. Values in parentheses are standard errors on three replicates. Units without S.E. indicate isolate was recovered from sole replicate after 10 cycles (see Fig. 4.2).

		Mean AULPC (±S.E)			
		DL43B_A1		**CW13C_A2**	
Line	**RB copy number**	**C1**	**C10**	**C1**	**C10**
MP100_4	5	85.95 (20.04)	0.00	515.63 (22.37)	344.51
MP100_5	3	201.36 (51.21)	1226.27	759.31 (41.56)	931.89 (51.14)
MP100_6	1	317.03 (45.83)	903.51	678.37 (31.14)	1055.85
MP100_9	1	54.27 (4.14)	1003.65 (44.82)	562.31 (31.58)	984.77
Maris Peer	0	647.19 (24.20)	1105.67 (27.52)	789.55 (42.47)	1050.96 (16.62)
DES RB4	1	787.90 (35.26)	1187.89 (9.53)	727.61 (42.22)	1158.23 (72.29)
Desiree	0	833.52 (18.49)	1082.26	772.43 (49.29)	1279.65

The number of sites showing amino acid substitutions varied among isolates but ranged from none in both isolates cycled through untransformed potato cultivars to four sites in an isolate cycled through transgenic line MP100_4 which had five copies of the resistance *RB* gene. BLAST results on the sequences showed that both original isolates DL4-3B_A1 and CW1-3C_A2 had IPI-O variants which showed 100% similarity with IPI-O11 (NCBI accession number gb ACU56993.1). IPI-O11 belongs to class I IPI-O that is widely distributed among the European *P. infestans* population and is recognized by *RB* (Champouret *et al*., 2009). Additionally, the isolates retained 98-99% similarity to the IPI-O11 variant despite cycling through the potato hosts and the occurrence of the listed amino acid substitutions. Only classes I and II but not class III of IPI-O were detected in a subset of 2009 Irish *P. infestans* population in a preliminary study based on sequencing of PCR products of primers flanking the *ipiO* gene (Appendix 4.1).

Maximum likelihood analysis of the sequences using a codon based Muse-Gaut model indicated no significant deviation (P≤0.05) from the null hypothesis of strict neutrality implying mutations in *ipi*O (including the sites that showed amino acid substitutions) occurred at the same rate as the synonymous substitutions (Table 4.2). Similarly, pair wise comparisons of each recovered isolate and the corresponding original isolate for adaptive change using the method Nei-Gojobori method (Nei and Gojobori, 1986) revealed no significant deviation from the null hypothesis of strict neutrality (Table 4.3).

#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
AA	N	A	D	E	K	R	S	L	R	G	D	Y	N	N	E	V	T	K	E	P	N	T	S	D	E	E	R	A	F	S	I	S	K	S	A	E	Y	V	K	M	V	L	Y	G	F	K	L	G	F	S	P	R	T	Q
CW13C_A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MP100_4A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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DL43B_A1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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#	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107
AA	S	K	T	V	L	R	Y	E	D	K	L	F	T	A	L	Y	K	L	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	G	S	V	F	F	N	R	F	K	K	W	Y	D	K	N	V	G	P	S
CW13C_A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MP100_4A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	A	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MP100_5A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MP100_6A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MP100_9A2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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DL43B_A1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MP100_5A1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
MP100_6A1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
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DES4A1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.
DESWTA1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Fig. 4.5: RXLR effectors (*ipiO*) of original *P. infestans* isolates DL43B_A1 and CW-13C_A2 compared to thirteen isolates after ten passages through seven potato hosts with or without a race non-specific blight resistance *RB* gene from *S. bulbocastanum*. Positions of amino acids (AA) coding the effector protein are numbered 1 to 107 and the amino acids highlighted in orange indicate polymorphism. 6-11 RSLRGD motif; 24-27 dEER motif and 65-98 C-terminal domain

Table 4.2: Codon-by-codon[a] Maximum Likelihood analysis for adaptive selection on *Ipi*O gene sequences of *P. infestans* isolates after ten passages through potato hosts with or without the transgenic *RB* resistance gene.

Codon#	Syn (s)	Nonsyn (n)	Syn sites (S)	Nonsyn sites (N)	dS	dN	dN-dS	P-value
72	1	3	1.00	2.00	1.00	1.50	0.50	0.59
77	1	0	0.84	2.16	1.19	0.00	-1.19	1.00
89	0	1	0.64	1.74	0.00	0.57	0.57	0.73
90	1	0	0.63	1.75	1.59	0.00	-1.59	1.00
98	1	0	0.63	2.22	1.59	0.00	-1.59	1.00

[a]For each codon, estimates of the numbers of inferred synonymous (s) and nonsynonymous (n) substitutions are presented along with the numbers of sites that are estimated to be synonymous (S) and nonsyonymous (N). A positive value for the test statistic indicates an overabundance of nonsynonymous substitutions. Maximum Likelihood computations of dN and dS were conducted using HyPhy software package (Kosakovsky Pond *et al*., 2005). The analysis involved 91 nucleotide sequences. There were a total of 107 positions in the final dataset. Analyses were conducted in MEGA5 (Tamura *et al*., 2011).

Table 4.3: Pair wise comparison of adaptive change between original isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans* and the corresponding isolates after ten passages through potato lines with or without the resistance *RB* gene

Control isolate	**Passaged isolate**	**p-distance**	**S.E.**
CW1-3C_A2	A2_MP100_4	1.000	0.000
CW1-3C_A2	A2_MP100_6	1.000	-0.656
CW1-3C_A2	A2_MP100_9	1.000	-0.641
CW1-3C_A2	A2_MP100_5	1.000	-1.599
CW1-3C_A2	A2_Maris Peer	0.155	1.018
CW1-3C_A2	A2_Desiree	0.155	1.018
CW1-3C_A2	A2_DES RB4	0.155	1.018
DL4-3B_A1	A1_DES RB4	1.000	-0.651
DL4-3B_A1	A1_MP100_9	1.000	-0.886
DL4-3B_A1	A1_MP100_5	1.000	-0.429
DL4-3B_A1	A1_Maris Peer	1.000	-0.651
DL4-3B_A1	A1_Desiree	1.000	-0.651

Values of P less than 0.05 are considered significant at the 5% level. The test statistic (dN - dS) was computed for each pair compared. The variance of the difference was computed using the bootstrap method (10000 replicates). Analyses were conducted using the Nei-Gojobori method (Nei and Gojobori, 1986). The analysis involved 14 consensus nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 107 positions in the final dataset. Analyses were conducted in MEGA5 (Tamura *et al*., 2011).

4.4.3 Analysis of genetic distance

A phylogenetic tree (Fig. 4.6) inferred from genetic distances using the Close-Neighbour Interchange algorithm indicated that the isolates recovered after cycling isolate CW1-3C_A2 through transgenic potato lines MP100_4, MP 100_5 and MP100_6 were the most genetically distant from the original uncycled isolates. However, contrary to expectation, the rest of the recovered isolates did not delineate with the respective original isolates (Fig. 4.5).

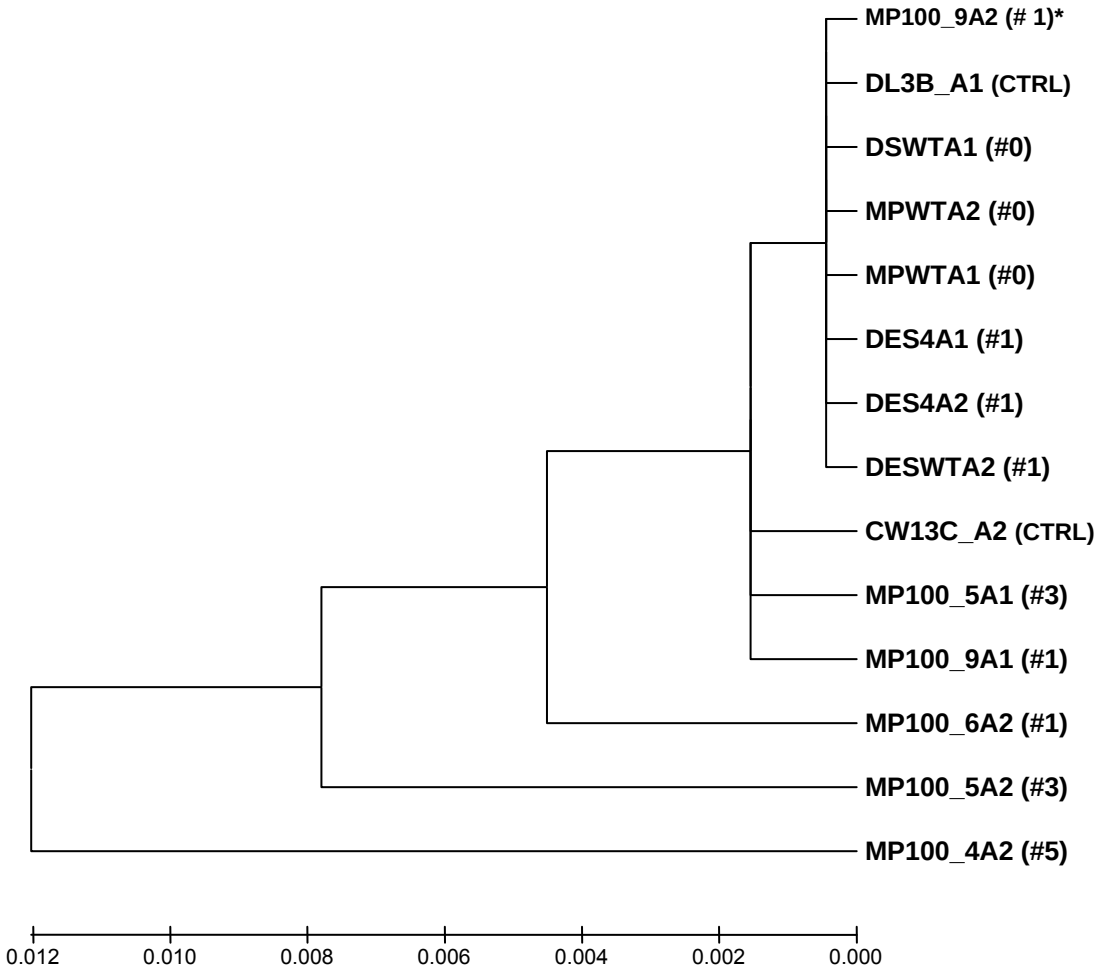


Fig. 4.6: Genetic distance between *P. infestans* isolates after ten passages through hosts with or without blight non-race resistance *RB* gene. The tree was drawn to scale, with branch lengths in the same units as those of the genetic distances based on DNA sequences used to infer the phylogenetic tree

(Rzhetsky and Nei, 1992) in MEGA5 (Tamura *et al*., 2011). Parenthesis **(#n**) denotes the corresponding number of *RB* inserts.

4.4.4 Post-selection assessment of *P. infestans* isolates for aggressiveness on cultivar Cara

Isolates recovered from the passaging trial were grown on potato discs of cultivar Kerr's Pink to produce inoculums for infecting leaflets of cultivar Cara. Some of the isolates had reduced ability to colonize host tissue and therefore did not produce sufficient inoculum to permit subsequent aggressiveness on cultivar Cara and were therefore excluded from the experiment. (Appendix 4.2 shows the ability of recovered isolates to infect and proliferate on tuber discs of cultivar Kerr's Pink)

An estimate of gain or loss of aggressiveness in cycled isolates relative to the original isolates DL4-3B_A1 and CW1-3C_A2 was determined by their latent period and the AULPC recorded on detached leaflets of cultivar Cara 4.2.7 (Table 4.4). Data showed no significant differences ($P< 0.37$) among isolates with respect to the latent period which ranged from mean values of 2.72 for MP100_6A1 to 3.00 for MP100_6A2. However, significant differences ($P< 0.001$) were observed among isolates regarding the size of lesion they caused on infected leaflets as measured by AULPC which, when averaged across the two experiments, varied from 1210 to 2335 for MP100_6A2 and DL4-3B_A1 respectively (Table 4.4; Appendix 4.3). Similarly, AULPC values of individual isolates varied significantly ($P< 0.001$) between the experiments. Separation of means using this test indicated that with the exception of isolates DL43B_A1 and MP100_6A2 on the extreme high and low ends of ranking by AULPC respectively, the AULPC values differed marginally among the rest of the isolates (Table 4.4). Notably, isolates (MP100_4A2, MP100_9A1, MP100_5A2, MP100_6A1 and MP100_6A2) cycled through transformed Maris Peer lines induced smaller lesions than MPWT_A1 cycled through un-transformed Maris Peer.

Table 4.4: Mean AULPC and LP values of 12 isolates inoculated onto leaflets of cultivar Cara in two replicate experiments to assess their aggressiveness after ten passages through potato lines bearing the blight resistance *RB* gene

Isolate (n=12)	**[a]Mean AULPC**	**Mean LP**
[b]DL43B_A1 (**CTRL**)*	2335a	2.92a
[c]CW13C_A2 (**CTRL**)	1657bcd	2.81a
MPWT_A1 **(#0**)	1995ab	2.83a
DESWT_A2 **(#0**)	1892abc	2.75a
DESR4_A2 **(#1**)	1838abc	3.00a
MP100_9A2 **(#1**)	1822abcd	2.92a
MP100_4A2 **(#5**)	1625bcd	2.83a
MP100_9A1 **(#1**)	1623bcd	3.00a
MPWT_A2 **(#0)**	1577bcd	3.00a
MP100_5A2 **(#3**)	1444bcd	3.00a
MP100_6A1 **(#1)**	1357cd	2.72a
MP100_6A2 **(#1)**	1210d	3.00a
Isolate	(P<0.001)	(P=0.19)
Experiment	(P=0.11)	(P=0.22)
LSD (P=0.05)	353.5	0. 35

[a]Values of the grand mean followed by the same letter do not differ significantly (P=0.05). [b,c] original isolates which were not cycled through host were included in assessment as controls. *Parenthesis **(#n)** denotes the corresponding number of *RB* inserts.

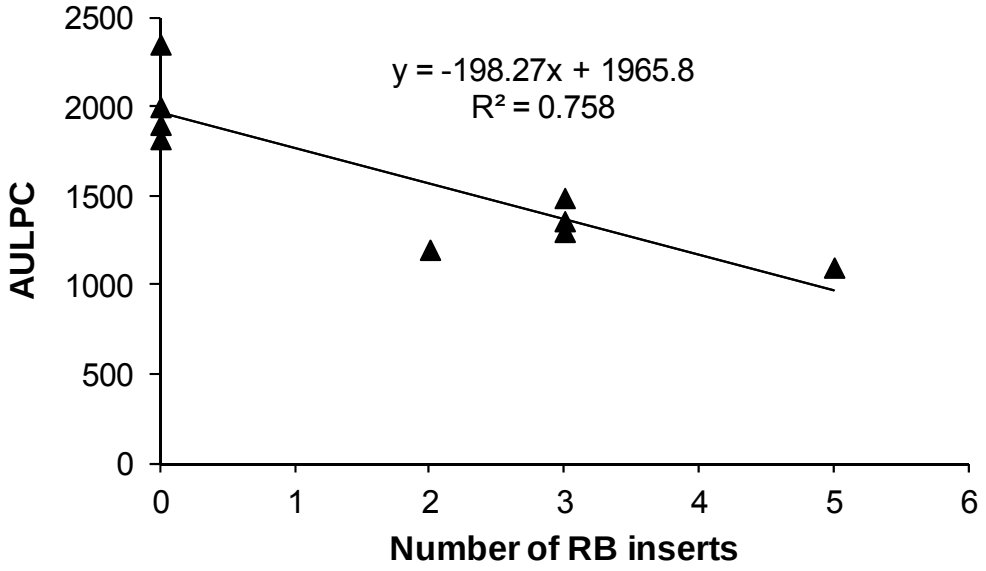


Fig. 4.7: Number of blight resistance (RB) gene inserts plotted against respective mean values of area under lesion progressive curves for respective host induced by *P. infestans* on cultivar Cara following ten cycles of cycling

Values of AULPC (Y-axis) for the respective potato lines plotted against the number of *RB* inserts (X-axis) (Fig. 4.7) showed a highly linear (p <0.002) correlation between the two variables and 75.8% of variation could be accounted for by the model. There was a corresponding decrease in disease quantity with increase in the number of inserts of the resistance gene (*RB*) implying a dosage effect on host resistance.

Additionally, utilizing as baselines the AULPC values of the un-cycled isolates in this experiment, percentage change in aggressiveness of cycled isolates was determined (Fig. 4.8). Results show that passing isolate CW1-3C_A2 repeatedly through untransformed cultivar Desiree, transgenic Desiree line with a single copy of *RB* or transgenic line MP100_9 also with a single copy insert of *RB* led to 14.18%, 10.92% and 9.96% increase in AULPC respectively. However there was 4.83%, 12.85%, 26.98% or 1.93% decrease in AULPC following ten clonal generations on untransformed cultivar Maris Peer, and on transgenic lines MP100_5, MP100_6, or MP100_4 respectively. Similarly,

14.56%, 30.49% or 41.88% decrease in AULPC was noted following cycling of DL43B_A1 repeatedly through MPWT, MP100_9, or MP100_6 respectively (Fig. 4.8).

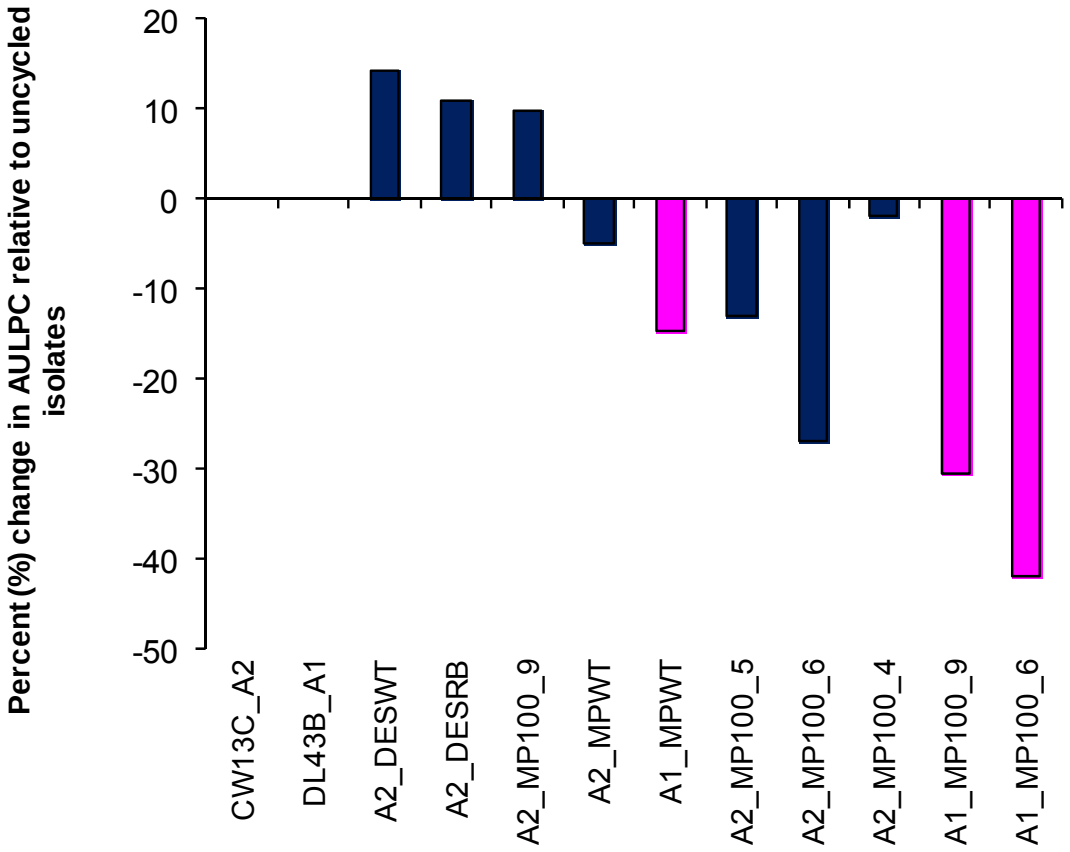


Fig. 4.8: Percent increase/decrease in AULPC on cultivar Cara for cycled isolates relative to un-cycled CW1-3C_A2 and DL4-3B_A1 isolates. AULPC values for un-cycled isolates DL4-3B_A1 and CW1-3C_A2 of *P. infestans* obtained on cultivar Cara in same experiment as the passages isolates was used as baseline in each respective case.

4.5 Discussion

One of the primary challenges in regard to using host resistance to tackle late blight disease is the ability of the pathogen to rapidly adapt to newly released resistant cultivars and to render their resistance ineffective (McDonald and Linde, 2002). The discovery of the *RB* resistance gene (Song *et al*., 2003) that confers broad-spectrum resistance boosted efforts towards quick deployment of host resistance against *P. infestans*. Since *RB* confers the disease-rate-limiting form of host resistance rather than immunity, the pathogen can complete multiple clonal generations on the host. Hence the evolutionary pressure on the pathogen is not as great as would be experienced with complete immunity.

However, the long-term implication of deploying this novel resistance gene into commercial breeding programmes is the likely adaptation of the pathogen and eventual breakdown of host resistance (Fry, 2008). An investigation into potential *RB* breakdown was approached by monitoring changes in aggressiveness data of two *P. infestans* clonal isolates of the opposite mating types during and after repeated exposure to hosts bearing one, three and five copies of inserts of *RB* gene. Additional insights were provided by the molecular data on mutation in the *RB* compatible IPI-O effector of the investigated isolates.

Varied outcomes were discerned from the virulence responses of the two *P. infestans* isolates during continuous interactions with the *RB* gene bearing hosts. Firstly, there was a decline in the disease by both isolates on transgenic line MP100_4 with five copy inserts of the resistance gene, which ultimately resulted in defeat of isolate DL4-3B_A1 in all three replicates in the fifth cycle of inoculation on this host. Secondly, the study showed infection and induction of expanding and sporulating lesions by both isolates on hosts bearing single or three copies of *RB* gene. The fluctuation in pathogenicity of the isolates over the cycles could be attributed to host physiological conditions including slight differences in the age of the leaflets.

One possible explanation to account for the occurrence of disease on *RB* bearing hosts is delayed or ineffective triggering of the hypersensitive response (HR) (Vleeshouwers *et al*., 2000) or its inactivation (Chen *et al*., 2012). Chen *et al*., (2012) have shown data to demonstrate disruption of the *RB* function by IPI-O effectors through interaction with the *RB* coiled coil (CC) domain.

From the host perspective, several aspects are known to contribute to the efficacy of *RB* mediated resistance. Wendt *et al*., (2012) demonstrated that *RB* transcriptional activity in transgenic lines challenged with *P. infestans* increased and peaked at 5 days following inoculation with the disease agent and further that transcription was abundant in transgenic lines with multiple inserts compared to single or no insert.

Crucially, levels of blight resistance in transgenic lines conferred by *RB* correlates with transcript levels (Kramer *et al*., 2009). Further evidence suggesting involvement of additional factors, including native promoters, the *RB* gene has higher basal transcription and transcript accumulation upon inoculation with *P. infestans* in its native *S. bulbocastanum* than in potato transgenic lines (Kramer *et al*., 2009). Additionally, the *RB* confers strong partial resistance in the form of disease rate reducing phenotype which is different from immunity (Song *et al*., 2003). As such, delayed or ineffective HR is inevitable and allows escape and proliferation of pathogen hyphae which are necessary for establishment of the biotrophic phase of parasitism (Vleeshouwers *et al*., 2000).

