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INHERITANCE OF COLD TEMPERATURE TOLERANCE IN PROGENY ISOLATES OF PHYTOPHTHORA INFESTANS FROM MATING

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INHERITANCE OF COLD TEMPERATURE TOLERANCE IN PROGENY ISOLATES OF *PHYTOPHTHORA INFESTANS* FROM MATING

By

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ABSTRACT

INHERITANCE OF COLD TEMPERATURE TOLERANCE IN PROGENY ISOLATES OF *PHYTOPHTHORA INFESTANS* FROM MATING

By

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Late blight of potato, caused by *Phytophthora infestans*, remains a threat to the production of high quality potatoes. Optimal temperature for mycelial growth is around 10°C with maxima and minima of 30 and 3°C, respectively. Recent studies have indicated that some isolates of different genotypes of *P. infestans* can survive about 5 days continuous exposure to -3°C. To observe more variation in the tolerance to cold temperature, isolates were tested for tolerance at -5°C for 5 days. This phenotypic trait has serious implications for potato production and disease protection. In this project mating type is used as a biological tool for enhancing the mutation in the pathogen. Crosses were carried out between isolates that were highly variable for temperature tolerance and identified as tolerant, sensitive or intermediate in tolerance of exposure to -5°C. To determine if temperature tolerance is a genetically inherited trait or occurs as a result of physiological adaptation of different *P. infestans* genotypes the progeny isolates from these crosses were assessed by using the phenotypic and genotypic characteristics of progeny such as mating type, isozyme analysis, Simple Sequence Repeats, metalaxyl sensitivity, virulence and pathogenicity tests. Progeny isolates from crosses performed have indicated that cold tolerance is a continuous variable showing both inheritance and hybridity. These data illustrate the potential variation in different characteristics of the pathogen after recombination or mutation occurs. Survival potential of inoculum may be increased under recent reported climatic changes in north-central US production areas.

Dedicated to:

My daughters Laya and Kavya,
My Pride and Joy

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1 Literature review

1.1 Introduction

Phytophthora infestans, the causal agent of late blight remains a major threat to potato production in the U.S. This is particularly true in Michigan where conditions are typically ideal for its spread, with periods of high humidity, frequent rainfall and moderate temperatures (Kudwa, 1999). Michigan is a major producer of potatoes in the U.S. In 1999, 18,000 ha of potatoes were planted in Michigan, of which 82% were round white cultivars used for table production or potato chipping. Currently the majority of the commercially available cultivars are susceptible to late blight and control measures can cost over $287.8 million, or $507 per hectare (Guenthner *et al.*, 2001). Potato late blight is notoriously difficult to manage resulting in tens of billions of dollars of losses annually (Erwin and Ribeiro, 1996).

1.2 Host

The potato is believed to have originated in the Andes mountains of Peru where it was first noted to be a major food source for the Incas around 400 BC (Schumann, 1991). The potato tubers grow underground and originate from the swollen, underground stems of the plant. It is a complete food that is rich in carbohydrates, proteins and vitamins. In the 16th century, potatoes were brought to Europe by Spanish explorers who traveled to South America in search of treasure. On their return trip home they brought potatoes with them (Schumann, 1991). The potato was introduced to Spain around 1570 and from there was distributed to Europe and England. The potato became a staple food crop in Europe during the 17th and 18th century because it was nutritious, easy to grow, and a large yield could be obtained from a relatively small plot of land. They were first

introduced into North America in the 17th century when European settlers brought potato tubers back to the New World. Potatoes have become a staple food crop in Europe. By the 1800's the Irish were totally dependent upon the potato for their food. Unfortunately, it was this dependence that led to the famine that occurred in the mid 1850's.

1.3 Pathogen

The pathogen was first described and named by Anton de Bary in 1861 (De Bary, 1876) as the cause of potato late blight. One of the main epidemics caused by *Phytophthora infestans* was the Irish potato famine of the 1840's which killed over 1.5 million people and almost the same numbers of people migrated from Europe to the United States and other parts of the world as a result of severe failure of the primary crop (Bourke, 1964). Late blight is readily transmitted from seed tubers or volunteer tubers in the fields or from discarded cull-piles. *P. infestans* survives in tubers as mycelium stored at about 3°C and produces spores on the tuber surface when temperature increases in storage or in soil. Optimal temperature for the growth of mycelium and spore production in tubers is 10°C. Distribution of *P. infestans* varies worldwide. *Phytophthora infestans* is a diploid, heterothallic organism believed to have originated and evolved in the central highlands of Mexico (Niederhauser, 1991). Until the early 1980's, A_1 was the only mating type found in most of the world, but in Central Mexico both A_1 and A_2 isolates coexisted in a 50:50 ratio and later spread world-wide. In the U.S, until the mid 1990s, A_1 mating type, US-1 isolates were predominant. The A2 mating type was likely introduced to the United States and Canada in the early 1990s. The US-8 (A_2) genotype is widespread in the United States (Fry 1997). The more aggressive nature of US-8 genotype made it the most common genotype, surpassing US-1. The different genotypes prevalent in North America

are US-1, US-6, US-7/16, US-8, US-10, US-11, US-12, US-14, and US-17. Mating of *P. infestans* threatens potato industries worldwide due to the possible emergence of recombinant genotypes with increased aggressiveness and wider virulence.

The probability that the disease will emerge from an infected tuber as a seed or from a cull pile is difficult to estimate because several factors (e.g. temperature) can influence, survival of mycelium and oospores. Oospores of oomycetous fungi are endogenously dormant, highly effective survival structures that allow *Phytophthora spp.* to survive adverse environmental conditions for relatively long periods of time in plant debris and in soil (Madden *et al.,* 1991). The production of oospores by *P. infestans* in the field and their survival under natural conditions has been intensively studied since the introduction of A_2 mating type isolates and the emergence and spread of new genotypes in North America.

1.4 Taxonomy

Phytophthora species are classified taxonomically in the kingdom Chromista, phylum Oomycota, order *Peronosporales*, family *Peronosporaceae* and genus *Phytophthora* (Birch, 2001). Oomycetes have often been confused by taxonomists with the true fungi such as ascomycetes and basidiomycetes, because of the filamentous growth. *Phytophthora* spp. differ from true fungi because the cell walls are composed of β– 1, 3 glucan polymers and cellulose rather than mostly chitin. Studies of phylogeny based on sequencing of the small subunit ribosomal DNA (Förster *et al.*, 1990) suggest the class shares little taxonomic affinity with filamentous fungi and should be classified instead with the chrysophytes, diatoms and brown algae (Judelson, 1997).

1.5 Host range

Pathogenesis, which involves asexual growth, displays a high degree of biotrophy and host specificity, with successful infections mainly limited to potato, tomato, and several less well known *Solanaceous* species although more distantly related species such as petunia may also be hosts (Erwin and Ribiero, 1996). The disease characteristics of *P. infestans* are therefore more reminiscent of the downy mildews than most other *Phytophthora* species, which are necrotrophs and often exhibit broad host ranges (Erwin and Ribiero, 1996).

1.6 Disease cycle

Primary infection often occurs after emergence of the pathogen in the spring. Epidemics are usually initiated by the dispersal of sporangia and zoospores. The source of primary inoculum has been widely debated. However, most studies show that cull piles are the major cause of primary infection (Bonde and Schultz, 1943, Zwankhuizen *et al.*, 1998, Kirk 2003a). Sources of early infection in potato fields have been traced to cull piles, infected seed tubers and volunteer plants and infested organic potato fields were also found to be a source of mid-season inoculum in Netherlands (Zwankhuizen *et al.*, 1998).

P. infestans is an aerial pathogen capable of infecting all parts of the plant with the exception of the roots (Fehrmann and Dimond, 1967). Sporangia are formed on aerial portions of infected plants and transferred to uninfected plants where direct germination may occur from a germ tube or by indirect germination by producing zoospores that swim to an infection site (Judelson, 1997). The germ tube then differentiates into an appressorium that produces narrow hyphae also called penetration pegs and degrading

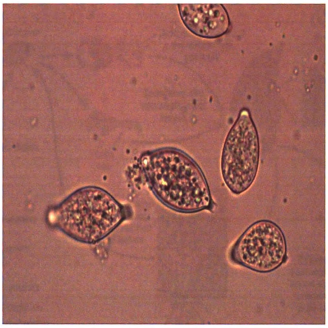


Figure 1.1 Reproduction structure of *Phytophthora infestans*: Sporangia and sporangia releasing zoospores

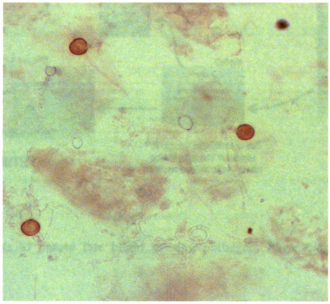


Figure 1.2 Reproduction structure of *Phytophthora infestans*: Oospores with Antheridum and oogonium

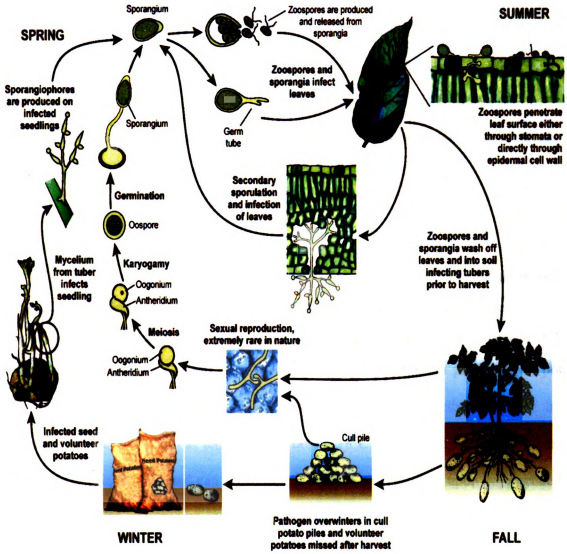


Figure 1.3 Life cycle of potato late blight causing pathogen, *Phytophthora infestans*. (© P.S. Wharton)

enzymes that enable invasion of the epidermis of the plant. Once inside the plant cell, the penetration pegs differentiate into primary infection hyphae, which then colonize host-plant tissue. *P. infestans* has a well distinguished life cycle and infection process. It can reproduce both asexually and sexually in plants and also *in vitro*. However, the most predominant means is by asexual reproduction with spores surviving over winter in infected plant material (Pethybridge, 1911).

1.6.1 Asexual reproduction

Asexual reproduction occurs via formation of sporangia. They are borne on stalk-like sporangiophores (Blackwell, 1949). Sporangia are lemon-shaped, semipapillate, tapered to the base and caducous. Average size ranges from 36 x 22 μm to 29 x 29 μm and formation occurs at an optimum temperature of 18-22°C, where relative humidity exceeds 90% (Erwin and Ribeiro, 1996). Sporangia produced on potato plants can be dispersed over large distances by wind (Hirst and Stedman, 1960). In the presence of free water and at temperatures below 18°C biflagellate zoospores produced within sporangia emerge and can swim in leaf surface or soil water upon emergence to a new infection site. Zoospores can move from cull piles to field plants in dense fog, rain puddles, or wind-blown rain (Bonde and Stedman, 1943). The pathogen therefore favors wet weather with high humidity and temperatures between 16 and 21°C for continual sporangial production and zoospore release. The main source of inoculum in an asexually reproducing population is therefore by infected tubers from the previous season, with survival possible from infected seed (Pethybridge, 1911), discarded tubers present in cull piles (Bonde and Schultz, 1943) and from volunteers or groundkeepers (Hirst and Stedman, 1960).

1.6.2 Sexual reproduction

P. infestans the causal agent of late blight in potatoes is a heterothallic oomycete, with two mating types or compatibility types and is able to reproduce sexually. Mating types differ in hormone production rather than in sexual form, with male and female gametangia (oogonia and antheridia) produced in response (Judelson, 1997). Sexuality is independent of mating type with both mating types capable of producing either type of sexual structure (Brasier, 1992), though isolates will preferentially form either oogonia or antheridia depending on mating partner (Gallindo and Gallegly, 1960). The mechanism by which mating type is determined appears to be governed by a single locus, where heterozygosity results in the A_1 mating type and homozygosity results in the A_2 mating type (Judelson *et al.*, 1995). In this system an equal number of A_1 and A_2 progeny would be expected from any mating; however, non-Mendelian segregation has been observed (Judelson *et al.*, 1995). As a result of sexual reproduction, where haploid nuclei from oogonia and antheridia fuse, they produce diploid thick-walled oospores. Oospores are spherical in shape and have a thick inner wall composed largely of β-1,3-glucans (Erwin and Ribeiro, 1996). Oospores are highly effective survival structures that allow *Phytophthora* spp. to survive adverse environmental conditions for relatively long periods of time in plant debris and in soil (Madden *et al.*, 1991). The optimum temperature for oospore production is 10°C but can occur anywhere between 5 and 25°C (Drenth *et al.*, 1995). Under high humidity from 8 to 23°C (Cohen *et al.*, 1997), oospores can germinate and form one or more germ tubes which either commence mycelial growth directly or produce a sporangium which will proceed to reproduce asexually (Erwin and Ribeiro, 1996). Oospores of *P. infestans* have been found to survive in soil during a

winter season in Europe and oospores also have been reported to survive temperatures between -80 and 35°C (Drenth *et al.*, 1995).

P. infestans can also be homothallic. Oospore production as a result of 'self-fertilization' has been well documented, with the first report in pure culture as early as 1911 (Clinton, 1911). Such an occurrence may result from non-disjunction (Fyfe and Shaw, 1992) or the presence of extra copies of the mating type locus in one parent (Judelson, 1996) or by autogamy (Smart *et al.*, 1998) whereby both male and female sexual structures are produced. It would appear though that the tendency for self-fertilization is isolated or even genotype specific (Groves and Ristaino, 2000) with multiple studies suggesting self-fertility is particularly common in one particular lineage named US-8 (Smart *et al.*, 1998, Groves, 2002), although reports from other genotypes are common e.g. from the UK (Tantius *et al.*, 1986) and Japan (Mosa *et al.*, 1989). Oospores may act as a long-term inoculum source with survival demonstrated for up to two years (Perches and Galindo, 1967). Additionally sexual recombination of genetic factors may generate genetically more diverse progeny, which could be more pathogenic or aggressive than parental isolates, leading to drastic changes in genetic structure of the population (Levin *et al.*, 2001). However, studies of populations where the A_1 and A_2 mating types are known to co-exist have demonstrated oospores are produced in only small numbers in the canopy (Drenth *et al.*, 1995). In most parental combinations, a characteristic mating region was present at the interaction zone between two isolates, where oospores are formed. Self-fertility could be due to the presence of an extra chromosome containing the mating type locus (Mortimer *et al.*, 1977). In a study of A_1 and A_2 mating types of *P. infestans* strains

from Japan, Mosa and colleagues reported that the A_2 isolates could form oospores in pure culture if the culture was grown in the dark for one month (Mosa *et al.*, 1989). In a study, Goodwin *et al.*, (1994) observed that a *P. infestans* strain of the US-8 clonal lineage was self-fertile in pure culture.

1.7 Non-chemical and chemical Control

For controlling late blight, adequate disease management strategies before, during and after planting and use of appropriate potato cultivars containing some resistance to potato blight are required (Garrett and Dendy, 2001). Cultural control usually refers to non-chemical methods which decrease a population by reduction in survival, dispersal and reproduction (Kirk *et al.*, 2005). A mixture of non-chemical and chemical measures can be incorporated into decision support systems (DSS), which integrate information of a variety of factors to achieve better control and more appropriate fungicide application with reduced input and longer time intervals between applications (Schepers, 2002).

For non-chemical control, good quality, preferably certified tubers should be used to minimize infection from planting of infected seed (Bumbawale, *et al.*, 1989). Volunteer plants should be destroyed by herbicide application or mechanical measures (Leach *et al.*, 1986) and un-harvested potatoes removed (Sato, 1980). Crop rotation and fallowing have traditionally been used in potato production to control a variety of diseases and pests such as the potato cyst nematode (Thurston, 1990). Intercropping with immune crop plants can also decrease reproduction, usually by planting resistant tubers along with a susceptible crop either as a mixture, or in blocks can greatly reduce late blight incidence by presenting a barrier to spread of inoculum, particularly in areas where sporangial

levels are relatively low (Garrett and Mundt, 2000). In addition over-fertilization and early nitrogen application should be avoided with multiple studies observing increasing infection of *P. infestans* under such conditions (Phukan, 1993).

For chemical control there are three types of fungicides used. They are protectants, systemic and translaminar fungicides. Protectants or contact fungicides such as chlorothalonil, mancozeb, maneb, fluazinam and the copper-based compounds act by direct contact with fungi through interference with several chemical processes e.g. cell wall disruption (Kuhn *et al.*, 1991). Both translaminar and systemic fungicides are single-site inhibitors and are therefore at high risk for resistance development (Gisi *et al.*, 2002), although as of yet only metalaxyl resistance has been documented in *P. infestans*.

1.8 Breeding for resistance

Although the production of late blight resistant cultivars is a priority for potato-breeding programs, no commercial cultivars adapted to North American growing conditions with good foliar resistance to modern genotypes of *P. infestans* have been adopted (Douches *et al.*, 1997). Only a small number of cultivars with some level of general resistance survived the epidemics of the 1840's leading to a narrow genetic base for modern potato breeding (Umaerus *et al.*, 1983). Most potato cultivars grown prior to the epidemics of the 1840's were highly susceptible to *P. infestans* and were derived mainly from Chilean *Solanum tuberosum* Group (Gp) Tuberosum with contributions from Gp Andigena (Pavek *et al.*, 2001). From 1851 to 1910, 380 new cultivars were developed based on general field resistance which during early potato breeding was the only type available to the breeder (Stuart, 1905; Davidson, 1928), although none were more resistant than the traditional 'Champion' a cultivar characterized as resistant even up until 1950 (Akeley,

1966). Many species have since been used, but the closely related *S. demissum* has been by far the most important source particularly during the decades of 1930-1960 along with the discovery of specific resistance (Salaman, 1929), with many cultivars incorporating specific R genes into new cultivars such as Pentland Dell in the UK, released in 1961 and containing three R genes for specific resistance. A cultivar bred in Michigan named Jacqueline-Lee with high foliar resistance to *P. infestans* has also shown high agronomic potential due to high yield, excellent cooking qualities and attractive appearance with bright golden skin, yellow flesh and oval shape (Douches *et al.*, 2001).

1.9 Characterization techniques

Phenotypic and genotypic characterizations of the pathogen are necessary to understand the pathogen and its population dynamics. There is no single system that can adequately describe all variation present. There are a range of markers that are applied for characterizing *P. infestans* including simple mating type reaction to sophisticated nuclear DNA analysis. The following section summarizes a few markers that were used for characterizing *P. infestans* in this study.

1.10 Phenotypic markers

Phenotypic markers were the first tests used to characterize *P. infestans* before more specific genetic markers became prevalent. Phenotypic markers are used to describe morphological characteristics of the pathogen.

1.10.1 Mating type

Mating type is one of the easiest techniques used to differentiate between two isolates and is determined when oospores are produced when a tester isolate is paired with an unknown isolate on media plates. Oospores may be produced by self mating and are termed "self-oospores". If the oospores result from apomixis, then oospores contribute nothing to variability in pathogen population but selfed oospores resulting from meiosis and recombination are a potential source of variation in this species (Shattock *et al.*, 1985). The role of selfed oospores is unknown, but they create a mechanism for genetic variability (Shattock *et al.*, 1985). The genetic basis of sexual reproduction is unclear despite much investigation. It would appear mating type is controlled by a single locus and subject to non-Mendelian segregation (Gallindo and Gallegly, 1960; Judelson *et al.*, 1995). Although it has been proposed mating type is controlled by nuclear genes, a study of *Phytophthora parasitica* and *Phytophthora capsici* supports the view that mating type in *Phytophthora spp.* is under control of mitochondrial gene (Gu and Ko, 2005). Mating type differences can also be detected due to differences in DNA polymorphisms, as demonstrated by studies using enzyme restriction of amplified fragment length polymorphisms (AFLP; Kim and Lees, 2002) and amplification of a sequence characterized amplified region (SCAR) marker (Xuan-Zhe *et al.*, 2006). These techniques are complicated and time-consuming.

1.10.2 Effects of temperature on the disease

Van Everdingen's conditions (Dutch rules) for causing an outbreak of potato late blight epidemics are as follows (Beaumont, 1947): 1. Minimum temperature not less than 17°C below the dew point. 2. Minimum temperature not less than 10°C. 3. Sunshine of the

following day not more than five hours. 4. Rainfall of the following day at least 0.1 cm. The disease has been associated historically with "cool (near 20°C) wet weather" (Harrison 1992). The effects of temperature, humidity, precipitation, wind and irradiation on the development of late blight in potato foliage were described by Harrison (1992). Most developmental structures of *P. infestans* cannot survive temperatures above 30°C (Harrison, 1992), but the pathogen is more robust when inside plant tissue and has survived temperatures of 40°C in stem tissue (Kable and Mackenzie, 1980). Temperatures below an organism's growth optimum will also cause reduced growth and temperatures near 0°C can induce ice nucleation or cellular dehydration causing stress or death (Pearce, 2001). Humidity, precipitation, wind, and irradiation are environmental factors that have an effect on leaf wetness and therefore effect germination and infection (Harrison, 1992).

Economic losses due to late blight result from foliar defoliation as well as tuber infection, and severe storage losses can occur after tubers infected with *P. infestans* are held for processing at temperatures in excess of 7°C (Kirk *et al.*, 2001). Late blight epidemics are associated with wet conditions and moderate temperatures (Harrison, 1995). There is a wide variation in the temperature effect on different life stages of *P. infestans* (Harrison 1992). The optimal temperature for growth of hyphae in foliage is 15-20°C (Harrison 1992). Kadish and Cohen (1992) found that the rate of sporangial development decreased at temperatures below 10°C. Temperature can influence spore germination, mycelial growth rate, inoculum production and survival. Potato tubers for seed or table stock are typically stored at 3°C, and processing tubers at 7 to 10°C (Kirk *et al.*, 2001b).

Tubers stored at 10°C are at greater risk for development of late blight because *P. infestans* has been shown to develop maximally at this temperature rather than at 3 or 7°C (Kirk *et al.*, 2001b). Tubers are usually held at 10°C for up to 6 months; therefore colonization of individual tubers poses a greater risk than spread of disease in storage by sporangia (Kirk *et al.*, 2001b).

There is generally a greater incidence of soil freezing in Michigan during warm dry winters due to recurring freeze-thaw episodes (Isard and Schaetzl, 1998). Isolates of *P. infestans* vary in their ability to survive winter freezing (Shattock, 1976; Kadish and Cohen, 1992; Kirk 2003b). More aggressive isolates of *P. infestans* are able to colonize potato tissue faster and pose a larger threat during the growing season than their less aggressive counterparts; however, the aggressive isolates may be at a selective disadvantage when it comes to over wintering (Shattock, 1976).

Temperatures lower than 20°C, with the optimum between 12-13°C, favor indirect germination of sporangia, formation of zoospores (5-10 per sporangium) and temperatures higher than 20°C, with the optimum temperature at 24°C, favor direct germination, i.e., one sporangium gives rise to a germ tube (Mizubuti and Fry, 1998). Infection of susceptible plant tissue by *P. infestans* takes place most readily when zoospores are formed, and this occurs only when there is a layer of moisture on the leaf. Zoospores were found to be motile for 22 hours at 5-6°C, while at 24-25°C zoospores were motile for only 19 minutes (Melhus, 1915a). In different genotypes of *P. infestans,*

temperature may affect sporangial germination and developmental rates which could have important implications for epidemic development and disease management.

The over wintering of *P. infestans* is an important epidemiological factor in temperature agroecosystems (De Bruyn, 1926). Mycelium of *P. infestans* can initiate crop infections over successive years by over wintering as mycelium in infected potato tubers or within cull piles or volunteer tubers. Tolerance of freezing temperatures by mycelium of *P. infestans* may be an ecologically important survival mechanism and the increased tolerance of US-8 and US-14 may explain their predominance in cooler climates such as north-central United States (Kirk, 2003). It is difficult to estimate the probability that infected potato stems or foliage will emerge from an infected tuber, as there are several factors that could influence the fate of infected tuber, temperature being one of the most important (Monteith, 1977, Kato *et al.*, 1997). Perches and Galindo (1969) found soil to be infectious 2-years after a late blight infection. Many investigators have used in vitro and soil assays to study the optimal and lethal thermal temperatures for growth of *Phytophthora spp.* (Bollen, 1985, Coelho *et al.*, 2000). No other studies that systemically examine the ability of *P. infestans* mycelium to survive at temperatures below 0°C exist, although De Bruyn showed that a mycelial colony was capable of withstanding temperatures as low as -25°C for up to 5-days in manure-based substrate and survival at such low temperatures was enhanced in dried substrates (De Bruyn, 1926). A few *P. infestans* isolates with different genotypes were killed by exposure to temperatures of -20°C and -10°C for more than one hour (Kirk, 2003). Pathogen growth was better after a sudden change in temperature, from cold to warm than after a gradual change. *P.*

infestans survived best when grown on manure because it encouraged good growth and strong resistance to cold (De Bruyn, 1926).

Oospores were found to survive in soil for 5 to 7 months at temperatures between 0 and 20°C (Pittis and Shattock, 1994). Temperature is an underpinning factor in both host and pathogen development and host by pathogen interactions. As the climate changes it is likely that soil temperature will increase to close to freezing in Northern U.S. production areas over winter. As it has been shown that *P. infestans* is tolerant to temperature close to freezing (Kirk, 2003), this therefore creates a situation for both the host and the pathogen to survive over winter. This could result in an increase in the emergence of potato plants carrying initial inoculum that could start a seasonal epidemic. Mizubuti and Fry (1998) studied the effect of temperature on sporangial germination from US-1, US-7, US-8 isolates of *P. infestans*. Their results indicate that US-1 isolates germinate better at higher temperatures (15-25°C) than US-7 or US-8, which germinated better at 10°C (Mizubuti and Fry, 1998).

Oospores of other *Phytophthora* species have shown similar response to temperature. Oospores of *P. cactorum* (homothallic) and *P. cinnamomi* (heterothallic) were not viable after exposure at 45°C for 30 min while oospores of a heat resistant strain of *P. megasperma* (homothallic) survived this treatment Juarez-Palacios *et al.*, 1991).

1.10.3 Fungicide resistance to metalaxyl as a phenotypic marker

The problems caused by potato late blight were exacerbated by the development of resistance to the most widely used group of systemic fungicides, the phenylamides, and

the appearance of new, highly aggressive strains of *P. infestans* (Gisi and Cohen, 1995). Phenylamides were the first compounds to be systemically active against oomycetes, and the development of resistance has been a major factor in the contemporary severity of potato late blight (Gisi and Cohen, 1995). The phenylamide metalaxyl (methyl-N- (2-furoyl)-N-(2,6-xylyl)-DL-alaninate; Ridomil®, Syngenta, Basle, Switzerland) was introduced by Ciba-Geigy in 1977 (Urech *et al.*, 1977) for control of *P. infestans* because of the high protective, curative and eradicative activity of the fungicide (Schwinn and Staub, 1987). Currently, the predominant isolates of *P. infestans* in North America are resistant to metalaxyl. Resistance to metalaxyl is now widespread globally with most populations outside North America containing both sensitive and resistant isolates (Deahl *et al.*, 1993; Koh *et al.*, 1994). Metalaxyl-resistant isolates have been shown to infect more tubers, but tubers infected with metalaxyl-sensitive isolates were more likely to produce infected plants because they were not completely destroyed by the disease (Walker and Cooke, 1990). The genetic control of metalaxyl sensitivity is unclear and multiple genes are likely involved (Shattock, 1988; Shaw, 1991; Lee *et al.*, 1999). If as suggested by Shattock, (1988) resistance is controlled by a single locus exhibiting incomplete dominance, heterozygous isolates with both resistant and sensitive alleles would exhibit intermediate resistance. However backcrosses have not segregated as expected by this model (Shattock, 1988) and the precise mechanism of inheritance is unknown. Since resistance is a relatively stable and heritable trait the method can be used to characterize individual isolates within a population (Forbes *et al.*, 1998).

1.10.4 The hypersensitive response

Disease resistance processes in plants are diverse, occurring at the sub-specific or varietal level (host specific resistance) or at the species or genus level (nonhost resistance). The hypersensitive response (HR) of plants is often associated with disease resistance (Dangl, *et al.*, 1996). The HR occurs as a rapid, localized necrosis, a form of programmed cell death. Early infection by *P. infestans* is similar in both susceptible and resistant hosts. Typically it starts when zoospores encyst and germinate on root or leaf surfaces. They penetrate the epidermal cell by germ tubes and subsequent formation of infection vesicles. However unlike in sensitive plants, feeding structures known as haustoria are formed expand from the site of penetration to neighboring cells through the intercellular space. In resistant plants the major defense reaction is the hypersensitive response (HR). The extent and timing of the reaction will depend on the interaction of pathogen and plant genotype and ranges from death of only a few cells with no macroscopic symptoms i.e. in non-host interactions, to potato cultivars containing R genes or displaying high general resistance where a group of cells are involved with later retardation of the pathogen and appearance of macroscopic brownish-black spots (Kamoun *et al.*, 1999). Although the HR is most often associated with expression of R gene resistance, the response has been associated with all forms of resistance including partial resistance, although in these cases the response of the host can be ineffective, with numerous hyphae escaping where the pathogen remains ahead of the response known as trailing HR (Kamoun *et al.*, 1999). This would suggest recognition occurs independently of the type of resistance (Vleeshouwers *et al.*, 2000).

Elicitins (extracellular protein elicitors), have also been identified in *P. infestans* which may play a role in host recognition (Kamoun *et al.*, 1997). It has been suggested the HR reaction follows recognition by the host of signal molecules known as elicitors, encoded by avirulence genes in the pathogen (Staskawicz *et al.*, 1995). Signal transduction may be associated with cytoplasmic aggregation as observed by acceleration of protoplasmic strand production in incompatible reactions (Tomiyama, 1956). Cytoplasmic aggregation has been connected with actin filament association, which when repressed inhibits accumulation of the phytoalexin rishitin, so suggesting the cytoskeleton is of importance in plant defense responses (Furuse *et al*, 1999).

Resistance has also been associated with induced deposition of structural barriers such as callose and extracellular globules containing phenolic compounds which may function in cell wall strengthening (Vleeshouwers *et al.*, 2004) and with a rapid increase in other compounds such as phytoalexins and glycoalkaloids (Andreu *et al.*, 2001). Other observed reactions include generation of superoxide anions (Doke *et al.*, 1991), an increase in chitinase and beta-1,3-glucanase and accumulation of pathogenesis related proteins (Taylor *et al.*, 1990).

1.10.5 Virulence phenotype

Even though R genes are thought to be ineffective in the field over long periods of time, there are plausible hypotheses that suggest these genes could mediate durable resistance (Kamoun *et al.*, 1997). The term 'virulence' of *P. infestans* has been used to refer to compatibility with single R genes introgressed into the host from wild *Solanum* species (primarily *Solanum demissum*) and corresponding virulence genes in the pathogen

(Malcolmson and Black, 1966). This gene for gene recognition system was first proposed by Black *et al.*, (1953) and to date eleven R genes have been elucidated, although it has been acknowledged more may exist (Trognitz, 1998). Compatibility with these known R genes will determine an isolates 'race' or 'virulence' phenotype (Van der Plank, 1963) and is assessed by inoculating eleven genetically defined 'differential' potato genotypes, each possessing a single R gene with subsequent recording of compatibility (Cooke *et al.*, 2004). The 'guard hypothesis' suggests that normal resistance occurs when a complex of the plant virulence target and the pathogen *Avr* gene product is recognized by a R gene product, with mutation to a state which avoids host recognition causing virulence to the gene (Dangl and Jones, 2001). Populations have been monitored for virulence phenotype since break-down of resistance was first recorded in commercial cultivars incorporating such R genes (Malcolmson, 1969; Shattock *et al.*, 1977). Multiple studies have demonstrated an increase in virulence complexity in recent years along with increasing sexual recombination (Drenth *et al.*, 1994; Cohen, 2002), with virulence against all eleven R genes reported (Goodwin *et al.*, 1995b). Since it is a long and laborious method of population characterization, virulence testing has been criticized due to inaccuracies between and within laboratories (Stewart, 1990). Improved understanding of the molecular nature of R genes, new methods, such as PCR-based approaches to the isolate analogs of R genes from resistant plants, in combination with reverse genetics and complementation in to the host crop, should facilitate the cloning and identification of novel R genes (Kamoun *et al*, 1997).

1.10.6 Pathogenicity testing

Pathogen response to resistance in tubers may be genotype specific (Kirk *et al.,* 2001a). The susceptibility of potato tubers is extremely variable and depends on the interaction between the cultivar and the isolate of *P. infestans* involved in addition to environmental properties and tuber age. Infection of tubers is similar to that of foliage with germ-tubes penetrating at lenticels, eyes, stolons, buds, wounds or any site incompletely suberized (Bain and Möller, 1999). Dowley and O'Sullivan (1991) found that healthy tubers became infected with *P. infestans* from sporangia after being stored with diseased tubers at 20°C for up to 35 days. Several studies of blighted tubers in storage have demonstrated *P. infestans* spreads fastest and sporulates at highest levels at around 10°C (Kadish and Cohen, 1992; Walker and Cooke, 1988). Research has shown that the newer US-8 genotype is more aggressive in potato tubers than the US-1 genotype, causing more surface necrosis and deeper lesions (Medina *et al.*, 1999). Spread of disease between whole tubers in storage is insignificant because of the barrier of intact skin and because tuber surfaces are usually dry, inhibiting production of sporangia (Lambert *et al.,* 1998). A study by Lambert reinforced the results from Medina and showed that some US-6 and US-7 isolates of *P. infestans* also cause faster infection spread than US-1 (Lambert and Currier, 1997). Potato tubers are conventionally held in storage at 10°C for up to six months to enhance sugar accumulation; spread of disease is enhanced under these conditions (Burton, 1992). Several studies of blighted tubers in storage have demonstrated *P. infestans* spreads fastest and sporulates at highest levels at around 10°C (Walker and Cooke, 1988; Kadish and Cohen, 1992).

Gees and Hohl (1987) suggested that the mechanisms by which late blight development is slowed are different between tubers and foliage, and they may be related to histological and/or cytological variations in tuber or canopy, biochemical defense responses or a combination of these and other factors. Studies of tuber tissue degradation in the whole tubers due to late blight infection have concentrated on the pathogenicity of biotypes as affected by tuber age and duration of storage periods (Grinberger *et al.*, 1995).

Infected tubers normally carries mycelium of the pathogen and when such tubers are planted in soil the fungus may produce sporangia through buds and lenticels, which may attack stems emerging from the infected tuber. A study from Grinberger *et al.*, (1995), has shown that no oospores were formed in young tubers, reaffirming that some physical and physiological-biochemical factors of the tubers prevents the fungus from mating and making oospores. Oospore formation in tuber tissue is very limited but increases with tuber aging which may be related also to pathogen aging. The number of oospores produced in tuber tissue was lower compared with that in leaf tissue regardless of the origin of isolates used (Levin *et al.*, 2001). Viability of oospores as measured by plasmolysis declined slightly over a period of 18 months whether they were stored in water at 4°C, in soil under natural field conditions (Mayton *et al.*, 2000).

1.11 Genetic markers

Although the use of phenotypic or biologically significant markers for distinguishing between isolates and assessing diversity within populations has been ongoing over the past number of decades and remains highly useful, it is only with advances in genomics (Shaw, 1991) that more powerful discriminatory tests capable of tracking isolates within

a population have become available. Such techniques have become fundamental for population studies and investigation of mechanisms such as selection, migration and competition.

1.11.1 Isozyme analysis

Characterization of allozyme loci were the first unambiguous genetic markers for investigation of populations of *P. infestans* and have been important for the characterization and tracking of isolates as well as other investigations including the study of diploidy (Tooley *et al.*, 1985) and identification of sexually reproduced isolates (Shattock *et al.*, 1986). *GPI* isozyme pattern is the most widely used isozyme approach to characterize isolates of *P. infestans*, in which *GPI* isozymes occur in multiple combinations, as shown by starch gel and cellulose-acetate electrophoresis analyses (Goodwin *et al.*, 1995a). Isozymes are defined as multiple molecular forms of a single enzyme and are usually differentiated only by variation in amino acid composition due to differences in the nucleotide sequence of DNA coding for that protein (Smithies, 1955). However, this will only occur where there is a difference in net charge (usually due to large differences in size and shape or substitution of a basic for an acidic amino acid), or some other change affecting electrophoretic mobility such as a change in tertiary structure (Micales and Bond, 1995). It has been suggested only one third of all single amino acid substitutions are electrophoretically detectable and thus use of isozyme analysis is a very conservative estimate of genetic variability (Micales and Bond, 1995). As a result gel bands are described as an enzyme phenotype rather than genotype (Hubby and Lewontin, 1966). The usefulness of isozyme analysis depends on the assumption of heritability and co-dominant expression i.e. all alleles at any one locus will be expressed

(Shaw, 1965). Polymorphic loci encoding glucose-6-phosphate isomerase *(GPI),* Peptidase *(PEP),* malic enzyme *(ME),* and xanthine dehydrogenase *(XDH)* have been successfully utilized to unequivocally differentiate multiple individuals within a sexual population (Tooley *et al.*, 1985) but it is in the *GPI* and *PEP* loci where polymorphisms are most commonly observed (Ospina-Giraldo *et al.*, 2003).

Previous studies have revealed that at least six genotypes can be found in sexual populations of *P. infestans* (Tooley *et al.*, 1985), although many more are possible (Grunwald *et al.*, 2001). These genotypes are known as 83/100, 86/86, 86/100, 100/100, 100/122 and 122/122. These alleles are named according to the electrophoretic mobility of the isozyme it encodes, in relation to the most common one, which is 100. Each allele codes for a structurally distinct polypeptide chain with a monomeric enzyme consisting of only one and a multimeric enzyme with more than one chain and will usually be either dimeric (two chains) or tetrameric (four chains). Over 50 different isozyme systems have been tested for *P. infestans* (Spielman *et al.*, 1990) with the first characterization made by Tooley *et al.* (1985) using starch gel electrophoresis. A genetic locus within a population is described as monomorphic where only one allele is present or polymorphic where more than one allele is present, with a polymorphic isozyme known as an allozyme. The number of alleles will depend on ploidy number, genetic make-up (i.e. homozygous or heterozygous) and nuclear condition (i.e. monokaryotic or dikaryotic), with complexity of resultant banding patterns varying accordingly (Micales and Bond, 1995). Differences in form may be distinguished either by number of bands, with complex patterns emerging from multimeric heterozygous isoforms due to formation of intermediate 'hybrid' bands,

or by intensity of bands, with differences in ploidy levels distinguishable due to the additive effects of subunits (Danzman and Bogart, 1982).

Until the late-1980's, the populations in North America was mostly represented by single clonal lineages known as US-6, which carried the 100/100 genotype, and US-1, which carried the 86/100 genotype (Goodwin *et al.*, 1994). In the early 1990's two additional lineages, US-7 and US-8 (*GPI* genotypes 100/111 and 100/111/122, respectively) were also found in the United States and Canada (Goodwin *et al.*, 1995b). In addition *GPI* genotype 100/122, found in US-14 and US-17, frequently observed between 1995 and 1997, and significantly decreased by 1998 (Daayf and Platt, 2000). Much in vitro evidence suggests new genotypes possess higher aggressiveness and have more efficient colonization of potato than the 'old' US-1 genotype (Goodwin *et al.*, 1995; Miller *et al.*, 1998; Flier *et al.*, 1998). However as new genotypes such as US-8 and many of the European genotypes are metalaxyl resistant this in itself may have allowed a competitive advantage against the phenylamide sensitive US-1 (Goodwin *et al.*, 1996; Cooke *et al*, 2006). It is generally accepted that metalaxyl resistance in the U.S. is the result of migration rather than mutation and subsequent selection (Goodwin, 1997). Although most of these genotypic changes have been attributed to migration rather than mutation (Goodwin *et al.*, 1994), the widespread occurrence of the newest genotypes might suggest a selective advantage for such genotypes and perhaps, a role for sexual recombination (Ospina-Giraldo *et al.*, 2003).

1.11.2 Nuclear DNA polymorphisms

Alternate forms of a chromosomal locus, either differing in nucleotide sequence or by variable numbers of repeated nucleotide units are known as DNA polymorphisms. Differences can arise due to insertions, deletions and substitution of individual nucleotides, or by chromosomal inversions or translocations (Goodwin, 1997). Polymorphism can be detected using a range of nuclear DNA markers.

1.11.2.1 Simple Sequence Repeats (SSR's)

SSR's are tandemly repeated motifs of one to six bases found in the nuclear genomes of all eukaryotes tested and are often abundant and evenly dispersed (Tautz & Renz, 1984; Lagercrantz *et al.*, 1993). These differences between numbers of repetitive units are known as satellites, minisatellites or microsatellites. Microsatellite sequences are usually characterized by a high degree of length polymorphism, and are ideal single-locus co-dominant markers for genetic studies (Lees *et al.*, 2006). Once SSR's have been identified through DNA sequencing, appropriate primers flanking the microsatellite can be developed to amplify the region through PCR (Cooke *et al.*, 2004). DNA fragments can then be separated on a gel under electrophoretic conditions and sequenced for number of repeats. Alleles (usually two per locus) appear either as one peak where the locus is heterozygous or two where heterozygous (Lees *et al.*, 2006).

Although a recent development, with few studies incorporating its use, fifteen polymorphic SSR markers have now been published for *P. infestans* (Knapova and Gisi, 2002; Lees *et al.*, 2006), although some show limited variation, with 68 different genotypes and an average of 3.9 alleles per locus detected amongst fourteen markers

tested on a subset of 90 isolates (Lees *et al.*, 2006). SSR analysis is highly specific usually focusing on specific loci allowing for easier interpretation (comparable with allozyme genotype) unlike other DNA based techniques, but is also highly powerful, unambiguously defining length variation (Cooke *et al.*, 2004). There is some suggestion though that high mutation rate may decrease heritability and therefore usefulness for certain functions such as tracking within populations (Weber and Wong, 1993). As commented by Cooke and Lees (2004), a greater emphasis must be put on studies of *P. infestans* biology, these studies should include improvements in our understanding of the relative contributions and rates of mutation, recombination, natural selection, gene flow, random genetic drift and migration (Burdon and Silk, 1997) to the generation and maintenance of variation in populations (relative importance of asexual v. sexual reproduction; Lees *et al.*, 2006).

1.11.2.2 Chromosome ploidy levels

Ploidy is the number of homologous sets of chromosomes in a biological cell. Laser flow cytometry and RFLP (Restriction Fragment Length Polymorphism) analyses allows quantitative measurement of nuclear DNA content. Substantial variation exists within populations of *P. infestans* with diploid, triploid, tetraploid and aneuploid isolates identified through the use of chromosome counting, cytophotometric counting and inheritance studies (Sansome and Brasier, 1974; Sansome, 1977; Tooley and Therrien, 1991). Early cytogenetic studies in *P. infestans* identified roughly 9 to 12 chromosomes in some isolates, but twice that amount in others (Sansome and Brasier, 1973; Sansome, 1977). These observations increase confusion in deciding if the genus is naturally haploid or diploid. It has been suggested that polyploid isolates may have arisen through

selection of autotetraploid nuclei, produced occasionally during rapid nuclear division (Sansome, 1977). These isolates may have been selected through better adaptation to environmental conditions or by higher fitness due to possession of a higher number of virulence genes in comparison to diploids (Sansome, 1977). It would appear from in vitro studies that mating between ploidy levels is viable, although with variable chromosome content (Whittaker, *et al.*, 1991). However, studies of natural populations would suggest such matings are rare (Tooley and Thierren, 1987; Spielman *et al.*, 1991) with an intraspecific post-zygotic barrier to mating hypothesized (Miller and Johnson, 2000). Difference in ploidy between mating partners is not a major barrier to sexual fertility, although such crosses may yield offspring with varied chromosome content (Whittaker *et al.*, 1991). This technique may not be feasible for the larger sized *P. infestans* chromosomes. Variable ploidy levels could lead to irregularities in segregation ratios and confusion in inheritance studies.

1.12 Objectives

Although it is highly likely that *P. infestans* can mutate asexually the rate of production of distinct phenotypes can be accelerated by the use of enforced sexual recombination utilizing the different mating types. To test this hypothesis several phenotypic and genotypic characters were selected some with immediate relevance in epidemiology and disease management including thermal tolerance, metalaxyl sensitivity, mating type and aggressiveness and further investigated in terms of genotypic markers such as isozyme analysis, microsatellite and ploidy analyses.

- Evaluate survival potential of mycelium of different genotype isolates of *P. infestans* exposed to temperature at -5°C for 5 days *in vitro* using a digital imaging technique.
- Carry out crosses between *P. infestans* isolates (A_1 and A_2 mating types) that are highly variable for temperature tolerance, to determine if temperature tolerance is inherited or occurs as a result of physiological adaptation of different genotypes of *P. infestans.*
- The progeny isolates from these crosses will be assessed for temperature tolerance at -5°C and data will be analyzed to estimate the inheritance of this trait.
- Evaluate other phenotypic characteristics of progeny such as mating type, isozyme analysis, metalaxyl sensitivity and virulence assessment.
- Evaluate the progeny using Microsatellite markers to confirm whether they are selfs or hybrid progeny.
- Evaluate if the progeny isolates are pathogenic in potato tubers.

2 Genotypic characteristics of *Phytophthora infestans* from mating: Determination of hybridity or selfing by isozyme analysis and microsatellite markers

2.1 Introduction

The Chromistan Oomycetous fungus *Phytophthora infestans*, the causal agent of potato late blight remains poorly understood relative to fungal pathogens. Since Oomycetes do not have a close taxonomic affinity with genetically well-characterized fungi such as Ascomycetes and Basidiomycetes, it is likely that study of *P. infestans* will yield novel biological findings (Judelson, 1997). In order to assess and follow selection within an epidemic it is important to possess the ability to identify and assess both gene and genotypic diversity. *P. infestans* causes the devastating late blight disease of potato and tomato. Re-emergence of late blight during the late twentieth century ensured that this pathogen continues to be an important threat to agriculture (Fry and Goodwin, 1997). *P. infestans* is an obligate biotroph which usually reproduces asexually, therefore the probability of extinction overwinter is high in most commercial systems (Milgroom, 1996) unless the pathogen overwinters as mycelium in seed, culled or volunteer tubers (Kirk 2001, 2003a, 2003b). As a result gene and genotypic diversity within local populations can vary considerably, both temporally and spatially as new sources of inoculum may be introduced sporadically (Fry and Goodwin, 1997; Day and Shattock, 1997). It is important to have the capability to assess both gene and genotypic diversity to monitor selection during an epidemic. Gene diversity refers to the frequency of alleles at individual loci in a population while genotypic diversity refers to the frequency of genetically distinct individuals within a population (McDonald and Linde, 2002). Assessment of such diversity is dependent on the availability and potential of precise and abundant genetic and phenotypic markers (Fry *et al.*, 1992). Cellulose-acetate

electrophoresis (CAE) provides an excellent resolution of allozyme genotypes of *P. infestans* and has many advantages over traditional starch gel analyses. CAE is faster, more reproducible, requires less amount of enzyme for an analysis, and can be useful as a diagnostic tool in field situations.

Allozyme genotyping by cellulose acetate electrophoresis, first used for this pathogen by Tooley *et al.*, (1985), is one of the easiest and cheapest methods and has now become the most commonly used technique to characterize populations (Goodwin *et al.*, 1995; Ospina-Giraldo and Jones, 2003). Polymorphic loci encoding glucose-6-phosphate isomerase *(GPI),* Peptidase *(PEP),* malic enzyme *(ME),* and xanthine dehydrogenase *(XDH)* have been successfully utilized to unequivocally differentiate multiple individuals within a sexual population (Tooley *et al.*, 1985) but it is in the *GPI* and *PEP* loci where polymorphisms are most commonly observed (Ospina-Giraldo *et al.*, 2003). Despite the importance of *GPI* in *P. infestans* studies, the gene encoding this enzyme has not yet been characterized (Ospina-Giraldo *et al.*, 2003). Although allozyme polymorphisms are used widely these markers have been criticized, because migration is expressed in relative terms and the genetic change involved is largely unknown (Cooke and Lees, 2004).

Amplified Fragment Length Polymorphism (AFLP) and microsatellites or Simple Sequence Repeats (SSR) markers are unambiguous nuclear tools considered to be highly useful for population investigations (Knapova *et al.,* 2002). In European studies using AFLP markers, up to 50% of isolates are shown to have discriminatory patterns

(Knapova and Gisi, 2002; Cooke *et al.*, 2003). However AFLP genotyping is also laborious, time-consuming, and may produce excessive data difficult to interpret in comparisons among strains (Cooke and Lees, 2004). Additionally AFLP markers are dominant so that heterozygotes cannot be distinguished and therefore many markers are required for thorough investigations (Jorde *et al.*, 1999). Although the supporting data are limited, there has been speculation that *P. infestans* may contain a variable and plastic genome. This has been proposed to explain the sectoring for traits such as growth rate, colony morphology, avirulence, and pathogenic aggressiveness reported for certain single cultures (Caten, 1971; Erwin *et al.*, 1963; Jeffrey *et al.*, 1962; Jinks and Grindle, 1963; Abu el Samen *et al.*, 2003). An increase in genetic variation at the molecular level and a more complex structure were observed in new *P. infestans* populations in Europe (Drenth *et al.*, 1993b; Knapova and Gisi 2002). SSR's have been used successfully in recent investigations to characterize populations in France and Switzerland (Knapova *et al.*, 2002). SSRs are short fragments of DNA composed of tandemly repeated motifs of 1-6 base pairs, characterized by a high degree of length polymorphism (Lees *et al.*, 2006). SSR markers focus on individual loci and have been proven to be highly specific (Lees *et al.*, 2006). Furthermore due to their co-dominant nature homozygotes and heterozygotes are easily distinguished, although the possible presence of null alleles at heterozygous loci may be problematic (Knapova *et al.*, 2002).

A genetic locus within a population is described as monomorphic where only one allele is present or polymorphic where more than one allele is present, with a polymorphic isozyme known as an allozyme. The number of alleles will depend on ploidy number,

genetic make-up (i.e. homozygous or heterozygous) and nuclear condition (i.e. monokaryotic or heterokaryotic), with complexity of resultant banding patterns varying accordingly (Micales and Bond, 1995). Differences in number of bands, with complex banding patterns may result from multimeric heterozygous isoforms due to formation of intermediate 'hybrid' bands, or by intensity of bands, with differences in ploidy levels producing the additive effects of subunits (Danzman and Bogart, 1982). The chromosome number of diploid nuclei is 8-12 and in tetraploid nuclei 14-20 (Whittaker *et al.*, 1991). Knowledge of ploidy is important both for population characterization and all types of inheritance investigations, since variation can lead to irregular segregation ratios (Judelson, 1997). Determination remains problematic however as chromosomes are too small for accurate counting using conventional cytological techniques (Shaw and Shattock, 1991). As commented by Cooke and Lees (2004), a greater emphasis must be put on studies of *P. infestans* biology. These studies should include the relative contributions and rates of mutation, recombination, natural selection, gene flow, random genetic drift and migration (Burdon and Silk, 1997) to the generation and maintenance of variation in populations (relative importance of asexual v. sexual reproduction; Lees *et al.*, 2006).

Oomycetes including *P. infestans* are recognized as diploid. However, *P. infestans* often exists in populations that may be heteroploid or polyploid (Lee *et al.*, 2004; Sansome, 1985; Tooley & Therrien, 1987, 1989). The genomic size of *P. infestans* is approximately 237 Mb (Tooley and Therrien, 1987) with at least 51% repeated DNA sequences (Judelson and Randall, 1998). Two independent analyses indicate that the genome has

about 52% GC content. Microscopic analyses indicate that *P. infestans* has 8-10 chromosomes (Sansome and Brasier, 1973). Due to this considerable genomic size and complexity, early *P. infestans* genomics efforts focused on cDNA sequencing and generated collections of expressed sequence tags (ESTs) from a variety of developmental, stress, and infection conditions (Kamoun *et al.*, 1999 and Randall *et al.*, 2005). Most of the extra genome mass has recently been identified as "junk" DNA that is not transcribed into ribosomes or transcribed and translated into proteins. The DNA content of nuclei of various isolates of *P. infestans* and progeny from controlled matings and progeny from asexual propagation by zoospore formation was assessed by laser flow cytometry. Variation of chromosomal size among isolates was evaluated with southern hybridizations, and some virulence genes were located on chromosome fragments.

Sexual crosses have been made *in vitro* for *P. infestans* to investigate inheritance of several genotypic characteristics using allozyme markers (Spielman *et al.*, 1990), or related microsatellite markers (Knapova and Gisi 2002). The characteristic of mating type is generally considered to be stable, heritable and easily detectable and is a commonly used and important marker for characterizing and assessing the potential for sexual reproduction within a population (Brasier, 1992). Although generally considered robust, mutation, mitotic recombination and treatment with certain fungicides can result in segregation of the opposite mating type (Smart *et al.*, 1998; Groves, 2002). Additionally self-fertility can occur amongst US lineages (specifically the US-7 and US-8 lineages), although fewer oospores are produced than between opposite mating types (Smart *et al.*,

1998; Groves 2002). How far these factors may confound use of mating type as a marker in selection studies is unknown.

The objective of this study was to determine whether the phenotypic character of temperature tolerance was genetically inherited or occurred as a result of physiological adaptation. *P. infestans* progeny isolates that were highly variable for temperature tolerance generated from A_1 and A_2 mating types were evaluated and phenotypes compared with inheritance of markers for mating type, isozyme analysis, microsatellites, and characterization of nuclear conditions and ploidy levels.

2.2 Materials and methods

2.2.1 Selection of isolates

In this study, the procedure for selection of isolates to use as parents for crossing to produce progeny isolates and determine the inheritance was described in chapter 4. Selected isolates were shown in Table 2.1.

2.2.2 Isozyme analysis

Cellulose acetate electrophoresis (CAE) was used to determine the polymorphic allozyme loci. All US parental isolates were heterozygous for the *GPI* (Glucose-6-phosphate isomerase) allozyme genotype and as a result only this marker (and not PEP) was used for isolate characterization in this study. *GPI* was conducted using the protocols of Goodwin *et al.*, (1995). Isolates used for isozyme analysis were 14 days old cultures, grown on Rye agar (Appendix 1). Mycelium was scraped from the culture plates using a rubber policeman and transferred to 1.5 ml micro-centrifuge tubes and 150-µl sterile distilled water was added to each tube. Proteins were extracted by grinding the mycelium

in double distilled water using a hand held homogenizer for 1 minute. Tubes were briefly vortexed and centrifuged at 13,000g for 10 minutes at 4°C. Supernatant was decanted to leave a pellet and a residual volume of 100 µl. The supernatant containing proteins was pipetted to a new tube, labeled and stored on ice or at -20°C if not used immediately. Parent isolates were used as standards for comparison with progeny isolates. Extracted protein was sufficiently stable for 3 – 4 electrophoretic assays prior to onset of enzyme degradation.

2.2.2.1 Cellulose acetate electrophoresis

10 X Tris Glycine (TG) buffer was prepared by adding 0.25 M (30g) Tris base and 0.192 M (144 g) Glycine to 1 L double distilled water, and adjusting the pH to 8.5 (by adding either sodium hydroxide pellets or hydrochloric acid), using a pH meter (Orion PerpHect Meter, Orion Research, INC., Beverly, MA 091915-6199, USA). 180 ml of 1X TG (100ml 10 X TG in 900ml sterile distilled water) was added to each side of the electrophoresis chamber (Zip Zone® Electrophoresis Chamber; Helena Laboratories, PO Box 752, Beaumont TX 77704-0752). Paper wicks (220 mm x 30 mm; Helena Laboratories) were placed in contact with gel plate edge and bridging buffer tray over the two rails separating the buffer trays for later contact with gel plates. Wicks were reused until the buffer became contaminated. Cellulose acetate gel plates (94mm x 76 mm; Titan® III; Helena Laboratories) were soaked for at least twenty minutes in appropriate 1 x TG electrode buffer. Plates were used immediately or were soaked for several days before use in a refrigerator (4°C). Buffer was reused until cloudiness appeared. Gel plates were blotted between sheets of blotting paper and fixed to the center of the aligning base of the loading applicator (Super Z-12 Applicator System; Helena

Table 2.1 Phenotypic and genotypic characteristic of isolates of *Phytophthora infestans* used in crosses.

Isolate of *Phytophthora infestans*	Thermal Tolerance[a]	*GPI* isozyme genotype[b]	Mating Type[c]	Metalaxyl Response[d]	R gene phenotype[e]
Pi 41-02[f]	T	100/100	A_1	R	1,3,4,5,6,7,8,9,10,11
Pi 02-007[g]	T	100/111/122	A_2	R	1,2,3,4,5,6,7,8,9,10,11
Pi S1-3[g]	T	100/111/122	A_2	I	1,2,3,4,5,6,7,8,9,10,11
Pi 98-1[g]	I	100/122	A_2	R	1,4,5,9,10.11
Pi 62-02[f]	T	100/100	A_1	I	1,2,3,4,5,6,7,8,9,10,11
Pi 95-3[g]	S	86/100	A_1	S	5
Pi Atlantic 2N[g]	S	100/111/122	A_2	I	1,3,4,5,6,7,8,9,10,11
Pi 4-19[h]	S	100/100	A_1	I	1,3,4,5,6,7,8,9,10,11

[a] Comparison of growth of isolates of cultures of *Phytophthora infestans* for 10 days at 18°C after exposure at -5°C for 5 days measured by an image analysis technique comparing the growth of exposed isolates to a positive control (exposed to 18°C for 5 days) or a negative non-inoculated control with no growth. T= Tolerant; growth not significantly different from the growth of the positive control cultures of the same isolate; I= Intermediate; growth significantly less than that of the growth of the positive control cultures of the same isolate but significantly greater than the negative control; S= Sensitive; growth not significantly different from the negative control.

[b] Glucose -6- phosphate isomerase allozyme genotype; 86/100 (US1), 100/100 (US-1.7), 100/111/122 (US-8), 100/122 (US-14); genotype as designated by Goodwin *et al.*, (1995).

[c] Mating type determined by production of oospores when grown with isolates of *P. Infestans* of known mating type.

[d] Sensitivity to the fungicide metalaxyl, R = resistant, I= intermediate, S = sensitive determined by comparing EC_{50} response of the isolates grown on media amended with different concentrations of metalaxyl with EC_{50} of known resistant and susceptible isolates.

[e] Virulence pathotype as defined by Muller and Black (1952).

[f] Isolates of *P. infestans* from Northern Ireland from collection of Dr. Louise Cooke, Plant Pathology, Queens University, Northern Ireland.

[g] Michigan isolates of *P. infestans* from collection of Dr. W. Kirk, Plant Pathology, Michigan State University, East Lansing, MI, USA.

[h] A progeny isolate obtained from the 4th cross, used as a parent because of its thermal sensitive phenotype (as defined above in [a])

Laboratories). The cellulose acetate side was oriented so that it faced upwards, and the bottom side was marked using a permanent marker, for identification. 10 µl of each protein extract sample was added to a reservoir of the applicator system (Super Z-12 Applicator Systems; Helena Laboratories). The multi-well applicator was pressed into the reservoirs three times. The sample was then transferred onto the surface of the cellulose acetate plate by depressing the multi-well applicator for 10 seconds. Gels were placed cellulose acetate side downwards onto the electrophoresis chamber in contact with wicks and the sample side oriented towards the cathodal rail. Microscope cover slips were placed on top of the gel to ensure contact to the wicks. Current was applied for 30 min at 200 V by using a power supply (E-C. Apparatus Corp., St. Peterburg, Fla., 33709, USA).

2.2.2.2 Gel overlay preparation and staining

Genotypes of the isolates were determined by staining with agar overlays, prepared from stock solutions of reagents (Sigma Aldrich Chemical Company, St. Louis, MO-63178). *GPI* overlay contained 1.5 ml of Tris-HCl (0.05M, pH 8), 5 drops of Fructose-6-phosphate (20 mg/ml in double distilled water, stored at -20°C), 1 ml of nicotinamide adenine dinucleotide (3 mg/ml in double distilled water, stored at-20°C), 5 drops of Methyl thiazole blue (10 mg/ml in double distilled water, stored at 4°C), 5 drops of Phenazine methosulphate (2 mg/ml in double distilled water, stored at 4°C) and 3 µl of Glucose-6-phosphate dehydrogenase (1 U/µl, stored at 4°C). Photosensitive chemicals were added last, prior to staining. Finally all the above reagents were mixed in a glass test tube with 2 ml of pre-melted 1.6 % agar. This was poured over the cellulose side of the cellulose acetate plate. Enzymatic activity stained the gel at the location of the allozyme migration creating bands that started appearing in 1-2 minutes and were then scored.

Plates were washed in water to prevent over staining, blotted, and images of the gels were recorded by scanning them facing down on a flat bed scanner (Epson Perfection 4870 PHOTO; Epson Inc. USA) and the scanned image was imported into Photoshop (Adobe Inc.), and saved in the JPEG file format.

2.2.2.3 Scoring of bands

Genotypes were determined by comparing the bands of unknowns with those of known standard isolates, including *Pi 02-007 (US-8, GPI 100/111/122), Pi 41-02 (GPI 100/100), Pi 62-02 (GPI 100/100), Pi S1-3 (US-8, GPI 100/111/122), Pi 98-1 (US-14 GPI 100/122), Pi Atlantic 2N (US-8, GPI 100/111/122), Pi 95-3 (US-1, GPI 86/100),*and *Pi 4-19 (GPI 100/100)* (Figure 2.1 and 2.2).

2.2.3 Microsatellite analysis

Simple sequence repeat (SSR) markers are used to identify polymorphisms at specific microsatellite loci and characterize sample isolates. All the homothallic isolates and few random isolates (5 from each cross), along with the parents were selected for SSR testing. A method modified from Knapova and Gisi (2002) and Lees *et al.,* (2006) was used.

2.2.3.1 Microsatellite Primers

Two SSR markers (Pi02, and 4B) shown to be polymorphic for specific alleles of DNA from *P. infestans* provided by Cooke *et al.*, (2006) were used in this study. These were derived from sequences documented in the *Phytophthora* Genome Consortium database (Waugh, 2000) and by Knapova and Gisi (2002). Primers were labeled at the 5' end with the fluorescent dye HEX (4,7,2,4,5,7-hexachloro-6-carboxyfluorescein) supplied by

Integrated DNA technologies (IDT, Inc., Coralville, IA, 52241, USA). Sequences of the primer pairs are:

Name	Sequence	Size range (bp)	No. of alleles
Pi 02 (forward)	5'CAGCCTCCGTGCAAGA- 3'	142 -166	8
Pi 02 (reverse)	5' AAGGTGCGCGAAGACC- 3'		
Pi 4B (forward)	5' AAAATAAAGCCTTTGGTTCA- 3'	205 - 217	3
Pi 4B (reverse)	5' GCAAGCGAGGTTTGTAGATT- 3'		

2.2.3.2 Amplification and Sequencing of Alleles

Amplification was done as follows for all primer combinations: 1-10 ng of genomic DNA (extracted using QIAGEN DNeasy Mini kit, QIAGEN Inc., 27220 Turnberry Lane, Valencia, Suite 200, CA91355, USA)) was mixed with 20 µl mastermix (Eppendorf MasterMix (2.5x), Brinkmann Instruments, Inc., One Cantiague Road, P.O. Box 1019, Westbury, New York 11590-0207, USA). 25 mM microsatellite primers were added, and finally sterile distilled water was added to make it to total volume of 50 µl. PCR conditions were as follows; 1 cycle of 95°C for 120 s, 33 cycles of 94°C for 40 s; 56°C for 40 s and 72°C for 20 s followed by one cycle of 72°C for 10 min. Reactions were held at 4°C following amplification. All amplifications were performed using a Thermocycler (Eppendorf Mastercycler, Brinkmann Instruments, Inc., One Cantiague Road, P.O. Box 1019, Westbury, New York, USA). A negative control (sterile distilled water instead of DNA) was included in each batch of samples. Samples were submitted to Genomics Technology Support Facility (GTSF, Michigan State University, 178 Plant Biology Building, S-18, E. Lansing, MI, USA). Genotypic allocations were based on

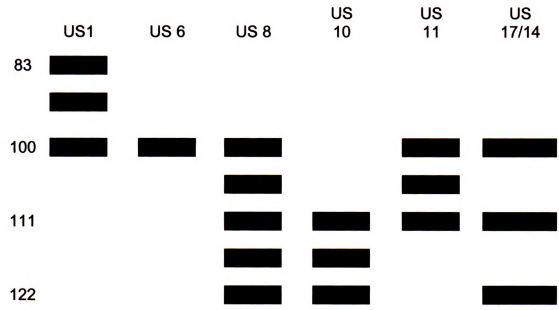


Figure 2.1 Banding patterns of isolates of *Phytophthora infestans* for differing allozyme genotypes, and accompanying US designations (Goodwin *et al.*, 1995) at the *GPI* locus, as revealed by cellulose acetate electrophoresis.

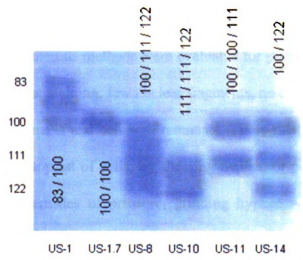


Figure 2.2 Standard *GPI* band patterns of *Phytophthora infestans* isolates ran on a cellulose acetate gel for 30 min at 200V.

electropherogram analysis (denoted by peaks at specific alleles) for individual loci and were described as having either a homozygous or heterozygous genotype.

2.2.4 Nuclear condition and ploidy levels

2.2.4.1 Sample preparation and flow cytometry

P. infestans isolates, and cultures derived from single zoospore progeny were grown at 18°C on Rye A agar (Caten & Jinks 1968) in Petri plates. The surface of each plate was flooded with 10 ml sterile water and a sterile cotton swab was employed to dislodge mycelium and sporangia. One milliliter of the resulting suspension was used to inoculate 9 cm diameter Petri plates containing 20 ml of pea broth (125 g/L frozen fresh peas, boiled for 1 h, filtered, and autoclaved). After 5-10 days depending on the growth rate of the isolate, mycelium was harvested and centrifuged at 5000 rpm for 10 min. The liquid was discarded and residual liquid removed by pressing the mycelium against the centrifuge tube wall. Numerous methods were evaluated for preparation of nuclei of high quality with stable nuclear staining, few nuclear fragments, no clumping of nuclei, and an absence of nuclear fragments and cell wall remnants. These methods included releasing nuclei by enzymatic protoplast of cells, by shearing hyphae or protoplasts in a pressure bomb using various intensities of pressure, grinding hyphae with and without liquid nitrogen and filtering the released nuclei, and chopping hyphae with a scalpel blade and filtering the released nuclei. By trial and error, the most reliable method of nuclear preparation was found to result from chopping hyphae with a scalpel blade and filtering the released nuclei. Additionally, pre-staining the suspension of nuclei with propidium iodide yielded more stable fluorescence than adding the stain just prior to injecting the suspension into the flow cytometer. Approximately 200 mg of fresh hyphae was chopped

with a sterile scalpel blade for 3-4 min in ice-cold 1ml PBS (pH 7.4) containing 0.1 % Triton X-100. Cellular debris was filtered through a 30 μm mesh nylon membrane to capture wall debris, and the nuclei passing through the filter were retained. RNase A (50 μg/ml) and 50 μg/ml propidium iodide (Molecular Probes, Inc., Eugene, Oregon) in PBS (pH 7.4) were added to the suspension of nuclei. The suspension was incubated in the dark for two hours at room temperature prior to injection into the flow cytometer. Commercially prepared Singlet Chicken Red Blood Cells (CRBC; BioSure, Grass Valley, CA) were used as standard nuclei of known DNA content. The CRBC were added to the oomycete nuclear preparation in quantity to account for 3-5 % of the total fluorescent nuclei, and stained simultaneously. Additionally, aliquots of the propidium iodide solution were fed into the flow cytometer sample delivery system prior to sample analysis. This minimized small fluctuations in sample DNA content caused by leaching of DNA into the cytometer. The pre-staining and pre-injection of stain yielded flat measurements of fluctuations in DNA fluorescence over time for both oomycete and (CRBC) nuclear DNA.