The present study implies a decrease in pathogenicity of both isolates of *P. infestans* on the potato hosts as the *RB* inserts increase. This result is in agreement with earlier findings which reported an association between the level of resistance and the number of *RB* inserts (Bradeen *et al*., 2009).

However, the expressed phenotype still contrasts with the complete defense typical of *S. bulbocastanum* accessions and which is thought to result from different *RB* genes working in cooperation and aided by native promoters (Chen and Halterman, 2011; Kramer *et al*., 2009).

This study has also provided further insights into the effect of continuous exposure of *P. infestans* to *RB*-mediated resistance by assessing the aggressiveness of isolates recovered after ten clonal generations on transgenic material. Obtained data (via detached leaf assay) showed that despite completing ten generations on resistant hosts, in comparison with their respective baseline AULPC values (original un-cycled isolates), the isolates produced smaller disease lesions when inoculated onto detached leaflets of non-*RB* bearing moderately resistant cultivar Cara. Evidence exists to support pathogenicity to *RB* by *P. infestans* (Forch *et al*., 2010; Fry, 2008). The reported cases of elevated pathogenicity of *P. infestans* on hosts carrying *RB* have involved laboratory trials on isolates with variants of IPI-O that are not recognized by *RB* (Champouret *et al*., 2009; Halterman *et al*., 2010). It is conceivable that under high selection pressure analogous to what is simulated in this study, *P. infestans* activates extra effectors of host-unrecognizable configuration (e.g. CRNs) to defeat the previously tolerant host (Haas *et al*., 2009).

Sequence analysis of the isolates recovered after ten clonal generations on the *RB*-carrying potato hosts revealed occurrence of amino acid substitutions in the IPI-O protein. In all cases however, the recovered isolates had the same IPIO-11 variant belonging to class I (Champouret *et al*., 2009). This result contradicts a recent study that found no mutation in IPI-O following twenty passages of a single isolate of *P. infestans* through a potato line carrying *RB* (Halterman and Middleton, 2012).

It is instructive to note that in the current study, the amino acid changes in the IPI-O protein occurred in the carboxyl terminal portions of the effectors known to be responsible for activation or suppression of host cell death executed within the host cytoplasm (Liu *et al*., 2011; Oh *et al*., 2009). Unsurprisingly, these amino acids have previously been shown to be responsible for polymorphisms in IPI-O variants among *P. infestans* (Champouret *et al*., 2009). This indicates that they are under diversifying selection and possibly involved in changes in virulence. Champouret *et al*., (2009) has already linked the variation in this region among the IPI-O family of effectors to virulence against *RB*.

Either reduction or increase in pathogen pathogenicity is a possible outcome of mutation. Consequently, it is logical to assert that that exertion of selection pressure on multiple sites of the effector coding sequences is likely to result in forms of mutation associated with fitness penalty on the pathogen (Leach *et al*., 2001). While mutations appear randomly, it has been hypothesized that mutation from avirulence to virulence persists in the fittest isolates only (Pilet *et al*., 2005). It seems from the present data that as few as ten clonal generations of *P. infestans* cycled through a transgenic potato bearing *RB* are sufficient for mutation involving multiple amino acids in the IPI-O to occur. In other systems however, relatively longer periods are required for detection of mutation although silent mutations may be occurring in the interim (Gaut *et al*., 1993).

Pathogenicity data on cycled isolates obtained from the lesion sizes they induced on cultivar Cara may be indicative of the ability of these isolates to complete at least several clonal generations on potato hosts including those with intermediate resistance to blight. Virulence genes occur in natural pathogen populations and it is an established fact that these genes are maintained principally by mutations but regulated by a multiplicity of factors including sexual recombination and parasexual processes (Day, 1978).

It is probable that when *P. infestans* completes multiple generations on a host with partial resistance under field conditions, the pathogen can undergo mutations at higher rates than observed on detached leaflets in the present study.

The fact that mutation in *P. infestans* occurs so rapidly poses immense challenges to breeding for cultivars with durable resistance. Several studies have demonstrated the capacity of *P. infestans* to overcome host resistance following an extended period of colonization (Wastie, 1991). And depending on the set of circumstances, a range of outcomes have been achieved in various investigations on *P. infestans* adaptations to potato hosts. For instance it has been shown that *P. infestans* is able to adapt and acquire virulence to cultivars with partial resistance in foliage and tubers (Bjor and Mulelid, 1991; Carlisle *et al*., 2002). Seemingly the distribution of virulent strains tends to fit patterns of the most common genotype at local and regional scales (Montarry *et al*., 2008). And so monitoring of *P. infestans* populations at local and regional levels for strains with virulence against novel R genes becomes a necessary component in the prediction of durability of host resistance.

Attempts at prediction of the performance of a host resistance phenotype prior to cultivar release have been aided a great deal by the advent of the concept of effector genomics (Vleeshouwers *et al*., 2008). For previously, durability of resistance could be confirmed only after a resistant cultivar was deployed over considerably large cropping areas for several years. Now, it is possible to explore *P. infestans* populations for the prevalent effector variants and apply these data for decision making in spatio-temporal deployment of the *R* gene form of resistance (Vleeshouwers *et al*., 2011).

For instance, monitoring of *P. infestans* populations during the period between 2006-2008 in the Netherlands has found isolates with virulence on the new R gene, *RB* (*Rpiblb1*) and *Rpiblb2* (Forch *et al*., 2010). Employing a detached leaflet assay, the study also demonstrated that genetic background can reduce the frequency of isolates with virulence against *Rpiblb1* or *Rpiblb2* and that only

a single isolate was virulent on a potato line with pyramided *Rpiblb1* and *Rpiblb2* (Forch *et al*., 2010).

Helpfully, despite the rapidly changing pathogen population, biological stability of resistance to *P. infestans* lasting at least four years can be achieved through conventional breeding (Tatarowska *et al*., 2011). It is clear that conventional breeding techniques are not adequately suited to the need for a more rapid release of cultivars with resistance to *P. infestans.* For example a four-decade-long effort to exploit sources of resistance in wild *Solanum* species finally yielded two resistant cultivars Bionica and Toluca in 2005 (Haverkort *et al*., 2009).

Alternatives that continue to gain currency include pyramiding of *P. infestans* resistance genes to confer durable and broadspectrum resistance using conventional breeding and or transgenesis (Tan *et al*., 2010; Zhu *et al*., 2011). Importantly, biotechnology-based tools are increasingly finding utility in these renewed efforts to generate new cultivars with resistance to blight in tandem with the rapidly adapting *P. infestans* (Mullins *et al*., 2006). An illustration of these is the recent research work that utilized host germplasm responses to a set of RXLR effectors to finally determine that the resistance of cultivar Sarpo Mira is based on four pyramided qualitative *R* genes and a quantitative *R* gene for field resistance (Rietman *et al*., 2012). Whereas conventional breeding is arduous, a quicker and more efficient delivery of cultivars with stacked novel *R* genes by genetic engineering is now a reality (Zhu *et al*., 2011). However, large scale deployment of transgenic cultivars has to withstand challenges arising from legislative hurdles and public sensitivity to transgenesis (O'Brien and Mullins, 2008). A form of genetic modification based on cisgenesis that essentially involves generation of germplasm by transfer of genes between two crossable species has been advocated in an attempt to justify exemptions from the strict requirements of Directive 2001/18/EC of the European Union (Jacobsen, 2008).

For instance, a cisgenesis approach based “project to create a potato with **D**urable **R**esistance against ***P****hytophthora*: DuRPh” is ongoing in the Netherlands and envisages delivering stacked *R*-genes in various combinations. Further, the DuRPh project proposes a deployment strategy to spatially and temporally separate the cultivars comprising the various combinations of stacked *R*-genes thus promoting their durability (Haverkort *et al*., 2009).

Chapter Five

Towards the development of a real-time PCR technique for the quantitative detection of *Phytophthora infestans* DNA in asymptomatic potato tubers

5.1 Background

Changes in the population structure of *P. infestans* in Ireland pose dramatic challenges to disease management strategies. As previously detailed in chapter 3, such changes have an effect on disease epidemiology particularly in regards to the threat of oospore production and survival in the soil. Secondly, aggressive strains infect more host foliar tissue in the field which increases the chance of sporangia reaching the soil to cause tuber blight. In turn, infected tubers become a source of inoculum for the next blight epidemics (Andrivon, 1995).

Therefore, the detection of infected tubers is a major step towards controlling blight epidemics. The simplest method for detecting blighted tubers is by visual examination but this can fail to arrest the seed lots with low frequency or latent forms of infection by late blight. Such tubers pose an even greater risk of spreading blight than those with severe infection which typically just rot away before they have the opportunity to spread disease.

PCR-based techniques, on the other hand, provide levels of sensitivity unattainable with visual assessment. Conventional PCR assays have been developed employing a variety of approaches to uniquely detect the DNA of *P. infestans* and related species. These approaches include those based on the use of satellite DNA sequences occurring in tandem repeats (Niepold and Schober-Butin, 1995), mitochondrial DNA (Forster *et al*., 1990) and ribosomal DNA (rDNA) (Maniatis *et al*., 2002). In fact, compared to mitochondrial DNA, the internal transcribed spacer (ITS) region of the rDNA has been used more often in the on-going search for better *P. infestans* detection assays. For example, a PCR assay based on ITS 2 of rDNA of *P. infestans* and *P. erythroseptica* detected blight in tubers 72 hrs after inoculation although the tubers showed no blight symptoms (Tooley *et al*., 1998). Another assay (Tooley *et al*., 1997) also based on the ITS 2 of rDNA was able to discriminate between three *Phytophthora* species pathogenic to potato (*P. infestans, P. erythroseptica and P. nicotianae*). Species-specific assays based on ITS rDNA have been

published describing the detection of blight in tubers and soil (Hussain *et al*., 2005; Wangsomboondee and Ristaino, 2002).

Conventional PCR-based techniques are, however, prone to failure for the detection of target DNA in very small quantities. Hussain *et al*., (2005) for instance used nested PCR to improve the sensitivity of their assay and achieved detection limits of 5fg of *P. infestans* DNA in the second round of amplification with the same primers whereas they could only detect 500fg in the first round PCR. Nested PCRs are time consuming and prone to error as a result of multiple steps of sample handling. Based on criteria such as sensitivity, specificity and time taken to complete the assay, virtually all the conventional PCR techniques fail to meet the requirements of an ideal technique for routine testing of seed quality under commercial settings.

Quantitative real time PCR (qPCR) techniques on the other hand easily fulfill these essential requirements. They are fast and allow for simultaneous detection and quantification of target DNA (Martin *et al*., 2000). The most widely used qPCR technologies are SYBR Green and TaqMan® (Bustin, 2005). A SYBR Green real time qPCR assay with sensitivity of 2pg was described for monitoring *P. infestans in planta* (Llorente *et al*., 2010) based on primers (Eschen-Lippold *et al*., 2007). These primers detect highly repetitive sequences from the *P. infestans* genome (Judelson and Tooley, 2000), but the specificity of this assay remains unknown not having been performed against other *Phytophthora* species. The detection system used in SYBR Green qPCR is based on fluorescent intercalating dyes which are monitored in real time at every amplification cycle (Becker *et al*., 1996). In contrast, the detection system of TaqMan® qPCR is based on dual-labeled probes specifically designed to bind to the DNA sequence. A fluorescent signal is generated at each amplification cycle when the polymerase hydrolyzes the probe (Whitcombe *et al*., 1999). A real time qPCR assay using TaqMan® technology for *P. infestans* based on primers that detect the ITS1 of *Phytophthora* species described (Lees *et al*., 2012) was reported to detect down to 100fg of *P. infestans* DNA and to be

sufficiently sensitive to quantify pathogen propagules in tubers 24 hrs after inoculation but could not detect sexual inocula in soil samples.

Comparative phylogenetic analyses of *Phytophthora* species based on ITS1 and ITS2 regions (Cooke *et al*., 2000) and different coding genes of nuclear and mitochondrial encoded cytochrome (Kroon *et al*., 2004) sequences suggest that in some circumstances, ITS fail to discriminate between closely related taxa. Indeed, the assay described by Lees *et al*., (2012) was specific to *P. infestans* and not the closely related but non-potato pathogens *P. mirabilis, P. phaseoli* and *P. ipomea*. Comparatively, introns and intergenic portions of the genome are sufficiently variable and may be useful targets for development of diagnostic tools with improved specificity (Schena and Cooke, 2006).

The objective of this chapter was to explore efforts towards the development of a qPCR technique based on primers flanking an intron of the *Ypt*1 gene and employing TaqMan® technology to try and detect *P. infestans* in symptomless tubers. This research built on a study by Jonathan Thompson (Thompson, 2008) carried out as part of his doctoral research supervised by Dr Louise Cooke (AFBI, Belfast, Northern Ireland) in which the coding primers of the *Ypt1* sequence of *P. infestans* were identified (with the assistance of Caoimhe Fleming-Archibald, Queen's University, Belfast) and their specificity and sensitivity tested.. For the qPCR assay developed here, sensitivity of the assay was evaluated using DNA samples extracted using standard extraction procedures in comparison to a rapid low cost DNA extraction procedure. The results in this chapter are discussed in light of the previously reported procedures for detection of *P. infestans* including the qPCR assay of Lees *et al.,* (2012) which was published when this work was near completion and with which there are a number of similar features.

5.2 Materials and methods

5.2.1 *P. infestans* isolates

P. infestans isolates DL4-3B_A1 (6_A1), DL12-5B_A1 (5_A1), DL1-5A_A1 (8_A1) and CW1-3C_A2 (13_A2), were retained from studies in the preceding

chapters of this thesis and maintained on pea agar cultures for use in studies described in this chapter.

5.2.2 Potato material

Cultivar British Queen was selected based on its high tuber blight susceptibility. Initial seed tubers were acquired from the Teagasc Potato Breeding and Seed Programme. To produce a sufficient supply of tubers free of blight for this study, well sprouted seed tubers were planted in plastic pots containing potting mixture and maintained in the glasshouse at 18-23^{o}C with natural lighting and regular watering for three months before the tubers were subsequently harvested.

5.2.3 Primers and probe design

Primers and probe design were based on the single copy *Ypt*1 gene whose deduced amino acid sequence predicts a polypeptide with features typical of GTP-binding proteins of the Ras family of proteins. These proteins are essential for vesicle transport of material between the endoplasmic reticulum and cis-Golgi compartments (Chen and Roxby, 1996) during secretion or cell wall synthesis at the hyphal tips. The *Ypt*1 gene has been shown to have a high level of polymorphism essential for development of markers for nearly all *Phytophthora* species (Schena and Cooke, 2006). Using the criteria such as melting temperature, GC content and self-complementarity, the actual primer and probe design was performed using Primer3 software (Rozen and Skaletsky, 2000) on the nucleotide sequence for the *Ypt*1 gene retrieved from the NCBI database. Primer sequences were forward primer: 5' TGT CTA ACA TAT TTT ACG CCA 3' and reverse primer: 5' ACA AGA CGA GCG CAC CTA TC 3' with an amplification product of 359bp and the TaqMan® probe: 5' AGG TCT AGA TTG CCA TTA CA 3'. The probe was labeled at the 5'-end with a fluorescent reporter dye 6-FAM (6 carboxy-fluoroscein), while the 3'-end was modified with a Minor Grove Binding (MGB) molecule (Applied Biosystems).

5.2.4. DNA extraction from isolate CW1-3C_A2 of *P. infestans*

Approximately 50mg of *P. infestans* mycelia were harvested from 10-day-old cultures of isolates DL4-3B_A1, DL12-5B_A1, DL1-5A_A1 and CW1-3C_A2

growing on pea agar (Hollomon, 1965) using sterile scalpels. The harvested mycelia were transferred into 2ml Eppendorf tubes, freeze dried for 24 hrs and pulverized using a mixer mill with sterile glass beads. DNA extraction was performed on the pulverized samples using the procedure of Raeder and Broda (1985) described in section 4.2.3. The extracted DNA in each sample was quantified using a fluorescent Qubit DNA Assay (Invitrogen, USA) and standardized to 25ng prior to use in PCR reactions. The extracted DNA was stored at -20^{o}C and thawed on ice prior to use in subsequent reactions.

5.2.5 Optimization of primer annealing temperature using gradient PCR

A gradient PCR was performed to determine the optimal annealing temperature for the designed primers. The reactions were performed in 20µl reactions consisting of 25ng genomic DNA from isolate CW1-3C_A2 as template, 1X Thermal Buffer (Promega), 0.2mM dNTP, 0.3µM of each forward and reverse primers and 1.5U of Taq DNA polymerase (Promega). The PCR reactions were performed on a Biometra Thermo cycler (USA) with an initial denaturation of 10 min at 95^{o}C followed by 35 cycles for 30 sec at 95^{o}C, 30 sec at 58-65^{o}C and 40 sec at 72^{o}C, with a final 5 min at 72^{o}C. PCR products were loaded onto 1% (w/v) agarose gel stained with ethidium bromide (1µg/ml) submerged in 0.5M TBE buffer. A 100bp ladder (NEB, UK) was loaded alongside the samples and the gel run for 30 min at 70V. Bands were visualized and the image captured using Kodak Imager (Image Station 440 CF, Kodak Digital Science^{TM}, USA).

5.2.6 Amplification of the Ypt1 gene in representative isolates of different genotypes of *P. infestans*

Once the optimum annealing temperature of the primers was determined, PCRs were performed to assess the primers for their capability to detect the target sequence in representative isolates (DL4-3B_A1, CW1-3C_A2, DL1-5A_A1 and DL12-5B_A1) of the four most prevalent genotypes of *P. infestans* in Ireland. Separate experiments were conducted for each isolate using the conditions described in section 5.2.5 with an annealing temperature of 60^{o}C. In each reaction 25ng of template DNA of an appropriate isolate was used. Post

amplification visualization of amlicons was carried out as described in section 5.2.5.

5.2.7 Optimization of the primer and TaqMan probe conditions

To determine the optimal primer and probe conditions for qPCR, primer concentrations (200, 250, 300, 350nM) and TaqMan® probe concentrations (150, 200, 300, 350nM) were evaluated in separate experiments on a LightCycler® 480 II platform (Roche Applied Science, Germany) using the thermocycling program of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min in a final reaction volume of 25µl (Tables 5.1 and 5.2). In each reaction of either primer or probe concentration optimization, 1ng of template DNA from isolate CW13C_A2 was used. The optimized assay was used for detection of *P. infestans* propagules in artificially inoculated tuber tissues.

Table 5.1: qPCR components for primer optimization to detect *P. infestans*. The first column shows the components of each qPCR, first row shows combination of forward and reverse primer concentrations (nM) in each reaction respectively, values in rows 2-7 are volumes (µl) of reaction component. Row 8 shows reaction volumes of combined reaction components for each technical replicate.

component	200/ 200	200/ 250	200/ 300	200/ 350	250/ 200	250/ 250	250/ 300	250/ 350	300/ 200	300/ 250	300/ 300	300/ 350	350/ 200	350/ 250	350/ 300	350/ 350	No primer
UMMix	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
FD Primer	0.5	0.5	0.5	0.5	0.63	0.63	0.63	0.63	0.75	0.75	0.75	0.75	0.88	0.88	0.88	0.88	0
RV Primer	0.5	0.63	0.75	0.88	0.5	0.63	0.5	0.88	0.5	0.63	0.75	0.88	0.5	0.63	0.75	0.88	0
Probe	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Template	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Water	9.87	9.74	9.62	9.49	9.74	9.61	10.63	9.36	9.62	9.49	9.37	9.24	9.49	9.63	9.24	9.11	11.87
Total	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0

Table 5. 2: qPCR components for probe optimization to detect *P. infestans*. The first column shows the components in each qPCR, first row shows probe concentrations (nM) evaluated, values in rows 2-7 are volumes (µl) reaction components. Row 8 shows reaction volumes of combined reaction components for each technical replicate.

component	150	200	250	300	350	No probe
UMMix	12.5	12.5	12.5	12.5	12.5	12.5
FD* Primer	1.5	1.5	1.5	1.5	1.5	1.5
RS Primer**	1.5	1.5	1.5	1.5	1.4	1.5
Probe	0.44	0.5	0.63	0.75	0.88	0
Template	1	1	1	1	1	1
Water	8.06	8.0	9.37	7.75	7.72	8.5
Total	25	25	25	25	25	25

*FD-forward primer; **RS-reverse primer

5.2.8 *Ypt*1 gene amplification and purification

The *Ypt*1 gene was PCR amplified from genomic DNA of isolate CW1-3C_A2 of *P. infestans* and later isolated for downstream application in the generation of standard curves. PCRs were performed in a 40µl volume containing 25ng of standardized (25ng/µl) DNA, 1X Thermal Buffer (Promega), 0.2mM dNTP, 0.3µM of each forward and reverse primers and 1.5U of Taq DNA polymerase (Promega). The PCR reactions were performed on a Biometra Thermo cycler (USA) with an initial denaturation of 10 min at 95^{o}C followed by 35 cycles for 30 sec at 95^{o}C, 30 sec at 60^{o}C and 40 sec at 72^{o}C, with a final 5 min at 72^{o}C. PCR products were loaded onto 1% (w/v) agarose gel stained with ethidium bromide (1µg/ml) submerged in 0.5M TBE buffer. A 100bp ladder (NEB, UK) was loaded alongside the samples and the gel run for 30 min at 70V. Bands were visualized and the image captured using Kodak Imager (Image Station 440 CF, Kodak Digital Science^{TM}, USA). Once amplified, the PCR product bands were extracted and purified using the procedures described in section 4.2.4.

5.2.9 Cloning *Ypt*1 gene into *E. coli* with pGEM-T® Easy vector system, DNA extraction and sequence confirmation of stable integration

Extracted DNA was ligated into pGEM-T® Easy vector (Fig. 4.1) following the manufacturer's instructions (Promega, Madison, USA) and procedures described in section 4.2.5. Ligated products were used to transform chemically competent *E coli* Top 10 cells (Invitrogen, USA), using the procedure described in section 4.2.5. Colonies of transformed *E. coli* cells were picked for plasmid DNA extraction using a mini-prep procedure described in section 4.2.6. Stable presence or absence of the *Ypt*1 gene was confirmed by PCR and subsequent sequencing. For sequence confirmation, eight plasmid DNA samples (Pi-01-M13, Pi-02-M13, Pi-03-M13, Pi-04-M13, Pi-05-M13, Pi-06-M13, Pi-07-M13 and Pi-08-M13) were sequenced in one direction using vector specific primer M13 (Fig. 4.1). Sequencing was performed at GATC (Germany) and obtained sequences were analyzed for similarity with *Ypt*1 gene sequence on the NCBI data base using the BLAST facility (Altschul *et al*., 1997).

5.2.10 Detection limits and reference standards for quantification of propagules of *P. infestans* in samples

Recovered DNA from sample Pi-08-M13 was quantified using a fluorescent Qubit DNA Assay (Invitrogen, USA) following the manufacturer's instructions. DNA in the sample was standardized to 10^{10} molecules/µl (See section 5.3.1 for copy number determination). Ten-fold serial dilutions were performed on the sample to yield 10^6 to 10^1 *Ypt*1 molecules per µl. To generate a standard curve, qPCR was performed for each dilution in MicroAmp Optical 96-well Plates in a LightCycler® 480 II (Roche Applied Science, Germany). Each reaction had a total volume of 25µl containing 300nM of forward primer, 300nM of reverse primer, 250nM of the probe, 12.5µl of Taqman® Universal Master Mix (Applied Biosystems), 1 µl of sample and 7.87µl of molecular grade water (Sigma, Aldrich). The thermal cycling was: denaturation at 95^{o}C for 10 min, followed by 40 cycles of denaturation at 95^{o}C for 15 sec and annealing and extension at

60°C for 1 min. Fluorescence signal acquisition was set at 60°C in each amplification cycle. Three technical replicates were performed for each reaction.