Prepared and stained nuclei of *P. infestans* and standardized CRBC were delivered into a Vantage SE laser flow cytometer (BD Bioscience, San Jose, CA). A Spectra-Physics 377G laser (Spectra-Physics, Mountain View, CA) set to a 100 mw 488 argon line excited the propidium iodide stained nuclei, and fluorescence was collected using a 630/22 band pass filter. CellQuest ver. 3.3 software (BD Bioscience, San Jose, CA) was used for acquisition and processing of fluorescence signals from the detectors. DNA fluorescence was collected in linear scaling using single CRBCs as an internal standard

for determination of oomycete nuclear DNA content. Fluorescent data for 20,000 oomycete nuclei were collected at a fluorescence threshold excluding only signal noise while allowing the detection of the lowest fluorescence from oomycete nuclei. DNA fluorescence was statistically analyzed using Modfit LT ver. 3 software (Verity Software House, Topsham, ME) to distinguish complexities in the population of nuclei, such as G1, G2, and M phase nuclei present in the cell cycle of eukaryotes. A count gate was set over the oomycete DNA fluorescence region to insure the ability to count a minimum of 12,000 nuclei.

DNA content of singlet *P. infestans* nuclei was calculated with comparison of the oomycete G1 peak fluorescence to the G1 peak fluorescence of standard singlet CRBC nuclei. The Modfit LT calculated the G2/G1 ratio for each sample to optimize the G1 peak to G2+M peak fit. Additionally, DNA area fluorescence versus sample run time data plotting was monitored to insure that no fluctuation in DNA fluorescence was occurring during a run (any deviation from a flat DNA fluorescence versus time curve). DNA integrated area fluorescence versus DNA width fluorescence (time of flight) was plotted in order to delimit DNA analysis to the optimum fluorescence data for singlet oomycete nuclei. The plotting produced a histogram of DNA quantity based on area of fluorescence. The data was further analyzed with the program Modfit LT to determine the number of nuclear populations present, and then the data was parsed based on best statistical fit into representative peaks. The numbers of cell cycles present (i.e., diploid, aneuploid, heteroploid nuclear populations) was determined by analyzing the numbers of G1 peaks present which also showed corresponding G2 peaks. The G1/G2 peaks were

compared to those of the typical diploid peaks. The Modfit LT program automatically detected the additional cell cycles after the typical diploid cell cycle peaks were designated manually. A color code was assigned to each peak using the Modfit LT software to aid in visual recognition of the nuclear populations in each *P. infestans* isolate and progeny. Graphical output was written to a PDF file writer and edited in Adobe Photoshop CS for display (Adobe Systems Incorporated).

Each flow cytometry analysis of a parent isolate or progeny was repeated three times using suspensions of nuclei independently prepared from replicate cultures in order to have statistically supported means for calculations of DNA content per nucleus.

2.3 Results

2.3.1 Isozyme Analysis

The electrophoretic patterns at the *GPI* loci were identified for all the isolates of parents and progeny (n=300) isolates from nine crosses (Table 2.2). From the nine crosses, none of the progeny isolates produced banding patterns consistent with Mendelian ratios expected from hybridization (sexual recombination) between two parents. Single oospore cultures gave banding patterns identical to one or the other of the parents (homothallic reproduction). Parent isolate, Pi 41-02 (with 100/100 *GPI* banding pattern, A_1 mating type, from Northern Ireland) was involved in 6 crosses, 204 of 295 progeny produced this single banded phenotype, while the rest of them were identical to the other parent. Parent isolate, Pi 02-007 (with 100/111/122 *GPI* banding pattern, A_2 mating type) was involved in 4 crosses, 71 of 295 progeny produced this five-banded phenotype, and the remaining were identical to the other parent. Parental isolate, Pi S1-3 (with 100/111/122 *GPI*

banding pattern, A_2 mating type) was involved in 2 crosses, 13 of 101 progeny produced this five-banded phenotype. Parental isolate, Pi Atlantic2N (with 100/111/122 *GPI* banding pattern, A_2 mating type) was involved in 2 crosses, and out of 18 progeny none produced this five-banded phenotype. Parental isolate Pi 98-1 with 100/122 *GPI* banding pattern, when crossed with Pi 41-02, produced 7 out of 45 progeny with the double-banded phenotype and the rest had a 100/100 genotype. In cross 7 (Pi 95-3 X Pi 02-007), there were no progeny with the phenotype identical to the parent Pi 95-3 (96/100 *GPI*). Parental isolate, Pi 62-02 (with 100/100 *GPI* banding pattern, A_1 mating type, from Northern Ireland) was involved in 1 cross, 17 of 25 progeny produced this single-banded phenotype. *GPI*-banding of progeny isolates indicted that that the *GPI* genotype is heritable, as only the parental type alleles were identified and hybrids were not produced. Isolate Pi 41-02 was homozygous for the most common allele (100/100 *GPI*) and majority of the progeny possessed this allele.

2.3.2 Microsatellites

Of the two microsatellite markers used to characterize parental isolates, marker Pi 4B differentiated well between the isolates (Table 2.3). Parents selected for crosses in this study have heterozygous alleles using microsatellite markers (Pi 4B and Pi 02; Table 4.3). Using the marker Pi 4B, 8 out of 25 progeny isolates tested, showed patterns of alleles that were recombinants, differing from the pattern of either parent. Seventeen progeny isolates had alleles identical to the parents, and therefore many of these offspring may have resulted from apomixis (selfing). Progeny isolate 2_29 has the alleles at 201,

212, 214, 216 and 223 bp that were produced from both the parents Pi 41-02 (201, 212, 214 bp) and Pi 02-007 (210, 223 bp; Fig. 2.5).

The Pi 02 microsatellite marker showed more variation in the banding pattern of the progeny isolates than marker Pi4B. Out of 25 progeny, only 8 showed banding patterns identical to a parent and are assumed to be the result of selfing, whereas, 17 progeny were recombinants (hybrids), Allelic patterns for a few isolates are shown in Fig. 2.5. Offspring 2_35, 3_50, 5_12, and 5_37 (from crosses 2, 3, and 5, respectively) were observed as hybrids using both the markers.

2.3.3 Nuclear condition and ploidy levels

Examination of field isolates of *P. infestans* using laser flow cytometry revealed that of 16 isolates, 10 contained one nuclear population (G1 peak) that was presumably diploid. The other 6 isolates were heterokaryotic, containing more than one nuclear population in their thallus (Table 2.5). Among the typical (homokaryotic) isolates, the DNA content per nucleus varied considerably among isolates, ranging from a mean of 0.48537 to 0.70260, $\pm$ 0.02200 s.d. (Tables 2.5 and 2.6). In heterokaryotic isolates, the nuclear population with a DNA content similar to the nuclei in the homokaryotic isolates was designated as "diploid" and other nuclear populations present were designated aneuploid if they had less DNA content or heteroploid if greater in DNA content (Table 2.5). Five isolates contained two populations of nuclei (G1 peak) in their thallus, two of these were from Michigan, one from Minnesota and another from Mauritius. This is the first discovery that field isolates in Michigan may be heterokaryons. One isolate from Mauritius

contained 3 nuclear populations in its thallus, aneuploid, diploid and heteroploid nuclear populations (G1 peak). No isolate could be demonstrated to be polyploid (i.e. 4N).

Single-zoospore derived cultures of isolate MYA1114 also varied statistically in their mean DNA content per nucleus, ranging from 0.41 to 0.50 $\pm$ 0.007 s.d. (Table 2.4). At least 3 single-zoospore isolates were statistically significantly smaller in DNA content than the parent. These isolates are presumably aneuploids. Additionally, laser flow cytometry revealed that the nuclear populations present in the heterokaryotic parents were not inherited equally by the single-zoospore derived isolates (asexual progeny). Segregation of nuclear types during asexual propagation was unexpected and represents a new mechanism of variation in *P. infestans* field populations that are primarily clonal. Differences in nuclear condition among field isolates are represented graphically in Figure 2.6 with G1 peaks color-coded to distinguish aneuploid (yellow), diploid (red), and heteroploid (orange) nuclear populations. The percentage of single-zoospore derived isolates of a heterokaryotic parent that inherited the same nuclear condition of the parent, or just one or the other of the two nuclear populations, are represented in Table 2.5 for isolates MYA-1114 and isolate Pi 3768.

2.4 Discussion

Two systems of genetic markers were used to study inheritance in field isolates of *P. infestans*. They are SSR or microsatellite markers (Pi 4B and Pi 02), and the allozyme markers or *GPI*. Additionally, DNA content of nuclei and nuclear condition of isolates was examined with laser flow cytometry to clarify interpretation of inheritance patterns. The parental isolates chosen for this study from the US population were composed of

three differing genotypes for the *GPI* locus and this variation in GPI patterns was sufficient among the parents and progeny for use of allozymes as markers for a study of sexual inheritance. However, due to the lack of variation in the *Pep* locus this allozyme marker was not used in our study and isolates homozygous (100/100) for the *GPI* locus were excluded from the study. All Northern Ireland parental isolates were homozygous *100/100* for the *Gpi* allozyme genotype and as in this study there are no other parental isolates with this genotype, *PEP* marker was not used in this study. *P. infestans* is a diploid organism that is usually heterothallic in sexuality and some populations have been reported to be polyploid (Tooley & Therrien, 1991). When A_1 and A_2 mating type isolates are paired, each isolate may produce antheridia, oogonia or both, which later fuse to form hybrid oospores from outcrossing , however, such a pairing also can initiate a proportion of self-fertilization events, or inbreeding events (Judelson, 1997).

Outcrossing and selfing can both occur within mating cultures (Judelson, 1997). In our crosses, approximately 10% of oospore-derived progeny were recombinants (automixis or sexual reproduction) based on inheritance patterns of codominant SSR markers. The remaining progeny were products of self fertile oospores (homothallic reproduction or apomixis). The application of isozyme analysis enabled identification of progeny from mating cultures of *Phytophthora infestans* (Cooke & Lees, 2004). In this study, oospore-derived progeny from all crosses yielded banding patterns and presumed genotypes consistent with one or the other of the parents but no recombinant patterns. When a parental isolate with a homozygous allele pattern was crossed with a parent having the heterozygous three-banded pattern, the homozygous allele was inherited predominantly

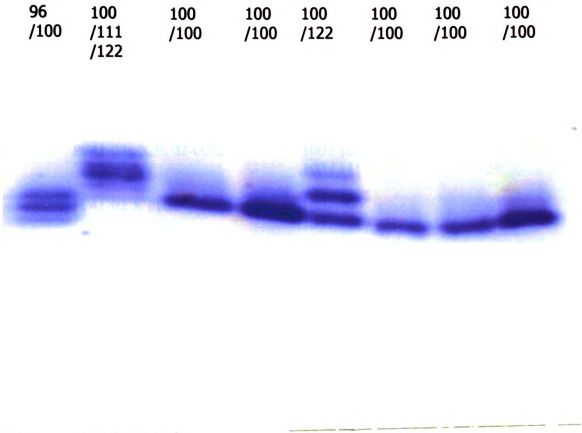


Figure 2.3 Isozyme analysis (*GPI*) of isolates of *Phytophthora infestans* parents and progeny; From Left to right, Lane 1: 96/100, Pi 95-3; Lane 2: 100/111/122, Pi 02-007; Lane 3: 100/100, Pi 41-02; Lane 4: 100/100, 2-35; Lane 5: 100/122, Pi 98-1; Lane 6: 100/100, 2-2; Lane 7: 100/100, 2-10; Lane8: 100/100, 2-15.

Table 2.2 Presumed genotypes at the Glucose phosphate isomerase (*GPI*) locus in parental and single oospore progeny isolates of *Phytophthora infestans*.

Parents[a]	Genotype[b]	Progeny[c]
Pi 41-02 X Pi 02-007	100 / 100	13
	100 / 111 / 122	24
Pi 41-02a X Pi 02-007a	100 / 100	31
	100 / 111 / 122	39
Pi 41-02 X Pi S1-3	100 / 100	43
	100 / 111 / 122	8
Pi 41-02a X Pi S1-3	100 / 100	45
	100 / 111 / 122	5
Pi 41-02 X Pi 98-1	100 / 100	38
	100 / 122	7
Pi 02-007 X Pi 62-02	100 / 111 / 122	7
	100 / 100	18
Pi 95-3 X Pi 02-007	96 / 100	0
	100 / 111 / 122	8
Pi Atlantic2N X Pi 41-02	100 / 111 / 122	0
	100 / 100	16
Pi Atlantic2n X Pi 4-19	100 / 111 / 122	0
	100 / 100	2

[a] Isolates of *Phytophthora infestans* used as parents in the crosses to produce progeny isolates derived from single oospores

[b] Genotypes of parent isolates

[c] Number of progeny having a respective genotype from each cross

Table 2.3 Different allelic patterns of parent and progeny isolates of *Phytophthora infestans* at microsatellite, Pi4B and Pi02 loci.

Crosses	Isolate	Pi 4B marker	Pi 02 marker
Parent 1	Pi 02-007	210,223	157,159
Parent 2	Pi 41-02	201,212,214	150,160,161
Progeny	2_29	201, 210,212,214,223	nd
Progeny	2_35	201,210,212,214,223	147,158,160,161
Progeny	2_20	201,210,212,213,223	158,160,145
Progeny	1_13	201,212,214,215,223	145,158,160
Progeny	2_25	201,212,214,223	nd
Progeny	1_9	201,212,215	145,156,159
Progeny	2_51	201,212,214	145,161
Progeny	2_46	210,214,223	nd
Progeny	2_13	210,223	145,158
Progeny	2_21	210,223	145,160
Progeny	2_3	210,223	145,156,159
Progeny	2_9	210,223	143,145,158,159
Progeny	2_33	nd	145,158,159
Progeny	2_2	210,223	145,160
Progeny	2_31	210,223	145,160
Progeny	1_6	210,223	145,156,159
Progeny	1_4	210,223	145,156,159
Progeny	1_7	210,223	145,156,159
Progeny	2_19	210,223	nd
Progeny	2_7	210,223	nd
Parent 1	Pi 02-007	210,223	157,159
Parent 2	Pi S1-3	210,224	nd
Progeny	3_4	202,213,215	146,160,161
Progeny	3_13	202,213,215	146,160,161
Progeny	3_50	202,213,215	146,160,161
Progeny	4_37	nd	147,161
Progeny	4_11	201,212,214	nd
Parent 1	Pi 02-007	210,223	157,159
Parent 2	Pi 95-3	210,214	145,157,159
Progeny	7_8	202,213,215	145,146,160,161
Progeny	7_17	202,213,215	145,146,160,161
Parent 1	Pi Atlantic 2N	210,223	145,157,159
Parent 2	Pi 95-3	210,214	145,157,159
Progeny	8-1	202,213,215	145,146,160,161

nd = not detected

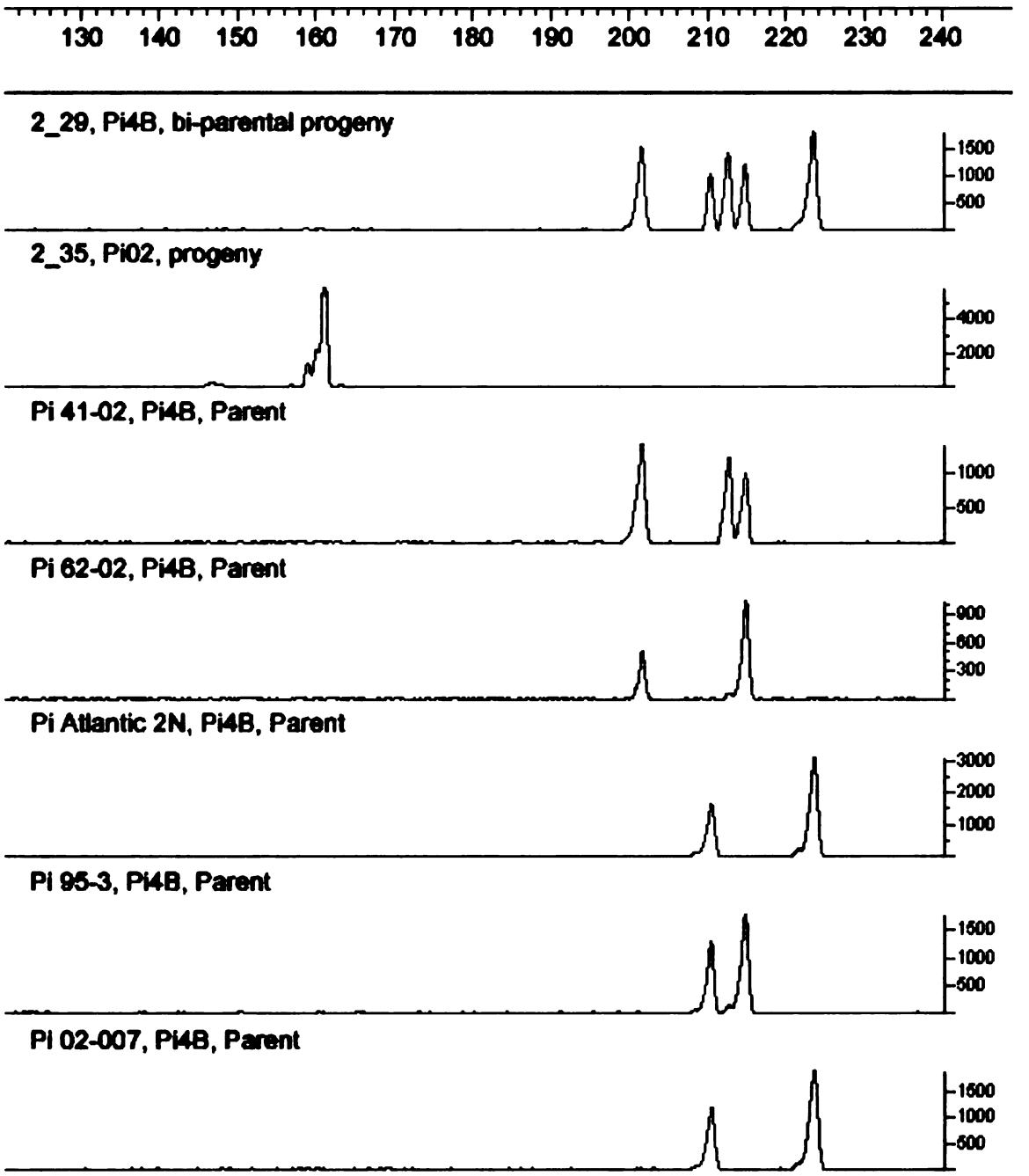


Figure 2.4 Electropherograms illustrating isolates heterozygous for the Pi4B and Pi02 loci at alleles from 200 to 226 bp and 160bp respectively. The Y axis represents relative fluorescence unit (RFU) and the X axis molecular size of the allele marker in base pairs (bp) size.

Table 2.4 *Phytophthora infestans* showing variation in the DNA content of nuclei among different single zoospores (asexual progeny) of the isolate PI-1055P-MYA1114

Isolates	DNA content (pg/nucleus)	Standard deviation	Statistical difference	Isolates	DNA content (pg/nucleus)	Standard deviation	Statistical difference
PI-105P-MYA1114 (P[a])	0.49	0.007	P	PI-105P-MYA1114-SS20	0.47	0.003	NSD
PI-105P-MYA1114-SS1	0.49	0.005	NSD	PI-105P-MYA1114-SS21	0.48	0.002	NSD
PI-105P-MYA1114-SS2	0.50	0.006	NSD	PI-105P-MYA1114-SS22	0.50	0.002	NSD
PI-105P-MYA1114-SS3	0.49	0.006	NSD	PI-105P-MYA1114-SS23	0.50	0.009	NSD
PI-105P-MYA1114-SS4	0.41	0.002	A	PI-105P-MYA1114-SS24	0.50	0.003	NSD
PI-105P-MYA1114-SS5	0.50	0.010	NSD	PI-105P-MYA1114-SS25	0.50	0.002	NSD
PI-105P-MYA1114-SS6	0.49	0.004	A	PI-105P-MYA1114-SS26	0.48	0.005	NSD
PI-105P-MYA1114-SS7	0.46	0.003	NSD	PI-105P-MYA1114-SS27	0.48	0.004	NSD
PI-105P-MYA1114-SS8	0.49	0.008	NSD	PI-105P-MYA1114-SS28	0.47	0.001	NSD
PI-105P-MYA1114-SS9	0.46	0.005	NSD	PI-105P-MYA1114-SS29	0.48	0.004	NSD
PI-105P-MYA1114-SS10	0.46	0.001	NSD	PI-105P-MYA1114-SS30	0.48	0.003	A
PI-105P-MYA1114-SS11	0.46	0.005	NSD	PI-105P-MYA1114-SS31	0.48	0.004	NSD
PI-105P-MYA1114-SS12	0.47	0.004	NSD	PI-105P-MYA1114-SS32	0.46	0.001	NSD
PI-105P-MYA1114-SS13	0.49	0.007	NSD	Pi 02-007-Parent-1	0.56	0.006	D
PI-105P-MYA1114-SS14	0.48	0.005	NSD	Pi 41-02-Parent-2	0.67	0.008	D
PI-105P-MYA1114-SS15	0.48	0.002	NSD	Progeny-1-13	0.67	0.007	NSD
PI-105P-MYA1114-SS16	0.48	0.003	NSD	Progeny-2-29	0.55	0.023	NSD
PI-105P-MYA1114-SS17	0.49	0.004	NSD	Progeny-2-35	0.68	0.008	NSD
PI-105P-MYA1114-SS18	0.50	0.006	NSD	Progeny-2-20	0.67	0.020	NSD
PI-105P-MYA1114-SS19	0.47	0.004	NSD	Progeny-5-37	0.70	0.012	NSD

[a] Proportion of diploid vs aneuploid in each single zoopore culture varies from one single spore to another; [a] P= parent, A= Aneuploid (Highly significantly different than the parent in DNA content per nucleus); NSD= Not significantly different from the parent (contains nuclei similar to parental isolate); D= Diploid, two other parental isolates tested

Table 2.5 Different isolates of *Phytophthora infestans* showing variation in levels of nuclear condition, karyotype and sexuality

Isolates	*GPI*	Aneuploid[a]	Diploid[a]	Heteroploid[a]	Nuclear state	Nuclear condition[b]	Sexuality[c]
ATCC1114	100/111/122	57%	43%		$2N^- + 2N^e$	Heterokaryotic	Heterothallic
PI 95-7	100/111/122	36.3%	63.7%		$2N^- + 2N^e$	Heterokaryotic	Heterothallic
PI Atlantic 2N	100/111/122	57%	43%		$2N^- + 2N^e$	Heterokaryotic	Heterothallic
PI US940489	100/111/122		100%		$2N^f$	Homokaryotic	Homothallic
PI Centerville	100/111/122	35.6%	35.6%	64.3%	$2N^- + 2N + 2N^{+g}$	Heterokaryotic	Heterothallic
PI 02-007	100/111/122		100%		$2N^f$	Homokaryotic	Heterothallic
PI 41-02	100/100		100%		$2N^f$	Homokaryotic	Heterothallic
PI 3765	100/100	33.5%	32.9%	33.6%	$2N^- + 2N + 2N^{+g}$	Heterokaryotic	Heterothallic
PI 3768	100/100	53%	47.0%		$2N^- + 2N^h$	Heterokaryotic	Heterothallic
1-13[d]	100/111/122		100%		$2N + 2N^h$	Heterokaryotic	Homothallic
2-29[d]	100/100		100%		$2N + 2N^h$	Heterokaryotic	Homothallic
2-35[d]	100/100		100%		$2N + 2N^h$	Heterokaryotic	Homothallic
5-37[d]	100/100		100%		$2N + 2N^h$	Heterokaryotic	Homothallic

[a] based on groupings of single nuclei by differences in DNA content out of greater than 30,000 nuclei
[b] presumed number of different types of nuclei in the thallus
[c] based on oospore formation behavior in crosses and on markers for mating type alleles
[d] progeny isolates that had inherited all the microsatellite markers (alleles) of both parents
[e] Aneuploid + Diploid
[f] Diploid
[g] Aneuploid + Diploid + Heteroploid
[h] Diploid + Diploid , recombinants (alleles from both the parents)

. When a parental isolate with a two-banded pattern was crossed with a parent having the three-banded pattern, the two-banded pattern was predominantly inherited. There were no progeny with recombinant or hybrid patterns. This is interpreted to imply that the oospore derived progeny were the result of selfing (homothallic sexuality). Mating types are now widely distributed (Deahl *et al.*, 1991) and it is probably only a matter of time before sexual recombination alters the population genetics of *P. infestans* in North America. Currently, the clonal populations show correlations between allozyme genotype, mating type, and fungicide sensitivity (Goodwin *et al.*, 1995).

Mating type markers, isozyme analysis and SSR markers of many of the progeny were identical to their parents and confirmed that these progeny had resulted from selfing. In our studies, isozyme analysis could not differentiate the variation as well as SSR markers (Cooke and Lees 2004), whereas, SSR markers showed variation in some of the progeny confirming that they were recombinants (hybrids). SSR markers proved to be important nuclear tools useful in identifying and characterizing the segregation patterns in progeny (Knapova *et al.*, 2001; Knapova and Gisi, 2000).

SSR markers are important nuclear tools useful to identify and characterize the segregation patterns in F1 progeny. Isozyme analysis couldn't differentiate the variation as well as SSR markers. SSR markers indicated some variation in the progeny. Microsatellites have shown that some of these isolates may have resulted from selfing. The isolates that were identified as hybrids using SSR markers were not differentiated using isozyme analysis. The application of SSR markers has distinguished hybrids from

selfs. Mating type, isozyme analysis and SSR genotype of the selfs were identical to their parents. The presence of three bands is indicative of trisomic linkage and trisomic progeny were shown to be pathogenic (van der Lee *et al.*, 2001, 2004).

Progeny isolates showed comparatively minor variation with either a band missing or presence of an extra band from standard fingerprints. Our data support the conclusion that *P. infestans* has the potential to be homothallic and have the ability to self under certain conditions such as stimulation to form antheridia and oogonia by pairing with the opposite mating type. Additionally, our data support the conclusion that a limited amount of sexual recombination occurred which produced progeny with different banding pattern than the parents by meiotic recombination. Alternatively, these recombinants could be due to non-mechanisms of variation such as mutation, mitotic recombination or parasexual recombination in the nuclear DNA. Mutation usually involves the appearance of new alleles and has likely occurred where an extra single band is detected in the DNA fingerprint (Drenth *et al.*, 1994; Goodwin *et al.,* 1995). Previous investigations indicate mutation rates in the nuclear DNA are at a detectable level in *P. infestans* (Goodwin 1997). The other selfed isolates having *GPI* and SSR marker patterns similar to a parent could be a result of apomixis or asexual reproduction.

Microsatellite markers and isozyme markers differentiated well between parental isolates. A combination of the two microsatellite primers Pi 02 and Pi 4B were able to distinguish between the isolates and could be useful in future population studies. The variation between the isolates using the SSR markers did not correlate between the phenotypic and

genotypic characteristics (data not shown in this chapter). These results agree with previous work which has shown no correlation between SSR allele identification and phenotypic markers including mating type, virulence, aggressiveness and metalaxyl sensitivity (Knapova *et al.*, 2002).

As previously noted, although progeny have identical allozyme genotypes, differing alleles in SSR markers were detected, which indicated these isolates were recombinants. Although one study has investigated inheritance during sexual reproduction with microsatellite markers (Knapova *et al.,* 2002) none have investigated inheritance during asexual reproduction in *Phytophthora infestans* or variation between individual zoospore isolates with SSR's. However, such studies have been conducted for inheritance of virulence and metalaxyl sensitivity using AFLP markers (Abu-El Samen *et al.*, 2003). As distinguished from other genetic markers, SSR analysis can differentiated among parental isolates thus it would appear that microsatellite markers are easily identifiable, relatively robust and stable over time. Additionally as co-dominant markers, SSR's could be extremely useful in population genetics analysis for to track populations, temporally and spatially. The usefulness of SSR genotyping is dependent on the heritability and stability of the marker. Also, the usefulness of SSR markers will depend also on the ability of such markers to distinguish between clonal lineages and the rate of mutation or crossing over events which occur.

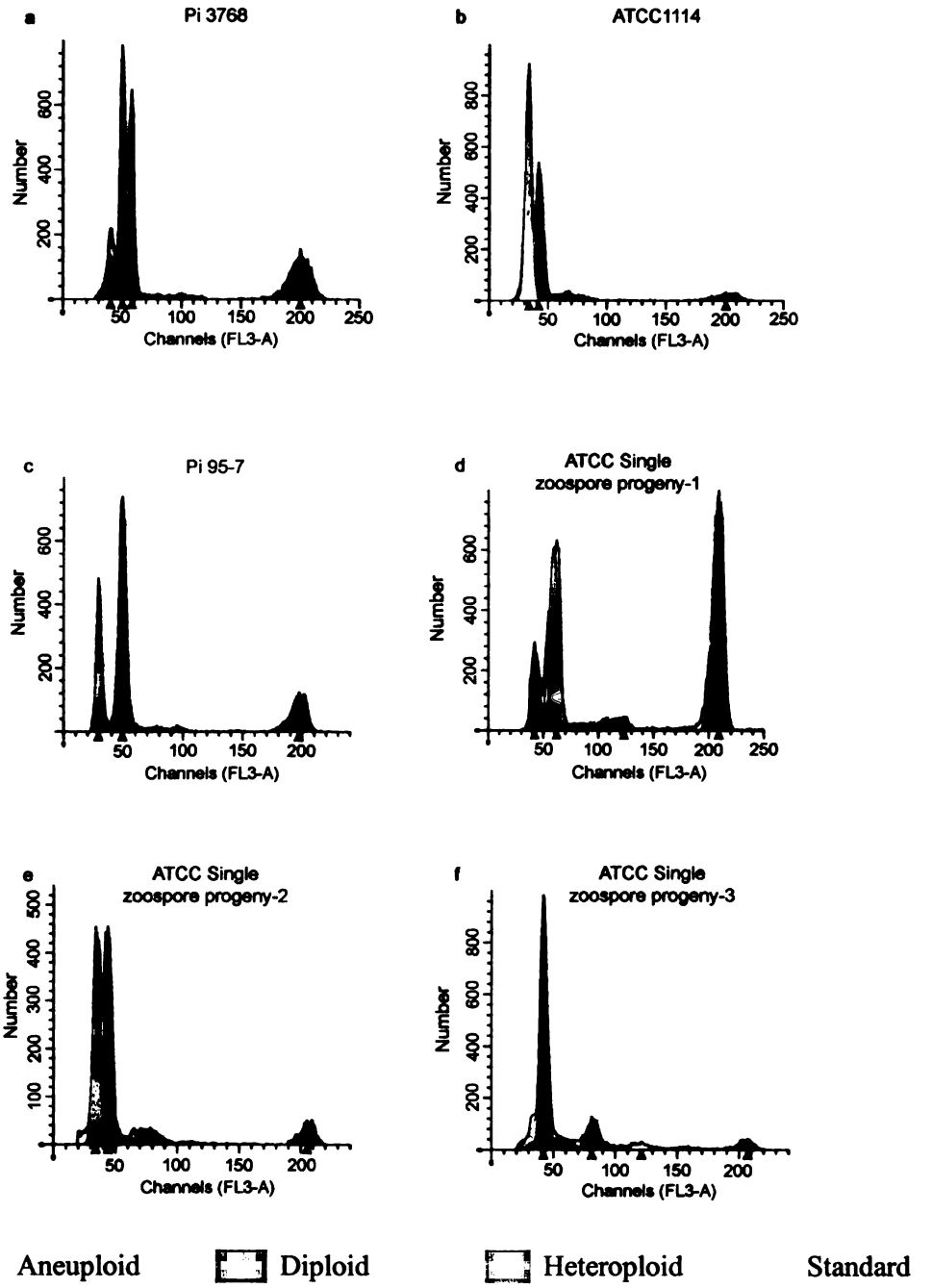
The inheritance of the progeny with respect to the measured tratits in this study did not follow Mendelian segregation. The reasons for the distortion are not understood fully, but

there are several possibilities described in the literature, including linked deleterious, recessive alleles, mitotic irregularities, chromosome rearrangements and unsuspected epistatic loci (Spielman *et al.*, 1990), or something as simple as contamination. This could be a reason that some progeny showed recombination in one SSR marker but not the other SSR markers. The variation among single zoospores of an isolate could explain the undergoing diversity of *P. infestans* isolates in nature. Our studies revealed that field isolates varied in DNA content of nuclei which would be assumed to be either due to variation in number of copies of chromosomes or different amounts of accumulated "junk" DNA within a genotype. Previous studies in *P. infestans* suggested that most hybrids were stable polyploids or parasexual recombinants rather than heterokaryons (Shattock & Shaw, 1976), however, our results suggest many hybrids are the result of heterokaryosis, differences in the balance among nuclei in similar heterokaryons, or in unbalanced inheritance of nuclear types in asexual progeny of heterokaryons. Single-oospore derivatives of interstrain crosses often display the genetic markers of only one parent (Shattock *et al.*, 1986; Whittaker, Assinder & Shaw 1994) and such strains are commonly described as a result of selfing, a type of asexual reproduction. However, this asexual reproduction method is enabled in mating cultures by the bisexuality of *P. infestans* (Judelson, 1997) behavior where hormonal signals can initiate antheridia and oogonia in both of the paired isolates.

Other mechanisms of variation may act during controlled and natural pairings. Mating may begin in the normal manner with the multiple nuclei present within each of an adjacent antheridium and oogonium undergoing meiosis, then defective transmission of

the signal that normally induces simultaneous meiosis in both gametangia (Win-Tin & Dick, 1975) might allow diploid nuclei to pass into the oosphere in which karyogamy might subsequently occur. Another mechanism might include errors occurring in the oosphere such that rather than one viable diploid zygote forming and the other nuclei degenerating, the other nuclei could participate in forming heterokaryons, or participate in karyogamy and spawn polyploids (Judelson *et al.*, 1998). Within the oomycetes there is substantial diversity in patterns of gametangial differentiation and nuclear behaviour (Dick, 1995) and the assumption that all oosporic nuclei within a species are produced by the same process may be invalid (Judelson *et al.*, 1998). Further genetic studies of polyploids might contribute to a better understanding of mating type determination by indicating the effect of allele dosage on phenotype, since polyploid by diploid crosses might yield progeny of variable ploidy (Judelson *et al.*, 1998). The generation of heterokaryons and polyploids may contribute to the evolution and adaptation of natural populations of *Phytophthora*. Heterokaryosis and polyploidy also represent a means to increasing the variability and adaptability of *P. infestans* through the mechanism of mitotic crossing-over. There is some suggestion that high mutation rate may decrease heritability and therefore usefulness for certain functions such as tracking within populations (Weber and Wong, 1993). Hybrid isolates of *Phytophthora infestans* may vary in fungicide sensitivity, virulence and temperature tolerance and enable the development of strains which could potentially increase damage in potato crop production. These processes might be rare in nature but if they occur they could result in shifts within localized populations of *P. infestans* and produce epidemics that are difficult to manage.

Figure 2.5 Representative histograms of the nuclear condition of parent and progeny isolates of *Phytophthora infestans*. The typical diploid nuclei of most isolates of P. infestans produces a fluorescence intensity of 48 – 52 units on the X-axis (on channel FL3-A) when stained with Propidium iodide. The units are relative and compared to the standar (Gray peak) of stained chicked red blood cell nuclei.



3 Mating type, metalaxyl resistance, and virulence characteristics of *Phytophthora infestans* from mating isolates with variable thermal tolerance

3.1 Introduction

Heterothallic species of *Phytophthora* are bisexual and capable of self-mating and producing oospores. De Bary (1881) was the first to report the germination of oospores of a *Phytophthora* species. Compared to the number of genotypic markers available there are relatively few phenotypic tests for *Phytophthora infestans* (Shaw and Shattock, 1991). Of these the most reliable and commonly used are mating type, resistance to metalaxyl and virulence complexity. *Phytophthora infestans* has a mating system that is representative of most heterothallic oomycetes, with two known mating types existing, A_1 and A_2. Mating type plays a major role in the phenotypic development of populations. Each mating type is distinguished by the production of a specific pheromone and subsequent response to the pheromone of the opposite mating type (Ko, 1988). Mating type is generally considered to be stable, heritable and easily detectable and as such is a commonly used and important test for characterizing and assessing the potential for sexual reproduction within a population (Brasier, 1992). Mating type is controlled by a single locus, with A_1 being heterozygous and A_2 homozygous. Most isolates are bisexual but display relative sexuality, preferentially forming antheridia or oogonia depending on their mating partner (Gallindo and Gallegly, 1960; Judelson, 1997).

The genetics of mating type in *P. infestans* is complex and the A_1 : A_2 ratio in progeny can vary significantly, ranging from close to 1 : 1 to an excess of A_1 over A_2 (Shaw 1991; Judelson *et al.*, 1995). Oospores are the sexual structures that are produced when two mating type fuse. Oospores are diploid, contain one viable nucleus and are produced

when two haploid nuclei fuse in the gametangia. Germinating oospores are considered as progeny of the A_1 or A_2 type. Self-fertile progeny are sometimes recovered as well, and these may result from nondisjunction (Fyfe and Shaw, 1992) or the presence of extra copies of the mating type locus in one parent (Judelson, 1996b). Efforts to recover progeny of *P. infestans* from crosses began in earnest only a few decades ago (Gallindo and Gallegly, 1960). Most oospore-derived cultures are true progeny although some may arise from selfing or apomixis (asexual reproduction; Shaw, 1991). If oospores result from apomixis, then selfed oospores contribute nothing to variability in the pathogen population. If on the other hand, selfed oospores result from meiosis and recombination, they would be a potential source of variation in this species (Shattock *et al.*, 1985). Sexual crosses have been made in vitro for *P. infestans* in order to investigate inheritance of several phenotypic characteristics such as resistance to metalaxyl (Shattock 1998; Lee *et al.*, 1999); allozyme markers (Spielman *et al.*, 1990); mating type (Judelson *et al.*, 1995) and for avirulence genes (Al-Kherb *et al.*, 1995; Lees *et al.*, 2001). A system with a single locus, where the alternate mating types are homozygous and heterozygous, has been proposed for *Phytophthora* spp. although the mating type ratios observed in the progeny of crosses are frequently inconsistent with this model (Gallegly 1968; Shattock *et al.*, 1986; Spielman *et al.*, 1990; Shaw, 1991). Although generally considered robust, mutation or mitotic recombination as well as treatment with certain fungicides can result in segregation of the opposite mating type (Smart *et al.*, 1998). Additionally, self-fertility can occur amongst US lineages (specifically the US-7 and US-8 lineages), although fewer oospores are produced than between opposite mating types (Smart *et al.*, 1998).

How far these factors may influence use of mating type as a marker in selection studies is unknown.

The problems caused by potato late blight were exacerbated by the development of resistance to the most widely used systemic fungicide group (phenylamide, FRAC group 4) and the appearance of new, highly aggressive strains of *P. infestans* (Gisi and Cohen, 1995). Major improvements and sophisticated procedures have significantly enhanced the understanding of the biology and pathogenic strategies of *P. infestans*. Phenylamides were the first compounds discovered to be systemically active against oomycetes, and the development of resistance has been a major factor in the contemporary severity of potato late blight (Gisi and Cohen, 1995). Currently, the predominant isolates of *P. infestans* in North America are resistant to the widely used fungicide metalaxyl. Metalaxyl [methyl-N-(2-furoyl)-N-(2,6-xylyl)-DL-alaninate (Ridomil®, Syngenta, Basle, Switzerland)] belongs to a class of fungicides known as the phenylamides and was introduced by Ciba-Geigy in 1977 (Urech *et al.*, 1977) for control of *P. infestans* due to a high protective, curative and eradicative activity (Schwinn and Staub, 1987). Metalaxyl-resistant isolates have been shown to infect tubers, but tubers infected with metalaxyl-sensitive isolates were more likely to produce infected plants because they were not completely destroyed by the disease (Walker and Cooke, 1990). Pathogen response to resistance in tubers may be genotype specific (Kirk *et al.*, 2001a). For example, observations by Flier *et al.* (2001) indicate that the gene-for gene pathosystem in potato is not solely responsible for the differential interaction in tuber infection. A resistance response occurs when an incompatible race of *P. infestans* attempts to infect a potato plant containing appropriate

R genes. The genetic control is unclear though and multiple genes are likely involved (Shattock, 1988; Shaw, 1991; Lee *et al.*, 1999). If as suggested by Shattock, (1988) resistance is controlled by a single locus exhibiting incomplete dominance, heterozygous isolates with both resistant and sensitive alleles would exhibit intermediate resistance. However backcrosses have not segregated as expected by this model (Shattock, 1988) and the precise mechanism of inheritance is unknown. Since resistance is a relatively stable and heritable trait the method can be used to characterize individual isolates within a population (Forbes *et al.*, 1998).

Much *in vitro* evidence suggests new genotypes possess higher aggressiveness and have more efficient colonization of potato than the 'old' US-1 (96/100) genotype (Goodwin *et al.*, 1995; Miller *et al.*, 1998; Flier *et al.*, 1998). However as new genotypes such as US-8 (100/111/122) and many of the European genotypes are metalaxyl resistant this in itself may have allowed a competitive advantage against the phenylamide sensitive US-1 (100/100) genotype (Goodwin *et al.*, 1996; Cooke *et al.*, 2006). It is generally accepted that metalaxyl resistance in the U.S. is the result of migration rather than mutation and subsequent selection (Goodwin, 1997). Sensitivity to the fungicide metalaxyl has been used to characterize isolates since the first detection of resistance in 1980 (Davidse *et al.*, 1981). Resistance is qualitative in practice with isolates being either sensitive or resistant with intermediate sensitivity usually infrequent (Gisi and Cohen, 1996). The nature of this resistance is uncertain though and it is now considered likely that multiple genes are involved (Judelson and Roberts, 1999). The trait is believed to have originated from 'naturally' resistant strains present in the population at a low level prior to introduction of the fungicide (Gisi and Cohen, 1996).

Genetic analysis of resistance derived from wild Solanum species has demonstrated the existence of a gene-for gene interaction between the host (*Solanum tuberosum*) and the pathogen (*Phytopthora infestans*) with single genes, termed R genes corresponding to specific virulence genes in the pathogen (Malcolmson and Black, 1966). Patterns of inheritance support the hypothesis of Mendelian inheritance with avirulent alleles being dominant and virulent alleles recessive (Al-Kherb *et al.*, 1995). However, it has been demonstrated that isolates of a single genotype, as well as single zoospores from a single isolate can have a high level of variability as regards compatibility with R genes (Goodwin *et al.*, 1995; Abu-El Samen *et al.*, 2003). To date eleven R genes have been elucidated, although it has been acknowledged more may exist (Trognitz, 1998). Detached leaflet assays for quantitative assessments of virulence in *P. infestans* have been commonly used in number of studies. Virulence refers to the ammount of pathogenicity of an organism, or it can also be defined as the relative ability of a microbe to cause disease. These techniques have been used in preference to whole-plant assays because of the ease of testing multiple isolates simultaneously, less contamination from airborne spread of inoculum, and ability to control the environmental conditions in replicated tests consistently. Test isolates of the pathogen which cannot be released into the field can also be tested in these conditions. Legard *et al.*, (1995), observed that the detached leaflet assay was a reasonably accurate predictor of the behaviour of isolates under field conditions.

The objectives of this study are:

1. To assess the different phenotypic characteristics a) mating type, b) metalaxyl sensitivity and c) virulence assessment on the progeny isolates generated from the nine crosses described in chapter 4.
2. Estimate the inheritance of these traits.

3.2 Materials and methods

3.2.1 Selection of isolates

Parental isolates were selected on the basis of their temperature tolerance at -5°C and the compatibility type as described in Chapter 2. Two out of seven parental isolates are from Northern Ireland, UK and the rest from the United States (Appendix 2). Progeny were isolated from single oospore cultures as described in chapter 2.

3.2.2 Mating type

Mating type determination was accomplished by growing the isolates on non-amended rye agar media (Appendix 1), poured thinly on 15 x 60 mm Petri dishes for a final volume of 25.0 ml (as described in Chapter 4). Briefly, each sample isolate was paired with known A_1 and A_2 isolates of *P. infestans* referred to as testers. Mycelial plugs (4 mm diameter) were placed 2 cm apart on the agar and incubated at 18°C in the dark for 14 days. Isolates were then assessed microscopically for oospore production where the two colonies interacted. An isolate was designated A_1 if oospores were produced when paired with the A_2 tester and *vice versa*. Pure cultures of the same test isolate were also observed under the microscope, to test for any evidence of self-fertilization.

3.2.3 Metalaxyl sensitivity

All the isolates were tested for the sensitivity to the systemic fungicide, metalaxyl (new isomer mefenoxam; Syngenta, Research Triangle Park, NC U.S.A.). The method followed was as described by Sujkowski *et al.*, (1995) and as modified by Stein and Kirk (2003). Stock solutions were prepared by adding metalaxyl (Ridomil Gold EC, 47.6% active ingredient) to the double distilled water. For making 1000 ppm (parts per million) stock solution, 2.10g metalaxyl was added to 1 L double distilled water. Serial dilutions were continued to make 100, 10, 5, 1, 0.1 ppm stock solutions. Stock solutions (100, 10, 5, 1, and 0.1 ppm) were added to molten media (10 ml/990 mL) after the media was cooled to 55°C. All stock solutions were sterilized by passing them through 0.22 µM millipore filters (Millipore Corp., Bedford, MA, U.S.A.) using 10 ml syringes. Plates containing 10 ml/liter of filtered water were used as a control (0 ppm). Rye B agar amended with 0, 0.1, 1, 5, 10 and 100 ppm of fungicide were made and compared with growth on medium without fungicide (0 ppm); (Fig. 3.1).

Mycelial plugs of the sample isolates of *P. infestans* (2 mm diameter) were removed from the edge of an actively growing colony on Rye RAN agar (Appendix 1) and placed on the center of fungicide amended rye agar. The experiment was replicated three times and repeated twice. The cultures were incubated at room temperature for 8-days and the radius of each colony was measured. A separate EC_{50} was produced for each isolate within all three experiments. The mean EC_{50} was determined for each isolate using all three experiments. Percent inhibition for each concentration of metalaxyl was calculated relative to the mean of the control plates within each experiment. EC_{50} for hyphal growth (diameter) was calculated using the Pharmacology function standard curves analysis

feature of SigmaPlot (Version 9.01, Systat Software, Inc., Chicago, IL, USA). The sensitivity of the isolates was classified by comparing EC_{50} response of the isolates grown on media amended with different concentrations of metalaxyl with EC_{50} of known resistant and susceptible isolates. Isolates were termed as resistant (R) when the EC_{50} was not significantly different at $p = 0.05$ from the growth of the resistant reference isolate; intermediate in resistance (I) when the EC_{50} was significantly less than that of the EC_{50} of the resistant reference isolate but significantly greater than that of the sensitive reference isolate; and sensitive (S) when the EC_{50} was not significantly different from the sensitive reference isolate.

3.2.4 Virulence assessment

All parent and progeny isolates were analyzed for virulence phenotype by the detached leaf method using differential plants LB1 to LB11 (micro-plants of the differentials were obtained from the USDA Potato Germplasm Unit, WI, US and grown in tissue culture until needed) carrying single genes for resistance (R1-R11) and Craig's Royal (R0) containing no R genes was used as the susceptible variety or control. Plants were grown for about 8 weeks in six 1 L pots (one tuber per pot) containing sterilized soil. They were maintained in temperature-controlled environment chambers (Environmental Growth Chambers, Chagrin Falls Ohio, USA) at 18°C, 16/8 h photoperiod and >80% RH and the illumination was provided by 500 W Philips-HPIT lamps. Greenhouses were also used to grow the plants and they were maintained at 20°C under light. Healthy secondary leaflets were cut from the compound leaves and used for all the tests. Isolates were tested on three replicate leaflets of each differential cultivar (LB1 to LB11) and control (Craigs Royal). Inoculum was prepared from 12-14 day old cultures grown on Rye agar.

Sporangia were collected by adding 10 ml sterile distilled water to each isolate. Mycelium was scraped from the flooded culture using a rubber-policeman and then the solution was filtered through three layers of cheesecloth. Sporangia were counted and the suspension was standardized to approximately 2 x 10^4 sporangia ml^{-1} using a hemacytometer. The spore suspension was incubated in a refrigerator at 4°C for 90 minutes to induce zoospore release. Transparent plastic boxes (13 x 18 x 5 cm) were used for the assay. A moistened thick paper towel was placed in the bottom of the box to maintain the humidity at >90%, and then a plastic mesh was placed over the towel, so that the leaflets did not come into contact with the wet paper towel and predispose the leaflets to bacterial wet-rot caused by pectolytic bacteria. Leaflets about the same size (2 cm long x 2 cm at the widest point) were excised from potato plants generated from tissue culture and placed abaxial side up on the plastic mesh. Five leaflets per R gene differential genotype were inoculated with a droplet of the suspension (20 µl) placed close to the center of each leaflet (Fig. 3.2). The inoculated leaflets were incubated in a growth chamber at 18°C and 95% humidity with a 12-h light:dark cycle and the illumination was provided by 500 W Philips-HPIT lamps. Leaflets were scored after seven days for presence or absence of a sporulating lesion, by giving the numbers 1 and 0, respectively. Each test was repeated twice for a total of 15 leaflets per differential per parental and progeny isolate tested. The corresponding Avr gene was considered absent if >85% of leaflets had a sporulating lesion.

3.3 Results

3.3.1 Compatibility type

Phytophthora infestans was primarily heterothallic, though some isolates appeared to be self-fertile. The number of oospores germinated in matings between A_1 and A_2 compatibility types of *P. infestans* was significantly different in each cross. Oospores were produced in all crosses between A_1 and A_2 compatibility types, the manner of germination was similar for oospores from all crosses, but the amount of germination significantly differed among crosses. Germination rate of oospores from some crosses was greater than 75% and in some crosses the germination rate was less than 5%. Progeny from each cross had a different segregation in the compatibility type, and some self-fertile progeny were also observed. About 215 oospores germinated when both parents with the thermal phenotype character "tolerant" were crossed (crosses 1, 2, 3, 4, and 6; Table 3.1); 45 oospores germinated when thermal tolerant with intermediately tolerant were crossed (Cross5; Table 3.1); 24 oospores germinated when thermal tolerant with thermal sensitive parent isolates were crossed (crosses 7 and 8; Table 3.1); 2 oospores germinated when thermal sensitive with sensitive parent isolates were crossed (cross 9, Table 3.1). In total, 300 progeny isolates were collected from the nine crosses.

3.3.1.1 Inheritance of mating type after 2 weeks of incubation

In cross 1 and cross 2 (Pi 41-02 X Pi 02-007) high numbers of progeny were established and the ratio of A_1:A_2 was approximately 1:2. There were eight self-fertile isolates (oospores found in both the testers) present out of 88 total isolates from cross 1 and cross 2 (Table 3.1). In cross 3 (Pi 41-02 and Pi S1-3) and cross 7 (Pi 95-3 X Pi 02-007) A_1: A_2 progeny were in the ratio of approximately 1:1. In cross 4 (Pi 41-02 X Pi S1-3), and cross

5 (Pi 41-02 X Pi 98-1) A_1: A_2 progeny were in the ratio of approximately of 2:1. In cross 6 (Pi 62-02 X Pi 02-007) A_1: A_2 progeny were in the ratio of approximately of 1:2. There were three self-fertile isolates out of 51 from cross 3 and one from cross 5 out of 35 isolates. Oospores formed in pure culture were indistinguishable from those produced in mating involving A_1 and A_2 mating type strains. Progeny isolates in general had thick fast growing mycelium, abundant sporangia and oospores compared to the parents. Self-fertility (oospore production) was found in the progeny isolates from crosses where one of the parents was the 100/111/122 genotype. Compared to the US-8 genotype, progeny isolates generated from other genotypes did not form as many self-fertile progeny isolates in pure culture (Table 3.1).

3.3.1.2 Variation of mating type after 1 month of incubation

All the pure cultures (not crossed with testers) were observed under the microscope for the presence of oospores (Table 3.2). The number of self-fertile oospores in the progeny isolates increased with time. In cross 1, 18 out of 27 progeny isolates; cross 2, 27 out of 39; cross 3, 8 out of 19; cross 4, 12 out of 29; cross 5, 7 out of 32; cross 6, 11 out of 18; cross 7, 3 out of 6; cross 8, 3 out of 6 had self-fertile oospores; and in cross 9, there were no self fertile oospores observed.

3.3.2 Metalaxyl sensitivity

Parents in this study were either resistant, intermediate in sensitivity or sensitive to metalaxyl (Table 3.3). Variation was observed in the progeny isolates in metalaxyl phenotype. From the crosses 1, 2, and 5 (metalaxyl resistant X resistant cross), out of 133 isolates, 67 progeny isolates were identical to the parents; 64 isolates were intermediate

and 2 isolates were sensitive to metalaxyl and were hybrid progeny. Parents in the crosses 3, 4, 6, and 8 were resistant and intermediate in resistance to metalaxyl. Out of 101 isolates from crosses 3 and 4 (same parents), 18 isolates were resistant and 61 isolates were intermediate in metalaxyl sensitivity (identical to the parents) and 22 isolates were hybrids and had a sensitive metalaxyl sensitivity phenotype (Table 3.4). Out of 41 isolates from crosses 6, and 8, recombination was observed in 3 isolates that were sensitive to metalaxyl. 12 isolates were resistant and 26 isolates were intermediate (Table 3.4). In cross 7, parents were resistant and sensitive to metalaxyl and produced 7 resistant, 1intermediate and 0 sensitive isolates (Table 3.4). In cross 9, both the parents were intermediate in resistance to metalaxyl and produced two isolates also intermediate (Table 3.4). In total, 117 isolates were determined as hybrids out of total of 285 progeny isolates.

3.3.3 Virulence assessment

Virulence testing was completed for all the parents and several progeny isolates selected at random from the crosses. From crosses 1 through 6, 5 to 10 progeny isolates were chosen per cross. Among these a few isolates were identical to the parents and some were recombinants (Table 3.4). The parental isolates chosen were similar in race structure and by having common virulence genes. Pi 02-007, Pi 62-02 and Pi S1-3 were virulent on all 11 R genes (LBR1 to LBR11). Pi 98-1 was virulent on 1, 4, 5, 9, 10, and 11. Pi 41-02 had the virulence factors 1, 3, 4, 5, 6, 7, 8, 9, 10 and 11. Pi 95-3 had only virulence factor 5. Pi Atlantic2N had virulence factors 2, 3, 5, 6, 7, 8, 10, and 11. Progeny were all pathogenic on at least more than seven LBR differential lines. About 80% of the isolates were pathogenic on all 11 R gene differentials. All the isolates were shown to be

pathogenic by infecting the R0 differential and were able to overcome at least one virulence factor. Variation was observed in the progeny isolates, by having different R genes. Isolates with extensive variation because of the absence of R genes were 1_04, 1_05, 1_06, 1_29, 3_14, 3_37, 4_01, 5_12, 5_14, 5_38, 5_41, 5_45, 6_11, and 6_19.

3.4 Discussion

In these studies, progress in understanding the patterns of inheritance was severely hampered by difficulties in germinating oospores and establishing single-oospore cultures. For example, Romero and Erwin, 1969 detected high levels of oospore production in some crosses, but established only 34 single oospore cultures. In another study with siblings, only 4 of 620 produced oospores were established in pure culture, thus preventing a study of inheritance in F2 progeny (Romero and Erwin, 1969). Variation among *P. infestans* populations in phenotypic characteristics such as growth rate, colony morphology, aggressiveness and virulence were recognized in the clonal populations of *P. infestans*, collected prior to migrations in the 1990's which were restricted to asexual reproduction (Graham, 1955; Caten and Jinks, 1968; Zarzycka, 1996). The sources of this variability are not well understood in the absence of sexual reproduction and genetic markers. Direct observation of germination of selfed oospores of heterothallic species of *Phytophthora* has only been reported once before (Brasier *et al.*, 1975). When A_1 and A_2 mating type isolates are paired, each may produce antheridia, oogonia or both, which later fuse to form hybrid oospores (Judelson, 1997). Outcrossing and selfing can both occur within mating cultures (Judelson, 1997). In this study, 12 isolates produced oospores without the presence of the opposite mating type, which were determined to be self-fertile or homothallic isolates. The genetics of mating type in *P.*

Table 3.1 Number of progeny isolates in compatibility type phenotype categories resulting from oospores generated by crossing parental isolates of *Phytophthora infestans* with different compatibility types and tolerance to temperature.

Cross[a]	Thermal tolerance type of parent[b]	Number of progeny isolates in Compatibility type categories[a]		
		A_1[c]	A_2[d]	Self fertile (SF)[e]
1. Pi 41-02 X Pi 02-007	T X T	9	23	5
2. Pi 41-02 X Pi 02-007	T X T	17	31	3
3. Pi 41-02 X Pi S1-3	T X T	27	21	3
4. Pi 41-02 X Pi S1-3	T X T	36	14	0
5. Pi 41-02 X Pi 98-1	T X I	29	15	1
6. Pi 62-02 Pi 02-007	T X T	8	17	0
7. Pi 95-3 X Pi 02-007	S X T	4	4	0
8. Pi 41-02 X Pi Atlantic2N	T X S	14	2	0
9. Pi 4-19 X Pi Atlantic2N	S X S	2	0	0

[a] *P. infestans* isolates used as parents to produce single oospore progeny (A_1 X A_2).

[b] Comparison of growth of isolates of cultures of *Phytophthora infestans* for 25 days at 18°C after exposure at -5°C for 5 days measured by an image analysis technique comparing the growth of exposed isolates to a positive control (exposed to 18°C for 5 days) or a negative non-inoculated control with no growth. T= Tolerant; growth not significantly different from the growth of the positive control cultures of the same isolate; I= Intermediate; growth significantly less than that of the growth of the positive control cultures of the same isolate but significantly greater than the negative control; S= Sensitive; growth not significantly different from the negative control.

[c] Number of isolates produced that were A_1 compatibility type.

[d] Number of isolates produced that wereA_2 compatibility type.

[e] Oospores that were produced with both A_1 and A_2 tester isolates and also in pure cultures.

Table 3.2 Different progeny and parent isolates showing either presence of self-fertile oospores in pure cultures after one month of incubation resulting from oospores generated by crossing parental isolates of *Phytophthora infestans* with different compatibility types and tolerance to temperature.

Cross 1		Cross 2		Cross 3		Cross 4	
1-01	0[a]	2-02	+	3-01	0	4-01	+
1-02	+	2-03	+	3-05	+	4-02	0
1-03	+	2-05	0	3-06	0	4-03	0
1-06	+	2-06	+	3-07	0	4-04	+
1-07	0	2-07	+	3-09	0	4-05	0
1-08	+	2-08	+	3-11	+	4-06	+
1-10	0	2-09	+	3-12	+	4-07	0
1-13	0	2-10	+	3-13	+	4-08	+
1-14	+	2-11	+	3-14	0	4-09	+
1-15	+	2-12	0	3-21	+	4-10	+
1-16	+	2-17	+	3-27	0	4-11	+
1-17	0	2-19	0	3-37	+	4-12	+
1-19	+	2-20	+	3-41	+	4-13	0
1-20	+	2-23	+	3-42	0	4-14	0
1-21	+	2-24	0	3-44	0	4-15	0
1-22	+	2-25	+	3-46	0	4-16	0
1-23	+	2-26	0	3-47	0	4-17	0
1-24	+	2-27	+	3-48	0	4-18	+
1-26	+	2-28	0	3-49	+	4-29	0
1-27	0	2-30	+			4-32	0
1-28	+	2-32	+			4-34	0
1-29	+	2-33	0			4-36	0
1-30	+	2-34	+			4-40	0
1-31	0	2-35	+			4-41	+
1-34	0	2-36	+			4-44	0
1-36	+	2-37	+			4-45	0
1-A	0	2-38	+			4-46	0
		2-39	+			4-48	+
		2-40	0			4-51	+
		2-41	+				
		2-44	+				
		2-46	0				
		2-47	+				
		2-48	0				
		2-49	+				
		2-50	0				
		2B.A	0				
		2B.B	+				
		2B.C	+				

[a] "+" = Self-fertile oospores present in pure cultures, "0" = Self-fertile oospores absent in pure cultures

Table 3.2 continued

Cross 5		Cross 6		Cross 7		Cross 8		Cross 9	
5-04	0	6-06	+	7-01	+	8-02	+	9_01	0
5-05	0	6-07	+	7-03	0	8-03	0	9_02	0
5-06	0	6-08	+	7-05	0	8-05	0		
5-07	0	6-11	0	7-08	+	8-06	+		
5-08	0	6-12	+	7-09	+	8-08	+	**Parents**	
5-10	0	6-13	+	7-18	0	8-09	0	Pi 41-02	0
5-11	0	6-14	0					Pi S1-3	0
5-12	0	6-15	0					Pi 62-02	0
5-14	0	6-16	0					Pi98-1	0
5-15	+	6-17	0					Pi 95-3	0
5-16	0	6-18	+					Pi 4-19	0
5-17	+	6-19	+					Pi 02-007	1
5-19	0	6-20	+						
5-20	+	6-22	+						
5-21	0	6-23	+						
5-23	0	6-21	+						
5-24	0	6-24	0						
5-25	0	6-25	0						
5-26	0								
5-27	0								
5-29	+								
5-31	0								
5-32	0								
5-33	+								
5-35	0								
5-36	+								
5-38	+								
5-39	0								
5-40	0								
5-41	0								
5-42	+								
5-43	0								
5-46	0								

[a] "+" = Self-fertile oospores present in pure cultures, "0" = Self-fertile oospores absent in pure cultures

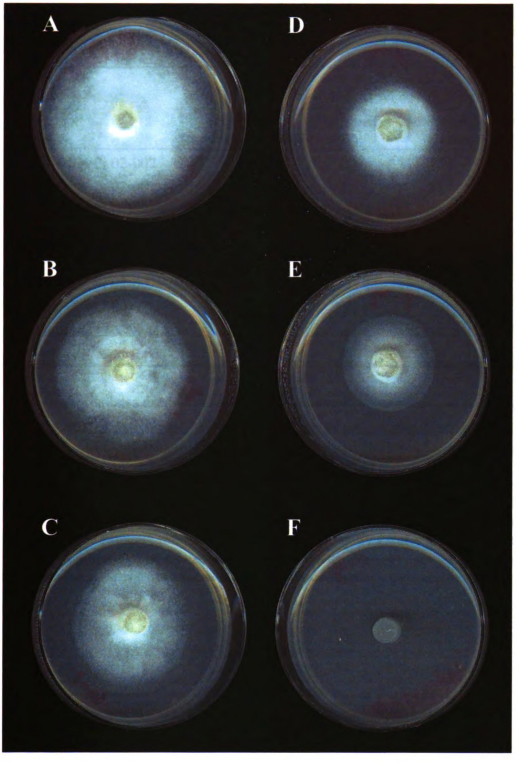


Figure 3.1 An isolate of *P. infestans* showing levels of sensitivity to metalaxyl at different concentrations: A= Control or 0; B= 0.1; C= 1; D= 10; E= 100; F= 1000 PPM metalaxyl.For example isolate 1-4 is intermediately resistant to Metalaxyl with EC_{50}: 5 PPM

Table 3.3 Number of progeny isolates in metalaxyl sensitivity phenotype categories resulting from oospores generated by crossing parental isolates of *Phytophthora infestans* with different metalaxyl sensitivity.

Cross[a]	Metalaxyl Sensitivity Parent[b]	Number of progeny isolates in metalaxyl sensitivity categories[b] Resistant (R)	Intermediate (I)	Sensitive (S)
1. Pi 41-02 X Pi 02-007	R X R	20	15	2
2. Pi 41-02 X Pi 02-007	R X R	38	13	0
3. Pi 41-02 X Pi S1-3	R X R	10	31	10
4. Pi 41-02 X Pi S1-3	R X R	8	30	12
5. Pi 41-02 X Pi 98-1	R X I	9	36	0
6. Pi 62-02 X Pi 02-007	R X I	8	16	1
7. Pi 95-3 X Pi 02-007	S X R	7	1	0
8. Pi Atlantic2N X Pi 41-02	I X R	4	10	2
9. Pi Atlantic2N X 4-19	I X I	0	2	0

[a] *P. infestans* isolates used as parents to produce single oospore progeny (A_1 X A_2).

[b] Metalaxyl sensitivity phenotypes; R= Resistant; I= Intermediate; S=Sensitive; the sensitivity of the isolates was classified by comparing EC_{50} response of the isolates grown on media amended with different concentrations of metalaxyl with EC_{50} of known resistant and susceptible isolates. Isolates were termed as resistant (R) when the EC_{50} was not significantly different from the growth of the resistant reference isolate. Isolates were termed as intermediate in resistance (I) when the EC_{50} was significantly less than that of the EC_{50} of the resistant reference isolate but significantly greater than that of the sensitive reference isolate; Isolates were termed sensitive (S) when the EC_{50} was not significantly different from the sensitive reference isolate.

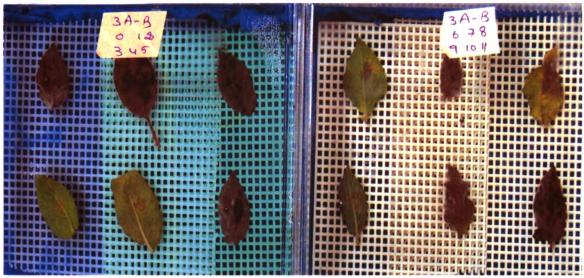


Figure 3.2 Example of a single replication for assessing the virulence pathotype. LBR gene leaflets (LBR0 to LBR11) showing lesions symptomatic of infection with *P. infestans* incubated at 18°C in a controlled growth chamber for eight days. The virulence pathotype indicated that isolate 3A lacked the avirulence genes 0, 1, 2, 5, 7, 8, 10, and 11

Table 3.4 Parental and progeny isolates of *Phytophthora. infestans* showing virulence on R gene potato differential genotypes LB 0 to LB 11.

Isolate name[a]	R gene phenotype[a]	Isolate name	R gene phenotype[a]
Pi 02-007	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	3A	0,1,2,3,5,7,8,10,11
Pi 98-1	1,4,5,9,10.11	4_01	1,3,5,9,11
Pi 41-02	1,3,4,5,6,7,8,9,10,11	4_11	0,1,3,4,5,7,9,10,11
Pi 62-02	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	4_12	0,1,2,3,4,5,6,7,8,10,11
Pi Atlantic2N	0,2,3,5,6,7,8,10,11	4_29	0,1,2,3,4,5,6,7,8,9,10,11
1_01	0, 1, 2, 3, 6, 10, 11	5_01	0,1,2,3,4,5,6
1_03	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	5_02	1,2,3,4,5,6,7,10,11
1_04	0	5_09	1,2,3,4,5,6,7,10
1_05	0, 1, 2, 3, 4, 5	5_12	0,1,2,3,4,5,6
1_06	1,2	5_14	3,5,7,8,9
1_10	0,1,2,3,4,5,6,7,8,9,10,11	5_16	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11
1_13	0,1,3,4,5,6,10,11	5_19	0,1,2,5,6,7,8,9,11
1_18	0,1,2,3,4,6,7,8,11	5_28	0,1,2,3,4,5,7,8,9,10,11
1_29	3,5,6,7,9,10,11	5_30	0,1,2,3,4,5,6,7,9,10,11
2_01	0,1,2,3,4,5,6,7,8,9,10,11	5_37	1,3,5,6,8
2_07	0,1,2,3,4,5,6,7,9,10,11	5_41	1
2_13	1,3,5,6,7,8,10,11	5_45	1,4
2_31	0,1,2,3,4,5,6,7,8,9,10,11	6_08	0,1,2,3,4,5,6,7,8,9,10,11
3_01	0, 1, 2, 3, 4, 5, 6, 7, 9, 10, 11	6_11	1,3,5,7,10,11
3_06	1,2,3,4,5,7,8,10,11	6_19	3,4,6,7,8,10,11
3_14	0,1,2,3,6	Pi 95-3	5
3_37	0,1,2,3,5,6	Pi S1-3	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11

[a] Pi in isolate identification indicates a parental isolate otherwise the first number of the isolate identifies the parental cross as defined in Table 2.1. The isolates were selected at random for virulence phenotype testing.