5.2.11 Inoculation of tuber discs with *P infestans*

Tubers harvested from 13 week old plants of cultivar British Queen grown in the glass house provided tuber discs (8mm diameter x 2mm thick) which were used for inoculation with *P. infestans*. Isolate CW1-3C_A2 of *P. infestans* was used to produce pathogen inoculum by growing it *in vitro* on pea agar in a 9cm diameter Petri dish for ten days followed by harvesting sporangia in 2 ml of sterile distilled water. Prior to inoculation, the suspension was filtered through two layers of cheese cloth and the resulting suspension observed under a light microscope for quantification of sporangia. The concentration was adjusted to 25,000 sporangia/ml followed by stimulating them to produce zoospores by incubating at 4°C for 2 hours. Six tuber discs were prepared for each of the five time points [1 days post inoculation (dpi), 2dpi, 3dpi, 4dpi, 5dpi]. Inoculation was performed by depositing a single droplet of 10µl at the centre of an individual tuber slice disc placed in inverted 9 cm Petri dishes lined with moist Whatmann filter papers. Six un-inoculated tuber slice discs were placed in a Petri dish lined with Whatmann filter paper to serve as controls. Petri dishes containing tuber slice discs were wrapped in clear polythene bags and incubated at 18°C for between 1 and five days (n=36).

5.2.12 Extraction of DNA from tuber discs inoculated with *P infestans*

After incubation, the tuber discs from each time point were separated into two duplicate sets of three tuber discs one for each of the two extraction procedures. In the first method, fresh tuber disc samples were ground in a mortar using a pestle and the resulting paste was used in subsequent steps of DNA extraction using a GenElute™ Plant Genomic DNA MiniPrep (Sigma Aldrich) commercial kit following the manufacturer's instructions.

In the second method, fresh tuber slice samples were homogenized by grinding in a 0.5M NaOH lysis solution contained in a mortar. Grinding was performed

using a pestle and a volume of 1ml of 0.5M NaOH was used to every 10mg of fresh tuber slice sample. The resulting homogenates were transferred into 15 ml tubes centrifuged at 4,000 rpm for 20 min and the supernatants transferred into fresh tubes before 1 in 1000 dilutions were obtained per respective sample.

5.3 Data Analysis

5.3.1 Absolute Quantification and standard curves

Crossing point (Cp) values were determined by the in-built LightCycler® 480 SW 1.5 software (Roche Applied Science, Germany). Cp is the cycle number at which the concentration of the target DNA exceeds the threshold and is dependent on its initial concentration. Using the absolute quantification and Fit Point methods, a standard curve was derived which is a linear regression line through plotted data points with log of initial target DNA concentration on the X-axis and Cp in cycles on the Y-axis. The slope of the standard curve provided the efficiency (E) of the amplification reactions (following $E= [(10^{(-1/slope)})-1] \times 100$.

The standard curve generated in the step above was utilized in the LightCycler® 480 SW 1.5 software as an external standard to measure quantities of target DNA (*Ypt*1) in the samples in all subsequent reactions. However, a reaction with a template of 10^3 molecules of *Ypt*1 was included in each run to act as reference to facilitate 'domestication' of the external standard curve. Values of Cp and initial concentration of template in each sample were obtained at the end of each experiment. Copy number for qPCR was based on the following formula: Weight in Daltons (g/mol) = [(bp size of plasmid + insert) (330 Da x2 nucleotides/bp)]. Hence copy number (g/molecule) =g/mol/Avogadro's number (6.02×10^{23}). Plasmid size was 3015 bp and size of *Ypt*1 gene insert was 359bp. Additional analyses and visual presentation of the data were performed in Windows Excel (Microsoft, USA). Where experiments did not require quantification of target DNA in the sample, Cp values were collected and their means were used to perform comparisons among treatments.

5.4 Results

5.4.1 Optimal annealing temperature for the *Ypt*1 gene-specific primers

Qualitative PCR performed at thermocycling conditions involving an annealing temperature ranging from 58°C to 65°C produced bands of the predicted size (359bp) according to the sequence of the *Ypt*1 gene (Fig. 5.1, and Fig. 5.4). Faint bands of the predicted size were produced by reactions carried out at annealing temperatures of 62.5°C (lane 11) and 63°C (lane 12) but annealing temperatures above 63°C led to no amplicon production (Fig. 5.1).

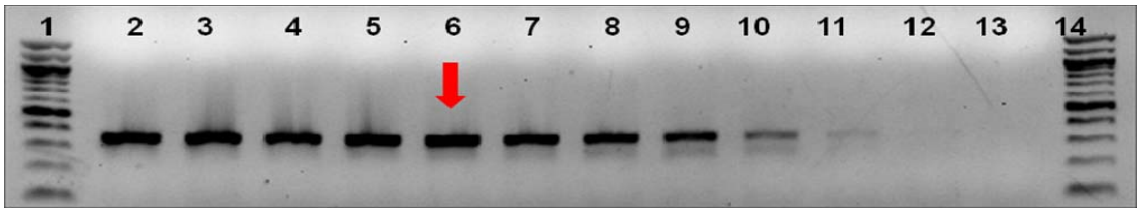


Fig. 5.1: Agarose analysis of gradient PCR to determine the optimal annealing temperature for the *Ypt*1 specific primer pair. Lanes 1 and 14 show 100bp molecular ladder (NEB, UK). Lanes 2 to 14 show presence or absence of bands of predicted product (359bp) from reactions with annealing temperatures ranging from 58°C to 65°C respectively. Red arrow indicates a band from reaction with an annealing temperature of 60°C which was considered optimum and used in subsequent reactions.

5.4.2 Detection of *Ypt*1 gene in four isolates of *P. infestans* with differing genotypes

Amplicons of the expected size (359bp) were observed in each PCR with template DNA from representative isolates DL4-3B_A1, CW1-3C_A2, DL1-5A_A1 and DL12-5B_A1 for each of the four genotypes of *P. infestans* assessed 6A_1, 13_A2, 8_A1, 5_A1 respectively (Fig. 5.2).

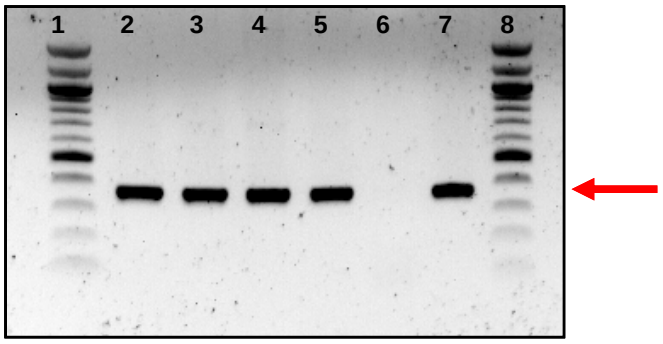


Fig. 5.2: Agarose analysis of PCR amplicons of the *Ypt*1 gene. Amplification was performed using the designed primers specific to the *Ypt*1 gene. Lanes 1 and 8 show a 100bp molecular ladder (NEB, UK). Lanes 2, 3, 4 and 5 are isolates DL4-3B_A1 (6A1), CW1-3C_A2 (13A2), DL1-5A_A1 (8A1) and DL12-5B_A1 (5A1). Lane 6 is a non-template control and lane 7 is DNA derived from *E. coli* transformed with pGEMT-Easy carrying *Ypt*1 gene (sections 5.2.9). Red arrow indicates amplicon size (359bp).

5.4.3 Optimization of primer concentrations for *Ypt*1 gene amplification

Manipulation of primer concentrations in the qPCR produced significant differences ($P<0.01$) in the Cp thresholds. Mean Cp values ranged from 23.48 to 23.81 the former value was achieved in reactions in 350nM was used for both forward and reverse primers while the later was obtained in reactions in which forward and reverse primer concentrations were 200nM and 300nM respectively. Although the reproducibility of detection of target DNA across replicates was comparable for all combinations of primer concentration tested as indicated by the Cp values (Table 5.3), 300nM was deemed to be the most appropriate concentration for both forward and reverse primers (mean Cp 23.48) and was used in subsequent qPCRs. Additionally, reactions with similar concentrations for both primers are easier to setup.

Table 5. 3: Crossing points (Cp) during qPCR amplification of template target gene *Ypt*1 of *P. infestans* using different primer concentrations

Forward/reverse primer concentration	Mean Cp*	
200F/300R	23.81	
300F/250R	23.75	
200F/200R	23.71	a
250F/250R	23.67	b
200F/250R	23.67	b
300F/200R	23.64	
300F/300R	23.48	c
350F/250R	23.46	c
250F/200R	23.43	d
350F/200R	23.42	d
250F/350R	23.42	d
200F/350R	23.34	e
250F/300R	23.32	e
300F/350R	23.26	
350F/300R	23.21	
350F/350R	23.14	
Mean	23.48	
Reverse Primer (P<0.01)		
Forward Primer (P<0.01)		
ForwardxReverse (P<0.01)		
LSD (P=0.05)	0.01	

*Values are mean Cp values of three technical replicates for each respective primer combination; Replicated non-template reactions were included as control. No amplification in reactions without template DNA, no values for Cp recorded.

5.4.4 Optimization of probe concentration for real-time qPCR detection of *P. infestans*

Increasing the probe concentration led to a significant increase (P<0.01) in fluorescence obtained as replicated in decreased Cp values. However, keeping the cost of the assay in perspective and despite the fact that there were significant differences (P<0.01) between probe concentrations (Table 5.4), 250nM was deemed appropriate and used in subsequent qPCRs.

Table 5. 4: Crossing points (Cp) during qPCR amplification of template target gene *Ypt*1 of *P. infestans* using different probe concentrations

Probe concentration	Mean Cp	
150nM	24.76	
200nM	24.42	
250nM	24.17	a
300nM	23.72	b
350nM	23.71	b
Mean	24.15	
Concentration (P<0.01)		
LSD (P=0.05)	0.02	

5.4.5 PCR verification of stable integration of *Ypt*1 in *E. coli*

E. coli cells in white colonies growing on LB medium amended with 100µg/ml of ampicillin following plating after transformation were presumed to have acquired the vector or both the vector and the *Ypt*1 gene insert. However, a preliminary PCR test using cells for each white colony directly as template was performed in order to eliminate false positives (white *E. coli* colonies bearing vector but not *Ypt*1 gene inserts). Of the 13 colonies selected for PCR with *Ypt*1 specific primers, all produced a band of the expected size (359bp), confirming the presence of the *Ypt*1 target sequence (Fig. 5.3).

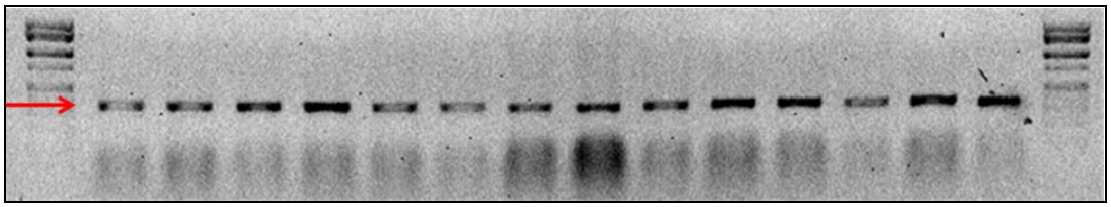


Fig. 5.3: Post cloning confirmation of *P.* infestans *Ypt*1 gene inserts by PCR in which cells obtained from white colonies of *E. coli* growing on LB+ampicillin selective medium were used as template. Red arrow indicates 359bp product. From left, first and last lane show 100bp ladder. Lanes 2-15 show bands confirming presence of *Ypt*1 gene inserts in the respective *E. coli* colony sampled.

5.4.6 Sequencing confirmation of the *Ypt*1

The sequencing of the eight plasmid DNA samples from these colonies identified an identical sequence of 450 bases. Sequence alignment with the *Ypt*1 gene sequence retrieved from the NCBI data base under accession number DQ162961.1 (Schena and Cooke, 2006) using CLUSTAL W software (Chenna *et al*., 2003), gave 100% homology against a consensus of the amplified sequence (Fig. 5.4).

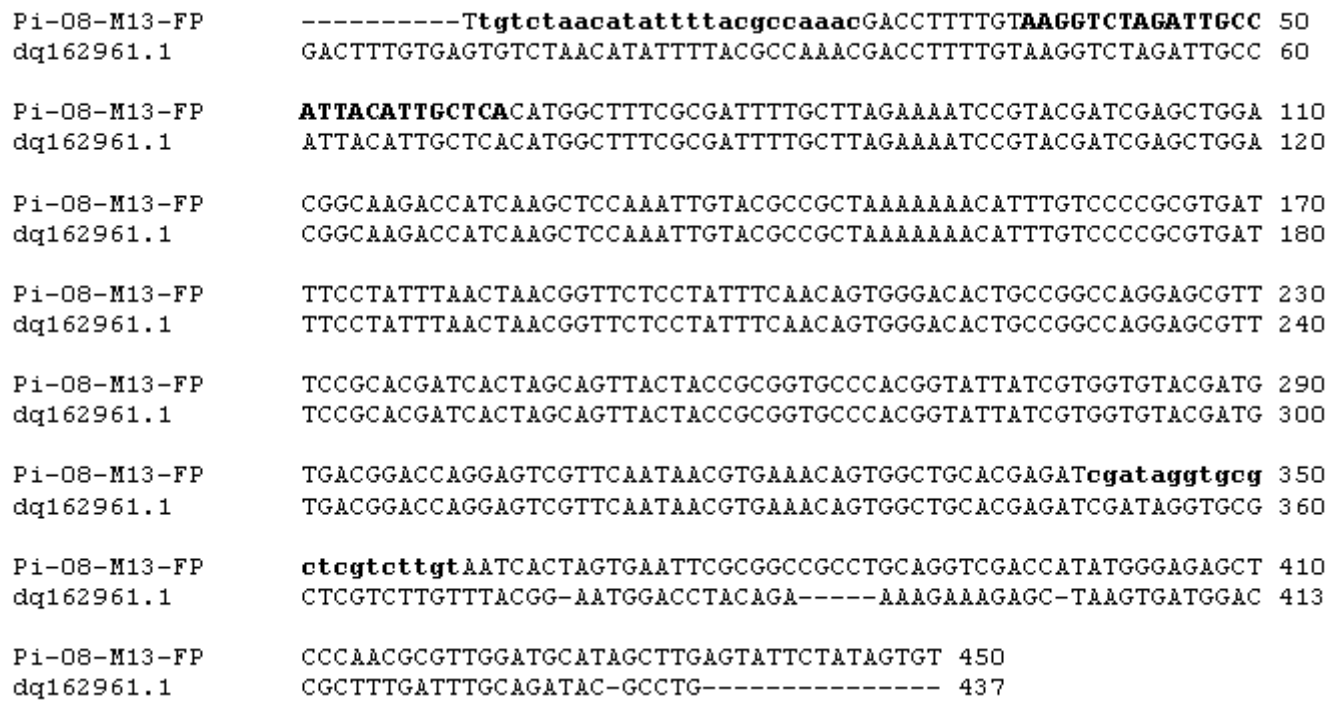


Fig. 5.4: Sequence amplified with pUC/M13 forward sequencing Primer from clone Pi-08-M13-FP aligned with sequence for *P. infestans Ypt*1 gene under accession number DQ162961.1 (Schena and Cooke, 2006) NCBI database. Alignment was performed using CLUSTAL W (Chenna *et al*., 2003) multiple sequence alignment program. Bold lower case in the sequence shows the position of forward and reverse primers used to amplify the *Ypt*1 gene. Bold upper case shows the position of the probe sequence.

5.4.7 Testing the sensitivity of qPCR assay on a cloned sequence target

The linear regression delivered automatically from assessment of the standard curves by the Fit Point Method implemented in the LightCycler® 480 SW 1.5 program (Roche Applied Science, Germany) showed that with every increase in the quantity of the initial target plasmid DNA with *Ypt*1 sequence in the qPCR, there was a corresponding and reproducible decrease in the number of amplification cycles needed to reach the threshold for detection (Cp values) of *Ypt*1 sequence. Similarly, the high linearity (R^2=0.99) of Cp for standard curves plotted against log concentration (y axis plotted against x axis) imply a strong positive correlation between the initial concentration of target DNA and threshold of *Ypt*1 gene detection (Fig. 5.5). The efficiency of the qPCR calculated from the slope of the standard curves was (E>95%) (Fig. 5.5).

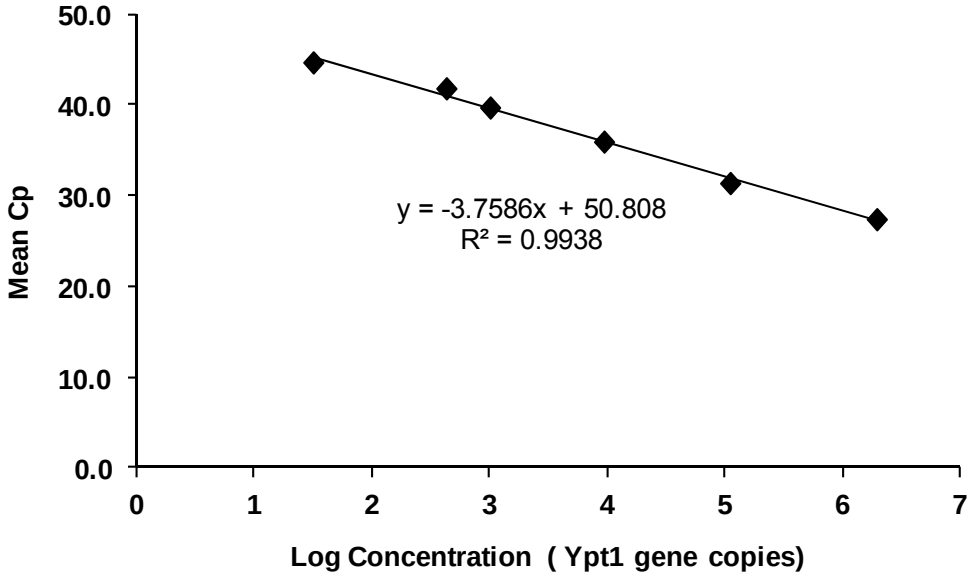


Fig. 5.5: Mean Cp values of standard curves plotted against Log concentration in qPCRs with plasmid *Ypt*1 DNA of *P. infestans* as template. Serial dilutions were performed to yield samples with final concentrations ranging from 10^6 to 10^1copies of *Ypt*1 prior to qPCR.

5.4.8 Effect of the DNA extraction method on sensitivity of the qPCR for *P. infestans* detection

The described qPCR technique was capable of detecting propagules of *P. infestans* in potato tuber discs beginning at 2 days post inoculation (dpi) when DNA was extracted from the samples using a GenElute commercial kit (Sigma Aldrich). When DNA was extracted using the NaOH based protocol, the assay permitted detection of *P. infestans* beginning at 3 dpi. The quantity of the *Ypt*1 genes detected increased progressively each day thereafter ranging from 25 to 300 copies of the *Ypt*1 gene at 2 dpi and 5dpi respectively for samples extracted using the GenElute kit. In comparison, *Ypt*1 gene copy numbers detected in tuber slice samples extracted using the NaOH based procedure ranged from 27 to 200 at 3 and 5dpi respectively. Therefore, irrespective of the length of incubation of the sample after inoculation, the qPCR detected higher quantities of *P. infestans* DNA in samples extrated using the GenElute Kit than using the NaOH based procedure (Fig. 5.6). These results demonstrate that the qPCR technique described is capable of detecting target *P. infestans* DNA (*Ypt*1 gene) down to 25 copies (93.3fg) in artificially infected tuber tissues extracted using the GenElute commericial kit.

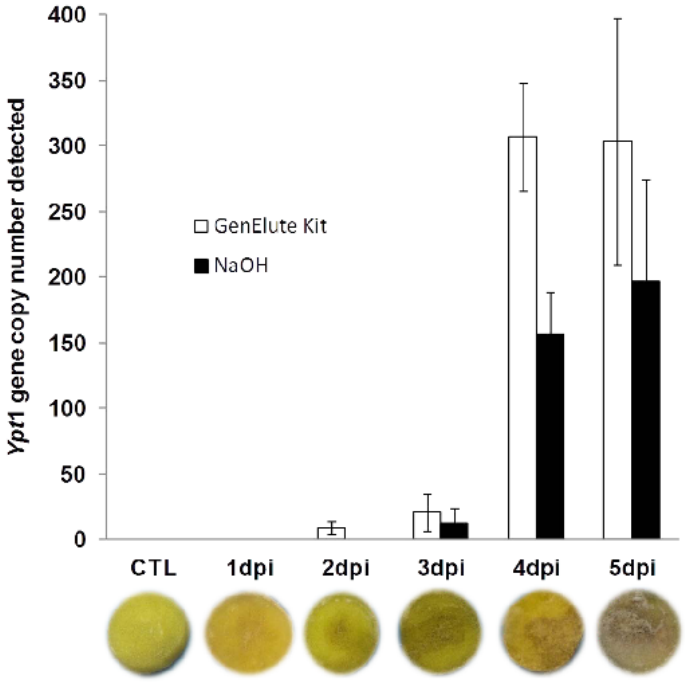


Fig. 5.6: Copy numbers of *Ypt*1 gene detected in DNA samples extracted from tuber slices following 1 to 5 days of incubation post inoculation (dpi) with *P. infestans* isolate CW1-3C_A2. Error bars are standard deviations on means of three biological replicates of each sample. The discs at the bottom are representative tuber slice samples showing the progression of *P. infestans* within the tissues starting from 1 day of incubation after inoculation (1dpi) through to 5dpi. CTL was an un-inoculated tuber disc included as control.

5.5 Discussion

As well as acting as a source of inoculum for epidemics in a new potato crop, the escape of infected asymptomatic tubers through trading channels contributes to the incursions by novel strains of *P. infestans* into new territories. For that reason, early detection of infected tubers in seed lots reduces not only the incidence of blight epidemics instigated by infected seed tubers but also the risk of dispersal of new *P. infestans* strains.

Previous techniques for the detection of *P. infestans* in infected tubers have reported varied levels of sensitivity. Tooley *et al.,* (1997) reported detection of *P. infestans* using ITS based primers in samples 72 hours after artificial inoculation. In another study, Hussain *et al.,* (2005) improved the sensitivity of their technique from detection of 0.5pg to 5fg of *P. infestans* when they used the products from previous amplifications as templates in subsequent PCRs. In yet another technique, detection of 10pg of target *P. infestans* DNA was achieved based on randomly amplified DNA (RAPD) markers (Kim and Lee, 2002). Although, each of these methods demonstrated detection of *P. infestans* at relatively small quantities, all of them are time consuming and have low reproducibility. Additionally, they do not support high throughput application.

In the present study, successful detection of *P. infestans* in plasmid DNA samples and artificially inoculated tubers was demonstrated using the qPCR based assay. The results of this assay are in accordance with those of the qPCR method described by Lees *et al.,* (2012) who achieved detection of *P. infestans* at the lowest limit of 100fg on tuber samples just 24 hours after they were artificially inoculated. Comparatively, both the detection assay described in this chapter and the qPCR method of Lees *et al.,* (2012) gave better sensitivity compared to the SYBR Green qPCR method of Llorente *et al.,* (2012) who achieved the detection limit of 2pg of *P. infestans* DNA.