[b] Virulence pathotype as defined by Muller and Black (1952).

infestans is complex and the A_1 : A_2 ratio in progeny can vary significantly, ranging from close to 1:1 to an excess of A_1 over A_2 (Shaw 1991; Judelson *et al.*, 1995). It was quite difficult to understand if the progeny isolates were self fertile or hybrid, as they have a tendency to change their compatibility type, from being heterothallic (A_1 or A_2) to homothallic. In the first two weeks after isolation, about 70% were hybrids with characteristics of the parents and the remainders were self-fertile. After one month, 50% of the progeny were selfs or homothallic and the other 50% were heterothallic. Further differentiation of these oospores generated from from outcrossing and from cultures arising from residues of parental mycelia was attempted by the segregation of SSR markers (Chapter 2).

In previous studies the frequency of oospore-derived cultures that resulted from selfing ranged from low (Shattock *et al.*, 1986) to more than half of all cultures (Whittaker *et al.*, 1994). When antheridia and oogonia from the same parent fuse they form self-fertile oospores. The gene control of pathogenicity and virulence is not yet defined and the mechanisms of variation are still ambiguous (Shattock *et al.*, 1985). Early studies proposed that one locus determined mating type (Galindo and Gallegly, 1960), but this could not be reconciled with the widely varying ratios of A_1 and A_2 types observed in crosses (Shaw, 1991). Later studies using linked DNA markers confirmed the involvement of a single locus and indicated that it displayed non-Mendelian inheritance in most isolates (Judelson *et al.*, 1995). In cross 5, the A_1 mating type was dominant in progeny isolates. When this phenotypic character was compared with the genotypic character for this cross (*GPI* isozyme type), they both showed that the parent, Pi 41-02

(100/100) was more prevalent than Pi 02-007 (Chapter 2). The progeny selfs were either intermediate or resistant as were the parents, and true sexual recombinants had a sensitive response to metalaxyl.

Variation between isolates in their resistance to fungicide, aggressiveness on R gene foliage, and pathogenicity on tubers may lead to an understanding of the different factors that plants use for defense. In the evolution of field isolates and genetic diversity of populations, not only outcrossing but also selfing should be considered. The latter process may be a more likely event in populations, assuming that selfing is similar in frequency *in planta* as observed *in vitro* (Knapova *et al.*, 2002).

Inheritance of resistance to metalaxyl has been described as being controlled by one single, incompletely dominant gene (Shaw and Shattock, 1991). In another report, resistance to metalaxyl was associated with one semi-dominant locus combined with the effect of several minor loci resulting in a continuous segregation for sensitivity in F1 progeny (Fabritius *et al.*, 1997). In a separate study, one parent was heterozygous with one single gene carrying a dominant allele, and the segregation was 1s: 1r in F1 progeny and 1s:3r in F2 progeny (Lee *et al.*, 1999). The distortion from the Mendelian distribution may be explained by the effects of minor loci (Fabritius *et al.*, 1997) or the presence of more than one major locus for metalaxyl resistance (Judelson and Roberts, 1999). It is not known how frequent the various resistant alleles are in resistant individuals of different origins, but it is apparent that sensitive offspring will always appear after recurrent sexual recombination in subsequent generations, and resistant individuals will not disappear

independently of the model of inheritance described above (Knapova *et al.*, 2002). In this study, the progeny from the crosses had different inheritance ratios and did not follow Mendelian segregation, agreeing with Judelson and Roberts (1999), implying that there is more than one locus for metalaxyl resistance.

Virulence is usually considered as the most important phenotypic marker in studying most plant pathogens; however virulence/avirulence loci represent only a small portion of the total genetic variation that might exist among different races within a plant pathogen population. The term complex virulence has generally been used to describe isolates carrying more than one virulence gene (Umaerus *et al.*, 1983). Multiple population studies have reported an increase in virulence complexity in association with the emergence of 'new' populations of *Phytophthora infestans* (Goodwin 1997; Dorrance *et al.*, 1999), although others suggest all known virulence factors were present at a much earlier stage (Day and Shattock 1995). Isolates from the US-8 and US-14 groups contained more than six virulence factors, with US-10 containing five and other genotypes contain less than three. These results agree with other studies where US-8 and US-14 genotypes on average contain higher numbers of virulence factors than 'older genotypes' such as US-11 (Dorrance *et al.*, 1999) and US-6 (Goodwin *et al.*, 1995). Isolates originating from Northen Ireland Pi 41-02 and Pi 62-02 are aggressive and also had 10 and 11 virulence factors, respectively. Since most parents from this study contained highly complex isolates, any effect of incompatibility with R genes is likely to be small. Just like the parents, US-8 progeny isolates had all the 11 virulence factors; several progeny from this parent had all the eleven virulent factors, which could be

because of selfing, as they had the same virulence phenotype. From Van der Plank's theory of stabilizing selection, races with the fewest unnecessary virulence genes will be the most fit (Van der Plank, 1968). Since the majority of planted commercial cultivars in Europe and the United States contain few if any R genes (Fry and Goodwin, 1997) most virulence genes would be deemed unnecessary and therefore very simple races (such as 100/122 and 86/100 from this study), should be fittest. In disagreement with the theory Tooley *et al.*, (1986) assessed fitness on simple hosts (i.e. containing few R genes) using sexual and asexual populations of *P. infestans* and detected no higher fitness associated with fewer unnecessary virulence genes. More recent studies have also detected no evidence of any correlation between fitness and virulence phenotype (Schober and Turkensteen, 1992; Lebreton *et al.*, 1999). Although an important phenotypic test for selection studies, R gene testing has certain weaknesses as regards general population and tracking investigations. Multiple studies have highlighted the importance of evaluation conditions, with inoculum concentration, temperature, age of plant and state of the pathogen all shown to influence results (Swiezynski *et al.*, 2000). Findings have been inconsistent between laboratories and even between individual isolates (Schick and Schick, 1959; Schober and Turkensteen, 1992). Additionally variability in virulence among sexual progeny (most likely due to mitotic recombination) has led to the hypothesis that *P. infestans* has the capacity for continuous change in R gene compatibility (Abu-El Samen *et al.*, 2002). For all of these reasons only a few progeny isolates were tested for compatibility with R genes as a means of tracking inheritance in this study.

Sexual recombination will produce new genetic combinations, creating the possibility of unusually fit or pathogenic individuals or individuals with novel ecological characteristics. This possibility has been termed the "Red Queen hypothesis (Barton *et al.*, 1998) and is particularly important for organisms that reproduce both asexually and sexually (Mayton *et al.*, 2000). Shattock *et al.*, (1985) presented data that indicated that selfed oospores of A_1 isolates of *P. infestans* produced in vitro by intraspecific stimulation are viable and will germinate to produce mycelial colonies. If interspecific stimulation occurs in natural populations of *Phytophthora*, then selfing may generate variation in populations of *P. infestans* of a single compatibility type. Oospores formed at the beginning of epidemics could therefore contribute to the emergence of novel phenotypes within a growing season as well as between seasons (Drenth *et al.*, 1995).

4 Cold temperature tolerance of mycelium in progeny isolates of *Phytophthora infestans* from mating

4.1 Introduction

Potato late blight epidemics caused by *Phytophthora infestans* are associated with wet conditions and moderate temperatures (Harrison, 1992). Temperature can influence spore germination, mycelial growth rate, inoculum production and survival of *P. infestans*. The range of temperature at which predominant genotypes of *P. infestans* in the US and Europe can develop and grow is relatively wide and recent estimates indicate that the range for mycelium may be as extensive as -5 to 35°C (Zwankhuizen *et al.*, 1998; Kirk, 2003b). There have been fewer studies of the temperature range for spore development and other components of epidemic development for these predominant genotypes and current estimates are known mainly for pan-global clonal lineages (US-1; Goodwin *et al.*, 1995) although this lineage is seldom recovered in nature. The thermal profile for this pan-global lineage was reported by Harrison (1992); indirect sporangial germination [formation of zoospores (5 to 10 per sporangium)] was favored below 20°C with the optimum between 12 to 13°C and direct germination was favored at above 20°C, (optimum 24°C; Mizubuti and Fry, 1998). Recent reports suggest that predominant genotypes of *P. infestans* have variability in the dynamics of thermal responses may affect epidemic development (Kirk 2003a, 2003b). Many investigators have used *in vitro* and soil assays to study the optimal and lethal upper temperatures for growth of *Phytophthora* spp. (Bollen, 1985; Juarez-Palacios *et al.*, 1991; Coelho *et al.*, 2000; Snider *et al.*, 2000). The survival of *P. nicotianae* chlamydospores in soil was reduced considerably when temperatures were raised above 45°C (Coelho *et al.*, 2000). Few studies that systematically examine the ability of *P. infestans* mycelium to survive at

temperatures below 0°C exist although De Bruyn (1926) showed that a mycelial colony was capable of withstanding temperatures as low as -25°C for up to 5 days in manure-based substrate and that in dried substrates survival at such low temperatures was enhanced and Kirk (2003b) showed that several genotypes of *P. infestans* could withstand exposure to temperature close to freezing for at least 5 days.

The overwintering of *P. infestans* is an important epidemiological factor in temperature agroecosystems (De Bruyn, 1926). Mycelium of *P. infestans* can initiate crop infections over successive years by over wintering as mycelium in infected potato tubers or within cull piles or volunteer tubers (Easton, 1982; Zwankhuizen *et al.*, 1998). Economic losses due to late blight result from foliar defoliation as well as tuber infection, and severe storage losses can occur after tubers infected with *P. infestans* are held for processing at temperatures in excess of 7°C (Kirk *et al.*, 2001). Tolerance of freezing temperatures by mycelium of *P. infestans* may be an ecologically important survival mechanism and the increased tolerance of US-8 and US-14 may explain their predominance in cooler winter climates such as north-central United States (Kirk, 2003a). It is difficult to estimate the probability of infected potato stems or foliage will emerge from an infected tuber, as there are several factors that could influence the fate of infected tubers, temperature being one of the most important (Monteith, 1977; Kato *et al.*, 1997). Many investigators have used in vitro and soil assays to study the optimal and lethal thermal temperatures for growth of *Phytophthora* spp. (Bollen, 1985; Coelho *et al.*, 2000). Perches and Galindo (1969) found soil to be infectious 2-years after a late blight infection. For example,

oospores were found to survive in soil for 5 to 7 months at temperatures between 0 and 20°C (Pittis and Shattock, 1994).

An increased risk of potato late blight epidemics in the same region has been suggested over the period from 1950 to 2000 (Baker *et al.*, 2005). If, seasonal soil temperature is moderating as speculated (and measured), especially over the winter period the risk of survival of volunteer tubers in the soil, in culls and in rock piles will increase in Northern U.S. production areas over winter and the risk of tuber-vectored potato late blight will increase. As it has been shown that *P. infestans* is tolerant to temperature close to freezing (Kirk, 2003a), this therefore creates a situation for both the host and the pathogen to survive over winter. This could result in an increase in the emergence of potato plants carrying initial inoculum that could start a seasonal epidemic.

Weather conditions such as temperature and precipitation will affect the survival of *P. infestans* during winter months and can potentially affect disease development in the summer (Easton, 1982). Climate data illustrate that, in Michigan, warmer winters are accompanied by lower winter precipitation (Isard and Schaetzl, 1998). There is generally a greater incidence of soil freezing in Michigan during warm dry winters due to recurring freeze-thaw episodes (Isard and Schaetzl, 1998). Isolates of *P. infestans* vary in their ability to survive winter freezing (Shattock, 1976; Kadish and Cohen, 1992; Kirk, 2003b). It has been suggested that the pathogen population and variability is severely reduced during winter, but the variability is regenerated each year within the expanding population (Shattock, 1976).

The production of oospores by *P. infestans* in the field and their survival under natural conditions has become important since the introduction of A_2 mating type isolates. Oospores of oomycetous fungi are, in general, endogenously dormant, tolerant of adverse conditions, and capable of long-term survival (Ribiero, 1983). Oospores of *P. infestans* have been found to survive in soil during a winter season in Europe and oospores also have been reported to survive temperatures between -80 and 35°C (Drenth *et al.*, 1995). Genetic recombination in *P. infestans* through the sexual process (production of oospores) has been shown to occur (Lees *et al.*, 1997). The presence of oospores in the soil as a source of inoculum will drastically change present disease management strategies (Umaerus and Umaerus, 1994). Oospores of *Phytophthora* spp. are insensitive to fungicides (Duncan, 1985b) but sensitive to heat treatments of 45°C (Duncan, 1985a). Despite many studies (Ribiero, 1983), factors affecting germination of oospores are not well understood. The objective of the project was to conduct an *in vitro* study to evaluate the inheritance of cold temperature tolerance in the oospore generated isolates (progeny) after mating of *P. infestans* because of the convenience of using oospores as a tool for studuyng this inheritance. Given these scenarios the objectives of this study were to investigate:

- The survival potential of mycelium of different genotype isolates of *P. infestans* exposed to temperature at -5°C for 5 days *in vitro* using a digital imaging technique
- Carry out crosses between *P. infestans* isolates (A_1 and A_2 mating types) that were highly variable for temperature tolerance, and determine if temperature tolerance

was inherited or occured as a result of physiological adaptation of different genotypes of *P. infestans*

4.2 Information from previous studies (Kirk lab) that helped in this research

Previous research conducted at the Kirk lab at the department of Plant Pathology, Michigan State University was utilized to expedite the project.

4.2.1 Substrate optimization and digital image analysis

Sterol-free clarified rye agar (SFCRA) was prepared by washing 100g organically produced rye seed, boiling for 1 h in dH_2O, straining the suspension through four layers of cheese cloth and adding 15g agar, 7.5g sucrose and dH_2O to bring the solution to 1L final volume. The optimal volume of sterol-free clarified rye agar (SFCRA) that could be added to 60 mm diameter Petri dishes in order to measure the reflectance characteristics by digital image analysis (described below) was determined. Volumes of 5, 10, 15 and 20 ml SFCRA were poured into Petri dishes. The reflectance intensity of the Petri dishes with different volumes of SFCRA was then determined by digital image analysis. The scanned images were the SFCRA Petri dishes without cover lids. The Petri dishes were placed open-surface down on a glass plate, 40 x 30 x 0.2 cm thick and digital images generated and analyzed as adapted from Niemira *et al.*, (2001). The average reflective intensity (ARI) of all the pixels within the image gave a measurement of the plate without any growth of the sample. The calibrations resulted in optimal agar volumes of 10 ml substrate/60 mm diameter Petri dishes with mean ARI = 90.

4.2.2 Temperature optimization

Temperature exposure studies were performed at different temperatures close to 0°C in Peltier chambers [PTC-1 Peltier-effect temperature cabinet (PTC-1) controlled by a PELT-3 Peltier-effect temperature controller (PELT-3); Sable Systems International, 2887 Green Valley Parkway #299, Henderson, NV 89014]. Temperature equilibration was measured after the door of the PTC-1 cabinet was opened, and at 0°C set temperature, temperature rose to 5°C after the door was opened but then dropped to -0.3°C in 1.5 hr. At -3, -5, -10 and -20°C set temperatures, temperature rose to -1.2, -1.5, -3.5 and -4.8°C and recovered in 1.1, 1.3, 1.5 and 1.4 h, respectively. Sample cultures of *P. infestans* were placed in the PTC-1 cabinets and exposure times were measured from when the set temperature was reached. Sample cultures were removed after exposures of 1, 3, 5, and 10 days. Temperature treatments were 0, -3, -5, -10 and -20°C. After plates were removed from the PTC-1 cabinet they were stored in the dark at 18°C. After 25 days the samples were scanned to determine amount of growth of mycelium. *P. infestans* cultures survived -3°C for 5 days, and a few cultures have survived at -5°C after 1 day (Kirk, 2003b).

There was not much variation noticed in the temperature tolerance when the isolates were tested at -3°C for five days and -5°C for one day. Based on these results, to observe more variation the temperature exposure used in this study to determine temperature tolerance was -5°C for 5 days.

4.2.3 Determination of developmental stage of sporangia on mycelial plugs after transfer from parent cultures

As the objective of the experiment was to determine the influence of duration of exposure of temperatures to -5°C on mycelial survival (described below), it was necessary to determine the length of time for all sporangia to germinate after transfer of mycelial plugs from parent cultures. *P. infestans* cultures of each genotype were grown on Sterol-Free Clarified Rye Agar (SFCRA) for 14 days in the dark at 18°C. Ten 5 mm-diameter cores were removed from the growing edge of each of 10 plates. The cores were placed into 10 ml distilled H_2O in test tubes and stirred on a magnetic stirrer for 1 hour to dislodge sporangia. The suspension was strained through four layers of cheesecloth and the concentration of sporangia was calculated using a hemacytometer. The number of non-germinated and germinated sporangia was counted at the time of transfer and again two days after incubation at 4°C. After two days only empty sporangia were observed in any of the genotypes tested and a pre-treatment of two days incubation at 4°C in the dark was imposed on all freshly transferred cores prior to exposure to temperature treatments.

4.2.4 Relation between digital image of samples and mycelial weight

Mycelium of *P. infestans* is white and the average reflectance intensity (ARI) of a digital image of a mature culture measures about 150 - 170 LIU. To determine the relation between ARI of cultures of *P. infestans* growing on SFCRA and fresh weight of the cultures, a further calibration was attempted. Sterile cellophane discs (40 mm diameter) were placed over SFCRA and 5 mm-diameter plugs of mycelium were transferred to the center of the plates (n = 12 per genotype tested). Sample plates were incubated in the

dark at 12°C and every two or three days after the transfer until 28 days after the initial transfer, two plates of each isolate were harvested. The ARI was determined for each plate. The weight of the culture was determined by weighing the total mycelium plus cellophane and initial transfer plug and subtracting the weight of the cellophane plus initial transfer plug. ARI was expressed as a function of the fresh weight of the culture for each genotype to determine the ARI at which no growth occurred. This threshold was used as an indication to determine qualitatively if cultures were alive. The relation between the ARI and weight of individual cultures was determined by linear regression (SigmaStat ver. 2.03, Jandel Scientific, San Rafael, CA).

4.3 Materials and methods

4.3.1 Isolate descriptions

Cultures of different genotypes of *P. infestans* were originally isolated from foliage of potato plants grown in north central/Great Lakes region of the US with symptoms of late blight and maintained in the Kirk collection at Michigan State University (MSU). The identity of the genotypes was determined by isozyme analysis (chapter 2), metalaxyl sensitivity and mating type (Chapter 3). The isolates were maintained on sterile rye seed stock cultures. For each experiment, isolates were re-propagated from the long-term storage stock culture. The different isolates tested for the temperature tolerance were shown in Table 4.1.

In order to increase diversity of the phenotypic characteristics of isolates of *P. infestans* some isolates collected from Northern Ireland, UK were used. These isolates were from the collection of G. Young and were maintained under USDA PPQ permit number P526-

061106-014 in the MSU collection in the Kirk laboratory. These isolates had been previously described and were A_1 mating type (Chapter 3).

4.3.2 Determination of thermal tolerance

An image analysis technique developed by Kirk (2003b) was used to determine the thermal tolerance of the isolates of *P. infestans*. Several procedures and stages were followed including substrate optimization; determining the thermal exposure treatments, and generation of the images; analyses of the images and quantification of the responses. Cultures were prepared using 10 ml SFCRA in each Petri dish (Appendix1) so that the distance between the scanner surface and media in the plate was the same throughout all experiments. This was important to ensure that the brightness of the images was consistent throughout the experiment since radial growth of the fungus in the images was to be assessed using image analysis and optimizing the image clarity during the image analysis process (see section below).

Temperature exposure experiments were done over a five-day period. All the plates were labeled with culture ID numbers and exposure times, bound together with Parafilm. For every isolate 15 cultures were prepared; 10-Petri dishes were inoculated with a 5 mm diameter plug of agar plus mycelium from a stock culture (described above). Plates were incubated at 4°C for two days in the refrigerator as an acclimatization period. Test isolates were moved to -5°C in the PTC-1 cabinets for five days. Five of these plates were incubated at 18°C in an incubator (Advanced Intellus Environmental Controller, Percival Scientific, Inc. Perry, IA 50220 USA) for 30 days as positive controls for statistical comparisons; five cultures (the test isolates) were placed together on a plastic loading

tray and then incubated in PTC-1 cabinets set at -5°C located either in a cold-room (set at 4°C) or in a growth chamber set at 4°C. Five negative control plates (non-inoculated) were prepared by adding a 5-mm diameter plug of SFCRA without any mycelium and incubated along with the test isolates. On the sixth day, culture-plates were removed from -5°C environment and moved to the 18°C incubator with the control cultures. All the cultures were incubated for a further 25 days.

The PELT-3 was set for the exposure temperature 2 h prior to the start of the experiment (Figure 4.1). The tray with the culture-plates was placed into the PTC-1 cabinet quickly to minimize temperature increase. Temperature equilibration was measured after the door of the chamber was opened, and at 0°C set temperature, temperature rose to 5°C after the door was opened but then dropped to -0.3°C in 1.5 hr. At -5°C set temperature, temperature rose to -1.5 and recovered in 1.3h. Exposure time was measured from when the set temperature was reached.

After 25 days the samples were scanned to determine amount of growth of mycelium. The experiment was repeated twice over the period from December 2005 to February 2006 for a total of 15 estimations of thermal survival for each test-isolate of *P. infestans*.

4.3.3 Image analysis technique

Images of the cultures were scanned and digitally analyzed to measure the Average Reflective Intensity (ARI) in light intensity units (LIU). The lids of the plates to be assessed (with and without growing cultures) were removed and placed face down on a flat bed scanner (Epson Perfection 4870 PHOTO; Epson Inc.) and the image was

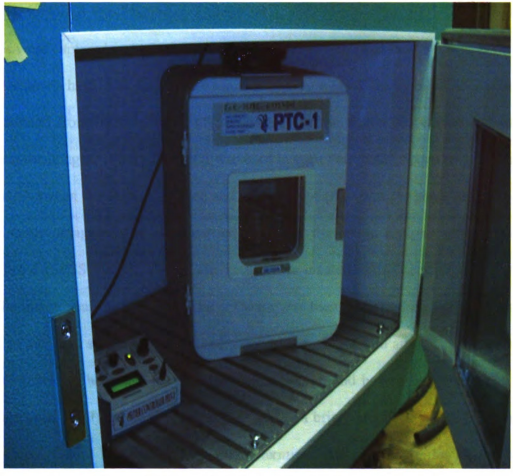


Figure 4.1 C-1 Peltier-effect temperature cabinet (PTC-1) and a PELT-3 Peltier-effect temperature controller in a controlled growth chamber used to maintain the temperature in the Peltier chamber at -5°C.

scanned, imported into Photoshop (Adobe Inc.), and saved in the JPEG file format. All images were captured at 11.8 pixels per mm, which gave a final image 2547 pixels wide by 3509 pixels high. The plates were scanned against a black background so that images were formed from light reflected off the surface of the media. To ensure consistency between the images, 10 ml of SCFRA was used in each Petri dish (Appendix1) so that the distance between the scanner surface and media in the plate was the same throughout all experiments. Image analysis was carried out using the image analysis software Sigma Scan Pro 5 (SPSS Inc. IL, USA). The image files created with Photoshop were loaded into the image analysis software. The software was then calibrated on the basis that the width of the glass screen of the flat bed scanner was 219.5 mm and this was equivalent to 2547 pixels. The image pixels in the black background have a brightness of 0 light intensity units (LIU), while pure white pixels have a brightness of 255 (LIU). Using the "eye dropper" tool in Photoshop it was determined that the brightness of the pixels making up the image of the PDA media were no brighter than 90 LIU, while the fungal hyphae had a brightness above 91 LIU. So the area selection cut-off threshold in the Sigma Scan Pro 5 software was set to 90 LIU. This effectively allowed the software to exclude all parts of the image darker than 80 LIU (e.g. the Rye media surrounding the hyphal growth). By selecting the "fill" tool and clicking on the fungal colonies within the image all the pixels with an LIU above 80 were selected. The scans were digitally analyzed to measure the ARI in LIU using Sigma Scan software and this value was then automatically added to a spreadsheet by the image analysis software

4.3.4 Production of progeny isolates of *Phytophthora infestans* from mating

4.3.4.1 Production of oospores

Oospores were produced from the crosses among cold tolerant (T), cold intermediate (I) and cold sensitive isolates (S). These isolates used to produce oospores were referred to as parental isolates and were transferred to Petri plates (100 X 15mm diameter) containing rye agar amended with 0.1g/ltr beta-sitosterol (Spectrum Chemical MFG. Corp., CA 90248). Mycelial plugs (3 mm diameter) of each of the two selected isolates in a cross were placed on opposite sides of 100 x 15mm Petri dishes containing 25 ml rye agar. Crosses were replicated five times for each cross. Three agar plugs of 2mm diameter were taken from a fast growing colony (parental isolate 1) and were placed at the edge of the agar on the Petri plate. Three mycelial plugs from the parental isolate 2 were placed about 3 cm apart at 90, 180 and 270° from parental isolate 1. Using three plugs of parental isolate 2 increased the potential for oospore production. Plates were sealed with parafilm and were incubated in dark for 1 to 2 months and were examined weekly for the presence of mature oospores in the zone of contact between the two colonies using an Olympus BX41 System microscope (Olympus America Inc., NY 11747-3157, USA).

4.3.4.2 Extraction of oospores

Plates with abundant oospores were prepared for extraction. The mating region was marked with a permanent marker on the bottom of the Petri dish and the mating region was excised using a scalpel. The agar pieces containing the mating region were transferred to sterile 50 ml Eppendorf centrifuge tubes, containing 9 ml sterile distilled water and then ground with a homogenizer (IKA-Ultra-Turrax, T 25 basic, with the

dispersing tool, S25N – 8G, IKA WORKS, INC. SE, Wilmington, NC 28405) for 2-min in the laminar flow cabinet, using standard sterile technique. Probes were sterilized for 30-s at 20,000 rpm by mixing in a beaker containing 96% ethanol. The probe was then rinsed twice with double distilled water. The agar pieces containing the mating region were blended for 60-s at 20,000 rpm, making sure that the mixture did not spill out. The oospores were purified by digesting mycelia with a lysing enzyme (L1412, Sigma; from *Trichoderma harzianum*). Lysing solution was prepared by mixing 0.1g of lysing enzyme (L1412 Sigma) in 10 ml double distilled water and 1 ml of the lysing solution was added to 9 ml of the oospore suspension. Lysing enzyme was used to digest sporangia and mycelial fragments. Tubes were incubated at 18°C for 40-h, in the dark. The solution of oospores was washed three times by adding 8 ml water each time, spinning down the oospores using a centrifuge (RC5C, DuPont, Sorvall instruments, Newton, CT, USA) for 5-min at 5,000 rpm. Supernatant was poured off carefully after each spin and finally, the pellet containing oospores was re-suspended with 10 ml double distilled water. Oospore concentration was quantified by counting oospores in three 50 µl aliquots using a hemacytometer. The concentration of the solution of oospores was adjusted to 1 x 10^3 spores/ml and used to inoculate water agar plates with 1 ml of the diluted suspension. Plates were incubated under continuous fluorescent light at 22°C for 5-d. Plates were examined for germinating oospores using the BX41 System microscope and were transferred on to a fresh rye agar plate. Single oospore cultures (50) were collected from each cross. Each isolate was numbered and the first number referred to the cross it came from e.g. cross 3 isolate 06 = 3_06, 5_11. These isolates were referred to as progeny isolates.

4.3.4.3 Oospore germination

A sample of 1 ml was drawn from the oospore suspension and spread on a 100 x 15mm Petri plate containing 15 ml water agar (2% agar, 5g/L) amended with 20mg/L Rifampicin*; 50mg/L polymixin B*; 50mg/L Vancomycin*; 100mg/L Ampicillin*; and 100mg/L Benomyl* (* = dissolved in 100% DMSO). Plates were bound with parafilm and incubated for 14-d at 20°C under cool blue fluorescent light. Oospore germination was assessed by inverting the plate and observing if germination had occurred using the Olympus BX41 System microscope at a magnification of 10X. Germinated and well-separated oospores were marked and transferred to fresh Rye B plates, and then pure cultures were obtained. Each germinating oospore (Fig. 4.2 and 4.3) was considered as a single progeny isolate. From each cross 50 oospores were isolated and labeled from 1 to 50, along with the cross number. All the progeny isolates from each cross were tested for mating type and temperature tolerance and the genotype was determined by isozyme analysis (Chapter 2). Additional tests such as metalaxyl sensitivity, virulence tests, and pathogenicity tests were also completed on parental and progeny isolates (Chapter 3 & 5).

4.3.5 Data analysis

To determine if mycelium of *P. infestans* isolates survived exposure to the thermal treatment, the ARI of treated mycelium was compared to the ARI of the non-inoculated control (negative control) and the inoculated control (positive control) grown at 18°C for comparable periods to determine the relative degree of survival. Combining these comparative outcomes resulted in a qualitative measure of survival; if the ARI of a sample was not significantly different at P = 0.01 (NSD) from the negative control the culture was considered dead and sensitive to exposure (thermally sensitive; S); if NSD

form the positive control the culture was considered unaffected by either temperature treatment or exposure period (thermally tolerant; T); if significantly different from both the negative and positive controls the culture was considered affected by the treatment but not killed (thermally intermediate response; I). The qualitative outcome of temperature treatments resulted in assignment of the parental isolate to a category for future crossings with other *P. infestans* isolates of opposite mating types. Comparisons of survival potential were made among isolates exposed to different temperatures for variable durations. An index value was assigned for each progeny isolate based on ARI of each isolate (as described above) in relation to the negative and positive control and was described by the following equation:

$$\%\text{RARI} = \left[1 - \frac{\left(\bar{x}_{pos} - x_i\right)}{\left(\bar{x}_{pos} - \bar{x}_{neg}\right)}\right] * 100$$

where the variation of each replicate's ARI value, x_i, with respect to the average positive control, $\bar{x}_{pos}$, is compared with the total ARI range between the positive and negative controls, $\bar{x}_{pos} - \bar{x}_{neg}$. When this ratio was equal to 1, the replicate ARI value is equal to the mean negative control value. By subtracting the ratio from 1 and multiplying the difference by 100, the percent relative ARI is calculated (%RARI). This percent relative ARI quantifies the percentage by which the total range in values between average positive and negative controls is greater than the variation difference between the replicate value and the average positive control. Index values of temperature tolerance for progeny isolates from each cross were analyzed by ANOVA to determine if the %RARI was significantly different from isolates previously characterized as thermally tolerant or intolerant as described for ARI above.

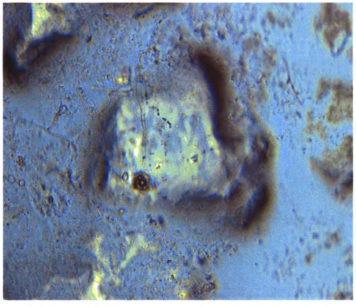


Figure 4.2 A germinating oospore of *Phytophthora infestans* forming mycelium under 10 X magnification. From Cross 3; ID 3_06.

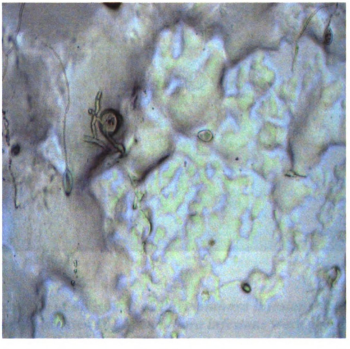


Figure 4.3 An oospore of *Phytophthora infestans* producing germ tube and sporangia under 10 X magnification

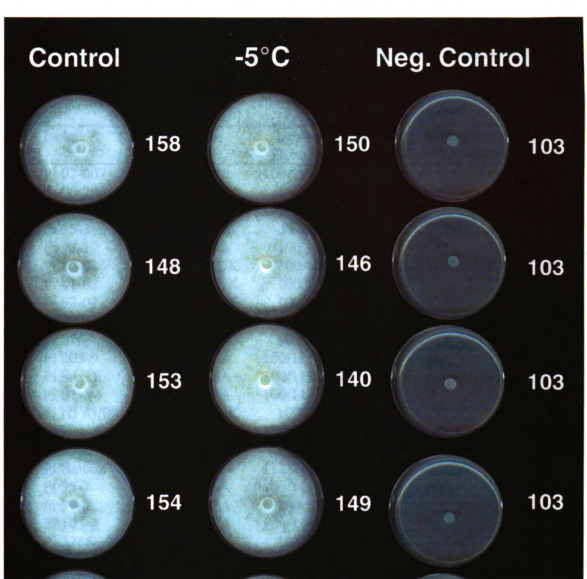


Figure 4.4 An example of an isolate of *Phytophthora infestans* showing tolerance to -5°C. JPEG images of isolates of *Phytophthora infestans* were produced in Photoshop on an Epson Perfection 4870 flatbed scanner. Images were digitally analyzed to measure the Average Reflective Intensity (ARI) in Light Intensity Units (LIU) using Sigma Scan software. Left Side: Positive controls, exposed to 18°C; Center: Test Isolates, exposed to -5°C for 5 days; Right side: Negative controls, non-inoculated Petri dish.