Being a TaqMan based real time PCR assay, the detection technique for *P. infestans* described in this chapter offers several advantages: (i), the assay lends itself to high throughput applications like routine testing of seed stocks for latent infection of *P. infestans* following additional work. This is because it permits relatively rapid analysis and doesn't require post amplification handling including gel visualization of amplicons. (ii), unlike SYBR Green based techniques which have a chelating fluorescent dye that may indiscriminately bind on dsDNA present in a sample, the specificity of TaqMan® assay is ensured by exclusive binding of the probe to the target DNA sequence. (iii), the detection system of the described assay is based on primers flanking an intron of the *Ypt*1 gene specific to *P. infestans.* As a result, it has more potential for specificity compared with detection methods based on ITS regions that fail to discriminate between *P. infestans* and other *Phytophthora* species (Cooke *et al*., 2000; Schena *et al*., 2006).

It is foreseeable that in the practical applications of this assay to seed testing, the probability of detecting infection by *P. infestans* in potato tubers will be dependent on the size of the seed-lot, the number of tubers in the seed lot with infection and the number of tubers sampled for assessment.

Due to time constraints, these aspects were not investigated during this study. There is, however, ample evidence in the literature about the relationships between each of these variables and the sensitivity of blight detection using molecular techniques. For example, it has been shown that to achieve a 90% probability of detecting a single blight infected tuber when 5 tubers are infected in a seed-lot of 400 tubers, 147 tubers are to be sampled.

And if 40 tubers are blight infected in the same size of seed-lot, a sample of 24 tubers is sufficient to achieve 90% probability of detecting a single blight infected tuber (Wangsomboondee and Ristaino, 2002).

The second aspect with significant effect on detection is the method of DNA extraction adopted. The present study has shown that better detection results were achieved with a commercial kit compared to the in-house prepared lysis buffer NaOH for DNA extraction.

The findings of this study support the view that use of this rapid extraction method can be adopted for use on samples which are incubated further at conditions favourable to blight so as to facilitate build-up of pathogen load to compensate for the drop in sensitivity. This is a precautionary measure that should eliminate the risk of arriving at a false negative result for a seed lot with infected tubers whose pathogen load is below the detection threshold.

Several considerations warrant attention during practical application of the technique for commercial seed testing. Principally, the possibility of identifying seed lots as infected when the *P. infestans* propagules detected are not viable or do not reach the threshold inoculums density for transmission or symptom development.

Where as it may be acknowledged that it is dicey trying to determing the ‘safe’ levels of a positive result, such seed lots should be used with caution in commercial potato crops and early fungicide applications should be adopted as a precautionary measure against blight outbreaks that may occur.

Due to time constraints, the qPCR assay described in this chapter was not tested on oospore samples. Detection of oospores of *P. infestans* in soil using previous molecular assays has proved to be more difficult compared to detection of vegetative propagules in infected tubers based on these procedures. Lees *et al.,* (2012) reported a lack of success with their method in the detection of oospores.

They attributed this failure to problems of DNA isolation from oospores. Wangsomboondee and Ristaino (2002) achieved detection of *P. infestans* on oospores samples previously picked individually and DNA extracted by either CTAB or NaOH lysis methods.

In addition, Hussain *et al.,* (2005) reported detection of oospores by a single round PCR on soil samples artificially inoculated with 12-15 oospores per 0.5g of soil. They further reported detection of 10 oospores in 0.5g of soil by nested PCR. So far, a technique for detection of oospores in naturally infected soils with reproducible results is still lacking. Progress towards this goal has been hampered by the low viability and germination rates of oospores in naturally infected soils (Pittis and Shattock, 1994).

Commercial kits are relatively expensive, especially in the context of commercial seed testing on a routine basis (Llorente *et al*., 2010). Estimation of the cost for a single qPCR run in 96 well plate suggests a €0.7 and €0.4 for samples using GenElute Kit and NaOH Lysis solution respectively (Appendix 5.1) Accordingly, extraction of DNA from samples using NaOH solution is not only faster but it is also cheaper compared to the GenElute commercial kit. However, it is probable that the residual tissue material in the sample post extraction using NaOH lysis solution impacts on the quality of the DNA requiring additional amplification cycles to achieve detection of target sequence.

Chapter Six

General Discussions and Conclusions

The over-arching objective of this study was to explore the potential impact of the novel A1 and A2 strains on the Irish *P. infestans* population and the resulting implications for potato blight management efforts. To undertake this, key tasks were executed to determine the genetic makeup of the prevailing *P. infestans* population and afterwards to investigate selected isolates for potential change through sexual recombination or host resistance associated adaptive change. Consequently, a subset of the isolates from a 2009 nationwide sampling was studied (chapter 2) to elucidate the population structure using simple sequence repeat (SSR) markers which revealed: (i) a moderate diversity within a population; (ii) two co-existing clusters which delineated with mating type as well as metalaxyl phenotypes and an (iii) on-going displacement of existing A1 clonal lineages.

In using SSR markers to explore population structure, the present study avoided the problems of limited resolving power associated with isozyme and RFLP techniques as used previously to study *P. infestans*. Besides allowing for comparison between samples at multiple loci, SSR markers are co-dominant and are not linked (Lees *et al*., 2006; Montarry *et al*., 2010). All of which are features that make these markers ideal for tracking the frequency of alleles or their segregation within populations. Furthermore, the evidence for enhanced genetic diversity within the Irish *P. infestans* population is supported by the consensus between three complementary approaches (i.e. Bayesian clustering, principal component analysis and analysis of relatedness based on Jaccard's distance co-efficients). Significantly, the change in the composition of mating types and metalaxyl phenotypes mirrors the changes observed in many countries in mainland Europe (Détourné *et al*., 2006; Gisi *et al*., 2011; Montarry *et al*., 2010) and the UK (Cooke *et al*., 2010; Cooke *et al*., 2008) over the past decade. In some instances, upsurge in metalaxyl insensitive strains has followed increased usage of metalaxyl fungicides leading to suggestions that selective pressure has led to adaptation by some genotypes which became dominant. The blue 13 genotype illustrates this best, because apart from being insensitive to metalaxyl, its aggressiveness to

potato foliage even at low temperatures means it is unlikely to be ousted by other less fit genotypes (Cooke *et al*., 2010). In the first three years since the occurrence in Ireland of the blue 13 strains, the initial outbreak of blight in commercial potato crops was in Mid June. In 2012 however, the first blight outbreak was reported in early May. Monitoring of blight epidemics at Oak Park in Ireland indicate that when blight outbreaks occur in June, severe crop losses of up to 80% in unsprayed plots usually follow where as mild crop losses result from delayed blight outbreaks (i.e. September) (Dowley *et al*., 2002; Griffin *et al*., 2002). Early blight outbreaks imply additional fungicide sprays at shorter intervals are required to give sufficient control of blight. However, as this may merely provide suitable conditions for further resistance selection when metalaxyl or other phenylamide fungicides are used, the stage appears set for renewed exploitation of host resistance to reduce fungicide usage (Naerstad *et al*., 2007). Indeed the data in chapter 2 of this thesis indicates that conventional resistant cultivars retain an unambiguous effectiveness against 'old' *P. infestans* genotypes in addition to restricting disease induced by some isolates with typical 'new' genotypes. This discrimination provides an opportunity for deployment of host diversity to reduce severity of the epidemics likely to be caused by the new *P. infestans* genotypes (Mundt, 2002) and alter the conditions that favour *in planta* oospore production (chapter 3). Currently, most cultivars grown in Ireland are susceptible to late blight, partly because there is a ready market for these cultivars. In fact, the rate of adoption of resistant cultivars by farmers is influenced to a great extent by the success of the new cultivars in the market. Lack of adoption of resistant cultivars constitutes a significant hindrance to the deployment of host resistance at an individual farm level. As an alternative, deliberate efforts are needed to alter cultivar composition of potatoes grown in the country in each season which will lead to host diversification at regional level while reducing the risks of large scale epidemics (Phillips *et al*., 2005).

An alternative response to the surge in insensitivity to metalaxyl with *P. infestans* in Ireland is through a changeover to fungicide formulations that do not select for

resistance in *P. infestans* under field conditions (Hollomon and Thind, 2011). Towards this effort, a standard protocol has been proposed for assessing new fungicide formulations for effectiveness to control foliar and tuber blight in Europe. This protocol takes into account and rates a product based on: Firstly, its effectiveness to control blight on potato foliage, new growth, stems, and tubers; secondly: its mode of action (whether it is a protectant, curative or antisporulant); three, its rain fastness; and four, its mobility within the plant (Bain, 2009). Based on these evaluations, a number of fungicide formulations including, mandipropamid, propamocarb-HCl+chlorothalonil and propamocarb-HCl+fluopicolide have shown effectiveness against potato blight under field conditions (Bain, 2009; Huggenberger and Knauf-Beiter, 2009).

Of the fungicides listed above, mandipropamid, a mandelic acid amide fungicide belonging to the carboxylic acid amide (CAA) class of fungicides has emerged as an alternative to metalaxyl by exhibiting effective control of foliar late blight (Cohen and Gisi, 2007). More importantly, evidence from mutagenesis of *P. infestans* for resistance to CAA and phenylamide indicates that all mutants that exhibited initial resistance to CAA later showed erratic and unstable resistance which diminished through one to eight asexual infection cycles and failed to grow on CAA amended medium. On the other hand mefenoxam (phenylamide) resistant mutants maintained stable resistance in both in vitro and *in planta* (Rubin *et al*., 2008). Similarly, forced selection using mandipropamid has failed to produce stable field resistance in *P. infestans* (Cohen *et al*., 2007).

Further evidence suggests that field selection for fungicide resistance is unique to phenylamides, all the other fungicide classes used to control *P. infestans* including cyanoactemide-oximes, benzamides, QiIs, QoIs, dinitroanilines, carboxylic acid amide (CAA) and carbamates do not select for resistance in *P. infestans* under field conditions (Kuck and Russell, 2006). This variation is derived from the mode of action of the fungicide product as well as the genetics of resistance to a particular fungicide. Fungicides with a site-specific mode of action are more

susceptible to pathogen resistance than those that target multiple sites (Levy *et al*., 1983). Whilst resistance to metalaxyl is conditioned by a locus with a major effect in *P. infestans* (Fabritius et al., 1997; Judelson, 1997a), resistance to CAA in *P. viticola* is conferred by recessive genes which would require multiple generations of genetic recombination to be fixed in a resistant strain of *P. infestans* (Gisi *et al*., 2007).

Having established the composition and genetic structure of the studied *P. infestans* population from Ireland, the next objective was to investigate the likelihood of sexual recombination to occur between the co-existing opposite mating types of recent genotypes and to ultimately establish the possibility of the resulting oospores to act as a primary source of inoculum and variation in *P. infestans.* Typically, outbreaks of blight from volunteers acting as the primary source of inoculum occur later in a growing season because the volunteers tend to establish after the main crop has fully emerged. For this reason, blight outbreaks early in the season have been taken to indicate the involvement of oospores in the Nordic countries where both mating types of *P. infestans* are present (Hannukkala *et al*., 2007). As indicated, the same conditions prevail in Ireland and therefore the results of a systematic assessment of the risk of sexual recombination become invaluable in the design of blight management strategies. Findings of the complementary *in vitro* and *in vivo* trials described in chapter 3 are significant as elaborated below.

Experimental data obtained from oospore biology in this study provide lucid evidence for genetic recombination in *P. infestans* and suggests its potential as a source of variation. A number of possible outcomes of this genetic recombination and their implications to agriculture in Ireland are considered. Firstly, there is likelihood for increased adaptive capacity of *P infestans* to existing potato hosts including resistant cultivars (Barton and Charlesworth, 1998; Heitman, 2006; Wills, 2003) as a result of genetic recombination and disruption of linkages between loci that exist in clonal populations (Anderson and Kohn, 1995) and secondly,

enhanced fitness in some of the genotypes due to genetic recombination (Gavino *et al*., 2000; Lee *et al*., 2002; Smart and Fry, 2001). Both outcomes impact adversely on durability of host resistance. However these mechanisms are mediated by oospores which must remain viable. Oospore viability is a necessary condition in their role of facilitating fixation of inherited traits in a population through initiation of blight epidemics in subsequent host crops and further recombination cycles.

In view of this it was logical to choose oospore germination rate as an ideal and objective parameter for making inferences about oospore associated risks. This approach has been used to assess risks of field resistance in pathogens against fungicide (Gisi *et al*., 2007). Yet, the objective of this study was satisfied by data obtained from the *F*1 generation therefore it was not necessary to track the fixation of traits which typically requires the completion of multiple reproductive generations of the studied organism. Other studies which investigated inheritance of metalaxl resistance in *P. infestans* in the *F*2 population have reported inheritance patterns consistent with Mendelian segregation patterns for the trait (Knapova *et al*., 2002). Theoretically each viable oospore from any sexual cross represents a unique genotype and a large number of novel genotypes are expected to occur when an equivalent number of oospores germinate. In contrast, the present study recorded a low number of unique genotypes of *P. infestans* from potato crops growing in an oospore infested polytunnel. These findings effectively highlight the likelihood of oospores to act as a source of primary inoculum. Additionally the results demonstrate a lack of abundance of novel genotypes occurring from recombination events. Furthermore, the study illustrates segregation patterns in foliar blight aggressiveness among the *F*1 progeny resulting in genotypes which are as fit or less fit compared to the parental aggressive genotypes.

Over the years, concerted efforts have gone into developing resistant potato cultivars as a way of coping with the potato blight menace. The drawback to these efforts has remained the ability of *P. infestans* to rapidly adapt and overcome the

newly released resistant cultivars. Durable resistance is now sought by isolating major genes from resistant wild tuber bearing potato species and incorporating them into cultivated potato (Haverkort *et al*., 2009; Wendt *et al*., 2012). Chapter 4 of this thesis investigated the potential for *P. infestans* to overcome a major resistance R-gene (*Rpiblb1*). This was undertaken by allowing two isolates of *P. infestans* to complete multiple clonal generations on transgenic potato host carrying the *Rpiblb1* gene and afterwards analyzing them for changes at the corresponding avirulence gene (IPI-O). The choice of a single host whose resistance is conferred by a single gene ensured a gene for gene interaction. This was considered crucial in light of the fact that resistance breaking events results are mediated at the gene level (Dangl *et al*., 1996). Most studies on pathogen adaptation on the host have involved many hosts or a single host with polygenic resistance (Andrivon *et al*., 2007). The downside to these approaches continues to be the lack of direct association between the observed changes and the source of variation.

While taking advantage of the direct interaction between the host resistance gene and pathogen avirulence gene, changes in the selected isolates were tracked. The size of the disease lesions induced on host leaflets at each clonal generation and changes in amino acids coding for the effector protein provided a suitable basis for estimating the changes in each isolate. Remarkably, each of the two isolates of *P. infestans* tested (DL43B_A1 and CW13C_A2) showed capacity to adapt to hosts carrying *Rpiblb1* within ten clonal generations of repeated exposure. Significantly, the presence of a single copy or two copies of the resistance *Rpiblb1* gene did not inhibit the development of blight disease caused by the investigated isolates. In contrast, the presence of five copies of the resistance *Rpiblb1* enabled the host to defeat or substantially reduce the amount of blight disease caused. In each of the two isolates, these changes were mediated by aminoacid substitutions occurring in the carboxylic terminal of the effector protein which has previously been shown to be prone to adaptive varation (Champouret *et al*., 2009). Additionally isolates of *P.*

infestans which had adapted to a host with a major gene displayed reduced fitness on a potato host lacking the particular major resistance gene.

Recent advances in molecular biology have contributed significantly to the isolation and cloning of blight resistance genes (Bradeen, 2011). The exploitation of these resistance genes will be boosted significantly by the availability of tools for predicting their durability prior to large scale release in agriculture. Profiling of prevalent avirulent gene classes within local and regional populations of *P. infestans* forms an essential part of these efforts and has already been undertaken (Vleeshouwers *et al*., 2011). The findings of the present study permit insights into dynamics of *P. infestans* adaptations that support the stacking of genes and alternation of hosts with different R genes in a cropping system to delay resistance breakdown.

Infected tubers form a significant source of blight epidemics. Over the years, the introduction of novel genotypes into regions where they were previously absent has been accomplished mainly through transfer of infected tubers (Shaw *et al*., 1985). Occasionally, the introduced strains have driven major shifts in the structure of local populations. For example available evidence indicates that the changes within the European populations of *P. infestans* in the mid 1970s were instigated by arrival of A2 mating types on the continent possibly through imported tubers (Fry *et al*., 1993). According to analysis revealed by recent investigations (Kildea *et al.,* 2010) and the current study, *P. infestans* populations in Ireland presently conform to patters of recent introductions of the new genotypes which are displacing the old genotypes. It is speculated that these migrant genotypes got into Ireland through potato seeds from continental Europe or the UK where these genotypes had previously been reported (Day et al., 2004). A reliable detection technique is required in order to curb spread of blight through infected tubers. Chapter 5 describes a sensitive real time quantitative PCR detection assay for *P. infestans* propagules in potato tubers. This technique offers possibilities for application to routine use in seed testing for tuber blight. The result achieved in this study in

regards to the disease detection of blight in symptomless tubers provides impetus to progress towards a commercially feasible assay for routine application in seed testing.

Future work

The questions which were posed at the beginning of this study have been addressed in chapters 2 to 5 of this thesis. However, the study has also opened up further questions which remain unanswered and should form the basis for further enquiry.

1. The present study demonstrated production of oospores under *in vitro* and the polytunnel environments and provided strong indications of oospore production under field conditions. These findings together with evidence from the assessment of the population structure and genetic diversity within *P. infestans* in Ireland provide a basis for field scouting for oospore production.

2. The *F*1 progeny from the *in vitro* and polytunnel crosses as well as isolates from repeated passaging through GM and non-GM potato lines were screened for aggressiveness on a single potato genotype, Cara. However, as different host genotypes (including those which are widely grown in Ireland like Rooster, Kerr's Pink and British Queen) are likely to respond differently to blight caused by such a diverse population of *P. infestans,* controlled studies need to be carried out to investigate presence or absence of host-pathogen interactions. Additionally, further research is required to assess the response of the various *P. infestans* genotypes emerging from sexual recombination to a range of fungicide formulations currently in use against late blight.

3. The *F*1 population of *P. infestans* generated in this study provides a bench mark upon which to base the tracking of effector diversity within the Irish population of *P. infestans.* A beginning point would be to elucidate the

contribution of genetic recombination to fitness in *P. infestans* by profiling the avirulence genes of the *F*1 individuals in comparison to the parental isolates.

4. Finally, further work needs to be carried out to test the efficacy of the described qPCR detection assay on naturally infected tubers and soils for oospore infestation.

Bibliography

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Anderson J.B., Kohn L.M. (1995) Clonality in soilborne, plant-pathogenic fungi. Annual Review of Phytopathology 33:369-391.

Andersson B., Sandström M., Strömberg A. (1998) Indications of soil borne inoculum of *Phytophthora infestans*. Potato Research 41:305-310.

Andrivon D. (1995) Biology, ecology, and epidemiology of the potato late blight pathogen *Phytophthora infestans* in soil. Phytopathology 85:1053-1056.

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

Andrivon D., Pilet F., Montarry J., Hafidi M., Corbière R., Achbani E.H., Pellé R., Ellissèche D. (2007) Adaptation of *Phytophthora infestans* to partial resistance in potato: evidence from French and Moroccan populations. Phytopathology 97:338-343.

Armstrong M.R., Whisson S.C., Pritchard L., Bos J.I.B., Venter E., Avrova A.O., Rehmany A.P., Böhme U., Brooks K., Cherevach I. (2005) An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. Proceedings of the National Academy of Sciences 102:7766-7771.

Arora R.K. (1989) Distribution of healthy and late blight infected tubers in potato ridges. Indian Journal of Mycology and Plant Pathology 19:232-233.

Bain R.A. (2009) Report of fungicide sub-group: Discusssion of potato late blight fungicides, their properties and ratings, PPO-Special Report No. 13. pp. 177-182.

Bain R.A., Möller K. (1999) Factors influencing potato tuber infection by *Phytophthora infestans*, in: H. Scheppers and E. Bouma (Eds.), Applied

Research for Arable Farming and Field Production of Vegetable., Lelystad, The Netherlands. pp. 210-227.

Bain R.A., Fairclough R.W., Holmes S.J., Ligertwood G.L. (1997) The role of *Phytophthora infestans* inoculum from stem lesions in the infection of tubers, in: E. Bouma and H. Schepers (Eds.), Proceedings of the Workshop on the European Network for the Development of an Integrated Control Strategy of Potato Late Blight. pp. 98-105.

Bajpai R., Yang F., Curran D.P. (2007) On the structure of the *Phytophthora* α1 mating hormone: synthesis and comparison of four candidate stereoisomers. Tetrahedron Letters 48:7965-7968.

Bartnicki-Garcia S. (1968) Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annual Reviews in Microbiology 22:87-108.

Barton N.H., Charlesworth B. (1998) Why sex and recombination? Science 281:1986-1990.

Becker A., Reith A., Napiwotzki J., Kadenbach B.A. (1996) A quantitative method for determining initial amounts of DNA by polymerase chain reaction cycle titration using digital imaging and novel DNA stain. Analytical Biochemistry 237:204-207.

Bednarek P., Kwon C., Schulze-Lefert P. (2010) Not a peripheral issue: secretion in plant-microbe interactions. Current Opinion in Plant Biology 13:378-387.

Berkeley M.J. (1948) Observations, Botanical and Physiological, on the Potato Murrain, Phytopathological Classica 8, American Phytopathological Society, Michigan, USA.

Birch P.R.J., Rehmany A.P., Pritchard L., Kamoun S., Beynon J.L. (2006) Trafficking arms: oomycete effectors enter host plant cells. Trends in Microbiology 14:8-11.

Bjor T., Mulelid K. (1991) Differential resistance to tuber late blight in potato cultivars without R-genes. Potato Research 34:3-8.

Black W. (1970) The nature and inheritance of field resistance to late blight (*Phytophthora infestans*) in potatoes. American Journal of Potato Research 47:279-288.

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

Bohs L. (2007) Phylogeny of the Cyphomandra clade of the genus *Solanum* (Solanaceae) based on ITS sequence data. Taxon:1012-1026.

Boller T., Felix G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology 60:379-406.

Bolstein D., White R., Skolnick M., Davis R. (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 3:314-331.

Bourke P.M.A. (1964) Emergence of potato late blight 1845-46. Nature 203:805-808.

Bourke P.M.A. (1993) 'The visitation of God'? The potato and the great Irish Famine. The Lilliput Press Ltd, Dublin, Ireland.

Bradeen J.M. (2011) Cloning of late blight resistance genes: strategies and progress, in: J. M. Bradeen and K. Chittaranjan (Eds.), Genetics, Genomics and Breeding of potato, CRC Press, New York, USA. pp. 153-183.

Bradeen J.M., Iorizzo M., Mollov D.S., Raasch J., Kramer L.C., Millett B.P., Austin-Phillips S., Jiang J., Carputo D. (2009) Higher copy numbers of the potato RB transgene correspond to enhanced transcript and late blight resistance levels. Molecular Plant-Microbe Interactions 22:437-446.

Brurberg M.B., Elameen A., Le V.H., Naerstad R., Hermansen A., Lehtinen A., Hannukkala A., Nielsen B., Hansen J., Andersson B., Yuen J. (2011) Genetic diversity of *Phytophthora infestans* populations in the Nordic

European countries reveals high genetic variability. Fungal Biology 115:335-342.

Bustin S.A. (2005) Real-time, fluorescence-based quantitative PCR: a snap shot of current procedures and preferences. Expert Review of Molecular Diagnostics 5:493-498.

Campbell C.L., Madden L.V. (1990) Introduction to plant disease epidemiology. John Wiley and Sons, New York.

Carlisle D.J., Cooke L.R., Watson S., Brown A.E. (2002) Foliar aggressiveness of Northern Ireland isolates of *Phytophthora infestans* on detached leaflets of three potato cultivars. Plant Pathology 51:424-434.

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

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

Caten C.E., Jinks J.L. (1968) Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variations. Canadian Journal of Botany 46:329-248.

Champouret N., Bouwmeester K., Rietman H., van der Lee T., Maliepaard C., Heupink A., van de Vondervoort P.J.I., Jacobsen E., Visser R.G.F., van der Vossen E.A.G., Govers F., Vleeshouwers V.G.A.A. (2009) *Phytophthora infestans* isolates lacking class I ipiO variants are virulent on Rpi-blb1 potato. Molecular Plant-Microbe Interactions 22:1535-1545.

Chen Y., Roxby R. (1996) Characterization of a *Phytophthora infestans* gene involved in vesicle transport. Gene 181:89-94.

Chen Y., Halterman D.A. (2011) Phenotypic characterization of potato late blight resistance mediated by the broad-spectrum resistance gene RB. Phytopathology 101:263-270.

Chen Y., Liu Z., Halterman D.A. (2012) Molecular determinants of resistance activation and suppression by *Phytophthora infestans* effector IPI-O. PloS Pathogens 8:e1002595.

Chen L.Q., Hou B.H., Lalonde S., Takanaga H., Hartung M.L., Qu X.Q., Guo W.J., Kim J.G., Underwood W., Chaudhuri B. (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. Nature 468:527-532.

Chenna R., Sugawara H., Koike T., Lopez R., Gibson T.J., Higgins D.G., Thompson J.D. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research 31:3497-3500.

Cohen Y., Gisi U. (2007) Differential activity of carboxylic acid amide fungicides against various developmental stages of *Phytophthora infestans*. Phytopathology 97:1274-1283.

Cohen Y., Rubin E., Hadad T., Gotlieb D., Sierotzki H., Gisi U. (2007) Sensitivity of *Phytophthora infestans* to mandipropamid and the effect of enforced selection pressure in the field. Plant pathology 56:836-842.

Collier S.M., Moffett P. (2009) NB-LRRs work a “bait and switch” on pathogens. Trends in Plant Science 14:521-529.

Colon L.T., Turkensteen L.J., Prummel W., Budding D.J., Hoogendoorn J. (1995) Durable resistance to late blight (*Phytophthora infestans*) in old potato cultivars. European Journal of Plant Pathology 101:387-397.

Cooke D.E.L., Lees A.K. (2004) Markers, old and new, for examining *Phytophthora infestans* diversity. Plant Pathology 53:692-704.

Cooke D.E.L., Drenth A., Duncan J.M., Wagels G., Brasier C.M. (2000) A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology 30:17-32.

Cooke D.E.L., Lees A.K., Shaw D.S., Bain R.A., Ritchie F., Taylor M. (2010) Survey of GB blight populations. Potato Council Final Report Agriculture and Horticulture Development Board.

Cooke D.E.L., Lees A.K., Shaw D.S., Taylor M.C., Prentice M.W.C., Bradshaw N.J., Bain R.A. (2008) The status of GB blight populations and the threat of oospores, Crop Protection in Northern Britain. pp. 217-222.

Cooke D.E.L., Young V., Birch P.R.J., Toth R., Gourlay F., Day J.P., Carnegie S.F., Duncan J.M. (2003) Phenotypic and genotypic diversity of *Phytophthora infestans* populations in Scotland (1995–97). Plant Pathology 52:181-192.

Cooke L.R. (1981) Resistance to metalaxyl in *Phytophthora infestans* in Northern Ireland, Proceedings of the Brighton Crop Protection Council Conference-Pests and Diseases. pp. 641-649.

Cooke L.R., Swan R.E., Currie T.S. (1995) Incidence of the A2 mating type of *Phytophthora infestans* on potato crops in Northern Ireland. Potato Research 38:23-29.

Cooke L.R., Carlisle D.J., Donaghy C., Quinn M., Perez F.M., Deahl K.L. (2006) The Northern Ireland *Phytophthora infestans* population 1998–2002 characterized by genotypic and phenotypic markers. Plant Pathology 55:320-330.

Cooke L.R., Little G., Armstrong C., Thompson J.M., Griffin D., Dowley L.J., Kildea S., Perez F.M., Deahl K.L. (2009) Recent changes in the *Phytophthora infestans* population in Northern Ireland and first results from a new all Ireland blight project. PPO-Special Report No.13:183-190.

Cooke L.R., Kildea S.,Mehennie-Cis, J., Quinn, L., Little G., Hutton F., Perez F.M., Deahl K.L. Griffin D. (2011) Ongoing changes in the Irish potato blight population. PPO-Special Report No.15:75-79.

Cooke L.R., Schepers H.T.A.M., Hermansen A., Bain R.A., Bradshaw N.J., Ritchie F., Shaw D.S., Evenhuis A., Kessel G.J.T., Wander J.G.N., Andersson B., Hansen J.G., Hannukkala A., Naerstad R., Nielsen B.J. (2011) Epidemiology and Integrated Control of Potato Late Blight in Europe. Potato Research 54:183-222.

Dangl J.L., Jones J.D.G. (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826-833.

Dangl J.L., Dietrich R.A., Richberg M.H. (1996) Death don't have no mercy: cell death programs in plant-microbe interactions. The Plant Cell 8:1793.

Davidse L.C. (1995) Phenylamide fungicides: biochemical action and resistance, in: H. Lyr (Ed.), Modern selective fungicides. 2nd edition, Gustav Fischer Verlag, Jena. pp. 347-354.

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

Day J.P., Wattier R.A.M., Shaw D.S., Shattock R.C. (2004) Phenotypic and genotypic diversity in *Phytophthora infestans* on potato in Great Britain, 1995–98. Plant Pathology 53:303-315.

Day P.R. (1978) The genetic basis of epidemics, in: J. G. Horfall and E. B. Cowling (Eds.), Plant Disease. An advanced Treatise. Vol 2. How disease Develops in popuations, Academic Press. pp. 263-285.

de Bary A. (1876) Researches Into the Nature of the Potato-fungus (*Phytophthora Infestans*). Journal of the Royal Agricultural Society of England 12:239-269.

Deahl K.L., Cooke L.R., Black L.L., Wang T.C., Perez F.M., Moravec B.C., Quinn M., Jones R.W. (2002) Population changes in *Phytophthora infestans* in Taiwan associated with the appearance of resistance to metalaxyl. Pest management science 58:951-958.

Détourné D., Duvauchelle S., Dubois L. (2006) The evolution of the population of *Phytophthora infestans* in France (Epidemiology and phenotypic markers), PPO-Special Report No. 11. pp. 115-155.

Dieringer D., Schlotterer C. (2003) Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. Molecular Ecology Notes 3:167-169.

Dodds P.N., Rathjen J.P. (2010) Plant immunity: towards an integrated view of plant–pathogen interactions. Nature Reviews Genetics 11:539-548.

Dorrance A.E., Inglis D.A. (1997) Assessment of greenhouse and laboratory screening methods for evaluating potato foliage for resistance to late blight. Plant Disease 81:1206-1213.

Dou D., Kale S.D., Wang X., Jiang R.H.Y., Bruce N.A., Arredondo F.D., Zhang X., Tyler B.M. (2008) RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. The Plant Cell Online 20:1930-1947.

Dowley L., Griffin D., O'sullivan E. (2002) Two decades of monitoring Irish populations of *Phytophthora infestans* for phenylamide resistance. Potato Research 45:79-84.

Dowley L.J., O'Sullivan E. (1981) Metalaxyl-resistant strains of *Phytophthora infestans* (Mont.) de bary in ireland. Potato Research 24:417-421.

Dowley L.J., O'Sullivan E. (1991) Sporulation of *Phytophthora infestans* (Mont.) de Bary on the surface of diseased potatoes and tuber to tuber spread of infection during handling. Potato Research 34:295-296.

Dowley L.J., Grant J., Griffin D. (2008) Yield losses caused by late blight (*Phytophthora infestans* (Mont.) de Bary) in potato crops in Ireland. Irish Journal of Agriculture and Food Research 47:69-78.

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

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

Ebel J., Cosio E.G. (1994) Elicitors of plant defense responses. International Review of Cytology 148:1-36.

Erselius L.J., Shaw D.S. (1982) Protein and enzyme differences between *Phytophthora palmivora* and *P. megakarya*: evidence for self-fertilization in pairings of the two species. Transactions of the British Mycological Society 78:227-238.

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

Eschen-Lippold L., Rothe G., Stumpe M., Gobel C., Feussner I., Rosahl S. (2007) Reduction of divinyl ether-containing polysaturated fatty acids in transgenic potato. Phytochemistry 68:797-801.

Evanno G., Regnaut S., Goudet J. (2005) Detecting the number of structure using multilocus software structure: a simulation study. Molecular Ecology 14:2611-2620.

Evenhuis B., Turkensteen L.J., Raatjes P., Flier W.G. (2007) Monitoring Primary Sources of Inoculum of *Phytophthora infestans* in The Netherlands 1999-2005. PPO-Special Report No. 12:357-363.

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

Falush D., Stephens M., Pritchard J.K. (2003) Inference of population structure using multilocus data: linked loci and correlated allele frequencies. Genetics 164:1567-1587.

Flier W.G., Turkensteen L.J. (1999) Foliar aggressiveness of *Phytophthora infestans* in three potato growing regions in the Netherlands. European Journal of Plant Pathology 105:381-388.

Flier W.G., Van Den Bosch G.B.M., Turkensteen L.J. (2003) Stability of partial resistance in potato cultivars exposed to aggressive strains of *Phytophthora infestans*. Plant Pathology 52:326-337.

Flor H.H. (1942) Inheritance of pathogenicity in *Melampsora lini*. Phytopathology 32:653-669.

Food and Agriculture Organization of the United Nations (2010). http://faostat.fao.org/site/339/default.aspx.

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

Forbes G.A., Chacón M.G., Kirk H.G., Huarte M.A., van Damme M., Distel S., Mackay G.R., Stewart H.E., Lowe R., Duncan J.M., Mayton H.S., Fry W.E., Andrivon D., Ellisseche M., Pelle R., Platt H.W., Mackenzie G., Tarn T.R., Colon L.T., Budding D.J., Lozoya-Saldana H., Hernandes-Vilchis A., Capezio S. (2005) Stability of resistance to *Phytophthora infestans* in potato: an international evaluation. Plant Pathology 54:364-372.

Forch M., van Den Bosch T., van Bekkum P., Evenhuis B., Vossen J., Kessel G. (2010) Monitoring the Dutch *Phytophthora infestans* population for virulence against new R-genes, PPO-Special Report No. 14. pp. 45-50.

Forster H., Oudemans P., Coffey M.D. (1990) Mitochondrial and nuclear DNA diversity within six species of *Phytophthora*. Experimental Mycology 14:18-31.

Fry W. (2008) *Phytophthora infestans*: the plant (and R gene) destroyer. Molecular Plant Pathology 9:385-402.

Fry W.E., Goodwin S.B. (1997) Resurgence of the Irish potato famine fungus. Bioscience 47:363-371.

Fry W.E., Cooke D.E.L., McLeod A., Forbes G.A., Cao K. (2009) Population genetics and population diversity of *Phytophthora infestans*. Oomycete Genetics and Genomics:139-164.

Fry W.E., Drenth A., Spielman L.J., Mantel L., Davidse L.C., Goodwin S.B. (1991) Population genetic structure of *Phytophthora infestans* in the Netherlands. Phytopathology 81:1130-1336.

Fry W.E., Goodwin S.B., Matuszak J.M., Spielman L.J., Milgroom M.G., Drenth A. (1992) Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30:107-130.

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

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

Galindo A., Gallegly M.E. (1960) The nature of sexuality in *Phytophthora infestans*. Phytopathology 50:123-28.

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

Gaut B.S., Clegg M.T., Muse S.V. (1993) Relative rates of nucleotide substitution in the chloroplast genome. Molecular Phylogenetics ans Evolution 2:89-96.

Gavino P.D., Fry W.E. (2002) Diversity in and evidence for selection on the mitochondrial genome of *Phytophthora infestans*. Mycologia 94:781-793.

Gavino P.D., Smart C.D., Sandrock R.W., Miller J.S., Hamm P.B., Lee T.Y., Davis R.M., Fry W.E. (2000) Implications of sexual reproduction for *Phytophthora infestans* in the United States: Generation of an aggressive lineage. Plant Disease 84:731-735.

Gebhardt C., Valkonen J.P.T. (2001) Organization of genes controlling disease resistance in the potato genome. Annual Review of Phytopathology 39:79-102.

Gisi U., Cohen Y. (1996) Resistance to phenylamide fungicides: a case study with *Phytophthora infestans* involving mating type and race structure. Annual Review of Phytopathology 34:549-572.

Gisi U., Waldner M., Kraus N., Dubuis P.H., Sierotzki H. (2007) Inheritance of resistance to carboxylic acid amide (CAA) fungicides in *Plasmopara viticola*. Plant Pathology 56:199-208.

Gisi U., Walder F., Resheat-Eini Z., Edel D., Sierotzki H. (2011) Changes of genotype, sensitivity and aggressiveness in *Phytophthora infestans* isolates collected in European countries in 1997, 2006 and 2007. Journal of Phytopathology 159:223-232.

Goodwin S.B. (1991) DNA polymorphisms in *Phytophthora infestans*: the Cornell experience Cambridge University Press, Cambridge.

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

Goodwin S.B., Cohen B.A., Fry W.E. (1994) Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. Proceedings of the National Academy of Sciences 91:11591.

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

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

Goodwin S.B., Sujkowoski L.S., Fry W.E. (1995) Rapid evolution of pathogenicity within clonal lineages of potato late blight disease fungus. Phytopathology 85:667-676.

Gotoh K., Akino S., Kiyotoshi T., Naito S. (2005) Sexual mating preferences in in vitro and in planta among Japanese isolates of *Phytophthora infestans*. Journal of General Plant Pathology 71:29-32.

Griffin D., O'Sullivan E., Harmey M.A., Dowley L.J. (2002) DNA fingerprinting, metalaxyl resistance and mating type determination of the *Phytophthora infestans* population in the Republic of Ireland. Potato Research 45:25-36.

Griffin D., Dowley L.J., Cooke L.R., Shaw D.S., Nyongesa M., Mullins E., Milbourne D., Kildea S. (2010) Recent changes in *Phytophthora infestans* populations in Ireland challenge available cultivar resistance. Potato Research 53:212-213.Griffith G.W., Shaw D.S. (1998) Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. Applied and Environmental Microbiology 64:4007-4014.

Groves C.T., Ristaino J.B. (2000) Commercial fungicide formulations induce in vitro oospore formation and phenotypic change in mating type in *Phytophthora infestans*. Phytopathology 90:1201-1208.

Haas B.J., Kamoun S., Zody M.C., Jiang R.H.Y., Handsaker R.E., Cano L.M., Grabherr M., Kodira C.D., Raffaele S., Torto-Alalibo T., Bozkurt T.O., Ah-Fong A.M.V., Alvarado L., Anderson V.L., Armstrong M.R., Avrova A., Baxter L., Beynon J., Boevink P.C., Bollman S.R., Bos J.I.B., Bolune V., Cai G., Cakir C., Carrington J.C., Chawner M., Conti L., Costanzo S., Ewan R., Fahlgren N., Fischbach M.A., Fugelstad J., Gilroy E.M., Gnerre S., Green P.J., Greenville-Briggs L.J., Griffith J., Grunwald N.J., Horn K., Horner N.R., Hu C.-H., Huitema E., Jeong D.-H., Jones A.M.E., Jones J.D.G., Jones R.W., Karlsson E., Kunjeti S.G., Lamour K., Liu Z., Ma L., MacLean D., Chibucos M.C., McDonald H., McWaters J., Meijer H.J.G., Morgan W., Morris P.F., Munro C.A., O'Neill K., Ospina-Giraldo M., Pinzon A., Pritchard L., Ransahoye B., Ren Q., Restrepo S., Roy S., Sadanandom A., Savidor A., Schornack S., Schartz D.C., Schumann U., Schwessinger B., Seyer L., Sharpe T., Silvar C., Song J., Studholme D.J., Sykes S., Thines M., van der vondervoort P.J.I., Phuntumart V., Wawra S., Weide R., Win J., Young C., Zhou S., Fry W., Meyers B.C., van West P., Ristaino J., Govers F., Birch P.R.J., Whisson S.C., Judelson H.S., Nusbaum C. (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature 461:393-398.

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. pp. 95-98.

Halterman D.A., Middleton G. (2012) Presence of the potato late blight resistance gene RB does not promote adaptive parasitism of *Phytophthora infestans*. American Journal of Plant Sciences 3:360-367.

Halterman D.A., Chen Y., Sopee J., Berduo-Sandoval J., Sánchez-Pérez A. (2010) Competition between *Phytophthora infestans* effectors leads to increased

aggressiveness on plants containing broad-spectrum late blight resistance. PloS one 5:e10536.

Hamed B.H., Gisi U. (2012) Generation of pathogenic F1 progeny from crosses of *Phytophthora infestans* isolates differing in ploidy. Plant Pathology. DOI: 10.1111/j.1365-3059.2012.02655.x.

Hannukkala A.O., Kaukoranta T., Lehtinen A., Rahkonen A. (2007) Late-blight epidemics on potato in Finland, 1933–2002; increased and earlier occurrence of epidemics associated with climate change and lack of rotation. Plant Pathology 56:167-176.

Hansen J.G., Koppel M., Valskyte A., Turka I., Kapsa J. (2005) Evaluation of foliar resistance in potato to *Phytophthora infestans* based on an international field trial network. Plant Pathology 54:169-179.

Hanson K., Shattock R.C. (1998) Formation of oospores of *Phytophthora infestans* in cultivars of potato with different levels of race-nonspecific resistance. Plant Pathology 47:123-129.

Haverkort A.J., Struik P.C., Visser R.G.F., Jacobsen E. (2009) Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. Potato Research 52:249-264.

Haverkort A.J., Boonekamp P.M., Hutten R., Jacobsen E., Lotz L.A.P., Kessel G.J.T., Visser R.G.F., van der Vossen E.A.G. (2008) Societal costs of late blight in potato and prospects of durable resistance through cisgenic modification. Potato Research 51:47-57.

Hawkes J.G. (1979) Genetic poverty of the potato in Europe, in: A. C. Zeven and A. M. van Harten (Eds.), Proceedings of the Conference on Broadening the Genetic Base of Crops, Pudoc, Wageningen, The Netherlands. pp. 19-27.

Hawkes J.G. (1990) The potato: evolution, biodiversity and genetic resources Belhaven Press.

Hein I., Birch P.R.J., Danan S., Lefebvre V., Achieng Odeny D., Gebhardt C., Trognitz F., Bryan G.J. (2009) Progress in mapping and cloning qualitative

and quantitative resistance against *Phytophthora infestans* in potato and its wild relatives. Potato Research 52:215-227.

Heitman J. (2006) Sexual reproduction and the evolution of microbial pathogens. Current Biology 16:711-725.

Helgeson J.P., Pohlman J.D., Austin S., Haberlach G.T., Wielgus S.M., Ronis D., Zambolim L., Tooley P., McGrath J.M., James R.V., Stevenson W.R. (1998) Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theoretical and Applied Genetics 96:738-742.

Henfling J.W. (1987) Late blight of potato, Technical Information Bulletin No. 4, International Potato Centre, Lima, Peru. pp. 17.

Hermsen J.G.T., Ramanna M.S. (1969) Meiosis in different F1-hybrids of *Solanum acaule* Bitt. x *S. bulbocastanum* Dun. and its bearing on genome relationship, fertility and breeding behaviour. Euphytica 18:27-35.

Hermsen J.G.T., Boer A.J.E. (1971) The effect of colchicine treatment on *Solanum acaule* and *S. bulbocastanum*; a complete analysis of ploidy chimeras in S. bulbocastanum. Euphytica 20:171-180.

Hill A.E., Grayson D.E., Deacon J.W. (1998) Suppressed germination and early death of *Phytophthora infestans* sporangia caused by pectin, inorganic phosphate, ion chelation and calcium modulating treatments. European Journal of Plant Pathology 104:367-376.

Hohl H.R., Iselin K. (1984) Strains of *Phytophthora infestans* from Switzerland with A2 mating type behaviour. Transactions of the British Mycological Society 83:529-530.

Hollomon D.W. (1965) A medium for the direct isolation of *Phytophthora infestans* Plant Pathology 14:34-35.

Hollomon D.W., Thind T.S. (2011) New modes of action contribute to resistance management, in: T. S. Thind (Ed.), Fungicide Resistance in Crop Protection: Risk and Management, CABI, Wallingford, Oxon, UK. pp. 104-115.

Howard H.W. (1970) Genetics of the potato: *Solanum tuberosum*. Logos Press London, UK.

Huamán Z., Spooner D.M. (2002) Reclassification of landrace populations of cultivated potatoes (*Solanum* sect. *Petota*). American Journal of Botany 89:947-965.

Huggenberger F., Knauf-Beiter G. (2009) Mandipropamid-protection of new growth from infection with late blight (*Phytophthora infestans*) in potatoes, PPO-Special Report No. 13. pp. 153-158.

Hussain S., Lees A.K., Duncan J.M., Cooke D.E.L. (2005) Delelopment of species-specific and sensitive detection assay for *Phytophthora infestans* and its application for monitoring of inoculum in tubers and soil. Plant Pathology 54:373-382.

IFA. (2011) Trends and challenges in Irish potato production, Irish Potato Farmers' Association, Dublin, Ireland. pp. 20.

Jackson S.A., Hanneman R.E. (1999) Crossability between cultivated and wild tuber-and non-tuber-bearing *Solanums*. Euphytica 109:51-67.

Jacobsen E. (2008) An ethical assessment of cisgenesis in breeding late blight resistant potato. Potato Research 51:59-73.

Jarne P., Lagoda P.J.L. (1996) Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution 11:424-429.

Johnson R. (1984) A critical analysis of durable resistance. Annual Review of Phytopathology 22:309-330.

Jombart T., Pontier D., Dufour A.B. (2009) Genetic markers in the playground of multivariate analysis. Heredity 102:330-341.

Jones J.D.G., Dangl J.L. (2006) The plant immune system. Nature 444:323-329.

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

Judelson H.S. (1997a) The Genetics and Biology of *Phytophthora infestans*: Modern Approaches to a Historical Challenge. Fungal Genetics and Biology 22:65-76.

Judelson H.S. (1997b) Expression and Inheritance of Sexual Preference and Selfing Potential in *Phytophthora infestans*. Fungal Genetics and Biology 21:188-197.

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

Judelson H.S., Tooley P.W. (2000) Enhanced PCR methods for detecting and quantifying *Phytophthora infestans* in plants. Phytopathology 90:1112-1119.