Table 4.1 Isolates of *Phytophthora infestans* tested for thermal tolerance. Isolates were exposed to -5°C for 5-days and regenerated for selection as potential parental isolates with different thermal tolerance phenotypes.

Isolate	Origin	Isozyme Genotype	Mating Type	Thermal Tolerance[a]
Pi 02-007	MI, USA	100/111/122	A2	T
Pi 96-2	MI, USA	100/100	A1	I
Pi 458	ND, USA	100/111/122	A2	T
Pi 95-3	MI, USA	96/100	A1	S
Pi 98-1	MI, USA	100/122	A2	I
Pi 30-01	Ireland, UK	100/100	A1	T
Pi 41-02	Ireland, UK	100/100	A1	T
Pi 62-02	Ireland, UK	100/100	A1	T
Pi 107-1	MI, USA	100/111/122	A2	T
Pi 3768	Mauritius	100/100	A1	T
Pi 203-9	MI, USA	100/111/122	A2	T
Pi S4-11	MI, USA	100/111/122	A2	T
Pi S1-3	MI, USA	100/111/122	A2	T
Pi NI 43/02	Ireland, UK	100/100	A1	T
Pi 284	ND, USA	100/100/111	A1	I
Pi 365	ND, USA	100/100	A1	I
Pi Banam	AK, USA	111/111/122	A2	T
Pi 95-7	MI, USA	100/111/122	A2	I
Pi 99-2	MI, USA	100/122	A2	I
Pi 103-4	MI, USA	100/111/122	A2	T
Pi 3765	Mauritius	100/100	A1	T
Pi 407-6	MI, USA	100/111/122	A2	T
Pi Atlantic 2N	MI, USA	100/111/122	A2	S
Pi 46-02	Ireland, UK	100/100	A1	S

[a] T= tolerant; I= intermediate; S= sensitive; Isolates are considered as Tolerant when the growth is not significantly different from the growth of the positive control cultures of the same isolate. Isolates are considered as Intermediate when the growth is significantly less than that of the growth of the positive control cultures of the same isolate but significantly greater than the negative control. Isolates are considered as sensitive when the growth is not significantly different from the negative control.

Table 4.2 Origin, mating type, thermal tolerance, and *GPI*-isozyme phenotypes of isolates of *Phytophthora infestans* selected for crossing studies. Progeny from these crosses were isolated to identify if thermal phenotype character was inherited.

Cross Number	Isolate[a]	Origin	Thermal Tolerance
1	Pi02-007	US	T
	Pi 41-02	NI	T
2	Pi 02-007	US	T
	Pi 41-02	NI	T
3	Pi S1-3	US	T
	Pi 41-02	NI	T
4	Pi S1-3	US	T
	Pi 41-02	NI	T
5	Pi 98-1	US	I
	Pi 41-02	NI	T
6	Pi 02-007	US	T
	Pi 62-02	NI	T
7	Pi02-007	US	T
	Pi 95-3	US	S
8	Pi Atlantic 2N	US	S
	Pi 41-02	NI	T
9	Pi Atlantic 2N	US	S
	Pi 4-19	US	S

[a] The first isolate in the cross is the A_2 isolate and the second is the A_1 isolate of *P. infestans*.

Table 4.3 Phenotypic and genotypic characteristic of isolates of *Phytophthora infestans* used in crosses

Isolate of *Phytophthora infestans*	Thermal Tolerance[a]	*Gpi* Isozyme Genotype[b]	Mating Type[c]	Metalaxyl Response[d]	R gene Phenotype[e]
Pi 41-02[f]	T	100/100	A_1	R	1,3,4,5,6,7,8, 9,10,11
Pi 02-007[g]	T	100/111/122	A_2	R	1,2,3,4,5,6,7 8,9,10,11
Pi S1-3[g]	T	100/111/122	A_2	I	1,2,3,4,5,6,7, 8,9,10,11
Pi 98-1[g]	I	100/122	A_2	R	1,4,5,9,10,11
Pi 62-02[f]	T	100/100	A_1	I	1,2,3,4,5,6,7, 8,9,10,11
Pi 95-3[g]	S	86/100	A_1	S	5
Pi 4-19[h]	S	100/100	A_1	I	1,3,4,5,6,7,8, 9,10,11

[a] Comparison of growth of isolates of cultures of *Phytophthora infestans* for 25 days at 18°C after exposure at -5°C for 5 days measured by an image analysis technique comparing the growth of exposed isolates to a positive control (exposed to 18°C for 5 days) or a negative non-inoculated control with no growth . T= Tolerant; growth not significantly different from the growth of the positive control cultures of the same isolate; I= Intermediate; growth significantly less than that of the growth of the positive control cultures of the same isolate but significantly greater than the negative control; S= Sensitive; growth not significantly different from the negative control.

[b] Glucose -6- phosphate isomerase allozyme genotype; 86/100 (US1), 100/100 (US-1.7), 100/111/122 (US-8), 100/122 (US-14); genotype as designated by Goodwin *et al.*, 1995.

[c] Mating type determined by production of oospores when grown with isolates of *P. infestans* of known mating type.

[d] Sensitivity to the fungicide metalaxyl, R = resistant, I= Intermediate, S = sensitive determined by comparing EC_{50} response of the isolates grown on media amended with different concentrations of metalaxyl with EC50 of known resistant and susceptible isolates.

[e] Virulence pathotype as defined by Muller and Black 1952.; [f] Isolates of *P.infestans* from Northern Ireland from collection of Dr. Louise Cooke, Plant Pathology, Queens University, Northern Ireland; [g] Michigan isolates of *P. infestans* from collection of Dr. W. Kirk, Plant Pathology, Michigan State University, East Lansing, MI, USA.

[h] A progeny isolate obtained from the 4th cross, used as a parent because of its sensitive phenotype.

4.4 Results

4.4.1 Testing of thermal tolerance for potential parental *P. infestans* isolates

There was variation in the tolerance of parental isolates to exposure to temperature for different durations at -5°C. Out of 24 parental isolates tested 15 were positive for tolerance to -5°C (Table 4.1). Six isolates were intermediately tolerant to -5°C and only three were sensitive, completely dead when exposed to -5°C for five days. The following crosses (Table 4.3) were selected and tested for the following phenotypes; 1) opposite mating type; 2) difference in thermal tolerance; 3) different genotype; 4) different tolerance to metalaxyl.

4.4.2 Thermal tolerance

Parental isolates were variable in thermal tolerance at -5°C (Table 4.1 and 4.2). The range in thermal tolerance measured as % RARI of the parental isolates was shown in Figure 2.5. Pi 02-007, Pi 41-02, Pi 62-02, and PiS1-3 were tolerant to -5°C and were used as thermally tolerant parents. Pi 98-1 was used as an intermediately tolerant isolate. Pi 95-3 and Pi Atlantic 2N were thermally sensitive isolates. Pi 02-007 and Pi 95-3 were used in all analyses as standards of thermal tolerant and sensitive isolates, respectively for comparisons of thermal responses. Pi 02-007 had %RARI value of 100.0 and Pi 95-3 had %RARI value of 1.35 (Fig. 4.5). The isolates used for the crosses were selected based on mating type and thermal tolerance. All isolates grew at expected rates and densities in isolation and formed sporangia (data not shown).

The crosses were selected using a selection of thermally tolerant (T) x T crosses; thermally sensitive (S) x T crosses; thermally intermediate (I) x T; S x T crosses; and S x S crosses (Table 4.2). Formation of oospores on Rye B agar occurred after 2 weeks of

incubation for most crosses. The number of oospores germinated *in vitro* varied greatly among the nine crosses tested. The highest number of oospores were produced in cross 2, followed by cross 3, 4, 5, 1, 6, 7, 8 and 9, respectively. Cross 9 (S X S) produced about 20 oospores but only two germinated.

Forty percent of the progeny isolates had intermediate temperature tolerance, but the other 60% were identical in response to one of the parents. The highest number of germinated oospores was from the T x T crosses (1 and 2). The number of oospores that germinated decreased as thermal tolerance decreased. Generally, the crosses that produced the greatest number of viable oospores were the most tolerant to -5°C. A total of 305 progeny isolates were produced from 9 crosses together. Crosses 1, 2, 3, 4, and 6 were the crosses among T x T parental isolates and produced 206 progeny isolates. Cross 5 was a cross between T and I and produced 33 progeny isolates. Crosses 7 and 8 between T and S parental isolates produced total of 24 isolates. Cross 9 between S x S parental isolates produced only 2 progeny isolates. Other crosses such as intermediate with intermediate, intermediate with sensitive were attempted, but did not produce any viable oospores.

In general, the effects of temperature exposure, isolates and their interactions on %RARI were significant at $P = 0.01$ in all the crosses. There was variation in the tolerance between progeny isolates from similar crosses of T x T isolates and had different ratios of tolerances, even though they were similar crosses. In almost all the crosses, more than 50% of the progeny retained thermal phenotypes identical to their parents. In cross 5, T x

I, the percentage of thermally sensitive progeny isolates was higher than that of thermally tolerant and intermediately tolerant isolates.

In cross 1 (T x T), there was no significant difference among isolates with %RARI values from 123.4 to 77.3, 75.9 to 60.7, 58.8 to 52.8 and 40.9 to 0 (Fig 4.6). The isolates that were defined as thermally tolerant had RARI values not significantly different (NSD) from Pi 02-007 (%RARI = 100). The isolates that were defined as thermally sensitive had RARI values NSD from Pi 95-3 (%RARI = 1.35); the same standards were maintained throughout the analyses. Out of a total of 29 isolates from cross 1, there were 23 x T isolates, 2 x I isolates and 4 x S isolates (Table 4.4). There was 21% variation in cross 1 and 79% of the progeny isolates were identical in temperature tolerance (Fig. 2.6). Cross 2 (T x T), there was no significant difference among isolates with %RARI values from 107.2 to 53.4, 50.3 to 45, 46.0 to 1.3 (Appendix 4). Out of total 50 isolates from cross 2, there were 40 x T isolates, 1 x I isolates and 9 x S isolates. There was 20% variation occurred in cross 2 and 80% of the isolates were inherited (Appendix 4). Cross 3 (T x T); there was no significant difference among isolates with %RARI values from 111.2 to 59.5, 49.5 to 44, 44.2 to 1.3 (Appendix 4). Out of total 52 isolates from cross 3, there were 37 x T, 1x I and 14 x S isolates. There was 29% variation occurred in cross 3 and 71% of the isolates were inherited (Appendix 4). Cross 4 (T x T), there was no significant difference among isolates with %RARI values from 105.3 to 47.5, and 47.5 to 1.3 (Appendix 4). Out of total 50 isolates from cross 4, there were 37 x T and 13 x S isolates. There was 1 intermediate isolate (4_49) produced which shared %RARI value with both tolerant and sensitive control isolates. There was 26% variation occurred in this cross and

74% of the isolates were inherited. In Cross 5 (T x I), there was no significant difference among isolates with %RARI values from 100 to 96.4, 45.1 to 36.1, and 24.6 to -1.5 (Fig 4.7). Out of total 29 isolates from cross 5, there was 1 x T, 2 x I and 26 x S isolates. There was 89% variation occurred in this cross and 11% of the isolates were inherited. Isolate 5_04 has %RARI value not significantly different from the control intermediate isolate (Pi 98-1) and sensitive isolates (Pi 95-3). Cross 6 (T x T), there was no significant difference among isolates with %RARI values from 104.1 to 58.9, 53.2 to 53.1, and 43.9 to 0.0 (Appendix 4). Out of a total of 19 isolates from cross 6, there were 5 x T, 2 x I and 12 x S isolates. There was 74% variation occurred in this cross and 26% of the isolates were inherited. Cross 7 (T x S), there was no significant difference among isolates with %RARI values from 115.2 to 82.8, and 14.4 to 0.0 (Fig 4.8). Out of 10 isolates from cross 7, there were 4 x T and 6 x S isolates. There were no intermediate progeny isolates. There was no variation occurred in this cross, they all were inherited from one parent or the other. Cross 8 (T x S), there was no significant difference among isolates with %RARI values from 100 to 50.6, and 42.9 to 1.3 (Appendix 4). Out of 10 isolates from cross 8, there were 7 x T and 3 x S progeny isolates. Once again there were no intermediate progeny isolates. There was no variation occurred in this cross, they all were inherited from one parent or the other (Appendix 4). Cross 9 (S x S), there was no significant difference among isolates with %RARI values from 100 to 87.91, 33.38 to 9.7 and 9.84 to 1.35 (Fig 4.9). Out the two progeny isolates in this cross, one was tolerant and the other was sensitive.

4.5 Discussion

Temperature is an important environmental factor that can affect both host and pathogen. The temperature trends in the Great Lakes region of the United States have displayed an increase in the annual mean temperature for the area (Baker *et al.*, 2002). Temperature is an underpinning factor in both host and pathogen development and host by pathogen interactions. In response to changes in temperature, microorganisms like fungi adjust the fatty acid composition of membrane phospholipids. As temperatures decrease, the fatty acid chains undergo a change in state from fluid to a more ordered crystalline array of fatty acid chains (Berry and Foegeding, 1997). Cold adapted fungi respond to freezing temperatures by incorporating fatty acids with lower melting points into their membranes, lower the temperature of lipid phase transition, and thus maintain membrane fluidity and cell function. Cold tolerant fungi generally have higher proportions of unsaturated fatty acids in their membrane phospholipids, and there is a correlation of minimum growth temperature and fatty acid composition (Berry and Foegeding, 1997). Plants that undergo cold acclimation experience changes in accumulation of solutes such as sugars, proline, betaines, and proteins called dehydrins. Solutes and dehydrins have been proposed to stabilize membranes by interaction with membrane surfaces or by interaction with surrounding water (Pearce, 2001).

Table 4.4 Number of progeny isolates in thermal tolerance phenotype categories resulting from oospores generated by crossing parental isolates of *Phytophthora infestans* with different thermal tolerance to -5°C.

Parents[a]	Thermal tolerance of Parent[b]	Number of progeny isolates in thermal tolerance categories[a]		
		Tolerant (T)[c]	Intermediate (I)[d]	Sensitive (S)[e]
Pi 41-02 X Pi 02-007	T X T	23	2	4
Pi 41-02 X Pi 02-007	T X T	40	1	9
Pi 41-02 X Pi S1-3	T X T	37	1	14
Pi 41-02 X Pi S1-3	T X T	37	1	13
Pi 41-02 X Pi 98-1	T X I	1	2	26
Pi 02-007 X Pi 62-02	T X T	5	2	12
Pi 95-3 X Pi 02-007	S X T	4	0	6
Pi 41-02 x Pi Atlantic2N	S X T	7	0	3
Pi Atlantic2n X Pi 4-19	S X S	1	0	1

[a] *P. infestans* isolates used as parents to produce single oospore progeny (A_1 x A_2)
[b] Thermal tolerance phenotype of the parents
[c] Number of isolates produced were tolerant to -5°C temperature
[d] Number of isolates produced were intermediately tolerant to -5°C temperature
[e] Number of isolates produced were sensitive to -5°C temperature

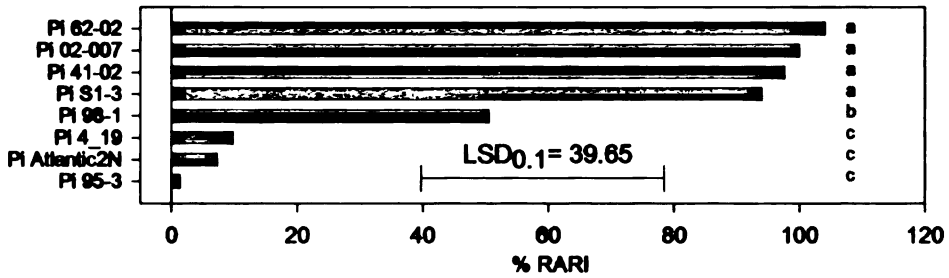


Figure 4.5 Differences in thermal tolerance of parental isolates used for mating to produce progeny isolates. *Phytophthora infestans* growth was measured as %RARI after exposure of isolates to -5°C for 5 days. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.

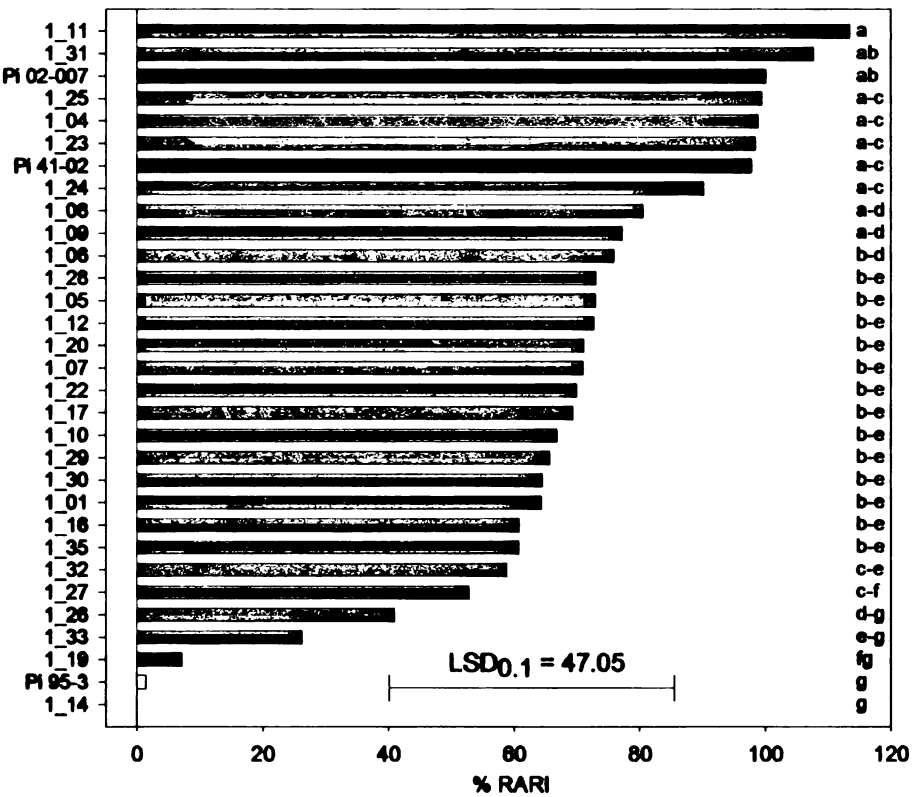


Figure 4.6 Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of *Phytophthora infestans* Pi 41-02 (A_1, thermally tolerant) x Pi 02-007 (A_2, thermally tolerant). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 1). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively; Pi 02-007 was a parent in this cross. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.

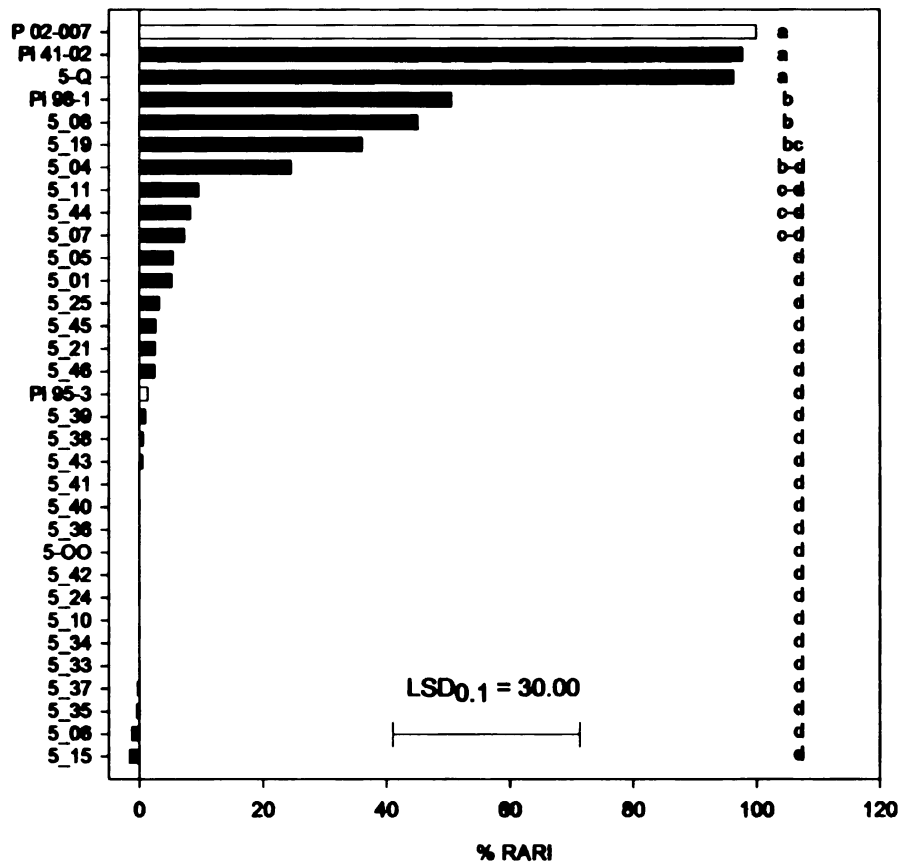


Figure 4.7 Differences in thermal tolerance of progeny isolates from mating of thermally tolerant with intermediately tolerant parental isolates of *Phytophthora infestans* Pi 41-02 (A_1, thermally tolerant) x Pi 98-1 (A_2, thermally intermediate). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 5). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.

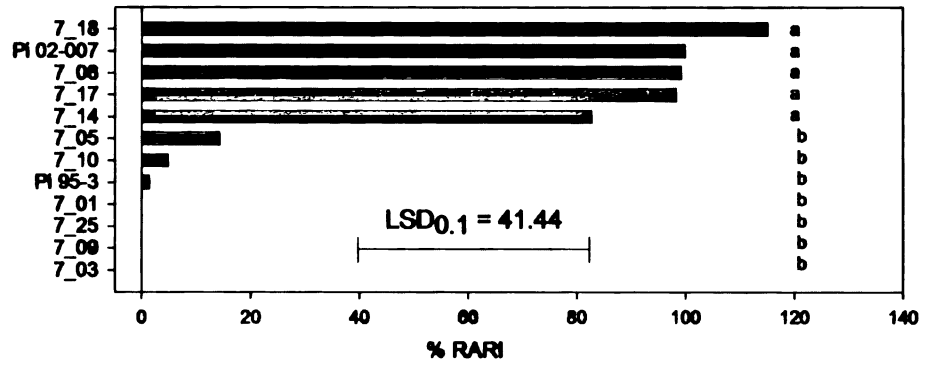


Figure 4.8 Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of *Phytophthora infestans* Pi 02-007 (A_2, thermally tolerant) x Pi 95-3 (A_1, thermally sensitive). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 7). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively; Pi 02-007 and Pi 95-3 are parents in this cross. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.

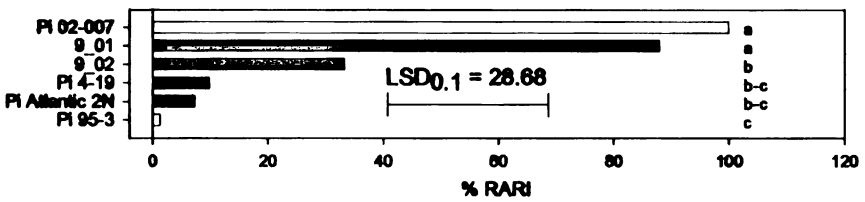


Figure 4.9 Differences in thermal tolerance of progeny isolates from mating of thermally sensitive parental isolates of *Phytophthora infestans* Pi 4-19 (A_1, thermally sensitive) x Pi Atlantic 2N (A_2, thermally sensitive). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 9). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.

Volunteer tubers are therefore protected from reduction in soil temperature below the freezing point of water (and tuber tissue). Temperatures at the center and base of cull piles found in Michigan range from 10°C to 0°C during the winter months (Kirk 2003a). In addition, insulation of the soil by early snow in northern potato producing states of the US may result in soil temperatures that only decrease to temperatures at which both tubers and mycelium of *P. infestans* can survive. The ability of *P. infestans* to survive cold temperatures is therefore important in understanding the possible sources of primary inoculum. In recent years in northern US potato producing areas the occurrence of soil temperatures below -5°C at 6 cm depth has been rare (Michigan Automated Weather Network (MAWN), www.agweather.geo.msu.edu/mawn/) and re-growth from volunteer tubers has been common throughout MI since 1997 (Kirk pers. comm.).

De Bruyn (1926) reported that mycelium can survive exposure to at least -25°C however this may be due to a physiological mechanism that is different from that which enables survival at temperatures closer to the freezing point of water and may be of little epidemiological significance either in terms of survival of mycelium between seasons or of long term ecological implications of the thermal tolerance of populations. *P. infestans* cultures survived -3°C for 5 days, and a few cultures have survived at -5°C after 1 day (Kirk, 2003b). There was not much variation noticed in the temperature tolerance when the isolates were tested at -3°C for five days and -5°C for one day. To observe more variation the temperature exposure used in this study to determine temperature tolerance was -5°C for 5 days. Thermal tolerance of isolates of *P. infestans* was defined in this study as the ability of mycelium to re-grow after exposure to -5°C for a duration of 5

days. These considerations and limitations to survival of substrate for the mycelium of *P. infestans* dictated the parameters of the current study. This definition may underestimate the ability of some of the isolates recovered to tolerate even lower temperatures or longer exposure to -5°C.

The isolates tested by Kirk (2003) had a narrow range of mating types and for the purposes of this study a wider range of thermal tolerance phenotypes were required that could also potentially mate, i.e. A_1 and A_2 mating types were required. In the current study a wider set of isolates originating from different locations in the US and internationally representing different genotypes and mating strains (n = 24) were selected and tested in replicated experiments at -5°C in order to identify isolates with tolerance to sub-zero temperatures. Both the Mauritius isolates Pi 3765 and Pi 3768 were tolerant to -5°C and 80% of isolates from Northern Ireland were temperature tolerant and only Pi 46-02 was sensitive to exposure to -5°C for five days. Two of the North Dakota isolates were intermediate and one was tolerant. Alaskan isolate, Pi Banam was tolerant. There were sensitive, intermediate and tolerant isolates in Michigan. There was no pattern between the habitat from where the isolates originated and thermal phenotype. There were cultures from cold and warm regions such as Mauritius that were tolerant to exposure to -5°C for five days. It can be speculated that thermal tolerance is a variable phenotype, and can occur in isolates from any location.

Differences in epidemiological factors such as latent period, growth rate and potential sporangial production among clonal lineages of *P. infestans* are likely to be greater than differences among isolates of the same lineage than within distinct lineages (Mizubuti *et*

al., 1998). In this study, all the potential parental *P. infestans* genotypes tested survived exposure to 0°C for any exposure period but survival was varied after exposure to -3°C and -5°C, up to 24h exposure. A more stringent exposure period was therefore selected and thermal tolerance was defined as the ability of an isolate to re-grow after exposure of 5 days to -5°C. This resulted in a diverse group from which to choose parents however the ability of these genotypes to mate was severely restricted and in many cases oospore production was not observed. For example, in order to find a thermally sensitive A_2 mating type one of the progeny isolated from cross 4 was selected (Pi 4_19) and used in cross 9; thermally sensitive x sensitive,

In this study, oospores were mostly formed in agar cultures after two weeks of incubation. Although the parents were of opposite mating type a few crosses did not produce oospores, and a few crosses produced few oospores. Non-viability in progeny may be caused by balanced lethal due to a deficiency in essential genes (Judelson *et al.*, 1995). Furthermore, germination of oospores was low or zero from some crosses. It could have depended on the combination of parental isolates and tolerance to freezing temperatures, as crosses with isolates sensitive to temperature produced less progeny isolates. Continuous variation in thermal tolerance was observed in most of the crosses however variation was also observed in crosses involving the same parents e.g. cross 1 and 2 although essential they produced similar propoetions of thermally tolerant, intermediate and sensitve progeny isolates. Physiological mechanisms conferring cold tolerance in fungi are complex; they include increases in intracellular trehalose (important storage compound in fungal vegetative cells and spores) and polyol

concentrations and unsaturated membrane lipids as well as secretion of antifreeze proteins and enzymes active at low temperatures (Robinson, 2001).

Age of culture, mycelial growth rate, origin of the culture, might also play an important role in the production and germination of oospores. Multiple studies have highlighted the importance of evaluation conditions, with inoculum concentration, temperature, age of plant and state of the pathogen all shown to influence results (Swiezynski *et al.*, 2000). It is not understood which factors control fertility in *P. infestans* isolates, but origin and fitness of parents are likely to play a major role. In general, sexual recombination in *P. infestans* may yield a high diversity in progeny isolates which could lead to either tolerance or sensitiveness to lower temperatures.

As the rate of growth of cultures originating from single spores can vary five replicates were used in every repetition of the experiment, and each of the experiments was repeated twice. In total, 252 progeny isolates were successfully revived from a total 300 isolates that were originally produced. Among all the crosses, there were a total of 155 thermally tolerant isolates; 9 intermediate in tolerance isolates and 88 sensitive isolates. The range in thermal tolerance in progeny isolates from many crosses was continuous indicating a wide range in thermal tolerance even though the definitions used in the study were statistically precise. Thermally tolerant isolates may be more likely to survive moderately cold winters and may initiate epidemics the following season. However, the epidemiological implications of thermal tolerance in the population of *P. infestans* are unclear as survival potential is closely linked with the thermal tolerance of the host tuber

which may vary within the same cultivar depending on the physiological age or physical condition e.g. size and shape of the tuber which may in turn vary with cultivar. Some cultivars that mature later or have different dry matter accumulation potential may have better survival than tubers of early maturing low dry matter tubers which are prone to rotting. In addition, parameters such as the timing of infection of the tuber in the soil and the number or position of infections relative to meristematic tissue may also be more important in determining the fate of the tuber or ultimately of an epidemic the following year. The observation that there is potential for variation in thermal tolerance within *P. infestans* suggests that there may be epidemiological consequences resulting from these phenotypes.

The causes of variation in the progeny isolates of *P. infestans* could have occurred because of mating, mutation, mitotic crossing over, chromosome number and ploidy condition. Variation of *P. infestans* from mating studies has been reported to vary in terms of fungicide sensitivity (Gisi and Cohen, 1996), and virulence phenotype (Abu el Samen *et al.*, 2003). In this study the same genetic causes may underpin the wide variation observed among progeny isolates in terms of temperature tolerance. Whether or not thermal tolerance poses an increased damage to agriculture is difficult to determine.

Temperature tolerance of pathogen has ecological significance, especially in agricultural regions where the climate is warming and becoming more conducive for late blight (Baker *et al.*, 2002). Since 1950, soils have become more likely to freeze in the Great Lakes region due to climate change, but only to a depth of 5 cm (Isard *et al.*, 1998),

which is above the depth that tubers may be buried (Kirk, 2003). Oospores produced from US strains of *P. infestans* will apparently survive temperatures encountered in moist soils in the United States (Fry *et al.*, 1997). Sexual reproduction results in the formation of oospores which serves as a source of initial inoculum and may with an additional genetic variation, rendering oospore-infested soil dangerous to potato crops. The effects of varied temperature on mycelial survival and sporangia germination could have important implications for epidemic development and disease management. Reports of increased annual mean daily temperature in the Great Lakes region of the United States increases the potential for survival of volunteer potatoes in field and cull piles (Baker *et al.*, 2002; Kirk, 2003). The interaction between climate change, soil temperature during winter months, thermal tolerance of mycelium of *P. infestans* and physiological parameters of tubers of the potato may require more intensive evaluations to determine the future of potato production in the Great Lakes region and other northern regions of North America in relation to epidemic potential of potato late blight.

5 Pathogenicity of progeny isolates from crosses of thermally variable *Phytophthora infestans* on potato tubers

5.1 Introduction

The final determinant of the significance of thermal tolerance was to determine a profile of aggressiveness of progeny and parental isolates of *Phytophthora infestans*, used and generated in this project. Infection of tubers is similar to that of foliage with germ-tubes penetrating at lenticels, eyes, stolons, buds, wounds or any site incompletely suberized (Bain and Möller, 1999). To understand if the progeny isolated from several temperature phenotype crosses are pathogenic, a pathogenicity test was done. Potato tuber tissue infected with late blight (*Phytophthora infestans*) was quantified using a digital scanner and image analysis software. The average reflective intensity of light reflected from the cut surface of sample tubers measures the darkened, diseased potato tuber tissue in conjunction with lighter, late blight-free tissue. Qualitative disease rating systems often assess the disease level on the surface of whole tubers or tuber slices (Douches *et al.*, 1997, Kirk *et al.*, 1985). The change in the reflective intensity of exposed tuber surfaces can be determined by digital scanning. This method allows comparing pathogenicity along with virulence.

Infection of a potato is extremely variable and it depends on the cultivar and the isolate of *P. infestans* involved. The pathogen can undergo various changes (recombination), but the final goal is to identify if the progeny both inherited and recombinants were pathogenic. In Chapter 2, we found that the phenotype, thermal tolerance inherited a wide range of variability but we don't know if this variability was related to the ability to cause disease. Pathogenicity testing can validate if variation has caused the progeny isolates to

be more aggressive. Biotypes of *P. infestans* that have become dominant since 1992 can infect tubers more effectively than biotypes of the fungus present in North America prior to 1992 (Fry and Goodwin, 1997; Lambert and Currier, 1997). Even though we did not notice any recombination in the genotypic character, isozyme analysis (Chapter 4); it would be interesting to find out if the progeny isolates are pathogenic. This could be a result of recombination occurring in nature, which shows that it is important to study recombination and it's effects on pathogenicity, to prepare us with better management strategies. We noticed that the isolates from thermal tolerance crosses are also variable in terms of mating type, metalaxyl sensitivity and virulence phenotype (Chapter 3), but we don't know if there is variability in terms of pathogenicity.

The objective of this study was to evaluate if the progeny isolates from the different thermal phenotype crosses are pathogenic, and to estimate if the progeny isolates have inherited the parental characteristics or if variation has occurred in this phenotype.