Judelson H.S., Blanco F.A. (2005) The spores of *Phytophthora*: weapons of the plant destroyer. Nature Reviews Microbiology 3:47-58.

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

Kadish D., Cohen Y. (1988) Competition between metalaxyl-sensitive and metalaxyl-resistant isolates of *Phytophthora infestans* in the absence of metalaxyl. Plant Pathology 37:558-564.

Kadish D., Cohen Y. (1989) Population dynamics of metalaxyl-sensitive and metalaxyl resistant isolates of *Phytophthora infestans* in untreated crops of potato. Plant Pathology 38:271-276.

Kaeuffer R., Reale D., Coltman D.W., Pontier D. (2007) Detecting population structure using STRUCTURE software: effect of background linkage disequilibrium. Heredity 99:374-380.

Kale S.D., Gu B., Capelluto D.G.S., Dou D., Feldman E., Rumore A., Arredondo F.D., Hanlon R., Fudal I., Rouxel T. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142:284-295.

Kalinowski S.T., Taper M.L., Marshall T.C. (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099-1106.

Kamoun S. (2006) A catalogue of the effector secretome of plant pathogenic oomycetes. Annual Review of Phytopathology 44:41-60.

Kamoun S., Hraber P., Sobral B., Nuss D., Govers F. (1999) Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. Fungal Genetics and Biology 28:94-106.

Kaschani F., Shabab M., Bozkurt T., Shindo T., Schornack S., Gu C., Ilyas M., Win J., Kamoun S., van der Hoorn R.A.L. (2010) An effector-targeted protease contributes to defense against *Phytophthora infestans* and is under diversifying selection in natural hosts. Plant physiology 154:1794-1804.

Keeling P.J., Burger G., Durnford D.G., Lang B.F., Lee R.W., Pearlman R.E., Roger A.J., Gray M.W. (2005) The tree of eukaryotes. Trends in Ecology and Evolution 20:670-676.

Keen N.T. (1990) Gene-for-gene complementarity in plant-pathogen interactions. Annual review of genetics 24:447-463.

Kildea S., Cooke L.R., Quinn L., Little G., Armstrong C., Hutton F., Dowley L.J., Griffin D. (2010) Changes within the Irish potato late blight population. PPO-Special Report No. 14:147-150.

Kim K.J., Lee Y.S. (2002) Genetic DNA marker for the A2 mating type in *Phytophthora infestans*. Journal of Microbiology 40:254-259.

Klarfeld S., Rubin A.V., Cohen Y. (2009) Pathogenic fitness of ooporic progeny isolates of *Phytophthora infestans* on late blight-resistant tomato lines. Plant Disease:947-953.

Knapova G., Gisi U. (2002) Phenotypic and genotypic structure of *Phytophthora infestans* populations on potato and tomato in France and Switzerland. Plant Pathology 51:641-653.

Knapova G., Schlenzig A., Gisi U. (2002) Crosses between isolates of *Phytophthora infestans* from potato and tomato and characterization of F1 and F2 progeny for phenotypic and molecular markers. Plant Pathology 51:698-709.

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

Kosakovsky Pond S.L., Frost S.D.W. (2005) Not So Different After All: A Comparison of Methods for Detecting Amino Acid Sites Under Selection. Molecular Biology and Evolution 22:1208-1222.

Kosakovsky Pond S.L., Frost S.D.W., Muse S.V. (2005) HyPhy: hypothesis testing using phylogenies. Bioinformatics 21:676-679.

Kosman E., Leonard K.J. (2005) Similarity coefficients for molecular markers in studies of genetic relationships between individuals for haploid, diploid, and polyploid species. Molecular Ecology 14:415-24. DOI: 10.1111/j.1365-294X.2005.02416.x.

Kothe E. (2008) Sexual attraction: on the role of fungal pheromone/receptor systems (A review). Acta Microbiologica et Immunologica Hungarica 55:125-143.

Kramer L.C., Choudoir M.J., Wielgus S.M., Bhaskar P.B., Jiang J. (2009) Correlation between transcript abundance of the RB gene and the level of the RB-mediated late blight resistance in potato. Molecular Plant-Microbe Interactions 22:447-455.

Krämer R., Freytag S., Schmelzer E. (1997) In vitro formation of infection structures of *Phytophthora infestans* is associated with synthesis of stage specific polypeptides. European Journal of Plant Pathology 103:43-53.

Kroon L.P.N.M., Bakker F.T., van der Bosch G.B.M., Bonants P.J.M., Flier W.G. (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genetics and Biology 41:766-782.

Kuck K., Russell P. (2006) FRAC: Combined resistance risk assessment, in: R. J. Bryson, et al. (Eds.), Fungicide Resistance: Are We Winning the Battle but Losing the War?, Scottish Agricultural College, Edinburgh, UK. Aspects of Applied Biology 78. pp. 3-10.

Kuiken C., Yusim K., Boykin L., Richardson R. (2005) The Los Alamos hepatitis C sequence database. Bioinformatics 21:379-384.

Lacey J. (1967) The role of water in the spread of *Phytophthora infestans* in the potato crop. Annals of Applied Biology 59:245-255.

Lagercrantz U., Ellegren H., Andersson L. (1993) The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. Nucleic Acids Research 21:1111-1115.

Lambert D.H., Currier A.I., Olanya M.O. (1998) Transmission of *Phytophthora infestans* in cut potato seed. American Journal of Potato Research 75:257-263.

Lapwood D.H. (1977) Factors affecting the field infection of potato tubers of different cultivars by blight (*Phytophthora infestans*). Annals of Applied Biology 85:23-42.

Latijnhouwers M., de Wit P.J.G.M., Govers F. (2003) Oomycetes and fungi: similar weaponry to attack plants. Trends in Microbiology 11:462-469.

Latin R.X., MacKenzie D.R., Cole Jr H. (1981) The influence of host and pathogen genotypes on the apparent infection rates of potato late blight epidemics. Phytopathology 71:82-85.

Leach J.E., Vera Cruz C.M., Bai J., Leung H. (2001) Pathogen fitness penalty as a predictor of durability of disease resistance genes. Annual Review of Phytopathology 39:187-224.

Lebreton L., Andrivon D. (1999) Aggressiveness and competitive fitness of *Phytophthora infestans* collected from potato and tomato in France. Phytopathology 89:679-686.

Lee S.-J., Rose J.K.C. (2010) Mediation of the transition from biotrophy to necrotrophy in hemibiotrophic plant pathogens by secreted effector proteins. Plant Singnaling and Behavior 5:769-772.

Lee T.Y., Simko I., Fry W.E. (2002) Genetic control of aggressiveness in *Phytophthora infestans* to tomato. Canadian Journal of Plant Pathology 24:471-480.

Lees A., Cooke D.L.E., Stewart J.A., Sullivan L., Williams N.A., Carnegie S.F. (2009) *Phytophthora infestans* population changes: implications. PPO-Special Report No. 13:55-60.

Lees A.K., Sullivan L., Lynott J.S., Cullen D.W. (2012) Development of a quantitative real time PCR assay for *Phytophthora infestans* and its applicability to leaf, tuber and soil samples. Plant Pathology. DOI: 10.1111/j.1365-3059.2011.02574.x.

Lees A.K., Wattier R., Shaw D.S., Sullivan L., Williams N.A., Cooke D.E.L. (2006) Novel microsatellite markers for the analysis of *Phytophthora infestans* populations. Plant Pathology 55:311-319.

Lehtinen A., Hannukkala A. (2004) Oospores of *Phytophthora infestans* in soil provide an important new source of primary inoculum in Finland. Agricultural and Food science 13:399-410.

Leonard R.T. (2003) Evaluation of decision support systems and air-assisted spraying as means of reducing fungicide input, spray drift and costs in the control of late blight of potatoes, PhD thesis, University College Dublin. pp. 262.

Levin A., Baider A., Rubin E., Gisi U., Cohen Y. (2001) Oospore formation by *Phytophthora infestans* in potato tubers. Phytopathology 91:579-585.

Levy Y., Levi R., Cohen Y. (1983) Buildup of a pathogen subpopulation resistant to a systemic fungicide under various control strategies: A flexible simulation model. Phytopathology 73:1475-1480.

Liu T., Ye W., Ru Y., Yang X., Gu B., Tao K., Lu S., Dong S., Zheng X., Shan W., Wang Y., Dou D. (2011) Two host cytoplasmic effectors are required for pathogenesis of *Phytophthora sojae* by suppression of host defenses. Plant Physiology 155:490-501.

Llorente B., Bravo-Almonacid F., Cvitanich C., Orlowska E., Torres T.N., Flawia M.M., Alonso G.D. (2010) A quantitative real time PCR method for in planta monitoring of *Phytophthora infestans* growth. Letters in Applied Microbiology 51:603-610.

Malcolmson J.F., Black W. (1966) New R genes in Solanum demissum Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. Euphytica 15:199-203.

Malcolmson J.F. (1976) Assessment of field resistance to blight (*Phytophthora infestans*) in potatoes. Transactions of the British Mycological Society 67:321-325.

Maniatis T., Fritsch E.F., Sambrook J. (2002) Molcular cloning, Cold Spring Habour, New York.

Marshall T.C., Slate J., Kruuk L.E.B., Pemberton J.M. (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology 7:639-655.

Martin R.R., James D., Levesque C.A. (2000) Impacts of molecular diagnostic technologies on plant disease management. Annual Review of Phytopathology 38:207-239.

May J.K., Ristaino J.B. (2004) Identity of the mtDNA haplotype(s) of *Phytophthora infestans* in historical specimens from the Irish Potato Famine. Mycological Research 108:471-479.

Mayton H., Griffiths H., Simko I., Chen S., Lorenzen J., de Jong W., Fry W.E. (2010) Foliar and tuber blight resiatnce in a *Solanum tuberosum* breeding population. Plant Breeding 129:197-201.

McDonald B.A., Linde C. (2002) Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review of Phytopathology 40:349-379.

Mendoza H.A. (1993) Experiences, difficulties and prospects for durable disease resistance breeding in potatoes, in: T. H. Jacobs and J. E. Parlevliet (Eds.), Durability of disease resistance. International Symposium on the Durability of Disease Resistance in Plants, Kluer Academic Publishers, Dordrecht, Netherlands. pp. 249-257.

Meyers B.C., Kozik A., Griego A., Kuang H., Michelmore R.W. (2003) Genome-wide analysis of NBS-LRR–encoding genes in Arabidopsis. The Plant Cell Online 15:809-834.

Miller J.S., Johnson D.A., Hamm P.B. (1998) Aggressiveness of *Phytophthora infestans* from the Columbia Basin of Washington and Oregon. Phytopathology 88:190-197.

Montarry J., Glais I., Corbiere R., Andrivon D. (2008) Adaptation to the most abundant host genotype in an agricultural plant–pathogen system–potato late blight. Journal of Evolutionary Biology 21:1397-1407.

Montarry J., Andrivon D., Glais I., Corbiere R., Mialdea G., Delmotte F. (2010) Microsatellite markers reveal two admixed genetic groups and an ongoing displacement within the French population of the invasive plant pathogen *Phytophthora infestans*. Molecular Ecology 19:1965-1977.

Mullins E., Milbourne D., Petti C., Doyle-Prestwich B.M., Meade C. (2006) Potato in the age of biotechnology. Trends in Plant Science 11:254-260.

Mundt C.C. (2002) Use of multiline cultivars and cultivar mixtures for disease management. Annual Review of Phytopathology 40:381-410.

Muse S.V., Gaut B.S. (1994) A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. Molecular Biology and Evolution 11:715-724.

Naerstad R., Hermansen A., Bjor T. (2007) Exploiting host resistance to reduce the use of fungicides to control potato late blight. Plant Pathology 56:156-166.

Nei M. (1972) Genetic distance between populations. The American Naturalist 106:283-292.

Nei M. (1973) Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences 70:3321-3323.

Nei M., Gojobori T. (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Molecular Biology and Evolution 3:418-426.

Nei M., Kumar S. (2000) Molecular Evolution and Phylogenetics Oxford University Press, New York.

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

Niederhauser J.S. (1991) Phytophthora infestans: the Mexican connection Cambridge University Press, Cambridge.

Niederhauser J.S., Mills W.R. (1953) Resistance of *Solanum* species to *Phytophthora infestans* in Mexico. Phytopathology 43:57.

Niepold F., Schober-Butin B. (1995) Application of the PCR technique to detect *Phytophthora* in potato tubers and leaves. Mycological Research 150:379-385.

Nürnberger T., Brunner F. (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Current Opinion in Plant Biology 5:318-324.

O'Sullivan E., Dowley L.J. (1991) A note on the occurrenece of the A2 mating type and self-fertile isolates of *Phytophthora infestans* in the Republic of Ireland. Irish Journal of Agricultural Research 30:67-69.

O' Sullivan E., Cooke L.R., Dowley L.J., Carlisle D.J. (1995) Distribution and significance of the A2 mating type of *Phytophthora infestans* in Ireland., in: L. J. Dowley, et al. (Eds.), *Phytopthora infestans*, Boole, Dublin. pp. 232-238.

O'Brien M., Mullins E. (2008) Relevance of genetically modified crops in the light of future environmental and legislative challenges to agri-environment. Annals of Applied Biology. DOI: 10.1111/j.1744-7348.2008.00304.x.

Oh S.K., Young C., Lee M., Oliva R., Bozkurt T.O., Cano L.M., Win J., Bos J.I.B., Liu H.Y., van Damme M., Morgan W., Choi D., van der Vossen E.A.G., Vleeshouwers V.G.A.A., Kamoun S. (2009) *In planta* expression screens of Phytophthora infestans RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. The Plant Cell 21:2928-2947.

Ojika M., Molli S.D., Kanazawa H., Yajima A., Toda K., Nukada T., Mao H., Murata R., Asano T., Qi J. (2011) The second *Phytophthora* mating hormone defines interspecies biosynthetic crosstalk. Nature Chemical Biology 7:591-593.

Ordoñez M.E., Forbes G.A., Trognitz B. (1998) Relationship between ineffective R-genes and expansion rate of lesions on potato leaves, caused by *Phytophthora infestans*. Plant Pathology 47:130-136.

Panstruga R., Dodds P.N. (2009) Terrific protein traffic: the mystery of effector protein delivery by filamentous plant pathogens. Science 324:748.

Paquin B., Laforest M.J., Forget L., Roewer I., Wang Z., Longcore J., Lang B.F. (1997) The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression. Current Genetics 31:380-395.

Park T., Vivianne A.A.A.G.V., Kim J.B., Hutten R.C.B. (2005) Dissection of foliage and tuber blight resistance in mapping populations of potato. Euphytica 143:75-83.

Parlevliet J.E. (1983) Can horizontal resistance be recognized in the presence of vertical resistance in plants exposed to a mixture of pathogen races? . Phytopathology 73:379-385.

Pavlicek A., Hrda S., Flegr J. (1999) Free-Tree: Freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of genus Frenkelia. Folia Biologica (Praha.) 45:97-99.

Payne R.W., Harding S.A., Murray D.A., Soutar D.M., Baird D.B., Glaser A.I., Channing I.C., Weilham S.J., Gilmour A.R., Thompson R., Webster R. (2008) GenStat 11th Edition. Release 11.1. Reference Manual. VSN International Ltd., Hertfordshire, UK.

Peakall R., Smouse P.E., Huff D.R. (1995) Evolutionary implications of allozyme and RAPD variation in diploid population of dioecious buffalograss (Buchloe dactyloides (Nutt.) Engelm.). Molecular Ecology 4:135-137.

Peakall R., Smouse P.E. (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6:388-295.

Petti C., Wendt T., Meade C., Mullins E. (2009) Evidence of genotype dependency within *Agrobacterium tumefaciens* in relation to the integration of vector backbone sequence in transgenic *Phytophthora infestans*-tolerant potato. Journal of Bioscience and Bioengineering 107:301-306.

Phillips S.L., Shaw M.W., Wolfe M.S. (2005) The effect of potato variety mixtures on epidemics of late blight in relation to plot size and level of resistance. Annals of Applied Biology 147:245-252.

Pieterse C.M.J., van West P., Verbakel H.M., Brasse P.W.H.M., van den Berg-Velthuis G., Govers F. (1994) Structure and genomic organization of the ipiB and ipiO gene clusters of *Phytophthora infestans*. Gene 138:67-77.

Pilet F., Pelle R., Ellisseche D., Andrivon D. (2005) Efficacy of the R2 resistance gene as a component for the durable management of potato late blight. Plant Pathology 54:723-732.

Pipe N.D., Azcoitia V., Shaw D.S. (2000) Self-fertility in *Phytophthora infestans*: heterokaryons segregate several phenotypes. Mycological Research 104:676-680.

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

Platt H.W. (1999) Response of solanaceous cultivated plants and weed species to inoculation with A1 or A2 mating type strains of *Phytophthora infestans*. Canadian Journal of Plant Pathology 21:301-307.

Porter L.D., Johnson D.A. (2004) Survival of Phytophthora infestans in surface water. Phytopathology 94:380-387.

Pritchard J.K., Stephens M., Donnelly P. (2000) Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Purvis A.I., Pipe N.D., Day J.P., Shattock R.C., Shaw D.S., Assinder S.J. (2001) AFLP and RFLP (RG57) fingerprints can give conflicting evidence about the

relatedness of isolates of *Phytophthora infestans*. Mycological Research 105:1321-1330.

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

Reddick D. (1939) Whence came *Phytophthora infestans*. Chron. Bot 5:410-412.

Rietman H., Bijsterbosch G., Cano L.M., Lee H.-R., Vossen J.H., Jacobsen E., Visser R.G.F., Kamoun S., Vleeshouwers V. (2012) Qualitative and quantitative Late blight resistance in the potato cultivar Sarpo Mira is determined by the perception of five RXLR effectors. Molecular Plant Microbe Interactions 25:910-919.

Ristaino J.B. (2002) Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*. Microbes and Infection 4:1369-1377.

Ristaino J.B., Groves C.T., Parra G.R. (2001) PCR amplification of the Irish potato famine pathogen from historic specimens. Nature 411:695-697.

Ross H. (1986) Potato breeding-problems and perspectives. Fortschritte der Pflanzenzüchtung.

Rozen S., Skaletsky H. (2000) Primer3 on the WWW for general users and for biologist programmers, in: S. Krawetz and S. Misener (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology, Humana Press, Totowa, NJ, USA. pp. 365-386.

Rubin A., Gotlieb D., Gisi U., Cohen Y. (2008) Mutagenesis of *Phytophthora* infestans for resistance against carboxylic acid amide and phenylamide fungicides. Plant Disease 92:675-683.

Rzhetsky A., Nei M. (1992) A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution 9:945-967.

Salaman R.N. (1985) The history and social influence of the potato Cambridge University Press.

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

Sezonov G., Joseleau-Petit D., D'Ari R. (2007) Escherichia coli physiology in Luria Bertani broth. Journal of Bacteriology 189:8746-8749.

Schena L., Cooke D.E.L. (2006) Assessing the potential of regions of the nuclear and mitochondrial genome to develop a “molecular tool box” for the detection and characterization of *Phytophthora* species. Journal of Microbiological Methods 67:70-85.

Schena L., Hughes K.J.D., Cooke D.E.L. (2006) Detection and quantification of *Phytophthora ramorum, P. kernoviae, P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. Molecular Plant Pathology 7:365-379.

Schornack S., van Damme M., Bozkurt T.O., Cano L.M., Smoker M., Thines M., Gaulin E., Kamoun S., Huitema E. (2010) Ancient class of translocated oomycete effectors targets the host nucleus. Proceedings of the National Academy of Sciences 107:17421-17426.

Schwinn J.E., Margot P. (1991) Control with chemicals, in: D. S. Ingram and P. S. Williams (Eds.), Advances in Plant Pathology- Vol 7. *Phytophthora infestans*, The cause of Late blight of Potato, Academic Press Ltd, London, UK. pp. 225-265.

Shannon C.E., Weaver W. (1949) The Mathematical Theory of Communication. University of Illinois Press, Urbana, Illinois US.

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

Shattock R.C., Tooley P.W., Fry W.E. (1985) Genetics of *Phytophthora infestans*: determinants of recombination, segregation, and selfing by isozyme analysis. Phytopathology 76:410-413.

Shattock R.C., Tooley P.W., Fry W.E. (1986) Genetics of *Phytophthora infestans*: Characterization of single-oospore cultures from Al isolates induced to self by intraspecific stimulation. Phytopathology 76:407-410.

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

Shaw C.R. (1965) Electrophoretic variation in enzymes. Science 149:936-943.

Shaw D.S. (1991) Genetics., in: D. S. Ingram and P. H. Williams (Eds.), *Phytophthora infestans*, the cause of late blight of potato., Academic Press (Advances in Plant Pathology 7), New York. pp. 131-170.

Shaw D.S., Shattock R.C. (1991) Genetics of *Phytophthora infestans*: the Mendelian approach., in: J. A. Lucas, et al. (Eds.), Cambridge University Press Cambridge. pp. 218-230.

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

Shaw D.S., Evans D., Buffe C., Cooke D.E.L. (2009) Can UK populations mate?, in: H. T. A. M. Schepers (Ed.), Proceedings of the Eleventh Euroblight Workshop, Lelystad, Netherlands, Hamar, Norway. pp. 317-318.

Singh P.B., Gupta J., Roy S., Rana D.K. (2004) Production of *Phytophthora infestans* oospores *in planta* and inoculum potential of *in vitro* produced oospores under temperate highlands and sub-tropical plains of India. Annals of Applied Biology 144:363-370.

Skidmore D.I., Shattock R.C., Shaw D.S. (1984) Oospores in cultures of *Phytophthora infestans* resulting from selfing induced by the presence of *P. drechsleri* isolated from blighted potato foliage. Plant Pathology 33:173-183.

Smart C.D., Fry W.E. (2001) Invasions by the late blight pathogen: renewed sex and enhanced fitness. Biological Invasions 3:235-243.

Smart C.D., Willmann M.R., Mayton H., Mizubuti E.S.G., Sandrock R.W., Muldoon A.E., Fry W.E. (1998) Self-Fertility in Two Clonal Lineages of *Phytophthora infestans*. Fungal Genetics and Biology 25:134-142.

Smithies O. (1955) Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochemical Journal 61:629.

Sneath P., Sokal R. (1973) Numerical Taxonomy. W.H. Freeman, San Francisco, USA.

Song J., Bradeen J.M., Naess S.K., Raasch J.A., Wielgus S.M., Heberlach G.T., Liu J., Kuang H., Austin-Phillips S., Robin Buell C., Helgeson J.P., Jiang J. (2003) Gene RB cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proceedings of the National Academy of Sciences 100:9123-9133.

Spielman L.J., McMaster B.J., Fry W.E. (1992) Relationships among measurements of fitness and disease severity in *Phytophthora infestans*. Plant Pathology 41:317-324.

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

Spooner D.M., Hijmans R.J. (2001) Potato systematics and germplasm collecting, 1989–2000. American Journal of Potato Research 78:237-268.

Spooner D.M., van der Berg R.G., Rodriquez A., Bamberg J., Hijmans R.J., Lara-Cambrera S. (2004) Wild potatoes (*Solanum* section Petota) of North and Central America. Systematics and Botanical Monographs 68:201-209.

Spooner D.M., Núñez J., Trujillo G., del Rosario Herrera M., Guzmán F., Ghislain M. (2007) Extensive simple sequence repeat genotyping of potato landraces supports a major reevaluation of their gene pool structure and classification. Proceedings of the National Academy of Sciences 104:19398-19403.