5.2 Materials and methods

5.2.1 Pathogenicity test on tubers

Five isolates were selected at random from each cross along with the parents for pathogenicity and aggressiveness testing. Methods developed by Niemira *et al.*, (1999) and Kirk *et al.*, (2003 b) using whole tubers were adapted for this study. Tubers of cv. FL1879 were obtained from Walthers and Sons Potato Farms, Newberry, Michigan. Potato tubers were harvested during the previous growing seasons and were stored at 3°C in the dark at 90% relative humidity until used. Tubers for the experiments were within the size grade range 50-150 mm diameter (any plane). Visual examination of a random

sample of tubers (n = 20) for disease symptoms indicated that tubers were free from late blight and other diseases such as Fusarium dry rot. The sample was further tested with the ELISA immuno-diagnostic Alert Multi-well kit (Alert Multiwell Kit - *Phytophthora sp.* Neogen Corporation, Lansing, MI, USA); *P. infestans* was not detected in any of the tubers. Prior to inoculation, all tubers were washed with water to remove soil. The tubers were then surface sterilized by soaking in 2% sodium hypochlorite (Clorox) solution for 30 min and then rinsed with water. Tubers were dried in a controlled environment with continuous airflow (5950 l min^{-1}) at 15°C in dry air (30% relative humidity) for four hours prior to inoculation.

The sample isolates of *P. infestans* (parents and progeny) selected for testing were grown on Rye agar plates (Appendix 1) for 14 to 20 days. A sample from each of the cultures was measured to determine sporangial production using a hemacytometer. Each culture produced about 2 x 10^{-5} sporangia per ml of solution indicating that the cultures were viable and of similar maturity. Non-growing cultures were discarded and were not used for the evaluation. The washed, surface-sterilized tubers were inoculated by removing a tuber plug of 5mm diameter x depth from the apical end of the tuber about 0.5 cm from the dominant apical sprout at the bud end of the tuber to a maximum depth of 5 mm using a sterile cork borer. A plug (4 mm diameter) was removed from the colony edge of the sample culture and placed in the plug-hole of the tuber and the tuber plug was re-placed over the inoculum. The non-inoculated control tubers were inoculated with a sterile plug of rye agar. Vaseline was placed over the wound to prevent the plug from falling out and desiccating the inoculum. Aerated plastic boxes (46 x 28 cm Akro-N.S.T boxes, Akro-Mils, Akron, Ohio 44309, USA) were prepared by placing a wet paper towel in the base

of the boxes followed by placing a plastic mesh to separate sample tubers from the wet towel to avoid direct contact between the wet towel and the tubers and minimize the contamination or rotting of the tubers. Five tubers were inoculated for each isolate and the whole experimented was repeated twice to give a total of 15 tubers for the estimation of aggressiveness. The tubers were stored in a temperature-controlled environment chamber, 1.8 m^3 volumes (Environmental Growth Chambers, Chagrin Falls Ohio, USA) at 10°C for 30 days. Relative humidity was maintained at 90% within the chamber. Disease development rates within tubers in relation to storage temperature were known from previous experiments (Kirk *et al.*, 2001) and a single sampling date was selected about 30 days after inoculation (DAI).

A digital image analysis technique was used to assess tuber tissue infection similar to the method described for estimation of growth of cultures of *P. infestans* on Petri dishes (Chapter 4). The scanned surface was the cut face of a sample tuber. A sharp knife was used to ensure a smooth cut face. Fresh-cut tuber sections were placed cut surface down on a glass plate, 40 x 30 cm and 2 mm thick. The glass plate was used to prevent surface contamination of the scanner glass and permitted multiple samples to be prepared and moved to the scanner for image production. The plate was transferred to a flatbed scanner (HP ScanJet 4c, Hewlett-Packard Co., Houston, TX) controlled by an IBM-compatible PC. Scanner control software (Desk Scan II ver. 2.4, Hewlett-Packard, Co., Houston, TX), generated an image of the cut tuber surfaces against a black background. The image was formed from light reflected from the cut tuber surfaces.

The brightness value of the image controlled the light intensity of every pixel in the image. The contrast value controlled the differences between light and dark regions of the image. While the scanner control software was able to automatically adjust the brightness and contrast of the image by comparing the relative size of the pale tuber surfaces against the black background, the settings were manually set to 180 units (brightness) and 200 units (contrast) to ensure consistent readings. A photograph-quality image was taken and stored for analysis (e.g. Fig. 5.1). A typical image in Tagged Image Format (*.tif) occupies 1 megabyte. Typical ARI values for a range of infected and uninfected cut tuber surfaces were shown on Table 5.2. The image files created with the scanner software were loaded into the image analysis software (SigmaScan ver. 3.0, Jandel Scientific, San Rafael, CA). The black background has 0 light intensity units (LIU), while pure white has 255 LIU. Disease-free and blemish-free tuber tissue is pale. Diseased or blemished tuber tissue is darkened. The image of the cut tuber surface was selected for analysis, and isolated from the adjacent regions of the image. The image was carefully cropped for irregularly shaped tubers to remove the image of the adjacent tuber skin, and the image of the cut surface was unedited. The area was selected with the fill tool, which encompassed all pixels within a given area brighter than the cut-off threshold. The area selection cut-off threshold was set to 10 LIU, effectively allowing the software to exclude all parts of the image darker than 10 LIU, e.g. the black background. The average reflective intensity (ARI) of all the pixels within the image gave a measurement of disease severity of the tuber tissue of each sample. The ARI was measured in sections from the apical, middle and basal regions of the tuber, approximately 25% (apical), 50%

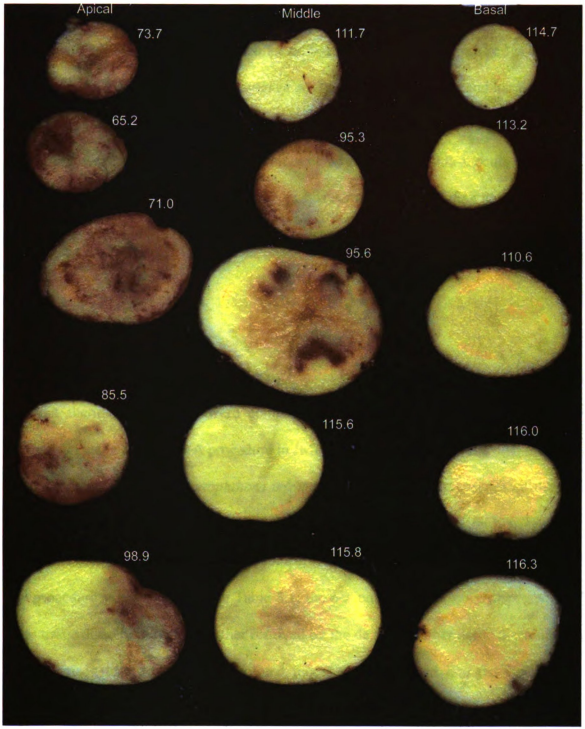


Figure 5.1 Late Blight Development in FL1879 tuber tissue caused by *Phytophthora infestans* (isolate Pi 5-16), incubated at at 10°C for 30 days.

(middle) and 75% (basal) of the length of the tuber (respectively) as measured from the apical end. The amount of late blight infected tissue per tuber was expressed as a single value (Mean ARI) calculated as the average ARI of the apical, middle and basal sections (total images, n = 45 per Mean ARI). The lower Mean ARI values indicate relatively greater disease severity than those with values close to that of the non-inoculated control. The presence of *P. infestans* in sample tubers was confirmed by isolating pure cultures of *P. infestans* from the infected tuber tissue and successful re-inoculation of potato tubers and leaves.

5.2.2 Data analysis

Pathogenicity and virulence of the progeny isolates of *P. infestans* produced from mating different thermal phenotypes were compared using a technique based on statistical comparison using the ANOVA procedure in JMP (version 5.01; SAS). The quantification of virulence of isolates of *Phytophthora infestans* was based on the presence or absence of significant differences measured at P = 0.05 in tuber tissue darkening between non-inoculated control tubers and positive control tubers inoculated with a virulent isolate. The procedure was summarized in Table 5.1; the outcomes were absence or presence of tuber tissue discoloration, and further resolved by the degree of tuber tissue discoloration in comparison to the controls. Discolored tubers were classified into three categories dependent on the comparison of the Mean ARI values to those of the negative and positive controls, non-inoculated and inoculated with Pi 02-007, respectively. The negative and positive controls had discrete values for comparisons at the apical, middle and basal regions of the test tubers. Numerical values 0, 1, 2 or 3 (severity scores) were assigned to the isolates dependent on classification using the criteria described in Table

5.1. The total severity value (TSV) for each isolate was attained by adding the severity score calculated from each of the three regions on the test tuber.

5.2.3 Compiling data

Data on temperature tolerance from Chapter 2 (temperature tolerant (A), intermediate (B) or sensitive (C) to -5C); Mating type (A1, A2, or self fertile); metalaxyl sensitivity (Resistant (R), intermediate (I), or sensitive (S) to fungicide metalaxyl), and virulence assessment on foliage (LBR 1 to 11 genes) from Chapter 3; isozyme analysis (*GPI*) from Chapter 4; and pathogenicity test (TSV = 0 – 4) from Chapter 5 were all put together in Appendix 4. Finally, using the parental and progeny isolates of *P. infestans* selected for the pathogenicity tests comparisons of their other phenotypic and genotypic characteristics were compiled in a single table (Table 5.4). If the progeny has the phenotype or genotype character from either of the parent, it was given a value = 0, meaning the character was inherited, and if it had the character different from the parents, it was given a value = 1, meaning that it was a recombinant and isolates with value = -1 meant that isolates were less virulent than the parents. (Table 5.4).

5.3 Results

As an example, tubers of the FL1879 inoculated with *Phytophthora infestans* isolate Pi 5-16, (US8 genotype) incubated at 10°C for 30 days resulted in significant tuber infection and range of ARI values (Figure 5.1). The mean ARI values of the negative control (non-inoculated) and the positive control (Pi 02-007) were 112.2, 114.8 and 117.3 (TSV = 0) and 90.2, 107.9 and 115.7 (TSV = 2) at the apical, middle and basal sections of the

sample tubers 30 days after inoculation, respectively (Table 5.3). The range in susceptibility of the tubers to late blight was heterogenous (Table 5.3) over the two executions of the experiment and the data were therefore combined by taking an average. The most aggressive isolates, 2-20 had apical, middle and basal ARI values of 69.3, 79.2 and 100.5 and isolate 3-14 had apical, middle and basal ARI values of 80.7, 92.7, 97.9. The parental isolates Pi 62-02, Pi 95-3 and Pi Atlantic 2N were pathogenic (showing some darkening on some tubers) but were avirulent using the criteria of the pathogenicity and virulence test. Pi 02-007, Pi 41-02 and Pi S1-3 were moderately virulent (TSV = 2). Out of total 49 isolates tested, 11 were pathogenic but were avirulent, showing little tuber tissue darkening around the plug core, and had TSV = 0. The other 38 isolates had varying degrees of virulence, with TSV = 1-4. Two isolates (2-20 and 3-13) were the most virulent isolates, with TSV = 4, more than the positive control (TSV=2).

We observed that, out of 21 isolates tested for mating type, thermal tolerance phenotype, metalaxyl sensitivity, virulence phenotype and *Gpi* isozyme genotype, 2 (9.5%), 11 (52.4%), 9 (42.9%), 16 (76.2%), 12 (57.1%) isolates showed recombination respectively (Table 5.4). The isolates inherited discrete characteristics from the parent for example an isolate may have a different thermal tolerance phenotype but not differ in mating type or metalaxyl sensitivity. Therefore there is continuous heterozygosity which means a very diverse population. So, if clonality exists for one phenotype or genotype it is unlikely to be clonal with respect to the other phenotypes. In total, 139 isolates have attained phenotypes that are not inherited from either parent. 49% progeny were recombinants (139 / 284 isolates (Appendix 4). All the parents in the 9 crosses were heterothallic

isolates which produced out of a total of 285 progeny isolates 13 self-fertile or homothallic isolates (oospores were produced in both testers). Recombination occurred in 4.5% of the isolates (13 / 284 isolates, Appendix 4). After one month, all the pure cultures (parental and progeny) were observed under microscope, and about 50% of the isolates produced self-fertile oospores (Table 3.3, Chapter 3). In the metalaxyl sensitivity test, out of 284 isolates, 117 (41%) had a metalaxyl sensitivity phenotype different from the parents indicating that many of the progeny were recombinants (Appendix 4). In the virulence phenotype assessment, out of 37 isolates tested, 22 (59.4%) of them had different virulence from parents and that progeny may have been recombinants (Appendix 4). In the isozyme analysis, all the progeny inherited the genotype from the parents and from this trait recombination did not occur. Out of 54 progeny isolates tested for pathogenicity, 31 (57%) were more or less aggressive than the parents, suggesting that 43% of the progeny were recombinants.

5.4 Discussion

The variability in pathogenicity and virulence of both parental and progeny isolates of *Phytophthora infestans* with different thermal tolerance phenotypes was clear from this study. The observation that three of the parental isolates and nine of the progeny isolates were non-pathogenic on tubers of FL1879 suggests a strong varietal component and a potential confounding factor of this technique. Susceptibility of foliage and tubers to the same genotype of *P. infestans* can vary within the same cultivar (Kirk *et al.*, 2003). Therefore when conducting this type of analysis it would be prudent to use tubers of at least three cultivars known to be susceptible to *P. infestans*. The pathogenicity and virulence technique utilized the response of tuber tissue darkening during development of

tuber blight. By introducing the inoculum to only the bud or apical end of the tuber it was possible to measure the spread of the disease through the sample tubers by sampling at three points through the length of the tuber from the apical to the basal end with ARI generally increasing with distance from the inoculation site. Niemira *et al.,* (1999) optimized this technique and reported that using three surfaces cut across the longitudinal plane of the tuber about 25, 50 and 75% of the distance from the apical to the basal end gave equivalent results to taking as many as 10 slices (Kirk pers. comm.). Differences in section thicknesses and shapes resulted in significant, though small, differences. Thinner tuber sections may allow greater transmission of light through the section, thereby reducing the light reflected from the section surface. This would return a lower (darker) ARI rating than sections which reflect light more effectively. The optimal thickness for slices was reported to be >5 mm (Niemira *et al.*, 1999). Darker infected tissue results in lowered average reflective intensity because of the reduced light reflectance. In this study, the use of replication and repetition resulted in 15 estimations of tuber disease severity for each isolate tested. Pathogenicty of the test isolates was confirmed by the presence of at least a single cut surface showing darkening typical of successful infection. The strict statistical conditions imposed to measure virulence indicated that many of these isolates although pathogenic were nearly avirulent. To improve this technique, an increased range of potato cultivars should be used and also a greater sample size for the estimations. However, to put the technique into perspective the number of tubers used for a comparison of 50 isolates was 750 and 2250 cut surfaces were evaluated. Some of the non-pathogenic progeny even from crosses between two pathogenic parental isolates could be the result of significant chromosomal heterogeneity or many recessive

Table 5.1 Differences between the two executions of pathogenicty and virulence of parental and progeny isolates of *Phytophthora infestans* with different thermal tolerance phenotypes based on the Average Reflective Intensity (ARI) of inoculated potato tubers cv. FL1879.

Isolate	Replicates	Isolate	Replicates
1_06	A	5_43	B
1_07	A	5_46	B
1_13	A	6_06	B
1_20	A	6_11	B
1_36	A	6_14	A
2_03	A	6_19	B
2_09	B	6_20	A
2_20	A	7_01	A
2_33	A	7_18	B
2_34	A	7_03	A
3_01	A	7_08	B
3_05	B	7_09	A
3_13	A	8_02	A
3_41	B	8_05	B
3_41	A	8_08	A
4_01	B	8_09	A
4_05	B	9_01	B
4_09	A	9_02	A
4_12	A	Pi Atlantic 2n	B
4_19	B	-ve Control	A
4_21	A	Pi 02-007	A
4_29	A	Pi 95-3	B
5_16	A	Pi S1-3	A
5_19	A	Pi 41-02	A
5_36	A	Pi 62-02	B

The first digit of the isolate name is the cross number and the 2-digits following the hyphen are the numbers of the single oospore-generated progeny isolate.
Isolate names starting with Pi are parents.
A= No significant difference in disease severity in between the two replicates at p=0.05;
B= significantly different in between the two repetitions at p=0.05.
-ve control = Non-inoculated tubers.

Table 5.2 Quantification of virulence of isolates of *Phytophthora infestans* using a tuber inoculation technique based on differences in tuber tissue darkening between non-inoculated control tubers and positive control tubers inoculated with a virulent isolate.

Condition	Comparison criteria based on ARI[a] values of tuber tissue from apical, middle and basal tuber sections		Severity Value[d]
	Non-inoculated (-ve control)[b]	Inoculated (+ve control)[c]	
No tissue discoloration	NSD[e] (ARI = -ve control)		0
Tissue discolored	SD[f] (ARI < -ve control)	SD (ARI > +ve control)	1
Tissue discolored		NSD (ARI = +ve control)	2
Tissue discolored		SD (ARI < +ve control)	3

[a] ARI = Average Reflectance Intensity [measured as Light Intensity Units (LIU)] of all the pixels within the scanned cut tuber surface image gave a measurement of severity of tuber tissue darkening due to *P. infestans* from each sample.

[b] The non-inoculated control had a specific ARI value to which all ARI values of inoculated tuber slices could be compared (a unique value was measured for apical, middle and basal sections).

[c] The positive control was inoculated with a known aggressive isolate of *P. infestans* (Pi 02-007) which produced a specific ARI value to which all ARI values of inoculated tuber slices could be compared (a unique value was measured for apical, middle and basal sections).

[d] The numerical value was assigned dependent on the outcome of the statistical comparisons and each value generated from the outcome criterion form apical, middle and basal evaluations was summed and used to produce an overall severity score with maximum value of 9.

[e] NSD = not significantly different from the critical ARI value measured at $p = 0.05$.

[f] SD = significantly different from the critical ARI value measured at $p = 0.05$.

Table 5.3 Pathogenicty and virulence of parental and progeny isolates of *Phytophthora infestans* with different thermal tolerance phenotypes measured using a severity scale based on the Average Reflective Intensity (ARI) of inoculated potato tubers cv. FL1879 based on comparison to negative and positive controls.

Isolate	Apical ARI	Severity	Middle ARI	severity	Basal ARI	Severity	Total severity
1_01	71.7	2	91.0	1	104.5	0	3
1_05	74.0	2	92.4	1	104.2	0	3
1_10	87.0	2	102.4	0	106.9	0	2
1_06	83.3	2	99.8	0	100.6	0	2
1_07	97.0	0	106.2	0	94.9	0	0
1_13	82.2	2	104.2	0	105.9	0	2
1_18	75.9	2	91.2	1	107.7	0	3
1_20	71.7	2	91.0	1	104.5	0	3
1_29	93.7	1	103.6	0	108.1	0	1
1_36	78.4	2	91.9	1	107.7	0	3
2_03	76.4	2	105.5	0	110.8	0	2
2_09	75.7	2	90.2	1	108.7	0	3
2_13	76.8	2	94.5	1	110.6	0	3
2_20	69.3	2	79.2	2	100.5	0	4
2_33	79.2	2	89.9	1	103.6	0	3
2_34	79.9	2	99.9	0	107.4	0	2
3_01	83.6	2	95.8	1	106.1	0	3
3_06	88.0	2	102.7	0	107.9	0	2
3_14	80.7	2	92.7	1	97.9	1	4
3_37	82.9	2	92.9	1	105.9	0	3
3_41	82.6	2	92.4	1	100.6	0	3
3_42	84.9	2	93.3	1	104.2	0	3
3_51	81.5	2	93.5	1	104.9	0	3
4_01	79.4	2	99.1	0	110.5	0	2
4_05	89.6	2	100.5	0	111.4	0	2
4_09	87.0	2	97.9	0	110.1	0	2
4_12	90.4	2	103.3	0	106.6	0	2
4_19	88.1	2	106.0	0	103.1	0	2
4_21	91.3	1	99.0	0	111.6	0	1
4_29	82.3	2	98.3	0	101.1	0	2
5_12	82.9	2	91.4	1	99.6	0	3
5_16	89.8	2	110.7	0	117.9	0	2
5_19	100.8	0	116.0	0	111.5	0	0
5_36	87.4	2	101.0	0	111.3	0	2
5_37	79.8	2	93.8	1	106.8	0	3
5_41	91.7	1	103.0	0	109.1	0	1
5_45	104.2	0	116.9	0	112.9	0	0
6_06	84.1	2	104.8	0	107.4	0	2
6_11	92.1	0	107.1	0	108.8	0	0
6_14	77.8	2	92.5	1	111.6	0	3
6_19	82.5	2	93.9	1	106.7	0	3
6_20	88.9	2	107.0	0	110.3	0	2
7_01	92.6	0	105.9	0	115.3	0	0
7_02	92.4	0	105.4	0	113.8	0	0
7_03	82.7	2	95.1	1	106.2	0	3
7_08	88.8	2	106.4	0	108.7	0	2

Continued

Table 5.3 Continued

Isolate	Apical ARI	Severity	Middle ARI	severity	Basal ARI	Severity	Total severity
7_09	98.3	0	105.6	0	109.3	0	0
8_02	80.8	2	94.9	1	107.8	0	3
8_05	86.8	2	104.1	0	108.9	0	2
8_07	86.4	2	104.0	0	110.3	0	2
8_08	86.8	2	104.6	0	110.1	0	2
9_01	80.5	2	90.2	1	107.4	0	3
9_02	103.7	0	111.2	0	101.2	0	0
Parental and reference isolates							
PiAtlantic2N	108.2	0	118.3	0	113.5	0	0
Pi 41-02	84.1	2	104.8	0	106.1	0	2
Pi 62-02	104.5	0	103.5	0	109.8	0	0
Pi 95-3	107.4	0	115.1	0	108.0	0	0
Pi S1-3	91.0	2	107.5	0	107.5	0	2
Pi 02-007 (+ve control)	90.2	2	107.9	0	115.7	0	2
-ve control	112.2	0	114.8	0	117.3	0	0

Total severity = Apical severity + Middle severity + Basal severity.

Table 5.4 Progeny isolates of *Phytophthora infestans* selected from some crosses showing inheritance and recombination for phenotypic and genotypic characteristics.

Isolate[a]	Mating type[b]	Temperature Tolerance[c]	Metalaxyl Sensitivity[d]	Virulence Assessment[e]	Pathogenicity[f]	Genotype[g]
1_01	0[i]	0	0	1	1	0
1_05	0	0	1	1	1	0
1_06	1	0	0	1	0	0
1_10	0	1	1	0	0	0
1_13	1	1	0	1	1	0
1_18	0	1	0	1	1	0
1_29	0	0	1	1	-1[h]	0
2_13	0	1	1	1	1	0
3_01	0	0	0	0	1	0
3_37	0	1	1	1	1	0
3_51	0	0	0	1	1	0
4_01	0	1	1	1	0	0
4_12	0	1	1	0	0	0
4_29	0	1	1	0	0	0
5_12	0	0	0	1	1	0
5_16	0	0	0	0	0	0
5_41	0	0	0	1	-1[h]	0
5_45	0	1	0	1	0	0
6_11	0	1	1	1	0	0
6_19	0	1	0	1	1	0
6_20	0	0	0	1	0	0
Total	2	11	9	16	12	0
%	9.5	52.4	42.9	76.2	57.1	0

[a] The first digit of the isolate name is the cross number and the 2-digits following the hyphen are the numbers of the single oospore-generated progeny isolate.

[b] Mating type, determined by production of oospores when grown with isolates of *P. infestans* of known mating type (testers).

[c] Temperature tolerance, Comparison of growth of isolates of cultures of *P. infestans* for 25 days at 18°C after exposure at -5°C for 5 days.

[d] Metalaxyl sensitivity determined by comparing EC_{50} response of the isolates grown on media amended with different concentrations of metalaxyl with EC_{50} of known resistant and susceptible isolates.

[e] Virulence assessment, Virulence pathotype as defined by Muller and Black, 1952.

[f] Pathogenicity where 1 indicated more virulent than parental isolates..

[h] Isolates were less virulent than the parents.

[g] Genotype is the genotypic characteristic Glucose -6- phosphate isomerase allozyme genotype; designated by Goodwin *et al.*, 1995.

[i] For all assessments "0" = the respective character was inherited; and "1" = the respective character was not inherited (recombinant) from either of the parental isolates.

defective alleles. Significant chromosomal heterogeneity might also exist within the species. This would also explain why the frequencies of oospore germination in different crosses ranged from nearly 0 to 100% and why mating between pathogenic parents may yield significant numbers of nonpathogenic progeny or progeny with decreased virulence (Al-Kherb *et al.*, 1995).

6 Executive Summary

The digital method of assessment of survival of mycelium of *P. infestans* in vitro is based on light reflectance from developing mycelium (Kirk, 2003). Image analysis is quantitative and objective and scanned images can be stored for future references (Kirk, 2001). The pathogenicity and virulence testing were vital to check if progeny isolates differed from the parental isolates in their ability to cause disease and if they were more or less aggressive than parental isolates. *P.infestans* has been shown to have several variable characteristics such as those tested in this study and such variations may have important survival and disease management implications. In this study, the main goal was to evaluate variation in several phenotypic and genotypic characteristics after mating between parental isolates with similar or different thermal tolerance phenotypic characteristics. The continuous variation in thermal tolerance among progeny isolates from tolerant to sensitive to thermal exposure to -5°C for five days indicated that there was a high likelihood that progeny isolates could readily adapt to exposure to temperature. The phenotypes with most variability were e.g. tempareture tolerance, virulence phenotype and the most conserved was genotype. The implications of this finding are serious in that it is likely that given this improved thermal tolerance within the population of the pathogen during times when climate change studies indicate warmer aerial and therefore soil temperatures in potato growing areas of the north central region of the US, that the pathogen will likely survive during winter. Even if oospores were produced in natural conditions in soil or in the plant material (tubers, stem, leaves), it is hard to speculate and differentiate or characterize the parents that are producing the oospores, as there could be only one or more than one genotype present. To eliminate this problem, and understand more about this pathogen this study was done *in vitro.* Even

though our main objective was to check the inheritance of thermal tolerance, the research has interested us to include few other phenotypic and genotypic tests like compatibility or mating type, metalaxyl sensitivity, virulence on foliage, pathogenicity and aggressiveness on tubers, isozyme analysis. Microsatellites, DNA content and ploidy level tests were also tested on some isolates, to prove that recombination had occurred and to reveal the complexity of DNA content in *P. infestans*.

The thermal tolerance study indicated that if mating occurred, producing progeny that are more tolerant to cold temperature (-5°C) they could also be more aggressive to potatoes. In terms of survival and initiation of epidemics these characteristics may balance as more aggressive isolates are likely to kill the host tubers quickly and therefore reduce the potentially infected meristematic tissue that will survive to the following season. Alternatively, if isolates can tolerate reduced soil temperatures (within host tuber tissues) freezing conditions but are moderately aggressive then the risk of surviving winter is greater and the initiation of epidemics the following season more likely. As noted in Chapter 3, the continuous variation noted in terms of metalaxyl sensitivity is interesting in that Ridomil-based products are really used for control of potato late blight in North America as it is widely assumed that all isolates are insensitive to metalaxyl. The wide variation in sensitivity may result in populations in the future developing that are even partially sensitive to metalaxyl and may justify the re-use of this class of fungicides.

P. infestans is likely to continue surviving, so it is important to expand our knowledge of the survival potential of different *P. infestans* genotypes under different temperature

settings. It is essential to predict changes in *P. infestans* population structure over time, especially when extraordinary weather conditions prevail.

6.1 Future research

1. Expand our knowledge to find out the biochemical basis and physiological basis of thermal tolerance in *Phytophthora infestans*.
2. Characterize genes that are involved for the complexity of *Phytophthora infestans*, in mating type, thermal tolerance, and metalaxyl sensitivity.

7 Appendix

Appendix 1

Rye A Agar

Rye A Agar was used for the isolation of *Phytophthora infestans*, modified from (Caten and Jinks, 1968 #35). For 1 liter media, 100.0g of pesticide free rye seed (Soldan's Feed & Pet Supplies, 5200 S Martin L King Jr. Blvd, Lansing, MI 48911) was washed before use by placing seed into a beaker, covering the opening with cheesecloth secured with a rubber band and running under water for 10-15 minutes. Washed seed was placed into a stainless steel container with an appropriate amount of distilled water and was boiled for one hour. Adequate amount of water is necessary to prevent burning.

After boiling, solution was transferred into a graduated cylinder by filtering through two layers of cheesecloth (equals four layers) placed over the mouth of a funnel. All the seed was brought to the beaker and additional distilled water is added and procedure is repeated until desired final volume is attained. Rye broth is evenly distributed among the different proper sized flasks. Following chemicals were added to the broth before it's autoclaved. 10.0g of sucrose (Sigma-Aldrich, Inc., 3050 Spruce Street, St. Louis, MO-63103 USA) and 15.0g of Baccto Agar (Sigma-Aldrich, Inc., 3050Spruce street, St. Louis, MO-63103 USA) were added to the media. For enhancing sporulation, 0.05g of Beta-sitosterol (Spectrum Chemical MFG. Corp., CA 90248) was added. Media was kept in the autoclave for 30 minutes for the sterilization. Hot liquid media was poured into 60 X 15mm Petri plates (Falcon Petri dish, Becton Dickinson Lab ware, Becton Dickinson and company, Franklin Lakes, NJ – 07417-1886, USA) with a 10ml pipette tip, using the automatic pipettor (Eppendorf Easypet 4421, Eppendorf North America, One Cantiague

Road, P.O. Box 1019, Westbury, New York 11590-0207, USA). While pouring the agar the flask is placed on a stirrer plate to keep agar homogenous.

To eliminate contamination, 500ul RAN antibiotic stock solution was added for 1 Liter Rye media, which is selective for Oomycetes containing 20 mg/ml Ampicillin, 75 mg/ml Nystatin, and 75mg/ml Rifamycin B (Sigma-Aldrich Co., P.O.Box 14508, St.Loius MO-63178, USA) in 1ml DMSO (Dimethylsulfoxide, JT Baker, Mallinckrodt Baker, Inc., Phillipsburg, NJ 08865). RAN was added to media after autoclaving and prior to pouring in to the plates. Sterile Rye agar plates amended with RAN were stored in the refrigerator, with no lights since the antibiotics are both heat and light sensitive. Aliquots of RAN were stored in the freezer, -20°C.

Long-term storage of isolates
Cultures were stored for periods of up to five to six months in three methods.

1. In 60 X 15mm Petri plates, each plate containing 25 ml Rye A agar (based on Caten and Jinks, 1968). Antibiotics were included in Rye A agar as needed. Isolates were maintained at 18°C, in the dark.
2. In 25ml vials (Research Products International Corp.) with 8gms of Rye seed (See above) and 10 ml sterile distilled water. After autoclaving add 2 plugs (3mm) of freshly growing *P. infestans* mycelium. After observing the growth, store them at 10°C in the dark.
3. Cultures were also maintained in long-term storage on slopes of Rye A agar using 25 ml vials. Isolates were maintained at 10°C, in the dark.

Isolates were sub-cultured as required by removing a section of mycelium from the actively growing edge of a fungal colony, using a No. 3 cork borer and a scalpel and transferring to another fresh medium.

Appendix 2

Origin, mating type, thermal tolerance, and *GPI*-isozyme phenotypes of isolates of *Phytophthora infestans* selected for crossing studies.

Cross number	Isolate	Origin	Mating type	Thermal tolerance
1	Pi02-007	US	A_2	T[a]
	Pi 41-02	NI[d]	A_1	T
2	Pi 02-007	US	A_2	T
	Pi 41-02	NI	A_1	T
3	Pi S1-3	US	A_2	T
	Pi 41-02	NI	A_1	T
4	Pi S1-3	US	A_2	T
	Pi 41-02	NI	A_1	T
5	Pi 98-1	US	A_2	I[b]
	Pi 41-02	NI	A_1	T
6	Pi 02-007	US	A_2	T
	Pi 62-02	NI	A_1	T
7	Pi02-007	US	A_2	T
	Pi 95-3	US	A_2	S[c]
8	Pi Atlantic 2N	US	A_2	S
	Pi 41-02	NI	A_1	T
9	Pi Atlantic 2N	US	A_2	S
	Pi 4-19	US	A_1	S

Origin, mating type and thermal tolerance of different parent isolates used in nine different crosses. Progeny from theses crosses were isolated to identify if thermal phenotype character is inherited. [a] = Tolerant; [b] = Intermediate; [c] = Sensitive; [d] = Northern Ireland

Appendix 3

Phenotypic and genotypic characteristics of *Phytophthora infestans* used in crosses.

Isolate	Thermal tolerance[a]	*Gpi* isozyme genotype[b]	Mating type[c]	Metalaxyl response[d]	R gene phenotype[e]
Pi 41-02[f]	T	100/100	A_1	R	1,3,4,5,6,7,8, 9,10,11
Pi 02-007[g]	T	100/111/122	A_2	R	1,2,3,4,5,6,7 8,9,10,11
Pi S1-3[g]	T	100/111/122	A_2	I	1,2,3,4,5,6,7, 8,9,10,11
Pi 98-1[g]	I	100/122	A_2	R	1,4,5,9,10,11
Pi 62-02[f]	T	100/100	A_1	I	1,2,3,4,5,6,7, 8,9,10,11
Pi 95-3[g]	S	86/100	A_1	S	5
Pi 4-19[h]	S	100/100	A_1	I	1,3,4,5,6,7,8, 9,10,11

[a] Comparison of growth of isolates of cultures of *Phytophthora infestans* for 25 days at 18°C after exposure at -5°C for 5 days measured by an image analysis technique comparing the growth of exposed isolates to a positive control (exposed to 18°C for 5 days) or a negative non-inoculated control with no growth . T= Tolerant; growth not significantly different from the growth of the positive control cultures of the same isolate; I= Intermediate; growth significantly less than that of the growth of the positive control cultures of the same isolate but significantly greater than the negative control; S= Sensitive; growth not significantly different from the negative control.

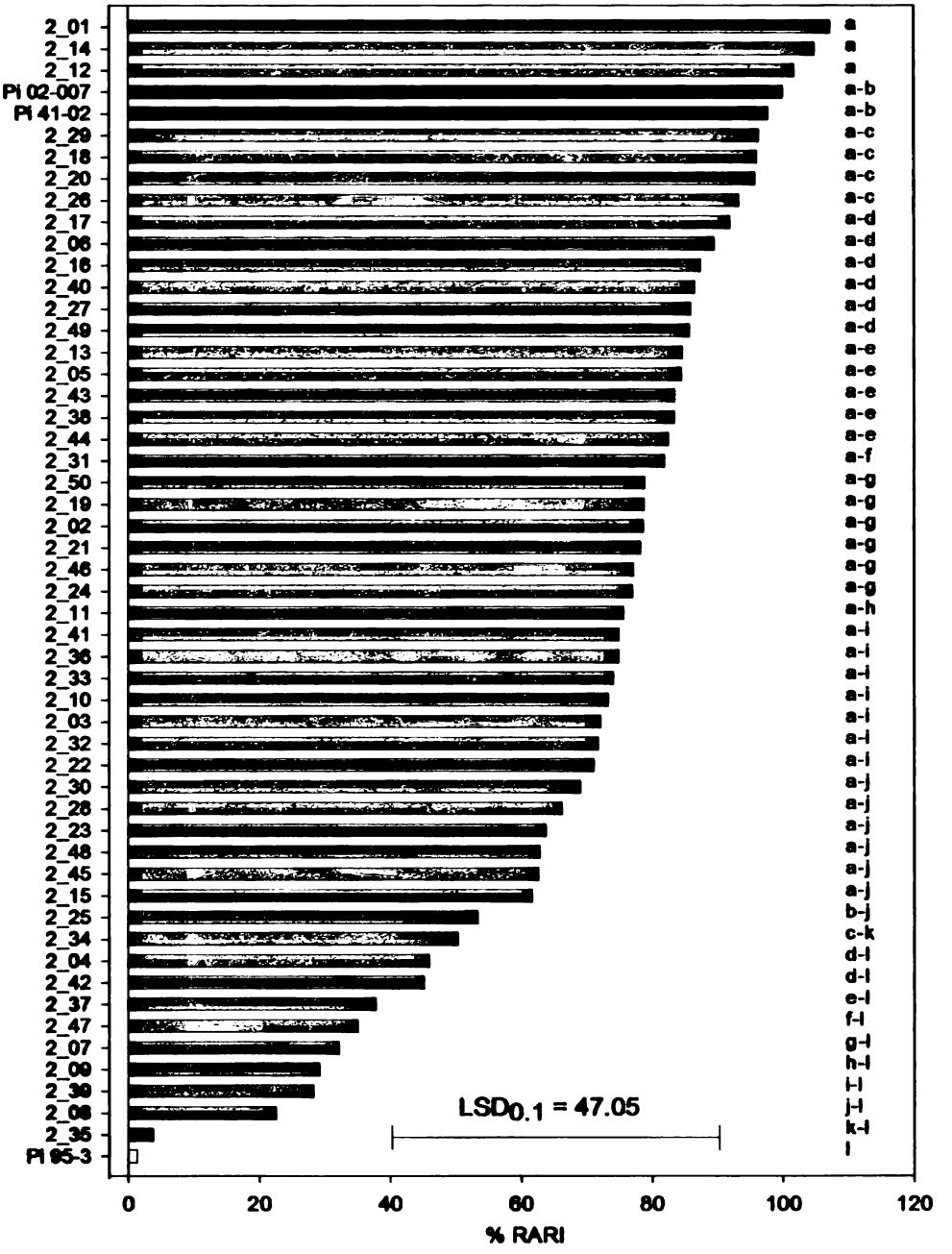
[b] Glucose -6- phosphate isomerase allozyme genotype; 86/100 (US1), 100/100 (US-1.7), 100/111/122 (US-8), 100/122 (US-14); genotype as designated by Goodwin *et al.*, 1995.

[c] Mating type determined by production of oospores when grown with isolates of *P. infestans* of known mating type.

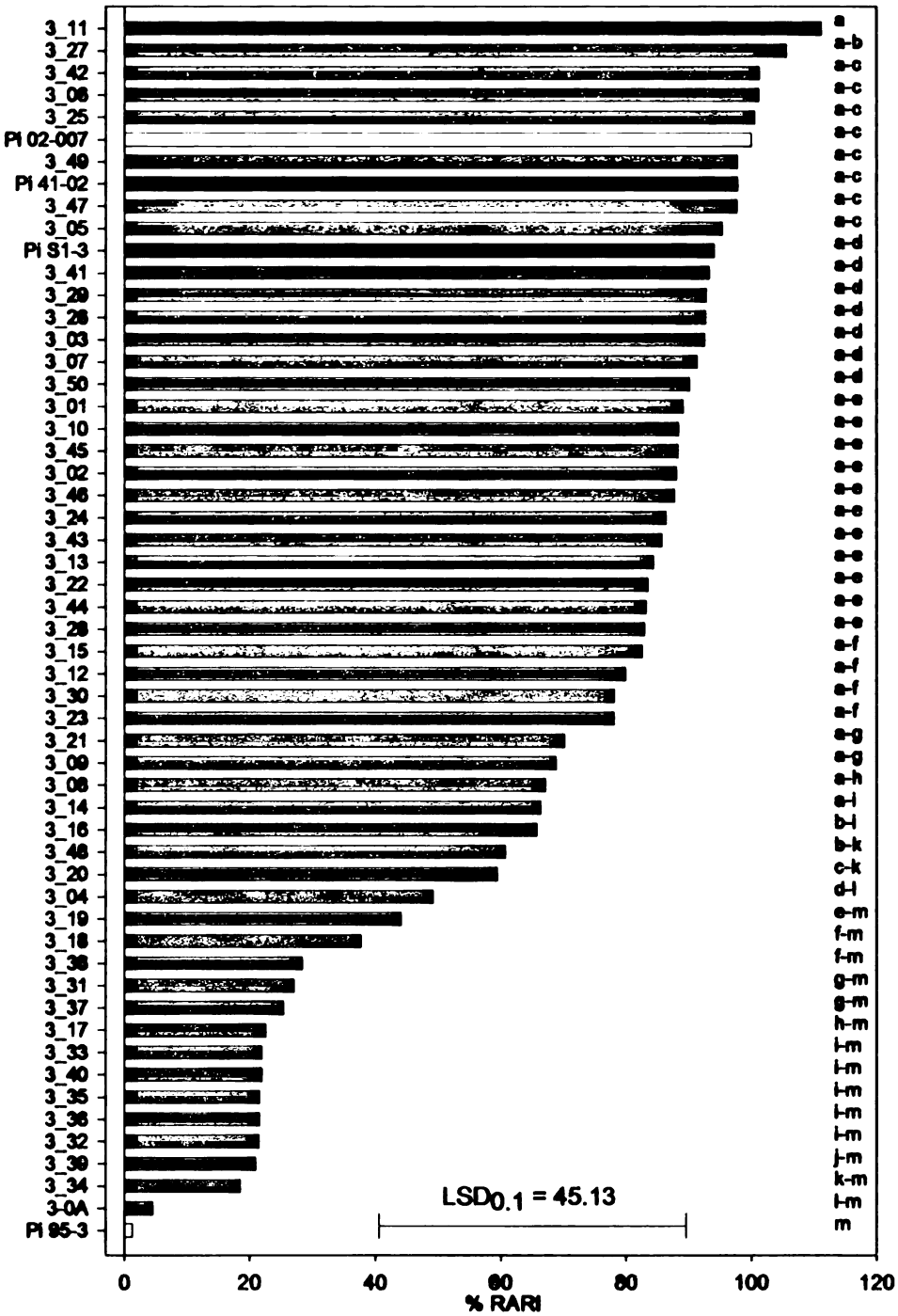
[d] Sensitivity to the fungicide metalaxyl, R=resistant, I=Intermediate, S=sensitive determined by comparing EC_{50} response of the isolates grown on media amended with different concentrations of metalaxyl with EC50 of known resistant and susceptible isolates.

[e] Virulence pathotype as defined by Muller and Black 1952. [f] Isolates of *P.infestans* from Northern Ireland from collection of Dr. Louise Cooke, Plant Pathology, Queens University, Northern Ireland. [g] Michigan isolates of *P. infestans* from collection of Dr. Kirk, Plant Pathology, Michigan State University, East Lansing, MI, USA. [h] A progeny isolate obtained from the 4th cross, used as a parent because of its sensitive phenotype.

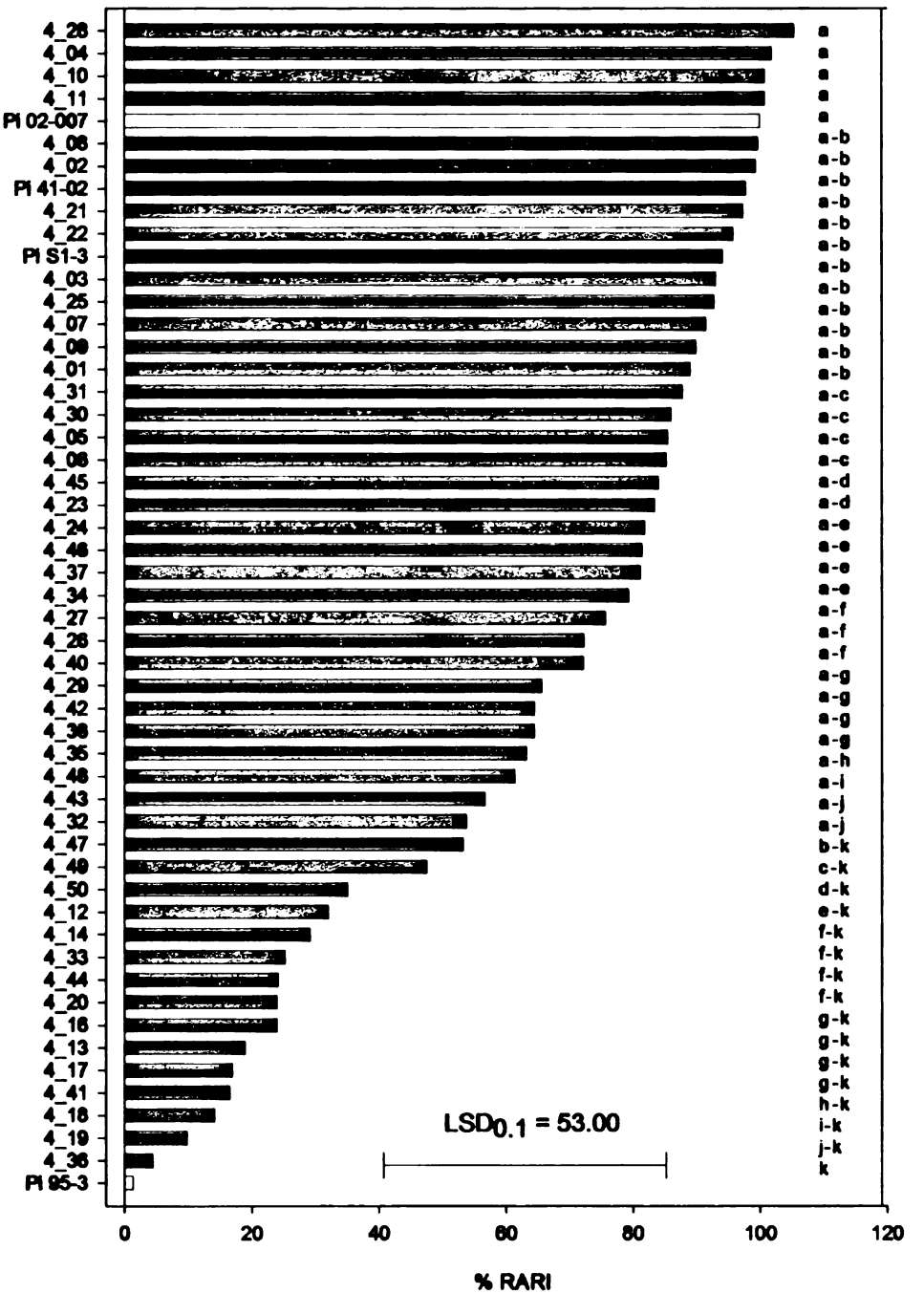
Appendix 4



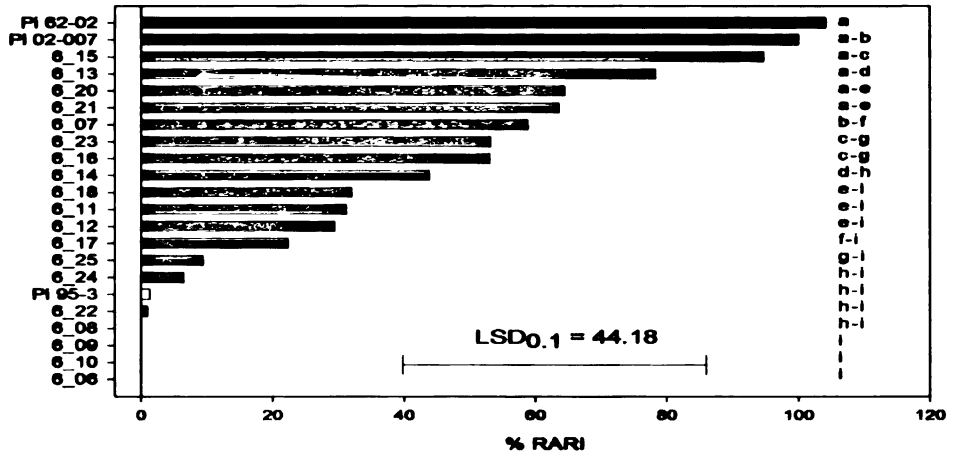
Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of *Phytophthora infestans* Pi 41-02 (A_1, thermally tolerant) x Pi 02-007 (A_2, thermally tolerant). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 2). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively; Pi 02-007 was a parent in this cross. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.



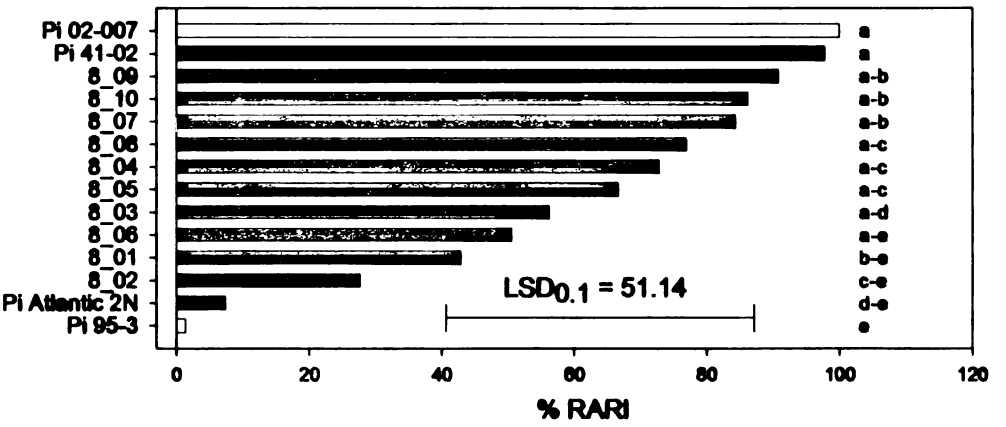
Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of *Phytophthora infestans* Pi 41-02 (A_1, thermally tolerant) x Pi S1-3 (A_2, thermally tolerant). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 3). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.



Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of *Phytophthora infestans* Pi 41-02 (A_1, thermally tolerant) x Pi S1-3 (A_2, thermally tolerant). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 4). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.



Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of *Phytophthora infestans* Pi 62-02 (A_1, thermally tolerant) x Pi 02-007 (A_2, thermally tolerant). Growth was measured as %RARI after exposure of isolates to -5°C for 5 days (Cross 6). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively; Pi 02-007 was a parent in this cross. %RARI values followed by the same letter are not significantly different at $p = 0.1$ (Tukey Multiple Comparison); $LSD_{0.10}$ indicated by bar.



Differences in thermal tolerance of progeny isolates from mating of thermally tolerant parental isolates of Phytophthora infestans Pi 41-02 (A1, thermally tolerant) x Pi Atlantic 2N (A2, thermally sensitive). Growth was measured as %RARI after exposure of isolates to -5oC for 5 days (Cross 8). The black bars represent the parental isolates and the white bars indicate thermal sensitive (Pi 95-3) and insensitive (Pi 02-007) standards, respectively. %RARI values followed by the same letter are not significantly different at p = 0.1 (Tukey Multiple Comparison); LSD0.10 indicated by bar.

Appendix 5

Phenotypic and genotypic characteristic of progeny isolates of *Phytophthora infestans* produced from 9 different thermal phenotype crosses.

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
1-01	A1	A	R	0,1,2,3,5,6,10, 11	3	100/100
1-02	A2	A	R	Nd	Nd	100/100
1-03	A1	A	R	0,1,2,3,4,5,6,7 ,8,9,10,11	Nd	100/100
1-04	A1,A2	A	I	0	Nd	100/111/122
1-05	A2	A	I	0,1,2,3,4,5	3	100/111/122
1-06	A1,A2	A	R	1,2	2	100/111/122
1-07	A1,A2	A	I	Nd	1	100/111/122
1-08	A2	A	I	Nd	Nd	100/111/122
1-09	A1,A2	B	I	Nd	Nd	100/100
1-10	A2	B	I	0,1,2,3,4,5,6,7 ,8,9,10,11	2	100/111/122
1-11	A2	B	I	Nd	Nd	100/100
1-12	A2	B	R	Nd	Nd	100/100
1-13	A1,A2	B	R	0,1,3,4,5,6,10, 11	2	100/111/122
1-14	A1	C	R	Nd	Nd	100/111/122
1-15	A2	A	R	Nd	Nd	100/111/122
1-16	A2	A	R	Nd	Nd	100/111/122
1-17	A2	A	R	Nd	Nd	100/111/122
1-18	A1	C	R	0,1,2,3,4,6,7,8 ,11	3	100/111/122
1-19	A2	B	R	Nd	Nd	100/111/122
1-20	A1	A	S	Nd	3	100/111/122
1-21	A2	A	S	Nd	Nd	100/111/122
1-22	A1	B	I	Nd	Nd	100/111/122
1-23	A1	A	I	Nd	Nd	100/111/122
1-24	A2	A	I	Nd	Nd	100/111/122
1-25	A2	B	I	Nd	Nd	100/111/122
1-26	A2	C	R	Nd	Nd	100/111/122
1-27	A2	A	R	Nd	Nd	100/111/122
1-28	A2	A	R	Nd	Nd	100/111/122

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
1-29	A2	A	I	3,5,6,7,9,10,11	1	100/100
1-30	A1	A	R	Nd	Nd	100/100
1-31	A2	A	R	Nd	Nd	100/100
1-32	A2	B	I	Nd	Nd	100/100
1-33	A2	B	R	Nd	Nd	100/111/122
1-34	A2	A	I	Nd	Nd	100/100
1-35	A2	B	R	Nd	Nd	100/111/122
1-36	A2	A	I	Nd	3	100/100
1-51	A1	B	R	Nd	Nd	100/100
2-01	A1	A	I	0,1,2,3,4,5,6,7,8,9,10,11	Nd	100/100
2-02	A1	B	R	Nd	Nd	100/100
2-03	A1,A2	B	R	Nd	2	100/100
2-04	A2	B	R	Nd	Nd	100/100
2-05	A1	B	I	Nd	Nd	100/100
2-06	A1	A	R	Nd	Nd	100/100
2-07	A2	B	R	0,1,2,3,4,5,6,7,9,10,11	Nd	100/100
2-08	A2	B	R	Nd	Nd	100/100
2-09	A1,A2	C	R	Nd	3	100/100
2-10	A1	A	R	Nd	Nd	100/100
2-11	A2	A	R	Nd	Nd	100/100
2-12	A2	A	R	Nd	Nd	100/100
2-13	A2	B	I	1,3,5,6,7,8,10,11	3	100/111/122
2-14	A2	A	R	Nd	Nd	100/111/122
2-15	A2	A	R	Nd	Nd	100/100
2-16	A1	B	R	Nd	Nd	100/100
2-17	A2	A	R	Nd	Nd	100/100
2-18	A1	A	I	Nd	Nd	100/100
2-19	A1	B	I	Nd	Nd	100/100
2-20	A1	A	R	Nd	4	100/100
2-21	A2	B	R	Nd	Nd	100/100
2-22	A2	B	R	Nd	Nd	100/100
2-23	A2	B	R	Nd	3	100/100
2-24	A2	A	I	Nd	2	100/100
2-25	A2	B	I	Nd	Nd	100/100
2-26	A2	A	I	Nd	Nd	100/111/122
2-27	A1	A	R	Nd	Nd	100/100

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
2-28	A1	B	R	Nd	Nd	100/100
2-29	A2	A	R	Nd	Nd	100/100
2-30	A2	B	R	Nd	Nd	100/111/122
2-31	A2	B	R	0,1,2,3,4,5,6,7 ,8,9,10,11	Nd	100/100
2-32	A2	B	R	Nd	Nd	100/111/122
2-33	A1,A2	B	R	Nd	3	100/100
2-34	A1	B	I	Nd	2	100/100
2-35	A2	C	R	Nd	Nd	100/111/122
2-36	A2	B	I	Nd	Nd	100/111/122
2-37	A2	B	R	Nd	Nd	100/111/122
2-38	A1	B	R	Nd	Nd	100/100
2-39	A2	B	I	Nd	Nd	100/111/122
2-40	A1	B	R	Nd	Nd	100/100
2-41	A2	A	R	Nd	Nd	100/111/122
2-42	A2	B	I	Nd	Nd	100/111/122
2-43	A1	B	R	Nd	Nd	100/111/122
2-44	A2	B	R	Nd	Nd	100/111/122
2-45	A2	A	R	Nd	Nd	100/111/122
2-46	A2	A	R	Nd	Nd	100/111/122
2-47	A2	C	R	Nd	Nd	100/111/122
2-48	A1	B	R	Nd	Nd	100/111/122
2-49	A2	A	R	Nd	Nd	100/111/122
2-50	A2	A	I	Nd	Nd	100/111/122
2-51	A1	C	R	Nd	Nd	100/111/122
3_0A	A1	A	R	0,1,2,3,5,7,8,1 0,11	3	100/111/122
3-01	A1	A	R	0,1,2,3,4,5,6,7 ,9,10,11	3	100/100
3-02	A2	A	I	Nd	Nd	100/111/122
3-03	A1	A	R	Nd	Nd	100/100
3-04	A1,A2	B	R	Nd	Nd	100/100
3-05	A1	A	I	Nd	2	100/100
3-06	A2	A	R	1,2,3,4,5,7,8,1 0,11	Nd	100/100
3-07	A2	A	I	Nd	Nd	100/100
3-08	A2	A	I	Nd	Nd	100/100
3-09	A2	B	I	Nd	Nd	100/100
3-10	A2	A	I	Nd	Nd	100/100
3-11	A2	A	I	Nd	Nd	100/100

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
3-12	A2	B	I	Nd	Nd	100/100
3-13	A1,A2	A	I	Nd	Nd	100/100
3-14	A2	A	S	0,1,2,3,6	4	100/100
3-15	A1	B	I	Nd	Nd	100/100
3-16	A1	B	S	Nd	Nd	100/100
3-17	A2	C	S	Nd	Nd	100/100
3-18	A1	A	I	Nd	Nd	100/100
3-19	A1	B	S	Nd	Nd	100/100
3-20	A1	A	S	Nd	Nd	100/100
3-21	A2	A	I	Nd	Nd	100/100
3-22	A1	A	R	Nd	Nd	100/100
3-23	A1	A	I	Nd	Nd	100/100
3-24	A1	B	I	Nd	Nd	100/100
3-25	A1	A	I	Nd	Nd	100/100
3-26	A1	A	S	Nd	Nd	100/100
3-27	A1	A	I	Nd	Nd	100/100
3-28	A1	B	I	Nd	Nd	100/100
3-29	A1	A	I	Nd	Nd	100/100
3-30	A1	A	I	Nd	Nd	100/100
3-31	A1	B	I	Nd	Nd	100/100
3-32	A2		I	Nd	Nd	100/100
3-33	A2	B	I	Nd	Nd	100/100
3-34	A1	B	I	Nd	Nd	100/100
3-35	A1	B	I	Nd	Nd	100/100
3-36	A2	B	S	Nd	Nd	100/100
3-37	A2	B	S	0,1,2,3,5,6	3	100/111/122
3-38	A2	B	I	Nd	Nd	100/111/122
3-39	A2	B	I	Nd	Nd	100/111/122
3-40	A1	B	I	Nd	Nd	100/111/122
3-41	A1	A	I	Nd	3	100/100
3-42	A2	A	R	Nd	3	100/111/122
3-43	A1	B	I	Nd	Nd	100/100
3-44	A1	B	S	Nd	Nd	100/100
3-45	A1	A	S	Nd	Nd	100/100
3-46	A2	B	R	Nd	Nd	100/111/122
3-47	A2	A	R	Nd	Nd	100/100
3-48	A2	B	R	Nd	Nd	100/100
3-49	A1	A	I	Nd	Nd	100/100
3-50	A1,A2	A	I	Nd	Nd	100/100
4-01	A1	B	I	1,3,5,9,11	2	100/100

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
4-02	A2	A	I	Nd	Nd	100/100
4-03	A1	A	I	Nd	Nd	100/100
4-04	A1	A	I	Nd	Nd	100/100
4-05	A1	A	R	Nd	2	100/100
4-06	A1	A	R	Nd	Nd	100/100
4-07	A1	A	R	Nd	Nd	100/100
4-08	A1	A	R	Nd	Nd	100/100
4-09	A1	A	S	Nd	2	100/100
4-10	A2	A	R	Nd	Nd	100/100
4-11	A1	C	I	0,1,3,4,5,7,9,10,11	Nd	100/100
4-12	A1	C	I	0,1,2,3,4,5,6,7,8,10,11	2	100/100
4-13	A1	B	I	Nd	Nd	100/100
4-14	A1	B	I	Nd	Nd	100/100
4-15	A1	C	R	Nd	Nd	100/100
4-16	A1	B	I	Nd	Nd	100/100
4-17	A1	B	I	Nd	Nd	100/100
4-18	A1	B	I	Nd	Nd	100/100
4-19	A1	C	I	Nd	2	100/100
4-20	A1	B	I	Nd	Nd	100/100
4-21	A1	A	S	Nd	1	100/100
4-22	A1	A	S	Nd	Nd	100/100
4-23	A1	B	I	Nd	Nd	100/100
4-24	A1	B	I	Nd	Nd	100/100
4-25	A1	A	S	Nd	Nd	100/100
4-26	A1	A	I	Nd	Nd	100/100
4-27	A1	B	I	Nd	Nd	100/100
4-28	A1	A	I	Nd	Nd	100/100
4-29	A2	B	S	0,1,2,3,4,5,6,7,8,9,10,11	2	100/111/122
4-30	A2	A	S	Nd	Nd	100/100
4-31	A1	A	R	Nd	Nd	100/100
4-32	A2	B	I	Nd	Nd	100/100
4-33	A1	B	I	Nd	Nd	100/100
4-34	A2	B	I	Nd	Nd	100/100
4-35	A2	A	I	Nd	Nd	100/100
4-36	A1	A	S	Nd	Nd	100/100
4-37	A2	A	S	Nd	Nd	100/100
4-38	A1	A	S	Nd	Nd	100/100

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
4-39	A2	A	S	Nd	Nd	100/100
4-40	A1	A	I	Nd	Nd	100/100
4-41	A1	B	S	Nd	Nd	100/100
4-42	A1	B	I	Nd	Nd	100/100
4-43	A2	B	I	Nd	Nd	100/111/122
4-44	A1	C	I	Nd	Nd	100/100
4-45	A1	B	I	Nd	Nd	100/100
4-46	A2	A	I	Nd	Nd	100/100
4-47	A2	B	S	Nd	Nd	100/111/122
4-48	A2	B	R	Nd	Nd	100/100
4-49	A1	B	I	Nd	Nd	100/111/122
4-50	A2	B	I	Nd	Nd	100/111/122
4-51		B		Nd	Nd	
5-01	A1	B	R	0,1,2,3,4,5,6	Nd	100/122
5-02	A1	B	I	1,2,3,4,5,6,7,10,11	Nd	100/100
5-03	A1	C	I	Nd	Nd	100/100
5-04	A1	B	I	Nd	Nd	100/100
5-05	A2	C	I	Nd	Nd	100/100
5-06	A2	B	R	Nd	Nd	100/100
5-07	A1	A	I	Nd	Nd	100/100
5-08	A2	A	I	Nd	Nd	100/122
5-09	A1		I	1,2,3,4,5,6,7,10	Nd	100/100
5-10	A1	C	R	Nd	Nd	100/100
5-11	A1	B	R	Nd	Nd	100/100
5-12	A1		I	0,1,2,3,4,5,6	3	100/100
5-13	A2	A	I	Nd	Nd	100/100
5-14	A1	C	I	3,5,7,8,9	Nd	100/100
5-15	A2	A	R	Nd	Nd	100/100
5-16	A1	A	R	0-11	2	100/100
5-17	A1		I	Nd	Nd	100/100
5-18	A2		R	Nd	Nd	100/100
5-19	A1	C	I	0,1,2,5,6,7,8,9,11	0	100/100
5-20	A1		I	Nd	Nd	100/100
5-21	A2	C	I	Nd	Nd	100/100
5-22	A1		I	Nd	Nd	100/100
5-23	A1		I	Nd	Nd	100/100
5-24	A2	C	R	Nd	Nd	100/122

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
5-25	A1	A	I	Nd	Nd	100/100
5-26	A1	A	I	Nd	Nd	100/100
5-27	A2		I	Nd	Nd	100/100
5-28	A1	A	I	0,1,2,3,4,5,7,8 ,9,10,11	Nd	100/122
5-29	A2	A	I	Nd	Nd	100/100
5-30	A1,A2	A	I	0,1,2,3,4,5,6,7 ,9,10,11	Nd	100/100
5-31	A1		I	Nd	Nd	100/100
5-33	A1	A	I	Nd	Nd	100/100
5-34	A1	C	I	Nd	Nd	100/100
5-35	A1	C	I	Nd	Nd	100/100
5-36	A1	A	I	Nd	2	100/100
5-37	A2	C	I	Nd	3	100/122
5-38	A1	B	I	1,3,5,6,8	Nd	100/100
5-39	A2	C	I	Nd	Nd	100/100
5-40	A1	C	I	Nd	Nd	100/122
5-41	A2	A	I	1	1	100/100
5-42	A1	C	I	Nd	Nd	100/100
5-43	A2	B	I	Nd	1	100/100
5-44	A1	B	R	Nd	Nd	100/100
5-45	A2	C	I	1,4	0	100/122
5-46	A1	C	I	Nd	0	100/100
6-01	A1	B	R	Nd	Nd	100/100
6-02	A1	B	I	Nd	Nd	100/100
6-03	A1	B	I	Nd	Nd	100/100
6-04	A1	B	R	Nd	Nd	100/100
6-05	A2	B	I	Nd	Nd	100/111/122
6-06	A2	A	R	Nd	2	100/111/122
6-07	A2	B	I	Nd	Nd	100/111/122
6-08	A2	B	I	0,1,2,3,4,5,6,7 ,8,9,10,11	Nd	100/111/122
6-09	A2	C	I	Nd	Nd	100/111/122
6-10	A2	B	R	Nd	Nd	100/111/122
6-11	A1	B	S	1,3,5,7,10,11	0	100/100
6-12	A2	C	R	Nd	0	100/111/122
6-13	A2	A	R	Nd	Nd	100/111/122
6-14	A2	C	I	Nd	3	100/111/122

Continued

Isolate	Mating type	Temp-erature	Metal-axyl	Virulence	Pathogenicity	Isozyme
6-15	A2	A	I	Nd	Nd	100/111/122
6-16	A2	B	I	Nd	3	100/111/122
6-17	A2	B	I	Nd	2	100/111/122
6-18	A2	B	I	Nd	Nd	100/111/122
6-19	A1	B	I	3,4,5,6,7,8,10, 11	3	100/111/122
6-20	A1	A	R	0,1,2,3,5,7,10, 11	2	100/100
6-21	A1	B	I	Nd	Nd	100/100
6-22	A2	C	R	Nd	Nd	100/111/122
6-23	A2	A	I	Nd	Nd	100/111/122
6-24	A2	C	I	Nd	Nd	100/111/122
6-25	A2	C	I	Nd	Nd	100/111/122
7-01	A2	A	R	Nd	0	100/111/122
7-02	A2	B	R	Nd	0	100/111/122
7-03	A2	C	R	Nd	3	100/111/122
7-04	A2	B	R	Nd	Nd	100/111/122
7-05	A1	A	R	Nd	Nd	100/111/122
7-06	A1	C	R	Nd	Nd	100/111/122
7-07	A1	C	R	Nd	0	100/111/122
7-08	A1	A	I	Nd	2	100/111/122
8-01	A1	B	R	Nd	Nd	100/100
8-02	A1	C	I	Nd	3	100/100
8-03	A1	B	S	Nd	Nd	100/100
8-04	A1	A	I	Nd	Nd	100/100
8-05	A1	A	S	Nd	2	100/100
8-06	A1	B	I	Nd	Nd	100/100
8-07	A1	A	I	Nd	Nd	100/100
8-08	A1	B	R	Nd	2	100/100
8-09	A1	A	I	Nd	2	100/100
8-10	A1	A	I	Nd	Nd	100/100
8_11	A2	A	I	Nd	Nd	100/100
8_12	A1	C	R	Nd	Nd	100/100
8_13	A1	C	R	Nd	Nd	100/100
8_14	A1	A	I	Nd	Nd	100/100
8_15	A2	A	I	Nd	Nd	100/100
8_16	A1	C	I	Nd	Nd	100/100
9_01	A1	T	I	Nd	3	100/100
9_02	A1	S	I	Nd	0	100/100

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