Stein J.M., Kirk W.W. (2002) Containment of existing potato late blight (*Phytophthora infestans*) foliar epidemics with fungicides. Crop Protection 21:575-582.

Stewart H.E. (1990) Effect of plant age and inoculum concentration on expression of major gene resistance to *Phytophthora infestans* in detached potato leaflets. Mycological Research 94:823-826.

Stewart H.E., Bradshaw J.E., Pande B. (2003) The effect of the presence of R-genes for resistance to late blight (*Phytophthora infestans*) of potato (*Solanum tuberosum*) on the underlying level of field resistance. Plant Pathology 52:193-198.

Stol W., De Koning G.H.J., Haverkort P.L., Van Keulen H., Penning de Vries F.W.T. (1991) Agro-ecological Characterization for Potato Production: A Simulation Study at the Request of the International Potato Centre (CIP), Lima, Peru Center for Agrobiological Research (CABO-DLO).

Suzuki Y., Gojobori T. (1999) A method for detecting positive selection at single amino acid sites. Molecular Biology and Evolution 16:1315-1328.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731-2739.

Tan M.Y.A., Hutten R.C.B., Visser R.G.F., van Eck H.J. (2010) The effect of pyramiding *Phytophthora infestans* resistance genes RPi-mcd1 and RPi-ber in potato. Theoretical and Applied Genetics 121:117-125.

Tani S., Judelson H. (2006) Activation of zoosporogenesis-specific genes in *Phytophthora infestans* involves a 7-nucleotide promoter motif and cold-induced membrane rigidity. Eukaryotic Cell 5:745-752.

Tatarowska B., Flis B., Zimnoch-Guzowska E. (2011) Biological stability of resistance to *Phytophthora infestans* (Mont.) de Bary in 22 Polish cultivars evaluation in field experiments. American Journal of Potato Research. DOI: 10.1007/s12230-011-9222-3.

Thieme R., Darsow U., Gavrilenko T., Dorokhov D., Tiemann H. (1997) Production of somatic hydrids between *S. tuberosum* L. and late blight resistant Mexican wild potato species. Euphytica 97:189-200.

Thompson J.M. (2008) *Phytophthora infestans* in potato tubers: infection and transmission, The Queen's University of Belfast, Belfast, UK. pp. 259.

Tooley P.W., Fry W.E., Gonzalez M.J. (1985) Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. Journal of Heredity 76:431.

Tooley P.W., Therrien C.D., Ritch D.L. (1989) Mating type, race composition, nuclear DNA content, and isozyme analysis of Peruvian isolates of *Phytophthora infestans*. Phytopathology 79:478-481.

Tooley P.W., Carras M.M., Lambert D.H. (1998) Application of a PCR-based test for detection of potato late blight and pink rot in tubers. American Journal of Potato Research 75:187-194.

Tooley P.W., Bunyard B.A., Carras M.M., Hatziloukas E. (1997) Development of PCR primers from internal transcribed spacer region 2 for detection of *Phytophthora* species infecting potato. Applied and Environmental Microbiology 63:1467-1475.

Tooley P.W., Therrien C.D., Sim J.H., O'Sullivan E., Dowley L.J. (1993) Mating type, nuclear DNA content and isozyme genotypes of Irish isolates of *Phytophthora infestans*. Mycological Research 97:1131-1134.

Turkensteen L., Flier W., Wanningen R., Mulder A. (2000) Production, survival and infectivity of oospores of *Phytophthora infestans*. Plant Pathology 49:688-696.

Turkensteen L.J. (1993) Durable resistance of potatoes against *Phytophthora infestans*, in: T. H. Jacobs and J. E. Parlevliet (Eds.), Durability of Disease Resistance, Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 115-124.

Turkensteen L.J., Flier W.G. (2002) Late blight: Its global status in 2002 and beyond, in: C. Lizarraga (Ed.), Global Initiatives on Late Blight Conference, CIP, Humburg, Germany. pp. 11-13.

U. N. Secretariat. (2009) World population prospects, The 2008 revision, The Department of Economic and Social Affairs, United Nations, New York.

Umaerus V., Linhell D. (1976) A laboratory method for measuring the degree of attack by *Phytophthora infestans*. Potato Research 19:91-107.

Umaerus V., Umaerus M., Erjefalt L., Nilsson B.A. (1981) Control of *Phytophthora* by host resistance: Problems and progress, in: D. C. Erwin, et al. (Eds.), *Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology, American Phytopathological Society, St. Paul, Minnesota. pp. 315-326.

van der Biezen E.A., Jones J.D. (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends in Biochemical Sciences 23:454.

van der Hoorn R.A.L., Kamoun S. (2008) From Guard to decoy: a new model for perception of plant pathogen effectors. Plant Cell 20:209-217.

van der Lee T., Testa A., Robold A., van‘t Klooster J., Govers F. (2004) High-density genetic linkage maps of *Phytophthora infestans* reveal trisomic progeny and chromosomal rearrangements. Genetics 167:1643-1661.

van der Vossen E., Sikkema A., Hekkert B.L., Gros J., Stevens P., Muskens M., Wouters D., Pereira A., Stiekema W., Allefs S. (2003) An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. The Plant Journal 36:867-882.

Villesen P. (2007) FaBox: an online toolbox for fasta sequences. Molecular Ecology Notes 7:965-968.

Visker M. (2005) Association between late blight resistance and foliage maturity type in potato: Physiological and genetic studies, PhD Thesis, Wageningen Agrcultural University. pp. 159.

Vleeshouwers V.G.A.A., van Dooijeweert W., Govers F., Kamoun S., Colon L.T. (2000) The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210:853-864.

Vleeshouwers V.G.A.A., Raffaele S., Vossen J.H., Champouret N., Oliva R., Segretin M.E., Rietman H., Cano L.M., Lokossou A., Kessel G., Pel M.A., Kamoun S. (2011) Understanding and exploiting late blight resistance in the age of effectors. Annual Review of Phytopathology 49:507-531.

Vleeshouwers V.G.A.A., Rietman H., Krenek P., Champouret N., Young C., Oh S.K., Wang M., Bouwmeester K., Vosman B., Visser R.G.F., Jacobsen E., Govers F., Kamoun S., van der Vossen E.A.G. (2008) Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. PloS one 3:e2875.

Vos P., Hogers R., Bleeker M., Reijans M., van der Lee T., Hornes M., Friters A., Pot J., Paleman J., Kuiper M. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Wang X., Hadrami E., Adam L.R., Daayf F. (2008) Differential activation and suppression of potato defence responses by *Phytophthora infestans* isolates representing US-1 and US-8 genotypes. Plant Pathology 57:1026-1037.

Wang M., Han C., Ferreira A.O., Yu X., Ye W., Tripathy S., Kale S.D., Gu B., Sheng Y., Sui Y., Wang X., Zhang Z., Cheng B., Dong S., Shan W., Zheng X., Dou D., Tyler B.M., Wang Y. (2011) Transcriptional Programming and functional interactions within the *Phytophthora sojae* RXLR effector repertoire. The Plant Cell 23:2064-2086.

Wangsomboondee T., Ristaino J.B. (2002) Optimization of sample size and DNA extraction methods to improve PCR detection of different propagules of *Phytophthora infestans*. Plant Disease 86:247-253.

Wastie R.L. (1991) Breeding for resistance. Advances in Plant Pathology 7:193-224.

Weir B.S., Cockerham C.C. (1984) Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

Wendt T., Doohan F., Mullins E. (2012) Production of Phytophthora infestans-resistant potato (*Solanum tuberosum*) utilizing Ensifer adherens OV14. Transgenic Research 21:567-578.

Whisson S.C., Boevink P.C., Moleleki L., Avrova A.O., Morales J.G., Gilroy E.M., Armstrong M.R., Grouffaud S., van West P., Chapman S., Hein I., Toth I.K., Pritchard L., Birch P.R. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450:115-8. DOI: 10.1038/nature06203.

Whitcombe D., Theaker J., Guy S.P., Brown T., Little S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotechnology 17:804-807.

Whittaker S.L., Assinder S.J., Shaw D.S. (1994) Inheritance of mitochondrial DNA in *Phytophthora infestans*. Mycological Research 98:569-575.

Widmark A.K., Andersson B., Sandström M., Yuen J.E. (2011) Tracking *Phytophthora infestans* with SSR markers within and between seasons–a field study in Sweden. Plant Pathology.

Widmark A.K., Andersson B., Cassel-Lundhagen A., Sandström M., Yuen J.E. (2007) *Phytophthora infestans* in a single field in southwest Sweden early in

spring: symptoms, spatial distribution and genotypic variation. Plant Pathology 56:573-579.

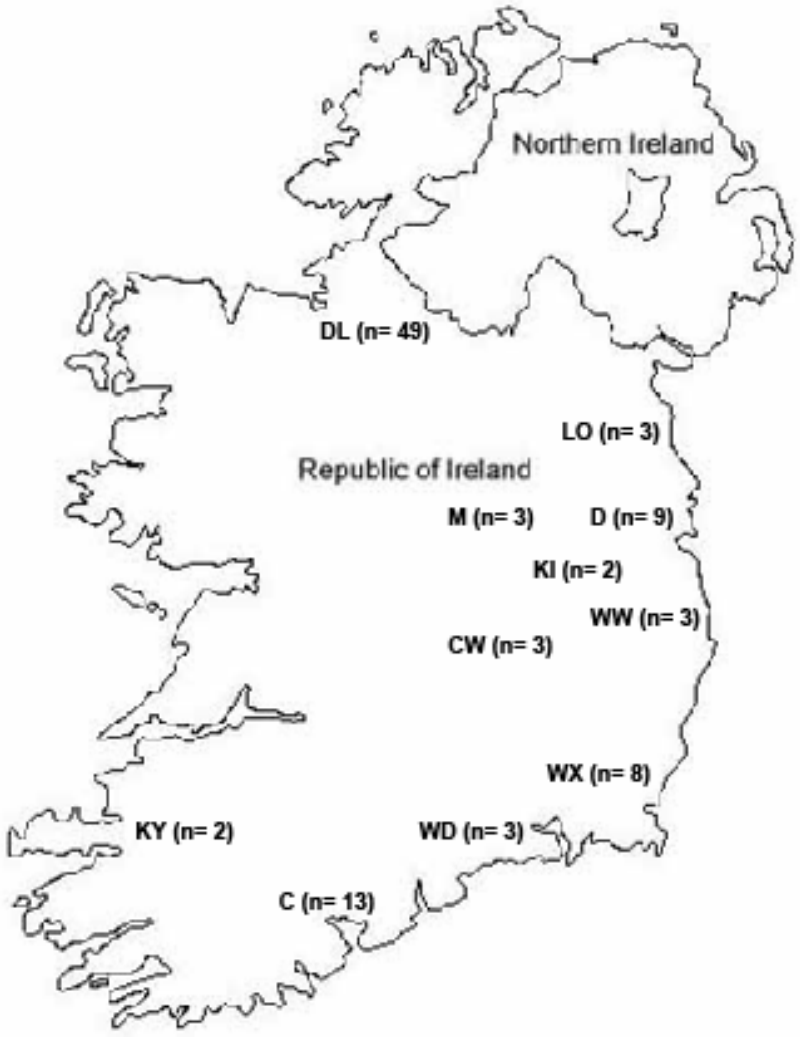
Wills C. (2003) The trouble with sex. New Scientist 180:44-47.

Yaeno T., Li H., Chaparro-Garcia A., Schornack S., Koshiba S., Watanabe S., Kigawa T., Kamoun S., Shirasu K. (2011) Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. Proceedings of the National Academy of Sciences 108:14682-14687.

Zhu S., Li Y., Vossen J.H., Visser R.G.F., Jacobsen E. (2011) Functional stacking of three resistance genes against *Phytophthora infestans*. Transgenic Research. DOI: 10.1007/s11248-011-9510-1.

Appendices

Appendix 2. 1: Single lesion leaflets (n=98) sampled for SSR finger printing, mating type and metalaxyl resistance determination from commercial potato farms in eleven potato producing counties in the Republic of Ireland. Names of counties* are abbreviated and figures in parentheses indicate characterized samples from individual counties.



*C= Cork; CW= Carlow; D= Dublin; DL=Donegal; KI= Kildare; KY= Kerry; LO= Louth; M= Meath; WD= Waterford; WW= Wicklow; WX= Wexford.

Appendix 2.2: Media preparation (pea agar based on Hollomon, 1965, Rye agar based on Caten and Jinks, 1968 and carrot agar based on Erselius and Shaw, 1982)

Pea agar

Frozen garden peas (160 g) were blended using a domestic blender and strained through a domestic sieve (1 mm^2 pore size) and made up to 1 L using SDW, with 10 g of agar added (Oxoid Technical Grade No. 3, Sigma Aldrich Inc. St. Louis, MO). The medium was autoclaved at 121^oC at 8psi for 15 min, left to cool to 50^oC and and dispensed into sterile Petri-dishes under a laminar flow chamber.

Rye A agar

Sixty grams of rye seed were immersed in 100 ml of SDW and autoclaved at 121^oC at 8psi for 15 min. Grains were subsequently ground using a domestic blender as described above. Agar (10 g) was added (Oxoid Technical Grade No. 3, Sigma Aldrich Inc.) to the resulting filtrate, which was made up to 1 litre using SDW. Media were autoclaved as described above, allowed to cool to 50^oC and poured, a laminar flow chamber, into either 9 cm Petri plates or 25 ml universal glass bottles, which were inclined to approximately 45^o, to produce slants.

Carrot agar

Two hundred grams of frozen fresh carrots were boiled in 600 ml of distilled water and blended as described above before being drained through muslin. Medium was then made up to 1 L using SDW, with 9.6 g of agar added (Oxoid Technical Grade No. 3, Sigma Aldrich Inc.) autoclaved and poured as described above.

Appendix 2.3: [a]SSR primer sequences their annealing temperatures, expected product sizes and repeat motifs

Marker	Primer Sequence	Tm (^{o}C)	Dye	Size range (bp)	Exp. size (bp)	Repeat motif
Pi02	F:CAGCCTCCGTGCAAGA	58	FAM	142-166	154	TG
	R:AAGGTGCGCGGAAGACC					
Pi04	F:AGCGGCTTACCGATGG	58	VIC	162-170	170	GT
	R:CAGCGGCTGTTTCGAC					
Pi16	F:CACAGCACGCGGAATC	58	VIC	174-178	177	GA
	R:ACGCCGAGTGTCCTGA					
Pi33	F:TGCCGACGACAAGGAA	58	NED	203-209	203	CAG
	R:CGGTCTGCTGCTGCTC					
Pi56	F:ACAACTATCTATCGGCGTGC	58	PET	174-178	176	AT
	R:AGTAGGCTTCACCGACCAGC					
Pi63	F:ATGACGAAGATGAAAGTGAGG	58	FAM	148-160	157	GAG
	R:ATTCATTATTGGCAATGTTGG					
Pi70	F:ATGAAAATACGTCAATGCTCG	58	FAM	189-195	195	AAG
	R:CGTTGGATATTTCTATTTCTTCG					
Pi89	F:GAGAACGCACAATGTAAGGC	58	NED	175-185	181	AT
	R:ACATAAATACACGCTGAACGG					
Pi4B	F:AAAATAAAGCCTTTGGTTCA	58	PET	205-217	215	TC
	R:GCAAGCGAGGTTTGTAGATT					
G11	F:TGCTATTTATCAAGCGTGGG	56	NED	142-166	156	TC
	R:TACAATCTGCAGCCGTAAGA					
D13	F:TGCCCCCTGCTCACTC	50	FAM	108-142	136	CT
	R:GCTCGAATTCATTTTACAGA					

[a]Adapted from Lees *et al*., (2006)

Appendix 2.4: *Blight resistance rating of potato cultivars

Cultivar	Blight resistance rating
Cara	5
Maris Peer	4
Maris Piper	4
Desiree	4
British Queen	4
Rooster	4
Sarpo Mira	7

*The British Potato Variety Database (http://varieties.potato.org.uk), Teagasc Crops Research Centre, Blight Field Trial data 2006-2009 provided rating for variety British Queen.

Appendix 2.5: SSR fingerprints of 98 Irish *P. infestans* isolates revealed by 6 markers (0 denotes absence of an allele at the respective locus).

	Isolate	Pi02	Pi02	Pi02	D13	D13	Pi16	Pi16	G11	G11	G11	Pi56	Pi56	Pi89	Pi89
1	DL71A	160	162	0	136	152	174	176	154	160	164	174	176	179	179
2	DL72A	160	162	0	136	156	176	178	154	160	164	174	176	179	179
3	DL64B	160	162	0	136	154	176	178	154	160	164	174	176	179	179
4	DL72B	160	162	0	136	140	176	178	154	160	0	174	174	179	179
5	DL61A	160	162	0	136	136	176	178	154	160	0	174	176	179	179
6	DL64D	160	162	0	136	154	176	178	154	160	164	174	176	179	179
7	DL82B	152	160	162	136	136	178	178	160	160	0	174	176	181	197
8	DL43B	152	160	162	136	136	176	178	160	160	0	174	176	181	197
9	DL74D	160	162	0	136	136	176	178	154	160	164	174	176	179	179
10	DL15A	160	162	0	136	136	176	178	0	0	0	174	176	179	179
11	DL73A	152	160	0	136	136	178	178	156	164	0	174	176	181	197
12	DL64A	160	162	0	136	136	176	178	154	160	164	174	176	179	179
13	DL165	152	160	162	136	136	178	178	160	160	0	174	176	181	197
14	DL115	152	160	162	118	136	176	178	160	160	0	174	176	181	197
15	DL72T	160	162	0	136	156	176	178	154	160	0	174	176	179	179
16	MN11	160	162	0	136	158	176	178	154	160	0	174	176	179	179
17	DL43C	152	160	162	0	0	178	178	160	160	0	174	176	181	187
18	DL125A	160	162	0	136	140	176	178	154	160	164	174	176	179	179
19	MN11D	160	162	0	136	140	176	178	154	160	0	174	176	179	179
20	DL112	160	162	0	136	140	176	178	154	160	164	174	176	179	179
21	KI14A	160	162	0	136	154	174	176	154	160	164	174	176	179	179
22	DB21B	160	162	0	136	136	174	176	164	164	0	174	176	179	179
23	DB55D	160	162	0	136	144	176	178	154	160	164	174	176	179	179
24	DB33A	160	162	0	136	144	176	178	154	160	164	174	176	179	179
25	DB53B	160	162	0	136	154	176	178	154	160	164	174	176	179	179
26	KI13A	160	162	0	136	136	176	178	154	160	164	174	176	179	179
27	LO22A	160	162	0	136	154	176	178	0	0	0	174	176	179	179
28	DB32A	160	162	0	136	144	176	178	154	160	164	174	176	179	179
29	DB32D	160	162	0	136	144	176	178	154	160	164	174	176	179	179
30	CW13C	160	162	0	136	154	176	178	154	160	0	174	176	179	179
31	CW14B	160	162	0	136	136	176	178	154	160	0	174	176	179	179
32	LOA2A	160	162	0	136	154	176	178	0	0	0	174	176	179	179
33	DB54D	160	162	0	136	136	176	178	154	160	164	176	176	179	179
34	DB63A	160	162	0	136	136	176	178	154	160	164	174	176	179	179
35	KI12A	160	162	0	136	154	178	178	154	160	164	174	176	179	179
36	CW13D	160	162	0	136	154	176	178	154	0	160	174	176	179	179
37	KI18A	160	162	0	158	158	176	178	154	0	160	174	176	179	179
38	LO41D	160	162	0	136	154	176	178	154	160	164	174	176	179	179
39	LO22D	160	162	0	136	140	176	178	154	160	164	174	176	179	179
40	WX43A	160	162	0	136	154	176	178	154	160	0	174	176	179	179
41	WX1B	160	162	0	136	154	176	178	154	160	0	174	176	179	179
42	WW11A	160	162	0	136	154	176	178	154	160	0	174	176	179	179
43	WD11A	160	162	0	140	144	176	178	138	150	0	176	176	179	179
44	WX61B	160	162	0	136	154	176	178	154	160	0	174	176	179	179
45	WX42B	160	162	0	136	154	176	178	154	160	0	174	176	179	179
46	WD11B	160	162	0	140	144	176	178	154	160	0	174	176	179	179
47	CK14C	160	162	0	136	136	176	178	154	160	0	174	176	179	179

Appendix 2.5: (Continued from previous page) SSR fingerprints of 98 Irish *P. infestans* isolates revealed by 6 markers (0 denotes absence of an allele at the respective locus).

	Isolate	Pi02	Pi02	Pi02	D13	D13	Pi16	Pi16	G11	G11	G11	Pi56	Pi56	Pi89	Pi89
48	WX51A	160	162	0	136	154	176	178	154	160	0	174	176	179	179
49	WX61A	160	162	0	136	154	176	178	154	160	0	174	176	179	179
50	WX71B	160	162	0	136	154	176	178	154	160	0	174	176	179	179
51	WX72B	160	162	0	136	154	178	178	154	160	164	174	176	179	179
52	DL32B	162	162	0	118	136	176	178	166	0	166	176	176	179	181
53	DL21B	160	162	0	136	136	176	178	156	162	0	176	176	179	181
54	DL21A	162	162	0	136	136	174	176	156	162	0	176	176	179	181
55	DL44B	160	162	0	118	136	174	176	162	162	0	176	176	179	181
56	DL63B	162	162	0	118	136	176	178	0	0	0	176	176	179	181
57	DL52C	162	162	0	136	136	176	178	156	162	0	176	176	179	181
58	DL42C	160	162	0	118	136	176	178	166	166	0	176	176	179	181
59	DL61B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
60	DL62B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
61	CK33C	162	162	0	118	136	178	178	166	166	0	174	176	179	181
62	DL72A	162	162	0	118	136	176	178	166	166	0	176	176	179	181
63	DL83B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
64	DL52B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
65	DL32A	162	162	0	118	136	176	178	166	166	0	176	176	179	181
66	DL52A	162	162	0	136	136	176	178	156	162	0	176	176	179	181
67	DL54A	162	162	0	136	136	176	178	156	162	0	176	176	179	181
68	DL82C	162	162	0	118	140	176	178	162	162	0	176	176	179	181
69	KY12C	162	162	0	118	142	176	178	0	0	0	176	176	179	181
70	DL62A	162	162	0	136	136	176	178	138	150	0	176	176	179	181
71	DL81B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
72	DL55B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
73	DL83A	162	162	0	118	140	176	178			0	176	176	179	181
74	DL42B	162	162	0	118	136	176	178	162	162	0	176	176	179	181
75	DL32D	162	162	0	118	136	176	178	166	166	0	176	176	179	181
76	DL81C	160	162	0	118	136	176	178	162	162	0	176	176	179	181
77	DL43C	162	162	0	118	136	176	178	162	162	0	176	176	179	181
78	DL54D	162	162	0	118	136	176	178	156	162	0	176	176	179	181
79	KY12A	162	162	0	118	142	176	178	0	0	0	176	176	179	181
80	DL81B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
81	DL53B	162	162	0	136	136	176	178	156	162	0	176	176	179	181
82	DL122	162	162	0	118	138	178	178	162	164	0	176	176	179	181
83	DL15A	162	162	0	118	136	176	178	162	162	0	176	176	179	181
84	MN12C	162	162	0	118	136	178	178	162	162	0	176	176	179	181
85	DL161	162	162	0	136	136	176	178	156	162	0	176	176	179	181
86	DL125	162	162	0	136	136	176	178	156	162	0	176	176	179	181
87	DL111	162	162	0	136	136	176	178	156	162	0	176	176	179	181
88	DL163	162	162	0	118	136	176	178	162	162	0	176	176	179	181
89	CW11C	162	162	0	118	136	176	178	166	166	0	176	176	179	181
90	C31B	162	162	0	118	136	176	178	166	166	0	174	176	179	181
91	CK31C	162	162	0	118	136	178	178	166	166	0	174	176	179	181
92	CK32D	156	162	0	118	136	176	176	162	162	0	176	176	179	181
93	CK34A	162	162	0	118	136	178	178	166	166	0	174	176	179	181
94	CK31B	162	162	0	118	136	176	178	166	166	0	174	176	179	181
95	CK11C	162	162	0	118	136	176	178	166	166	0	176	176	179	181
96	CK32B	162	162	0	118	136	178	178	166	166	0	174	176	179	181
97	CK12D	156	162	0	118	136	176	178	162	162	0	176	176	179	181
98	CK35A	162	162	0	118	136	178	178	166	166	0	174	176	179	181

Appendix 2.6: Tables showing the ANOVA statistics for parameters of aggressiveness of *Phytophthora infestans* isolates on the foliage and tubers of four potato cultivars Bionica, British Queen, Rooster and Sarpo Mira

a. ANOVA table of mean values of infection efficiency (IE) on detached leaflets of four potato cultivars inoculated with thirty isolates of *Phytophthora infestans*. Initial sporangial concentration in the inoculums was treated as a covariate in the analysis of variances

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**cov.ef.**	**F pr.**
Expt	1	1.24	1.24	0.06	1	0.813
Isolate	29	182017.28	6276.46	287.14	0.97	<.001
Expt.Isolate	29	1088.61	37.54	1.72	0.99	0.028
Cultivar	3	41115.6	13705.2	626.99	0.97	<.001
Isolate.cultivar	87	69378.74	797.46	36.48	0.99	<.001
Covariate	1	0.01	0.01	0		0.983
Residual	89	1945.43	21.86		0.99	
Total	239	295684.4				

b. ANOVA table of mean values of AULPC on detached leaflets of four potato cultivars inoculated with thirty isolates of *Phytophthora infestans*. Initial sporangial concentration in the inoculums was treated as a covariate in the analysis of variances

Source of variation	d.f.	s.s.	m.s.	v.r.	cov.ef.	F pr.
Expt	1	53813	53813	0.38	1	0.538
Isolate	29	122725801	4231924	30.12	0.97	<.001
Expt.Isolate	29	11667836	402339	2.86	0.99	<.001
Variety	3	110670205	36890068	262.54	0.97	<.001
Isolate.Variety	87	57431358	660131	4.7	0.99	<.001
Covariate	1	61427	61427	0.44		0.51
Residual	89	12505549	140512		0.99	
Total	239	315790245				

c. ANOVA table of mean values of latent period (LP) on detached leaflets of four potato cultivars inoculated with thirty isolates of *Phytophthora infestans*. Initial sporangial concentration in the inoculums was treated as a covariate in the analysis of variances

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	cov.ef.	F pr.
Expt	1		5.199	5.199	9.33	1	0.003
Isolate	29		408.8197	14.0972	25.29	0.96	<.001
Expt.Isolate	29		35.6967	1.2309	2.21	0.99	0.002
Cultivar	3		262.0356	87.3452	156.72	0.97	<.001
Isolate.cultivar	87		172.413	1.9818	3.56	0.99	<.001
Covariate	1		0.5315	0.5315	0.95		0.331
Residual	88	1	49.0467	0.5573		1	
Total	238	1	932.2186				

d. ANOVA table of mean values of sporulation density (SI) on detached leaflets of four potato cultivars inoculated with thirty isolates of *Phytophthora infestans*. Initial sporangial concentration in the inoculums was treated as a covariate in the analysis of variances

Source of variation	d.f.	s.s.	m.s.	v.r.	cov.ef.	F pr.
Expt	1	188	188	0.01	1	0.904
Isolate	29	37108401	1279600	99.41	0.97	<.001
Expt.Isolate	29	677513	23363	1.81	0.99	0.018
Cultivar	3	52481094	17493698	1359.04	0.97	<.001
Isolate.cultivar	87	41855874	481102	37.38	0.99	<.001
Covariate	1	1375	1375	0.11		0.745
Residual	89	1145619	12872		0.99	
Total	239	135824810				

e. ANOVA table of mean values of AULPC induced by four genotypes *Phytophthora infestans* on detached leaflets of four potato cultivars. Initial sporangial concentration in the inoculums was treated as a covariate in the analysis of variances

Source of variation	d.f.	s.s.	m.s.	v.r.	cov.ef.	F pr.
Expt	1	53813	53813	0.08	1	0.783
Genotype	3	32959167	10986389	15.59	0.99	<.001
Expt.Genotype	3	1958261	652754	0.93	1	0.429
Variety	3	110664760	36888253	52.36	0.99	<.001
Genotype.Variety	9	15133572	1681508	2.39	1	0.013
Covariate	1	1749621	1749621	2.48		0.116
Residual	219	154284989	704498		1.01	
Total	239	315790245				

f. ANOVA table of mean values of AULPC on detached leaflets of four potato cultivars inoculated reference isolate (DL16-1D) of *Phytophthora infestans*.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Experiment stratum	5	1672241	418060	0.59	
Leaflet.*Units* stratum					
Variety	3	56866683	18955561	26.78	<.001
Residual	64	45297733	707777		
Total	72	103836657			

g. ANOVA table of mean values of tuber blight lesion sizes (%) on whole tubers of four potato cultivars inoculated with ten isolates of *Phytophthora infestans*.

Source of variation	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr.**
expt	1	7073.2	7073.2	83.74	<.001
Isolate	9	5345.52	593.95	7.03	<.001
expt.Isolate	9	1960.85	217.87	2.58	0.025
Cultivar	3	519.29	173.1	2.05	0.128
Isolate.Cultivar	27	1767.65	65.47	0.78	0.747
Residual	30	2534.1	84.47		
Total	79	19200.62			

h. ANOVA table of mean values of tuber blight incidence (%) on whole tubers of four potato cultivars inoculated with ten isolates of *Phytophthora infestans*.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Expt	1	1983.4	1983.4	11.04	0.002
Isolate	9	1736.1	192.9	1.07	0.409
expt.Isolate	9	635	70.6	0.39	0.929
Cultivar	3	1002.6	334.2	1.86	0.158
Isolate.Cultivar	27	2015.1	74.6	0.42	0.988
Residual	30	5388.5	179.6		
Total	79	12760.8			

i. ANOVA table of mean values of tuber blight incidence (%) on whole tubers of four potato cultivars inoculated with four *Phytophthora infestans* genotypes

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Expt	1	1983.4	1983.4	14.45	<.001
Genotype	3	246	82	0.6	0.619
Expt.Genotype	3	346.3	115.4	0.84	0.477
Cultiva	3	1002.6	334.2	2.44	0.074
Genotype.Cultiva	9	947.9	105.3	0.77	0.647
Residual	60	8234.7	137.2		
Total	79	12760.8			

j. ANOVA table of mean values of tuber blight lesion size (%) on whole tubers of four potato cultivars inoculated with four *Phytophthora infestans* genotypes.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Expt	1	7073.2	7073.2	49.36	<.001
Genotype	3	1778.9	593	4.14	0.01
Expt.Genotype	3	776.7	258.9	1.81	0.156
Cultiva	3	519.3	173.1	1.21	0.315
Genotype.Cultiva	9	454.9	50.5	0.35	0.953
Residual	60	8597.7	143.3		
Total	79	19200.6			

Appendix 3.1: *SSR profiles of 96 isolates isolated from polytunnel in 2011 as revealed by 10 markers. Random single lesion leaf samples covering the area in the polytunnel were obtained during the final stages of the epidemic on the second season crops

Isolate	Pi02	Pi02	D13	D13	Pi33	Pi33	Pi04	Pi04	Pi4B	Pi4B	Pi16	Pi16	G11	G11	Pi56	Pi56	Pi63	Pi63	Pi89	Pi89
1-E1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G7	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-H1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-F6	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-D6	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-A2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-F4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-B6	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-E3	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-F1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-E8	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-D5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G8	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-D6	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-C2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-F4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-A4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-F8	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-A4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-H3	160	162	118	136	203	203	166	170	205	213	176	178	154	160	174	176	151	157	179	181
2-B1	160	162	118	136	203	203	166	170	213	213	178	178	154	160	174	176	151	157	179	181
2-A1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-B3	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-E2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-B8	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G6	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-B2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-C8	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-H3	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-G5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-F3	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-G2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-D8	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-F5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-H2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-E4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-E2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-B3	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-E7	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-E7	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-D1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-C5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
2-D3	160	162	118	136	203	203	166	170	205	213	178	178	154	160			151	157	179	181
2-E4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-F1	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176			179	181

*blank spaces indicate missing data

Appendix 3.1: Continued *SSR profiles of 96 isolates as revealed by 10 markers. Random single lesion leaf samples covering the area in the polytunnel were obtained during the final stages of the epidemic on the second season crops.

Isolate	Pi02	Pi02	D13	D13	Pi33	Pi33	Pi04	Pi04	Pi4B	Pi4B	Pi16	Pi16	G11	G11	Pi56	Pi56	Pi63	Pi63	Pi89	Pi89
2-F7	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-D2	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-H4	160	162	118	136	203	203	166	170	205	213	176	178	154	160	174	176	151	157	179	181
1-G4	160	162	118	136	203	203	166	170	205	213	176	178	154	160	174	176	151	157	179	181
2-B5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-A7	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-D4	160	162			203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-C3	160	162			203	203	166	170	205	213	178	178	154	160	174	176	151	157	179	181
1-A3	160	162					166	170	205	213			154	160	174	176	151	157	179	181
2-C5	160	162					166	170	205	213			154	160	174	176	151	157	179	181
2-E6	160	162					166	170	205	213			154	160	174	176	151	157	179	181
2-F2	160	162	118	136	203	203			205	213	178	178	154	160	174	176			179	181
1-H7	160	162	118	136	203	203			205	213	178	178	154	160	174	176			179	181
2-E1	160	162	118	136	203	203			205	213	178	178	154	160	174	176			179	181
2-A5	160	162	118	136	203	203			205	213	176	178	154	160	174	176			179	181
1-E5	160	162	118	136	203	203			205	213	178	178	154	160	174	176			179	181
2-C6	160	162	118	136	203	203			205	213	178	178	154	160	174	176	151	157	179	181
2-D7	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157		
1-H2	160	162	118	136	203	206	166	170	205	213	176	178	154	160	174	176	151	157		
2-C7	160	162	118	136	203	203	166	170			178	178	154	160	174	176	151	157		
1-F7	160	162	118	136	203	203	166	170	205	213	178	178	154	160			151	157		
1-F5	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157		
2-C4	160	162	118	136	203	203	166	170	205	213	178	178	154	160	174	176	151	157		
2-B7	160	162	118	136	203	203	166	170			178	178	154	160	174	176	151	157		
2-H7	160	162	118	136	203	203	166	170			178	178	154	160	174	176	151	157		
1-H5	160	162					166	170	205	213			154	160	174	176	151	157		
2-E5	160	162			203	203	166	170	205	213	178	178	154	160	174	176	151	157		
1-H8	160	162					166	170					154	160	174	176				
2-D5	160	162					166	170					154	160	174	176				
2-D4	160	162					166	170					154	160	174	176	151	157		
2-H1	160	162					166	170					154	160	174	176	151	157		
1-E6	160	162	118	136	203	206					176	178	154	160						
1-C6	160	162			203	203							154	160	174	176				
2-A3	160	162											154	160	174	176				
1-B3	160	162	118	136	203	203	166	170	205	213	178	178					151	157	179	181
2-F3	160	162	118	136	203	203	166	170	205	213	178	178					151	157	179	181
1-E8	160	162	118	136	203	203	166	170	205	213	178	178					151	157	179	181
1-E2	160	162	118	136	203	203	166	170	205	213	176	178			174	176			179	181
1-G7	160	162	118	136	203	206			205	213	176	178			174	176			179	181
2-A6	160	162	118	136	203	206	166	170			176	178					151	157		
1-D1	160	162	118	136	203	203	166	170	205	213	178	178					151	157		
1-D8	160	162					166	170												
1-D3	160	162													174	176				
2-G3			118	136	203	203	166	170			178	178	154	160	174	176				
1-A1			118	136	203	203	166	170			178	178					151	157		
1-D7			118	136	203	206	170	170			176	178			174	176				
1-A6			118	136	203	206					176	178			174	176				
1-B1											176	178								

*blank spaces indicate missing data

Appendix 3.2: Statistics for analysis of variances for various aspects of host-isolates interactions to assess effect of sexual reproduction in *P. infestans*

a. Number of lesion induced on each of the five cultivars (Bionica, Cara, Maris Piper, Sarpo Mira and Toluca) when they were simultaneously inoculated with isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans*.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Leaf disc stratum	39	13.195	0.3383	1.15	
Cultivar	4	32.22	8.055	27.45	<.001
Residual	156	45.78	0.2935		
Total	199	91.195			

b. Oospore production on each of the five cultivars (Bionica, Cara, Maris Piper, Sarpo Mira and Toluca) when they were simultaneously inoculated with isolates CW1-3C_A2 and DL4-3B_A1 of *P. infestans*.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Leaf_disc stratum	15	2.4875	0.1658	0.77	
Cultivar	4	303.45	75.8625	351.49	<.001
Residual	60	12.95	0.2158		
Total	79	318.8875			

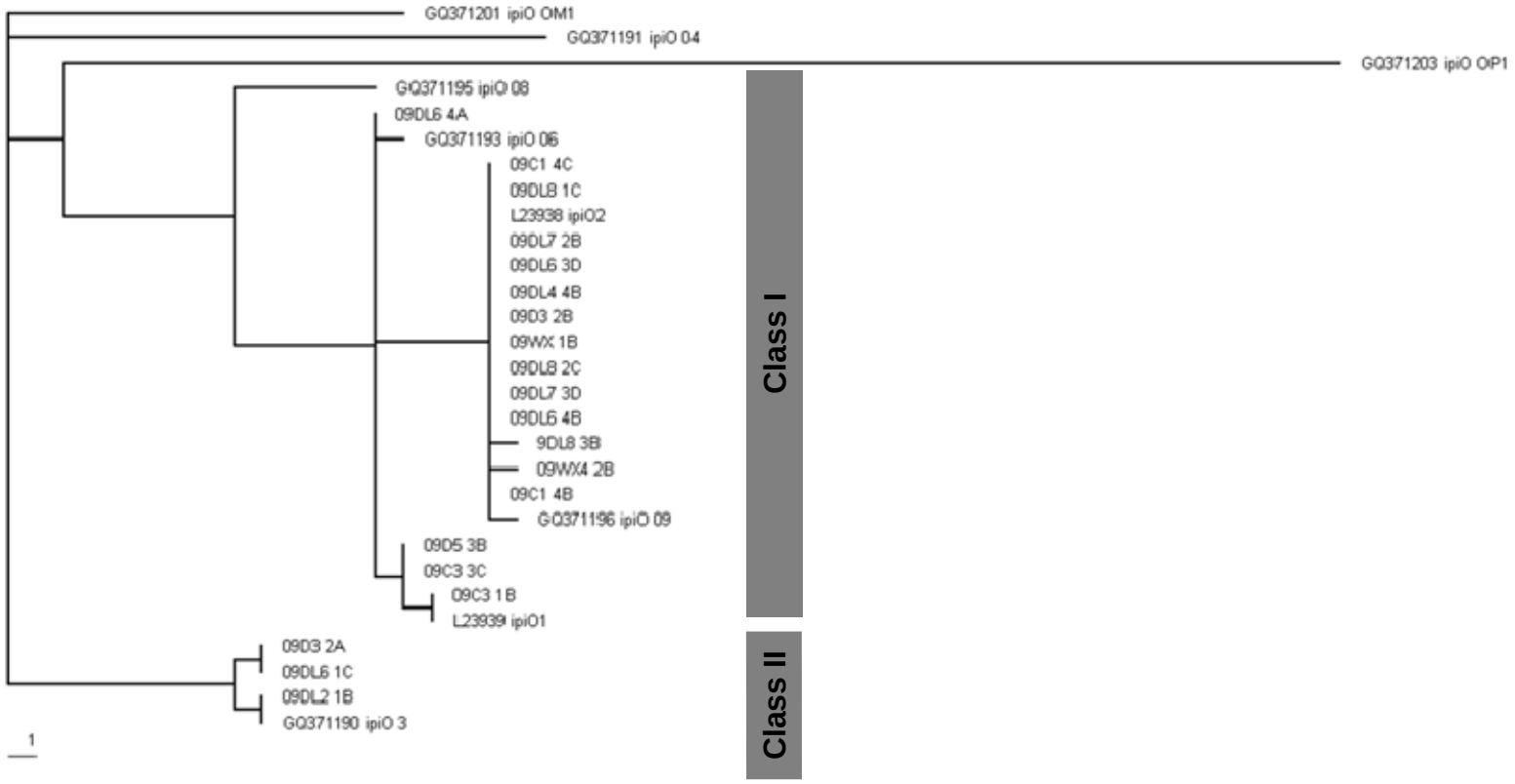
c. Area under Lesion Progress Curve (AULPC) induced on cultivar Cara by 12 isolates of *P. infestans* recovered from *in vitro* and polytunnel crosses (between isolates CW1-3C_A1 and DL4-3B_A1).

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Tray stratum	5		376302	75260	0.87	
Isolate	11		88417089	8037917	92.83	<.001
Expt	1		199223	199223	2.3	0.132
Isolate.Expt	11		8331381	757398	8.75	<.001
Residual	114	1	9870951	86587		
Total	142	1	106824652			

d. Latent Periods (LP) of 12 *P. infestans* isolates recovered from *in vitro* and polytunnel crosses (between isolates CW1-3C_A1 and DL4-3B_A1) when they were inoculated onto cultivar Cara.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Tray stratum	5	3.701	0.74	0.61	
Isolate	11	505.076	45.916	37.86	<.001
Expt	1	0.84	0.84	0.69	0.407
Isolate.Expt	11	17.576	1.598	1.32	0.224
Residual	115	139.465	1.213		
Total	143	666.66			

Appendix 4.1: Phylogenetic relationships among *P. infestans* isolates according to the *ipiO* effector gene. The minimum evolution tree was rooted with Ipio-Op1 (NCBI Accession number GQ371203. *IpiO* effector gene classes are shown (Champouret *et al.*, 2009). The tree is drawn to scale. Scale bar shows genotype divergence in percentage



Appendix 4.1: continued

The PCR products of *ipiO* gene were amplified from genomic DNA of isolates of *P. infestans* reflecting the representation of genotypes within the 2009 Irish population. The PCR products were sequenced (Gatc Biotech, Germany) and the sequence data were aligned together with *ipiO* nucleotide sequences deposited at NCBI GenBank under accession numbers GQ371190-GQ371203 (Champouret *et al*., 2009); L23938.1 (Pieterse *et al*., 1994) and AY961430.1 (Win *et al*., 2006) using ClustalW 2.0.12 Multiple Sequence Alignment Computer Software and clustering was done using Neighbour Joining algorithm. The Neighbour Joining Tree was rooted using IpiO-Op1. Phylogenetic analysis of *ipiO* sequences were conducted using Maximum Parsimony method in PAUP 4b program. The 28 isolates were classified in either Class I or Class II of the *ipiO* family of effectors both which are recognized by Rpi-blb1 resistance gene that is presently a major source of non-specific resistance to *P. infestans*

Appendix 4.2: Potato discs of cultivar Kerr's Pink inoculated with representative sets of [a]*Phytophthora infestans* isolates from three sources (i) clonal isolates passed ten times through leaves of transgenic potato lines equipped with *RB* gene for resistance to blight; (ii) isolates from *in vitro* crosses between isolates CW1-3C_A2 and DL4-3B_A1 (iii) isolates recovered from potato growing on in the polytunnel previously inoculated simultaneously with two isolates CW1-3C_A2 and DL4-3B_A1.

[a]Isolates from reiterated passaging through leaflets of transgenic and non-trangenic potato lines: MP100_9A1, MP100_4A2, MP100_6A2, MPWT_A2, MP100_9A1, MP100_A2, MP100_5A2 and unpassaged control isolates CW1-3C and DL4-3B; isolates from *in vitro* crosses: I-20, I-48, I-69, I-13, I-70, I-32, parental isolates were CW1-3C and DL4-3B; isolates recovered from the polytunnel potato crop: T4-8, T4/5, T4/10, T35 parental isolates previously introduced into the polytunnel were CW1-3C and DL4-3B.

Appendix 4. 3: Statistics for analysis of variances for area under lesion progress curve (AULPC) and Latent Period (LP) of 12 isolates of *P. infestans* inoculated onto leaflets of cultivar Cara in two replicate experiments to assess their aggressiveness after ten passages through potato lines bearing the blight resistance *RB* gene

a. AreaUnder Lesion Progress Curve (AULPC)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Tray stratum	5		3714007	742801	3.89	
Tray.*Units* stratum						
Isolate	11		12159710	1105428	5.79	<.001
Expt	1		499330	499330	2.61	0.109
Isolate.Expt	11		11455148	1041377	5.45	<.001
Residual	111	4	21206993	191054		
Total	139	4	48033512			

b. Latent Period (LP)

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
Tray stratum	5		0.29646	0.05929	0.63	
Isolate	11		1.44053	0.13096	1.39	0.187
Expt	1		0.1408	0.1408	1.5	0.224
Isolate.Expt	11		0.26529	0.02412	0.26	0.992
Residual	112	3	10.5463	0.09416		
Total	140	3	12.60993			

Appendix 5.1: Difference in the cost TaqMan qPCR detection of *P. infestans* in potato tuber samples prepared by two DNA extraction procedures (GenElute Kit and NaOH Lysis)

	Cost (€) for a single run of 96 well plate*	
Item	**GenElute**	**NaOH**
Universal Master Mix	23	23
*Ypt*1 specific Primers	0.1	0.1
TaqManProbe	3.4	3.4
LightCycler PCR plate	2.2	2.2
LightCycler Adhesive	0.5	0.5
GenElute Kit	30.7	n/a
NaOH	n/a	0.04
1.5ml Eppendorf tubes	n/a	0.8
2.0ml Eppendorf tubes	n/a	1.8
Blue filter tips	0.9	0.9
Yellow filter tips	2.5	2.5
White filter tips	2.7	2.7
Total (€)	**66.0**	**37.8**
Cost (€)per reaction	**0.7**	**0.4**

*****One qPCR run on a full 96 well plate completes 32 samples including, a non-template and a positive sample. Each of the 32 replicated three times.

Appendix 5.2: ANOVA table of mean crossing points (Cp) during qPCR amplification of template target gene *Ypt*1 of *P. infestans* using different primer concentrations

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.00002917	0.00001458	0.22	
FWD	3	0.66810833	0.22270278	3389.98	<.001
RVS	3	0.793625	0.26454167	4026.85	<.001
FWD.RVS	9	0.40305833	0.04478426	681.71	<.001
Residual	30	0.00197083	0.00006569		
Total	47	1.86679167			

Appendix 5.3: ANOVA table of mean crossing points (Cp) during qPCR amplification of template target gene *Ypt*1 of *P. infestans* using different probe concentrations

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.00017333	0.00008667	1.11	
Concentation	4	2.45677333	0.61419333	7840.77	<.001
Residual	8	0.00062667	0.00007833		
Total	14	2.45757333			